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Olfactory and electrophysiological response of cucumber moth *Diaphania indica* (Saunders) (Lepidoptera, Crambidae) to different plants

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ABSTRACT: The behavioural response of adults and larvae of cucumber moth, *Diaphania indica* (Saunders) (Lepidoptera, Crambidae), a serious pest of cucurbitaceous crops was evaluated on different cucurbitaceous and non-cucurbitaceous plants through olfactometer bioassay. The volatile organic compounds (VOCs) present in these plants were identified by gas chromatography-mass spectrometry. Further, electrophysiological response of *D. indica* to plant extracts and synthetic volatiles were recorded by electroantennographic detection. Larvae had the highest olfactory response to wild coccinia followed by bitter gourd, coccinia and cucumber. In GC-MS analysis, á-linolenic acid, palmitic acid and dotriacontane were identified as common components in leaf extracts, whereas, benzaldehyde was the major component in headspace of dried leaves. The antennae of gravid female moths were more sensitive to plant odours than unmated females and males. Gravid females showed highest response to extracts of bitter gourd and unmated females to cucumber extract. A synthetic volatile mixture of 10µL of benzaldehyde and 30µL of benzyl alcohol were found responsible for high antennal response. Significant orientation and landing response were shown by *D. indica* moths to a mixture of 10µL benzaldehyde (10%) and 20µL benzyl alcohol (10%). These findings suggest the above blend could be employed for the development of plant volatile based management strategies. © 2024 Association for Advancement of Entomology

KEY WORDS: Host plant volatile, kairomone, electroantennogram, olfactometer, cucurbits

INTRODUCTION

UN Food and Agricultural Organization (2021) estimates a 40 per cent annual loss of crops due to different pests and diseases worldwide. The cucumber moth *Diaphania indica* (Saunders) infests a variety of cucurbits and some non-cucurbits including cotton, cowpea, amaranth etc. Therefore, this insect is considered as a major insect pest. The pest is found throughout the tropics and subtropics, ranging from the Caribbean and northern South America to sub-Saharan Africa, Asia, and the Pacific (Patel and Kulkarny, 1956; Peter and David, 1991). Generally the early instars of the larvae scrape off the lower epidermis and later instars become voracious defoliators (Debnath *et al.*, 2020; Debnath *et al.*, 2021). Further, they may also eat tender shoots, flowers, and fruits under severe infestation which can result in severe crop loss and a decline in market value (Nagaraju *et al.*, 2018; Debnath *et al.*, 2022). It could become a serious and consistent problem in vegetable

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cultivation and to tackle this, farmers still rely on chemical insecticides (Debnath *et al*., 2023). The indiscriminate use of chemicals not only contaminate the produce with pesticide residue but also cause the development of resistance in pest, harm natural enemies and pollinators, pollute the environment and increase the cost of cultivation (Gharaei *et al.,* 2019). Several biopesticides are proven to control this pest, but none of these are practically utilized by the farmers due to its unavailability (Debnath *et al.*, 2020). Another safe option is semiochemicals which have been introduced in pest management since many years. Pheromone traps are available for many important pests; however, host plant volatiles are less studied and exploited. Plants use volatile organic compounds (VOCs), one of the most prevalent chemical signals, to interact with their environment. Insects detect these chemicals and aid in locating their habitat and host plant, evaluating the quality of their host, aggregation and herbivorous prey (Reinecke and Hilker, 2014). Based on the interaction, it could be kairomone, allomone, synomone or antimone (El-Ghany, 2019). The kairomones can attract herbivorous insects and stimulate oviposition (Baskaran *et al.,* 2018). Some insects obtain and utilize them as sex pheromones or sex pheromone precursors while, some others either produce or release sex pheromones in the presence of specific host plant volatiles (Reddy and Guerrero, 2004). It can even enhance the responsiveness to pheromones by triggering physiological and/or behavioural modifications (Landolt and Philips, 1997). Understanding the plant volatiles involved in the interaction of *D. indica* with its host plants and the nature of the response is necessary for devising efficient traps utilising attractants. Hence, this study aimed to evaluate plant extracts and synthetic volatiles for the behavioural and electrophysiological responses of *D. indica* and identify the components of the plant extract that can be used in plant volatile based control strategies.

