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ORIGINAL ARTICLE

Leaf waxes from *Lathyrus sativus*: short-range attractant and stimulant for nymph laying in a viviparous insect

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Abstract

Lathyrus sativus L. (Fabaceae) is an important pulse crop of Asia, Europe, and Africa. Infestation by the aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae) causes stunted growth of plants and reduces seed production. Females lay nymphs on the leaves and flowers of *L. sativus*. Hence, it is relevant to study the importance of leaf wax compounds (long-chain alkanes and free fatty acids) from two cultivars [BIO L 212 Ratan (BIO) and Nirmal B-1 (NIR)] of *L. sativus* as short-range attractant and stimulant for nymph laying in the aphid. The TLC, GC-MS and GC-FID analyses of *n*-hexane extracts from leaves of two cultivars revealed 18 *n*-alkanes from *n*-C_{15} to *n*-C_{36} and 14 free fatty acids from C12:0 to C22:0. Pentadecane was predominant among *n*-alkanes in both cultivars. Palmitoleic acid and pentadecanoic acid were predominant free fatty acids in leaf waxes of BIO and NIR, respectively. Females were attracted towards leaf waxes of both cultivars compared to the control solvent (*n*-hexane) in Y-tube olfactometer bioassays. A synthetic blend of either pentadecane, tridecanoic acid, and linoleic acid at similar amounts present in one leaf equivalent wax of BIO, or pentadecane, docosane, pentacosane, heptacosane, tritriacontane, and linoleic acid at similar amounts present in one leaf equivalent wax of NIR acted as short-range attractant and stimulated females to lay nymphs. But, the latter blend was more attractive and stimulated females to lay more nymphs than the former blend, and hence, this latter blend could be employed in the development of baited traps in pest management strategies.

Keywords *Aphis craccivora* · *Lathyrus sativus* · Leaf wax · Long-chain alkanes · Free fatty acids · Olfactometer bioassay · Viviparity assay

Introduction

Lathyrus sativus L., commonly known as grass pea, is a food, feed, and fodder crop belonging to the family Fabaceae, subfamily Papilionoideae and tribe Vicieae (Allkin et al. 1985). Grass pea is a crop of great agronomic and economic significance in India, Bangladesh, Pakistan, Nepal, and Ethiopia (Kumar et al. 2011; Grela et al. 2012). It is extensively naturalized in Central, Southern and Eastern Europe, Crete, Rhodes, Cyprus, and in West Asia and North Africa (Syria, Lebanon, Palestine, Egypt, Iraq, Afghanistan, Morocco, and Algeria) (Campbell et al. 1994; Grela et al. 2012).

The viviparous aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae) is a polyphagous pest of 400 plant species (Powell et al. 2006; Brady and White 2013) and remarkable for its wide geographical range as a serious pest of leguminous crops in all continents except the Antarctic (Obopile and Ositile 2010; Kamphuis et al. 2012). Both nymphs and adults of *A. craccivora* attack seedlings, leaves, flowers and pods of *L. sativus*, and cause direct damage to the plant by sucking cell sap. Adult viviparous females lay nymphs on leaves of *L. sativus*, and during severe infestation, females also lay nymphs on flowers. The nymphs suck cell sap of *L. sativus* for 5–6 days to complete four instars, and the adults subsequently suck cell sap for a further 9–11 days on this plant. A single female lays 2–8 nymphs in a single batch

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between 12 and 24 h of emergence and subsequently lay 40–50 nymphs in its lifetime (personal observation).

Insect herbivores recognize host plants by long-range volatile organic compounds and/or visual cues from the host plant, but the first physical contact between an insect and host plant occurs on the leaf surface (Fernández et al. 2019). If the host plant is suitable, insects lay eggs or nymphs on the leaves indicating that females may use sensory cues from leaf waxes of the host plant for egg or nymph laying. In addition, the survival of offsprings is dependent on the host selection process by females on which they lay eggs or nymphs (Calatayud et al. 2008). Hence, chemicals present in leaf waxes may act as low volatile cues in finding their host in its microhabitat (Eigenbrode and Espelie 1995; Müller and Hilker 2001; Schoonhoven et al. 2005; Manosalva et al. 2011; Mukherjee et al. 2014; Sarkar and Barik 2014, 2015; Malik and Barik 2015; Das et al. 2019). Therefore, understanding the importance of leaf wax compounds in the oviposition behaviour of insects could contribute to improving strategies in integrated pest management (e.g. development of bait traps), thus reducing the use of pesticides.

The amount and composition of leaf wax compounds such as long-chain alkanes, fatty acids, esters, aldehydes, primary and secondary alcohols vary widely within species or cultivars of a species (Stadler and Reifenrath 2009; Supapvanich et al. 2011; Haliński et al. 2012). The probing behaviour of *Chactosiphon fragaefolii* (Cockerell) (Homoptera: Aphididae) is stimulated when organic solvent extracts (acetone, chloroform, and petroleum ether) from strawberry, *Fragaria vesca* L. leaves are applied on artificial glass surfaces (Shanks and Finnigan 1970). Powell et al. (1999) showed that epicuticular lipids of oats stimulate the stylet penetration activity of the black bean aphid, *Aphis fabae* Scopoli while bean extract has no such effect, and suggested that host-plant selection by *A. fabae* is influenced by epicuticular lipids. Long-chain alkanes and free fatty acids from leaf waxes of *Ludwigia octovalvis* (Jacq.) Raven (Onagraceae) act as short-range attractant and stimulate oviposition in *Altica cyanea* Weber (Coleoptera: Chrysomelidae) (Mitra et al. 2017). Furthermore, long-chain *n*-alkanes and free fatty acids from leaf waxes of the Japanese knotweed *Fallopia japonica* (Houtt.) Ronse Decr. stimulate oviposition in the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Li and Ishikawa 2006). So, it is also of considerable interest to observe whether leaf waxes of *L. sativus* could act as short-range attractant and stimulate nymph laying in the aphid *A. craccivora*.

Thus, the objectives of the present study were to (1) evaluate whether dipping extracts from leaves of two different *L. sativus* cultivars (BIO L 212 Ratan and Nirmal B-1, from now on BIO and NIR, respectively) may act as a short-range attractant of adults and/or stimulant for nymph laying in the aphid *A. craccivora*, (2) identify and quantify long-chain alkanes and free fatty acids present in leaf waxes extracts of both cultivars, (3) assess whether synthetic blends mimicking *L. sativus* leaf waxes extracts of both cultivars can act as olfactory cues for adult females *A. craccivora*, and (4) evaluate whether the most attractive blend for females can also stimulate nymph laying. This research depicts how biologically active components of leaf waxes from two cultivars of *L. sativus* act as short-range signals to attract and stimulate nymph laying in *A. craccivora*, which helps to understand chemically mediated interactions between leaf waxes and the insect.

