



# Leaf Surface Wax Chemicals in *Trichosanthes anguina* (Cucurbitaceae) Cultivars Mediating Short-Range Attraction and Oviposition in *Diaphania indica*

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## Abstract

Larval *Diaphania indica* (Saunders) (Lepidoptera: Crambidae) cause complete defoliation of *Trichosanthes anguina* L. and reduce crop yield in India. Females lay eggs on the leaf surface, and therefore leaf surface waxes are potentially involved in host selection. Alkanes and free fatty acids are the major constituents of leaf surface waxes, so a study was conducted to determine whether these wax constituents from three *T. anguina* cultivars (MNSR-1, Baruipur Long, and Polo No.1) could act as short-range attractants and oviposition stimulants in *D. indica* females. Twenty *n*-alkanes from *n*-C<sub>14</sub> to *n*-C<sub>36</sub> and 13 free fatty acids from C12:0 to C21:0 were detected in the leaf surface waxes of these cultivars. Heptadecane and stearic acid were predominant among *n*-alkanes and free fatty acids, respectively, in these cultivars. Females showed attraction towards one leaf equivalent surface wax of each of these cultivars against solvent controls (petroleum ether) in Y-tube olfactometer bioassays. A synthetic blend of heptadecane, eicosane, hexacosane, and stearic acid, a synthetic blend of hexacosane and stearic acid, and a synthetic blend of pentadecane and stearic acid comparable to amounts present in one leaf equivalent surface wax of MNSR-1, Baruipur Long, and Polo No.1, respectively, were short-range attractants and oviposition stimulants in *D. indica*. Female egg laying responses were similar to each of these blends, providing information that could be used to developing baited traps in integrated pest management (IPM) programs.

**Keywords** *Diaphania indica* · Snake gourd · Surface wax · Long-chain alkanes · Free fatty acids · Olfactometer bioassay · Oviposition assay

## Introduction

*Trichosanthes anguina* L. (Cucurbitaceae), commonly known as snake gourd or serpent gourd, is an important crop in India, Sri Lanka, Nepal, Australia, North America, Nigeria, and Pakistan (Kage et al. 2009; Singh et al. 2017; Yusuf et al. 2007). The fruit is generally consumed as a vegetable due to its high nutritive value. The plant is a good source of functional ingredients such as carotenoids, flavonoids, phenolic acids, and vitamins A and E, which makes the whole plant pharmacologically and therapeutically active (Ojiako and Igwe 2008; Yusuf et al. 2007). The plant has been shown to possess antidiabetic

(Arawwawala et al. 2009), gastro protective (Arawwawala et al. 2010a), anti-inflammatory (Arawwawala et al. 2010b), lipid lowering properties (Arawwawala et al. 2011a), antioxidant (Arawwawala et al. 2011b), and antimicrobial activity (Ali et al. 2011).

The caterpillar, *Diaphania indica* (Saunders) (Lepidoptera: Crambidae), is a major pest of *T. anguina* that consumes leaves, flowers, and young fruits of the crop in India and Sri Lanka (Debnath et al. 2020; Roopa et al. 2014). Generally, young fruits are not the primary target, but when leaves are completely eaten then the larvae attack young fruits. The insect also consumes leaves of cucumber, melon, gherkin, bottle gourd, bitter melon, luffa, little cucumber, and cotton (Clavijo et al. 1995; Hosseinzade et al. 2014; Pandey 1977; Tripathi and Pandey 1973). Larvae of *D. indica* feed on the leaves of *T. anguina* for 9–12 days through five instars to complete their larval development, and after pupation (8–10 days), newly emerged male and female adults live for 6–8 and 9–10 days, respectively (Debnath et al. 2020).

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The first and second instars consume the lower epidermis of leaves, while third to fifth instars gregariously consume whole leaf tissue. Severe infestation by the larvae of *D. indica* on *T. anguina* plants causes complete defoliation, which results in loss of crop yield.

The influence of plant leaf surface waxes on insect host plant acceptance is an area of interest because after reaching the plant, the first contact between a plant and an insect takes place on the leaf surface (Müller 2006; Müller and Hilker 2001). For lepidopterans, host plant oviposition on a suitable host is essential for first instar survival, so chemicals on or near the plant surface, including surface waxes are vital oviposition cues. Hence, leaf surface wax chemicals serve a vital role in host acceptance by herbivorous insects. Therefore an assessment of leaf surface wax chemicals stimulating oviposition in insect pests of economic significance is significant due to their potential use in the manipulation of insect behavior in a crop field.

The leaf surface waxes are mainly comprised of long-chain alkanes, free fatty acids, esters, aldehydes, and primary and secondary alcohols, which differ within plant species as well as cultivars of a plant species (Tomasi et al. 2018). Long-chain alkanes and free fatty acids, the major components of surface wax, can be short-range attractants (Manosalva et al. 2011; Schiestl et al. 1999) and stimulate oviposition in females (Li and Ishikawa 2006; Müller 2006). Long-chain alkanes such as heneicosane, docosane, tricosane, tetracosane, pentacosane, hexacosane, and heptacosane along with other alkane compounds from the labelum extracts of orchid flower, *Ophrys sphegodes* Miller elicited attraction in the solitary bee, *Andrena nigroaenea* (Kirby) (Schiestl et al. 1999). Manosalva et al. (2011) elucidated that long-chain free fatty acids present in the root surface waxes of young red clover (*Trifolium pratense* L.) served as short-range attractant in *Hylastinus obscurus* (Marsham) (Coleoptera: Curculionidae). Five long-chain alkanes – hexacosane, heptacosane, octacosane, nonacosane, and tritriacontane, present in the epicuticular wax of corn (*Zea mays* L.) leaves stimulated oviposition in *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Udayagiri and Mason 1997). In another study, Li and Ishikawa (2006) demonstrated that long-chain *n*-alkanes and free fatty acids present in the surface waxes of Japanese knotweed *Fallopia japonica* (Houtt.) Ronse Decr. stimulated oviposition in *O. nubilalis*. According to Jiang et al. (2015), the sugarcane and maize leaf surface waxes comprised of alkanes and other chemicals stimulated oviposition in *O. furnacalis*. However, no study has demonstrated the role of leaf surface waxes in *D. indica*.

This investigation is conducted to: (i) determine the composition of *n*-alkanes and free fatty acids in the leaf surface waxes of three *T. anguina* cultivars (cv. MNSR-1, Baruipur Long, and Polo No. 1, three cultivars commonly cultivated

in West Bengal due to high yielding potential because they are well adapted to conditions there), (ii) observe whether the leaf surface waxes of these cultivars act as short-range attractants and stimulate oviposition in *D. indica*, (iii) study the behavioral responses of *D. indica* using Y-tube olfactometer bioassays towards the individual synthetic *n*-alkanes and fatty acids, and blends comparable to the amounts present in one leaf equivalent surface wax of these cultivars, and (iv) examine whether the most attractive synthetic blends (*n*-alkanes and free fatty acids) comparable to the amounts present in one leaf equivalent surface wax of these cultivars stimulate oviposition in *D. indica*.

## Methods and Materials

### Insects

Light traps were placed near a *Cucumis sativus* L. planting in the field to collect adults (males and females) of *D. indica*, and collected adults were kept on the same leaves for egg laying. Newly emerged first instars were fed on cucumber leaves, and they were reared at  $27 \pm 1$  °C,  $65 \pm 10\%$  relative humidity, and 12 L: 12 D photoperiod in a 'BOD' incubator. A moist piece of cotton was attached with the cut end of the petiole of each cucumber leaf and this was wrapped by aluminium foil to prevent water loss from the leaf. Fresh leaves were provided at 24 h intervals by replacing the previous ones. The second generation *D. indica* larvae were also fed on cucumber leaves. Gravid F2 females (2–3 days old, i.e., after emerging from pupa) were used for olfactory bioassays and oviposition assays. Gravid females were identified based on their swollen abdomens and slower movement of fusiform hairs at the end of the abdomen.

### Plant Materials

Three cultivars of *T. anguina* seeds, MNSR-1, Baruipur Long, and Polo No. 1 (hereafter MNS, BAR, and POLO, respectively) were grown at the Crop Research Farm (CRF) [soil organic matter  $5.3 \pm 0.2\%$  ( $\pm$  Standard Error), pH 7.7], University of Burdwan ( $23^{\circ}16' N$  &  $87^{\circ}54' E$ ), West Bengal, India during May, 2019. Mature leaves (six to seven-weeks old plants) were collected, and leaves were washed with deionised water followed by paper towelling. Mature leaves were collected because gravid females prefer to lay eggs on the abaxial surface of mature leaves than either young or senescent leaves.

### Extraction of Leaf Surface Waxes

Seventy-five grams of leaves of each *T. anguina* cultivar were separately collected five times ( $5 \times 75$  g MNS,  $5 \times 75$  g

BAR, and 5 × 75 g POLO), and the gum arabic method of Jetter and Schäffer (2001) was used to isolate leaf surface waxes from an intact leaf. Before the use of gum arabic (Roth, Karlsruhe, Germany), the contaminants of gum arabic were separated by soxhlet extraction with hot chloroform. An aqueous solution of ca. 0.1 ml [50% (w/w)] gum arabic was applied per cm<sup>2</sup> of leaf surface on the adaxial and abaxial surface of each leaf with a small paintbrush. After drying for 1 h, a thin whitish adhesive layer formed and was removed from each leaf with forceps, keeping the leaves as undamaged and intact (without damaging the epidermal and mesophyll tissue). The gum arabic fractions of adaxial and abaxial leaf surfaces were collected for each leaf and extracted with 10 ml each of water and chloroform (v/v). After vigorous agitation and phase separation, the organic solution was removed and the solvent was evaporated under reduced pressure.

