

Full Length Research Paper

Antenna elicitation and behavioral responses of oriental fruit moth, *Grapholita molesta*, to allyl cinnamate

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Female sex pheromones have been used in pest control since the 90s; attracting males to baited traps (mass-trapping and monitoring) or avoiding (or reducing) mating in fields under mating disruption. By contrast, little is done among the use of male sex pheromones in pest control. Allyl cinnamate was evaluated as potential oriental fruit moth (*Grapholita molesta*, Busck) (Lepidoptera: Tortricidae) behaviour modifier, after recording positive electroantennographical responses in both male and female moths. Females over-responded in front of sources of allyl cinnamate at short distances, and both male and female showed typical pre-mating behavioural responses at mid-distances (in a wind tunnel). Males responded showing its hair-pencils and wing fanning and females started with wing fanning, curling abdomen and locating position opposite the source of allyl cinnamate. The same effect was observed in front of *trans*-ethylcinnamate, the main component of *G. molesta* male sex pheromone. Results here indicate a putative role of male sex pheromones (or chemically related compounds as allyl cinnamate) in oriental fruit moth integrated pest control. Understanding the role of male sex pheromones and chemically related compounds could help in the development of new pest control strategies.

Key words: Electroantennography (EAG), male sex pheromone, allyl ester, *trans*-ethyl cinnamate, wind-tunnel, behaviour modifier.

INTRODUCTION

Grapholita molesta (Busck) (Lepidoptera: Tortricidae), the oriental fruit moth, is a key-pest in most stone-fruit productive areas worldwide (Rotschild and Vickers, 1991), and it is also known to cause damages in apples at the end of the season (Kovanci et al., 2004). They firstly feed shoots, and after in fruits, causing damage on young trees and

depreciating economic value of fruits (González, 2003). Moreover, feeding wounds are easily infected by brown rot (Garic et al., 2004; Holb, 2004), increasing fruit depreciation and reducing its marketability.

Chemical control is still being the most used technique to maintain *G. molesta* populations under economic

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thresholds, but mating disruption based on use of female sex pheromones, are being greatly spread as a way to avoid insecticide negative effects (Il'chev et al., 2004; Devine and Furlong, 2007; Kong et al., 2014).

Female sex pheromones are used to monitor pest populations aimed to do chemical treatments in the best moment, to bait traps in mass-trapping and also for attract-and-kill strategies (Damos et al., 2014). In all cases, females are not the target, although some authors have reported an effect on female behaviour by exposure to their own sex pheromone (Stelinski et al., 2006; Gökçe et al., 2007; Kuhns et al., 2012). To increase the attractive effect on females, some volatile compounds were successfully added to pheromonal blends (Natale et al., 2003, 2004; Piñero and Dorn, 2007).

By contrast, few Lepidopteran male sex pheromones were studied even playing an important role in mate acceptance (Landolt and Heath, 1989; Hillier and Vickers, 2011). In the case of oriental fruit moth, a male sex pheromone is described; *trans*-ethyl cinnamate which is the main component. It is emitted by *G. molesta* males on their hair-pencils as aphrodisiac (Birch and Hefetz, 1987), increasing mating success at short distances (Baker et al., 1981; Löfstedt et al., 1990).

The study of courtship behaviour and mate acceptance could help to improve pest control based in the use of synthetic pheromones. A highly stereotypic sequence of behaviours are described to occur before mating (Curkovic et al., 2006), so the interference in this sequence could reduce mating and, consequently, pest populations.

Allyl cinnamate, chemically related with oriental fruit moth male main component of pheromone (Figure 1), have been described as female attractant on other two Tortricidae pests and so, an effect in oriental fruit moth was be expected.

This work describes for the first time allyl cinnamate as antenna elicitor and behaviour modifier of *G. molesta* moths, and contributes to the knowledge of oriental fruit moth courtship behaviour aim to be used into an integrated pest management (IPM) program. Differential effect than observed in other Tortricidae species is suggested due to similarities of allyl cinnamate with the main component of *G. molesta* male pheromone.

MATERIALS AND METHODS

Insects

G. molesta laboratory strain established in IRTA Research Centre (Spain) in 2005 from an established colony from field-collected individuals (Dr. F. Molinari, Piacenza, Italy) was used in all bioassays. Larvae were reared on semi-artificial diet (Ivaldi-Sender 1974) under long photoperiod (16L: 8D) and 24±1°C. Pupae were sorted by sex and placed into plastic cages (d= 15 cm, H= 5 cm) in a separated chamber at 24±1°C under the same photoperiod to obtained virgin adults. To obtain mated female individuals for EAG recordings and wind-tunnel behaviour assay, 2-3 couples of pupae were kept in the same cage.