Methods

Insects

Adults of *A. craccivora* were collected from field bean plants, *Lablab purpureus* subsp. *bengalensis* (Jacq.) Verdc. growing in the Crop Research Farm (CRF), University of Burdwan (23° 16′ N and 87° 54′ E), West Bengal, India and maintained on same leaves. They were reared at 22 ± 1 °C, 65 ± 10% relative humidity and 12 L: 12 D photoperiod for two generations in a 'BOD' incubator. Natural condition of field bean leaves was maintained by attaching a moist piece of cotton around the petiole of leaves followed by wrapping with aluminum foil, and fresh leaves were given daily by replacing the previous ones. The aphids were not reared on *L. sativus* to avoid habituation to leaf waxes of this plant and cause a bias in the olfactometer and viviparity bioassays. Adult viviparous females between 12 and 24 h of emergence were used for olfactory and viviparity bioassays.

Plant materials

Six plots [each plot 20 ft × 20 ft] were prepared for cultivation of BIO and NIR seeds of *L. sativus* (these two cultivars are cultivated in West Bengal due to high yielding potential because genetic make-up of these cultivars are suitable in present conditions) in the CRF, University of Burdwan during end of September, 2018. Each cultivar was grown in three plots with a gap of 3 ft between two plots. There were ca. 300 plants of a cultivar in each plot. Four to six mature leaves were collected from a 7-weeks old plant at 8.00 a.m. during end of November, 2018. Seventy-five grams leaves of each cultivar were collected from a plot for extraction of leaf waxes. Three separate batches of 75 g leaves of each cultivar were collected from three different plots.

Extraction of leaf waxes

Each batch of BIO and NIR leaves were separately dipped in 1 L *n*-hexane for 1 min at room temperature for the

extraction of waxes, which yielded a light straw colored extract without the traces of chlorophyll (Supplementary Fig. 1a, b) (Das et al. 2019). The crude extract from each batch of either BIO or NIR leaves was divided into three sub-samples. The first, second and third sub-samples of each BIO or NIR crude extract were used, respectively, for (1) bioassays, (2) identification and quantification of alkanes, and (3) identification and quantification of free fatty acids. One mg heneicosane (n-C_{21}) was dissolved in 1 ml n-hexane, which was added as internal standard to the second sub-sample of each crude extract before evaporation for identification and quantification of alkanes, while tricosanoic acid (C23:0, 1 mg dissolved in 1 ml n-hexane) was added to the third sub-sample of each crude extract before evaporation for identification and quantification of free fatty acids. Each sub-sample of crude extract was filtered through Whatman No. 41 filter paper and evaporated to dryness at room temperature. Each sub-sample was equivalent to 25 g leaves [number of leaves for 25 g BIO and NIR were 432 ± 8 and 406 ± 6 (mean $\pm$ standard deviation), respectively].

Olfactometer bioassays of adult viviparous *A. craccivora* females

The olfactory responses of females were performed in a glass Y-tube olfactometer (1 cm internal diameter, common arm and two side arms 5 cm long, and 45° Y angle). The common arm of the olfactometer was attached with a porous glass vial (1 cm radius × 3 cm long) and a female was released into this porous glass vial. Each arm of the olfactometer was connected to a glass-made adapter fitted into a glass vial (1 cm radius × 3 cm long), which contained a piece (2×2 cm^2) of Whatman No. 41 filter paper moistened with 1 ml of the test sample (leaf waxes or individual synthetic compounds or synthetic blends), whilst the other glass vial contained a filter paper of the same size moistened with 1 ml of the control solvent (n-hexane). Charcoal-filtered air entered each arm of the Y-tube at 75 ml min^{-1}. All the connections between different parts of the set-up consisted of Teflon tubing.

All bioassays were performed in a climate room at 22 ± 1 °C, 70 ± 5% relative humidity (RH), and light intensity of 150 lx. Females were starved for 4 h prior to use in bioassays. Behavioural responses of females to the control solvent against clean air were neutral in preliminary assays. The behaviour of each female was observed for 2 min. A female was considered to have made a choice in case of reaching the end of one arm, and the choice of the 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 aphid was removed from the Y-tube. Females that did not enter any arm (right or left) of the Y-tube and remained in the common arm of the Y-tube for 2 min, were recorded as non-responders (Mukherjee et al. 2015; Sarkar et al. 2015). For each bioassay, responses of 90 naïve aphids were recorded excluding the number of aphids did not respond. 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 minimize any spatial effect on choices.

Behavioural responses of adult viviparous *A. craccivora* females by dual choice bioassays towards natural samples (for experimental design see Supplementary Table 1).

Bioassay 1: Responses of females towards a single BIO or NIR leaf were tested against the clean air flow to find whether females were attracted towards a single leaf of both cultivars.

Bioassay 2: Responses of females towards a single leaf of BIO and NIR were tested against each other to find whether a single leaf of a particular cultivar was preferred.

Bioassay 3: Responses of females towards one leaf equivalent wax of BIO or NIR (crude extract) were tested against the control solvent to find whether females were attracted towards crude leaf waxes of both cultivars (Supplementary Table 2).

Bioassay 4: Responses of females towards one leaf equivalent wax of BIO and NIR were tested against each other to find whether leaf wax from a particular cultivar was preferred.

Viviparity assays of *A. craccivora* females (for experimental design see Supplementary Table 3).

Glass-made I-tube (length of I-tube: 10 cm and internal diameter: 1 cm, 0.3 cm diameter hole in the middle of an I-tube where a female was released) having attached with glass vials (1 cm diameter × 3 cm long) was used for viviparity assays (Supplementary Fig. 2). One ml of the test sample and the control solvent were applied to separate filter paper pieces (each filter paper: 2×2 cm^2) and allowed to evaporate the solvent under fume hood, and these filter papers were separately placed in two glass vials. Females did not lay nymph on the filter paper or filter paper moistened with the control solvent in preliminary assays. At least, ten females were separately used for each viviparity assay. Each female was observed for 6 h after releasing in an I-tube, and when a female laid nymphs for the first time, then nymphs were counted and the female was discarded. If a female did not lay nymph within 6 h, it was also discarded. The viviparity assays towards natural samples were carried out in following combinations.