Each dried crude extract from 75 g leaves of each *T. anguina* cultivar was then dissolved in 30 ml chloroform and divided into three equal crude fractions [each 10 ml crude fraction was equivalent to ca. 25 g of leaves; the number of leaves for 25 g MNS, BAR, and POLO were 14 ± 1, 16 ± 1, and 18 ± 1 (mean ± standard error), respectively]. One mg heneicosane (*n*-C<sub>21</sub>) was added as internal standard to the second fraction of each crude extract for identification and quantification of alkanes, while tricosanoic acid (C23:0, 1 mg) was added to the third fraction of each crude extract for identification and quantification of free fatty acids. Each fraction of crude extract was filtered through Whatman No. 41 filter paper and evaporated to dryness at room temperature. The first, second, and third fractions were used for (i) olfactometer and oviposition bioassays, (ii) identification and quantification of alkanes, and (iii) identification and quantification of free fatty acids, respectively.

### Identification and Quantification of Alkanes

Alkanes were identified and quantified according to the protocol of Mitra et al. (2020a). The second fraction of each crude extract (equivalent to ca. 25 g of leaves) was fractionated by Thin Layer Chromatography (TLC) on silica gel G (Sigma St. Louis, MO, USA) layers (thickness 0.5 mm) with carbon tetrachloride as the mobile phase. A faint yellowish band appeared on the TLC plate, and the plate was air-dried under laboratory conditions. The single hydrocarbon band appeared in each TLC plate was eluted from the silica gel layer with chloroform. A total of 15 purified alkane samples (five alkane samples from each *T. anguina* cultivar) were prepared for gas chromatography-mass spectrometry (GC-MS) and GC-flame ionisation detector (GC-FID) for identification and quantification, respectively. Half portion of each sample was used for identification by GC-MS and

the remainder for quantification of alkane compounds by GC-FID.

For identification of alkanes, the samples were analyzed with Clarus 690 GC coupled to a SQ8C Mass Selective Detector using a SE-30 column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness). The oven temperature program was initially 170 °C held for 1 min, then raised at 4 °C/min to 300 °C, and finally held for 15 min (Sarkar et al. 2014). Helium was the carrier gas. The MS parameters were 280 °C at the interface, ionization energy 70 eV, scan speed 5 scans/sec and scanned over the mass range 40–600 mass units. The identity of compounds was confirmed by injections of a mixture of synthetic *n*-alkanes (*n*-C<sub>14</sub> to *n*-C<sub>36</sub>). All *n*-alkanes (≥ 99% purity) between *n*-C<sub>14</sub> and *n*-C<sub>36</sub> were purchased from Sigma Aldrich. Alkanes were verified by the comparison of diagnostic ions and GC retention times with those of respective authentic standards.

For quantification of compounds, five separate samples of each *T. anguina* cultivar (MNS, BAR, and POLO) were analyzed by a Techcomp GC (Em Macau, Rua De Pequim, Nos. 202A-246, Centro Financeiro F7, Hong Kong) model 7900 fitted with a SE-30 capillary column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness) and a flame ionization detector which was run under the same temperature conditions as mentioned in GC-MS analysis. The carrier gas was nitrogen with a flow rate of 18.5 ml/min. The volume of sample injected was 1 μl with a split ratio of 1:5. The amount of individual alkanes was computed from the GC peak areas and the areas of each peak were converted into quantities of *n*-alkanes based on internal standard heneicosane (*n*-C<sub>21</sub>).

### Identification and Quantification of Free Fatty Acids

Free fatty acids were identified and quantified according to the protocol of Mitra et al. (2020a). The third fraction of each crude extract obtained from each *T. anguina* cultivar (MNS, BAR, and POLO) (equivalent to ca. 25 g of leaves) was mixed with diethyl ether and filtered through Whatman No. 41 filter paper. The extract was purified by TLC on silica gel G layers (thickness 0.5 mm) with *n*-butanol: acetic acid: water (4:1:5; this mixture was shaken, and water was separated from this mixture by a separating funnel and discarded) as the mobile phase (Das et al. 2019). The band was eluted from the silica gel layer with diethyl ether, and diethyl ether was removed under reduced pressure leaving purified free fatty acids. The purified free fatty acids were esterified with 3 ml BF<sub>3</sub>-methanol followed by warming for 5 min in a hot water bath at 50–60 °C and cooled. Hexane (30 ml) was added to this mixture followed by washing twice with saturated NaCl in a separating funnel. The aqueous layer of each sample was discarded, and the hexane fraction was passed through 50 g anhydrous Na<sub>2</sub>SO<sub>4</sub> twice. One portion of each esterified sample (hexane fraction) was used for GC-MS

and another for GC-FID. The extraction of free fatty acids from each crude extract was separately repeated five times followed by esterification, and a total of 15 samples were prepared.

One portion of the esterified fatty acids was analyzed with an Clarus 690 GC coupled to a SQ8C Mass Selective Detector with a SE-30 column (Agilent, USA; length: 30 m × 0.32 mm × 0.25- $\mu$ m film thickness). The oven temperature program was initially held at 160 °C for 2 min, then raised at the rate of 3 °C/min to 220 °C and finally held at 220 °C for 18 min (Mukherjee et al. 2014; Sarkar and Barik 2015). Helium was the carrier gas. The MS temperature parameter was 280 °C at the interface, ionization energy 70 eV, scan speed 5 scans/sec, and scanned over the mass range 40–600 mass units. Fatty acids were verified by comparing the diagnostic ions and GC retention times with those of respective standard esterified fatty acids [methyl laurate (C12:0), methyl tridecanoate (C13:0), methyl tetradecanoate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl heptadecanoate (C17:0), methyl stearate (C18:0), methyl linoleate (C18:2), methyl linolate (C18:3), methyl nonadecanoate (C19:0), methyl arachidate (C20:0), and methyl heneicosanoate (C21:0)]. All standard esterified fatty acids (fatty acid methyl esters,  $\geq 99\%$  purity) were purchased from Sigma-Aldrich, Germany.

The remaining portion of esterified fatty acids (five separate samples of each *T. anguina* cultivar) were analyzed using a Techcomp Gas Chromatograph model 7900 fitted with a SE-30 capillary column (Agilent, USA; length: 30 m × 0.32 mm × 0.25- $\mu$ m film thickness) and a flame ionization detector, which was run under the same temperature conditions as described for GC–MS analysis. The injector port temperature was 280 °C. The carrier gas was nitrogen with a flow rate of 20 ml/min. The volume of the sample injected was 1  $\mu$ l with a split ratio of 1:5. The amount of individual free fatty acids was computed from the GC peak areas and the areas of each peak were converted into quantities of fatty acids based on reference standard methyl tricosanoate (C23:0).

## Olfactometer Bioassays

A glass Y-tube (internal diameter: 3 cm; common arm: 10 cm and each arm 10 cm; two lateral arms at an angle of 45°) was used to perform the olfactometer bioassays of gravid *D. indica* females (Supplementary Fig. 1). A glass adapter was attached with the end of each arm of the Y tube. Each adapter was fitted with a glass vial (3 cm diameter × 3 cm long). Charcoal filtered air (70 ml/min) was pushed through an inlet tube of each adapter, so the purified air could enter the vial and then exit through an outlet tube directed to an arm of the Y tube. One glass vial held a piece

(2 × 2 cm<sup>2</sup>) of Whatman No. 41 filter paper moistened with 1 ml of the test samples, while the other glass vial held a filter paper of the same size moistened with 1 ml of the control solvent (petroleum ether). All the connections between different parts of the set-up consisted of teflon tubing.

Bioassays were performed in the laboratory at 27 ± 1°C, 65 ± 10% relative humidity (RH), and 150 lx light intensity. In preliminary bioassays, the behavioral response of gravid females to the control solvent (petroleum ether) was neutral. One ml of the test sample and the control solvent was applied to separate filter paper pieces and the solvent was allowed to evaporate, and these filter papers were introduced into the glass vials before the first insect was released into olfactometer, for each experiment tested as a sample against control. Individual females were released into a porous glass vial (3 cm diameter × 3 cm long), which was then attached with the common arm of glass Y and exposed to a particular odor, consisting of 1 ml of the control solvent (petroleum ether) in one glass vial and 1 ml of the test sample (leaf surface waxes, individual synthetic alkanes, and fatty acids or synthetic blends comprised of alkanes and fatty acids compounds) in another glass vial. The behavioral response of each gravid female was observed for 3 min. A choice was recorded when a gravid female reached the far end of one arm, and the choice of insect was recorded as a positive (showed attraction to test samples) or negative (did not show attraction to test samples) response, respectively, and subsequently, the female was discarded. Gravid females that did not show any choice within 3 min or remained in the stem of the Y-tube were discarded and recorded as no response (Mitra et al. 2020b). For each bioassay, 60 naïve females were tested excluding those who did not respond. Each gravid female was tested once. After five insects had been tested, the olfactometer set-up was cleaned with petroleum ether followed by acetone, left to dry, and subsequently, the odor sources were switched between left and right arms to avoid positional bias.