MATERIALS AND METHODS

Larvae of *D. indica* were procured from the Instructional Farm, College of Agriculture, Vellayani. The larvae were reared on different cucurbitaceous leaves based on availability in plastic containers (diameter-10cm and height- 8cm) in the laboratory. All the pupae were carefully sexed and male and female pupae kept in seperate boxes to facilitate their emergence. Adults were provided with a 10 per cent honey solution soaked in cotton. Two days old male and female moths were paired in individual boxes to facilitate mating. Fresh leaves of either snake gourd or coccinia were furnished as oviposition sites and the fresh leaves were provided at 24 hours interval. These eggs were utilized for maintaining the culture.

Fresh and healthy leaf samples of bitter gourd (*Momordica charantia* L.), snake gourd (*Trichosanthus anguina* L.), salad cucumber (*Cucumis sativus* L.), cultivated and wild cultivar of coccinia (*Coccinia grandis* L.), culinary melon (*Cucumis melo* var. *acidulus* Naudin.), brinjal (*Solanum melongena* L.) and red amaranth (*Amaranthus tricolor* L.) were collected from Organic/ Instructional Farm, College of Agriculture, Vellayani or farmers' fields. Leaves were cleaned and shade-dried for about a week and then minced. Dried leaf extract was prepared by soaking one gram of dried leaf in 20ml of *n*-hexane for 24 hours with intermittent shaking and then filtered using Whatman No.1 filter paper. Also, fresh leaf extract was prepared by soaking one gram of chopped fresh leaves in 10 ml of n-hexane overnight. The extracts were filtered and stored in a refrigerator at 4ºC until use.

The olfactory response of 3 day old mated *D. indica* moths were examined using a Y-tube olfactometer with a stem of length 27cm and an internal diameter of 1.8cm. The arms of the Y-tube were 13.5cm long at an angle of 77° between them. A piece of Whatman No. 1 filter paper (2cm X 3cm) containing 0.1mL of leaf extract was kept as odour source after letting the solvent evaporate. The odour source and control were placed in glass chambers at the end of the arms provided with an airflow of 750ml/min in each arm using an air pump. Gravid female moths were starved for one hour prior to the experiment was released singly at the open end and then closed with a piece of cotton. The first choice that it made within an hour was recorded as the response. The response was regarded as valid only if it stayed for more than a minute. The odour source was changed after testing three insects. Each odour source was replicated three times each with a new set of moths. The experimental setup was rotated at 180° after testing three insects and the odour source and control were also alternated between the two arms. After each test, the Y-tube was cleaned and rinsed with warm water followed by acetone and dried properly. The same experiment was carried out during photophase and scotophase with fresh and crushed leaves also as odour source.

Apart from the Y-tube olfactometer which utilizes airflow, a modified type of dual-choice olfactometer was designed for the assay without airflow (Fig.1). The device was made up of an acrylic box with dimensions 50cm x7cm x 7cm. On both ends (square faces), openings (5cm x 5cm) were made to which another small cubic box could be attached. These small boxes called odour source chambers served the function of extract loading. An insect introduction chamber is attached to a hole (5cm x 5cm) at the exact middle of the long box, which can be closed using a slide mechanism. The whole setup was kept inside a black box to avoid any interference of light in the behaviour of the moths. The experiment was carried out as mentioned in the Y-tube olfactometer. Five moths were kept in the insect introduction chamber for 15 minutes before loading of odour source to acclimatize. After loading the odour source, the sliding door was opened and the movements of the moths were monitored. The experiment was replicated 6 times for each treatment. Plant extracts and combinations of synthetic volatiles were tested for behavioural responses in the modified olfactometer.

