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Host plant volatiles induce oriented flight behaviour in male European grapevine moths, *Lobesia botrana*

Martin von Arx, Daniela Schmidt-Büsser, Patrick M. Guerin *

Institute of Biology, Faculty of Sciences, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

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ABSTRACT

The European grapevine moth *Lobesia botrana* relies on a female produced sex pheromone for long-distance mate finding. Grapevine moth males compete heavily during limited time windows for females. The aim of this study was to investigate the perception of host plant volatiles by grapevine moth males and whether such compounds elicit upwind oriented flights. We compared five host plant headspace extracts by means of gas chromatography linked electroantennogram (EAG) recording. We identified 12 common host plant volatiles (aliphatic esters, aldehydes, and alcohols, aromatic compounds and terpenes) that elicit EAG responses from grapevine moth males and that occur in at least three of the host plant volatile headspace extracts tested. Subsequently the behavioural response of grapevine moth males to four these compounds presented singly and in mixtures (1-hexanol, 1-octen-3-ol, (*Z*)-3-hexenyl acetate and (*E*)-β-caryophyllene) was recorded in a wind tunnel. Grapevine moth males engaged in upwind flights to all of four compounds when released singly at 10,000 pg/min and to all, except 1-octen-3-ol, when released at 100 pg/min. A blend of the four host plant volatiles released at 10,000 pg/min and mixed at a ratio based on the analysis of *Vitis vinifera* cv. Solaris volatile emissions attracted significantly more males than any single compound. Grapevine moth males perceive and respond to host plant volatiles at biologically relevant levels indicating that host plant volatiles figure as olfactory cues and that *L. botrana* males can discern places where the likelihood of encountering females is higher.

1. Introduction

The grapevine moth *Lobesia botrana* Den. et Schiff. (Lepidoptera; Tortricidae) is the most damaging insect pest of the grapevine in the European wine-growing areas. As larvae feed on fruit this favours infestation with bacteria and fungi causing a considerable loss at harvest. Several studies have documented the constituents of the female sex pheromone and the behavioural response of grapevine moth males to mixtures at specific ratios of these products (Arn et al., 1988; El-Sayed et al., 1999b; Witzgall et al., 2005). The identification and industrial synthesis of the main pheromone components allowed the development of pheromone-based control methods for this pest by mating disruption (Charmillot and Pasquier, 2000).

L. botrana is a polyphagous insect, able to complete its life cycle on several host plants belonging to different plant families (Galet, 1982; Katerinopoulos et al., 2005; Thiery and Moreau, 2005). The perception of plant volatiles is important for *L. botrana*, in that such compounds can signal food, oviposition sites and places of refuge as has been generally suggested for other phytophagous insects (Visser, 1986). Host plant volatiles were already shown to affect the behaviour of grapevine moth females. Behavioural assays in a Y-tube olfactometer showed that organic solvent extracts from the host plant *Rosmarinus officinalis* (Lamiaceae) attracted unmated grapevine moth females over short distances (Katerinopoulos et al., 2005). Mated females showed upwind flights to grapevine, *Vitis vinifera* (Vitaceae), and to plant parts such as leaves, flowers, buds and grape berries in a wind tunnel (Masante-Roca et al., 2007; Tasin et al., 2005). Recently, a mixture of (*E*)-β-caryophyllene, (*E*)-β-farnesene and (*E*)-4,8-dimethyl-1,3,7-nonatriene, identified in headspace collections from grapes, was shown to attract mated females in a wind tunnel (Tasin et al., 2007; Tasin et al., 2006a). These studies demonstrate the importance of plant volatiles in host location by grapevine moth females.

The perception of host plant volatiles could be as equally important for male grapevine moths as for females. Female moths are likely to be found on host plants that can serve as mating sites, thus facilitating encounter of the sexes (Landolt and Phillips, 1997). In this situation, competition for females between male moths is probably high, with the first-arriving males usually mating with any calling female. Furthermore, *L. botrana* uses protandric emergence as a male mating strategy (Thiery and Moreau, 2005). Maximum numbers of matings by virgin grapevine moth males occur on the 3rd day after eclosion and delayed mating

* Corresponding author. Tel.: +41 32 718 30 66; fax: +41 32 718 30 01.
E-mail address: patrick.guerin@unine.ch (P.M. Guerin).

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reduces the reproductive output of grapevine moth females (Torres-Vila et al., 1995; Torres-Vila et al., 2002). In this scenario, mating success is probably highest in grapevine moth males that locate mating sites earliest (Miller et al. 2006). In line with this we have found that single host plant compounds increase the responses of grapevine moth males to their sex pheromone (von Arx et al., in preparation). We hypothesise that host plant volatiles can serve to guide males to mating sites even in the absence of the female sex pheromone. Furthermore, since *L. botrana* is polyphagous, it is probable that nonspecific host volatile organic compounds influence *L. botrana* behaviour rather than plant products that are unique to one host plant.

To test our hypothesis we first collected different host plant volatile bouquets to isolate plant volatile products that elicit antennal receptor cell responses in grapevine moth males using gas chromatography linked electroantennogram recordings. We subsequently tested four identified chemical stimulants singly and in mixtures in wind tunnel tests and show that plant volatiles attract grapevine moth males. This provides new insights into host plant finding in grapevine moth males and supports the hypothesis that the host plants could serve as rendez-vous sites for the sexes.

2. Material and methods

2.1. Insects

Grapevine moths were reared from insects received from a laboratory culture at the Research Station Agroscope Changins-Wädenswil ACW (Switzerland). The grapevine moth was maintained on a semisynthetic diet (Rauscher et al., 1984) under a 16L:8D photoperiod, 65% RH and 25 °C during the photophase and at 85% RH and 18 °C during the scotophase. Pupae were separated by sex and males emerged into cages (BugDorm, 30 × 30 × 30 cm, MegaView Science Education Services Co., Taiwan) with a sugar–water solution (10%) provided on top of the cage. Males used for electrophysiological and behavioural experiments were 3 days old.