## Dual Choice Bioassays with Gravid *D. indica* Females Towards Crude Surface Waxes

**Bioassay 1:** Behavioral responses of females towards one leaf equivalent surface wax (crude extract) of each *T. anguina* cultivar (MNS, BAR, and POLO) were tested against the control solvent (petroleum ether) to observe whether leaf surface waxes from each *T. anguina* cultivar attracted *D. indica* (Table 1, Supplementary Table 1).

**Bioassay 2:** Behavioral responses of females towards one leaf equivalent surface wax of *T. anguina* cultivars were tested against each other (MNS vs. BAR, MNS vs. POLO, and BAR vs. POLO) to observe whether the leaf surface wax of a particular *T. anguina* cultivar was more attractive to the

**Table 1** One leaf equivalent surface waxes of three *Trichosanthes anguina* cultivars, MNSR-1, Baruiपुर Long, and Polo No. 1 used for olfactory bioassays

Leaf	One leaf equivalent Amount (mg)
MNSR-1	3.02 <sup>a</sup>
Baruiपुर Long	2.26 <sup>b</sup>
Polo No. 1	1.75 <sup>c</sup>

<sup>a</sup>25 g MNSR-1 mature leaves equivalent to 14 leaves, and 25 g leaves yielded 42.28 mg surface waxes

<sup>b</sup>25 g Baruiपुर Long mature leaves equivalent to 16 leaves, and 25 g leaves yielded 36.18 mg surface waxes

<sup>c</sup>25 g Polo No. 1 mature leaves equivalent to 18 leaves, and 25 g leaves yielded 31.46 mg surface waxes

test insect among three *T. anguina* cultivars (Supplementary Table 1).

### Dual Choice Bioassays with Gravid *D. indica* Females Towards Individual Synthetic Compounds and Synthetic Blends

Bioassay 3: Behavioral responses of females towards individual synthetic compounds, comparable to the amounts of individual compounds present in one leaf equivalent surface wax of each *T. anguina* cultivar (MNS, BAR, and POLO) dissolved in 1 ml petroleum ether and tested against 1 ml control solvent to observe the role of individual compounds in *D. indica* (Supplementary Table 2a and 2b). Those compounds eliciting a response individually were then combined for bioassay in proportions representing those present on waxes of MNS (MNS blend 4), BAR (BAR blend 2), and POLO (POLO blend 2) and were tested against the control solvent (Supplementary Table 3).

Bioassay 4: One leaf equivalent surface wax of each *T. anguina* cultivar (MNS, BAR, and POLO) was tested against individual or synthetic blends comparable to the amounts present in one leaf equivalent surface wax of each *T. anguina* cultivar (MNS, BAR, and POLO) to observe whether crude surface wax and synthetic blends were equally attractive to the test insect (Supplementary Table 1).

Bioassay 5: Each of the three blends, MNS blend 4, BAR blend 2, and POLO blend 2 were tested against each of the other two (Supplementary Table 1). The above bioassays were conducted to observe whether a particular synthetic blend comparable to one leaf equivalent surface wax of a particular *T. anguina* cultivar was most attractive to the test insect compared to the other two synthetic blends.

Bioassay 6: Dose response bioassays of *D. indica* towards individual synthetic compounds were conducted to determine the lowest and highest doses where the female responded initially and showed the highest attraction. These

five compounds were tested against the control solvent because in preliminary tests females showed responsiveness (pentadecane: 30, 60, and 120 µg/ml petroleum ether; heptadecane: 150, 300, and 600 µg/ml petroleum ether; eicosane: 200, 400, and 800 µg/ml petroleum ether; hexacosane: 75, 150, and 300 µg/ml petroleum ether; and stearic acid: 45, 90, and 180 µg/ml petroleum ether).

### Oviposition Assays

Gravid females were used for oviposition assays. Glass chambers (20×20 cm<sup>2</sup>) were used for oviposition assays. To prevent egg laying on the floor and bottom of the glass chamber, coarse grade emery papers were placed along sides and bottom of each glass chamber. Filter papers (Whatman No. 41) of 2×2 cm<sup>2</sup> sizes were used for oviposition assays. In preliminary assays, females did not lay eggs on filter papers with or without the control solvent (petroleum ether). A test sample (1 ml) and the control solvent (1 ml) were applied to separate filter paper pieces, and allowed to evaporate the solvent. These filter papers were separately kept in two Petri dishes (1 cm diameter×3 cm long), and these two Petri dishes were placed in the glass chamber with a gap of 10 cm, and subsequently, a gravid female was released in a glass chamber. Ten gravid females were separately tested for each experiment apart from the insects that did not react. Each female was observed continuously for 6 h after releasing in a square glass chamber, and when a female laid eggs for the first time, eggs were counted and this female was discarded (Sambaraju et al. 2016). If a female did not lay eggs within 6 h, this female was also discarded.

### Oviposition Assays of Gravid *D. indica* Females Towards Leaf Surface Waxes

Oviposition assay 1: A single leaf of each *T. anguina* cultivar (MNS, BAR, and POLO) was tested against the dewaxed leaf of the same cultivar to find whether leaf surface waxes from each *T. anguina* cultivar stimulated females to lay eggs (Supplementary Table 4).

Oviposition assay 2: One leaf equivalent surface wax (crude wax) of each *T. anguina* cultivar (MNS, BAR, and POLO) vs. the control solvent (petroleum ether) was tested on filter papers to observe whether crude leaf surface waxes from each *T. anguina* cultivar stimulated females to lay eggs on filter papers (Supplementary Table 4).

Oviposition assay 3: A single leaf of each *T. anguina* cultivar was tested against each other (MNS vs. BAR, MNS vs. POLO, and BAR vs. POLO) to observe whether the leaf surface wax of a particular cultivar stimulated females to lay more eggs compared to the other two cultivars (Supplementary Table 4).

Oviposition assay 4: One leaf equivalent surface wax (crude wax) of each *T. anguina* cultivar was tested against each other (MNS vs. BAR, MNS vs. POLO, and BAR vs. POLO) on filter papers to observe whether leaf surface wax of a particular cultivar stimulated females to lay more eggs compared to the other two cultivars (Supplementary Table 4).

### Oviposition Assays of Gravid *D. indica* Females Towards Synthetic Blends

Oviposition assay 5: MNS blend 4 or BAR blend 2 or POLO blend 2 vs. the control solvent were conducted on filter papers to observe whether synthetic blends resembling one leaf equivalent surface wax of each *T. anguina* cultivar stimulated females to lay eggs on filter papers (Supplementary Table 4). Previously, it was observed whether one leaf equivalent surface wax from these cultivars could serve as oviposition stimulant in females on filter papers.

Oviposition assay 6: One leaf equivalent surface wax of MNS vs. MNS blend 4, one leaf equivalent surface wax of BAR vs. BAR blend 2, and one leaf equivalent surface wax of POLO vs. POLO blend 2 were performed on filter papers to observe whether crude surface waxes and synthetic blends were equally acting as stimulants in females to lay eggs on filter papers (Supplementary Table 4).

Oviposition assay 7: MNS blend 4 vs. BAR blend 2 or POLO blend 2, and BAR blend 2 vs. POLO blend 2 were conducted on filter papers to conclude whether females preferred a particular synthetic blend for oviposition on filter papers (Supplementary Table 4).

### Statistical Analyses

Levene's test for homogeneity of variance was applied on the data of total crude surface waxes, and individual as well as total amounts of alkanes and free fatty acids present in the leaf surface waxes of three *T. anguina* cultivars (MNS, BAR, and POLO), and the test confirmed that the data conformed to ANOVA assumptions so the Discriminant function analysis (DA) and ANOVA were conducted with untransformed data. The surface wax profiles (*n*-alkanes and free fatty acids) of the three *T. anguina* cultivars were compared using DA (XLSTAT version 13). One-way ANOVA followed by post hoc Tukey test were performed using SPSS software (version 16.0) to compare the treatment effects on individuals, and total alkanes and free fatty acids (Zar 1999). Data from Y-tube olfactometer bioassays and oviposition assays were analyzed based on the null hypothesis that the probability of scores for the test compound(s) or control solvent is equal to 50%, i.e., by a Chi-square test ( $H_0: P = 50\%$ ) (Karmakar et al. 2018).

## Results

### Leaf Surface Wax in *T. anguina* Cultivars

The DA revealed that the first factor F1 explained more than 75% of the variation, and the biplot represented the spatial orientation of response variables, MNS, BAR, and POLO cultivars against the two extracted factors (Fig. 1). As shown through the Fisher's distance, three cultivars varied significantly with respect to the leaf surface wax compounds (Fig. 1, Supplementary Table 5). Based on canonical correlations and the eigen values, F1 and F2 explained 100% of variation among the three cultivars (Supplementary Table 5). Discrimination by DA was significant (Wilk's  $\lambda < 0.001$ ,  $F_{8, 18} = 190.275$ ,  $P < 0.001$ ).

