

Olfactory Responses of Gravid Melon Fly Females and *Bactrocera cucurbitae* (Coquillett) to Selected Cucurbitaceous Fruit Volatiles

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ABSTRACT

The present study investigates olfactory responses of gravid females to selected cucurbitaceous host fruit volatiles viz., ridge gourd (*Luffa acutangula* L.), cucumber (*Cucumis sativus* L.), bitter gourd (*Momordica charantia* L.) and snake gourd (*Trichosanthes cucumerina* L.) at different phenological fruiting stages viz., mature (10 days prior to harvest) and immature (10 days after fruit set) fruits. Olfactometer assays revealed that mature fruit volatiles elicited positive behavioural responses compared to immature fruit volatiles across the hosts. Further, multiple choice assays with mature fruit volatiles revealed significant ($P < 0.0001$) positive behavioural response by female flies to ridge gourd followed by snake gourd, cucumber and bitter gourd. The gas chromatography coupled mass spectrometry (GCMS) analysis of host fruit volatile samples showed varied volatile organic compounds across the host fruits and also between fruit phenological stages. Principal Component Analysis of volatile compounds revealed that chemical cues like β -cis-ocimene, ethyl-benzaldehyde, ethanone, 1-(4-ethyl phenyl), p-diacetylbenzene and 2, 4-dimethylacetophenone were responsible for the major variation among the volatile profiles of host fruits. Further, electroantennogram (EAG) studies indicated significant ($P < 0.0001$) antennal response to ridge gourd fruit volatiles followed by snake gourd, cucumber and bitter gourd.

Keywords : Gravid females, melon flies, cucurbit host fruits, volatiles, host preference, GCMS

THE melon fly, *Bactrocera cucurbitae* (Coquillett) (Tephritidae : Diptera) is one of the most devastating pests of cucurbitaceous vegetable crops. They usually lay eggs into the developing fruit that hatch out as larvae, causing heavy economic losses to farmers around the world. Depending on the species of the cucurbitaceous crop and the season, the yield losses due to melon fly varied from 30 to 100 per cent (Dhillon *et al.*, 2005). The cucurbit fruits are harvested at frequent intervals making it difficult to opt for insecticidal management. Further, to date, the melon fly management mainly revolves around male annihilation technique using cue lure traps to attract and kill male flies. However, as gravid females essentially forage for host fruits to lay eggs, there is a scope to identify potential ovipositional attractants and kairomones that are efficient in trapping the female melon flies. Use of female attractants in melon fly management to date are limited to food bait attractants such as fermenting sugars, hydrolysed protein, and yeast with limited success. Exploring the use of host plant kairomones that are capable of attracting gravid

females can be a promising option for melon fly integrated management programmes. Nevertheless, very limited research attempts were made in this direction to identify potent host plant kairomonal cues for female melon flies (Sulaeha Th *et al.*, 2017 and Siderhurst & Jang, 2006). The first step in identifying potent host kairomonal cues for any polyphagous or oligophagous gravid female insect is to locate the highly preferred host for oviposition.

Suitable host finding by female melon flies to oviposit is critically guided by volatile chemical cues (= kairomones) released by host plants and such cues are being explored as potent attractants for detection and management in several tephritids like *Bactrocera dorsalis* (Hendel), *Bactrocera invadens* Drew, Tsura and White, *Rhagoletis pomonella* (Walsh) (Kamala Jayanthi *et al.*, 2012 and Kimbokota *et al.*, 2013).

Therefore, it is very clear that the most promising attractants for female tephritids may be of host plant kairomones that are preferably used by them while searching suitable oviposition sites (Kamala Jayanthi

et al., 2012). Melon flies have a close relationship with diversified species of cucurbits and its preference to multiple species of cucurbits like cucumber, bitter melon, ivy gourd and zucchini has been documented in earlier studies (Pintero *et al.*, 2006). However, isolation and identification of attractants from these cucurbit fruits is not attempted. Further, it is possible that oligophagous insect pests like melon fly may respond to a wider range of chemical cues than other fruit flies that have narrow host range such as the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Siderhurst and Jang, 2006). Therefore, the objective of the present study was to identify the highly attractive host fruit cues for gravid female melon fly using olfactometer and electrophysiological studies.

