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ORIGINAL ARTICLE

Plant volatiles challenge inhibition by structural analogs of the sex pheromone in *Lobesia botrana* (Lepidoptera: Tortricidae)

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Abstract. Plant volatiles can synergize the response to moth pheromone. Synthetic pheromone analogs, in turn, have the opposite effect in reducing pheromone attractiveness. To determine how these two types of stimuli interact and influence male moth behaviour, we performed wind tunnel experiments on the grapevine moth, *Lobesia botrana*. We noticed that a blend of host plant volatiles [(*E*)-β-caryophyllene, 1-hexanol, (*Z*)-3-hexenyl acetate and 1-octen-3-ol in a 100:20:10:5 ratio] significantly increased the response of males to an optimized blend of sex pheromone [(7*E*,9*Z*)-dodeca-7,9-dienyl acetate (E7,Z9-12:Ac), (7*E*,9*Z*)-dodeca-7,9-dienol (E7,Z9-12:OH) and (*Z*)-9-dodecenyl acetate (Z9-12:Ac)] in a 100:10:2 ratio. However, the response of males to the natural attractant was significantly reduced by two analogs [(9*E*,11*Z*)-tetradeca-9,11-dien-2-one (MK **2**) and [(9*E*,11*Z*)-1,1,1-trifluoro-tetradeca-9,11-dien-2-one (TFMK **3**)], of the major component of the sex pheromone of the insect (E7,Z9-12:Ac). When both stimuli were tested on males at pheromone:analog:plant volatile blend 1:100:1000 ratio, the plant blend offset the inhibitory effect induced by TFMK **3** but not that of MK **2**. Our results show for the first time that under laboratory conditions plant volatiles can prevent inhibition by a pheromone analog.

INTRODUCTION

Pheromones are an important element in integrated pest management (IPM) programs, where many insect pests are successfully monitored and controlled by mass trapping and/or mating disruption (Witzgall et al., 2010). Pheromone detection occurs against a background of plant odours and there is growing evidence that this background can alter pheromone perception (Reddy & Guerrero, 2004; Reinecke & Hilker, 2014; Knudsen & Tasin, 2015). Plant volatiles influence insect behaviour and complement sex pheromone management (Szendrei & Rodriguez-Saona, 2010; Knight et al., 2011). This effect is particularly important under mating disruption conditions, in which plant volatiles, alone or in combination with the sex pheromone, may be more efficient for monitoring than the sex pheromone alone (Knight et al., 2014). In this respect, it is known that plant volatiles synergize insect response to pheromones (Landolt & Phillips, 1997; Reddy & Guerrero, 2004; Deisig et al., 2014), and have the advantage of attracting both sexes, whereas sex pheromone traps attract only males.

The possibility of interfering with chemical communication in insects using pheromone analogs is an interesting approach with potential use in pest management programs (Prestwich, 1987; Renou & Guerrero, 2000; Plettner, 2002). Among the structural analogs, methyl ketones (MKs) and, particularly, trifluoromethyl ketones (TFMKs), in which the acetate group has been replaced by CH_3CO or CF_3CO, respectively, are good disruptants of pheromone perception in a number of lepidopteran species, such as *Spodoptera littoralis* Boisduval (Duran et al., 1993; Rosell et al., 1996), *Plutella xylostella* (L.) (Prestwich & Streinz, 1988), *Thaumetopoea pityocampa* Denis & Schiffermüller (Parrilla & Guerrero, 1994), *Sesamia nonagrioides* (Lefevbre) (Bau et al., 1999; Riba et al., 2001), *Mamestra brassicae* (L.) (Renou et al., 1997), *Ostrinia nubilalis* (Hübner) (Riba et al., 2005), *Bombyx mori* (L.) (Pophof et al., 2000), *Antheraea polyphemus* (Cramer) (Vogt et al., 1985), *Cydia pomonella* (L.) (Giner et al., 2009), *Zeuzera pyrina* (L.) (Muñoz et al., 2011), *Spodoptera frugiperda* Smith (Malo et al., 2013) and *Tuta absoluta* (Meyrick) (Dominguez et al., 2016). In the field, these chemicals induce a significant

decrease in the number of males caught in traps baited with mixtures of the pheromone and the antagonist compared with the pheromone alone (Riba et al., 2001, 2005; Giner et al., 2009). In this regard, there is a remarkable reduction in damage induced by *S. nonagrioides* and *O. nubilalis* in maize fields treated with an analog of the major component of *S. nonagrioides* pheromone (Solé et al., 2008a). However, and based on the effect of plant volatiles on pheromone perception (see above), the presence of plant volatiles in these field experiments may interfere with the inhibitory effect of these antagonists on the response of males.