2.2. Vine plants

Potted vine plants, *V. vinifera* cv. Solaris, were obtained from the Research Station Agroscope Changins-Wädenswil ACW (Switzerland). Vine plants were held under a 13L:11D photoperiod at 22 °C. Vine plants used for headspace collections measured 45 ± 5 cm with a fresh mass of leaves of 20 ± 2 g.

2.3. Standard chemicals

The standard plant compounds used in wind tunnel studies were 1-hexanol, (*Z*)-3-hexenyl acetate, (*E*)-β-caryophyllene (all >98%) supplied by Sigma–Aldrich Chemie (Buchs, Switzerland) and 1-octen-3-ol (>97%) from Merck (Munich, Germany). Plant compounds used for gas chromatography linked mass spectrometry included 1-hexanol, (*Z*)-3-hexenyl acetate, (*E*)-β-caryophyllene, (*E*)-2-hexenal, benzaldehyde, methyl salicylate, (*Z*)-3-hexenol, limonene and 6-methyl-5-hepten-2-one (all >98%) supplied by Sigma–Aldrich Chemie (Buchs, Switzerland), 1-octen-3-ol (>97%, Merck, Munich, Germany), (*E*)-β-farnesene (>90%) from Bedoukian Research (Danbury, CT, USA) and (*E,E*)-α-farnesene (>60%) from Givaudan (Vernier, Switzerland).

2.4. Headspace collections

Headspace collections of volatiles from plant parts were made from wild grapevine *V. vinifera sylvestris* (Vitaceae) male and female flowers, from European privet *Ligustrum vulgare* (Oleaceae) flowers, from olive tree *Olea europaea* (Oleaceae) leaves and from rosemary *R. officinalis* (Lamiaceae) leaves. Fresh material was cut off the plant and placed in a 250 ml gas-wash bottle. Charcoal-filtered air was drawn through the gas-wash bottle at 500 ml/min and over a 50 mg Porapak Q trap (80/100 mesh, Alltech, Deerfield, IL, USA). Prior to experiments, Porapak Q traps were conditioned under N_2 at 200 °C for 90 min. Trapped volatiles were eluted with 100 μl dichloromethane (DCM; SupraSolv, Merck, Germany) into glass ampoules that were sealed and stored in the freezer at −20 °C until analysis. For headspace constituent ratio determination, headspace collections from entire vine plants were collected from two *V. vinifera* cv. Solaris plants held under a glass cylinder (i.d. 10 cm, height 50 cm). A Teflon disc, with a hole in its centre for the plant stem, was placed at the bottom of the cylinder to cover the soil. Charcoal-filtered air was drawn through the glass cylinders at 400 ml/min and over a commercial Tenax™ GR cartridge (i.d. 4 mm, Gerstel, Switzerland) for 18 h. Prior to experiments, Tenax GR cartridges were conditioned as above. After volatile collection, cartridges were immediately submitted to thermal desorption and gas chromatography linked mass spectrometry (see below).

2.5. Gas chromatography linked electroantennogram recording

Gas chromatography linked electroantennogram detection (GC–EAG) was used to isolate host plant volatile headspace constituents eliciting male *L. botrana* olfactory receptor neuron responses (Arn et al., 1975). A grapevine moth male antenna was suspended between two glass electrodes filled with 0.1 M KCl and held in a humidified charcoal-filtered air stream (90–100% RH, 23 ± 2 °C) delivered at 1 m/s via a glass water-jacketed tube (7 mm ID) whose outlet was about 1 cm from the preparation. Constituents of headspace extracts were separated by means of a gas chromatograph (HRGC 5300, Carlo Erba Instruments, Italy), equipped with a polar high resolution capillary column (FFAP, length 30 m, i.d. 0.25 mm, film thickness 0.25 μm; BGB Analytik, Switzerland). Aliquots of 1 μl of the headspace samples were injected on-column with the oven at 40 °C for 5 min, then programmed at 10 °C/min to 230 °C and held for 5 min. The column effluent was split (50:50) between the flame ionisation detector (FID) and to a heated (230 °C) outlet from the GC that led to the humidified air stream passing over the antenna. The FID and the electroantennogram (EAG) responses were recorded simultaneously on a computer using Syntech hardware and software (Kirchzarten, Germany). The responsiveness of the antenna was tested with an air puff from 10 μg of (*Z*)-3-hexenol on a filter paper strip in a 5 ml stimulus syringe (Taneja and Guerin, 1997). If the antennal response to this 1s air puff (1 ml/s) from the stimulus syringe was less than 0.7 mV another antenna was mounted. All headspace extracts were tested on three different *L. botrana* male antennae from different individuals. Compounds eliciting an EAG response of at least 0.08 mV (signal to noise ratio of 4:1) from at least three antennae at the same retention time were considered electrophysiologically active. Kovats retention indices were established for the EAG-active constituents of the extracts. Electrophysiologically active compounds were identified when they co-occurred in at least three of the five different headspace extracts.

2.6. Gas chromatography linked mass spectrometry (GC–MS)

2.6.1. Headspace collections dissolved in dichloromethane

Identification of headspace volatiles was done on a Hewlett Packard 5890 Series II gas chromatograph equipped with an on-column injector and a polar column (same specifications as the column used for GC–EAG). The outlet of the GC was linked to a Hewlett Packard 5971A mass selective detector with electron

impact ionisation at 70 eV with helium (30 cm/s) as carrier gas. Aliquots of 1 μl of headspace collections were injected at a GC-starting temperature of 40 °C, held for 5 min at this temperature and was then programmed at 15 °C/min to 230 °C and held for 5 min. The scan range of the MS was set to 20–350 m/z.

2.6.2. Thermal desorption

For the analysis of solventless headspace samples collected from entire vine plants the GC was equipped with a thermal desorption unit (TDS 2, Gerstel, Germany) and a cooled injection system (CIS 3, Gerstel, Germany). Headspace volatiles initially trapped on a Tenax™ GR cartridge (see above) were thermally desorbed via the TDS 2 temperature programmed at 60 °C/min from 30 °C to 220 °C and held for 2 min with a total flow of helium of 50 ml/min. Analyses were carried out with the TDS 2 in split mode. A heated transfer line (240 °C) evacuated the volatiles to the CIS 3 cooled to −80 °C with liquid nitrogen where volatiles were cryotrapped. At the end of desorption plus an equilibration time of 0.2 min, the CIS was ballistically heated at 12 °C/s to reach 230 °C and held there for 3 min to permit transfer of the desorbed volatiles in splitless mode (1 min) onto the high resolution capillary column. The GC was programmed from 40 °C for 5 min, heated at 5 °C/min to 230 °C for 5 min. The scan range of the MS was set to 20–350 m/z.