MATERIAL AND METHODS

Plant and insect cultures

The selected host plants *viz.*, Ridge gourd (*Luffa acutangula* L.), Cucumber (*Cucumis sativus* L.), Bitter gourd (*Momordica charantia* L.) and Snake gourd (*Trichosanthes cucumerina* L.) (Family: Cucurbitaceae) were maintained in the experimental fields of ICAR-Indian Institute of Horticultural Research, Bangalore (12° 58' N, 77° 35' E, 890 MSL). The seed of Ridge gourd cv. Mallika, Bitter gourd cv. Palee, Snake gourd cv. Covai 951 of East-West Seed International and Cucumber cv. Malini of Monsanto holdings Pvt. Ltd. were procured and sown in the field following standard agronomic practices without any pesticide application. To avoid insect pest incidences, regular water sprays were given at frequent intervals. Two different phenological fruit growth stages namely immature (10 days after fruit set) and mature (10 days prior to harvest) were used for volatile collections.

The melon flies, *B. cucurbitae* were reared on ridge gourd in the laboratory. Fruits were exposed for 24 h to mixed populations of fruit flies (100 pairs) for oviposition. The oviposited fruits were kept in a plastic box containing fine sterilized sand. The sand was sieved after 10 days to facilitate pupal collection. The collected pupae were placed in wire mesh cages for adults to emerge at ambient room condition (27 ± 2°C, 75 per cent RH and 12:12 h dark: light cycle). The emerged adults were given 10 per cent honey solution

and yeast extract *ad libitum*. The gravid females of 15 days old were used for all behavioural as well as electrophysiological assays.

Volatile collection through air entrainment

Before collection of volatiles from fruit samples, all the glassware were washed with liquid detergent, rinsed with distilled water followed by acetone. Later, the glassware was dried in a hot air oven preheated to 200 °C for 2 h. The Porapak Q tubes (50 mg, 60/80 mesh; Supelco, Sigma Aldrich, India; length = 5 cm, I.D = 5 mm) used for capturing volatiles subjected to washing with redistilled diethyl ether and kept at 120 °C for 2 h under a stream of purified nitrogen to remove contaminants. Volatiles emanating from immature and mature fruits (n = 4) were collected by air-entrainment following Kamala Jayanthi *et al.* (2012). Volatiles of selected host fruits were collected by placing separately mature and immature stages inside a modified glass vessel with two parts (M/s. The Super Scientific, Bangalore, India). The upper portion was a lid having an inlet and outlet port, while the basal container supports in holding the fruit samples. Fruits were kept in the container secured with clips to flanges on the open ends of the vessel. Through the inlet port purified air was passed at the rate of 600 mL/min, the fruit volatiles were collected on to Porapak Q tubes fitted into the outlet port (800 mL/min). All connections were made with polytetrafluoroethylene (PTFE) tubing with ferrules and fittings. Fruit volatiles from the selected host plants were entrained for 24 h. Porapak Q tubes containing the volatiles were eluted with 750 µl of redistilled diethyl ether, making the test samples. Such volatile samples collected in glass vials were stored at -20 °C for further use.

Insect behavioural bioassays

Behavioural bioassays were conducted in two ways using four-arm olfactometer. Initially, the preference of *B. cucurbitae* gravid females to immature and mature fruit stages of selected host fruits was studied independently in single choice assays (n=12). Later using multiple choice assays was studied the preference of *B. cucurbitae* gravid female among the fruit volatiles of selected host fruits (n=12).