Olfactory response of larvae: The olfactory preference of *D. indica* larvae was studied using a multi-arm olfactometer in which all the treatment plants were tested simultaneously. Uniform sized third-instar larvae were used for this experiment and they were starved for 1hr prior to the experiment. Fresh leaf samples of each treatment plants were kept in each arms. 20 starved larvae were released at the centre of the olfactometer and the number of larvae entering each arm was counted after an hour. The experiment was replicated six times with randomly assigning treatment positions.

Chemical analysis: The volatile compounds present in the leaves of the treatment plants were identified through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. *n*-hexane extracts of fresh leaves were used for liquid sampling, whereas, dried leaf samples were used for headspace analysis. It was carried out using a GC-MS (Shimadzu Nexis GC-2030) equipped with an autosampler AOC-30/20i and a headspace analyser. The capillary column (SH-I-5Sil MS) was having a length of 30 m, an inner diameter of 0.25mm and a film thickness of 0.25µm. The oven temperature was set from 70 to 260°C at the rate of 8°C/min, held for 2 minutes and then again raised to 280°C at a rate of 4°C/min. The carrier gas was helium with a continuous flow rate of 1.2mL/min. The injection temperature, ion source temperature and interface temperature were 250°C, 220°C and 280°C, respectively. The pressure was maintained at 76.9 kPa. The total run time was 31.75 minutes. The system utilized the software GCMS Solutions and the library used was NIST 20. The major compounds were identified according to the peak area and retention time.

Electroantennographic Detection (EAD): The electrophysiological response of one-day-old unmated male and female as well as 3-day old gravid female *D. indica* to plant extracts and synthetic volatile compounds were recorded. The synthetic volatiles were chosen based of the chemical analysis and available literature. The response was measured as the antennal depolarization potential using an EAD system (Syntech) at Regional Agricultural Research Station, Pattambi, Kerala. The insect head was carefully cut and mounted on a dual electrode probe using a conductive gel (signa gel Parker) by placing the head on one electrode and the antennal tip on the other. The probe was then mounted onto the EAD system about half a centimetre away from the mouth of the airflow tube. A continuous flow of clean and charcoal-filtered air was maintained.

Fresh leaf extract (0.1ml) was loaded on a strip of Whatman No. 1 filter paper (0.5cm x 3cm) and left for a minute for the solvent to evaporate. It was then inserted into the Pasteur pipette and connected to the stimulus controller (Syntech CS-55) via a silicon tube. A pulse of air passed through the Pasteur pipette which carry the odour stimuli to the insect antennae. The pulse duration, continuous flow rate, pulse flow rate was maintained at 0.5s, 25 ml/s and 21 ml/s, respectively as suggested by Venugopal and Subaharan (2019). The probe measured the depolarization potential of the antennae and the signals were amplified by an amplifier (Syntech IDAC-2) and recorded using EAG software (GC-EAD 2010, Syntech). All eight treatments and control were tested in the same antennae with two puffs of each. A time gap of at least 10 seconds was given between two puffs of a treatment and 30 seconds between treatments for antennal recovery. The average of the two peaks of a treatment was taken as the response. The experiment was replicated with new insect antennae (five replications) for males, unmated females and gravid females. Experiments were also carried out with different concentrations and combinations of synthetic volatile compounds, benzaldehyde and benzyl alcohol. Benzaldehyde was chosen based on GC-MS analysis and benzyl alcohol purely based on literature.

The data of both EAD and larval olfactometer experiments were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test in the software KAU GRAPES Version 1.1.0 (Gopinath *et al*., 2020). Since only positive responses were recorded in the modified olfactometer experiment with synthetic blends, ANOVA was carried out for data analysis.