Viviparity assay 1: A single BIO leaf vs. a single dewaxed BIO leaf, and a single NIR leaf vs. a single NIR dewaxed leaf (for dewaxing of leaves, a single leaf was dipped in 30 ml n-hexane for 1 min at room temperature for the extraction of leaf waxes) were tested to find whether leaf waxes of both cultivars stimulated females to lay nymphs.

Viviparity assay 2: A single BIO and NIR leaf was tested against each other to find whether a single leaf of a particular cultivar was preferred by females to lay more nymphs.

Viviparity assay 3: One leaf equivalent wax of BIO or NIR was tested against the control solvent to observe whether crude leaf waxes of both cultivars stimulated females to lay nymphs.

Viviparity assay 4: One leaf equivalent wax of BIO and NIR was tested against each other to find whether leaf wax of a particular cultivar was preferred by females to lay more nymphs.

Identification and quantification of alkanes

The second sub-sample of each crude extract was fractioned by Thin Layer Chromatography (TLC) on silica gel G (Sigma St. Louis, MO, USA) layers (thickness 0.5 mm), which had been prepared using a Unoplan (Shandon, London) coating apparatus, with carbon tetrachloride as the mobile phase, and we followed rest of the procedure adapted from Das et al. (2019) (see Supplementary material S1).

Identification and quantification of free fatty acids

The third sub-sample of each crude extract was mixed with diethyl ether and filtered through Whatman No. 41 filter paper (Sarkar and Barik 2015). 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 (Mukherjee et al. 2014; Sarkar and Barik 2015), and we followed rest of the procedure adapted from Das et al. (2019) (see Supplementary material S2).

Behavioural responses of adult viviparous *A. craccivora* females by dual choice bioassays towards synthetic compounds or blends (for experimental design see Supplementary Table 1).

Bioassay 5: Responses of females towards individual synthetic compounds at similar amounts present in one leaf equivalent wax of BIO or NIR were dissolved in 1 ml *n*-hexane and were tested against 1 ml control solvent to find the role of individual compounds on females (Supplementary Table 4a, b). Further, responses of females to synthetic blends (comprised of those synthetic compounds to which *A. craccivora* showed behavioural responses or attraction) at similar amounts present in one leaf equivalent wax of BIO or NIR were conducted against the control solvent to find the role of blends on females, and to compare the results obtained in olfactometer bioassays of *A. craccivora* to one leaf equivalent wax of BIO or NIR against the control solvent.

Bioassay 6: Responses of females towards one leaf equivalent wax of BIO were tested against synthetic blends at similar amounts present in one leaf equivalent wax of BIO. Similarly, responses of females towards one leaf equivalent wax of NIR were tested against synthetic blends at similar amounts present in one leaf equivalent wax of NIR. These tests were conducted to find whether synthetic blends and leaf waxes of both cultivars were equally attractive to females.

Bioassay 7: A synthetic blend of 3 compounds at similar amounts present in one leaf equivalent wax of BIO (from now on BIO blend 3: 2.75 µg pentadecane + 1.34 µg tridecanoic acid + 1.18 µg linoleic acid were dissolved in 1 ml *n*-hexane, as the insect showed highest attraction in the Y-tube olfactometer bioassay compared to the control solvent) was tested against a synthetic blend of 6 compounds at similar amounts present in one leaf equivalent wax of NIR (from now on NIR blend 6: 5.70 µg pentadecane + 3.73 µg docosane + 0.43 µg pentacosane + 0.42 µg heptacosane + 0.17 µg tritriacontane + 0.72 µg linoleic acid were dissolved in 1 ml *n*-hexane as the insect showed highest attraction in the Y-tube olfactometer bioassay compared to the control solvent) to find whether any synthetic blend is more attractive to the female.

Bioassay 8: Dose response bioassays of females towards individual synthetic compounds were carried out to find out the lowest and highest doses where the insect started responding and showed highest ($P < 0.0001$) attraction. Dose response bioassays of *A. craccivora* to the 7 compounds were tested at different doses against the control solvent (pentadecane: 2, 4, and 8 µg were separately dissolved in 1 ml *n*-hexane, respectively; docosane: 1.5, 3, 6, and 12 µg were separately dissolved in 1 ml *n*-hexane, respectively; pentacosane or heptacosane: 0.2, 0.4, 0.8, and 1.6 µg were separately dissolved in 1 ml *n*-hexane, respectively; tritriacontane: 0.1, 0.2, 0.4, and 0.8 µg were separately dissolved in 1 ml *n*-hexane, respectively; tridecanoic acid: 0.7, 1.4, and 2.8 µg were separately dissolved in 1 ml *n*-hexane, respectively; linoleic acid : 0.5, 1, and 2 µg were separately dissolved in 1 ml *n*-hexane, respectively). This experiment was conducted to confirm the results obtained in olfactometer bioassays of *A. craccivora* towards these compounds at various doses.

Viviparity assays of *A. craccivora* females towards synthetic blends (for experimental design see Supplementary Table 3).

Viviparity assay 5: BIO blend 3 or NIR blend 6 was tested against the control solvent to find whether synthetic blends at similar amounts present in one leaf equivalent wax of BIO or NIR stimulated females to lay nymphs.

Viviparity assay 6: One leaf equivalent wax of BIO vs. BIO blend 3 and one leaf equivalent wax of NIR vs. NIR blend 6 were conducted to find whether synthetic blends and

leaf waxes of both cultivars were equally stimulated females to lay nymphs.

Viviparity assay 7: BIO blend 3 was tested against NIR blend 6 to find whether a particular synthetic blend stimulated females to lay more nymphs.

Statistical analyses

To observe whether data on total amounts of leaf waxes, alkanes and free fatty acids, and amounts of individual alkanes and free fatty acids present in leaf waxes of two cultivars of *L. sativus* were normally distributed, we conducted Levene's test for the homogeneity of variance. Data on total amounts of leaf waxes, alkanes and free fatty acids were subjected to Student's *t* test. Data obtained on olfactometer and viviparity bioassays of *A. craccivora* to the test samples 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: $P = 50\%$) (Adhikary et al. 2015; Karmakar et al. 2018). Insects that did not respond by selection either arm of the olfactometer were excluded from the analyses.

