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Behavioural responses of the female melon fly Bactrocera cucurbitae to male produced sex pheromone

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Abstract

Behavioral responses of female *Bactrocera cucurbitae* to the male sex pheromones were studied by using a double-choice olfactometer with an air flow. Sexually mature females responding to the male pheromone during dusk period showed various types of behavioral activities. The most prominent sexual responses of females observed during the study were, attraction to the males and probing of the ovipositor. Wing vibration of males which is considered to be a mating call was observed in *Bactrocera cucurbitae* only at dusk. Behavioral responses of females observed in the olfactometer confirm the storage and release of a male sex pheromone which is highly attractive to females. Previous mating reduced the responsiveness of females to the male pheromone, whereas it neither affected the release of the pheromone by males nor diminish the ability of males to attract females.

Key words: *Bactrocera cucurbitae,* behavioral responses, sex pheromone, attraction, ovipositor probing, olfactometer

Introduction

Bactrocera (=*Dacus*) *cucurbitae* (Coquillet), commonly known as the melon fly is a major pest of vegetables such as bittergourd (*Mormodica charantia*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita maximus*) etc. and some times it also acts as an occasional pest of *Anona reticulata, Capsicum* spp., *Psidium* spp. *and Phaseolus* spp.

The existence of male produced sex pheromones have been reported for several species of tephritid fruit flies including *B. tryoni, B. dorsalis, Anastrepha suspensa* and *Rhagoletis cerasi* (Fletcher, 1968: Nation, 1972: Katsoyannos,

1976: Kobayashi et al, 1978). Sexual activities in many sp. are restricted to the dusk period. During this period, the males release a sex pheromone which attracts females. Virgin females are reported to respond to these pheromones over short distances (Drew, 1987). The presence of a male pheromone in the melon fly was suggested and smoke emission was observed at dusk by sexually mature flies densely confined in a cage (Ohinata et al, 1982). Furthermore, pheromone clouds sprayed by male melon flies were visually detected by focusing a beam of light at them during dusk when the males were vibrating their wings (Kuba & Sokei, 1988). It is also reported that *B. carambolae*, another fruit pest of economic importance, release volatile constituents into the air as a visible smoke when confined in a glass chamber (Wee & Tan, 2005).

Glandular structures in the male melon fly which were suspected to be the sex pheromone glands were described by Schultz and Boush (1971). Kobayashi et al (1978) stated that the source of production or storage of the sex pheromone in *B. cucurbitae* is the rectal gland. It consists of two main structures, a reservoir and a secretary sac.

The present investigation was carried out to study the behavioural responses of the female *B. cucurbitae* to the male sex pheromone with the view of assessing the potential of this pheromone for future use as trap bait for monitoring and possible control programmes of this pest.

Materials and Methods

Maintenance of the laboratory culture

Melon flies were obtained from infected bittergaurd (*Mormodica charantia*) fruits from the field. These were placed in enamel basins and covered with white nylon net. After emergence the adult males and females were transferred from the enamel basins into wooden mesh cages (32×32×32cm) using a plastic battery aspirator. The adults were maintained on a diet of sugar, water and dried brewer's yeast. Females were allowed to lay eggs in bittergaurd fruits placed in cages and these were transferred into enamel basins with soil for further development. The cultures were maintained at a temperature of 29±2ÚC and a 78±2% relative humidity. A 10 hour artificial light period was supplied with a 2 hour dusk and 2 hour dawn period. When unmated flies were required, the adults were separated within the first 24hours after emergence. The females were held in a separate room to avoid exposure to males or male odours before experiments.

