Chem. Senses 24: 423–428, 1999

Actographic Analysis of the Effects of an Esterase Inhibitor on Male Moth Responses to Sex Pheromone

Michel Renou, Andrée Berthier, Laetitia Desbarats, Jan Van der Pers[1] and Angel Guerrero[2]

INRA Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint Cyr, F-78026 Versailles, France, [1]SYNTECH VDP Laboratories, PO Box 1547, 1200 BM Hilversum, The Netherlands and [2]Department of Biological Organic Chemistry, CID (CSIC), Jordi Girona 18–26, E-08034 Barcelona, Spain

Correspondence to be sent to: Dr Michel Renou, INRA Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint Cyr, F-78026 Versailles, France. e-mail: renou@versailles.inra.fr

Abstract

The effects of 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP), a trifluoromethyl ketone that inhibits antennal esterases, on male *Mamestra brassicae* responses to the main pheromone component have been investigated using an actograph. This actograph used a movement detector based on the Doppler effect. The signal from the detector was digitalized and analysed on a PC microcomputer to quantify male activity. When added to the air flowing through the observation chamber, OTFP inhibited the responses of male moths to the pheromone. The number of males responding to the pheromone and the intensity of the response were decreased by OTFP. The latency of the response was increased and its duration decreased. These effects on the kinetics of the behavioural response cannot be directly correlated to the inhibition of pheromone catabolism by OTFP and other targets must be involved. The high level of inhibition of behaviour observed in presence of OTFP demonstrates the interest of trifluoromethyl ketones as mating disruption agents for pest control.

Introduction

Male moth behaviour in response to their specific sex pheromone has been intensively investigated using a variety of experimental set-ups (Baker and Cardé, 1984; Wyatt, 1997). Specifically designed olfactometers and wind tunnels have been widely used to measure orientation in a pheromone plume (Baker and Linn, 1984). Qualitative observations are essential to divide the behaviour into units to records, such as take off, wing fanning, landing at the source, copulation attempts. Scoring of these elementary responses are used to quantify the level of activity triggered by a chemical stimulus. Further refinement of the method resulted from tracking walking or flying insects with a video camera, or recording locomotory paths with a servosphere (Kramer, 1975) or other locomotion compensators, combined with the automatic analysis of tracks by computer-assisted trajectometry. All these methods require a sophisticated and expensive equipment and/or demand a large number of insects to achieve quantification of behaviour.

In the course of our studies on the effects of pheromone analogues on olfaction (Parrilla and Guerrero, 1994; Renou *et al.*, 1997) we needed a simpler device to screen candidates for inhibition of pheromone communication in insects. The Doppler radar has already been successfully employed in the monitoring of spontaneous activity rhythms of flies and cockroaches (Buchan and Satelle, 1979; Buchan and Moreton, 1981). Thus, we designed a simple recorder using a cheap movement detector, also based on Doppler radar. In this paper we describe the system and its use to record moth responses to a chemical stimulus. The movements of the insect are converted in an analogue electrical signal by the radar detector with a high signal to noise ratio. This signal is connected to a computer via an analogue/digital card and analysed with dedicated software. The radar detector has been used to measure the responses of male *Mamestra brassicae* to the main component of the sex pheromone, (*Z*)-11-hexadecenyl acetate (Z11-16:Ac) (Descoins *et al.*, 1978) either in pure air or in air containing the esterase inhibitor 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP). *In vitro*, OTFP is a very potent inhibitor of the antennal esterases of the Egyptian armyworm, *Spodoptera littoralis* (Duran *et al.*, 1993), enzymes responsible for the catabolism of the pheromone (Quero, 1996). *In vivo*, OTFP has been shown to alter pheromone detection (Renou *et al.*, 1997; Pophof, 1998) and to inhibit behaviour in the wind tunnel after conditioning males to a OTFP-saturated atmosphere. In the present study we show that an aerial