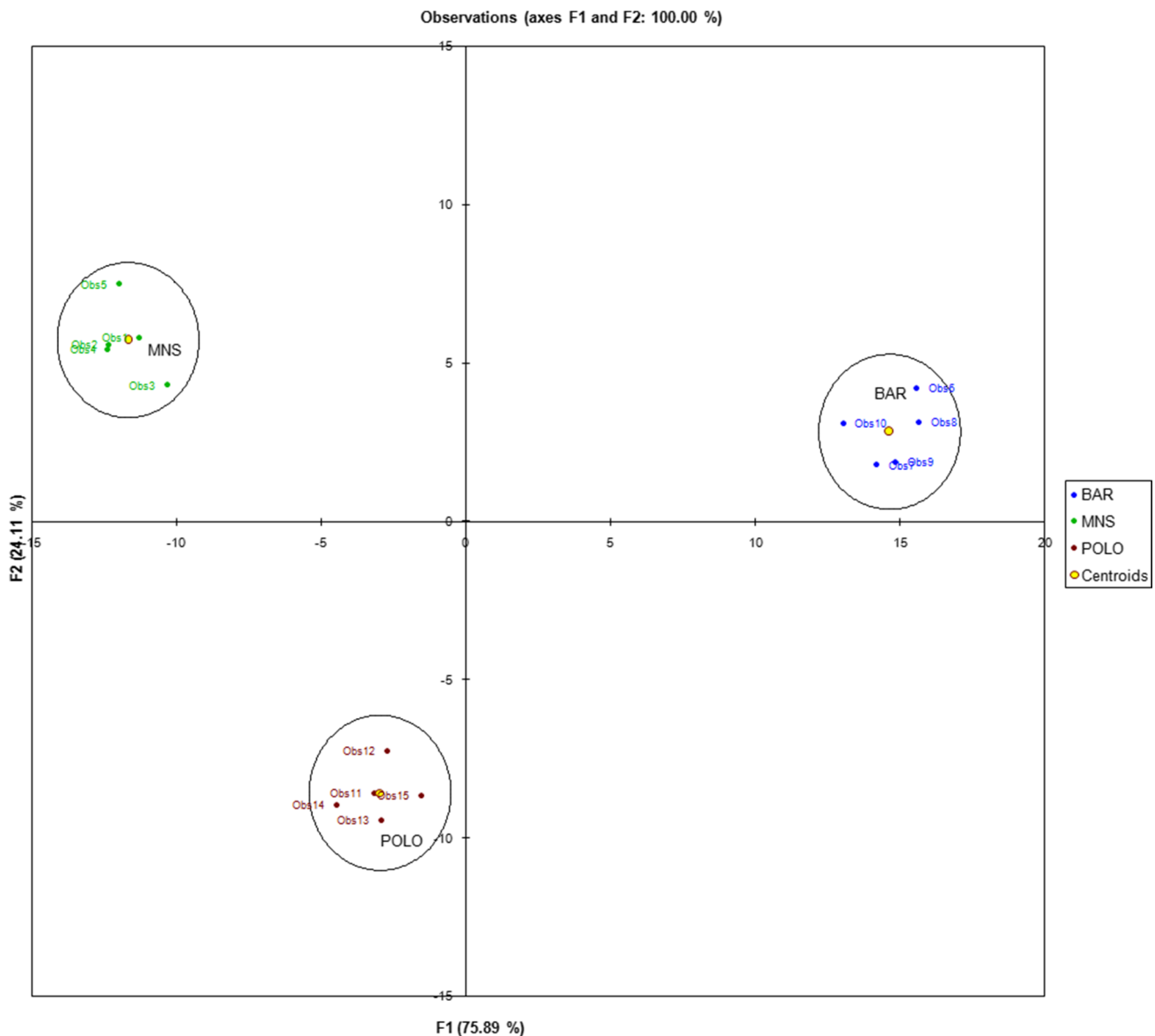
The amount of leaf surface wax was the highest in MNS ( $42.28 \pm 1.32$  mg) followed by BAR ( $36.18 \pm 1.13$  mg) and the lowest in POLO ( $31.46 \pm 1.07$  mg) ( $F = 21.772$ ;  $df = 2, 12$ ;  $P < 0.001$ ). Among the total amount of leaf surface waxes, alkanes represented for  $25.72 \pm 0.31$ ,  $22.45 \pm 0.44$ , and  $18.54 \pm 0.34$  mg (mean  $\pm$  SE) in MNS, BAR, and POLO, respectively; whereas free fatty acids accounted for  $5.63 \pm 0.18$ ,  $3.82 \pm 0.21$ , and  $4.06 \pm 0.17$  mg (mean  $\pm$  SE) in MNS, BAR, and POLO, respectively, with the balance consisting of unidentified surface wax compounds.

### Alkanes in Leaf Surface Waxes of Three *T. anguina* Cultivars

Total amounts of alkanes differed significantly among the leaf surface waxes of these cultivars, which were the highest in MNS, intermediate in BAR, and the lowest in POLO. Among the total amount of alkanes, the unidentified branched-chain alkanes represented for  $0.03 \pm 0.001$ ,  $0.01 \pm 0.001$ , and  $0.01 \pm 0.001$  mg (mean  $\pm$  SE) in MNS, BAR, and POLO, respectively. Twenty *n*-alkanes from *n*-C<sub>14</sub> to *n*-C<sub>36</sub> were detected in the leaf surface waxes of these cultivars (Supplementary Fig. 2, Table 2). Heptadecane (*n*-C<sub>17</sub>) and pentacosane (*n*-C<sub>25</sub>) were the predominant and least abundant in the leaf surface waxes of these cultivars.

### Free Fatty acids in Leaf Surface Waxes of *T. anguina* Cultivars

Total amounts of free fatty acids were higher in MNS compared to BAR and POLO, but there was no significant difference between BAR and POLO (Table 3). Thirteen free fatty acids from C12:0 to C21:0 were detected in the leaf surface waxes of these cultivars (Supplementary Fig. 3, Table 3). Stearic acid (C18:0) was the most abundant in the leaf surface waxes of these cultivars. Tetradecanoic acid (C14:0),



**Fig. 1** Discriminant function analysis (DA) using three *Trichosanthes anguina* cultivars, MNSR-1 (MNS), Baruipur Long (BAR), and Polo No.1 (POLO) as the response variables against the data set of

leaf surface wax chemicals (long-chain alkanes and free fatty acids) obtained from three *T. anguina* cultivars as explanatory variables

palmitoleic acid (C16:1), and heneicosanoic acid (C21:0) were the least abundant among the free fatty acids in MNS, BAR, and POLO, respectively (Table 3).

### Olfactometer Bioassays with Gravid *D. indica* Females Towards Crude Surface Waxes

More females were attracted towards one leaf equivalent of surface wax from each *T. anguina* cultivar against solvent controls (Fig. 2). Females did not show preference towards the leaf surface wax of a particular *T. anguina* cultivar when

the leaf surface waxes of three cultivars were tested against each other (Fig. 3).

### Olfactometer Bioassays with Gravid *D. indica* Females Towards Synthetic Blends

Among all the identified alkanes and free fatty acids present in the leaf surface wax of three *T. anguina* cultivars, females showed responses towards 10 individual synthetic compounds (pentadecane, heptadecane, eicosane, pentacosane, hexacosane, heptacosane, nonacosane, palmitoleic acid, palmitic acid, and stearic acid) comparable to the amounts

**Table 2** Composition of alkanes ( $\mu\text{g}/25$  g leaf) in the leaf surface waxes of three *Trichosanthes anguina* cultivars, MNSR-1 (MNS), Baruiapur Long (BAR), and Polo No. 1 (POLO)