Bioassay apparatus

The bioassay apparatus consisted of a dual - choice olfactometer (Figure 1) which was made of transparent perspex. This olfactometer consisted of three primary elements; the test cage, the chamber system and the bait cage. The test cage (16×16×16cm) held the test insects (i.e. females). One side of the test cage had a large hole to connect it with one end of the chamber system and the opposite side was covered with a nylon mesh. The other end of the chamber system was connected to the bait cage (16×16×16cm). The bait cage with its opposite side covered with a mesh held the males or the pheromone source. The chamber system consisted of three units (11.25×11.25×35cm length) which were connected together. All three units were about one meter long. The end of the chamber system which was connected to the bait cage was covered with a mesh to prevent the females contacting the males or the pheromone. A glass funnel was fixed into the other end of the chamber system to allow the responding flies to move towards the pheromone source. A fan blew an air stream through the olfactometer. It was adjusted to produce an air stream of 25 ml/min. The olfactometer was placed in a room with the temperature maintained at 29±2ÚC and Relative Humidity of 78±2%. The air stream carried the odour from the bait cage to the test cage through the opening of the funnel.

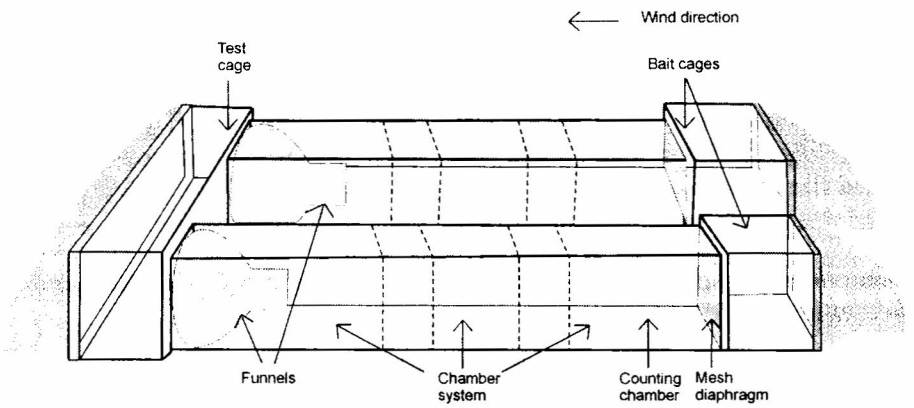

Figure 1 Dual choice olfactometer

Experimental protocol

The test flies (females) were introduced into the cage one hour before the commencement of the experiments in order to allow them time to adapt to the new environment. Just before the beginning of the bioassay, the bait cage containing the treatment (males or pheromone source) was connected to the chamber system and the air stream was generated.

All the experiments were conducted during the 'dusk' period between 18.00-20.00 hours and each test was carried out for 30 minutes. Males and females which were 6-8 days old were taken for all experiments as the intensity of their responses and sexual activities were highest during this period.

Two types of female responses were recorded during each test.

(i) Attraction

Identified as the number of flies moved into the counting chamber, after each observation period

(ii) Probing with ovipositor

As described by Fletcher and Giannakakis (1973) in which females probed the ovipositor through the mesh which separated them from the males or the pheromone in a manner similar to oviposition.

Twenty females were used for each experiment and at the end of each, the flies which moved into the counting chamber were returned to the test cage. Each experiment was repeated five times using a fresh batch of test flies. All the units of the olfactometer were cleaned after each test by washing them thoroughly with a mild detergent to prevent contamination.

Effects of the state of the flies on the responses of females

The experiment was designed using 20 males and 20 females in the following combinations.

Virgin females and virgin males
Virgin females and mated males
Mated females and virgin males
Mated females and mated males

Flies which mated once were used for this experiment. (Flies which stayed together for more than two hours after copulation were considered as mated flies.) They were used in the experiments 1-2 days after they mated. A control test without males was carried out for each combination.

Behavioral responses of virgin females to the male sex pheromone presented in different ways.

Virgin females were tested against different numbers of live males, male abdomens and pheromone glands to study their effects on the female responses.

(i) Effect of live males on the responses of virgin females

Unmated male flies were placed in an open-ended container made of cardboard (5×5×5cm). The open ends of this container were covered with white mesh for air to pass freely through and facilitate the distribution of the released pheromone. The container with the males was kept in the test cage. An identical empty container was used as the control. Observations were made using 20, 30, 40, 50, and 60 unmated live males with twenty virgin females in each experiment.

