

Chemical Ecology

Feeding Volatiles of Larval *Sparganothis pilleriana* (Lepidoptera: Tortricidae) Attract Heterospecific Adults of the European Grapevine Moth

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Abstract

Plants release volatiles in response to caterpillar feeding. These herbivore-induced plant volatiles (HIPVs) attract natural enemies of the herbivores and repel or attract conspecific adult herbivores in a tri-trophic interaction which has been considered to be an indirect plant defense against herbivores. Recently, we demonstrated the attraction of male and female European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) to a blend of phenylacetonitrile and acetic acid, two compounds identified as HIPVs in heterospecific apple-leafroller interactions. The ecological basis of our findings is not clearly understood. Thus, this work was undertaken to investigate HIPVs in the grapevine-leafroller interaction and study the response of heterospecific adults *L. botrana*, to these volatiles. We collected headspace volatiles emitted from uninfested grapevines and grapevines infested with larvae of a generalist herbivore, the grapevine leafroller moth, *Sparganothis pilleriana* (Denis & Schiffermüller), and analyzed them using gas chromatography/mass spectrometry. Infested grape leaves released three compounds (phenylacetonitrile, indole, and 2-phenylethanol) not found from uninfested leaves. Nine different blends, comprising a full factorial set of the three compounds with each blend containing acetic acid, were tested in a field-cage trial. Only lures containing phenylacetonitrile caused a significant increase in trap catches compared to the other lures and blank traps. Electroantennographic tests show that *L. botrana* can detect the compounds. The results confirm our hypothesis that phenylacetonitrile is released during grapevines infestation with herbivores, and attracts adult *L. botrana*.

Graphical Abstract



Key words: caterpillar-induced plant volatile, phenylacetone nitrile, 2-phenylethanol, acetic acid, *Lobesia botrana*

Insect herbivory often changes the quantity or quality of volatiles emitted from plants (Dicke and Baldwin 2010, Hare 2011). Natural enemies of insect herbivores, such as predators and parasitoids, may be attracted to these altered plant odors and this signal can increase their ability to locate their herbivore hosts (Alborn et al. 1997, Yoshinaga et al. 2010). This tri-trophic relationship has been hypothesized as an indirect plant defense strategy that recruits natural enemies to incapacitate the herbivores. The tri-trophic theory also has suggested that some herbivores would benefit by avoiding herbivore-induced odors that attract their predators and parasitoids. Thus, caterpillar feeding odors are reported to repel conspecific adult herbivores (De Moraes et al. 2001, Signoretti et al. 2012). In contrast, odors of other plant species infested with caterpillars can attract conspecific adults (El-Sayed et al. 2016, 2018; Suckling and El-Sayed 2017). Therefore, the advantages of the attraction of conspecific adult herbivores for both the herbivores and the plants are unclear. El-Sayed et al. (2016) suggested that herbivore-induced plant volatiles (HIPVs) signals might indicate a plant of lower resistance, hence more suitable to herbivores. For example, plant resistance has been linked to the higher production of secondary compounds, mainly monoterpenes and other compounds, in response to herbivory (Pare and Tumlinson 1999). An increase in the emission of monoterpenes and other compounds from infested plants could cause lower concentrations of these compounds in the foliage resulting in lower resistance to herbivore feeding and benefit herbivores (Trowbridge et al. 2014). Similarly, an increase in monoterpene release from storm-fallen pines is attractive to bark beetle herbivores probably because this indicates the tree is damaged and less resistant to attack (Byers et al. 1985).

In horticultural ecosystems, apple trees uniquely release several compounds, including acetic acid, benzyl alcohol, phenylacetone nitrile, indole, 2-phenylethanol, and (E)-nerolidol, only when infested by larvae of light brown apple moth (LBAM), *Epiphyas postvittana* (Walker), the eye-spotted bud moth (ESBM), *Spilonota ocellana* (Denis & Schiffermüller), the oblique-banded leafroller (OBLR), *Choristoneura rosaceana* (Harris), and the pandemis leafroller (PLR), *Pandemis pyrusana* (Kearfott) (El-Sayed et al. 2016, 2018; Suckling and El-Sayed 2017). El-Sayed et al. (2016) found that a binary blend of either the three HIPVs, phenylacetone nitrile and acetic acid, or 2-phenylethanol and acetic acid, attracted a significant number of conspecific male and female adult LBAM in New Zealand. Further investigation with other leafrollers (Tortricidae) in North America, including the ESBM, OBLR, and PLR revealed similar responses. Male and female adults of ESBM were most attracted to a blend of phenylacetone nitrile and acetic acid while male and female adults of OBLR and PLR were most attracted to a blend of 2-phenylethanol and acetic acid (El-Sayed et al. 2016, 2018; Suckling and El-Sayed 2017). In the same trials, the general predator, the common green lacewing, *Chrysoperla plorabunda* (Fitch) was also attracted to a ternary blend of phenylacetone nitrile and 2-phenylethanol + acetic acid (El-Sayed et al. 2018).