Moths were used the second or third day after emergence and in

behavioral assays 3 h before light off [peak of responsiveness of males to female sex pheromone and female gland extrusion (Baker and Cardé, 1979)].

The mating status of females was ascertained by the presence of spermatophore, which was determined after EAG recording or tunnel bioassay. Males maintained in the same case than females that were ascertained to be mated were differentially assessed than males that were kept in cages with no presence of females.

Chemicals

Allyl cinnamate and *trans*-ethyl cinnamate used in all bioassays were purchased by Sigma-Aldrich (www.sigmaaldrich.com). The main compound of *G. molesta* female sex pheromone was purchased by Pherobank (www.pherobank.com). All compounds had a minimum of 98% purity.

Electrophysiological assays

An EAG apparatus from Syntech (www.syntech.nl) was used. Signals after stimulus application (mV) were amplified (100×) and filtered (DC to KHz) with an ID-2 interface (Syntech), digitized on a PC and analysed with the EAG2000 program.

Antenna was carefully cut from an insect that was previously anesthetized with ice and then immobilized using a fine needle. Another cut was done at the end of the antenna using a scalpel. Then, antenna was placed between EAG electrodes, using electrode gel (www.parkerlabs.com/signagel.asp) to facilitate connexion between antenna and electrodes. Each stimulus was presented by first applying 0.1 µg to a piece of filter paper (2 × 2 cm). The piece of paper was then reinserted 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.

At least 10 antennae per sex and mating status were used in the bioassay. Five consecutive puffs (separated by 30 s) of the allyl cinnamate and control puffs with only solvent (acetone) were applied to each antenna in randomized order. No fatigue was observed in any antennae used in the bioassay.

For each antenna, the response to allyl cinnamate was calculated as the mean response to five puffs each, and was corrected by the mean of corresponding acetone puffs (EAG corrected = mean EAG compound – mean EAG control). Mean corrected EAG response of allyl cinnamate for each sex and state of mating were transformed [log (x + 1)] to normalize the data, and then compared by one-way ANOVA, followed by Tukey-Kramer HSD test ($P < 0.05$) using the JMP 8.0.1 program (www.jmp.com).

Behavioural assays

First assay was done to assess virgin female responses to allyl cinnamate at short distances. 0.1 micrograms of allyl cinnamate dissolved in acetone were applied in a filter paper (2 x 2 cm) and then, the filter paper was introduced in a Petri dish (10 cm diameter, 5 cm height) containing female moths (n= 10, three repetitions). Female behavioural responses were recorded during 10 min, and insects were maintained in Petri-dishes during 24 h. Viability of insects at the end of the assay was recorded. The same, but applying only acetone in the filter paper, was done as control (n= 10, three repetitions). Percent of insects showing pre-mating behaviour against allyl cinnamate or control, and mortality among treatment and control were compared by χ^2 test ($P < 0.05$) using the GraphPad program (www.graphpad.com).

Second assay aim to assess the effect of allyl cinnamate at mid-

distances was done in a wind tunnel (50 cm high, 200 cm long and 50 cm wide) situated in a room maintained at $23 \pm 2^\circ\text{C}$. Light was supplied by a fluorescent light situated on the ceiling of the room (200 lux) and two ventilators either on the side of the wind tunnel operated simultaneously, produced an air movement of 0.15 cm s^{-1} through the tunnel. At least 20 mated and 20 virgin female moths, and 20 naïve and 20 no-naïve male moths were observed in front of each stimulus for 3 min. Each insect was assessed individually and only once.

Red rubber septa of 8 mm (Sigma-Aldrich, Spain) situated 150 cm from the insect starting point and using a metal stand, held at a height of 20 cm, were used to place source, baited with $10 \mu\text{g}$ of stimulus. Septa with solvent alone ($10 \mu\text{L}$ acetone) were also assessed, as control. Percent of insect showing a specific behaviour for each group and stimulus was compared by χ^2 test using a contingency table ($P < 0.05$) with the GraphPad program.

The wind tunnel was cleaned with acetone after each experimental day and used material was washed with acetone and oven-dried at 200°C overnight.