2.7. Identification of the biologically active plant volatiles

Identification of an electrophysiologically active product in a headspace extract was based on the match of Kovats indices for the compound in GC–EAG and GC–MS analyses and of its mass spectrum with that of a known product stored in a computer-based library of the GC–MS (Nist98). The mass spectrum of the product at the Kovats index of the electrophysiologically active product was then compared with that of the library-proposed synthetic analogue injected under the same conditions. Full identification based on the mass spectrum was not always feasible because of the small amount of compound present and/or because of coeluting products that obscured the spectrum. The following chemicals were employed as standards in GC–MS: (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, benzaldehyde, methyl salicylate, 1-hexanol, (*Z*)-3-hexenol, 1-octen-3-ol, limonene, 6-methyl-5-hepten-2-one, (*E*)-β-caryophyllene, (*E*)-β-farnesene and (*E,E*)-α-farnesene.

2.8. Wind tunnel bioassay

The flight section of the wind tunnel measured 60:60:195 cm. Air was blown by a centrifugal fan (Fischbach GmbH, Neunkirchen, Germany) through a section of charcoal cartridges and a perforated steel screen (1 mm thick, 3 mm round holes, 51% of air passage, Schäfer, Neunkirchen, Germany) which resulted in a laminar airflow at 30 cm/s. The air issuing from the wind tunnel was sucked by another fan and cleaned by an additional set of charcoal filters. Overhead illumination was provided by high frequency fluorescent lights (36 W,>1 kHz, Philips) running the length of the tunnel. The light intensity was regulated to ca. 10 lux along the wind tunnel floor. Light was dispersed using a Perspex Prisma® crystal-clear plastic sheet under the fluorescent tubes and by placing crepe paper on the roof of the wind tunnel. Below the tunnel floor, black shapes of irregular sizes and forms placed on a white sheet served as visual cues. The wind tunnel was housed in a walk-in climate chamber (Schaller Uto AG, Bern, Switzerland) that allowed the air stream to be maintained at 18 ± 0.5 °C and 85 ± 2% RH. Olfactory stimuli were presented at the upwind end of the wind tunnel by means of a piezo sprayer (El-Sayed et al., 1999a) that permitted to release plant volatiles at known amounts. Solutions containing plant volatiles were fed by a motor-driven syringe (CMA 400, CMA Microdialysis AB, Solna, Sweden) at a constant rate of 10 μl/min into a glass capillary (i.d. 0.6 mm) with a drawn out tip (type GC100-10, Clark Electromedical Instruments, Pangbourne, England) connected to a piezo-ceramic disc (25 mm diameter, Philips PXE5 25/2.0). A frequency generator (Wavetek FG-5000A, Willtek Communications GmbH, Ismaning, Germany) producing a square-wave signal (ca. 80 kHz, 40 V amplitude) was connected to the piezo-ceramic disc inducing the tip of the capillary to oscillate and to produce an aerosol. A metal grid cylinder protected the capillary tip against damage by moths attempting to contact the chemical source. Ethanol (pro Analysis, Merck, Germany) was used as solvent. At the beginning of the scotophase, three day-old males were placed individually in glass tubes (125 mm long, 21 mm i.d.) and transferred into the climate chamber housing the wind tunnel where they had at least 10 min to adapt to the conditions. Male moths were individually presented to the odour source on a stand (height 30 cm) placed at the downwind end of the wind tunnel. Moth behaviour was scored for (1) no activation, (2) activation, (3) take off, (4) upwind flight, (5) passing the midline of the wind tunnel and (6) contacting the source by means of the OBSERVER software package (version 5.0, Noldus Information Technology, Wageningen, Netherlands). The behavioural response of male moths was recorded for 2 min and males that were not activated within 1 min or that landed on a wall (min. 5 s rest) were removed from the wind tunnel.

2.9. Statistical analysis

The responses of male *L. botrana* to different treatments were compared by fitting a generalised linear model (GLM) with a logit link function (logistic regression) to the responses that were assumed to be binomially distributed, using the statistical package R (Version 2.4.1). Analysis of deviance based on the asymptotic χ^2 distribution was used to test whether the flight responses were significantly dependent on the odour sources ($P < 0.05$). When the GLM was significant ($P < 0.05$) multiple comparisons (R-package: Multcomp) were made using Tukey-contrasts.

3. Results

3.1. Antennogram responses of L. botrana males to host plant headspace extract constituents

In a first step, the headspace extracts were analysed for host plant volatile constituents that repeatedly elicit similar EAG responses from grapevine moth male antennal preparations. A comparison of the headspace profiles showed that 14 EAG active plant volatiles co-occurred in at least three of the five host plant headspace extracts (Figs. 1 and 2). These commonly occurring plant volatiles were (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, benzaldehyde, methyl salicylate, 1-hexanol, (*Z*)-3-hexenol, 1-octen-3-ol, limonene, 6-methyl-5-hepten-2-one, (*E*)-β-caryophyllene, (*E*)-β-farnesene, (*E,E*)-α-farnesene (Table 1). Two of the 14 chemostimulants could not be identified. Headspace extracts of wild grapevine male and female flowers differed for six constituents that elicit EAG responses in *L. botrana* males. Sufficient amounts of product to elicit EAG responses to benzaldehyde, (*E,E*)-α-farnesene and methyl salicylate were only found in wild grapevine female flowers whereas (*Z*)-3-hexenol, limonene and one unknown constituent were only present in the wild grapevine male flower headspace extract. No entry for a product in a particular plant extract on Table 1 does not necessarily mean its absence from that extract series, for besides being absent the product could have been present at an insufficient quantity to elicit an EAG response.