Recently, the caterpillar-induced plant volatiles from the apple-leafroller system were tested in two vineyards in Spain and Hungary for their attractiveness to the European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Tortricidae: Olethreutinae) (El-Sayed et al. 2019). As in other Tortricinae species, a binary blend of phenylacetone nitrile + acetic acid attracted significantly more male and female *L. botrana* to traps than acetic

acid or blank lures. However, in this work, the ecological basis for the attraction of *L. botrana* to HIPVs found in the apple-leafroller system was not clear. Therefore, we conducted this present study to determine the HIPVs released from grapevine leaves infested with leafroller larvae. The leafroller selected for HIPV induction was *Sparganothis pilleriana* (Denis & Schiffermüller) whose larvae attack grape leaves and share habitat with *L. botrana*. We analyzed the headspace of healthy grape leaves and grape leaves infested with *S. pilleriana* larvae, and identified three unique compounds produced only by infested leaves. In addition, we conducted field cage trapping trials in conventional vineyards to investigate the direct responses of adult male and female *L. botrana* to HIPVs identified in the grapevine-leafrollers system. The goal is to elucidate the ecological basis underlying the attraction of *L. botrana* to HIPVs in the apple-leafroller system previously reported in El-Sayed et al. (2016), and to compare the similarities in HIPVs between the apple-leafrollers and grapevine-leafrollers systems.

Materials and Methods

Plants and Insects

All volatile collections and cage trials were conducted at the Julius Kühn-Institute (JKI) experimental vineyard in Siebeldingen, Germany. Grape leaves naturally infested with *S. pilleriana* were chosen based on visual observation prior to volatile collection. Plants infested with at least one or more larvae were selected for sampling. Healthy leaves were selected from uninfested neighboring plants. Infestations were characterized by the presence of feeding shelters either by the roll in a single leaf or two leaf surfaces sealed together by larval webbing. Infestation was confirmed by the presence of the larvae in the feeding shelter.

Chemicals

The chemical purity of the compounds used in the field experiments was as follows: Glacial acetic acid (99%), phenylacetone nitrile (99%), 2-phenylethanol (99%), and indole (99%). Glacial acetic acid was stored at ambient temperature while all other compounds were stored at -20°C until used. All compounds were purchased from Sigma-Aldrich (MO).

Air Entrainment of Volatiles Emitted from Grapevines Uninfested and Infested with Grapevine Leafroller Larvae

Volatile collections from infested grapevines ('Riesling') with grapevine leafroller larvae and uninfested grapevines were conducted in the field in Siebeldingen, Germany, using a dynamic headspace collection method, where air containing the odor was absorbed by a sorbent filter that was then extracted with hexane. Intact tree branches ($n = 6$) with either grapevine leaves infested with leafroller larvae or uninfested leaves were enclosed in a polyester oven bag (Glad NZ, 35 cm × 50 cm). A charcoal-filtered airstream was pulled over the enclosed leaves at 0.5 L/min, and the headspace volatiles were collected for 24 h on an adsorbent filter containing 50 mg of Tenax-GR 35/60 (Alltech Associates Inc.) in a 60 mm long × 6 mm diameter glass tube. For the collection of control samples, a charcoal-filtered airstream was pulled through an empty oven bag in the same vineyards. Samples were sealed

and shipped in dry ice to The New Zealand Institute for Plant & Food Research Limited (PFR) facility for Gas Chromatography/Mass Spectrometry (GC/MS) analysis. At the PFR laboratory, the Tenax filters were extracted with 0.5 ml of n-hexane (AnalaR BDH, Laboratory Supplies, Poole, United Kingdom). A subsample of 100 µl was reduced to 10 µl at ambient temperature under a stream of argon and 1 µl of the concentrated extract was injected in the GC/MS. Six samplings of volatile collections were performed on each of either infested leaves, uninfested leaves, or empty control bags. Different plants were used for each sample.

Analysis of Air-Entrainment Samples by GC/MS

The concentrated extracts of the air-entrainment samples were analyzed using GC/MS (Varian 3800 GC coupled to a Varian 2200 MS). Helium was used as the carrier gas (1 ml/min), and injections were splitless for 0.6 min. Transfer line and ion trap temperatures were 250 and 180°C, respectively. The GC injector temperature was set at

220°C, and the oven ramp was 40°C for 2 min, 4°C/min to 240°C, hold for 10 min, and then 15°C/min to 260°C, using a VF-5 MS capillary column (30 m × 0.25 mm inner diameter × 0.25 µm film) and a polar (30 m × 0.25 mm i.d. × 0.5 µm) VF23-MS capillary column; Varian, Inc., Walnut Creek, CA). The spectra were recorded at an ionization voltage of 70 eV over a mass range mass-to-charge (*m/z*) of 20–499. Kovats retention indices (KI) were calculated for the compounds (El-Sayed 2020; Table 1). Structural assignments of the compounds were made by comparing their mass spectra with MS library (NIST 2017), as well as by comparison with Kovats retention indices published in the literature (El-Sayed 2020). Identification of some volatiles was confirmed by comparison with authentic samples (Table 1).

Field Cage Experiment

Lobesia botrana in the experimental vineyards of JKI in Siebeldingen are managed with mating disruption for decades and this has resulted

Table 1. Relative amounts (% ± SEM) of the compounds identified in the headspaces of grapevines infested with *Sparganothis pilleriana* larvae and uninfested grapevines