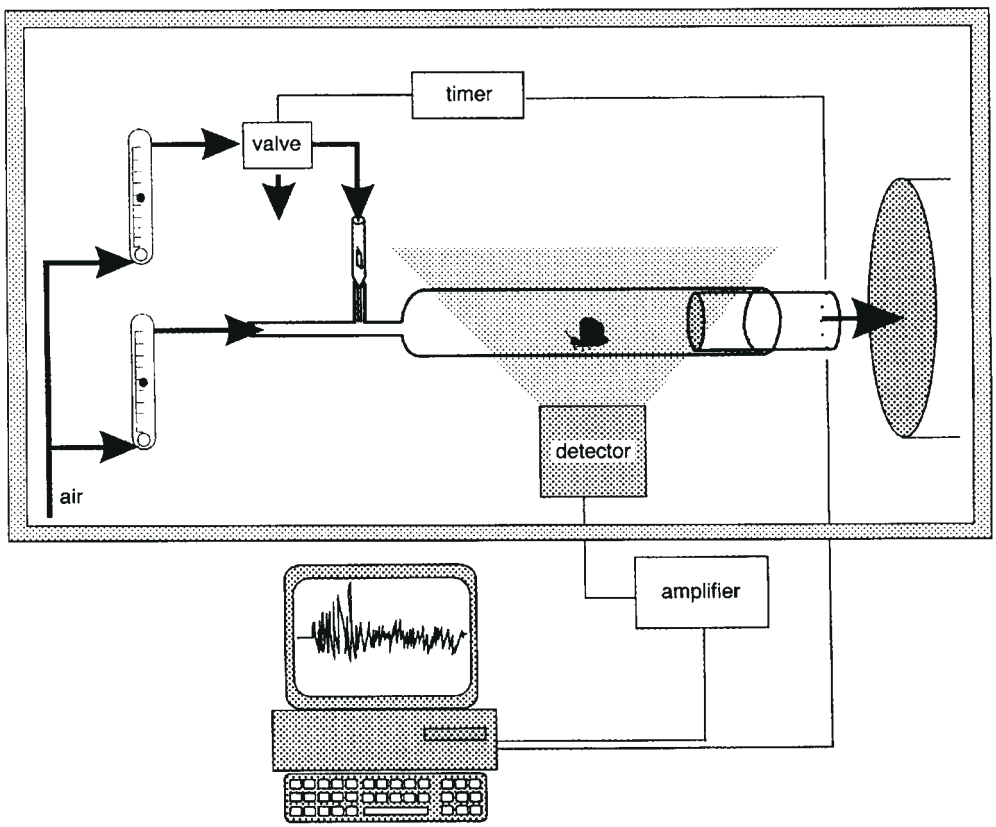

Figure 1 Schematic diagram of the experimental set-up.

background of OTFP during the test also affects the response to the pheromone. The effects of OTFP on the response kinetics are commented.

Material and methods

Insects

Mamestra brassicae were reared in the laboratory on an artificial medium. Male and female pupae were sorted and kept separately. Adult male moths were provided with a 10% sucrose solution and conditioned to a 16:8 photoperiod. Behavioural tests were performed on 3–5 day old males, during the last 3 h of the scotophase.

Compounds

OTFP was obtained by alkylation of the corresponding thiol with 3-bromo-1,1,1-trifluoropropan-2-one (Parrilla *et al.*, 1994). HPLC-purified Z11-16:Ac, free of alcohol, was prepared in the laboratory. Dilutions in hexane of both compounds were obtained at the appropriate concentrations.

Experimental set-up

To establish a homogeneous field of odourized air and to prevent the insect from moving out of the radar beam an observation chamber was made of a glass tube, 120 mm in length × 30 mm i.d., with an upwind end of smaller diameter (6 mm i.d.) and connected to a permanent source of charcoal-filtered air (85 l/h). A lateral branch, perpendicular to the main branch, was used to introduce a Pasteur pipette containing a piece of filter paper loaded with the pheromone. Air from the observation chamber was evacuated out of the room by an exhaust fan. The set-up (Figure 1) was enclosed in a cabinet installed in a climate room at 25°C. Red light provided by a 60 W incandescent lamp positioned above the observation chamber was adjusted at 0.3 lux. Stimulation was achieved by applying an air puff (3 s, 21 ml) through a Pasteur pipette containing a filter paper loaded with 0.1 μg of Z11-16:Ac. After each test, the observation chambers were washed overnight with a 5% solution of Decon (Prolabo, Paris), rinsed in distilled water and dried at 110°C, before being used again.