RESULTS AND DISCUSSION

Olfactometer bioassay: In both Y- tube and modified olfactometer, *D. indica* moths did not show any behavioural response to the plant extracts, while some random and inconsistent movements were shown with fresh or crushed leaf as odour source. The cause of failure of the experiment was expected to be the use of *n*-hexane extract as odour source and improper experimental conditions. But there are previous studies in which *D. indica* positively responded to the flower wax chemicals of *T. anguina* extracted using *n*-hexane (Debnath e*t al*., 2022) and to the VOCs collected from insect damaged and undamaged plants by headspace volatile collection method (Debnath *et al*., 2023). Other insects also responded to plant extracts as in case of *Choristoneura rosaceana* (Harris) which responded to five host plant extracts (Gokce *et al*., 2005) .

The olfactory preference of *D. indica* (Fig 2) larvae was assessed by the percentage of larvae attracted to each plant. Even though wild coccinia is not commonly found infested by *D. indica* in the field, this wild cucurbit attracted highest percentage of larvae (23.33%) out of 120 larvae tested ($P< 0.05$) in this experiment. This could be due to the previous feeding experience since the larvae were reared in wild coccinia leaves prior to this experiment. So keeping apart wild coccinia, the larvae shows olfactory preference to bitter gourd (10.83%), cultivated coccinia (9.17%), cucumber (8.33%), culinary melon (6.67%), snake gourd (5.00%) brinjal (4.17%), whereas, amaranthus did not attract any larvae. The preference to cucumber by *D. indica* larvae was also noted by Gharaei *et al*. (2019) while comparing watermelon, cucumber, melon and squash for the same.

Identification of volatile compounds: A total of 17 volatile compounds were identified in the GC-MS analysis of *n*-hexane extract of the treatment plants. Out of these, two fatty acids, (Z,Z,Z)-9,12,15-octadecatrienoic acid (á-linolenic acid) and n-hexadecanoic acid (palmitic acid) and an alkane, dotriacontane were detected in five or more plants (Table 1). These fatty acids were previously reported in the leaf surface wax of *T. anguina* by Debnath *et al*. (2021), however, the predominant alkane and free fatty acids in the wax were heptadecane and stearic acid, respectively. Palmitic acid and dotriacontane were detected in the ethanolic extracts of leaf callus of brinjal by Vanitha *et al*. (2016). These compounds have different kairomonal properties in other insects. These fatty

Table 1. Compounds detected in different plants by GC-MS analysis of leaf extracts

Name	*M. charantia*	*T. anguina*	*C. sativus*	*C. grandis*	*C. grandis* (wild)	*A. tricolor*	*S. melongena*	*C. melo* var. *acidulus*
9,12,15-octadecatrienoic acid, (z,z,z)-	✓	✓	✓	✓		✓	✓	✓
9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-		✓	✓					
Benzothiazole, 2-(2-hydroxyethylthio)-	✓		✓					
Dotriacontane	✓		✓		✓	✓		✓
Phytol	✓		✓					✓
N-hexadecanoic acid		✓	✓	✓	✓	✓	✓	✓
Octocrylene				✓	✓			✓

Table 2. Compounds detected in different plants by GC-MS headspace analysis

Name	*M. charantia*	*T. anguina*	*C. sativus*	*C. grandis*	*C. grandis* (wild)	*A. tricolor*	*S. melongena*	*C. melo* var. *acidulus*
3-hexen-1-ol		✓					✓	
2,6-dihydroxybenzoic	✓	✓	✓	✓	✓	✓		
acid, 3tms derivative								
Benzaldehyde	✓	✓	✓	✓		✓	✓	
Phosphonoacetic acid, 3TMS derivative	✓	✓	✓	✓	✓	✓		
Cyclohexanone, 4-hydroxy-4-methyl-	✓	✓	✓			✓		
2-cyclohexen-1-ol, 2,4,4-trimethyl-	✓			✓	✓	✓		
2-pyrroline, 1,2-dimethyl-		✓	✓	✓		✓		
2,6-dihydroxyacetophenone, 2TMS derivative		✓	✓	✓	✓	✓		
Cyclopentanol, 2-methyl-, trans-	✓		✓	✓	✓			
Z,Z-2,5-pentadecadien-1-ol				✓	✓	✓	✓	
1,3-cyclohexadiene-1-carboxaldehyde,2,6,6-trimethyl-	✓			✓	✓	✓	✓	