Compound	RI value ^a	% ^b	
		Infested	Uninfested
cis-3-Hexenyl acetate ^c	994	0.32 (0.07)	0.20 (0.12)
Hexyl acetate ^c	1,002	0.05 (0.02)	0.03 (0.03)
(-)-Limonene	1,012	0.13 (0.05)	0.15 (0.08)
cis-Ocimene	1,029	23.77 (3.90)	39.55 (9.23)
trans-β-Ocimene	1,040	47.01 (35.93)	48.83 (40.64)
Linalool	1,095	1.20 (0.55)	1.39 (1.16)
2-Phenylethanol ^c	1,115	0.80 (0.35)	nd
DMNT ^c	1,113	1.64 (1.39)	2.41 (1.09)
(3E,5E)-2,6-Dimethyl-1,3,5,7-octatetraene	1,119	0.42 (0.25)	0.94 (0.77)
(4E,6E)-2,6-Dimethyl-2,4,6-octatriene	1,126	1.17 (0.53)	1.01 (0.71)
Phenylacetonitrile ^c	1,142	1.06 (0.23)	nd
Ethyl benzoate ^c	1,165	0.04 (0.01)	0.01 (0.01)
Terpinen-4-ol	1,173	0.03 (0.01)	0.04 (0.04)
(E)-3-Hexenyl butyrate	1,186	0.26 (0.06)	0.13 (0.14)
Methyl salicylate ^c	1,192	0.05 (0.01)	0.03 (0.02)
Hexyl butyrate ^c	1,193	0.43 (0.17)	0.37 (0.33)
α-Terpineol	1,195	0.24 (0.10)	0.25 (0.25)
Decanal	1,206	0.10 (0.01)	0.08 (0.02)
(3Z)-3-Hexenyl 2-methylbutanoate	1,230	0.08 (0.02)	0.04 (0.05)
cis-3-Hexenyl isovalerate	1,236	0.13 (0.04)	0.04 (0.05)
Hexyl isovalerate	1,243	0.05 (0.01)	0.04 (0.05)
Indole ^c	1,288	3.69 (3.10)	nd
(-)-β-Bourbonene	1,378	0.30 (0.09)	0.11 (0.06)
β-elemene	1,390	0.12 (0.04)	0.08 (0.05)
Caryophyllene ^c	1,417	2.84 (0.54)	2.13 (1.20)
Germacrene D ^c	1,429	0.09 (0.02)	0.04 (0.04)
Humulene	1,455	0.72 (0.15)	0.42 (0.31)
(Z)-β-Farnesene	1,470	1.63 (1.69)	0.02 (0.03)
Germacrene-D ^c	1,485	0.85 (0.30)	0.14 (0.16)
(+)-Valencene	1,498	1.32 (1.11)	0.10 (0.11)
(Z,E)-α-Farnesene	1,514	1.87 (1.13)	0.32 (0.39)
α-Farnesene ^c	1,529	6.67 (5.48)	0.57 (0.24)
trans-Nerolidol	1,577	0.30 (0.15)	0.23 (0.16)
Hexyl benzoate	1,594	0.08 (0.04)	0.06 (0.07)
(-)-Caryophyllene oxide	1,597	0.19 (0.08)	0.10 (0.07)

nd: not detected.

^aKovats Retention Index (VF5-MS capillary column).

^bPercentage of total volatiles produced is given as the mean area of the GC peaks followed by the standard error (*n* = 6).

^cCompounds identified using authentic standards.

in a very low population. Therefore, instead of running an open field trial, we have conducted a field cage trial. Phenylacetonitrile, 2-phenylethanol, and indole were tested in the field cage trial. In this study, we could not verify the presence of acetic acid because of the limitations of the technique used to collect headspace volatiles. However, since acetic acid was produced only from the infested apple trees and was critical for the attraction of other related leaf-rollers in our previous work (El-Sayed et al. 2016), we hypothesized it would be also important for this species. The selection of the four compounds phenylacetonitrile, 2-phenylethanol, indole, and acetic acid for the field test was based on chemical analysis conducted in this work and our results from the previous study (El-Sayed et al. 2016). On the other hand, Suckling and El-Sayed (2017) showed that changing the ratio of HIPVs does not affect the response of adult herbivores. Accordingly, in this study compounds were tested at an equal ratio in multicomponent blends.

In the field cage trial, we compared three different lure combinations (PN lure: containing phenylacetonitrile, ID lure: containing indole, 2P lure: containing 2-phenylethanol) for their attractiveness to male and female *L. botrana*. In all, nine lure compositions were formulated, comprising a full factorial set of presence/absence of the three components. Each combination except the no-compound blank also included acetic acid (Table 2). This trial was carried out in an experimental vineyard at JKI site in Siebeldingen, Germany, using three aluminum field cages built over the canopy of two grapevines each (Hoffmann and Doye 2007, 2017). The cage dimensions used in this study were 2 m × 1.5 m × 2 m (L × W × H) and cages were arranged in a triangle, with each cage at least 50 m away from the next (with sides of 50–60 m). For each of four replicate runs, each cage

was set up with three traps placed in a different corner, each loaded with a different lure composition.

The lures tested in each cage for each run were selected according to a resolvable row-column design with “row” for each cage and “corner” for each position, with all lures used in each run. The design (Fig. 1) was Latinised by rows (across replicates) and was generated with CycDesign 5.1 (VSN International Ltd 2013). This trial is a choice test, with each of the nine lures representing a choice. It was not possible to use all nine lures within a cage, so this test is not a true choice between all of the lures, but instead, a choice between the three lures within each cage. At the start of a run, 50 insects (approximately half male) were released into each cage. Delta transparent traps (Trifolio-M GmbH, Lahnau, Germany) with sticky bases were

Table 2. Components used in each of the lures

Lures	Phenylacetonitrile	Indole	2-Phenylethanol	Quantities (µl)			AA (ml)
	PN	ID	2P	PN	ID	2P	
B 1	+	–	–	100	0	0	0.3
B 2	–	+	–	0	100	0	0.3
B 3	–	–	+	0	0	100	0.3
B 4	+	+	–	50	50	0	0.3
B 5	+	–	+	50	0	50	0.3
B 6	–	+	+	0	50	50	0.3
B 7	+	+	+	33	33	33	0.3
B 8	–	–	–	0	0	0	0.3
B 9	–	–	–	0	0	0	0