In this study, we test whether a plant volatile blend that increases the response of males to the pheromone can counteract the inhibitory effect of two pheromone analogs (see below). The work was done on the grapevine moth, *Lobesia botrana* (Denis & Schiffermüller), one of the main pests of grapes in Europe, and recently detected also in Argentina, California and Chile (Ioriatti et al., 2011). This pest can be successfully controlled by mating disruption, although pesticide applications are still the main method of control (Ioriatti et al., 2011). Host plant volatiles attract *L. botrana* females (Ioriatti et al., 2011) and males (von Arx et al., 2011, 2012), and also synergize the response of males to the pheromone (von Arx et al., 2012). The major pheromone component of *L. botrana* is (7*E*,9*Z*)-dodeca-7,9-dienyl acetate (**1**) and as pheromone analogs we have considered the ketones (9*E*,11*Z*)-tetradeca-9,11-dien-2-one (**2**), from now on "the methyl ketone analog or MK **2**", and (9*E*,11*Z*)-1,1,1-trifluoro-tetradeca-9,11-dien-2-one (**3**), from now on "the trifluoromethyl ketone analog or TFMK **3**".

In this paper we present the inhibitory effect of these compounds on the response of males in a wind tunnel and also demonstrate for the first time that this inhibition can be offset by a blend of plant volatiles when mixed with the pheromone in a specific ratio.

MATERIALS AND METHODS

Insects

The laboratory colony of *L. botrana* originated from insects collected in La Rioja, Spain in 2010. Larvae were reared on a modified version of the semi-synthetic diet used by Ivaldi-Sender (1974) under a 16L : 8D photo-regime at 25 ± 1°C. Pupae were separated by sex and placed in 4-L polypropylene containers provided with a cotton ball soaked in a 10% aq. solution of sugar. Adults were collected daily and used when they were 2–4 days old.

Chemicals

The components of the sex pheromone of *L. botrana*: (7*E*,9*Z*)-dodeca-7,9-dienyl acetate (E7,Z9-12:Ac, **1**), (7*E*,9*Z*)-dodeca-7,9-dienol (E7,Z9-12:OH), and (*Z*)-9-dodecenyl acetate (Z9-12:Ac), 11-dodecenyl acetate (11-12:Ac) and (*E*)-9-dodecenyl acetate (E9-12:Ac), with an isomeric purity > 93% were obtained from Pherobank (Wageningen, The Netherlands). The plant volatile blend was prepared by mixing (*E*)-β-caryophyllene, 1-hexanol, (*Z*)-3-hexenyl acetate and 1-octen-3-ol (>98% pure, Sigma-Aldrich, Madrid, Spain) in a 100:20:10:5 ratio according to von Arx et al. (2011).

The methyl ketone analog MK **2** and the trifluoromethyl ketone analog TFMK **3** were prepared in our laboratory (Gago, 2012) and stock solutions of both chemicals consisted of pure compounds in hexane (> 99% purity).

All the treatment blends in this study were prepared by mixing all the ingredients in a vial at the corresponding concentrations, using GC-grade n-hexane as a solvent. From these vials 10 µl aliquots were taken to load the stimulus filter paper dispenser.

Wind tunnel

The wind tunnel consisted of a 150 × 45 × 45 cm (length × height × width) glass cage with a solid white floor and a sliding door on one side. A 30-cm-diameter fan at the upwind end of the tunnel and a 20-cm-diameter exhaust vent at the downwind end created a 0.35 m s^{-1} wind flow of unfiltered room air through the tunnel that was vented outside the building after exiting the tunnel. Temperature inside the tunnel was 24 ± 1°C. The flight tunnel was illuminated from above by fluorescent light bulbs producing 10 lux of white light. Tests were carried out during the first 3 h of the insect's scotophase. Two to 3-day-old males were placed individually in 100 × 20 mm glass tubes with perforated aluminium lids covering both ends and were transferred to the flight tunnel room 30 to 120 min before the beginning of the test. Test odours were applied in 10 µl loads to 10 × 15 mm hexane-rinsed pieces of filter paper (Whatman® No. 1, Sigma-Aldrich, Barcelona, Spain). The filter paper was held by a 30-mm alligator clip and placed in a fume hood for 5–10 min to let it dry before transferring to a clean 20 ml vial, where it remained until tested in the flight tunnel 5 to 180 min later. The glass vial containing the test odour was opened and closed inside the flight tunnel to minimize contamination of the flight tunnel room. The base of the alligator clip was inserted vertically in the slot of a 25 mm binder clip, itself fixed to a 70 mm diameter aluminium metal plate located on top of a 25 cm tall metal-wire platform (0.5 cm mesh). The filter paper's flat surface faced the wind flow in order to obtain a sufficiently turbulent odour plume. Two to 4 males were flown individually to a filter paper in a given treatment before changing the filter paper for a new one and a different treatment. At the end of a test day a filter paper had been used for 2–8 males (mean = 5.5), so that filter papers were outside of a glass vial and exposed to the wind flow between approximately 8 and 32 min before being discarded. In a given day only one filter paper was used for each treatment. Males were used only once. The different treatments in each experiment were tested in random order for several days until the number of replications per treatment was ≥ 55 (for the exact N of each experiment see below). After placing the odour stimulus on the upwind platform, the male cage was placed on top of a metal-wire platform similar to the one used for the odour source 1.5 m downwind. The aluminium lids were removed and the following behaviour of the male recorded: taking flight, upwind zig-zagging oriented flight to the stimulus, and landing at the stimulus source. Taking-flight indicates that the insect has perceived the stimulus from a relatively long distance, but by itself does not demonstrate motivation to fly towards it. The zig-zagging upwind flight behaviour is an explicit olfactory response that shows not only that the signal has been perceived but that the insect is sufficiently stimulated to fly towards it. Oriented insects may or may not contact the odour source, which depends on further olfactory perception at close range. The time taken by males to engage in these behaviours during the 2-min observation period was also recorded. At the end of the day the interior of the flight tunnel was cleaned with ethanol, all glass and metal utensils were thoroughly rinsed in acetone and oven-dried at 200°C.