Table 3. EAG responses of *Diaphania indica* moths to different plant extracts

Plant extract	EAG responses (mV) (Mean ± SD)*		
	Male	Female	Gravid female
M. charantia	0.085 ± 0.02^{b}	0.137 ± 0.02^{ab}	**0.245 ± 0.07^{a}**
T. anguina	0.078 ± 0.01^{b}	0.107 ± 0.03^{ab}	0.162 ± 0.05^{bcd}
C. sativus	0.080 ± 0.01^{b}	**0.140 ± 0.01^{a}**	0.200 ± 0.04^{ab}
C. grandis	0.082 ± 0.01^{b}	0.120 ± 0.05^{ab}	0.178 ± 0.02^{bc}
C. grandis (wild)	0.088 ± 0.02^{b}	0.127 ± 0.03^{ab}	0.182 ± 0.05^{b}
A. tricolor	0.080 ± 0.01^{b}	0.120 ± 0.04^{ab}	0.168 ± 0.02^{bcd}
S. melongena	**0.105 ± 0.01^{a}**	0.113 ± 0.02^{ab}	0.155 ± 0.01^{bcd}
C. melo var. *acidulus*	0.073 ± 0.01^{b}	0.100 ± 0.03^{ab}	0.122 ± 0.01^{d}
Control (n-hexane)	0.043 ± 0.01^{c}	0.053 ± 0.01^{c}	0.050 ± 0.01^{e}
CD (0.05)	0.017	0.046	0.052

*Mean of 5 replications

acids and some alkanes present in the leaf surface wax of a weed *Ludwigia octovalvis* (Jacq.) were found to attract a chrysomelid beetle *Altica cyanea* (Weber) and also triggered oviposition (Mitra *et al*., 2017). Palmitic acid was found to involve in the larval preference of *Helicoverpa zeae* (Boddie) (Breeden *et al*., 1996). á-linolenic and palmitic acid also act as precursors for sex pheromones in various insects. Most of the type I pheromones of lepidopterans are synthesized from the precursor fatty acids such as palmitic acid and stearic acids (Ando *et al*., 2004) as in the case of *Helicoverpa armigera* (Hubner) (Wang *et al*., 2005). Whereas linoleic and linolenic acids are the precursors for the biosynthesis of Type II diene and triene compounds, respectively (Yamakawa *et al*., 2011) and their epoxy derivatives by decarboxylation (Matsuoka *et al*., 2008). The alkane, dotriacontane have reported insect-attracting properties in *Callosobruchus maculatus* (F.) (Adhikary *et al*., 2014) and an arctiid moth, *Diacrisia casignatum* Kollar (Roy and Barik, 2012). Breeden *et al*., (1996) identified the influence of dotriacontane on moth oviposition.

In the headspace GC-MS analysis of dried leaves, more than 25 compounds were detected from different treatment plants which included alcohols, aldehydes, ketones, cyclic amines, aromatic compounds, phenolic acids, esters etc. (Table 2). Benzaldehyde was identified in all samples except wild coccinia and culinary melon. While examining the peak area of benzaldehyde in the chromatogram of different treatments, the highest percentage of peak area (32%) was observed in bitter gourd. Benzaldehyde was detected in leaf extracts of bitter gourd (Sarkar *et al.*, 2015), fruit and leaf volatiles of brinjal (Nusra *et al*., 2021) and leaf volatiles of cucumber (Gharaei *et al*., 2020) previously.