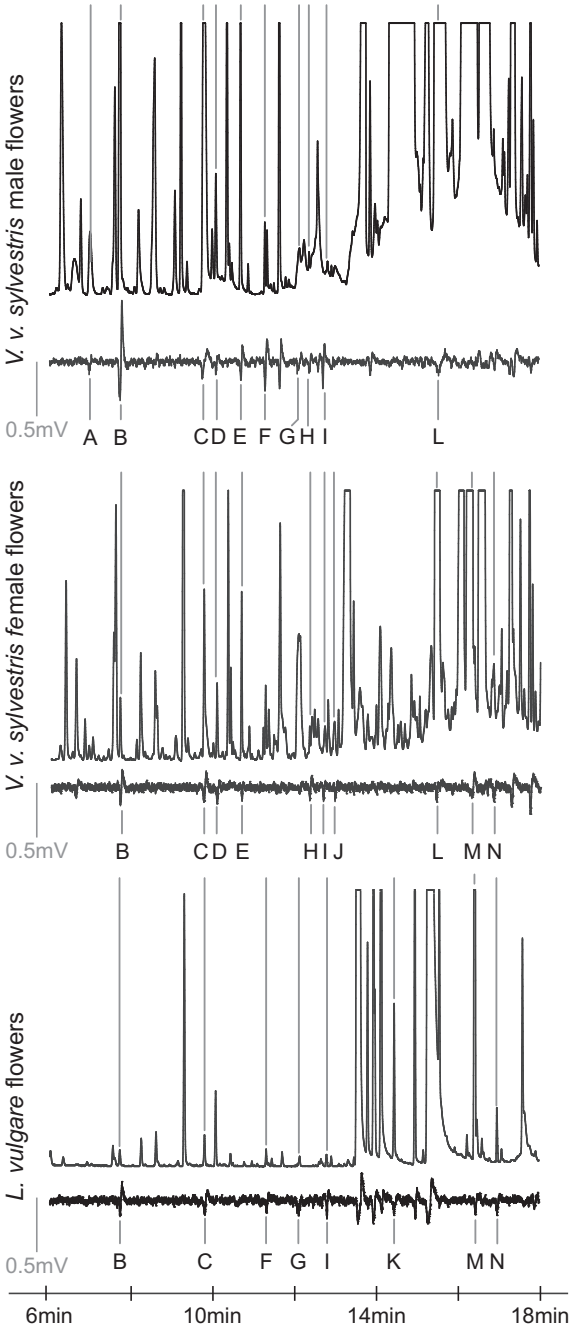

Fig. 1. Gas chromatography (GC) linked electroantennogram (EAG) responses of male *L. botrana* to constituents of the headspace extracts of *Vitis vinifera sylvestris* male flowers, *Vitis vinifera sylvestris* female flowers and *Ligustrum vulgare* flowers. Lettered constituents were found to elicit EAG responses in at least three out of the five different headspace collections tested: A limonene, B (*E*)-2-hexanal, C (*Z*)-3-hexenyl acetate, D 6-methyl-5-hepten-2-one, E 1-hexanol, F (*Z*)-3-hexenol, G unknown, H 1-octen-3-ol, I unknown, J benzaldehyde, K (*E*)-β-caryophyllene, L (*E*)-β-farnesene, M (*E,E*)-α-farnesene and N methyl salicylate. The GC is the upper trace and the EAG response is the lower trace for each analysis.

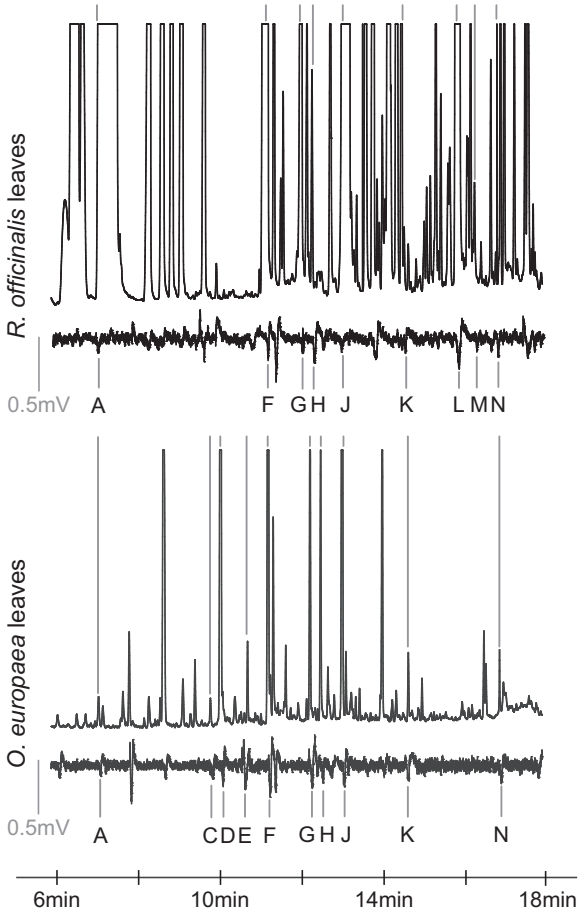

Fig. 2. Gas chromatography (GC) linked electroantennogram (EAG) responses of male *L. botrana* to constituents of the headspace extracts of *Rosmarinus officinalis* leaves and *Olea europaea* leaves. Lettered constituents were found to elicit EAG responses in at least three out of the five different headspace collections tested: A limonene, C (*Z*)-3-hexenyl acetate, D 6-methyl-5-hepten-2-one, E 1-hexanol, F (*Z*)-3-hexenol, G unknown, H 1-octen-3-ol, J benzaldehyde, K (*E*)-β-caryophyllene, L (*E*)-β-farnesene, M (*E,E*)-α-farnesene and N methyl salicylate. The GC is the upper trace and the EAG response is the lower trace for each analysis.