Experiments

The following experiments were run in succession as the information obtained from the first was needed to perform the fol-

lowing experiments. Several *L. botrana* pheromone blends are reported in the literature as male attractants in a wind tunnel, so the aim of our first experiment was to compare the activity of some of these blends under our laboratory conditions. A 100:20:5 blend of E7,Z9-12:Ac, E7,Z9-12:OH and Z9-12:Ac producing 80% (Arn et al., 1988), 63% (Gurba & Guerin, 2016), 57% (Witzgall & Arn, 1990) and 35% (El-Sayed et al., 1999) of male contacts in a wind tunnel, was tested under our conditions along with a 100:5:1 blend (70% contacts; El-Sayed et al., 1999) and a third intermediate blend (100:10:2). Each blend was tested at 10, 100 and 1000 ng doses (total blend quantity) to determine an optimal blend and dose for the rest of the tests. The sample size of this experiment was N = 55 for each blend × dose treatment combination. One of the most attractive blends resulting from this experiment (100 ng of the 100:10:2 blend, see Results) was used in the following experiments. In the second experiment the pheromone blend (100 ng) was mixed with MK **2** or TFMK **3** at 1:10 and 1:100 pheromone : analog ratios to determine the inhibitory activity of these analogs of the pheromone response in the wind tunnel. The sample size for the MK **2** and TFMK **3** experiments was N = 68 and N = 60, respectively. To determine the possible synergism of the plant volatile blend on the pheromone response, mixtures of the pheromone : plant volatile blend in 1:100, 1:1000 and 1:10000 ratios (amount of pheromone 100 ng) were also tested. In this experiment the pheromone blend alone (100 ng) and the plant volatile blend alone (100 µg) were considered as controls. The sample size in this experiment was N = 60 for each treatment. To determine if the plant blend counteracted the inhibition of the analogs, two ternary mixtures of pheromone : analog : plant volatile blend in 1:100:100 and 1:100:1000 ratios were tested against the 1:100 pheromone : analog blend. In this last experiment, the 1:100 and 1:1000 pheromone : plant volatile blends, the pheromone alone, and the plant volatile alone were used as controls. The sample size in this experiment was N = 130 for the pheromone alone and N = 127 for each of the other treatments.

Statistical analysis

The percentage of males that responded was analyzed using generalized linear models (GLM), the "glm" function and a binomial family link in the package "lm4" of R (R Development Core Team, 2015). A different model was run for each experiment and behavioural category. Times to take flight, flying to the source, and contact were not normally distributed so they were analyzed using GLM with a Gaussian family link if normality could not be restored by a log(x + 1) transformation, or with a linear model ("lm") if the transformation restored normality. When the models were significant, multiple or planned comparisons between treatment pairs were performed using the "glht" function in the "multcomp" package of R. Predicted values from the models were obtained using the "predictmeans" package, and these data are shown in the main figures. Raw data and R code scripts are provided as supplementary material (http://hdl.handle.net/10459.1/57678).

RESULTS AND DISCUSSION

Comparison of pheromone blends

Pheromone concentration affected the number of males flying, orienting in the plume and contacting the pheromone source, but blend type did not have any affect (Table 1, Fig. 1). Very few males contacted the source when the dose was 10 ng, whereas ca. 40–50% of the males suc-

Table 1. Parameters of the GLM and LM models analyzing the effect of pheromone, plant volatiles and pheromone analog on percent of males responding and time to respond in each of 5 experiments.

