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Differential electroantennogram response of male and female of *Dysdercus cingulatus* to okra plant volatiles

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Abstract
Different chemical compounds were identified from okra plant by gas chromatography-mass spectrometer (GC-MS) techniques. The male and female of *Dysdercus cingulatus* showed its characteristics EAGs response to okra extracts and to four common synthetic ((R)-(+)-Limonene, Methyl salicylate, Hexyl acetate and 4-Hydroxy-2, 5-dimethyl-3(2*H*)-furanone) plant compounds. Statistically no significant differences were observed in EAGs response between both sexes but the response of female was slightly higher than male. The response elicited by okra extracts was significantly higher than synthetic compounds. The dose-response analysis of selected compounds did not differ greatly in appearance according to the dose loaded on filter paper except (R)-(+)-Limonene. Electroantennogram study of *D. cingulatus* provides evidence that the antennal receptors were differentially sensitive to these compounds.

Keywords: *Dysdercus cingulatus*, electroantennogram, gas chromatography-mass spectrometer, okra volatile

1. Introduction
Okra, *Abelmoschus esculentus* is also known as lady finger, as herbaceous plant grown for its edible seeds. The crop is also used in paper industry as well as for the extraction of fiber [1]. A number of insect pests have been reported from okra ecosystem [2] and among them *Dysdercus cingulatus* (Fab.) commonly known as the red cotton stainer damage okra fruit by sucking sap which affects the yield as well as quality of fruit. A large number of volatile compounds have been reported from host plants which involved in insect-pests interaction [3]. In generals, volatiles compounds released from the plant can influence or guide insect-pests toward food sources [4-7] also help in selection of suitable oviposition site [8, 6] and to facilitate mate finding [9-12]. These plant volatiles can be employed to monitor pest population, mass trapping, or to interfere the reproduction by sexual confusion. They can also be used in manipulation of insect behaviour and their natural enemies [13-16]. The management of bugs by the use of plant volatiles is a better alternative to chemical pesticides that has shown it potentials for application in different crops [17, 18, 14]. The present study was conducted to investigate volatile compounds from okra plant (leaf and fruit) and their effect on *D. cingulatus* behaviour in okra ecosystem.

2. Materials and Methods
2.1 Insects
The male and female of red cotton bug, *Dysdercus cingulatus* were used for Electroantennogram (EAG) analysis. The okra plants used for present investigation were grown in research farm of the Indian Agricultural Research Institute (I.A.R.I) New Delhi.

2.2 Extraction of plant volatiles
The samples were collected from the okra plants which were grown in research farm of the Indian Agricultural Research Institute (I.A.R.I) New Delhi. The cotton plant extracts were prepared based on the protocol used by [19-21] with slight modification. Fresh plant free from infestation of pests and disease were selected for sample collection. The plant parts were washed thoroughly 2-3 times with water. Sample was taken from different part of plant viz. leaf, bud, and boll separately in 250ml conical flask. Each sample was immersed overnight in 250ml of chilled hexane in freeze followed by filtration through Whatman No. 1 filter paper.

The filtered elute was further subjected to cleanup by column chromatography. Elute from column was subjected to vacuum evaporator (25-30 ºC at 30-35 RPM) to evaporate solvent and final volume obtained (5 mL) was stored in -20 ºC for further use.

2.3 Gas Chromatography- Mass Spectroscopy (GC-MS) Analysis

Shimadzu QP 2000 equipped with Rtx-5ms column measuring 30×0.25mm composed of 95% Dimethyl polysiloxane. Helium with flow rate 1ml/min was used as carrier gas. One microliter volume of each sample was utilized. The injection temperature was maintained at 230 °C. The initial temperature of oven temperature was programmed at 40 °C for 4 min, then an increase to 220 °C and finally allowed to increase to 270 °C with ending rate of 15 °C for 1 min. The running time of sample was 45 min. The temperature for ion source was maintained at 200 °C. Electron impact ionization (EII) with 70eV was used for GSMS analysis and data was evaluated by TIC (Total ion count) for identification and quantification of compounds. The spectrums of each compound were compared with known stored data base of spectrum in GC-MS library (NIST14) and further data processing and peak areas measurement was done through software (Turbo-Mass-OCPTVS-Demo SPL).

Test stimuli: The final volume (5ml) of different extract obtained from vacuum evaporator and four host-related synthetic compounds were used in Electroantennogram study for both sexes of *D. cingulatus*. The purity and source of synthetic compounds are presented in Table 1.