(ii) Effects of the abdomens of males on the responses of virgin females

The abdomens were cut from anesthetized males just before the onset of the bioassay and were kept in chilled saline (0.9 NaCl) until required. They were crushed on the center of a filter paper disk immediately prior to the test to release the pheromone and placed in the test cage. 5, 10, 15, 20, 25 and 30 abdomens were used in the experiment. An untreated filter paper disk was tested against females as the control.

(iii) Effect of pheromone glands of males on the responses of virgin females

The rectal gland complexes of sexually mature males were removed under a dissecting microscope shortly before a bioassay. This was done by grasping the aedeagus with dissecting forceps and gently pulling the gland out, being careful to avoid puncturing the gland. To standardize the quantities, only the glands that were full of secretion were used for bioassays. Just before each test, these glands were squashed on a filter paper disk and paced inside the test cage. 6, 9, 12, 15 and 18 pheromone glands were tested against twenty female flies.

Statistical Analysis

Data were analyzed by using a one-way analysis of variance (1-way ANOVA). When the analysis indicated a significant difference between responses to different treatments, Duncan's multiple range test was carried out at $p=0.05$ level of significance to identify which of the treatments were significant. Student t-test was applied to test the significant differences between the treatment and corresponding control.

Results

Preliminary observations of female behavioral responses to male pheromone

At the beginning of the dusk period, the females were more active than during the day time. Just after the male flies were introduced into the bait cage, the behavior of the female changed considerably. They tried to fly towards the bait cage and showed various types of behavioral activities while gradually approaching the male cage. The observations clearly showed that the majority of female flies exhibited characteristic responses during dusk as their mating is restricted only to this period. These behavioral activities of the females and the males commenced within the dusk period and declined noticeably after dusk. Various types of female behaviors which occurred in response to the male pheromone were observed during the dusk period. Some of the significant behaviors observed during this period were, walking or running (locomotion), raising of both wings, cleaning of the wings, spinning and turning, female approaching the male (attraction), lateral rocking, fighting with other females, bobbing of the abdomen, cleaning of the ovipositor and probing with the ovipositor.

Effects of state of the flies on the responses of females

The mean responses of the females to different combinations of flies are presented in Figures 2 and 3. The responses (i.e. ovipositor probing and attraction) of virgin females were observed within 2-3 minutes after the introduction of mated or unmated males, but not with the mated female flies. The responses of the virgin females to unmated or mated males were considerably higher than those of mated females. Responses of virgin females to unmated males were not significantly different from those of to the mated males. Similarly responses of mated females to unmated and mated males were not significantly different from each other. Furthermore, female responses to all the treatments were significantly higher than those of the respective controls.