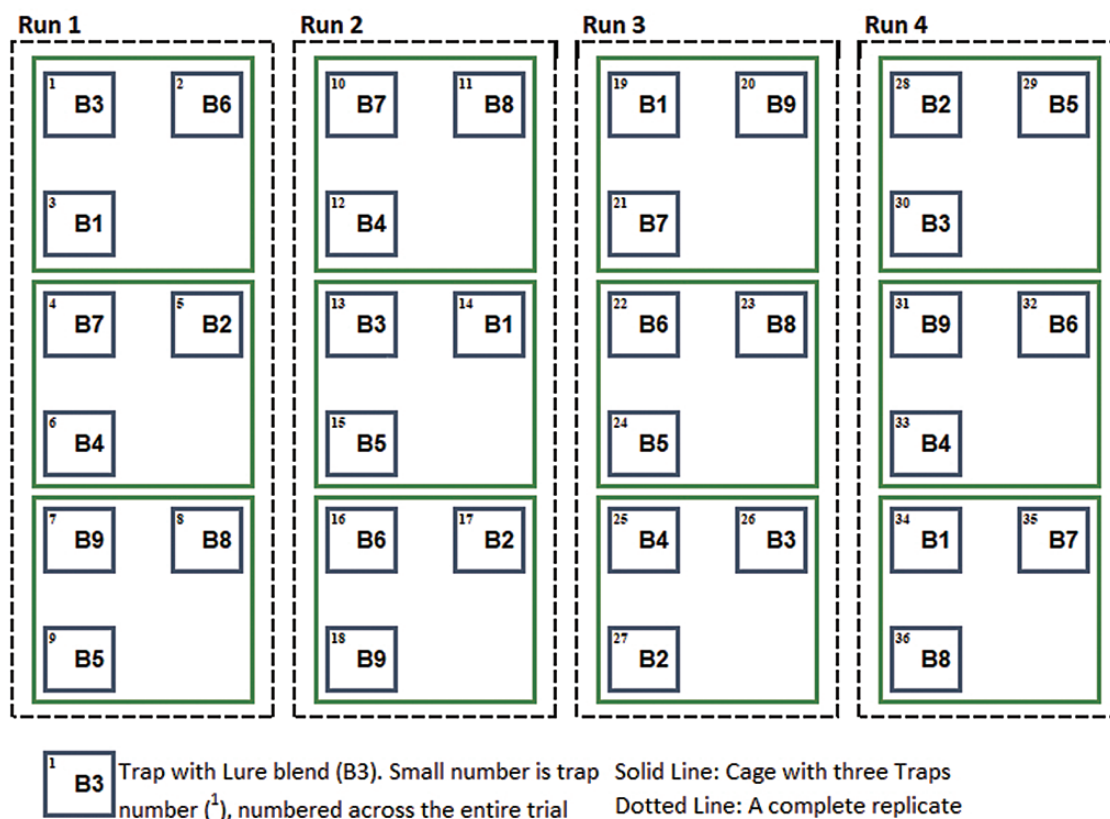


Fig. 1. Diagram showing layout of lures with cages.

Table 3. Estimated percentage catch on traps with each lure type (95% confidence limits)

ID	2P	PN-	%	PN+	%	Others	%
–	–	B8	3.2 (1.9,5.3)	B1	6.5 (4.0,10.1)		
–	+	B3	4.0 (2.4,6.5)	B5	6.0 (3.7,9.4)		
+	–	B2	3.0 (1.8,5.0)	B4	4.7 (2.8,7.5)		
+	+	B6	4.2 (2.5,6.8)	B7	4.3 (2.6,7.0)		
						B9, blank not on trap	0.5 (0.3,0.9) 63.6 (49.6,75.8)

Treatments are ordered to reflect their factorial structure.

used in the experiment. Sticky bases were removed after one week and the number of insects caught on each trap was recorded.

Electroantennographic Bioassay

Synthetic compounds (phenylacetone, 2-phenylethanol, indole, and acetic acid) were diluted in n-hexane, except for indole which was diluted in absolute ethanol. Dilutions were 10-fold, from 10^{-1} to 10^{-5} , which roughly corresponds to 100 μ g, 10 μ g, 1 μ g, and 100 ng/ μ l. Ten microliters of test stimuli were loaded on a piece of filter paper (Whatman #1, 1.5 cm², pre-cleaned with n-hexane). After 30-s solvent evaporation, the filter paper was inserted into a 1-ml disposable plastic pipette tip. Between uses, stimulus cartridges were stored in glass vials closed with screw-cap lids. A given pipette cartridge was not puffed more than eight times and they were disposed off at the end of the day. Antennae were stimulated first with solvent control followed by the test stimuli, with the order of the four odorants was randomized. Male and females 3–5 d of age were immobilized with CO₂ and introduced in a 200- μ l disposable pipette tip with the antennae exposed. Only one antenna per insect ($n = 8$ per sex) was used and each antenna was stimulated with all test odorants.

The head and the base of the antennae were restrained with melted dental wax. Electrodes were made with platinum filaments inserted in drawn-glass capillary tubes that were filled with saline solution (0.2M KCl). One electrode was inserted into the eye (reference electrode) and the other contacted the most distal segments of the antennae (recording electrode). A 0.5 l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation. Each stimulation (i.e., ‘puff’) generated by a stimulus controller (CS-55, Syntech, Germany) was delivered for 0.5 s at 0.2 l/min with an interstimulus interval of at least 30 s. The signal was pre-amplified (10x gain), high-pass filtered at 0.1 Hz, digitized and analyzed (Syntech, Buchenbach, Germany).