2.4 Electroantennogram responses

The Electroantennogram response was recorded from both male and female of *D. cingulatus*. Three replicates of both sexes were made for EAG (M/S Syntech, Germany) analysis. The antennae were cut excised at the base from the bug head by using micro scissors and part of distal segment was clipped off under 0.1M electrolyte solution for smooth electrical conductivity between electrodes. Then the basal portion of antenna was connected onto indifferent electrode and the tip portion of antenna was fixed with recording electrode by using electrical gel (Parker, spectra 360) (Fig. 1a). The ideal electrical conductivity of antennae between electrodes was indicated by a stable base line having minimum fluctuations (Fig. 1b). Antennal responses to various chemical stimuli were first recorded for a series of synthetic compounds representing known cotton volatiles. Then, the hexane extract of different part of cotton were tested. A second experiment for EAG tests were conducted including a dose-response series of selected compounds from first test. Serial dilutions of tested compounds were made in redistilled HPLC-grade hexane at dosages ranging from 1 to 0.0001 per cent. In each tests, 10 µl volume of extracts and tested were adsorbed on the filter paper (3×1 cm) disc and were left for 10 seconds for solvent evaporation. Subsequently, the filter paper with test stimuli was inserted inside the pasture pipette for air space saturation and subsequently stimuli puffed onto antennal preparation for 0.3 sec pulse duration. For the recovery of antenna, at least 1 minute time interval kept between subsequent stimulations. Continuous air flow over antennal preparation was maintained at 500ml/min. To ensure complete mixing of odour stimulus with continuous air flow, the stimulus was injected into the mixing tube through the side pore located at 10 cm distance from the antennal preparation. Each recording session was started through application of control stimuli (Hexane) followed by 10mg/ml doses of different test stimulus. Recording were analyzed through EAG 2000 software (version 2.7c, Syntech, Germany). The response of the (Z)-3-hexen-1-ol (standard) was used to normalize the test stimuli.

2.5 Statistical analysis

The obtained value of EAG amplitude (-mv) from antennal receptor of the red cotton bug, *D. cingulatus* for each stimuli at 10 µg dose was subjected to one way ANOVA (Analysis of variance) and Tukey honestly significant difference (HSD) test were used for separation of treatment mean using https://www.xlstat.com.

3. Results and Discussion

3.1 Identification and quantification of saturated hydrocarbons from okra plant

The extraction and analysis of plant volatiles play significant role in better understanding of host plant interaction. In the present study, different saturated hydrocarbons were identified and quantified from okra leaf and fruit through GC-MS techniques. The identified saturated hydrocarbons with their per cent area (concentration) are presented separately (Table 2). Totally 26 saturated hydrocarbons with carbon range C_8-C_{44} were identified from hexane extract of okra leaf and fruit. The hexane extract of okra fruit recorded highest numbers (24) of saturated hydrocarbons, whereas 20 saturated hydrocarbons were found in okra fruit extracts (Table 2). The major chemical constitutes okra leaf was Octadecane (14.13%), Tridecane, 6-methyl- (10.92), Dodecane (8.00), Hentriacontane (7.75) and Dotriacontane (5.48) while Octadecane (20.06), Tridecane, 6-methyl- (10.77), Hentriacontane (9.03), Dodecane (8.44), and Behenic alcohol (5.48) were the most abundant constitutes in fruit extracts (Table 2 and Fig. 3). Total six saturated hydrocarbons (Dotriacontane, 1-tridecane, Tetratetracontane, Nonane, n-Hexadecanoic acid) were not detected from okra fruit extracts, whereas 1, 7-Dimethyl-4-(1-methylethyl) cyclodecane and Cyclohexane, decyl- were absent in okra leaf extracts (Table 2). [22] have detected 20 volatile compounds through GC analysis with carbon number ranging from C_{10}-C_{30} in which C_{29}, C_{27}, C_{20} and C_{30} were the most abundant compounds that constituted more than 50% of the total extracts.

3.2 EAG response to crude extracts and synthetic compounds

The EAGs responses evoked by the compounds were negative, thereby indicating that the olfactory receptors contributing to them mostly responded to the stimulation by depolarization. In *Dysdercus cingulatus*, the hexane extract of okra plant (leaf, and fruit) were elicited higher responses from female antennae as compared to male. The mean EAGs response of okra leaf extract was significantly higher than fruit extract (Fig. 4). In response to synthetic compounds, the mean EAGs responses of female antennae were higher in all tested compounds except Hexyl acetate. The normalized responses of okra (leaf and fruit) extract were significantly higher than synthetic compounds (Tukey's HSD, $P < 0.05$) and this may be due to presence of more number of saturated hydrocarbons with high concentration in extracts than synthetic compounds. Statistically no significant differences in EAGs response were observed between both sexes (Student *t*-test, $P < 0.05$) (Fig. 2). Such similarity in EAGs response to host-related compounds between both sexes has been reported for several phytophagous insect species [23, 24].