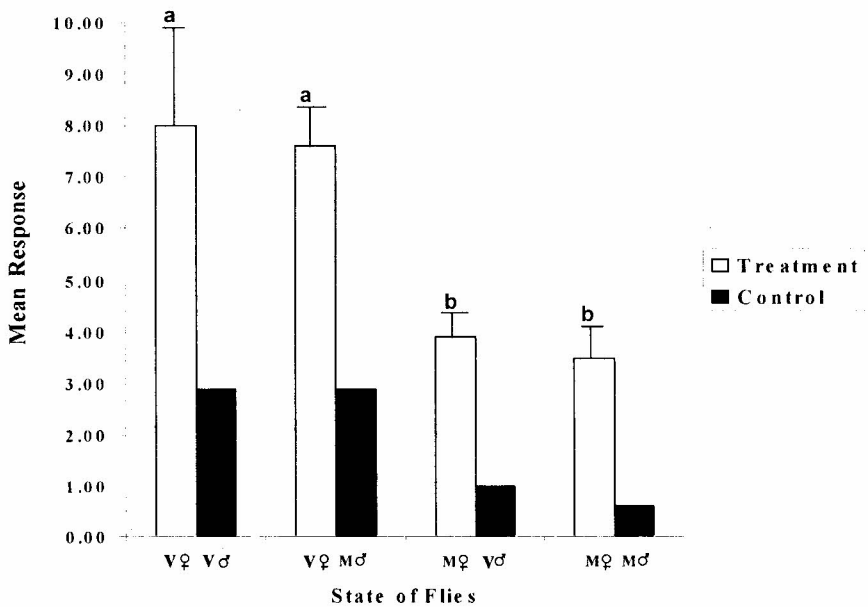

Figure2. Attraction response of female *Bactocera cucurbitae* with different state of flies

- Mean values followed by the same letters on each bar are not significantly different at 5% level (Duncan's Multiple Range Test); F Value = 37.56

- Mean responses for five replicates each
  Virgin females to Virgin males (V♀ - V♂)
  Virgin females to Mated males (V♀ - M♂)
  Mated females to Virgin males (M♀ - V♂)
  Mated females to Mated males (M♀ - M♂)

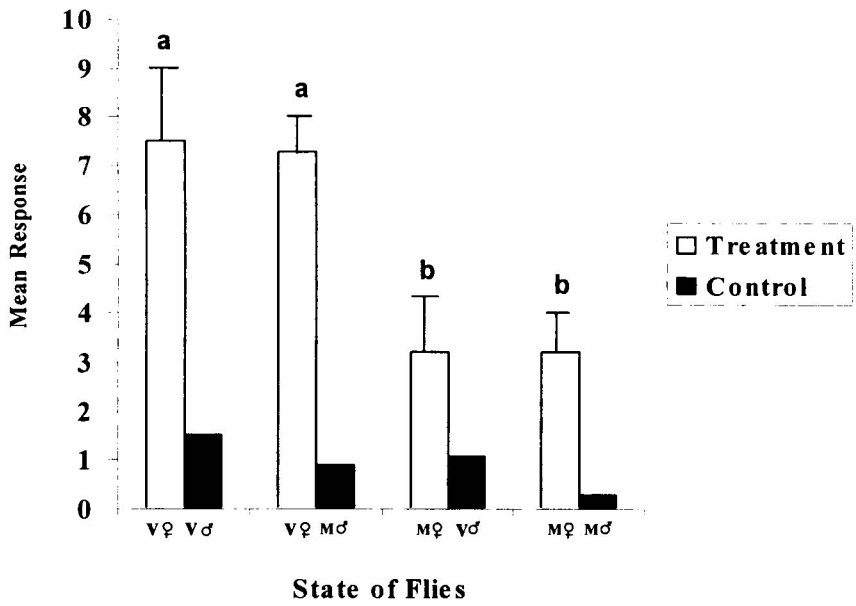

Figure3. Probing response of female *Bactocera cucurbitae* with different state of flies

- Mean values followed by the same letters on each bar are not significantly different at 5% level (Duncan's Multiple Range Test); F Value = 27.41

- Mean responses for five replicates each

Virgin females to Virgin males (V ♀ - V♂)
Virgin females to Mated males (V ♀ - M♂)
Mated females to Virgin males (M ♀ - V♂)
Mated females to Mated males (M ♀ - M♂)

Effects of live males on the responses of virgin females

Behavioral responses of virgin females increased with the increase of the number of males (Table 1). Both responses (attraction and probing) varied in a similar pattern. Females showed their highest responses, when they were tested against 50 and 60 males. These two responses were not significantly different from each other among all experiments.

Table 1. Behavioral responses of virgin *B. cucurbitae* females to live males

Number of males	Attraction		Probing with the ovipositor	
	Treatment	Control	Treatment	Control
10	5.80±0.83**a**	1.00±0.00	5.20±0.83**a**	1.00±0.70
20	8.00±0.54**b**	1.20±0.44	7.80±0.83**b**	1.00±0.00
30	10.00±0.71**b**	1.00±0.00	10.00±0.70**c**	1.80±0.83
40	12.00±0.71**b**	0.00±0.00	12.00±0.71**d**	2.00±0.00
50	15.20±0.83**e**	0.20±0.00	14.80±0.83**e**	2.40±0.54
60	15.40±0.54**e**	2.20±0.83	15.00±1.41**e**	0.60±0.89
F value	**99.59**		**99.27**	

N=100 (5 replicates); (Mean± S.E.)