Statistical Analysis

Field Cage Experiment

For each cage, the number of insects not caught on the traps was calculated. These numbers were included as a fourth choice for each cage. Thus, the data is multinomial, with ten choices in total. The standard method for multinomial data is a Poisson log-linear model (McCullagh and Nelder 1989; equivalent to the χ^2 test). However, for these data, there were potential random effects, so an extension to the standard model, a Poisson-gamma hierarchical generalized linear model (Chen and Kuo 2001, Lee et al. 2006) with log link was initially used. The potential random effects such as replicate were included with the gamma distribution. The fixed effects (Lure, including factors for the Lure components; and cage total) were included with a Poisson distribution. Inclusion of the cage total leads to the percentage of insects within a cage being assessed rather than the

counts. Random effects were assessed with a χ^2 test of the change in deviance on dropping a term, as implemented in Genstat’s HGRTEST procedure (GenStat Committee 2015). The importance of the lure components (main effects and all interactions) was assessed similarly, with the HGFIXED procedure. Of the random effects, only replicate was found to be important, so it was included in the final model.

Percentages and associated standard errors are technically very difficult to obtain from the multinomial model, particularly when random effects are included. Therefore, the data for each of the lures and those not on a trap were then analyzed using a standard binomial generalized linear model (McCullagh and Nelder 1989) with a logit link and no random effects, and with the dispersion set to that for the multinomial Poisson log-linear model. These models were used to obtain approximate confidence limits for the estimated percentage choosing each lure in a given cage. The predicted percentages and associated confidence limits were obtained on the link (logit) scale, and back-transformed. The estimates for the lures were then rescaled (divided by 3) to be as if all nine lures were run in each cage. These analyses were carried out with Genstat (Payne et al. 2017).

Electroantennographic Bioassay

The maximum negative electroantennographic (EAG) potential (mV) generated by each puff was analyzed. Four-term log-logistic models [drm() in the library drc of R, Ritz et al. 2015] were fitted to the dose–response curve for each individual and compound to estimate the dose at which the curve was at half height (i.e., the expected dose 50 or ED50). ANOVA models (glm() in R [R Core Team 2018]) tested the effect of sex and test compound on maximum EAG response and ED50.

Results

Analysis of Air-Entrainment Samples by GC/MS Volatiles

Analysis of the headspace of uninfested and infested grapevines indicated quantitative and qualitative differences in odor profiles. We identified a total of 33 compounds in the headspace of uninfested grapevines, and 36 compounds in the headspace of infested grapevines (Table 1). The infestation of grapevines with *S. pilleriana* larvae resulted in a change in the ratio of the compounds emitted from infested grapevines (Table 1). Trans- β -ocimene and cis-ocimene were the main compounds representing 87% and 70% of total volatiles produced in the headspace of uninfested and infested grapevines, respectively. The amounts of (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (Z)- β -farnesene, germacrene-D, (+)-valencene, (Z,E)- α -farnesene, and α -farnesene produced from the infested leaves were significantly higher than uninfested leaves (Table 1). In addition to quantitative differences,

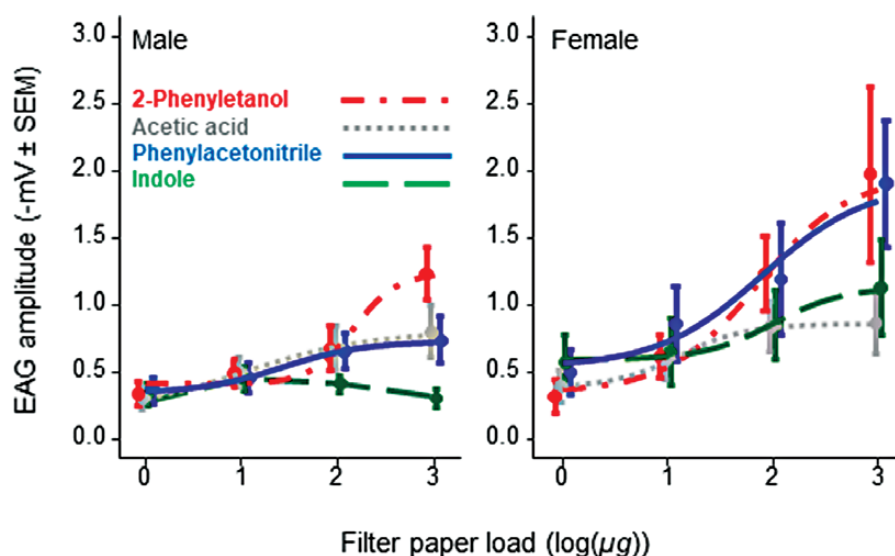


Fig. 2. Electroantennogram responses of *Lobesia botrana* antennae to four odorant stimuli. Symbols and error bars show the mean and SEM (they have been jittered along the x-axis for the sake of clarity). Curves represent log-logistic models fitted to the mean EAG responses, except for indole in males (hatched line) which could not be modeled.

a minor qualitative difference was observed between infested and uninfested plants. Three compounds (phenylacetonitrile, 2-phenylethanol, and indole) were present only in the headspace of infested grapevines.

Field Cage Experiment

The total number of males and females caught (and thus total not caught) in each cage varied noticeably between the four runs, with 40, 59, 63, and 91% not caught in runs 1–4, respectively. Overall, 42% of insects were caught in any trap ($X^2_1 = 34.3$; $P < 0.001$ compared with the 58% not caught). Of those caught on the traps (Table 3), the percentage on the blank trap was very low (0.5%; $X^2_1 = 18.5$; $P < 0.001$ compared with the ~38% caught on traps with lures). Of the lure components, only PN had a noticeable effect ($X^2_1 = 4.0$; $P = 0.046$ when comparing traps with PN to those without), with catches generally higher in the presence of phenylacetonitrile in the blend. In contrast, the presence of ID or 2P did not substantially affect the catch ($X^2_1 = 1.1$; $P = 0.296$ for ID and $X^2_1 = 0.1$; $P = 0.813$ for 2P). In addition, the presence of ID or 2P did not substantially alter the effect of adding PN ($X^2_1 < 0.4$; $P > 0.2$ for all interactions). It was not possible to determine the exact number of males and females caught in each treatment because of the low-quality insects due to the glue on the traps, which make it difficult for sex identification. However, for those that were identified, the ratio of male/female was around 1/2 in all treatments tested, a result to be interpreted with care.