Four-arm olfactometer bioassay

Behavioural responses of *B. cucurbitae* ovipositing female to selected host fruit odours were recorded using a Perspex four-arm olfactometer as suggested by Kamala Jayanthi *et al.* (2012). All the glassware used in bioassay were washed with non-ionic liquid detergent solution and then rinsed with acetone and distilled water. The materials were baked overnight at 100 °C in hot air oven. Perspex components were washed with non-ionic liquid detergent and rinsed with ethanol and distilled water and left to air dry. Experiments were conducted using an olfactometer (9 cm diameter) with four glass side arms leading into a central arena which was divided into four odour fields. The central area was fitted with a filter-paper base (Whatman No.1, 9 cm diameter) to facilitate movements of test insect. The olfactometer was illuminated by uniform lighting from the white fluorescent bulb (10 watts) covered with an opaque dome from above and encased with a black wall cage (0.62 × 0.62 × 0.62 m) to remove any extra visual stimuli. The gravid female *B. cucurbitae* flies were starved for 2 hours and then introduced singly through a hole on the top of the olfactometer and the test insect allowed to acclimatizing for 2 min. Next, the air was drawn through the central hole on the top of the olfactometer at the rate of 900 mL/min. The central arena of the olfactometer was divided into four discrete odour fields corresponding to each of four glass arms. For single choice assays, of the four glass arms, one contained the treatment and the other three arms served as controls. For multiple choice assays, all four arms served as treatments. Test samples (10 µl) were pipetted onto filter paper strips and the solvent was allowed to evaporate prior to their placement in the treatment arm. The filter paper strips with solvent (diethyl ether) served as control. Time spent by the insect in each olfactometer arm was recorded by Olfa software (F. Nazzi, Udine, Italy) for 10 min continuously.

Statistical analysis

The data of multiple choice bioassays were analyzed using ANOVA followed by Bonferroni's multiple comparison tests (Shivaramu *et al.*, 2017). Single choice assay data were subjected to paired *t*-test. All analyses were carried out using GraphPad Prism software (Ver. 7.03) for Mac OS X.

GC-MS analysis

Collected Porapak Q elutes chemical composition were analysed through GC-MS using Agilent 7890 B apparatus equipped with coupled MSD (Agilent 5977 B). A capillary column (HP-5 MS) of 30 m length and 0.25 mm ID and 0.25 mm film thickness was used to examine samples. Oven temperature was programmed at 60-280 °C with ramping at 10 °C/min for 40 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The MS detector was maintained at 280 °C and was in full scan mode (70 eV) and AMU ranged from 50 to 350. Sample (1 µl) was injected in split mode (1:20) with injection temperature at 270 °C following Kamala Jayanthi *et al.* (2012). Compounds were identified by comparing mass spectra of detected compounds using spectral libraries (NIST 2014 version). Identified compounds were authenticated by co-injecting standard synthetic compounds along with samples.

Electrophysiological studies

The head of gravid female *B. cucurbitae* (15 days old) anaesthetized by chilling was separated from the body with a micro scissor. The EAG preparations were made by placing the base of the head in the reference electrode and tip of antenna on the recording electrode of EAG probe holder (Syntech, Germany). A small amount of electrode gel (Sigma Gel, USA) was placed on the probe to aid in signal conductivity. Thus, prepared EAG probe with the live antenna was inserted into the pre-amplifier with a constant stream of humidified air over the antenna at 200 mL/min.