Results

Olfactometer bioassays with adult viviparous *A. craccivora* females towards natural samples

Females showed positive responses towards a single BIO ($\chi^2 = 25.6$, $df = 1$, $P < 0.0001$) or NIR ($\chi^2 = 40$, $df = 1$, $P < 0.0001$) leaf compared to the clean air flow (Fig. 1). Females did not discriminate between a single leaf of NIR and BIO ($\chi^2 = 2.18$, $df = 1$, $P = 0.14$) (Fig. 1). Females displayed positive responses towards one leaf equivalent wax of BIO ($\chi^2 = 16.04$, $df = 1$, $P < 0.0001$) or NIR ($\chi^2 = 25.6$, $df = 1$, $P < 0.0001$) compared to the control solvent (Fig. 2). Females could not distinguish between one leaf equivalent wax of NIR and BIO ($\chi^2 = 1.11$, $df = 1$, $P = 0.2919$) (Fig. 2).

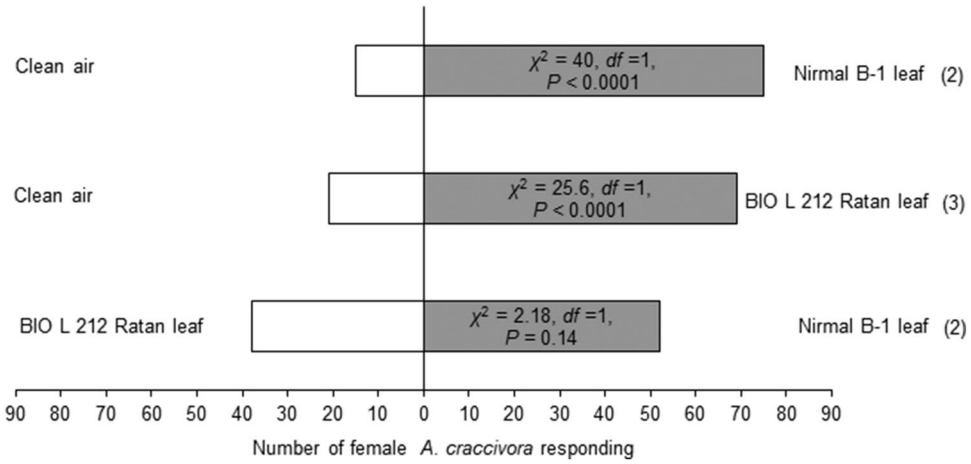

Fig. 1 Behavioural responses of *Aphis craccivora* females to a single leaf of BIO L 212 Ratan or Nirmal B-1 cultivars of *Lathyrus sativus* against the clean air, and a single leaf of BIO L 212 Ratan vs. Nirmal B-1 in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment

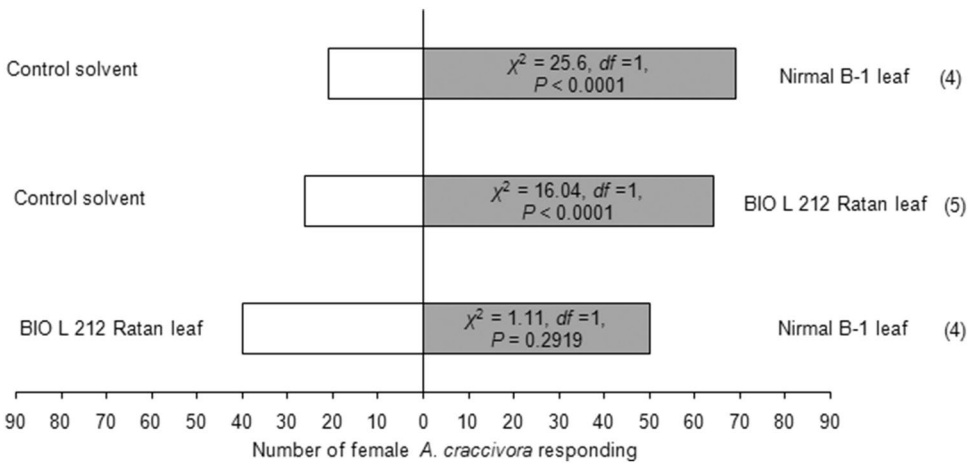

Fig. 2 Behavioural responses of *Aphis craccivora* females to one leaf equivalent wax of BIO L 212 Ratan or Nirmal B-1 cultivars of *Lathyrus sativus* against the control solvent (*n*-hexane), and one leaf equivalent wax of BIO L 212 Ratan vs. Nirmal B-1 in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment

Viviparity assays with adult viviparous *A. craccivora* females towards natural samples

Females laid significantly more nymphs on BIO ($\chi^2 = 31$, $df = 1$, $P < 0.0001$) or NIR ($\chi^2 = 40.09$, $df = 1$, $P < 0.0001$) leaves compared to the dewaxed leaves (Table 1). Females could not discriminate between NIR and BIO leaves for nymph laying ($\chi^2 = 0.8571$, $df = 1$, $P = 0.3545$) (Table 1). Females laid significantly more nymphs on one leaf equivalent wax of BIO ($\chi^2 = 29$, $df = 1$, $P < 0.0001$) or NIR ($\chi^2 = 41$, $df = 1$, $P < 0.0001$) compared to the control solvent (Table 1). Females could not distinguish between one leaf equivalent wax of NIR and BIO for nymph laying ($\chi^2 = 2.951$, $df = 1$, $P = 0.0858$) (Table 1).

Leaf waxes in BIO and NIR cultivars of *L. sativus*

The total amounts of crude waxes from leaves of two cultivars were homogeneously distributed as indicated by Levene's test (W) of homogeneity of variance ($W = 1.004$; $df = 1$, 4; $P = 0.373$). A total of 18.04 ± 0.61 and 24.36 ± 0.31 mg (mean ± SD) leaf waxes were obtained from the *n*-hexane extracts of 25 g mature BIO and NIR leaves, respectively. The total amount of crude waxes was higher in NIR compared to BIO ($t_{1,4} = -15.974$; $P < 0.001$). In BIO, alkanes and free fatty acids represented for 8.47 ± 1.14 and 3.70 ± 0.26 mg (mean ± SD), respectively, whereas alkanes and free fatty acids accounted for 15.27 ± 1.85 and 1.97 ± 0.29 mg (mean ± SD) in NIR, respectively, with the balance consisting of unidentified wax compounds.

Alkanes in leaf waxes of BIO and NIR cultivars of *L. sativus*

Total amount of alkanes was higher in leaf waxes of NIR compared to BIO (Table 2). The identified and unidentified branched-chain alkanes in leaf waxes of BIO represented for 8.37 ± 1.13 and 0.10 ± 0.01 mg (mean ± SD), respectively. In NIR, identified and unidentified branched-chain alkanes accounted for 15.06 ± 1.81 and 0.21 ± 0.04 mg (mean ± SD), respectively. Eighteen *n*-alkanes from $n\text{-}C_{15}$ to $n\text{-}C_{36}$ were identified in leaf waxes of both cultivars (Table 2, Supplementary Fig. 3 and Table 5). The amounts of individual alkanes were always higher in NIR compared to BIO (Table 2). Pentadecane ($n\text{-}C_{15}$) predominated in leaf waxes of both cultivars; whereas pentatriacontane ($n\text{-}C_{35}$) was identified in least amount in leaf waxes of both cultivars.