RESULTS

Allyl cinnamate elicited antenna of both male and female *G. molesta*. No differences in antenna elicitation among mated and un-mated female moths were recorded ($0.788 \pm 0.289 \text{ mV}$ and $0.822 \pm 0.379 \text{ mV}$, respectively) ($P > 0.05$). Neither among naïve and no-naïve males ($1.037 \pm 0.308 \text{ mV}$ and $1.429 \pm 0.289 \text{ mV}$, respectively) ($P > 0.05$).

When *G. molesta* female moths were introduced into Petri-dishes containing allyl cinnamate, typical behaviour preceding mating was triggered [insect movement, walking toward, hair-pencil extrusion and retraction, wing fanning, wing vibrating, flight and quick wing movement, cleaning antenna with legs, abdomen movement (Baker, 1989)]. Notable increase of wind fanning was observed in all individuals (in most cases, moth remain winging on the Petri-dish floor moving the abdomen) ($\chi^2 = 196$, $\text{df} = 1$, $P < 0.001$). The increase of moth activity conducted up to moth exhaustion; reflected by mortality observed in front of allyl cinnamate (100%) compared to controls ($< 10\%$) ($\chi^2 = 185$, $\text{df} = 1$, $P < 0.001$).

In wind-tunnel, more female moths showed the behaviour "clean antenna" in the presence of allyl cinnamate and ethyl cinnamate than in front of control sources. No oriented flight was recorded in front of control sources and significant lower number of females showed mating behaviour compared to sources baited with allyl cinnamate or ethyl cinnamate. If we compare the responses of female moth that start flight, no significant differences were observed among control, ethyl cinnamate and the lowest dose of allyl cinnamate tested. By contrast, a significant higher percent of females started flight in front of sources baited with $50 \mu\text{g}$ of allyl cinnamate, and most of them showed an oriented flight to the source, even no contacts were recorded (Figure 2).

All males cleaned its antenna, displayed typical behaviour that precedes mating and showed its hair-pencils in front of allyl cinnamate and the main component of sex (both male and female sex pheromone) in wind-tunnel, while not in front of control sources. Male fluttering was

only observed in the presence of female pheromone and allyl cinnamate, and mostly started flight in the presence of female sex pheromone and allyl cinnamate. Contacts were only recorded in front of main component of female sex pheromone (Figure 3).

DISCUSSION

To our knowledge, this is the first report describing *G. molesta* antenna elicitation by allyl cinnamate. Antenna elicitation by allyl cinnamate was independent of the state of mating, as previously observed in *C. pomonella* and *L. botrana* (not published data). *G. molesta* antenna elicitation was in the same range than recorded for the main components of sex pheromones giving an idea of the high affinity of allyl cinnamate to *G. molesta* antennae receptors.

Due to similarities in the chemical structure of allyl cinnamate and trans-ethyl cinnamate (main component of *G. molesta* male sex pheromone) an effect in mating process is suspected. Moths perceived allyl cinnamate, showing "cleaning antennae" behaviour, confirming results from EAG recordings, and all typical behaviours preceding mating were observed in presence of allyl cinnamate [insect movement, walking toward, hair-pencil extrusion and retraction, wing fanning, wing vibrating, flight and quick wing movement, cleaning antenna with legs, abdomen movement, female touch male abdomen, end-to-end position, wing directed to floor, oviposition movement] (Baker, 1989)]. The energy needed to maintained these movements during long time (24 h presence of allyl cinnamate) drives to the insect death due to exhaustion. This fact could be useful in pest control; all energy overused when allyl cinnamate is detected reduces time and energy that moth could actually use to find a mate, copulate, find a place to ovoposit and ovoposit. If one of these series is disrupted, a negative effect in the next generation (population reduction) is expected.

Ethyl cinnamate acts as aphrodisiac compound on females and it seems that males are attracted to ethyl cinnamate with the aim to 'sneak' copulations with females that are actively being courted by other males (Baker and Cardé, 1979). Allyl cinnamate effect could also be related with mating success, as a difference than ethyl-cinnamate - which acts at short distances (Baker et al., 1981); it seems that could be detected and conducted toward longer distances. Assays aim to discern the effect of both components at different ratios (female sex pheromone and allyl cinnamate) when applied together, and in field assays are necessary to ascertain this pest control proposal. If allyl cinnamate was joined to conventional mating disruption, an effect in female and male behaviour has to be expected; not attraction would be produced (as a difference than female sex pheromone), but a display of pre-mating behaviours. The time and energy spent on pre-mating behaviour could reduce the number of effective mating and so, population in next generations.