3.2. Behavioural response of grapevine moth males to plant volatiles

Flight responses in a wind tunnel showed that the host plant volatiles (*E*)-β-caryophyllene, 1-hexanol, (*Z*)-3-hexenyl acetate and 1-octen-3-ol significantly increase the behavioural response of *L. botrana* males to the female sex pheromone for the entire flight and had the strongest effects on the attractiveness of the pheromone among ten host plant volatiles tested (von Arx et al., in preparation). Based on this and the findings from the GC–EAG analyses of the host plant headspace extracts presented here, (*E*)-β-caryophyllene, 1-hexanol, 1-octen-3-ol and (*Z*)-3-hexenyl acetate were chosen to test the hypothesis that plant compounds presented singly or in mixtures could serve to guide grapevine moth males to host plants. (*E*)-β-caryophyllene, 1-hexanol and (*Z*)-3-hexenyl acetate released singly at 10,000 pg/min attracted grapevine moth males with 11.3%, 10.0% and 13.3% of the males contacting the odour source. These three compounds significantly increased the number of males responding for all behavioural elements compared to the control (Fig. 3A). Significantly more males were activated by (*E*)-β-caryophyllene (47.5%) than by 1-hexanol (28.3%) and 1-octen-3-ol (25.4%) at 10,000 pg/min. 1-octen-3-ol had the lowest effect on grapevine moth male behaviour released at 10,000 pg/min, significantly increasing only the initial steps of the flight behaviour (activation, take off and upwind flight). At the 100-fold lower release rate of 100 pg/min, a persistent behavioural effect of the host plant volatiles was observed on male *L. botrana*: 1-hexanol and (*Z*)-3-hexenyl acetate significantly increased the number of males responding under all behavioural elements from activation to source contact, and (*E*)-β-caryophyllene significantly increased the number of males responding under all behavioural elements except for source contact (Fig. 3B).

Table 1
Kovats indices (KI) of identified host plant constituents eliciting electroantennogram responses from *L. botrana* males and present in at least three different extracts based on gas chromatography linked electroantennogram recordings and gas chromatography linked mass spectrometry analyses. Criteria for identification of host plant volatiles were matching mass spectra and matching Kovats indices.

	V. vinifera sylvestris ♂ flowers	*V. vinifera sylvestris* ♀ flowers	*Ligustrum vulgare* flowers	*Rosmarinus officinalis* leaves	*Olea europaea* leaves	KI of plant extract constituents in GC–MS	KI of standards in GC-MS
Ester							
C (*Z*)-3-hexenyl acetate	1323	1322	1320		1322	1323	1324
Aldehydes							
B (*E*)-2-hexenal	1216	1215	1211			1214	1211
J benzaldehyde		1501		1503	1503	1502	1505
Aromatic compound							
N methyl salicylate		1775	1772	1772	1780	1778	1781
Aliphatic alcohols							
E 1-hexanol	1360	1361			1355	1354	1358
F (*Z*)-3-hexenol	1383		1383	1382	1378	1380	1380
H 1-octen-3-ol	1445	1445		1448	1447	1443	1446
Terpenes							
A limonene	1191			1195	1192	1192	1193
D 6-methyl-5-hepten-2-one	1338	1337			1340	1342	1336
K (*E*)-β-caryophyllene			1583	1586	1583	1589	1588
L (E)-β-farnesene	1672	1668		1663		1668	1661
M (*E,E*)-α-farnesene		1740	1739	1745		1743	1741
G unkown	1437		1434	1435	1434	–	–
I unkown	1478	1479	1476			–	–

1-octen-3-ol again affected grapevine moth male behaviour the least, significantly enhancing only male activation.

In subsequent experiments we studied the behavioural responses of grapevine moth males to combinations of the four compounds tested singly. In an attempt to admix the products at a ratio that approximates a natural blend, headspace volatiles from vine plants (*V. vinifera* cv. Solaris) were collected on an adsorbent and analysed by thermal desorption linked to GC–MS. The mass selective detector responses showed that (*E*)-β-caryophyllene, 1-hexanol, (*Z*)-3-hexenyl acetate and 1-octen-3-ol were present at a ratio of 1000:179:118:51 in these headspace samples (Table 2). Accordingly, the 4-component mixture was formulated at a ratio of 1000:200:100:50. A mixture of (*E*)-β-caryophyllene (10,000 pg/min), 1-hexanol (2000 pg/min), (*Z*)-3-hexenyl acetate (1000 pg/min) and 1-octen-3-ol (500 pg/min) attracted significantly more grapevine moth males than (*E*)-β-caryophyllene released on its own at 10,000 pg/min (Fig. 4). Hereafter, release rates refer to the content of (*E*)-β-caryophyllene in the mixture. A 100-fold decrease of the release rate (100 pg/min) of this 4-component mixture led to a significant decrease in the number of activated males and males that took off. The levels of responses under other behavioural elements were also reduced but not significantly. Additive effects of the three secondary host plant compounds with (*E*)-β-caryophyllene released at 100 pg/min were no longer significantly different from (*E*)-β-caryophyllene alone. A subtractive bioassay was used to identify possible key stimuli in the 4-component mixture. The 4-component blend (A) released at 10,000 pg/min elicited oriented upwind flights and close in on the odour source in 38% and 36% of males tested, respectively (Fig. 5). Blend A was arbitrarily split into blends B through D. Omission of (*E*)-β-caryophyllene (B) resulted in a significant reduction in the number of grapevine moth males engaging in upwind flights and closing in on the source. Presenting the main host plant volatile component (*E*)-β-caryophyllene alone (C) led also to a significant decrease in males responding under both behavioural elements. However, a 2-component blend consisting of (*E*)-β-caryophyllene and 1-hexanol (D) was as attractive as the complete 4-component blend (A) eliciting oriented upwind flights and close in by males on the odour source in 39% and 36% of the males tested, respectively.