The Doppler radar sensor (Alpha Industries, Type GOS2780) had a working frequency of 24 GHz and an output power of 3 μW. The output signal was amplified ×10 by a specially designed AC amplifier. The raw analogue signal was high-pass filtered, amplified ×10 and fed into an acquisition board IDAC-02 (Syntech, Hilversum) in a PC-based microcomputer. The board had a 16 bit A/D converter, a software controlled amplifier, programmable DSP and input–output FIFO buffer memory. Data acquisition was performed at 500 samples/s during 15 min. To

Table 1 Effects of increasing doses of OTFP on male *M. brassicae* activity after a 3 s stimulation with 100 ng of Z11-16:Ac, the main pheromone component

	n	% males activating at OTFP onset	% positive responses to Z11-16:Ac	Mean latency of male activation (s)	Mean activity amplitude over 60 s	Mean activity amplitude over 300 s
Control	58	18.4% c	77.6% a	69.1 ± 151.9 c	73.7 ± 58.2 a	60.4 ± 40.5 a
OTFP, 0.1 µg	30	36.7% b	80.0% a	81.9 ± 179.9 bc	82.7 ± 80.4 ab	54.4 ± 43.7 a
OTFP, 1 µg	26	96.0% a	23.1% ab	402.3 ± 278.2 a	11.4 ± 4.1 c	9.8 ± 13.2 b
OTFP, 10 µg	25	96.0% a	56.0% b	118.9 ± 182.7 bc	19.5 ± 22.5 bc	11.6 ± 8.6 b
OTFP, 100 µg	28	67.9% b	30.7% b	194.2 ± 231.5 ab	19.2 ± 27.4 bc	20.8 ± 24.5 b

Control insects received solvent only. Positive responses consider only the male moths that initiated their activity <60 s after the puff of Z11-16:Ac. Mean latency and mean activity amplitudes (± SD) are calculated over the whole population of tested insects. Numbers within columns followed by the same letter are not significantly different.

Table 2 Effects of OTFP on intensity and temporal parameters of the responses of male *M. brassicae* to a puff of 100 ng of Z11-16:Ac

	n	Mean response latency (s)	Duration of the first burst (s)	Mean activity amplitude during first burst	Total duration of response (s)	Mean activity amplitude during response
Control	45	5.7 ± 7.9 b	158.8 ± 145.6 a	109.1 ± 96.4 a	317.5 ± 154.3 a	75.1 ± 31.3 a
OTFP, 0.1 µg	24	11.8 ± 13.6 a	51.2 ± 37.0 b	124.9 ± 83.3 a	222.3 ± 167.9 ab	85.8 ± 40.5 a
OTFP, 1 µg	6	7.2 ± 3.6 ab	36.2 ± 28.0 b	49.5 ± 16.9 ab	56.5 ± 41.8 c	56.0 ± 27.7 ab
OTFP, 10 µg	14	11.0 ± 12.4 a	32.6 ± 22.2 b	46.1 ± 36.2 b	74.7 ± 77.0 c	34.8 ± 21.2 b
OTFP, 100 µg	10	15.5 ± 16.4 a	49.3 ± 47.9 b	58.7 ± 52.4 c	100.1 ± 102.2 bc	53.5 ± 38.9 ab

Behaviours of male moths that initiated their activity >60 s after the puff of Z11-16:Ac were not considered to calculate response parameters. Values are means ± SD. Numbers within a column followed by the same letter are not statistically different (Kruskal–Wallis, $P < 0.01$).

save memory space, software integration enabled us to save only 10 samples/s into a file on the hard disk. The software EAG for Windows (Syntech, Hilversum) was used to edit the recordings and to make direct measurements of the response parameters. Preliminary calibration experiments showed that maximum sensitivity was reached when the radar beam was focused perpendicularly to the chamber, the detector being placed in front of the middle of the observation chamber, 25 mm from the wall. These experiments showed that the amplitude of the signal from the radar depended on the intensity of the movement, its direction and the position of the insect relative to the radar beam. Positive peaks corresponded to movements of the insect toward and negative peaks away from the radar probe. Thus, the male locomotory activity could be easily quantified by summating the absolute values of the signal amplitude over the time.

Bioassays

A single male was introduced into a clean observation chamber and left to acclimate for 1 h in the experimental room. Then, OTFP-loaded air was applied into the chamber by introducing a filter paper loaded with the appropriate amount of OTFP, diluted in hexane, into the air inlet tube. After a minimum time of exposure to OTFP of 180 s, a 3 s puff of pheromone was applied. The insect movements were continuously recorded during the pre-stimulation period and 600 s after stimulation with Z11-16:Ac. Control experiments were performed using a filter paper loaded with pure hexane as a treatment and a puff of Z11-16:Ac as a stimulus. The following parameters were measured (Table 1): percentage of males activating at OTFP onset, mean latency of male activation after Z11-16:Ac stimulus, and mean amplitude of activity over 60 s and 300 s following the puff of Z11-16:Ac. The behaviours of male moths that initiated their activity later than 60 s after the puff of Z11-16:Ac were not considered as positive responses. Thus, the percentage of positive responses to Z11-16:Ac is given in Table 1 and the behaviour of male moths that met this criterion is analysed separately in Table 2. Data were submitted either to χ^2 tests for number of responses or to Kruskal–Wallis analyses for duration and amplitude of responses.