Free fatty acids in leaf waxes of BIO and NIR cultivars of *L. sativus*

Total amount of free fatty acids was higher in leaf waxes of BIO compared to NIR. Fourteen free fatty acids between C12:0 and C22:0 were detected in leaf waxes of both cultivars (Table 3, Supplementary Fig. 4 and Table 6). Palmitoleic acid (C16:1) and pentadecanoic acid (C15:0) predominated among all free fatty acids present in BIO and NIR, respectively (Table 3). The amounts of linoleic acid (C18:2), nonadecanoic acid (C19:0), arachidic acid (C20:0), and docosanoic acid (C22:0) were higher in leaf waxes of BIO compared to NIR (Table 3). Heptadecanoic

Table 1 Viviparity assays of *Aphis craccivora* females towards natural samples of BIO L 212 Ratan (BIO) or Nirmal B-1 (NIR) cultivars of *Lathyrus sativus* plants and synthetic blends at similar amounts present in BIO or NIR cultivars of *L. sativus* plants ($N = 10$ in each bioassay)

	Comparison		No. of insects laid nymphs		Nymphs laid		χ^2 ($df = 1$)	*P* values
	T1	T2	T1	T2	T1	T2		
Assay 1a	A single BIO leaf	A single dewaxed BIO leaf	10	0	31	0	31	< 0.0001
Assay 1b	A single NIR leaf	A single dewaxed NIR leaf	9	1	43	1	40.09	< 0.0001
Assay 2	A single NIR leaf	A single BIO leaf	5	5	24	18	0.8571	0.3545
Assay 3a	One leaf equivalent wax of BIO	Control solvent (*n*-Hexane)	10	0	29	0	29	< 0.0001
Assay 3b	One leaf equivalent wax of NIR	Control solvent	10	0	41	0	41	< 0.0001
Assay 4	One leaf equivalent wax of NIR	One leaf equivalent wax of BIO	6	4	26	15	2.951	0.0858
Assay 5a	BIO blend 3[a]	Control solvent	10	0	24	0	24	< 0.0001
Assay 5b	NIR blend 6[b]	Control solvent	9	1	36	1	33.11	< 0.0001
Assay 6a	One leaf equivalent wax of BIO	BIO blend 3[a]	5	5	21	16	0.676	0.411
Assay 6b	One leaf equivalent wax of NIR	NIR blend 6[b]	6	4	25	14	3.103	0.0782
Assay 7	NIR blend 6[b]	BIO blend 3[a]	8	2	29	7	13.44	0.0003

[a]Indicates a synthetic blend of 3 compounds (2.75 µg pentadecane + 1.34 µg tridecanoic acid + 1.18 µg linoleic acid) at similar amounts present in one leaf equivalent wax of BIO

[b]Indicates a synthetic blend of 6 compounds (5.70 µg pentadecane + 3.73 µg docosane + 0.43 µg pentacosane + 0.42 µg heptacosane + 0.17 µg tritriacontane + 0.72 µg linoleic acid) at similar amounts present in one leaf equivalent wax of NIR

Table 2 Composition of alkanes (µg/25 g leaf) in BIO L 212 Ratan and Nirmal B-1 cultivars of *Lathyrus sativus* leaves

Alkanes	Amount (µg) (mean ± SD)		t_4	*P* value
	BIO L 212 Ratan	Nirmal B-1		
Pentadecane (*n*-C_{15})	1189.58 ± 173.29	2312.56 ± 251.78	6.36	0.003
Hexadecane (*n*-C_{16})	1188.35 ± 162.02	2138.27 ± 252.70	5.48	0.005
Octadecane (*n*-C_{18})	1173.00 ± 166.30	2234.23 ± 221.85	6.63	0.003
Eicosane (*n*-C_{20})	1118.60 ± 148.38	2077.50 ± 236.50	5.95	0.004
Docosane (*n*-C_{22})	1042.53 ± 135.08	1514.01 ± 252.79	2.85	0.046
Tetracosane (*n*-C_{24})	707.91 ± 133.53	1242.58 ± 186.78	4.03	0.016
Pentacosane (*n*-C_{25})	44.89 ± 5.22	173.14 ± 26.44	8.24	0.001
Hexacosane (*n*-C_{26})	582.69 ± 106.92	964.62 ± 96.87	4.59	0.01
Heptacosane (*n*-C_{27})	75.3 ± 13.36	168.76 ± 21.47	6.40	0.003
Octacosane (*n*-C_{28})	349.54 ± 34.33	662.32 ± 101.90	5.04	0.007
Nonacosane (*n*-C_{29})	68.32 ± 5.98	180.03 ± 30.12	6.30	0.02
Triacontane (*n*-C_{30})	212.91 ± 23.08	384.31 ± 61.18	4.54	0.01
Hentriacontane (*n*-C_{31})	335.00 ± 36.11	490.75 ± 69.32	3.45	0.026
Dotriacontane (*n*-C_{32})	133.23 ± 18.02	212.74 ± 42.48	2.99	0.041
Tritriacontane (*n*-C_{33})	24.75 ± 3.78	70.68 ± 8.08	8.92	0.001
Tetratriacontane (*n*-C_{34})	67.09 ± 11.48	119.39 ± 20.06	3.92	0.017
Pentatriacontane (*n*-C_{35})	13.59 ± 2.71	51.26 ± 10.05	6.27	0.003
Hexatriacontane (*n*-C_{36})	41.39 ± 4.01	59.16 ± 10.32	2.78	0.05
Total	8368.66 ± 1126.10	15056.34 ± 1809.21	5.44	0.006

Table 3 Composition of free fatty acids (µg/25 g leaf) in BIO L 212 Ratan and Nirmal B-1 cultivars of *Lathyrus sativus* leaves