3.3 Dose-response analysis

No significant difference was found in EAGs response of male and female of *D. cingulatus* from all synthetic compounds except limonene where the response of both sexes decrease with increasing dose (Fig. 2). The antennae of female appeared to be relatively more sensitive to Limonene with significant EAGs elicited at the dose of 0.0001% and saturation reached at 0.001% dose in both sexes (Fig. 3). Variations observed in dose–response studies may be due to differences in the release rates of different compounds and the sensitivity of antennal receptors [25].

Table 1: List of chemicals used in EAG studies and their purity

Chemical	Purity (%)	Source
(R)-(+)-Limonene	97	Sigma
Methyl salicylate	99	Sigma
Hexyl acetate	99	Sigma
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	98	Sigma

Table 2: Chemical compounds identified from hexane extract of okra plant by GC-MS

S. No.	Compound (Carbon No.)	Area %	
		Leaf	fruit
1	3-Ethylheptane (C_9)	0.27	0.2
2	2,4-Dimethylhexane (C_8)	0.39	0.28
3	Nonane (C_9)	0.29	-*d*
4	1-Decene (C_{10})	2.32	1.75
5	Octane, 4-ethyl- (C_{10})	1.62	1.54
6	Hexadecane (C_{16})	1.28	0.87
7	Dodecane (C_{12})	8.00	8.44
8	Cyclopentane, 1,1,3,4-tetramethyl-, trans- (C_9)	0.5	0.63
9	Cyclohexane, hexyl- (C_{12})	1.15	1.49
10	3-Heptene, 2,2,3,5,5,6,6-heptamethyl- (C_{14})	0.47	0.54
11	Tetratetracontane (C_{44})	0.89	- *d*
12	1-Tridecene (C_{13})	4.63	- *d*
13	Tridecane, 6-methyl- (C_{14})	10.92	10.77
14	Cyclohexane, octyl- (C_{14})	1.36	1.96
15	Pentadecane (C_{15})	0.48	0.48
16	Hentriacontane (C_{31})	7.75	9.03
17	Heptadecane, 3-methyl- (C_{18})	0.32	0.42
18	Octadecane (C_{18})	14.13	20.06
19	Dodecylcyclohexane (C_{18})	062	0.94
20	n-Hexadecanoic acid (C_{16})	0.67	- *d*
21	Behenic alcohol (C_{22})	3.65	5.48
22	Tetracontane (C_{40})	4.74	- *d*
23	Pentacosane (C_{25})	0.65	1.21
24	Dotriacontane (C_{32})	5.48	- *d*
25	1,7-Dimethyl-4-(1-methylethyl)cyclodecane (C_{15})	- *d*	0.84
26	Cyclohexane, decyl- (C_{16})	- *d*	1.63

d =Not detected

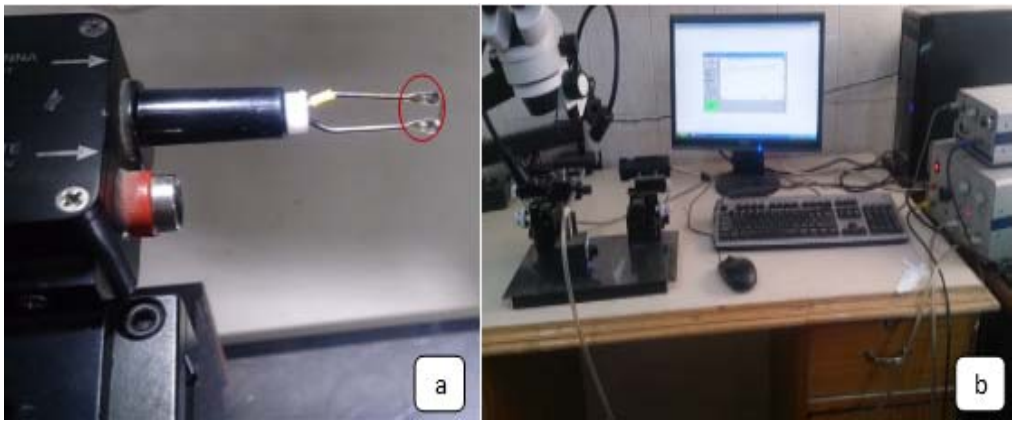

Fig 1: Electroantennogram setup: a. mounted antenna of *Dysdercus cingulatus* on gel electrode, b. application of stimuli over mounted antenna, IDAC, Microscope and computer system for EAG recording