4. Discussion

Male *L. botrana* show EAG responses to host plant volatiles that include aliphatic esters, aldehydes, and alcohols, aromatic compounds and terpenes. Four of these, (*Z*)-3-hexenyl acetate, 1-octen-3-ol, 1-hexanol and (*E*)-β-caryophyllene, elicit oriented upwind flights when presented singly and in mixtures in a wind tunnel. We show that the release rate of single plant compounds needed to elicit activation and an upwind response in grapevine moth males was as low as 100 pg/min. This is within the range of the optimal release rate of 10 pg/min of the main pheromone component (*E,Z*)-7,9-dodecenyl acetate needed to attract male *L. botrana* in a wind tunnel (Witzgall et al., 2005). Anfora et al. (2009) have determined headspace emission rates of flowers from two vine cultivars over different phenological stages and frequently found (*E*)-β-caryophyllene and 1-octen-3-ol to be released at rates around 100 pg/min. Upwind flights of male moths to host plant compounds presented singly have been documented for other moth species such as *Cydia pomonella* (Ansebo et al., 2004; Coracini et al., 2004; Yang et al., 2004), *Ephestia cautella* and *Plodia interpunctella* (Olsson et al., 2005), but only at high release rates ranging from 10,000–50,000 pg/min. To our knowledge, no previous example is known where male moths are shown to engage in upwind flights to single host plant compounds released in the range of 100 pg/min. Similar response thresholds to host plant volatiles have been recorded for *L. botrana* females: a 10-component host plant volatile blend containing methyl salicylate, 1-octen-3-ol, 2-ethyl-1-hexanol, (*E*)-β-caryophyllene, (*E*) β-farnesene, (*E,E*)-α-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, (*Z*)-linalool oxide (*E*)-linalool oxide, released at 180.3 pg/min (total amount of plant products) attracted grapevine moth females in a wind tunnel (Tasin et al., 2007; Tasin et al., 2006a). In this study we recorded oriented upwind flights by *L. botrana* males to one of these products ((*E*)-β-caryophyllene) and to two other host plant compounds ((*Z*)-3-hexenyl acetate and 1-hexanol) released at comparable levels (100 pg/min). This shows that *L. botrana* males respond partly to the same compounds as *L. botrana* females and that the response to plant volatiles is in the same dose range for the two sexes. Further investigations will probably show up even

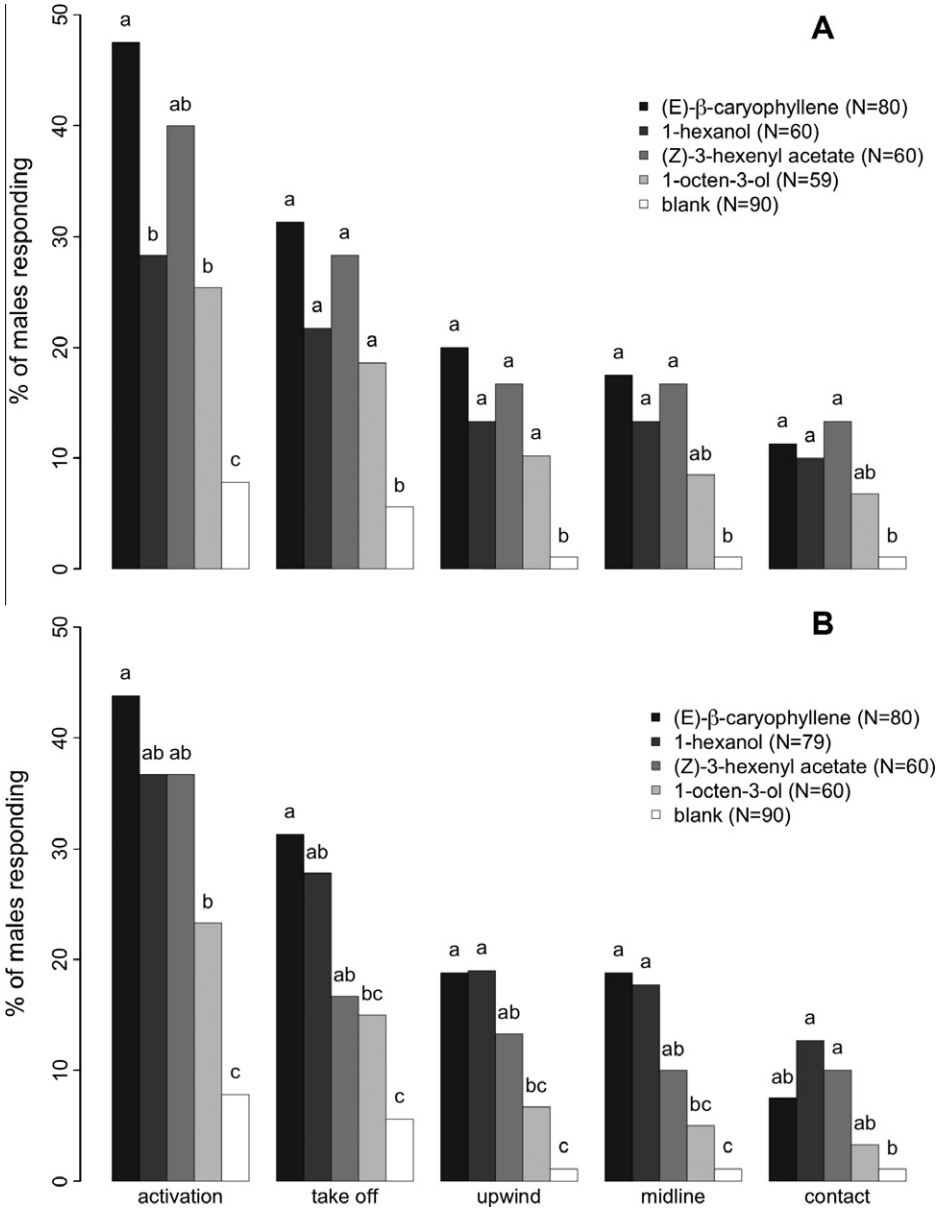

Fig. 3. Behavioural response of grapevine moth males to four plant volatiles released singly at 10,000 pg/min (A) and 100 pg/min (B) and to ethanol alone (blank). Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p < 0.05$); *N* is the number of moths tested for each treatment.

more extensive overlap between the sexes in their ability to detect and respond to host odours. The sites of convergence of antennal olfactory receptor neurons that detect individual host plant volatiles is on glomeruli within the olfactory lobe of the insect brain, and male and female *L. botrana* have been shown to possess very similar numbers of glomeruli (Masante-Roca et al., 2005).

