

Antennal and Behavioral Response of *Cydia pomonella* and *Lobesia botrana* Moths to Allyl Cinnamate

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Abstract

Electroantennographical (EAG) response to *allyl cinnamate* were assessed on virgin and mated *Cydia pomonella* and *Lobesia botrana* adults to determine whether this compound could be used within integrated management programs (IMP). Adult behavioral reaction was later assessed in a wind tunnel, with and without the main compound of the corresponding female sex pheromone.

Allyl cinnamate elicited antennae responses of *C. pomonella* and *L. botrana*, both males and females. *Allyl cinnamate* EAG response was as high as pheromone response, and it was not reduced after mating.

In wind tunnel assays, allyl ester itself was not attractive to *C. pomonella* males, but its presence did not interfere with the pheromonal action when the number of contacts was compared. For females, a higher proportion of codling moths moved towards the source when *allyl cinnamate* was in the wind-tunnel plume. No differences were recorded depending on the mating status of codling moth adults. The same trend was observed in *L. botrana* males and females.

Results suggest that *allyl cinnamate* acts as a female behavioral modifier, but more assays are required to determine its role in insect communication in field conditions before inclusion in integrated pest management.

Keywords: *Allyl cinnamate*; Behavioral response; *Cydia pomonella*; EAG; *Lobesia botrana*; Wind-tunnel

Introduction

Cydia pomonella (L.) (codling moth) and *Lobesia botrana* (Dennis and Schifferrmüller) (grapevine moth) (Lepidoptera: Tortricidae) are key pests in pome, pear and walnut orchards, and in vineyards, respectively [1]. Both species are pests of high-value crops and have low tolerance thresholds, leading to repeated insecticide treatments (with or without other pest control interventions) during the season. To solve negative effects of insecticide use [2,3], research has developed alternative pest control methods.

Mating disruption, based on the use of sex pheromones, is widely used in many countries to control codling and grapevine moths [4,5]. However, its application requires some specific field characteristics in order to succeed [6-8]. Other techniques based on the use of sex pheromones (mass-trapping and, attract and kill) are also available, but less used [9]. All these techniques focus in male moth control, but is also described an effect in female behavior by exposure to its own sex pheromone [10,11].

Tree fruit volatiles are usually added to pheromone traps to increase the number of moth captures [12-18]. Some of these plant volatiles synergize response to sex pheromone [19,20].

Our research group has been working on allyl ester synthesis using glycerol as starting material [21,22], which is produced in large amounts as a by-product in biodiesel production (<http://www.biodiesel.org/>). The insecticidal properties of some allyl esters have been assessed previously in order to give an added value to the surplus of glycerol [21,23,24]. As several volatile compounds from codling and grapevine moth host-plants are used by adults for host and mate localization [25-28], an effect in moth behavior due to allyl esters used as fruit aromas [29] was suspected. Moreover, some volatile compounds described as moth attractants [30-32] are chemically related to *allyl cinnamate*. This

fact drives to hypothesize that the latter may influence codling moth and grapevine moth adult behavior.

This paper describes for the first time the capacity of *allyl cinnamate* to elicit antennal response and to modify behavior from *C. pomonella* and *L. botrana* adults. Improved understanding of the role of this chemical compound may ultimately be incorporated into Tortricidae integrated pest management programmes (IPM).

Material and Methods

Insects

The experiments were conducted with a *C. pomonella* laboratory strain originated from a population collected in an unsprayed apple orchard in Lleida (north-east Spain), and with a *L. botrana* laboratory strain established in our laboratory from a mass-reared strain from INRA Bordeaux (France). Both strains were reared at the Crop Protection Laboratory of the UdL-IRTA Centre for Research and Development (Lleida, Spain) at 23 ± 2°C, with a photoperiod of 16:8 (L:D) and on agar-based semi-synthetic diet [33].

Cydia pomonella individuals were sexed as last instar larvae, whereas *L. botrana* individuals were sexed as pupae. The sexed individuals were kept separately by sex until adult emergence in the same conditions as

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the laboratory strains were. Next, four to six less than 24-h old adults of the same sex were transferred into plastic cages (diameter (d) = 15 cm, Height (H) = 5 cm), and maintained in the above mentioned conditions to obtain virgin individuals. To obtain mated adults, two or three couples were maintained together in the above mentioned plastic cages. Adults were used in electroantennogram (EAG) or wind-tunnel bioassays at second or third day after adult emergence. The mating status of females was ascertained by the presence of a spermatophore, by dissection of females after EAG recording or wind tunnel assay. Males were considered mated when females of their own group were ascertained to be mated.