Electroantennographic Bioassay

The response to n-hexane (males = 0.2175 ± 0.077 , females = 0.288 ± 0.131 mean, SEM) was not significantly different than the response to the lowest stimulus concentration (1 μ g; females: $df = 4,33$, $F = 0.612$, $P = 0.657$; males: $df = 4,35$, $F = 0.403$, $P = 0.804$). Average responses to the 1 mg dose of test compounds ranged between 1.97 mV (phenylacetonitrile, females) and 0.3 mV (indole, males; Fig. 2). Female antennae responded more strongly to test stimuli than male antennae (ANOVA, $df = 1,253$, $F = 18.16$, $P < 0.001$), and there were effects of odorant ($df = 3,250$, $F = 2.77$, $P = 0.042$). Sigmoidal functions

could be fitted to all the dose–response curves except for indole in males, which showed an atypical decrease in response as the concentration increased. For the other 3 compounds ED50 did not vary strongly with sex (ANOVA, $df = 1,34$, $F = 0.34$, $P = 0.56$) or odorant (ANOVA, $df = 2,33$, $F = 1.48$, $P = 0.24$). The estimated ED50s for 2-phenylethanol, phenylacetonitrile, and acetic acid were 74.26 ± 26.61 μ g, 78.41 ± 32.44 μ g and 134.41 ± 24.52 μ g, respectively.

Discussion

Qualitative differences in the emission of volatile organic compounds (VOCs) were observed between grapevines infested with grapevine leafroller moth larvae and uninfested grapevines. Infested plants uniquely produce three VOCs, phenylacetonitrile, 2-phenylethanol, and indole that were not present in uninfested plants. Although grapevine and apple belong to two different families, Vitaceae and Rosaceae, respectively, these three HIPVs were also produced by apple trees infested by other leafrollers including OBLR, ESBM, LBAM, and PLR larvae (El-Sayed et al. 2016, 2018). Acetic acid was reported in the headspace of apple trees infested with LBAM larvae (El-Sayed et al. 2016). In the previous studies, when these HIPVs were tested individually, few insects were caught in traps, and only a combination of aromatic HIPVs and acetic acid attracted male and female herbivores (El-Sayed et al. 2016, 2019). For that reason, phenylacetonitrile, 2-phenylethanol, and indole were not tested individually in this study.

In the present study, cages normally used to measure mating disruption efficacy in the field (Hoffmann and Doye 2007, 2017), which are equipped with a defined number of moths, proved to be suitable for the comparative measurement of the attraction effect of volatile substances. In the cage trial conducted in this study, only blends containing phenylacetonitrile caught a significant number of males and females *L. botrana*, than other blends without phenylacetonitrile. In contrast, the presence of 2-phenylethanol, and indole did not significantly enhance the attraction compared to acetic acid alone or synergized or antagonized the response to phenylacetonitrile. Similarly, a binary blend of phenylacetonitrile + acetic acid

was the most attractive blend to ESBM males and females (El-Sayed et al. 2016). In contrast, a binary blend of 2-phenylethanol + acetic acid was the most attractive blend for female OBLR (El-Sayed et al. 2016). In between these two species, a binary blend of phenylacetoneitrile + acetic acid, or 2-phenylethanol + acetic acid were equally attractive to PLR males and females (Suckling and El-Sayed 2017, El-Sayed et al. 2018). Also, similar to our results, a combination of these two compounds + acetic acid did not result in a significant increase in the numbers of males and females captured in these species (El-Sayed et al. 2016, 2018; Suckling and El-Sayed 2017).

About 38% of the total insects released in the field cage were caught in the traps baited with HIPVs compounds. More insects were caught in traps baited with blends containing phenylacetoneitrile. These results provide further support to the previous finding reported by El-Sayed et al (2019) that a blend of phenylacetoneitrile + acetic acid was attractive to male and female *L. botrana*. In addition, this study has demonstrated the production of these compounds from grape leaves infested with leafrollers. This might provide an explanation of the ecological basis underlying the attraction of adults *L. botrana* to these compounds. In our previous work on apple-leafrollers interaction, we have proposed that the attraction of the adult leafrollers to conspecific HIPVs signal might indicate a plant of lower resistance, hence more suitable host plants to herbivores. This study provides a further dimension in insect-plant interactions, that not only can adult herbivores detect conspecific HIPVs but also heterospecific adults can detect these volatiles possibly to gain information about host suitability. Thus, HIPVs in horticultural ecosystems may act as a common signal exploited at multitrophic levels including immature and mature conspecific and heterospecific herbivores and their natural enemies. Because of the physical status of insects caught in sticky bases, it was not possible to sex all insects. However, for those we were able to sex, the ratio was around 1:2 male: female. In the previous studies (El-Sayed et al. 2019), the ratio of males to females was around 50:50, while in other studies the ratio of females was higher than males (El-Sayed et al. 2018).