Electroantennographic bioassay (EAG)

Electroantennogram (EAG) recordings for different host fruit volatiles were made as per the procedure of Cork *et al.* (1990) with the empty air and honey as negative and positive controls, respectively. Host fruit volatiles (10 µl) impregnated on to individual filter paper strips (Whatman No. 1, 6 cm length x 0.5 cm breadth) served as olfactory stimuli. Before placing the filter paper inside the glass Pasteur Pipette (10 cm length and 6 mm outer diameter), the solvent was allowed to evaporate. The antennal preparation was stimulated by means of controlled airflow (300 mL/min) through the pipette with the filter paper. Odour stimulation was

administered, amplified by injecting a puff of purified air (0.5 sec) and recorded using AutoSpike software (Syntech EAG Model IDAC-4). Stimulus-response characteristics were measured by giving test stimuli successively with control stimulations interspersed between stimuli. The purified air was passed over the antennal preparation for at least 30 sec between stimulus presentations. The configuration in the Auto Spike properties tab for the channel with the EAG probe was set at a sampling rate of 100 and a filter of 0-32 Hz. The responses (amplitudes) to the host fruit volatiles were expressed as the mean of all recorded antennal depolarizations.

A total of five replicates were carried out for each host fruit stimulus and for each replicate, new insect antenna was used. Based on the downward deflection signal (in mV) antennal responses to all host fruit volatiles were recorded and analysed using a customized software package (SYNTECH, The Netherlands). In order to compare the responses among different host fruits, signal mean (in mV) of the gravid female antennal response for all host fruit volatiles were recorded. Thus obtained data were subjected to one way ANOVA using Graph pad prism (version 7.03) and means were differentiated using Critical Difference with α set at 0.05.

RESULTS AND DISCUSSION

The attraction of host volatiles collected from different phenological stages of fruits (mature and immature) independently to gravid females of *B. cucurbitae* was studied. There was no significant attraction to volatiles from immature fruits of ridge gourd (Treatment = 2.69 ± 0.14 min; Control = 2.49

± 0.04 min; mean \pm s.e.m; $t = 1.16$; $df = 11$; $P = 0.2701$), cucumber (Treatment = 2.62 ± 0.08 min; Control = 2.53 ± 0.06 min; $t = 0.99$; $df = 11$; $P = 0.3456$), bitter gourd (Treatment = 2.68 ± 0.07 min; Control = 2.51 ± 0.04 min; $t = 1.62$; $df = 11$; $P = 0.1337$) and snake gourd (Treatment = 2.74 ± 0.12 min; Control = 2.50 ± 0.04 min; $t = 1.51$; $df = 11$; $P = 0.1599$) (Table I) compared to their respective controls. However, *B. cucurbitae* responded positively to volatiles from mature fruits of ridge gourd (Treatment = 3.89 ± 0.26 min; Control = 1.99 ± 0.10 min; $t = 5.37$; $df = 11$; $P = 0.0002$), cucumber (Treatment = 3.74 ± 0.16 min; Control = 2.16 ± 0.05 min; $t = 7.78$; $df = 11$; $P < 0.0001$), bitter gourd (Treatment = 3.72 ± 0.13 min; Control = 2.18 ± 0.05 min; $t = 8.85$; $df = 11$; $P < 0.0001$) and snake gourd (Treatment = 3.80 ± 0.15 min; Control = 2.15 ± 0.08 min; $t = 8.04$; $df = 11$; $P < 0.0001$) (Table II) by spending significantly more time in treatment compared to control arm of the olfactometer. These results are comparable with Kimbokota *et al.* (2013), who reported significantly lower attraction of both male and female *B. invadens* flies to immature stages of mango fruits ($P < 0.05$). However, their attraction was equal to mature ripe as well as unripe fruits of mango.

Since, *B. cucurbitae* gravid females were highly attracted to volatiles of mature fruits of selected cucurbit host plants. Study aimed to know the behaviour of *B. cucurbitae* to all four mature cucurbit fruit volatiles. Therefore, a multiple-choice assay was conducted with volatiles from ridge gourd, cucumber, bitter gourd and snake gourd. In multiple-choice olfactometer bioassays, ridge gourd volatile elicited

TABLE I

Behavioural response of gravid female B. cucurbitae to immature cucurbitaceous fruit volatiles in olfactometer assay (N=12)