Fatty acids	Amount (µg) (Mean ± SD)		t_4	*P* value
	BIO L 212 Ratan	Nirmal B-1		
Lauric acid (C12:0)	288.93 ± 42.87	158.42 ± 21.94	− 4.69	0.009
Tridecanoic acid (C13:0)	580.97 ± 69.31	304.22 ± 46.01	− 5.76	0.005
Pentadecanoic acid (C15:0)	690.58 ± 98.21	374.31 ± 52.51	− 4.92	0.008
Palmitoleic acid (C16:1)	697.25 ± 86.94	331.01 ± 58.04	− 6.07	0.004
Palmitic acid (C16:0)	16.58 ± 2.36	5.95 ± 1.21	− 6.95	0.002
Heptadecanoic acid (C17:0)	1.84 ± 0.25	1.65 ± 0.19	− 1.02	0.364
Linolenic acid (C18:3)	2.09 ± 0.26	2.28 ± 0.42	0.65	0.551
Linoleic acid (C18:2)	509.95 ± 55.72	290.71 ± 40.83	− 5.50	0.005
Oleic acid (C18:1)	16.85 ± 1.74	9.61 ± 1.71	− 5.14	0.007
Stearic acid (C18:0)	2.14 ± 0.25	5.12 ± 0.63	7.65	0.002
Nonadecanoic acid (C19:0)	240.55 ± 26.78	143.99 ± 19.20	− 5.08	0.007
Arachidic acid (C20:0)	382.75 ± 43.62	196.70 ± 35.55	− 5.73	0.005
Heneicosanoic acid (C21:0)	31.45 ± 5.49	18.62 ± 1.89	− 3.82	0.019
Docosanoic acid (C22:0)	239.87 ± 21.50	131.08 ± 15.90	− 7.05	0.002
Total	3701.80 ± 256.56	1973.66 ± 290.50	− 7.72	0.002

acid (C17:0) was detected in least amount in leaf waxes of both cultivars (Table 3).

Olfactometer bioassays with adult viviparous *A. craccivora* females towards synthetic compounds or blends

Among the identified alkanes and free fatty acids present in BIO leaf waxes, females showed responses towards 8 individual synthetic compounds (pentadecane, docosane, pentacosane, heptacosane, nonacosane, tridecanoic acid, linoleic acid, and nonadecanoic acid) at similar amounts present in one leaf equivalent wax of BIO compared to the control solvent (Table 4). Females showed positive responses towards a synthetic blend of 8 compounds at similar amounts present in one leaf equivalent wax of BIO compared to the control solvent ($\chi^2 = 12.84$, $df = 1$, $P = 0.0003$). The insects showed clear positive responses

Table 4 Behavioural responses of *Aphis craccivora* females towards individual synthetic compounds or synthetic blends at similar amounts present in one leaf equivalent wax of BIO L 212 Ratan (BIO) and Nirmal B-1 (NIR) cultivars of *Lathyrus sativus* vs. the control solvent (*n*-hexane) ($N=90$ in each bioassay)

Comparison		Insects responded		Non-responders	χ^2 ($df=1$)	P values
T1	T2	T1	T2			
Synthetic compounds at similar amounts present in one leaf equivalent wax of BIO (µg/ml)	Control solvent					
a. Pentadecane (2.75)		55	35	5	4.44	0.035
c. Docosane (2.41)		53	37	6	2.84	0.0917
d. Pentacosane (0.10)		50	40	7	1.11	0.2919
e. Heptacosane (0.17)		47	43	9	0.18	0.6733
g. Nonacosane (0.16)		47	43	8	0.18	0.6733
i. Tridecanoic acid (1.34)		56	34	5	5.38	0.0204
j. Linoleic acid (1.18)		57	33	5	6.4	0.0114
k. Nonadecanoic acid (0.56)		51	39	8	1.6	0.2059
a+c+d+e+g+i+j+k		62	28	3	12.84	0.0003
a+i+j		58	32	4	7.51	0.0061
Synthetic compounds at similar amounts present in one leaf equivalent wax of NIR (µg/ml)						
a. Pentadecane (5.70)		59	31	4	8.71	0.0032
b. Octadecane (5.50)		53	37	6	2.84	0.0917
c. Docosane (3.73)		57	33	5	6.4	0.0114
d. Pentacosane (0.43)		55	35	5	4.44	0.035
e. Heptacosane (0.42)		55	35	6	4.44	0.035
f. Octacosane (1.63)		48	42	8	0.4	0.5271
g. Nonacosane (0.44)		52	38	7	2.18	0.14
h. Tritriacontane (0.17)		55	35	5	4.44	0.035
i. Tridecanoic acid (0.75)		53	37	6	2.84	0.0917
j. Linoleic acid (0.72)		55	35	5	4.44	0.035
k. Nonadecanoic acid (0.35)		48	42	9	0.4	0.5271
a+b+c+d+e+f+g+h+i+j+k		68	22	2	23.51	< 0.0001
a+c+d+e+h+j		66	24	3	19.6	< 0.0001

to 3 individual synthetic compounds [pentadecane ($\chi^2=4.44$, $df=1$, $P=0.035$), tridecanoic acid ($\chi^2=5.38$, $df=1$, $P=0.0204$), and linoleic acid ($\chi^2=6.4$, $df=1$, $P=0.0114$)] or BIO blend 3 ($\chi^2=7.51$, $df=1$, $P=0.0061$) compared to the control solvent (Table 4).

Females displayed responses to 11 individual synthetic compounds (pentadecane, octadecane, docosane, pentacosane, heptacosane, octacosane, nonacosane, tritriacontane, tridecanoic acid, linoleic acid, and nonadecanoic acid) at similar amounts present in one leaf equivalent wax of NIR compared to the control solvent (Table 4). Females showed positive responses towards a synthetic blend of 11 compounds at similar amounts present in one leaf equivalent wax of NIR compared to the control solvent ($\chi^2=23.51$, $df=1$, $P<0.0001$) (Table 4). Among 11 individual compounds, females showed positive responses to 6 individual synthetic compounds [pentadecane ($\chi^2=8.71$, $df=1$, $P=0.0032$), docosane ($\chi^2=6.4$, $df=1$, $P=0.0114$), and pentacosane or heptacosane or tritriacontane or linoleic acid ($\chi^2=4.44$, $df=1$, $P=0.035$) or NIR blend 6 ($\chi^2=19.6$, $df=1$, $P<0.0001$) compared to the control solvent (Table 4).

Females could not differentiate between one leaf equivalent wax of BIO and a synthetic blend of 8 compounds (pentadecane, docosane, pentacosane, heptacosane, nonacosane, tridecanoic acid, linoleic acid, and nonadecanoic acid) ($\chi^2=0.18$, $df=1$, $P=0.6733$) or BIO blend 3 (pentadecane, tridecanoic acid, and linoleic acid) ($\chi^2=0.71$, $df=1$, $P=0.3991$) (Table 5).