Alkanes	Amount ( $\mu\text{g}$ ) (Mean $\pm$ SE, $N=5$ )			$F_{2,12}$	$P$
	MNS	BAR	POLO		
Tetradecane ( $n\text{-C}_{14}$ )	126.24 $\pm$ 8.44 <sup>a</sup>	56.99 $\pm$ 3.75 <sup>b</sup>	161.22 $\pm$ 8.41 <sup>c</sup>	53.880	0.001
Pentadecane ( $n\text{-C}_{15}$ )	583.67 $\pm$ 33.42 <sup>a</sup>	491.26 $\pm$ 35.39 <sup>a</sup>	1145.00 $\pm$ 56.87 <sup>b</sup>	66.825	0.001
Hexadecane ( $n\text{-C}_{16}$ )	3017.61 $\pm$ 148.45 <sup>a</sup>	1293.83 $\pm$ 67.93 <sup>b</sup>	1922.88 $\pm$ 111.02 <sup>c</sup>	58.026	0.001
Heptadecane ( $n\text{-C}_{17}$ )	4274.26 $\pm$ 215.16 <sup>a</sup>	3602.60 $\pm$ 213.13 <sup>a</sup>	2523.01 $\pm$ 126.49 <sup>b</sup>	22.904	0.001
Octadecane ( $n\text{-C}_{18}$ )	292.66 $\pm$ 16.20 <sup>a</sup>	597.82 $\pm$ 32.26 <sup>b</sup>	111.43 $\pm$ 7.61 <sup>c</sup>	133.152	0.001
Eicosane ( $n\text{-C}_{20}$ )	4069.23 $\pm$ 196.93 <sup>a</sup>	3347.24 $\pm$ 195.84 <sup>b</sup>	2481.92 $\pm$ 119.99 <sup>c</sup>	20.642	0.001
Docosane ( $n\text{-C}_{22}$ )	3407.43 $\pm$ 178.58 <sup>a</sup>	3186.10 $\pm$ 207.33 <sup>a</sup>	2225.74 $\pm$ 122.17 <sup>b</sup>	13.109	0.001
Tricosane ( $n\text{-C}_{23}$ )	1251.34 $\pm$ 69.17 <sup>a</sup>	302.71 $\pm$ 15.43 <sup>b</sup>	348.76 $\pm$ 16.16 <sup>b</sup>	162.015	0.001
Tetracosane ( $n\text{-C}_{24}$ )	1899.88 $\pm$ 91.49 <sup>a</sup>	1911.47 $\pm$ 106.01 <sup>a</sup>	1193.81 $\pm$ 61.95 <sup>b</sup>	21.813	0.001
Pentacosane ( $n\text{-C}_{25}$ )	59.42 $\pm$ 5.67 <sup>a</sup>	40.32 $\pm$ 2.42 <sup>b</sup>	43.13 $\pm$ 2.76 <sup>b</sup>	6.957	0.010
Hexacosane ( $n\text{-C}_{26}$ )	1334.80 $\pm$ 87.65 <sup>a</sup>	1567.75 $\pm$ 86.31 <sup>a</sup>	1114.53 $\pm$ 77.46 <sup>b</sup>	7.273	0.009
Heptacosane ( $n\text{-C}_{27}$ )	70.73 $\pm$ 6.85	93.88 $\pm$ 7.21	83.71 $\pm$ 6.76	2.802	0.100
Octacosane ( $n\text{-C}_{28}$ )	806.18 $\pm$ 49.68 <sup>a</sup>	1242.34 $\pm$ 72.87 <sup>b</sup>	753.43 $\pm$ 55.69 <sup>a</sup>	19.820	0.001
Nonacosane ( $n\text{-C}_{29}$ )	73.53 $\pm$ 6.47 <sup>a</sup>	101.43 $\pm$ 6.12 <sup>b</sup>	111.53 $\pm$ 6.91 <sup>b</sup>	9.159	0.004
triacontane ( $n\text{-C}_{30}$ )	555.59 $\pm$ 44.35 <sup>a</sup>	888.93 $\pm$ 67.04 <sup>b</sup>	550.52 $\pm$ 46.95 <sup>a</sup>	13.018	0.001
Hentriacontane ( $n\text{-C}_{31}$ )	251.78 $\pm$ 16.29 <sup>a</sup>	263.11 $\pm$ 20.20 <sup>a</sup>	649.96 $\pm$ 38.72 <sup>b</sup>	70.990	0.001
Dotriacontane ( $n\text{-C}_{32}$ )	378.37 $\pm$ 31.40 <sup>a</sup>	572.66 $\pm$ 41.17 <sup>b</sup>	386.98 $\pm$ 26.63 <sup>a</sup>	10.744	0.002
Tritriacontane ( $n\text{-C}_{33}$ )	99.46 $\pm$ 9.06 <sup>a</sup>	121.41 $\pm$ 10.59 <sup>a</sup>	294.85 $\pm$ 14.91 <sup>b</sup>	82.613	0.001
Tetracontane ( $n\text{-C}_{34}$ )	214.33 $\pm$ 13.45 <sup>a</sup>	361.77 $\pm$ 29.72 <sup>b</sup>	210.46 $\pm$ 10.91 <sup>a</sup>	18.912	0.001
Hexatriacontane ( $n\text{-C}_{36}$ )	2927.53 $\pm$ 153.68 <sup>a</sup>	2397.71 $\pm$ 126.08 <sup>ac</sup>	2223.52 $\pm$ 145.72 <sup>bc</sup>	6.641	0.011
Total	25,691.66 $\pm$ 309.23 <sup>a</sup>	22,441.35 $\pm$ 443.03 <sup>b</sup>	18,535.78 $\pm$ 340.19 <sup>c</sup>	94.056	0.001

Within the rows means followed by different letters are significantly different ( $P < 0.05$ ) by Tukey's HSD test

**Table 3** Composition of free fatty acids ( $\mu\text{g}/25$  g leaf) in the leaf surface waxes of three *Trichosanthes anguina* cultivars, MNSR-1 (MNS), Baruiapur Long (BAR), and Polo No. 1 (POLO)

Fatty acids	Amount ( $\mu\text{g}$ ) (Mean $\pm$ SE, $N=5$ )			$F_{2,12}$	$P$
	MNS	BAR	POLO		
Lauric acid (C12:0)	959.68 $\pm$ 61.92 <sup>a</sup>	584.49 $\pm$ 46.11 <sup>b</sup>	307.81 $\pm$ 20.57 <sup>c</sup>	50.469	0.001
Tridecanoic acid (C13:0)	630.53 $\pm$ 31.84 <sup>a</sup>	388.96 $\pm$ 18.76 <sup>b</sup>	114.76 $\pm$ 7.06 <sup>c</sup>	140.756	0.001
Tetradecanoic acid (C14:0)	47.71 $\pm$ 2.56 <sup>a</sup>	197.25 $\pm$ 20.98 <sup>b</sup>	43.60 $\pm$ 3.92 <sup>a</sup>	49.784	0.001
Pentadecanoic acid (C15:0)	152.85 $\pm$ 15.17 <sup>a</sup>	61.76 $\pm$ 3.47 <sup>b</sup>	88.30 $\pm$ 6.34 <sup>c</sup>	23.318	0.001
Palmitoleic acid (C16:1)	64.10 $\pm$ 5.61 <sup>a</sup>	48.32 $\pm$ 2.95 <sup>b</sup>	57.13 $\pm$ 4.64 <sup>a</sup>	3.047	0.085
Palmitic acid (C16:0)	808.02 $\pm$ 44.03 <sup>a</sup>	563.15 $\pm$ 46.91 <sup>b</sup>	802.91 $\pm$ 73.93 <sup>a</sup>	6.125	0.015
Heptadecanoic acid (C17:0)	427.24 $\pm$ 35.40 <sup>a</sup>	257.44 $\pm$ 14.82 <sup>b</sup>	295.42 $\pm$ 20.85 <sup>b</sup>	12.531	0.001
Linolenic acid (C18:3)	230.19 $\pm$ 12.93 <sup>a</sup>	162.64 $\pm$ 13.32 <sup>b</sup>	119.31 $\pm$ 8.96 <sup>b</sup>	22.092	0.001
Linoleic acid (C18:2)	409.16 $\pm$ 22.76 <sup>a</sup>	171.58 $\pm$ 8.86 <sup>b</sup>	643.35 $\pm$ 44.57 <sup>c</sup>	64.669	0.001
Stearic acid (C18:0)	1103.9 $\pm$ 63.19	904.24 $\pm$ 65.18	956.50 $\pm$ 78.83	2.230	0.150
Nonadecanoic acid (C19:0)	554.80 $\pm$ 29.57 <sup>a</sup>	338.94 $\pm$ 16.88 <sup>b</sup>	473.48 $\pm$ 31.73 <sup>a</sup>	16.522	0.001
Arachidic acid (C20:0)	164.33 $\pm$ 12.25 <sup>a</sup>	70.19 $\pm$ 5.58 <sup>b</sup>	126.15 $\pm$ 6.87 <sup>c</sup>	29.546	0.001
Heneicosanoic acid (C21:0)	81.88 $\pm$ 4.91 <sup>a</sup>	69.64 $\pm$ 4.05 <sup>a</sup>	32.51 $\pm$ 2.57 <sup>b</sup>	42.187	0.001
Total	5635.09 $\pm$ 175.52 <sup>a</sup>	3819.15 $\pm$ 214.90 <sup>b</sup>	4061.24 $\pm$ 174.17 <sup>b</sup>	27.196	0.001

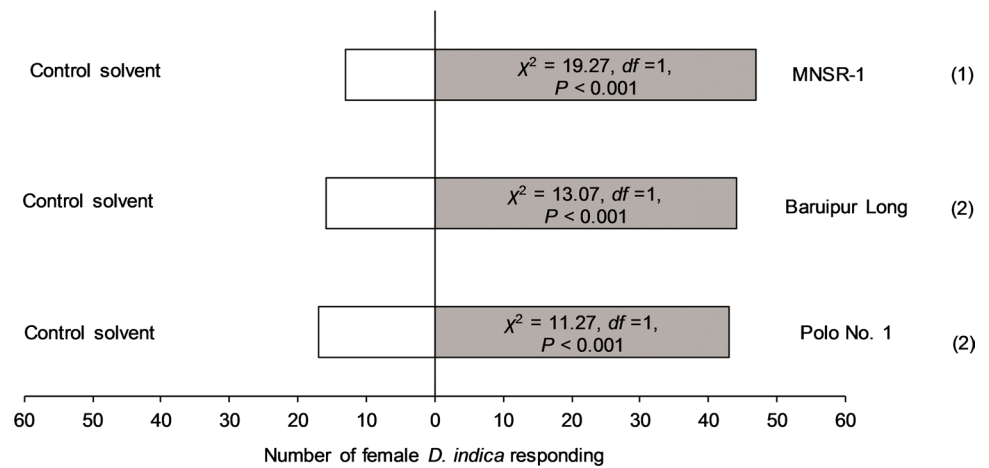
Within the rows means followed by different letters are significantly different ( $P < 0.05$ ) by Tukey's HSD test

present in one leaf equivalent surface wax of each *T. anguina* cultivar against solvent controls (Table 4). Females were attracted towards a synthetic blend of above 10 compounds