A. Percent response		Take flight					Orient					Contact				
Experiment	Term	DF	Dev	Res. DF	Res. Dev.	Pr(>Chi)	DF	Dev	Res. DF	Res. Dev.	Pr(>Chi)	DF	Dev	Res. DF	Res. Dev.	Pr(>Chi)
1. Effect of pheromone dose and composition	NULL			494	609				494	655				494	595	
	Blend	2	0.704	492	608	0.703	2	3.189	492	652	0.203	2	1.204	492	594	0.548
	Dose	2	90.95	490	517	<0.0001	2	125.17	490	527	<0.0001	2	76.44	490	517	<0.0001
2. Effect of analogs on pheromone	NULL			339	376				339	470				339	428	
	Treatment	4	27.2	335	348	<0.0001	4	18.1	335	452	0.0012	4	38.8	335	386	0.0006
3. Effect of plant on pheromone	NULL			239	181				239	320				239	331	
	Treatment	3	13	236	168	0.0046	3	21.9	236	298	<0.0001	3	17.4	236	314	0.0006
4. Effect of plant on MK	NULL			764	651				764	1031				764	996	
	Treatment	5	63.8	759	587	<0.0001	5	72.7	759	958	<0.0001	5	235	759	761	<0.0001
5. Effect of plant on TFMK	NULL			527	515				527	732				527	637	
	Treatment	5	40.2	522	475	<0.0001	5	62.7	522	669	<0.0001	5	105	522	533	<0.0001
B. Time to respond *		Take flight					Orient					Contact				
Experiment	Term	DF	Sum Sq	Mean Sq	F value	Pr>(F)	DF	Sum Sq	Mean Sq	F value	Pr>(F)	DF	Sum Sq	Mean Sq	F value	Pr>(F)
1. Effect of pheromone dose and composition	Blend	2	7.13	3.57	2.74	0.07	2	1.12	0.56	0.69	0.50	2	0.41	0.20	0.38	0.69
	Dose	2	0.03	0.02	0.01	0.99	2	2.09	1.05	1.60	0.28	2	1.02	0.51	0.95	0.39
	Residuals	339	441.96	1.30			181	146.05	0.81				74.13	0.54		
2. Effect of analogs on pheromone	Treatment	4	5.4	1.35	1.25	0.29	4	5.3	1.33	1.92	0.11	4	1.7	0.42	0.84	0.5
	Residuals	253	272	1.07			175	120.7	0.69			105	52.3	0.499		
		DF	Dev	Res. DF	Res. Dev.	Pr(>F)	DF	Dev	Res. DF	Res. Dev.	Pr(>F)	DF	Dev	Res. DF	Res. Dev.	Pr(>F)
3. Effect of plant on pheromone	NULL			227	74497				110	42495				147	59957	
	Treatment	3	1826.9	223	72670	0.2344	3	4965.4	107	37530	0.004	3	6452.1	144	53505	<0.0001
4. Effect of plant on MK	NULL			648	271545				457	196327				271	123724	
	Treatment	5	2098	643	269447	0.42	5	5981	452	190346	0.015	5	6366	266	117358	0.015
5. Effect of plant on TFMK	NULL			426	217156				265	136340				153	86521	
	Treatment	5	7684	421	209472	0.0095	5	7542	260	128798	0.011	5	6508	148	80013	0.039

* Analyzed with LM (experiments 1 and 2) or GLM (experiments 3–5).