The catch of males and females *L. botrana* in traps baited with 2-phenylethanol + acetic acid was not significantly different from traps baited with acetic acid alone. Similarly, in our previous trials in Spain and Hungary, the attraction to this blend was not significantly different from traps baited with acetic acid alone (El-Sayed et al. 2019). In contrast, a blend of 2-phenylethanol + acetic acid was attractive to *L. botrana* males and females in field trapping trials (Tasin et al. 2018, Larsson Herrera et al. 2020). Few possibilities might explain the disparity between the results in our study and the other two studies. One possible explanation for the disparity between our study and the other two studies could be geographical differences in the response of various populations of EGVM to HIPVs compounds. For example, strong variation in the response of OBLR across North America has been observed, where HIPVs are very attractive on the west coast but not attractive on the east coast (El-Sayed unpublished data). The 2-phenylethanol detected in the headspace of grape leaves infested with *S. pilleriana* larvae is also produced in grape berries inoculated with various bacteria (Tasin et al. 2018). In grape berries inoculated with microorganisms, 2-phenylethanol is produced in the microbial fermentation process by the transformation of L-phenylalanine (Hua and Xu 2011). 2-Phenylethanol can be further oxidized to phenylacetaldehyde and then further transformed to phenylacetic acid that reacts with ethanol to form ethyl phenylacetate. All these compounds were found in grape berries inoculated with microorganisms (Tasin et al. 2018). On the other hand, we could not find phenylacetaldehyde

and ethyl phenylacetate in grape leaves infested with *S. pilleriana* larvae. In black poplar, *Populus nigra* (L.) leaves, both phenylacetoneitrile and 2-phenylethanol are biosynthesized from the precursor L-phenylalanine by CYP79/CYP71 enzymes with phenylacetaldehyde as an intermediate (Irmisch et al. 2015).

In this study and our previous studies, common HIPVs from apple and grapevines infested with leafroller larvae have been identified, which attracted conspecific, heterospecific adult herbivores. Our current finding, together with our recently published results with other species (El-Sayed et al. 2016, Suckling and El-Sayed 2017), indicates that this phenomenon is widespread among leaf-feeding Tortricidae, some of which have very wide host ranges. Thus, this study provides a new pathway for the identification of kairomones for female moths of major agricultural pests and demonstrates the potential of HIPVs as a new tool in pest management. However, these results raise an important question: what are the advantages for conspecific and heterospecific adult herbivores of being attracted to infested plants? Infested plants might be more suitable oviposition sites for females because these plants may inherently have lower resistance or had resistance lowered by feeding and thus survival rates would be higher for offspring than with healthy uninfested plants (Williams and Myers 1984). There is no doubt that natural enemies, hyperparasitoids, conspecific herbivores, and heterospecific herbivores benefit from exploiting HIPVs in complex heterogeneously structured natural systems.

All HIPVs identified in this study elicited different levels of EAGs from both male and female antennae, with females having higher amplitudes at the higher dosages. Recently, Pérez-Aparicio et al. (2019) investigated the EAG response of male and female *L. botrana* to various host plant volatiles. Methyl salicylate elicited the highest response in females while (*E*)- β -farnesene elicited a higher response than several other plant odorants in both sexes (Pérez-Aparicio et al. 2019). Both compounds were found in both infested and healthy grape leaves in our study. However, the amount of (*E*)- β -farnesene present in the infested grape leaves was significantly higher than in healthy leaves. Future experiments should include these two EAG active compounds, in combination with the active blend identified in this study. The EAG response to HIPVs was much higher in the female compared to the male response. This agrees with the presence of a larger proportion of plant-sensitive sensilla in females than in males (De Cristofaro et al. 2008, Pérez-Aparicio et al. 2019).

The HIPVs reported in this study can be used to monitor the abundance and support control of these important tortricid pests in conventional vineyards. In addition, HIPVs can be used to manipulate natural enemy distributions in IPM of organic vineyards. It is not obvious that the phenomenon of HIPVs attraction of conspecific leaf-feeding herbivores would extend to attraction of heterospecific fruit-feeding frugivores. However, *L. botrana* does feed on different vegetative parts including inflorescences, developing fruit, and mature fruit (Ioriatti et al. 2011). Our findings suggest that attraction of herbivores to HIPVs may be extended across species and subfamily boundaries. In this study, adult *L. botrana* were attracted to binary mixtures of certain HIPV compounds together with acetic acid. We anticipate in the future, a more complex blend that incorporates other compounds will provide further improvements to the current blend. All compounds identified as HIPVs in this study are inexpensive commercially available, which would make area-wide implementation feasible. This kairomone could be used to monitor females within mating disruption treatments and our preliminary trials showed potential application in pest management. Further work with this attractant should involve trap efficiency and active space.