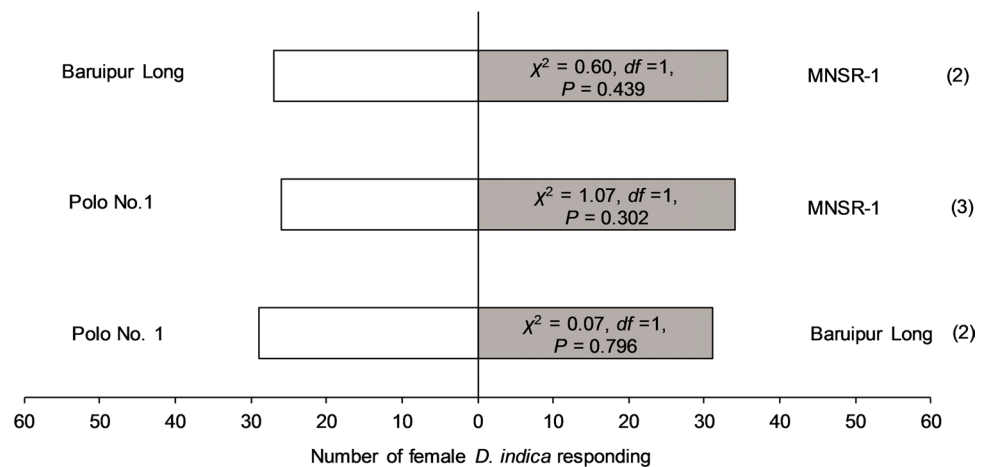
comparable to the amounts present in one leaf equivalent surface wax of each *T. anguina* cultivar against solvent controls (Table 4).



**Fig. 2** Behavioral responses of *Diaphania indica* females towards one leaf equivalent surface wax of three *Trichosanthes anguina* cultivars (cv. MNSR-1, Baruiapur Long, and Polo No.1) against solvent controls (petroleum ether) in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment



**Fig. 3** Behavioral responses of *Diaphania indica* females towards one leaf equivalent surface wax of three *Trichosanthes anguina* cultivars (cv. MNSR-1, Baruiapur Long, and Polo No.1) were tested against each other in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment



The insect showed attraction towards four individual synthetic compounds (heptadecane, eicosane, hexacosane, and stearic acid) comparable to the amounts present in one leaf equivalent surface wax of MNS against solvent controls. MNS blend 4 was attractive to the females against solvent controls (Table 4). Females were attracted towards two individual synthetic compounds (hexacosane and stearic acid) comparable to the amounts present in one leaf equivalent surface wax of BAR against solvent controls. BAR blend 2 was attractive to the females against solvent controls (Table 4). Females elicited attraction towards two individual synthetic compounds (pentadecane and stearic acid) comparable to the amounts present in one leaf equivalent surface wax of POLO against solvent controls. POLO blend 2 attracted females against solvent controls (Table 4).

Females could not differentiate between one leaf equivalent surface wax of each *T. anguina* cultivar and a synthetic blend of 10 compounds (pentadecane, heptadecane, eicosane, pentacosane, hexacosane, heptacosane, nonacosane, palmitoleic acid, palmitic acid, and stearic acid) comparable to the amounts present in one leaf equivalent

surface wax of each *T. anguina* cultivar (Table 5). Females could not distinguish between one leaf equivalent surface wax of MNS and MNS blend 4 (Table 5). Females could not discriminate between one leaf equivalent surface wax of BAR and BAR blend 2 (Table 5). Females could not make a distinction between one leaf equivalent surface wax of POLO and POLO blend 2 (Table 5). Females could not discriminate between MNS blend 4 and BAR blend 2 or POLO blend 2 (Fig. 4). Females also could not distinguish between BAR blend 2 and POLO blend 2 (Fig. 4).

In dose response bioassays, females started to demonstrate attraction towards pentadecane at 60 µg/ml petroleum ether and showed the highest attraction at 120 µg/ml petroleum ether (Table 6). Females were attracted towards heptadecane at 300 µg/ml petroleum ether and displayed the highest attraction at 600 µg/ml petroleum ether (Table 6). Females started to display attraction towards eicosane at 400 µg/ml petroleum ether and showed the highest attraction at 800 µg/ml petroleum ether (Table 6). Females started to show attraction towards hexacosane at 150 µg/ml petroleum ether and showed the highest

**Table 4** Behavioral responses of *Diaphania indica* females towards individual synthetic compounds or synthetic blends comparable to the amounts present in one leaf equivalent surface wax of MNSR-1 (MNS) or Baruiipur Long (BAR) or Polo No 1 (POLO) cultivars of *Trichosanthes anguina* vs. solvent controls (petroleum ether) ( $N=60$  in each bioassay)

Comparison	Insects responded		Non-responders	$\chi^2$ ( $df=1$ )	$P$ values
	T1	T2			
Synthetic compounds comparable to one leaf equivalent surface wax of MNS ( $\mu\text{g/ml}$ )	Control solvent				
a. Pentadecane (41.69)	35	25	4	1.67	0.197
b. Heptadecane (305.30)	39	21	2	5.40	0.020
c. Eicosane (290.66)	38	22	2	4.27	0.039
d. Pentacosane (4.24)	32	28	5	0.27	0.606
e. Hexacosane (95.34)	38	22	2	4.27	0.039
f. Heptacosane (5.05)	33	27	5	0.60	0.439
g. Nonacosane (5.25)	35	25	4	1.67	0.197
h. Palmitoleic acid (4.58)	32	28	6	0.27	0.606
i. Palmitic acid (57.72)	35	25	3	1.67	0.197
j. Stearic acid (78.85)	39	21	2	5.40	0.020
a+b+c+d+e+f+g+h+i+j	46	14	1	17.07	0.001
b+c+e+j	43	17	1	11.27	0.001
Synthetic compounds comparable to one leaf equivalent surface wax of BAR ( $\mu\text{g/ml}$ )					
a. Pentadecane (30.70)	34	26	5	1.07	0.302
b. Heptadecane (225.16)	37	23	3	3.27	0.071
c. Eicosane (209.20)	36	24	4	2.40	0.121
d. Pentacosane (2.52)	31	29	5	0.07	0.796
e. Hexacosane (97.98)	38	22	2	4.27	0.039
f. Heptacosane (5.87)	33	27	4	0.60	0.439
g. Nonacosane (6.34)	35	25	3	1.67	0.197
h. Palmitoleic acid (3.02)	32	28	5	0.27	0.606
i. Palmitic acid (35.20)	35	25	3	1.67	0.197
j. Stearic acid (56.52)	38	22	2	4.27	0.039
a+b+c+d+e+f+g+h+i+j	43	17	1	11.27	0.001
e+j	41	19	1	8.07	0.005
Synthetic compounds comparable to one leaf equivalent surface wax of POLO ( $\mu\text{g/ml}$ )					
a. Pentadecane (63.61)	38	22	2	4.27	0.039
b. Heptadecane (140.17)	34	26	4	1.07	0.302
c. Eicosane (137.88)	34	26	4	1.07	0.302
d. Pentacosane (2.40)	31	29	5	0.07	0.796
e. Hexacosane (61.92)	34	26	3	1.07	0.302
f. Heptacosane (4.65)	33	27	4	0.60	0.439
g. Nonacosane (6.20)	35	25	3	1.67	0.197
h. Palmitoleic acid (3.17)	32	28	5	0.27	0.606
i. Palmitic acid (44.61)	34	26	4	1.07	0.302
j. Stearic acid (53.14)	38	22	2	4.27	0.039
a+b+c+d+e+f+g+h+i+j	42	18	1	9.60	0.002
a+j	41	19	1	8.07	0.005

attraction at 300  $\mu\text{g/ml}$  petroleum ether (Table 6). Females started to display positive responses towards stearic acid at 90  $\mu\text{g/ml}$  petroleum ether and showed the highest attraction at 180  $\mu\text{g/ml}$  petroleum ether (Table 6).