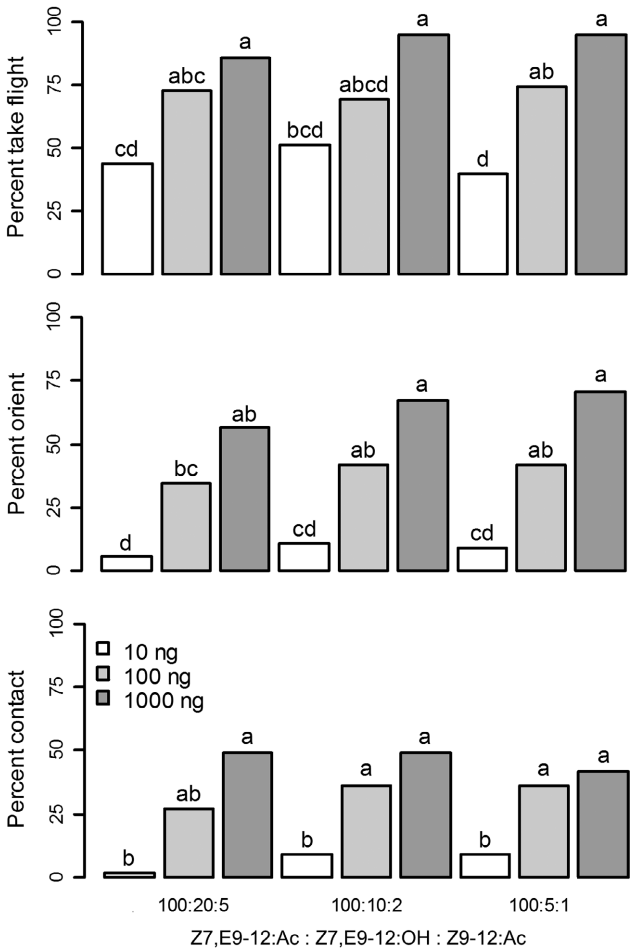

Fig. 1. Attraction of *L. botrana* males to mixtures of the pheromone components Z7,E9-12:Ac, Z7-E9-12:OH, Z9-12:Ac in different ratios at 10, 100 and 1000 ng doses in a wind tunnel. Percentage of males taking flight, orienting into the plume and contacting the pheromone source are the predicted values of GLM models. Different letters indicate significant differences among treatments (Multiple pairwise comparisons with Tukey's test, P < 0.05).

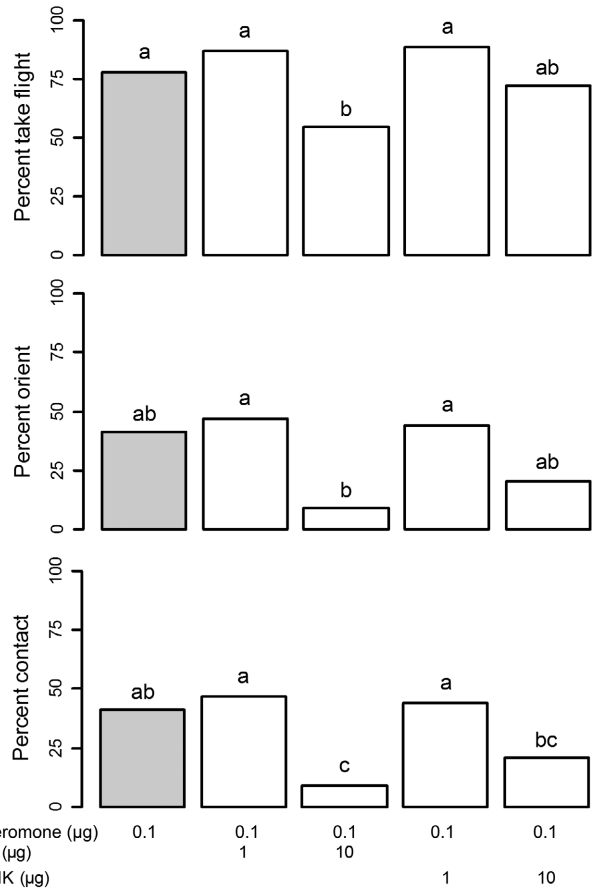

Fig. 2. Effect of analogs MK 2 and TFMK 3 on the attraction of *L. botrana* males to mixtures with the pheromone in 10:1 and 100:1 analog : pheromone ratio in a wind tunnel. Percentage of males taking flight, orienting and contacting the pheromone source are the predicted values of GLM models. Different letters indicate significant differences among treatments (Multiple pairwise comparisons with Tukey's test, P < 0.05).

cessfully made contact at 100 and 1000 ng doses of the 100:10:2 and 100:5:1 blends. No significant differences, however, were recorded between the last two groups (Fig. 1). The time males took to initiate flight, orient or contact the source was not affected by pheromone type or concentration (Table 1).

None of the three pheromone blends outperformed the others, which disagrees with a previous study in which the 100:5:1 blend was significantly better than the 100:20:5 blend (El-Sayed et al., 1999). We have used the three pheromone components that are most often cited in the literature, but additional synergistic compounds have also been reported (Witzgall & Arn, 1990; El-Sayed et al., 1999; Witzgall et al., 2005). Therefore, we compared the 100:10:2 blend with a blend containing two additional compounds (11-12:Ac and E9-12:Ac, Pherobank, The Netherlands) in a final ratio of 100:10:2:20:2. These compounds are known to enhance male responses when tested in a similar ratio (100:5:10:10:1) (El-Sayed et al., 1999). However, we found no significant differences between the 3-component and the 5-component blends (N = 101 and 99 males, respectively): Percentages of taking flight were 99% in both, oriented flight 70 and 72%, respectively, and contact 63 and 58%, respectively. The difference between our study and that of El-Sayed et al. (1999) could be the use of different methods of releasing the pheromone (Girling & Cardé, 2007) or different moth populations. Based on these results, we chose the 100:10:2 blend of E7,Z9-12:Ac: E7,Z9-12:OH:Z9-12:Ac at the 100 ng dose as the optimal test blend for the remaining experiments.