Females could not discriminate between one leaf equivalent wax of NIR and a synthetic blend of 11 compounds (pentadecane, octadecane, docosane, pentacosane, heptacosane, octacosane, nonacosane, tritriacontane, tridecanoic acid, linoleic acid, and nonadecanoic acid) ($\chi^2=0.04$, $df=1$, $P=0.8339$) or NIR blend 6 (pentadecane, docosane, pentacosane, heptacosane, tritriacontane, and linoleic acid) ($\chi^2=0.18$, $df=1$, $P=0.6733$) (Table 5).

Table 5 Behavioural responses of *Aphis craccivora* females to one leaf equivalent wax of BIO L 212 Ratan (BIO) and Nirmal B-1 (NIR) cultivars of *Lathyrus sativus* vs. individual synthetic compounds or synthetic blends at similar amounts present in one leaf equivalent wax of BIO and NIR ($N=90$ in each bioassay)

Comparison		Insects responded		Non-responders	χ^2 ($df=1$)	P values
T1	T2	T1	T2			
One leaf equivalent wax of BIO	Synthetic compounds or blends at similar amounts present in one leaf equivalent wax of BIO (µg/ml)					
	a. Pentadecane (2.75)	53	37	4	2.84	0.0917
	c. Docosane (2.41)	61	29	3	11.38	0.0007
	d. Pentacosane (0.10)	62	28	3	12.84	0.0003
	e. Heptacosane (0.17)	63	27	2	14.4	0.0002
	g. Nonacosane (0.16)	63	27	3	14.4	0.0002
	i. Tridecanoic acid (1.34)	52	38	4	2.18	0.14
	j. Linoleic acid (1.18)	51	39	4	1.6	0.2059
	k. Nonadecanoic acid (0.56)	63	27	2	14.4	0.0002
	a+c+d+e+g+i+j+k	47	43	2	0.18	0.6733
	a+i+j	49	41	2	0.71	0.3991
One leaf equivalent wax of NIR	Synthetic compounds or blends at similar amounts present in one leaf equivalent wax of NIR (µg/ml)					
	a. Pentadecane (5.70)	53	37	4	2.84	0.0917
	b. Octadecane (5.50)	67	23	2	21.51	<0.0001
	c. Docosane (3.73)	55	35	3	4.44	0.035
	d. Pentacosane (0.43)	56	34	3	5.38	0.0204
	e. Heptacosane (0.42)	56	34	4	5.38	0.0204
	f. Octacosane (1.63)	68	22	2	23.51	< 0.0001
	g. Nonacosane (0.44)	67	23	2	21.51	< 0.0001
	h. Tritriacontane (0.17)	56	34	4	5.38	0.0204
	i. Tridecanoic acid (0.75)	68	22	2	23.51	< 0.0001
	j. Linoleic acid (0.72)	56	34	3	5.38	0.0204
	k. Nonadecanoic acid (0.35)	68	22	2	23.51	< 0.0001
	a+b+c+d+e+f+g+h+i+j+k	46	44	2	0.04	0.8339
	a+c+d+e+h+j	47	43	2	0.18	0.6733

Females showed positive responses towards NIR blend 6 compared to BIO blend 3 ($\chi^2=7.51$, $df=1$, $P=0.0061$) (Fig. 3).

In dose response bioassays, females started to show positive responses towards pentadecane at 4 µg/ml ($\chi^2=6.4$, $df=1$, $P=0.0114$) and showed the highest positive responses

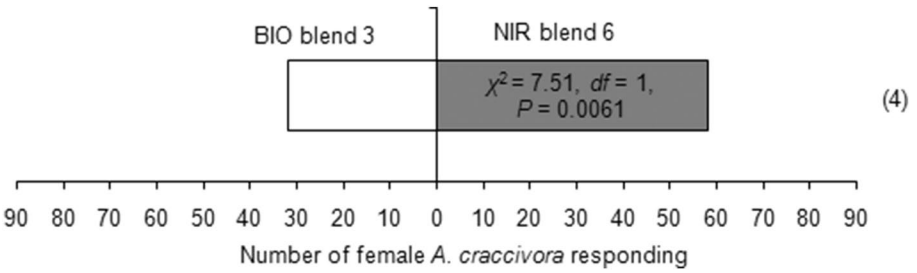

Fig. 3 Behavioural responses of *Aphis craccivora* females to NIR (Nirmal B-1) blend 6 (5.70, 3.73, 0.43, 0.42, 0.17 and 0.72 µg/ml of pentadecane, docosane, pentacosane, heptacosane, tritriacontane and linoleic acid, respectively) against BIO (BIO L 212 Ratan) blend 3 (2.75, 1.34, and 1.18 µg/ml of pentadecane, tridecanoic acid, and linoleic acid, respectively) in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment

at 8 µg/ml ($\chi^2 = 16.04$, $df = 1$, $P < 0.0001$) (Table 6). Females started to display positive responses towards docosane at 3 µg/ml ($\chi^2 = 4.44$, $df = 1$, $P = 0.035$) and showed the highest positive responses at 12 µg/ml ($\chi^2 = 19.6$, $df = 1$, $P < 0.0001$) (Table 6). Females started to show positive responses towards pentacosane or heptacosane at 0.4 µg/ml ($\chi^2 = 4.44$, $df = 1$, $P = 0.035$) and showed the highest positive responses at 1.6 µg/ml (pentacosane: $\chi^2 = 16.04$, $df = 1$, $P < 0.0001$, and heptacosane: $\chi^2 = 17.78$, $df = 1$, $P < 0.0001$). Females started to exhibit positive responses towards tritriacontane at 0.2 µg/ml ($\chi^2 = 5.38$, $df = 1$, $P = 0.0204$) and showed the highest positive responses at 0.8 µg/ml ($\chi^2 = 16.04$, $df = 1$, $P < 0.0001$) (Table 6). Females started to display positive responses towards tridecanoic acid at 1.4 µg/ml ($\chi^2 = 6.4$, $df = 1$, $P = 0.0114$) and showed the highest positive responses at 2.8 µg/ml ($\chi^2 = 16.04$, $df = 1$, $P < 0.0001$) (Table 6). Females started to demonstrate positive responses towards linoleic acid at 1 µg/ml ($\chi^2 = 6.4$, $df = 1$, $P = 0.0114$) and displayed the highest positive responses at 2 µg/ml ($\chi^2 = 17.78$, $df = 1$, $P < 0.0001$) (Table 6).