### Oviposition Assays with Gravid *D. indica* Females Towards Crude Surface Waxes

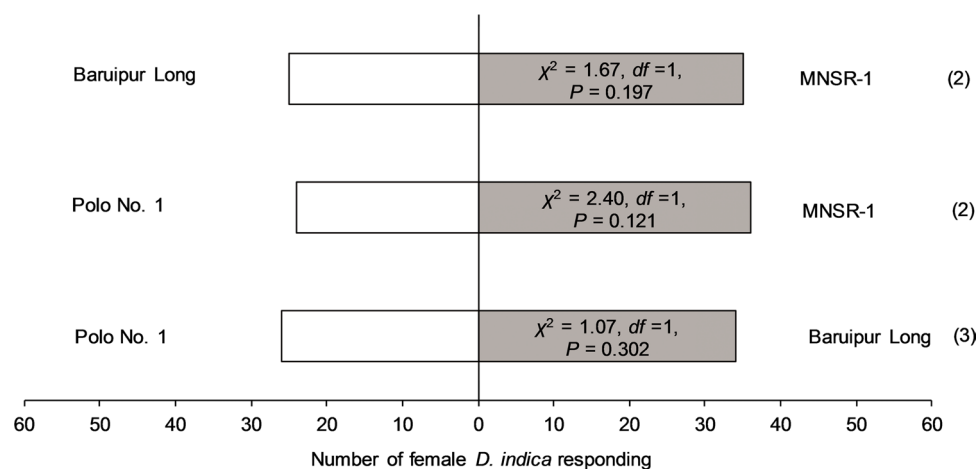
Females laid more eggs on filter paper treated with surface waxes from each *T. anguina* cultivar than on filter

**Table 5** Behavioral responses of *Diaphania indica* females towards one leaf equivalent surface wax of three *Trichosanthes anguina* cultivars, MNSR-1 (MNS), Baruipur Long (BAR), and Polo No. 1 (POLO) vs. individual synthetic compounds or synthetic blends comparable to the amounts present in one leaf equivalent surface wax of MNS, BAR, and POLO cultivars ( $N=60$  in each bioassay)

Comparison		Insects responded		Non-responders	$\chi^2$ ( $df=1$ )	$P$ values
		T1	T2			
One leaf equivalent surface wax of MNS	Synthetic compounds comparable to one leaf equivalent surface wax of MNS ( $\mu\text{g/ml}$ )					
	a. Pentadecane (41.69)	43	17	1	11.27	0.001
	b. Heptadecane (305.30)	38	22	2	4.27	0.039
	c. Eicosane (290.66)	39	21	2	5.40	0.020
	d. Pentacosane (4.24)	46	14	1	17.07	0.001
	e. Hexacosane (95.34)	39	21	2	5.40	0.020
	f. Heptacosane (5.05)	44	16	1	13.07	0.001
	g. Nonacosane (5.25)	43	17	1	11.27	0.001
	h. Palmitoleic acid (4.58)	46	14	1	17.07	0.001
	i. Palmitic acid (57.72)	43	17	1	11.27	0.001
	j. Stearic acid (78.85)	38	22	2	4.27	0.039
	a+b+c+d+e+f+g+h+i+j	31	29	1	0.07	0.796
	b+c+e+j	32	28	2	0.27	0.606
One leaf equivalent surface wax of BAR	Synthetic compounds comparable to one leaf equivalent surface wax of BAR ( $\mu\text{g/ml}$ )					
	a. Pentadecane (30.70)	42	18	1	9.60	0.002
	b. Heptadecane (225.16)	41	19	1	8.07	0.005
	c. Eicosane (209.20)	41	19	2	8.07	0.005
	d. Pentacosane (2.52)	43	17	1	11.27	0.001
	e. Hexacosane (97.98)	39	21	2	5.40	0.020
	f. Heptacosane (5.87)	42	18	1	9.60	0.002
	g. Nonacosane (6.34)	43	17	2	11.27	0.001
	h. Palmitoleic acid (3.02)	41	19	1	8.07	0.005
	i. Palmitic acid (35.20)	40	20	2	6.67	0.010
	j. Stearic acid (56.52)	39	21	2	5.40	0.020
	a+b+c+d+e+f+g+h+i+j	31	29	1	0.07	0.796
	e+j	33	27	2	0.60	0.439
One leaf equivalent surface wax of POLO	Synthetic compounds comparable to one leaf equivalent surface wax of POLO ( $\mu\text{g/ml}$ )					
	a. Pentadecane (63.61)	39	21	2	5.40	0.020
	b. Heptadecane (140.17)	41	19	1	8.07	0.005
	c. Eicosane (137.88)	41	19	1	8.07	0.005
	d. Pentacosane (2.40)	42	18	1	9.60	0.002
	e. Hexacosane (61.92)	41	19	1	8.07	0.005
	f. Heptacosane (4.65)	42	18	1	9.60	0.002
	g. Nonacosane (6.20)	40	20	1	6.67	0.010
	h. Palmitoleic acid (3.17)	42	18	1	9.60	0.002
	i. Palmitic acid (44.61)	41	19	1	8.07	0.005
	j. Stearic acid (53.14)	39	21	2	5.40	0.039
	a+b+c+d+e+f+g+h+i+j	31	29	1	0.07	0.796
	a+j	33	27	2	0.60	0.439

paper treated with solvent alone (Table 7). Oviposition assays revealed that wax extracts of each *T. anguina* cultivar elicited oviposition in *D. indica*, but there were no differences among the three cultivars (Table 7). Removal of waxes from each cultivar greatly reduced oviposition.

Oviposition did not differ on leaves of each cultivar or extracted leaf waxes of each cultivar in dual choice bioassays (Table 7).



**Fig. 4** Behavioral responses of *Diaphania indica* females towards a synthetic blend of four compounds (heptadecane, eicosane, hexacosane, and stearic acid) comparable to one leaf equivalent surface wax of MNSR-1 vs. a synthetic blend of two compounds (hexacosane and stearic acid) comparable to one leaf equivalent surface wax of Baruiपुर Long or a synthetic blend of two compounds (pentadecane and stearic acid) comparable to one leaf equivalent surface wax of

Polo No. 1, and a synthetic blend of two compounds (hexacosane and stearic acid) comparable to one leaf equivalent surface wax of Baruiपुर Long vs. a synthetic blend of two compounds (pentadecane and stearic acid) comparable to one leaf equivalent surface wax of Polo No. 1 were tested against each other in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment

### Oviposition Bioassays with Gravid *D. indica* Females Towards Synthetic Blends

Bioassays with synthetic blends (MNS blend 4, BAR blend 2, and POLO blend 2) showed that these stimulated oviposition similar to extracted waxes (Table 7). Synthetic blends of surface waxes of each cultivar elicited more oviposition on treated filter paper than solvent-treated controls (Table 7).

**Table 6** Responses of *Diaphania indica* females towards individual synthetic compound vs. the control solvent (petroleum ether) in the Y-tube olfactometer bioassay ( $N=60$  in each concentration bioassay)

Synthetic compounds	Concentration ( $\mu\text{g/ml}$ )	$\chi^2$ ( $df=1$ )	$P$ values of insects responded
Pentadecane ( $n\text{-C}_{15}$ )	30	1.07	0.302
	60	4.27	0.039
	120	15.00	0.001
Heptadecane ( $n\text{-C}_{17}$ )	150	1.07	0.302
	300	5.40	0.020
	600	15.00	0.001
Eicosane ( $n\text{-C}_{20}$ )	200	2.40	0.121
	400	8.07	0.005
	800	15.00	0.001
Hexacosane ( $n\text{-C}_{26}$ )	75	1.67	0.197
	150	9.60	0.002
	300	19.27	0.001
Stearic acid ( $\text{C}_{18:0}$ )	45	2.40	0.121
	90	8.07	0.005
	180	15.00	0.001

Oviposition did not differ between filter paper treated with synthetic blends vs. filter paper treated with extracted surface waxes of each cultivar, nor did it differ between synthetic blends from each cultivar presented in dual choice bioassays (Table 7).

### Discussion

The suitability of a host plant by monophagous or oligophagous insect herbivores depends on the chemical recognition of the host plant as an insect herbivore feeds and develops only on one or a few host plants within one plant family. Gravid lepidopteran females display a string of behavior to attain information from the epicuticular wax compounds of its host plant when a female touches on it, suggesting that sensory cues from the plant's leaf surface play an essential role for host acceptance (Müller 2006). The dichloromethane extract of maize seedlings consisting of linoleic acid, oleic acid, and stearic acid attracted the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Hibbard et al. 1994). The olfactometer bioassay of *D. indica* demonstrated that leaf surface waxes from the three *T. anguina* cultivars attracted females from short-range for oviposition. The oviposition assays showed that females of *D. indica* prefer to lay eggs on intact leaves containing surface wax chemicals compared to leaves from which surface waxes have been removed. Further, this was corroborated by the result that females of *D. indica* preferred to lay eggs on filter paper substrates treated with leaf surface wax extracts. The experiments on short Y-tube

**Table 7** Oviposition assay of *Diaphania indica* females towards three *Trichosanthes anguina* cultivars, MNSR-1 (MNS), Baruiपुर Long (BAR), and Polo No. 1 (POLO) ( $N=10$  in each bioassay)

Comparison		No. of insects laid eggs		Non-responders	Eggs laid		$\chi^2$ ( $df=1$ )	P values
T1	T2	T1	T2		T1	T2		
A single leaf of MNS	Dewaxed MNS leaf	9	1	1	206	20	153.08	0.001
A single leaf of BAR	Dewaxed BAR leaf	8	2	1	165	38	79.45	0.001
A single leaf of POLO	Dewaxed POLO leaf	7	3	2	137	45	46.50	0.001
One leaf equivalent surface wax of MNS	Control solvent (petroleum ether)	8	2	2	166	41	75.48	0.001
One leaf equivalent surface wax of BAR	Control solvent (petroleum ether)	8	2	2	157	33	80.93	0.001
One leaf equivalent surface wax of POLO	Control solvent (petroleum ether)	7	3	2	131	43	44.50	0.001
A single leaf of MNS	A single leaf of BAR	6	4	1	124	105	1.58	0.209
A single leaf of MNS	A single leaf of POLO	6	4	1	112	99	0.80	0.371
A single leaf of BAR	A single leaf of POLO	5	5	1	98	91	0.26	0.610
One leaf equivalent surface wax of MNS	One leaf equivalent surface wax of BAR	6	4	1	112	89	2.63	0.105
One leaf equivalent surface wax of MNS	One leaf equivalent surface wax of POLO	6	4	2	109	83	3.52	0.061
One leaf equivalent surface wax of BAR	One leaf equivalent surface wax of POLO	5	5	2	98	81	1.61	0.204
A synthetic blend of 4 compounds comparable to one leaf equivalent surface wax of MNS*	Control solvent (petroleum ether)	8	2	2	164	40	75.37	0.001
A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of BAR**	Control solvent (petroleum ether)	8	2	2	135	58	30.72	0.001
A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of POLO***	Control solvent (petroleum ether)	7	3	2	126	48	34.96	0.001
One leaf equivalent surface wax of MNS	A synthetic blend of 4 compounds comparable to one leaf equivalent surface wax of MNS*	5	5	1	107	104	0.04	0.841
One leaf equivalent surface wax of BAR	A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of BAR**	6	4	2	113	88	3.11	0.078
One leaf equivalent surface wax of POLO	A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of POLO***	6	4	2	104	85	1.91	0.167
A synthetic blend of 4 compounds comparable to one leaf equivalent surface wax of MNS*	A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of BAR**	6	4	1	112	89	2.63	0.105
A synthetic blend of 4 compounds comparable to one leaf equivalent surface wax of MNS*	A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of POLO***	7	3	2	105	82	2.83	0.092
A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of BAR**	A synthetic blend of 2 compounds comparable to one leaf equivalent surface wax of POLO***	6	4	2	98	79	2.04	0.153