Crops	Treatment (mean \pm SEM)	Control (mean \pm SEM)	P	t	df
Ridge gourd	2.69 ± 0.14	2.49 ± 0.04	0.2701	1.16	11
Cucumber	2.62 ± 0.08	2.53 ± 0.06	0.3456	0.99	11
Bitter gourd	2.68 ± 0.07	2.51 ± 0.04	0.1337	1.62	11
Snake gourd	2.74 ± 0.12	2.50 ± 0.04	0.1599	1.51	11

TABLE II
Behavioural response of gravid female *B. cucurbitae* to mature cucurbitaceous fruit volatiles in olfactometer assay (N=12)

Crops	Treatment (mean \pm SEM)	Control (mean \pm SEM)	P	t	df
Ridge gourd	3.89 \pm 0.26	1.99 \pm 0.10	0.0002	5.37	11
Cucumber	3.74 \pm 0.16	2.16 \pm 0.05	<0.0001	7.78	11
Bitter gourd	3.72 \pm 0.13	2.18 \pm 0.05	<0.0001	8.85	11
Snake gourd	3.80 \pm 0.15	2.15 \pm 0.08	<0.0001	8.04	11

a positive behavioural response. Gravid females of *B. cucurbitae* spent more time in the olfactometer arm containing ridge gourd (3.52 \pm 0.12 min; mean \pm s.e.m) compared to olfactometer arms containing cucumber (2.21 \pm 0.18 min), snake gourd (2.21 \pm 0.10 min) or bitter gourd volatiles (2.10 \pm 0.13 min) (Fig. 1). Results of the multiple choice assays, subjected to one-way ANOVA followed by Bonferroni's multiple comparison test, proved that *B. cucurbitae* spent significantly more time in olfactometer arm containing ridge gourd volatiles than cucumber, snake gourd or bitter gourd ($F = 24.86$; $df = 3,44$; $P < 0.0001$).

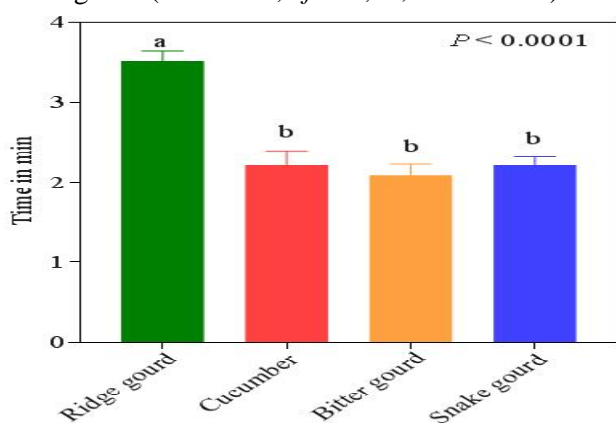


Fig. 1: Gravid female melon flies prefer volatiles from mature ridge gourd fruit followed by cucumber, snake gourd and bitter gourd. Multiple choice olfactometer assays were conducted between mature fruit volatiles of ridge gourd, cucumber, bitter gourd and snake gourd. Melon flies significantly attracted to ridge gourd volatiles.

The GC-MS analysis of fruit volatiles collected from both stages of ridge gourd, cucumber, bitter gourd and snake gourd revealed that difference in the volatile profile of analysed samples.

There was dissimilarity in the chemical composition of different compounds and their relative