Chemicals

Allyl cinnamate was purchased from Sigma-Aldrich (Madrid, Spain), and acetone for residue analysis was purchased from Panreac (Barcelona, Spain).

E,E-8,10-dodecadienol (codlemone) ($\geq 99.5\%$ purity), the main compound of *C. pomonella* sex pheromone, and E,Z-7,9-dodecadienyl acetate (EZ79Ac), the main compound of *L. botrana* sex pheromone ($\geq 99.5\%$ purity), were purchased from PheroBank (Wageningen, the Netherlands).

Electrophysiological assays

An EAG apparatus from Synthech (Hilversum, the Netherlands) was used to record electroantennographical responses of adults. Signals after stimulus application (mV) were amplified (100 \times) and filtered (DC to KHz) with an ID-2 interface (Syntech), digitized on a PC and analyzed with the EAG2000 program.

Each antenna was carefully cut from an insect that had previously been anesthetized with ice and immobilized using a fine needle. Another cut was done at the end of the antenna using a scalpel, and then the antenna was placed between EAG electrodes. Electrode gel (Parker, Orange, NJ) was used to facilitate connection between the antenna and electrodes.

Each chemical (stimulus) was presented by applying 0.1 μ g of it to a piece of filter paper (2 \times 2 cm). The piece of paper was then inserted into a Pasteur pipette, which was placed so that the tip of the pipette was 5 cm from the antenna. A puff of air (300 mL min⁻¹) through the pipette then carried the stimuli to the antenna.

Allyl cinnamate intrinsic activity

Twenty antennae per species (*C. pomonella* and *L. botrana*), sex (male and female) and mating status (virgin and mated) were used in the bioassay. Five consecutive puffs of allyl ester, pheromone and acetone (control puffs) were applied to each antenna in randomized order. Each puff was separated 30 s to minimize potential onset of antennae. No fatigue was observed in the antennae used in the bioassay.

The response to each stimulus was calculated as the mean response to the five puffs, and was compared to mean response to control puffs (acetone) by *t*-test ($P<0.05$). If significant greater response was observed to stimuli compared to control, mean response to stimuli was corrected as follow: corrected EAG response = mean EAG response to stimulus – mean EAG response to acetone. Corrected EAG responses were transformed [log (x + 1)] and analyzed by *t*-test or one-way ANOVA followed by Tukey-Kramer HSD test ($P<0.05$) for each species, sex and mating status. Statistical analysis was carried out with the JMP 8.0.1 program (SAS Institute, Cary, NC).

Synergism between Codlemone and Allyl cinnamate

Five consecutive puffs of codlemone (0.1 μ g), a mixture of codlemone and allyl cinnamate (0.05 + 0.05 μ g), allyl cinnamate alone (0.1 μ g) and control puffs (acetone) were applied to each antenna (N = 20) of *C. pomonella* virgin males and females.

Mean corrected EAG response (calculated as described in the intrinsic activity assay) for each treatment (codlemone, allyl cinnamate and mixture) were compared for each sex by one-way ANOVA followed by Tukey-Kramer HSD test ($P < 0.05$) using the JMP 8.0.1 program (SAS Institute, Cary, NC).

Wind-tunnel assay

The assay was conducted in a glass wind tunnel (H=50 cm, Long (L)=200 cm and wide=50 cm) situated in a room maintained at 23 \pm 2°C. Light was supplied by an incandescent light bulb situated on the ceiling of the room (2 lux for *C. pomonella* and 100 lux for *L. botrana*, following results from our research group). Two ventilators either side of the wind tunnel operated simultaneously, producing an air flow of 0.15 cm s⁻¹ through the tunnel (measured using an anemometer).

The assay was performed during the first two hours of scotophase, and at least 40 insects were used per treatment. Insects were tested individually and only once. Each individual was placed into a two-side open glass tube (d=2.5 cm, L=15 cm), which was oriented to the stimulus source and situated on a 20 cm high metal stand (the insect starting point). One 8 mm red rubber septum (Sigma-Aldrich, Spain) loaded with the main compound of the sex pheromone, allyl ester, or a mixture of pheromone and allyl ester in acetone (Table 1) was used as source of the stimulus. The source was situated on a 20 cm high metal stand located at 150 cm from the insect starting point. For solvent (acetone, code 0:0), 10 μ L were added to the septum.