Results

Responses to Z11-16:Ac in pure air

When stimulated with a puff of air from a source loaded with 0.1 µg of Z11-16:Ac, male *M. brassicae* became very active, performing wing fanning and intense locomotion in

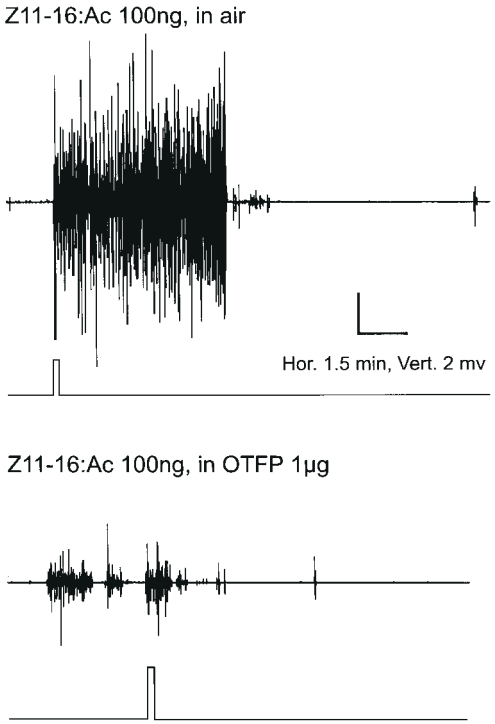

Figure 2 Examples of Doppler radar recorded responses of male *M. brassicae* to 100 ng Z11-16:Ac in pure air (upper recording) or in OTFP-loaded air (lower recording). Lower traces in both recordings indicate the puff of pheromone.

the observation chamber. Both activities are typical of male moth precopulatory behaviour and they were easily detected by the radar sensor, producing bursts of high-amplitude spikes (Figure 2). The male responses were recorded simultaneously with video and the radar actograph to determine if different behaviours produced different signals. When the insect was immobile the background signal amplitude was <0.5 mV. Movements of antennae, wings or legs produced signals of <1 mV. Displacements in the observation chamber produced signals comprised between 2 and 5 mV whereas the signal could reach 12 mV when locomotion was associated to wing fanning.

Most of the 58 male moths became active within a few s after the puff of Z11-16:Ac (n = 45, 77.6%). Nine males (15.5%) exhibited an intense activity after 60 s and four insects (7.0%) did not respond at all to the pheromone puff. The average amplitude of activity during the 60 s following the puff of Z11-16:Ac was 73.7 (Table 1, control). Males maintained a high level of activity for several minutes after the stimulus, as shown by the mean amplitude calculated over 300 s (60.4).

Behaviours of male moths that initiated their activity after 60 s were not considered as positive responses to the pheromone and temporal response patterns were analysed separately (Table 2) in the 45 male moths that were activated within 60 s after the puff of Z11-16:Ac. The mean latency of response in these males was of 5.7 s (Table 2). Furthermore, a male moth was considered to have completed its response when it was inactive for >60 s. According to these two criteria, the mean duration of the response to Z11-16:Ac in air was 317.5 s (Table 2). The mean activity amplitude during the whole response was 75.1. Short pauses within a response enabled the discrimination of individual bursts of activity (mean number: 4.3). The first burst of activity was generally longer than the subsequent one (mean duration: 158.8 s). The mean activity amplitude during this first burst reached 109.1.

Responses to OTFP

The introduction of variable concentrations of OTFP into the airflow elicited locomotory behaviour on males. At doses of OTFP between 1 and 100 µg, 67.9 to 96% of males were active during the pre-test period (Table 1), versus 18.4% in control insects exposed to pure air. At 0.1 µg, the percentage of males exhibiting pre-test activity was 36.7%, a value still significantly different from control (χ^2 = 24.36, P < 0.01).