In nature insects are more likely confronted with complex odour mixtures than with single host plant compounds, so it is not surprising that multicomponent blends evoke stronger behavioural responses than single plant components. This has been demonstrated for several moth species in both the laboratory and in the field (Ansebo et al., 2004; Bengtsson et al., 2006; Pinero and Dorn, 2007; Tasin et al., 2007) and in other phytophagous insects (Guerin et al., 1983). Our results confirm this as we found a 4-component plant volatile mixture released at 10,000 pg/min to be significantly more attractive to grapevine moth males than any of the host plant volatiles presented singly. The subtractive bioassays suggest that whereas certain constituents of the 4-component blend are in part behaviourally redundant, (*E*)-β-caryophyllene and 1-hexanol are semiochemicals that seem to play a key role in the suite of compounds studied here from vine plants. Nevertheless these two products may play a more fundamental role in the sensory ecology of *L. botrana* as (*E*)-β-caryophyllene attracts both males (this study) and females (Tasin et al., 2006b) at picogram levels in air, and 1-hexanol serves to attract grapevine moth males as shown here and grapevine moth larvae (Becher and Guerin, 2009).

The composition and complexity of the different headspace extracts analysed in our study showed qualitative and quantitative differences. This is not surprising since the extracts were obtained, on the one hand, from different plant parts with presumably quite different volatile emissions. Tasin et al. (2005) analysed headspace collections of leaves, flowers and fruits of the *L. botrana* host plant *V. vinifera* cv. Chardonnay and found that they differ significantly from one another. On the other hand, the host plants chosen for volatile headspace collections here belong to three different plant families. A comparison of electrophysiologically active compounds

Table 2
Kovats indices (KI), characteristic fragment ions and relative amounts of four host plant volatiles collected on an adsorbent from the headspace of *Vitis vinifera* cv. Solaris plants, thermally desorbed onto a high resolution capillary column and analysed by GC–MS. The proportions of the constituents of a blend of four host plant volatiles used in behavioral tests (column 6) was based on the ratios of the products in the *V. vinivera* headspace.

	KI of plant extract constituents in TDS–GC–MS	KI of standards in GC–MS	Corresponding m/z of constituents	Proportion in headspace	Proportion in synthetic mixture
(*Z*)-3-hexenyl acetate	1318	1324	54, 67, 82	118	100
1-hexanol	1357	1358	56, 69, 84	179	200
1-octen-3-ol	1440	1446	57, 72, 85, 99	51	50
(*E*)-β-caryophyllene	1592	1588	79, 93, 133, 204	1000	1000

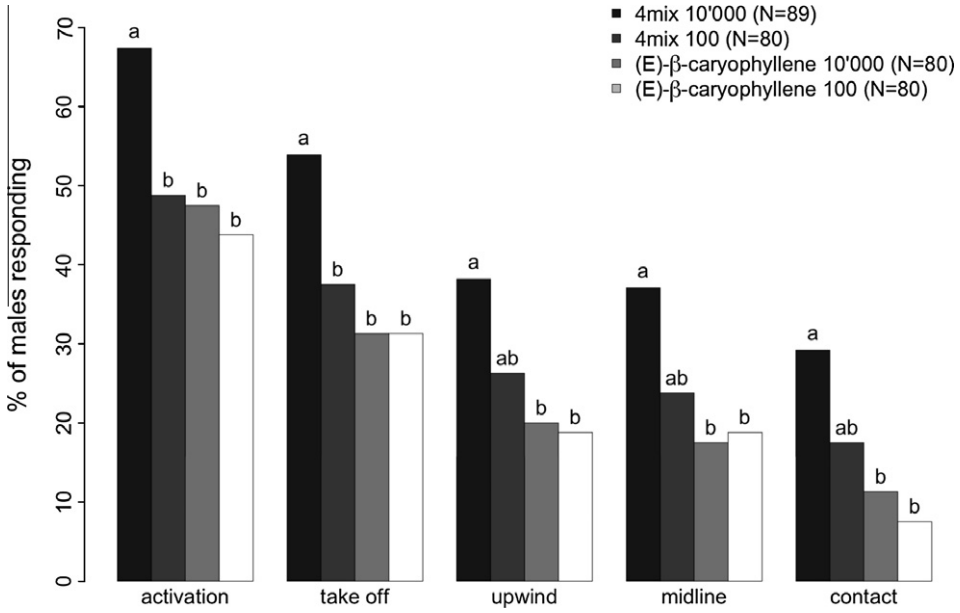

Fig. 4. Responses of *L. botrana* males to a 4-component mixture (4mix, (*E*)-β-caryophyllene, 1-hexanol, (*Z*)-3-hexenyl acetate and 1-octen-3-ol at a ratio of 1000:200:100:50, respectively) released at 10,000 pg/min and 100 pg/min of the main component and to (*E*)-β-caryophyllene alone released at 10,000 and 100 pg/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p < 0.05$); *N* is the number of moths tested for each treatment.

in headspace collections of *V. vinifera* (Vitaceae) and *Daphne gnidium* (Thymelaceae) revealed that six identified compounds were released from both plant species, four compounds were specific for *V. vinifera* and five compounds were found exclusively in *D. gnidium* (Tasin et al., 2010). In this study the odour bouquets of male and female flowers of the dioecious wild grapevine were found to differ for six constituents that elicit EAG responses in *L. botrana* males, four of which were identified. Dioecious plants often show sexual dimorphisms in attractive traits, including scent (Ashman, 2009). Scent variations between sexes can either be quantitative as it was observed in *Silene otites* (Jhumur et al., 2008) or qualitative as observed in *Laurus nobilis* (Flamini et al., 2002), and both factors could account for the variations of limonene, benzaldehyde, methyl salicylate and (*E,E*)-α-farnesene found in our headspace extracts of male and female flowers of wild grapevine. The 14 plant volatiles that elicited EAG responses and that co-occurred in at least three of the five host plant extracts are all commonly occurring plant volatiles emitted by a range of plants (Knudsen et al.,1993; Knudsen et al., 2006). Electroantennogram responses to 10 of these compounds has been reported for males of the sister species *Eupoecilia ambiguella* (Schmidt-Büsser et al. 2011) and to eight of them in *L. botrana* females (Tasin et al., 2005; Tasin et al., 2006a).