The effect of the allyl cinnamate alone or mixed to the pheromone at different proportions (Table 1) was tested on virgin males and females of *C. pomonella* and *L. botrana*. Moth behavior (activation, non-oriented flight, oriented flight and contact with source) in response to the stimulus was recorded for three minutes. The wind tunnel was cleaned with acetone after each experimental day and used material (glass tubes and metal stands) was washed with acetone and oven-dried at 200°C overnight. The percentage of insects that showed a specific behavior (activation, non-oriented flight, oriented flight and contact) for each species, sex and stimulus were compared by χ^2 test ($P<0.05$) using the GraphPad program (Graph Pad Software Inc., La Jolla, CA).

Stimulus	Code	Species	Dose (μ g / septum)	
			Pheromone	Allyl cinnamate
Pheromone*	1:0	<i>C. pomonella</i>	10.0	-
		<i>L. botrana</i>	2.5	-
Allyl cinnamate	0:1	<i>C. pomonella</i>	-	10.0
		<i>L. botrana</i>	-	2.5
Mixture of pheromone and allyl cinnamate	1:0.1	<i>C. pomonella</i>	10.0	1.0
		<i>L. botrana</i>	2.5	0.25
	1:1	<i>C. pomonella</i>	10.0	10.0
		<i>L. botrana</i>	2.5	2.5
	1:5	<i>C. pomonella</i>	10.0	50.0
		<i>L. botrana</i>	2.5	12.5

*Pheromone dose was fixed according to previous bioassays in our wind tunnel conditions (Giner et al., (2009) for *C. pomonella* [41] and Cruz -personal communication- for *L. botrana*).

Table 1: Composition of each stimulus used for each species in the wind tunnel assay.

Comparison between results obtained per sex on *C. pomonella* for the combination *codlemone*+*allyl cinnamate* and the sum of results for *codlemone* and *allyl cinnamate* separately were compared by *t*-test ($P < 0.05$) using the GraphPad program to ascertain synergism.

Results

Electrophysiological assays

Allyl ester intrinsic activity: *Allyl cinnamate* elicited virgin *C. pomonella* female antenna and EAG responses as great as the one produced by *codlemone*. The EAG response was maintained after mating (Table 2).

For *C. pomonella* males, *allyl cinnamate* also caused a significant EAG response (1.070 ± 0.460 mV), not significantly different from the response to *codlemone* (0.753 ± 0.068 mV). The EAG response to *allyl cinnamate* did not reduced after mating (0.756 ± 0.207 mV) ($P < 0.05$).

For *L. botrana* virgin males, EAG values regarding *allyl cinnamate* were not significantly different than the pheromone ones (0.635 ± 0.141 and 0.892 ± 0.403 , respectively). A significant reduction of response was recorded to pheromone after mating (0.323 ± 0.086 ; $P > 0.05$), but not to *allyl cinnamate* (0.237 ± 0.059 ; $P < 0.05$), as observed in *C. pomonella*. In the case of females, only virgins elicited a response followed by *allyl cinnamate* stimulation (0.305 ± 0.019 ; $P < 0.05$).

Synergism among *Codlemone* and *Allyl cinnamate*: No synergism was observed among *codlemone* and *allyl cinnamate* in *C. pomonella* antennae. No differences in EAG response were observed between *codlemone* - *allyl cinnamate* mixture and *codlemone* alone ($P_{\text{males}} = 0.91$; $P_{\text{females}} = 0.57$) or *allyl cinnamate* alone ($P_{\text{males}} = 0.76$; $P_{\text{females}} = 0.55$).

Wind tunnel: In the wind-tunnel, the *codlemone* caused the complete range of behaviors from *C. pomonella* males (Figure 1). The addition of *allyl cinnamate* to *codlemone* blend at different doses did not have a significant effect on activation and non-oriented flight behavioral steps when compared to the ones produced by *codlemone* alone. The addition of *allyl cinnamate* to *codlemone* at 1: 0.1 and 1: 5 caused some reduction on oriented flight and contact behavior, but not at 1:1 (Figure 1).

When *allyl cinnamate* was presented alone a significant reduction in percent of males that reach each behavioral step was recorded. If these values were compared to the solvent alone, a greater percent of males were activated and started the flight in the presence of the allyl ester, but few contacts were scored (Figure 1).