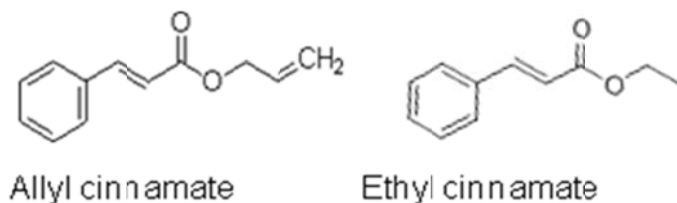


Figure 1. Chemical structures of allyl cinnamate and ethyl cinnamate.

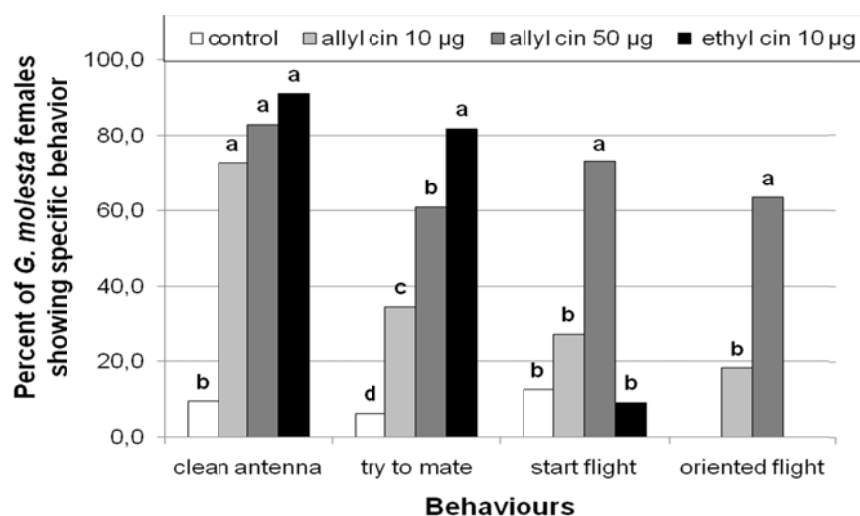


Figure 2. Percent of *G. molesta* virgin females showing a specific behaviour in wind-tunnel assay in front of septum containing 10 µg of allyl cinnamate, 10 µg ethyl cinnamate, 100 µg of allyl cinnamate or solvent during 3 min recording. cin = Allyl cinnamate; Columns within a specific behaviour followed by the same letter are not significantly different. X^2 test shows homogeneity, (2 x 2) $P < 0.05$.

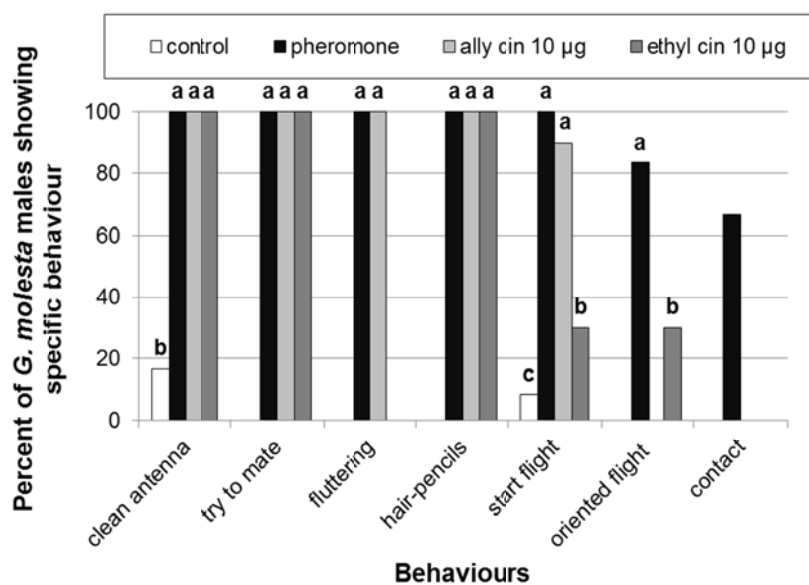


Figure 3. Percent of *G. molesta* virgin males showing a specific behaviour in wind-tunnel assay in front of septum containing 10 µg of main component of female sex pheromone, 10 µg of allyl cinnamate, 10 µg of ethyl cinnamate, or solvent during 3 min recording.

Additionally, allyl cinnamate is used as aroma (<http://eur-lex.europa.eu/>) so low toxic effects in non-target organisms are suspected and it could be synthesized from glycerol (Escribà et al., 2009, 2011), so its use could help to find an alternative to glycerol surplus (www.biodiesel.org).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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