Responses to Z11-16:Ac in the presence of OTFP

Male responses to a puff of Z11-16:Ac were measured in OTFP-loaded air. Pheromone triggered activity was strongly reduced in the presence of OTFP, as shown by significantly decreased amplitude of activity within 60 and 300 s after the stimulus (Figure 2 and Table 1). The number of male *M. brassicae* becoming active within 60 s after the pheromone puff (positive responses to Z11-16:Ac) was significantly reduced in the presence of OTFP. With 0.1 µg of OTFP most of the males still responded to the pheromone (80.0%); but with 1 and 100 µg of OTFP the numbers of positive responses were significantly smaller than the control, <30% of males showing post-stimulus activity (Table 1). With 10 µg of OTFP, 56% of males showed post-stimulus activity. However, the activity was low (19.5) and brief (74.7 s). Thus, this high score of males showing activity is probably due to the difficulty in discriminating between OTFP- and pheromone-triggered activities, OTFP itself triggering intense locomotory activity in the males.

The temporal characteristics of the response to Z11-16:Ac were also modified in the presence of OTFP. First, the latency of the response was significantly longer with 0.1, 10 and 100 µg (Table 2). Secondly, the duration of the first burst and the overall duration of the response were shorter than in control insects. Thirdly, the mean amplitude during the first burst and the overall mean amplitude were lower.

Discussion and conclusion

The radar sensor presents a number of advantages that make it an appropriate and interesting tool not only to monitor sustained spontaneous activity (Buchan and

Satelle, 1979; Buchan and Moreton, 1981) but also to record transient responses to stimulus. This is particularly true when the temporal patterns of the response are features of interest. First, it can work at low light intensity or in complete darkness, and the radar beam can pass through glass and plastic walls of insect containers with no interference regardless of the orientation of the beam with respect to the wall. Secondly, it can be focused on the target insect and has a low sensitivity to noise. Thirdly, it is cheap, simple and easy to handle. Moreover, it can provide very reliable recordings of the timing and sequence of the response, and can be used either with single individuals or with several insects. Further development aiming to improve quantification of the male responses is in progress.

The behavioural responses of male *M. brassicae* to the pheromone main component were reduced or even suppressed in the presence of the trifluoromethyl ketone OTFP in the air. These effects were dose-dependent. These results are consistent with former experiments in a wind tunnel that showed inhibition of the flight of male *Spodoptera littoralis* to a pheromone source after a 4 h exposure to air with a high concentration of OTFP (Quero, 1996). In the experiments presented here the inhibitory effects arose after a brief exposure and at lower concentrations of OTFP. Electrophysiological investigations (Renou *et al.*, 1997; Pophof, 1998) have shown that OTFP, like other trifluoromethyl ketones analogues of pheromone, inhibits the firing responses of the olfactory receptor neurones to pheromone components in different moth species, including *M. brassicae.* Thus, the effects of OTFP on behaviour are most probably due to its inhibitory activity on the peripheral sensory system. The high level of inhibition of behaviour observed in the presence of OTFP demonstrates the interest of trifluoromethyl ketones as mating disruption agents for pest control.

Flying male moths following an aerial pheromone trail exhibit very fast reaction times to fluctuations in pheromone concentration (Baker and Vickers, 1995). This outstanding capacity relies both on the functional ability of the olfactory neurones to resolve repetitive stimulus rates (Rumbo and Kaissling, 1989) and on efficient mechanisms of pheromone deactivation (Vogt *et al.*, 1985). Any factor altering one of these two mechanisms should also strongly affect moth behaviour. Thus, we expected that OTFP, which inhibits *in vitro* the degradation of pheromone components with an acetate function by the esterases contained in antennal extracts (Parilla and Guerrero, 1994; Parilla *et al.*, 1994), would increase *in vivo* the duration of the behavioural response. However, besides its effects on response amplitude, OTFP increased the latency of the response to Z11-16:Ac and strongly reduced the duration of the first burst and of the response. These two effects cannot be directly correlated to the inhibition of pheromone catabolism by OTFP, so other mechanisms must be involved. Potential molecular targets for OTFP are the receptors and pheromone binding proteins (PBP). Evidence for a reaction of OTFP with PBPs comes from *in vitro* binding experiments that show competition between tritiated Z11-16:Ac and its TFMK analogue for the binding sites of the PBPs (P. Nagnan, unpublished data). *In vivo*, due the role of PBPs as carriers for pheromone molecules, such a competition would reduce the availability of the stimulus for the receptor sites, resulting in a decrease of the response.

Acknowledgements

We thank Dr Jean-Pierre Rospars, Dr Arthur Vermeulen and Dr Pascal Chalande for helpful discussions, Dr Philippe Lucas for a critical reading of the manuscript, Mrs Martine Lettere for preparing Z11-16:Ac and Mr. Taylor Quadjovie for rearing the insects.

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Accepted April 16, 1999