\* A synthetic blend comprised of 305.30  $\mu\text{g}$  heptadecane, 290.66  $\mu\text{g}$  eicosane, 95.34  $\mu\text{g}$  hexacosane, and 78.85  $\mu\text{g}$  stearic acid

\*\* A synthetic blend comprised of 97.98  $\mu\text{g}$  hexacosane and 56.52  $\mu\text{g}$  stearic acid

\*\*\* A synthetic blend comprised of 63.61  $\mu\text{g}$  pentadecane and 53.14  $\mu\text{g}$  stearic acid

olfactometer bioassays and oviposition assays suggest that additional work would be required to decide whether the clear preference by the gravid females is due to attraction or oviposition-stimulatory effects.

The surface wax composition of each *T. anguina* cultivar is comprised of alkanes (ca. 27%), acids (ca. 19%), ketones (ca. 19%), alcohols (ca. 12.5%), aldehydes (ca. 4%), amines (ca. 4%), alkenes (ca. 4%), glycosides and triterpenes (ca.

4%), and other compounds (ca. 8%) (unpublished data). But, when *n*-alkanes and free fatty acids were extracted separately, 20 *n*-alkanes from *n*-C<sub>14</sub> to *n*-C<sub>36</sub> and 13 free fatty acids from C12:0 to C21:0 were identified in the leaf surface waxes of each *T. anguina* cultivar. Different alkanes and free fatty acids were predominant in the leaf surface waxes of various plant species (Dodoš et al. 2015; Piasentier et al. 2000). The wax biosynthesis has been found to occur by multiple elongation systems, which are both sequential as well as parallel reactions. The three pathways – decarbonylation, acyl-reduction, and β-ketoacyl-elongation are demonstrated as distinct and parallel pathways in the biosynthesis of wax. Through the decarbonylation pathway, aldehydes convert into odd chain alkanes followed by secondary alcohols and ketones (Post-Beittenmiller 1996). These three pathways occur in the epidermal tissue of most plants, but their relative proportions vary from organs to organs as well as species to species. In this study, low molecular weight compounds are abundant, indicating that odd chain alkanes are not so much transformed into secondary alcohols and ketones. Heptadecane and octanoic acid were the major wax components in pitted, ivyleaf, and palmleaf morningglory leaves (Chachalis et al. 2001). Among *n*-alkanes, heptadecane was predominant in the epicuticular waxes of *Ficus glomerata* leaves (Kundu and Sinhababu 2013). In the current investigation, heptadecane and stearic acid were the most abundant among alkanes and free fatty acids, respectively, in the three *T. anguina* cultivars. The current study revealed variations in the amounts of individual *n*-alkanes and free fatty acids in the leaf surface waxes of three *T. anguina* cultivars (MNS, BAR, and POLO). This study is in agreement with the hypothesis that variations in the compositions of leaf surface wax compounds might happen between plant species including within different cultivars of a plant species (Dodoš et al. 2015; Piasentier et al. 2000).

The present results obtained through olfactometer bioassays and oviposition assays suggested that the leaf surface waxes of three *T. anguina* cultivars played an important role in short-range attraction and stimulated females of *D. indica* to lay eggs. After coming close range of the host plants, leaf surface wax chemicals such as long-chain alkanes and free fatty acids serve a vital role in plant–insect interactions such as short-range attraction (Hibbard et al. 1994; Manosalva et al. 2011; Mitra et al. 2017; Schiestl et al. 1999) and stimulated females to lay eggs (Grant et al. 2000; Li and Ishikawa 2006; Parr et al. 1998; Phelan et al. 1991; Udayagiri and Mason 1997). This study also reveals that females of *D. indica* employ leaf surface wax chemicals such as alkanes and free fatty acids as olfactory cues, which stimulated females to lay eggs on the leaves of three *T. anguina* cultivars. But, females of *D. indica* could not make a distinction in olfactometer bioassays between MNS and BAR or POLO as well as BAR and POLO, and similarly, females did not

prefer to lay eggs on a particular *T. anguina* cultivar, which is indicative of that one leaf equivalent surface wax of the three *T. anguina* cultivars is equally stimulated females of *D. indica* to lay eggs. However, females of *D. indica* laid eggs on a synthetic blend of four compounds – heptadecane, eicosane, hexacosane, and stearic acid comparable to the amounts present in one leaf equivalent surface wax of MNS or a synthetic blend of two compounds – hexacosane and stearic acid comparable to the amounts present in one leaf equivalent surface wax of BAR or a synthetic blend of two compounds – pentadecane and stearic acid comparable to the amounts present in one leaf equivalent surface wax of POLO, suggesting that the gravid *D. indica* females could recognize the three *T. anguina* cultivars mainly by both the qualitative (by discrete chemical compounds) and quantitative (by an exact amount of compounds) as contact cues to lay eggs. In the present study, it is interesting to observe that the volatility of *n*-alkanes from tetradecane (*n*-C<sub>14</sub>) to nonacosane (*n*-C<sub>29</sub>) differs by more than two orders of magnitude, yet the longer chain alkane hexacosane (*n*-C<sub>26</sub>) is as active for attraction as or more active than heptadecane (*n*-C<sub>17</sub>). Further, some of the long-chain alkanes and free fatty acids might be ubiquitous in the leaf surface waxes of various plants, but the specific combination and the amounts of *n*-alkanes and free fatty acids differ between plants, by which an insect can distinguish between leaf surface waxes of host plants and non-host plants (Li and Ishikawa 2006). The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) employs long-chain fatty acids, particularly oleic acid and linoleic acid as ovipositional host-finding cues (Phelan et al. 1991). Fatty acids from C8 to C12, oleic acid (C18:1) and linoleic acid (C18:2) comparable to the amounts present in the surface wax of *Picea* and *Abies* spp stimulated oviposition in spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) (Grant et al. 2000). Further, a blend of pentacosane, heptacosane, nonacosane, hexatriacontane, palmitoleic acid, linolenic acid, and stearic acid, a synthetic blend of pentacosane, hexatriacontane, and stearic acid, and a synthetic blend of hexatriacontane, linolenic acid, and stearic acid comparable to the amounts present in one leaf equivalent surface wax of PDM 54, PUSA BAIKAKHI, and SAMRAT cultivars of green gram, respectively, stimulated *Spilosoma obliqua* Walker (Lepidoptera: Arctiidae) to lay eggs (Mobarak et al. 2020). The current study suggested that insects could respond to a particular combination of compounds as host-plant acceptance process to lay eggs, and the cues for egg laying behavior by the gravid female could weaken when the crucial compounds were altered (Li and Ishikawa 2006; Parr et al. 1998).

The present study concludes that the leaf surface wax chemicals of three cultivars, i.e., MNS, BAR, and POLO of *T. anguina* contain oviposition stimulants for the gravid

females of *D. indica*. Females of *D. indica* were attracted towards a synthetic blend of 305.30 µg heptadecane, 290.66 µg eicosane, 95.34 µg hexacosane and 78.85 µg stearic acid, a synthetic blend of 97.98 µg hexacosane and 56.52 µg stearic acid, and a synthetic blend of 63.61 µg pentadecane and 53.14 µg stearic acid comparable to the amounts present in one leaf equivalent surface wax of MNS, BAR, and POLO, respectively. This study propose that once volatile organic compounds (VOCs) from *T. anguina* plants causing long-range attraction of *D. indica* females have been determined then the above mentioned three synthetic blends along with the VOCs of leaves could be used as lures to developing baited traps in integrated pest management program (IPM) strategies.

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**Author Contributions** A.B. and R.D. designed experiments. R.D. and P.M. performed bioassays. R.D. and S.D. did chemical analyses. A.B. and R.D. analysed data. R.D. and P.M. made the Figs. A.B. wrote the manuscript. All authors edited the manuscript and approved the final version of the manuscript.

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**Data Availability** The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

**Code Availability** Not applicable.

## Declarations

**Ethics Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent for Publication** All authors agreed to the submission of the final manuscript.

**Conflict of Interest/Competing Interests** The authors declare that they have no conflict of interest/competing interests.

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