Effect of pheromone analogs

When mixed with the pheromone both pheromone analogs decreased the percentage of males responding to the pheromone at the highest dose tested (1:100, pheromone : analog ratio) (Table 1, Fig. 2). MK **2** had a more pronounced effect than TFMK **3** because it decreased all phases of the response, whereas TFMK **3** only reduced source contact. This was probably due to a different mode of action of both compounds, as discussed later. Intrinsically, the analogs had no effect on the time to take flight, orientation or contact (Table 1).

When mixed with the pheromone in 1:100 pheromone : analog ratio, the inhibition of the response of *L.*

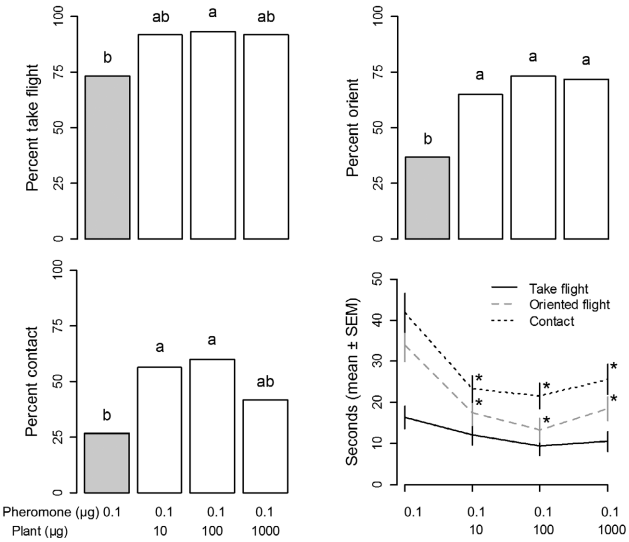

Fig. 3. Effect of a blend of plant volatiles on the attraction of *L. botrana* males to a source baited with pheromone : plant mixtures in 100:1, 1000:1 and 10000:1 ratio in a wind tunnel. Percentages of males taking flight, orienting and contacting the source, as well as the delay time for all these behaviours, are shown as the predicted values of GLM models. Different letters indicate significant differences among treatments (Multiple pairwise comparisons with Tukey's test, P < 0.05). For the time to respond (bottom right) asterisks indicate significant differences with the response to the pheromone (planned contrast, P < 0.05).

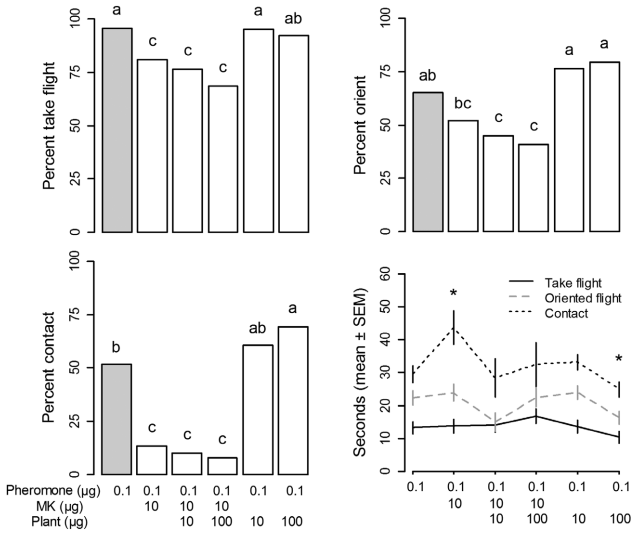

Fig. 4. Behavioural responses of *L. botrana* males flying towards a source baited with mixtures of pheromone : MK 2 : plant volatiles in several ratios relative to the pheromone in a wind tunnel. Percentage of males taking flight, orienting and contacting the source, as well as the delay time for all behaviours are shown as the predicted values of the GLM models. Different letters indicate significant differences among treatments (Multiple pairwise comparisons with Tukey's test, P < 0.05). For the time to respond (bottom right) asterisks indicate significant differences with the response to the pheromone (planned contrast, P < 0.05).

botrana elicited by TFMK **3** was relatively weaker than that of other TFMK analogs on other moth species. Thus, TFMK analogs of the pheromones of *S. nonagrioides* and *C. pomonella* inhibit pheromone response at 1:1 pheromone : analog ratio (Bau et al., 1999; Giner et al., 2009), whereas for *O. nubilalis* the corresponding analog was active at the minimum ratio of 1:5 (Riba et al., 2005; Solé et al., 2008b). In the processionary moth *T. pityocampa*, a structural TFMK analog, in which the acetate group of the pheromone was replaced by the CH_2COCF_3 group, a 5% blend with the pheromone decreased the close approach and source contact behaviour (Quero et al., 1995). The effect exerted by TFMK analogs was also evident when they were present in the proximity (1 cm apart) of a calling female of the leopard moth *Zeuzera pyrina* (L.) with 1 µg being sufficient to induce a highly erratic male flight to the source (Muñoz et al., 2011). With regard to the effect of MK analogs on behavioural responses, contradictory reports have appeared in the literature. Thus, while some chemicals exert a remarkable inhibition of male behavioural responses to the pheromone in a wind tunnel, e.g. in *Heliothis virescens* (F.) (Albans et al., 1984) and *O. nubilalis* (Solé et al., 2008b), other reports indicate modest electrophysiological inhibitory activity, as in the turnip moth *Agrotis segetum* Denis & Schiffermüller (Liljefors et al., 1984). In the processionary moth, the MK analog blocked the electroantennogram (EAG) responses after pre-exposure of males to vapour of the chemical (Parrilla & Guerrero, 1994).