Electrophysiological response: The antennal response of unmated male, unmated female and gravid female moths of *D. indica* to different plant extracts were measured as amplitude of depolarization of antennae in millivolts (Table. 3). The results demonstrate that the volatile compounds from the host plants influences the *D. indica* gravid females more than both male and female before mating. The gravid females are electro physiologically more active towards bitter gourd (0.245 mV) and cucumber (0.200 mV) while, unmated females were most responsive to cucumber (0.140 mV) which was on par with other treatment plants. Surprisingly, male moths elicited a greater response to the extract of brinjal (0.105 mV). The behavioural response to different volatiles and its magnitude is influenced by the physiological state of the insect including mating status and the

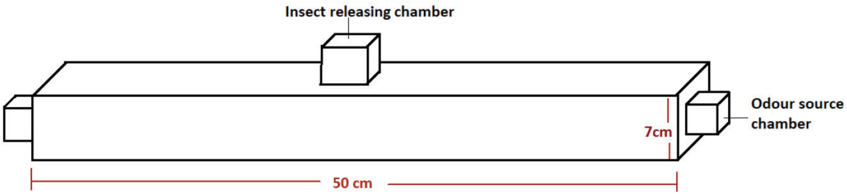


Fig. 1 Modified olfactometer

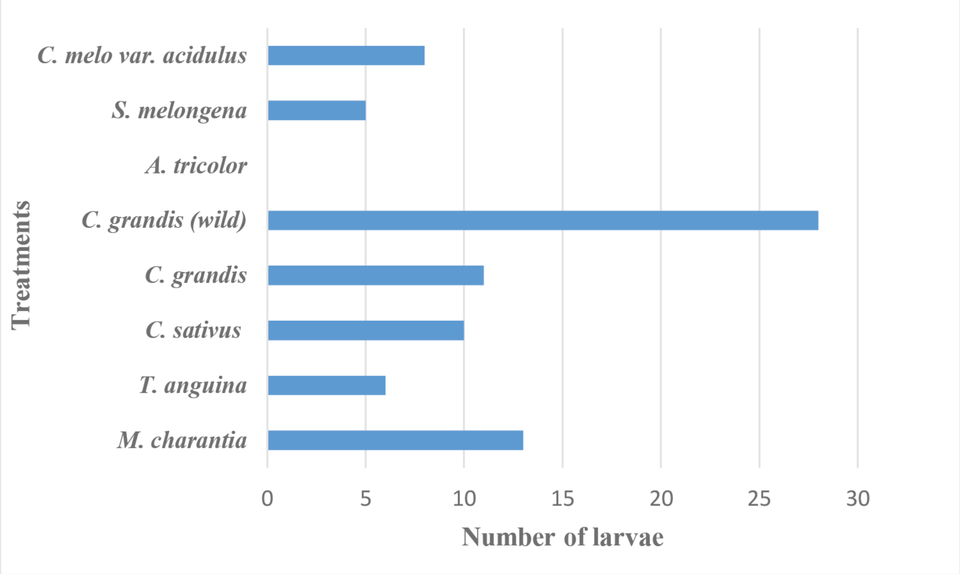
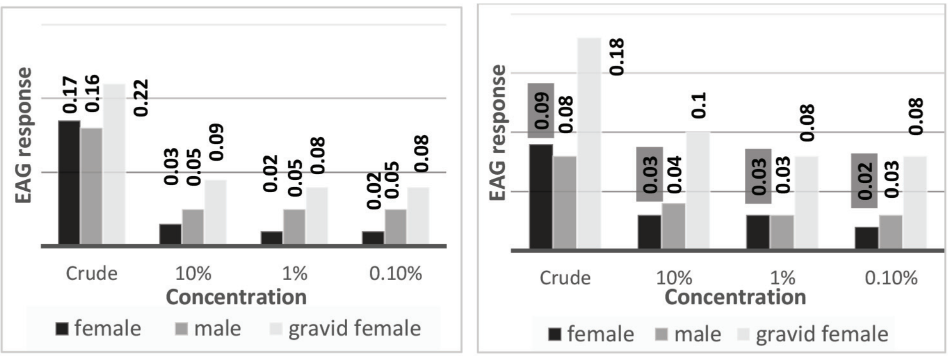