Mean values of treatment and control in each test, were significantly different at 5% level. (Student's t-test).

Means followed by same letters in each column were not significantly different at 5% level. (Duncan's Multiple Range Test).

Effects of abdomens of males on the responses of virgin females

Table 2 depicts the responses of virgin female flies to different numbers abdomens of males. The responses increased significantly with the increase of the number of abdomens. Maximum responses were reached when the females were exposed to 25 and 30 abdomens. These responses were significantly higher than the rest of the treatments. Also, all the treatments were significantly different from their respective controls.

Table2. Behavioral responses of virgin *B. cucurbitae* females to male abdomens

Number of abdomens	Attraction		Probing with the ovipositor	
	Treatment	Control	Treatment	Control
5	7.00±0.00**a**	0.40±0.20	4.40±1.14**a**	1.20±0.44
10	10.00±1.58**b**	0.20±0.15	8.80±0.83**b**	0.20±0.44
15	11.80±0.83**b**	1.40±0.53	10.60±1.51**c**	1.40±0.54
20	14.00±1.00**b**	1.80±0.44	12.80±1.87**d**	1.60±0.54
25	16.40±0.54**e**	0.20±0.00	15.20±0.83**e**	1.00±0.00
30	16.40±0.54**e**	0.20±0.00	15.00±0.70**e**	2.00±0.70
F value	**72.00**		**47.54**	

N=100 (5 replicates); (Mean± S.E.)
Mean values of treatment and control in each test, were significantly different at 5% level. (Student's t-test).
Means followed by same letters in each column were not significantly different at 5% level. (Duncan's Multiple Range Test).

Effect of pheromone glands of males on the responses of virgin females

The responses exhibited by virgin females to the male pheromone glands are presented in Table 3. The responses (attraction and probing) of virgin females increased immediately (after 1-2 seconds), when they were presented with a higher number of pheromone glands. Responses reached a constant level earlier (12 ph.glands) than in the previous experiments. Beyond that level, the responses did not change significantly. The controls were significantly different from their respective treatments.

Table3. Behavioral responses of virgin B. cucurbitae females to male pheromone glands

Number of pheromone glands	Attraction		Probing with the ovipositor	
	Treatment	Control	Treatment	Control
3	6.20±0.84a	0.80±0.45	4.40±1.14a	1.20±0.45
6	10.00±1.00b	1.60±0.89	9.20±0.84b	0.80±0.45
9	11.60±1.14c	2.20±0.45	11.00±0.71c	1.00±0.71
12	17.20±0.83d	2.20±0.45	14.60±1.14d	2.20±0.84
15	17.20±1.30d	2.40±0.55	15.20±0.84d	1.00±0.00
18	17.20±0.84d	2.20±0.45	15.40±0.55d	0.80±0.45
F value	**107.99**		**117.94**	

N=100 (5 replicates); (Mean± S.E.)
Mean values of treatment and control in each test, were significantly different at 5% level. (Student's t-test).
Means followed by same letters in each column were not significantly different at 5% level. (Duncan's Multiple Range Test).