In the case of *C. pomonella* females, *codlemone* did not cause significant behavioral effect but the presence of *allyl cinnamate* (alone or mixed with *codlemone*) did. A higher percent of virgin females were activated and flew to the source when *allyl cinnamate* was present (Figure 2). The same was observed in the case of mated females (data not shown). No differences were recorded in the response to *allyl cinnamate* between virgin or mated *C. pomonella* females ($P > 0.05$). No increase of attraction was observed in the case of mated males (data not shown), compared to virgins.

In *L. botrana*, *allyl cinnamate* did not produce an effect in males, and the addition to pheromone causes a significant reduction in percent of contacts with the source at 1:0.1 or 1:5 proportions, but not at 1:1 (Figure 3), as observed in *C. pomonella*. For females, *allyl cinnamate* caused a significant activation, non-oriented flight and oriented flight – compared to solvent – (Figure 4), but no contacts were observed.

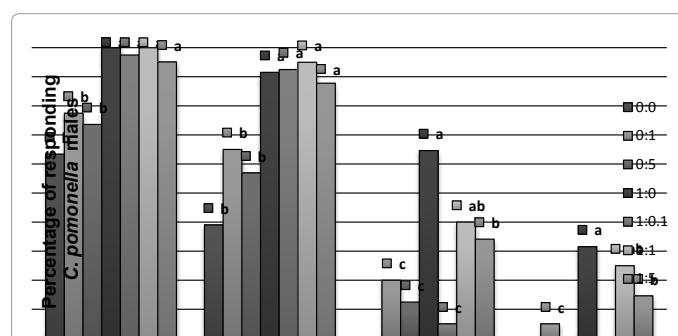
Chemical	<i>C. pomonella</i>		
	Virgin		Mated
Codlemone	0.520 ± 0.090 a		-
Allyl cinnamate	0.580 ± 0.036 a	ns	0.642 ± 0.166

Mean and SE, $n = 20$.

Values followed by the same letter in the same column are not significantly different (*t*-test, $P < 0.05$).

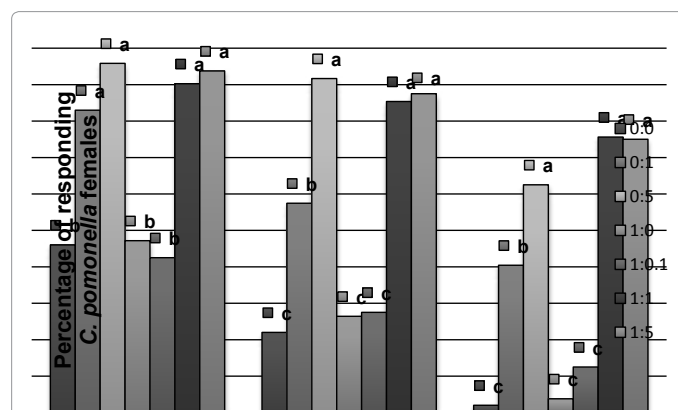
ns =not significant differences between virgin and mated responses (*t*-test, $P < 0.05$).

Table 2: Corrected (Response to chemical – response to acetone) EAG response (mV) of antennae of virgin and mated females of *Cydia pomonella* to 0.1 μ g of the main component of female sex pheromone (*codlemone*) and allyl cinnamate.



Different letters into each behavioral step indicate significant differences in moth response to different treatments (X^2 , $P < 0.05$).

Figure 1: Behavioral response of *C. pomonella* virgin males ($n = 40$, minimum) in the wind tunnel flying towards a source baited with pheromone (1:0) (10 μ g) and different proportions of pheromone: allyl cinnamate.



Different letters into each behavioral step indicate significant differences in moth response to different treatments (X^2 , $P < 0.05$).