Fig. 2 Olfactory response of *Diaphania indica* larvae

Table 4. EAG responses of gravid female *Diaphania indica* moths to different combinations of benzaldehyde and benzyl alcohol

Combinations	Response(mV) (Mean ± SD)*
10µL benzaldehyde (crude) + 10µL benzyl alcohol (crude)	$0.18^a \pm 0.03$
10µL benzaldehyde (10%) + 10µL benzyl alcohol (10%)	$0.09^b \pm 0.03$
10µL benzaldehyde (1%) + 10µL benzyl alcohol (1%)	$0.08^b \pm 0.03$
10µL benzaldehyde (crude) + 20µL benzyl alcohol (crude)	$0.20^a \pm 0.05$
20µL benzaldehyde (crude) + 10µL benzyl alcohol (crude)	$0.17^a \pm 0.04$
10µL benzaldehyde (crude) + 30µL benzyl alcohol (crude)	$0.21^a \pm 0.06$
30µL benzaldehyde (crude) + 10µL benzyl alcohol (crude)	$0.16^a \pm 0.06$
n-hexane 10µL	$0.05^b \pm 0.01$
CD (0.05)	0.07



Benzaldehyde Benzyl alcohol

Fig. 3 EAG response of *Diaphania indica* to different concentrations of synthetic volatiles

environmental conditions (Anton *et al*., 2007). The physiological changes following mating increase the response of female moths to host plant odours as proved in *Pectinophora gossypiella* (Saunders) in cotton (Wiesenborn and Baker, 1990).

In the electrophysiological evaluation of *D. indica* to different concentrations of benzaldehyde and benzyl alcohol, the antennal response was highest to the crude form of both the compounds for males, unmated and gravid females (Fig. 3). The response was reduced with a decrease in concentration and gravid females were the most responsive to both compounds. While testing different combinations of these compounds (Table 4), all the combinations with different proportions of the crude chemicals showed higher responses which were not significantly different. The synthetic volatile combination 10µl of crude benzaldehyde + 30 µl crude benzyl alcohol elicited a higher EAG response (0.21 mV) which was comparable with bitter gourd and cucumber. It could be inferred that not only benzaldehyde and benzyl alcohol, but some other volatiles in the host plants also have a role in the attraction of *D. indica*. The relative proportion and concentration of different volatiles will be critical for each pest and more specifically for each stage of an insect.

Behavioural response to volatile combinations: In this experiment conducted using modified olfactometer, synthetic volatile compounds at 10 per cent concentration produced significant reactions in *D. indica* moths. The mixture of 10µL benzaldehyde (10%) + 20µL benzyl alcohol (10%) produced the highest orientation and landing response. Out of 30 insects (6 replication x 5 insects per replication) exposed to this mixture, 66.67 per cent oriented towards it and 33.33 per cent landed on it. When 10µL of each of benzaldehyde and benzyl alcohol were combined, 60 per cent of the moths moved in the direction of the source and 30 per cent landed on it. Only 50 per cent of moths responded when benzaldehyde alone was used. In a similar sudy, Gharaei *et al.* (2020) reported a higher flight response of *D. indica* to a mixture of (E, Z)-2,6-nonadienal, (E)-2-nonenal, (Z)-6-nonenal, benzyl alcohol, benzaldehyde and 4,8-dimethyl-1,3,7-nonatriene (DMNT) in a wind tunnel but only the combination of benzyl alcohol, benzaldehyde and DMNT showed a landing response.

In conclusion, the results of the study revealed that *D. indica* has electrophysiological and larval olfactory preference to bitter gourd and cucumber. The results also show that the attractiveness is depended on plant species, the volatiles it emits, the mating status of insect, previous experience and environmental conditions. Important volatiles in the host plants having kairomonal properties have been identified and that of benzaldehyde and benzyl alcohol was proved in the study. An appropriate combination of host plant volatiles along with the pheromone components of *D. indica* can be used to device a semiochemicals based trap to manage this pest more efficiently.

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