Discussion

Several types of behavioural activities of mature females of *B. cucurbitae* were observed in response to males or the male pheromone. Before the introduction of males, the females were either motionless or walking slowly. An increase in their locomotor activities and preening accompanied by rapid vibration of the wings occurred within several seconds after the introduction of males or the pheromone. Also, responding females moved through the funnel towards the bait cage (attraction) and inserted their ovipositors through the net which separated them from the pheromone source. Kobayashi et al (1978) in their studies found that virgin females of *B. dorsalis* during the responding period clustered around the vials containing males and probed with their ovipositors through the holes in the walls. Karunaratne (1995) stated that the most prominent female sexual behaviour observed during mating of *B. dorsalis* was the probing of the plant with the ovipositor in a manner very similar to the

normal ovipositional behaviour. Similar observations had been made for other tephritid flies such as *B. tryoni* and *Ceratitis capitata* (Fletcher, 1987; Tychsen, 1977). As females display the ovipositor probing behaviour only during the dusk period when they are sexually excited, it is probably a copulatory response elicited by the presence of the male pheromone to indicate that they are receptive to the male.

During the dusk period, sexually mature males also increased their locomotory activities (rapid walking and erratic flying) and other courtship behaviour including wing fanning. Under laboratory conditions, they were observed on the walls of the cage vibrating their wings rapidly producing a high pitched 'buzz' which is audible to the human ear. This behavioural pattern is similar to that recorded for *B. cacuminata* where Drew et al (2008) reported that at dusk period males began rapid fanning of their wings in brief bursts and a distinct buzzing sound was also made during such wing fanning. Similarly Wee & Tang (2005) observed wing fanning associated with anal beating behaviour in *B. carambolae* during the dusk period. This wing fanning is considered to be a mating call (Monro, 1953).

Courtship behaviour is accompanied by the release of a substance which is secreted and stored in a gland associated with the rectum (Schultz & Boush, 1971; Nation, 1981; Wee & Tan, 2005). During the dusk period, this gland is presumed to secrete volatile compounds which act as a sex pheromone. The release of sex pheromones by males to attract conspecific females is common in many other species of tephritids (Nation, 1972; Prokopy, 1975; Kuba & Sokei, 1988; Perkins et al., 1990; Pike & Meats, 2003). The wing vibration of the male is thought to produce an auditory signal and/or to propel the male pheromone towards the female (Kuba & Sokei, 1988; Webb et al, 1983a). When the observations of the behavioural responses of *B. cucurbitae* are taken into account, it is evident that sexually mature males produce a pheromone which is highly attractive to mature virgin females. These results corroborate with the observations of other workers confirming the presence of a male sex attractant which controls the mating behaviour in the females. The restriction of female responsiveness and the release of the sex pheromone by the male at the dusk period showed that the timing of their response is controlled by an interaction between light intensity and a circadian rhythm (Tychsen & Fletcher, 1971).

Physiological factors such as mating have been shown to affect the responsiveness to the sex pheromone in many insect species. Studies on the responses of mated females indicated that soon after mating their attraction to the male pheromone decreases to a very low level. Kobayashi et al (1978) noted that previously mated oriental fruit fly females responded neither to live males nor rectal glands of the males. Studies on other tephritid fruit flies have also shown that mating strongly depressed responsiveness in females to the

male pheromone (Fletcher & Giannakakis, 1973; Robacker et al, 1965). It was suggested that re-mating by females probably occurs only when their viable sperm store depleted (Nakagawa et al, 1971). The observations of the present study on the other hand, suggest that previous mating does not affect the release of the sex pheromone or diminish the ability of males to attract females. Females increased their responses with the increase of the number of males, male abdomens and male pheromone glands. However, comparatively a lesser number of pheromone glands elicited the highest response in females. The comparatively lower responses to live males can be attributed to the fact that males may be releasing only the necessary amount of the pheromone which is sufficient for a successful mating during the dusk period. However, when pheromone glands were used, the whole amount which was stored in the glands was released to the environment, eliciting greater responses in the females.

The observations made during the study allow the conclusion to be drawn that the source of production or storage of the sex pheromone in *B. cucurbitae* males is the rectal gland. Also, the male sex pheromone elicited several types of behavioural activities associated with mating in females. Furthermore, the female responses to the male odorant observed in the olfactometer bioassays indicate that the male produced sex pheromone is a strong attractant with potential as trap bait for monitoring and possible control of this serious pest.

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