The headspace volatile profiles we studied here were considerably different and no constituent that elicited EAG responses occurred in all five headspace extracts. Despite this, populations of *L. botrana* are attracted to and found on *L. vulgare*, *O. europaea* and *R. officinalis* irrespective of their different volatile emission (Galet, 1982; Savopoulo-Soultani et al., 1998). Considering that *L. botrana* is polyphagous and has several generations per year (Bovey et al., 1972; Katerinopoulos et al., 2005; Thiery and Moreau, 2005) this insect needs to cope with plant volatile blends originating from different hosts, from different plant parts and additionally to quantitative and qualitative changes in host plant odour emissions throughout the season (Bengtsson et al., 2001; Tasin et al., 2005). Indeed, it is enigmatic that the first generation flight of *L. botrana* in western Europe occurs when the amount of foliage on the vine is very sparse. This indicates a certain degree of olfactory plasticity necessary to deal with the variability of headspace emissions. Plasticity in *L. botrana* responses to qualitative changes in odour composition was demonstrated in a wind tunnel where mated females were attracted just as well to mixtures of commonly occurring products as to compounds specific to two host plants (Tasin et al., 2010). Plasticity to blend constituent ratios was observed in the oligophagous *Cydia molesta*, with females being attracted to several host plant volatile blends with different concentrations of the constituent benzonitrile (Najar-Rodriguez et al., 2010). There are currently two accepted models concerning host-plant recognition in phytophagous insects: (1) species specific odour recognition (Fraenkel, 1959) and (2) ratio-specific odour recognition (Bruce et al., 2005). We show here that *L. botrana* males are equipped to perceive a broad range of plant volatiles with none of them restricted to *L. botrana* host plants and that a 4-component blend of host plant compounds, mimicking part of what is released by vine plant foliage, attracts grapevine moth males. This suggests that host recognition in *L. botrana* males is probably conferred

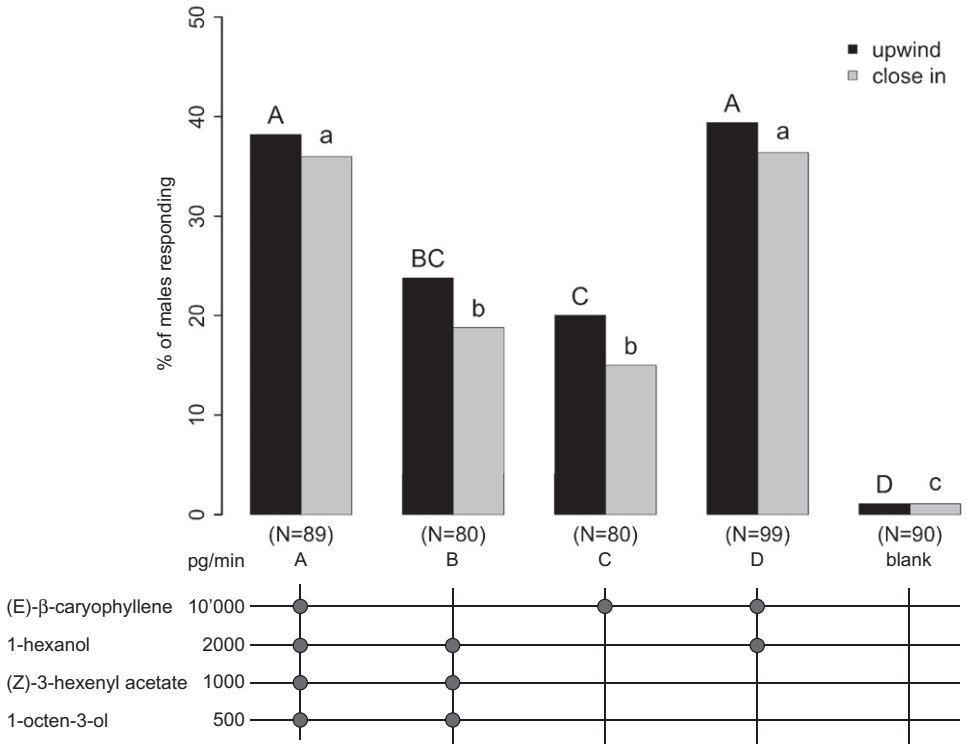

Fig. 5. Behavioural response of *L. botrana* males to (*E*)-β-caryophyllene and to combinations of host plant compounds with and without (*E*)-β-caryophyllene. Percentages of males flying upwind (dark bars) and closing in on the odour source (light bars). Ratios of products in mixtures are based on GC–MS analyses of grapevine headspace volatiles (see Table 2). Columns with the same letter are not significantly different (GLM, $p < 0.05$); *N* is the number of moths tested for each treatment.

through the ratios of compounds released than through products that are unique to a given host plant. This is in accordance with Tasin et al. (2006b) who found experimental evidence in a wind tunnel that appropriate blend ratios are critical in host finding by mated *L. botrana* females.

We have demonstrated that grapevine moth males engage in upwind flight to host plant volatiles presented singly and in mixtures. However, considering the range of host plant volatiles *L. botrana* males can detect with their sensory cells it is highly likely that mixtures containing the host plant attractants described here and/or other plant volatiles would also prove attractive to males. The host plant volatiles eliciting antennal responses and upwind flights in grapevine moth males are not specific to *L. botrana* host plants. Grapevine moth males are affected by very low amounts of plant volatiles, comparable to release rates of the sex pheromone necessary to induce a behavioural response in this moth species. This indicates how plant volatiles may play an essential role in the behavioural ecology of *L. botrana* males. Our results support the hypothesis that grapevine moth males could locate host plants in the absence of the sex pheromone to discern places where the likelihood of encountering females is higher. Furthermore, should the host plant bear a calling female, then her sex pheromone will be accompanied in the air by volatiles from the host plant on which she sits. We have found that individual host plant volatiles serve to enhance the attraction of the sex pheromone for *L. botrana* males (von Arx et al., in preparation), as is the case for *E. ambiguella* – The sister tortricid pest of the vine in Europe (Schmidt-Büsser et al., 2009).

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