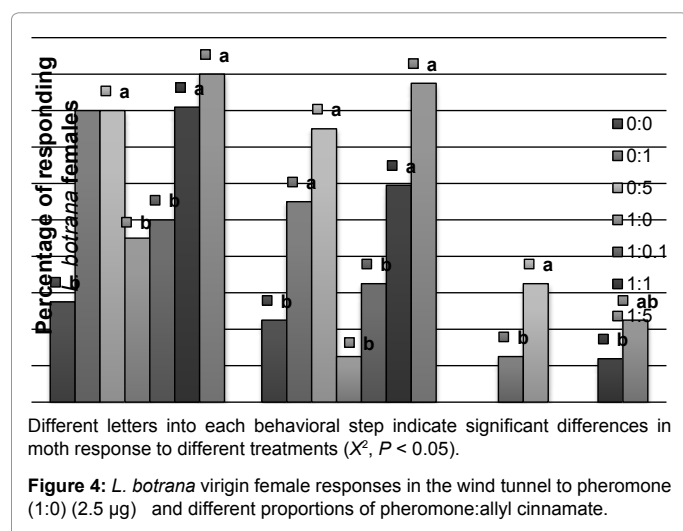
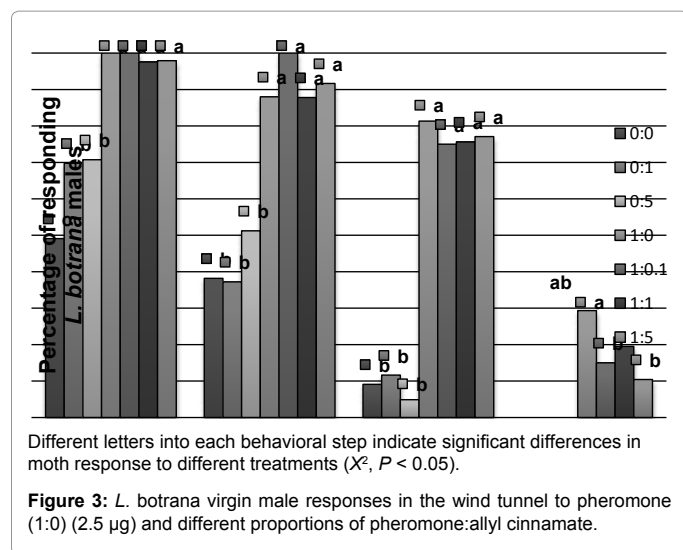
Figure 2: *C. pomonella* virgin female responses in the wind tunnel to pheromone (1:0) (10 μ g) and different proportions of pheromone: allyl cinnamate.

Discussion

To our knowledge, this is the first report of *allyl cinnamate* eliciting antennal response of *C. pomonella* and *L. botrana* moths and (or) causing a behavioral reaction in a wind tunnel.

Allyl cinnamate elicited an antenna response in both males and females of *C. pomonella* and *L. botrana*, independently of the state of mating. This indicates that its action would not be strictly related with the mating process but has to be involved in general activity (feeding, host-localization). This observation contrasts with the one produced by several plant volatiles, which are more attractive after mating [34,35].

It is interesting to note that the same EAG recordings were scored



by both codlemone and *allyl cinnamate* alone, or when blended, suggesting that the same receptors could be involved in the perception process. This possibility was previously described for plant volatiles [25,36-38]. In the case of *allyl cinnamate* this fact should be more deeply studied to be confirmed.

Although an effect of allyl esters of fatty acids could be suspected from similarities in chemical structure to butyl hexanoate (moth attractant) [15], no effect of alkyl allyl esters were recorded in *C. pomonella* or *L. botrana* (unpublished data).

It is known that the size of EAG response does not always indicate a behavioral response [34]. Even though male and female antenna elicitation was recorded, only females were attracted to sources baited with allyl cinnamate in the wind-tunnel. An increase of fluttering was observed in males and *allyl cinnamate* alone does not clearly produce a behavior of source contact in any case. This lack of contact could be related with the fact that the behavior was observed for three minutes. More assays should be done with increased observation time or semi-field assays to reaffirm the properties of *allyl cinnamate* as *Tortricidae* moth attractant.

No increase of attraction was observed in the wind-tunnel assay

after mating, reassuring the results from the EAG recordings and indicating the lack of *allyl cinnamate* effect on the mating process.

Only on *C. pomonella* females a synergism between pheromone and *allyl cinnamate* was suggested in the wind-tunnel. Other authors have described a similar synergism to plant volatiles [21,25]. This could be extremely interesting if confirmed in a field situation.

Allyl cinnamate is suggested as a candidate to bait pheromonal traps with the aim of increasing moth captures in pest monitoring, or in attract & kill and mass-trapping strategies in *Tortricidae* pest control.

The fact that allyl cinnamate is authorized for use as food aroma (<http://eur-lex.europa.eu/>) indicates its availability to use in ethologic control. Moreover, allyl esters can be synthesized from glycerol or fat wastes [21,22], what fosters the re-use of industrial sub products. However, more assays need to be done to fix the proportion of *allyl cinnamate* into the blend to improve the attractiveness of moths [39,40] and asses it in field conditions, as well as the fate of *allyl cinnamate* in field conditions.

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