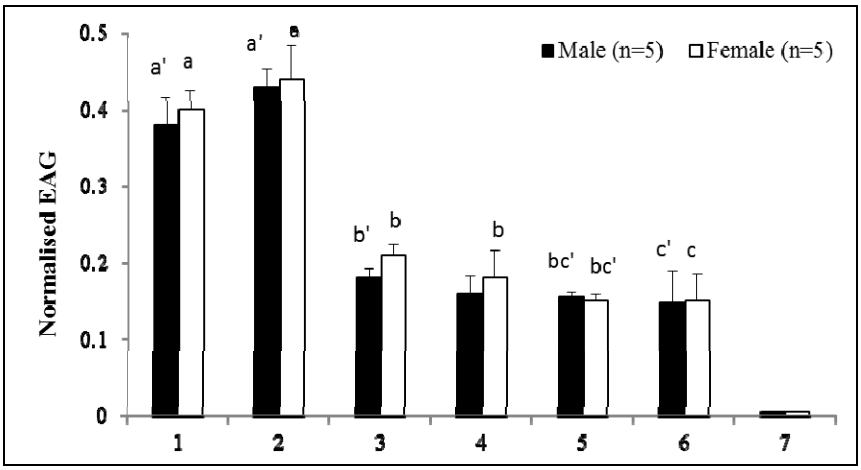

Fig 2: EAG response profile of *Dysdercus cingulatus* (Male and female) to hexane extract of okra plant and four synthetic compounds. Mean (± standard error) with no letters in common are significantly different (ANOVA followed by Tukey's HSD test $P < 0.05$). Code for each compound: 1. Okra fruit, 2. Okra leaf, 3. (R)-(+)-Limonene, 4. Methyl salicylate, 5. Hexyl acetate, 6. 4-Hydroxy-2, 5-dimethyl-3(2*H*)-furanone, 7. Hexane (control)

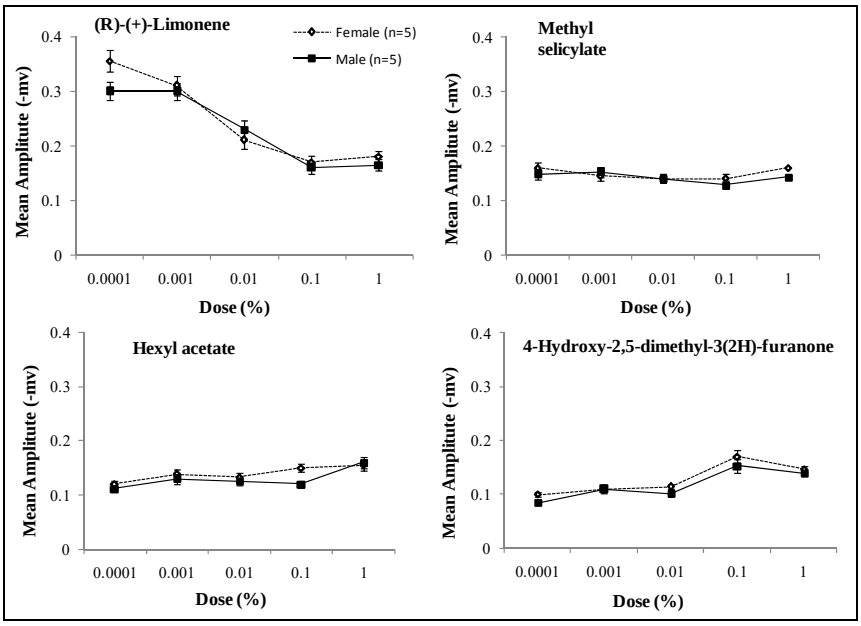

Fig 3: Dose-response curves constructed from EAGs of male and female of *Dysdercus cingulatus* to four synthetic green leaf volatiles

4. Conclusion

The use of semiochemicals has become increasingly important in the management of insect pest and to reduce the load of pesticides from the environment. The present study revealed that the volatile compounds identified from host plant can used in behavioural manipulation with suitable combination of synthetic compounds. The both sexes of *D. cingulatus* can distinguish and locate specific substrates by recognizing these compounds. Electroantennogram study of *D. cingulatus* provides evidence that the antennal receptors were differentially sensitive to these compounds.

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