There are few studies on the activity of MK analogs in the field. The MK analog of *O. nubilalis* pheromone is a good antagonist of the pheromone when mixed with natural attractant in 5:1 and 10:1 MK : pheromone ratios, although the MK analog of the processionary moth pheromone did not exhibit any inhibitory or synergistic action in comparison to the pheromone. In this case, a modest agonism of the pheromone activity is reported (Parrilla & Guerrero, 1994).

Effect of the plant volatile blend

In the experiments designed to determine the effect of the plant volatile blend, a few males (30%) took flight but none of them displayed an oriented flight or landed on the source (the plant blend alone) (data not shown). However, the plant volatile blend increased the number of males responding to the pheromone (Table 1, Fig. 3). The synergistic effect was also recorded in terms of taking flight at intermediate plant volatile concentrations, in oriented flight at the three plant volatile concentrations, and in source contact except at the 1 mg dose of the volatile blend (Fig. 3). The plant odour did not affect the time to take flight, but the time to orient and contact the pheromone source decreased significantly when it was mixed with the plant odour (Fig. 3).

Although the plant volatile blend did not stimulate oriented flight on its own, it was probably detected by the insect as it stimulated 25% of the males to take flight (the response to a blank stimulus was not tested in this experiment but it is typically less than 5% in this type of experiment (Varela et al., 2011). This same plant volatile blend induced approximately 30% of male contacts in a previous study (von Arx et al., 2011). The disagreement between these studies could result (a) from the use of different methods of releasing pheromone [aerosol in von Arx et al. (2011), and passive evaporation in our case] (Girling & Cardé, 2007),

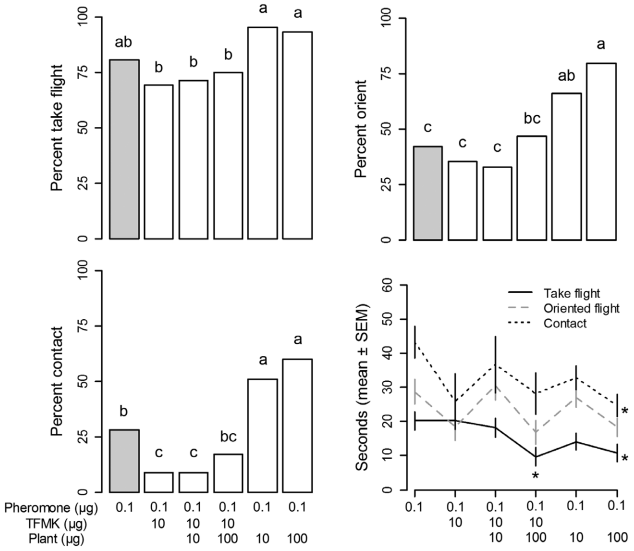

Fig. 5. Behavioural responses of *L. botrana* males flying towards a source baited with mixtures of pheromone : TFMK 3 : plant volatiles in several ratios relative to the pheromone in a wind tunnel. Percentage of males taking flight, orienting and contacting the source, as well as the delay time for all the behaviours are shown as the predicted values of the GLM models. Planned comparisons are analyzed using Tukey's test, $P < 0.05$. Different letters indicate significant differences among treatments (Multiple pairwise comparisons using Tukey's test, $P < 0.05$). For the time to respond (bottom right) asterisks indicate significant differences with the response to the pheromone (planned contrast, $P < 0.05$).

(b) from the use of different volatile concentrations, or (c) from genetic differences between the two moth populations. On the other hand, whereas von Arx et al. (2012) report pheromone synergism with each of the individual components of the plant volatile blend, we show that the complete blend also synergizes pheromone responses. It is interesting to note that pheromone-plant synergism is reported in the literature to occur at low doses of the plant volatiles, but not at high doses at which an inhibition of the pheromone response occurs (Varela et al., 2011; von Arx et al., 2012; Deisig et al., 2014; Yu et al., 2015).

Based on the results from this experiment, we chose the 1:100 and 1:1000 pheromone : plant volatile blend ratios for the following test.