abundance among the selected cucurbit host fruit volatiles. A total of 143 volatile organic compounds have been identified across the host fruits, in which terpenoids (both monoterpenes & sesquiterpenes) and aromatic hydrocarbons were in abundance. All compounds and their quantities present in different host fruits were depicted in Fig. 2. As mentioned earlier, among all host plant volatile compounds terpenoid group was found to be present in major quantities. Among the host fruits, ridge gourd and cucumber had higher terpenoid compounds than other host fruits. Within terpenoids, in ridge gourd fruit volatile sample, β -cis-ocimene (61.17 mg/mL) was abundant. Whereas, in cucumber isocaryophyllene (11.00 mg/mL) was abundant compared to other terpenoids. Further, caryophyllene was present in host fruits viz., snake gourd (8.16 mg/mL) and cucumber (7.90 mg/mL). Hydrocarbon group was found to be more prevalent after terpenoids. Among hydrocarbons, n-octadecane, eicosane and naphthalene were abundant in tested cucurbitaceous samples. However, n-octadecane was more in bitter gourd whereas it was absent in snake gourd and cucumber. Further, within bitter gourd, eicosane (11.58) was abundant over others. Aldehydes were next abundant group that followed terpenoids. In this group, ethyl benzaldehyde peak was observed in all the host fruit samples. Among aldehydes, n-decanal was present in higher quantities in ridge gourd followed by bitter gourd. Similar trend was reported earlier by Siderhurst and Jang (2010). Among ketones, ethanone, 1-(4-ethyl phenyl) was found to occur in all host fruits tested and was abundant in ridge gourd and bitter gourd fruits. Similarly, 2, 4-dimethyl acetophenone peak was observed in all host fruits except cucumber. Among

Mean depolarization achieved by *B. cucurbitae* gravid female antennae in response to host fruit volatiles of ridge gourd, cucumber, bitter gourd and snake gourd were presented in Fig. 4. Electroantennographic response (amplitude) was significantly higher than empty air [-0.03 ± 0.01 mV; mean amplitude \pm s.e.m; $F = 6.794$; $P < 0.0001$] in ridge gourd, cucumber, bitter gourd and snake gourd. Whereas among the host fruit volatiles tested, gravid

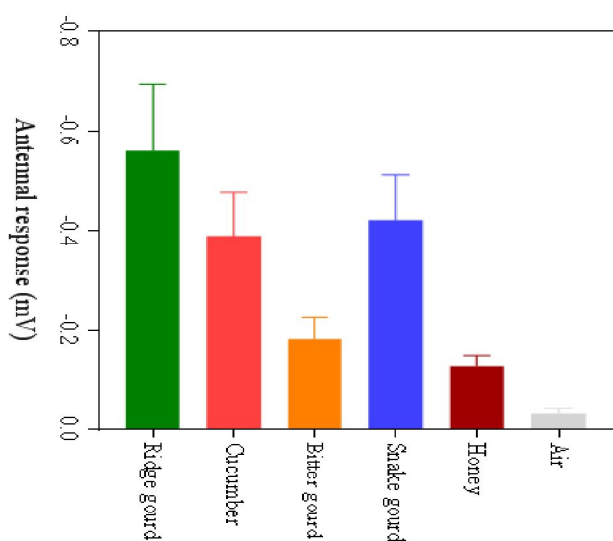


Fig. 4: Antennal response of *Bactrocera cucurbitae* gravid females to host fruits

female antenna showed maximum response to ridge gourd volatiles [-0.56 ± 0.13 mV; mean amplitude \pm s.e.m] followed by snake gourd [-0.42 ± 0.09 mV; mean amplitude \pm s.e.m], cucumber [-0.39 ± 0.09 mV; mean amplitude \pm s.e.m] and bitter gourd [-0.18 ± 0.04 mV; mean amplitude \pm s.e.m]. These results indicated that *B. cucurbitae* adults responded positively to preferred host fruit volatiles in a similar way as observed in olfactometer assays. The EAG response to fruit volatiles further critically endorsed that among the cucurbitaceous hosts tested, *B. cucurbitae* prefers ridge gourd fruit. These results were in agreement with the olfactometer bioassays.

Further, studies on isolation and identification of potent cues from ridge gourd fruit volatiles using GC-EAD (Siderhurst and Jang, 2006) will enable identification of specific compound(s) individually or in blends that can elicit positive behavioural responses in female melon flies. Such compounds can be utilized for developing female bait traps in future.

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