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References Cited

- Alborn, T., T. C. J. Turlings, T. H. Jones, G. Stenhagen, J. H. Loughrin, and J. H. Tumlinson. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276: 945–949.
- Byers, J. A., B. S. Lanne, F. Schlyter, J. Löfqvist, and G. Bergström. 1985. Olfactory recognition of host-tree susceptibility by pine shoot beetles. *Naturwissenschaften* 72: 324–326.
- Chen, Z., and L. Kuo. 2001. A note on the estimation of the multinomial logit model with random effects. *Am. Stat.* 55: 89–95.
- De Cristofaro, A., S. Vitagliano, G. Anfora, G. S. Germinara, M. Tasin, A. Lucchi, C. Ioriatti, and G. Rotundo. 2008. Olfactory cells responding to the main pheromone component and plant volatiles in *Lobesia botrana* (Den. & Schiff.): possible effects on monitoring systems. *IOBC/WPRS Bull.* 36: 245–249.
- De Moraes, C. M., M. C. Mescher, and J. H. Tumlinson. 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410: 577–580.
- Dicke, M., and I. T. Baldwin. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the ‘cry for help’. *Trends Plant Sci.* 15: 167–175.
- El-Sayed, A. M. 2020. The pherobase: database of pheromones and semi-chemicals. (<http://www.pherobase.com>) (accessed 20 November 2020).
- El-Sayed, A. M., A. L. Knight, J. A. Byers, G. J. Judd, and D. M. Suckling. 2016. Caterpillar-induced plant volatiles attract conspecific adults in nature. *Sci. Rep.* 6: 37555.
- El-Sayed, A. M., A. L., Knight, E. Basoalto, and D. M. Suckling. 2018. Caterpillar-induced plant volatiles attract conspecific herbivores and a generalist predator. *J. Appl. Entomol.* 142: 495–503.
- El-Sayed, A. M., A. Sporle, C. Gemenio, J. K. Jósai, G. S. Simmons, and D. M. Suckling. 2019. Leafroller-induced phenylacetone nitrile and acetic acid attract adult *Lobesia botrana* in European vineyards. *Z. Naturforsch. C. J. Biosci.* 74: 161–165.
- GenStat Committee. 2015. GenStat reference manual release 18—part: procedures. VSN International, Oxford, United Kingdom. pp 1351.
- Hare, J. D. 2011. Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annu. Rev. Entomol.* 56: 161–180.
- Hoffmann, C., and E. Doye. 2007. Käfigversuche zur Freilandprüfung der biologischen Wirksamkeit von Pheromonpräparaten zur Paarungsstörung. *Nachr. Dtsch. Pflanzenschutzd.* 59: 227–232.
- Hoffmann, C., and E. Doye. 2017. How to measure mating disruption efficacy of pheromone products for registration purposes in the field? Pheromones and other semiochemicals in integrated production. *IOBC-WPRS Bull.* 126: 59–70.
- Hua, D., and P. Xu. 2011. Recent advances in biotechnological production of 2-phenylethanol. *Biotechnol. Adv.* 29: 654–660.
- Ioriatti, C., G. Anfora, M. Tasin, A. De Cristofaro, P. Witzgall, and A. Lucchi. 2011. Chemical ecology and management of *Lobesia botrana* (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 104: 1125–1137.
- Irmisch, S., P. Zeltner, V. Handrick, J. Gershenzon, and T. G. Köllner. 2015. The maize cytochrome P450 CYP79A61 produces phenylacetaldoxime and indole-3-acetaldoxime in heterologous systems and might contribute to plant defense and auxin formation. *BMC Plant Biol.* 15: 128.
- Larsson Herrera, S., P. Rikk, G. Köblös, M. Szelenyi, M. Bela, T. Dekker, and M. Tasin. 2020. Designing a species-selective lure based on microbial volatiles to target *Lobesia botrana*. *Sci. Rep.* 10: 6512. doi:10.1038/s41598-020-63088-3.
- Lee, Y., J. A. Nelder, and Y. Pawitan. 2006. Generalized linear models with random effects: unified analysis via H-likelihood. Chapman & Hall/CRC Press, London, United Kingdom, pp. 416.
- McCullagh, P. and J. A. Nelder. 1989. Generalized linear models. Chapman & Hall, London, United Kingdom, pp. 511+xix.
- [NIST] National Institute of Standards and Technology. 2017. NIST/EPA/NIH mass spectral library with search program. Data version: NST02. NIST, Gaithersburg, MD.
- Pare, P. W., and J. H. Tumlinson. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121: 325–332.
- Payne, R., D. Murray, and D. Baird. 2017. The guide to the Genstat Command Language (Release 19). VSN International, Hemel Hempstead, Hertfordshire, United Kingdom.
- Pérez-Aparicio, A., L. M. Torres-Vila, and C. Gemenio. 2019. EAG responses of adult *Lobesia botrana* males and females collected from *Vitis vinifera* and *Daphne gnidium* to larval host-plant volatiles and sex pheromone. *Insects* 10: 281.
- R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Ritz, C., F. Baty, J. C. Streibig, and D. Gerhard. 2015. Dose-response analysis using R. *PLoS One* 10: e0146021.
- Signoretto, A., M. Penafior, L. Moreira, N. C. Noronha, and J. Bento. 2012. Diurnal and nocturnal herbivore induction on maize elicit different innate response of the fall armyworm parasitoid, *Campoletis flavicincta*. *J. Pestic. Sci.* 85: 101–107.
- Suckling, D. M., and A. M. El-Sayed. 2017. Caterpillar-induced plant volatiles attract adult Tortricidae. *J. Chem. Ecol.* 43: 487–492.
- Tasin, M., S. Larsson Herrera, A. L. Knight, W. Barros-Parada, E. Fuentes Contreras, and I. Pertot. 2018. Volatiles of grape inoculated with microorganisms: modulation of grapevine moth oviposition and field attraction. *Microb. Ecol.* 76: 751–761.
- Trowbridge, A. M., R. W. Daly, D. Helmig, P. C. Stoy, and R. K. Monson. 2014. Herbivory and climate interact serially to control monoterpene emissions from pinyon pine forests. *Ecology* 95: 1591–1603.
- VSN International Ltd. 2013. CycDesign 5.1 A package for the computer generation of experimental designs. Version 4.0. VSN International Ltd, Hertfordshire, United Kingdom.
- Williams, K. S., and J. H. Myers. 1984. Previous herbivore attack of red alder may improve food quality for fall webworm larvae. *Oecologia* 63: 16–70.
- Yoshinaga, N., H. T. Alborn, T. Nakanishi, D. M. Suckling, R. Nishida, J. H. Tumlinson, and N. Mori. 2010. Fatty acid-amino acid conjugates diversification in lepidopteran caterpillars. *J. Chem. Ecol.* 36: 319–325.