Effect of plant volatiles on the inhibition by pheromone analogs

The control experiments showed, again, that the pheromone analogs reduce the percentage of males responding to pheromone and increase their response time, while the blend of plant volatiles had the opposite effect. When mixed with the pheromone, MK **2** significantly reduced the percentage of males taking flight and contacting the source but not of those orienting to the bait (Table 1, Fig. 4). In the same tests the blend of plant volatiles synergized male response to pheromone only in terms of the percentage of contacts and only at the highest dose tested (pheromone : plant volatile blend 1:1000) (Table 1, Fig. 4). Despite the synergistic effect of the plant blend, it did not counteract, however, the antagonistic effect of the MK **2** in any of the three behavioural categories (Table 1, Fig. 4).The inhibitor induced an increase in the time to contact the pheromone source whereas the blend of volatiles decreased it (Table 1, Fig. 4). Like MK **2**, TFMK **3** also decreased the number of contacts with the pheromone and the blend of volatiles synergized the effect of the pheromone in the oriented flight and percentage of contacts (Table 1, Fig. 5). In addition, at the highest dose tested, the plant blend partially counteracted the antagonistic effect produced by TFMK **3** on oriented flight and source contact (Table 1, Fig. 5). Also, the highest plant dose reduced significantly the time for males to take flight, even in the presence of TFMK **3**, and it also reduced the time to contact (Table 1, Fig. 5).

Our tests show, for the first time, that a blend of plant volatiles is able to partially reverse the behavioural inhibition caused by a structural analog of the sex pheromone. Plant odours are normally sensed by moths by relatively plant-specific olfactory receptor neurons (pl-ORNs) whereas pheromone ligands are perceived by much more specific pheromone-specific olfactory receptor neurons (ph-ORNs) (Deisig et al., 2014; Ammagarahalli & Gemeno, 2015). Plant and pheromone stimuli travel via separate nerves to the brain where this information is integrated (Deisig et al., 2014). One interpretation of the reversal of the analog inhibition by plant volatiles is that these chemicals, which in the absence of inhibitors increased male response to the pheromone, also stimulated male response in the presence of the inhibitor, probably by stimulating pl-ORNs. A second possibility is that plant volatiles, in addition to stimulating pl-ORNs, also acted on ph-ORNs counteracting the effect of the pheromone analogs on the sensilla. Indeed, there is evidence that in several species of moths plant volatiles can alter the spike frequency of ph-ORNs with respect to stimulation with only the pheromone ligand, either by synergizing (Ochieng et al., 2002; Hillier & Vickers, 2011), or more often by inhibiting the response to the pheromone (Deisig et al., 2014; Ammagarahalli & Gemeno, 2015).

One question raised by our study is why the plant odour reversed the inhibition of only one of the analogs, the TFMK **3**, whereas it did not reverse the antagonism of MK **2**. If the counter-antagonism effect of the plant stimulus involved interactions within the ph-ORN sensillum, then it is possible that the different activity of the plant odour could be related to the different mode of action of each of these two analogs. The mode of action of MKs and TFMKs is not yet fully understood. TFMKs can bind to the pheromone binding proteins (PBPs), and therefore they would compete with pheromone molecules for transportation in the sensillum lymph (Feixas et al., 1995; Campanacci et al., 1999; Pophof et al., 2000). In addition, TFMKs reversibly inhibit in vitro the antennal esterases responsible for the catabolism of the pheromone (pheromone degrading enzymes, PDEs) in male olfactory tissues (Duran et al., 1993; Rosell et al., 1996; Riba et al., 2005). By binding to the PBPs or inhibiting the PDEs, the TFMK could alter the firing frequency of ph-ORNs, and this may result in altered pheromone sensing.

In *L. botrana*, it is possible that a putative competitive inhibition mechanism either for the PBPs and/or the pheromone receptors occurs, similar to the reported displacement of Z11-16:Ac, the major component of the pheromone of *M. brassicae* bound to a recombinant PBP1, by Z11-16:TFMK (Campanacci et al., 1999). In addition, because the major component of the pheromone of *L. botrana* is an acetate (ester), it is likely that TFMK **3** may act also as an inhibitor of the antennal PDEs (esterases) and block the catabolism of the pheromone. These processes may either under stimulate or over stimulate ph-ORN firing responses to the pheromone and, thus, reduce the response of males. The plant odour, in turn, may be able to rectify the alterations in the ph-ORN firing rates produced by the antagonist by mechanisms not yet known. In contrast to the TFMKs, to our knowledge no data are available on the mechanism of MKs at the receptor level. Nevertheless, because they are structurally very similar to the pheromone, the inhibitory activity of the MK could presumably be due to an overstimulation or adaptation of the receptor cells.

The results presented herein increase our scant knowledge about pheromone analogs and their potential use in IPM, in particular the possible interaction between pheromone analogs and plant volatiles that may take place in the field. Field experiments are required in order to determine if plant volatiles interact with pheromone analogs under natural conditions.

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