Table 6 Responses of *Aphis craccivora* females to individual synthetic compound vs. the control solvent (*n*-hexane) in the Y-tube olfactometer bioassay ($N = 90$ in each concentration bioassay)

Synthetic compounds	Concentration (µg/ml)	χ^2 ($df = 1$)	*P* values of insect responded
Pentadecane	2	3.6	0.0578
	4	6.4	0.0114
	8	16.04	< 0.0001
Docosane	1.5	0.4	0.5271
	3	4.44	0.035
	6	12.84	0.0003
	12	19.6	< 0.0001
Pentacosane	0.2	2.18	0.14
	0.4	4.44	0.035
	0.8	10	0.0016
	1.6	16.04	< 0.0001
Heptacosane	0.2	1.6	0.2059
	0.4	4.44	0.035
	0.8	11.38	0.0007
	1.6	17.78	< 0.0001
Tritriacontane	0.1	2.18	0.14
	0.2	5.38	0.0204
	0.4	10	0.0016
	0.8	16.04	< 0.0001
Tridecanoic acid	0.7	2.18	0.14
	1.4	6.4	0.0114
	2.8	16.04	< 0.0001
Linoleic acid	0.5	1.6	0.2059
	1	6.4	0.0114
	2	17.78	< 0.0001

Viviparity assays with adult viviparous *A. craccivora* females towards synthetic blends

Females laid significantly more nymphs on BIO blend 3 ($\chi^2 = 24$, $df = 1$, $P < 0.0001$) or NIR blend 6 ($\chi^2 = 33.11$, $df = 1$, $P < 0.0001$) compared to the control solvent (Table 1). Females could not distinguish between one leaf equivalent wax of BIO and BIO blend 3 for nymph laying ($\chi^2 = 0.676$, $df = 1$, $P = 0.411$) (Table 1). Females also could not discriminate between one leaf equivalent wax of NIR and NIR blend 6 for nymph laying ($\chi^2 = 3.103$, $df = 1$, $P = 0.0782$) (Table 1). Females laid significantly more nymphs on NIR blend 6 compared to BIO blend 3 ($\chi^2 = 13.44$, $df = 1$, $P = 0.0003$) (Table 1).

Discussion

We have recently demonstrated that flower waxes of two cultivars (BIO and NIR) of *L. sativus* act as short-range attractant and stimulate nymph laying in *A. craccivora* females (Mitra et al. 2019), and we hypothesized that *L. sativus* leaf waxes can act as cues for short-range attractant and stimulate nymph laying in the aphid *A. craccivora*. In this study, we demonstrated how short-range attraction and nymph laying behaviour of *A. craccivora* females are influenced by leaf waxes including long-chain alkanes and free fatty acids present in leaf waxes of two cultivars (BIO and NIR) of *L. sativus*.

The present Y-tube olfactometer bioassay results revealed clear olfactory responses of *A. craccivora* females to long-chain alkanes and free fatty acids present in leaf waxes of two *L. sativus* cultivars. Host-derived volatiles such as low molecular weight aldehydes, alcohols, ketones, esters, etc. might act as long-range cues for the aphid *A. craccivora* to locate host (Schoonhoven et al. 2005). After reaching within a close range to the host-plant, long-chain alkanes and free fatty acids might act as a short-range attractant and stimulate nymph laying in *A. craccivora* on *L. sativus* leaves. Long-chain alkanes and free fatty acids are major constituents in leaf waxes of numerous plant species and these compounds have been shown to play important role in plant–insect interactions such as attractant (Manosalva et al. 2011; Sarkar et al. 2013; Mukherjee et al. 2014; Malik and Barik 2015; Karmakar et al. 2016; Malik et al. 2017) and oviposition stimulant (Udayagiri and Mason 1997; Parr et al. 1998; Grant et al. 2000; Li and Ishikawa 2006; Mitra et al. 2017; Das et al. 2019).

n-Alkanes from C_{15} to C_{36} and free fatty acids from C12:0 to C22:0 are common components in leaf waxes of plants (Li and Ishikawa 2006; Sarkar et al. 2014; Mitra et al. 2017; Das et al. 2019). Twenty-one and 22 *n*-alkanes from *n*-C_{12} to *n*-C_{36} are detected in flower waxes of BIO

and NIR, respectively, while 12 free fatty acids from C12:0 to C22:0 are identified in both cultivars (Mitra et al. 2019). Pentadecane and tridecanoic acid are the most abundant *n*-alkane and free fatty acid in flower waxes of both cultivars, respectively. Nonadecane is the most abundant alkane in seed coat waxes of BIO and NIR (Adhikary et al. 2014), whereas palmitoleic acid, and palmitic acid and lauric acid are predominant free fatty acids in seed coat waxes of BIO and NIR, respectively (Adhikary et al. 2016). In the current study, pentadecane is predominant alkane in leaf waxes of BIO and NIR, while palmitoleic acid and pentadecanoic acid are dominant free fatty acids in leaf waxes of BIO and NIR, respectively. The present study suggested that the variations in the compositions of leaf wax compounds might occur among different plant species as well as within different cultivars of a same plant species including different plant parts (Piasentier et al. 2000; Dodoš et al. 2015; Wang et al. 2015).

The current research demonstrated that *A. craccivora* females are attracted towards a synthetic blend of pentadecane, tridecanoic acid, and linoleic acid at similar amounts present in one leaf equivalent wax of BIO, while a synthetic blend of pentadecane, docosane, pentacosane, heptacosane, tritriacontane, and linoleic acid at similar amounts present in one leaf equivalent wax of NIR, suggesting that females of *A. craccivora* could select BIO and NIR cultivars chiefly by both the qualitative (by distinct chemical compounds) and quantitative (by a specific ratio of compounds) contact cues. The above alkanes and free fatty acids are common in leaf waxes of various plant species and can act as short-range attractant for different insects (Schoonhoven et al. 2005; Li and Ishikawa 2006; Sarkar et al. 2013; Malik and Barik 2015; Karmakar et al. 2016; Mitra et al. 2017). Females of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) are attracted towards a synthetic blend of alkanes comprised of pentadecane, octadecane, nonadecane, heneicosane, tricosane, and pentacosane or a synthetic blend of free fatty acids comprised of myristic acid, palmitic acid, palmitoleic acid, and stearic acid at similar amounts present in seed coat waxes of BIO and NIR (Adhikary et al. 2014, 2016). Long-chain fatty acids, particularly oleic acid and linoleic acid act as ovipositional host-finding cue for the navel orangeworm, *Amyelois transitella* (Walker) (Phelan et al. 1991). A synthetic blend of alkanes including heneicosane, tricosane, pentacosane, heptacosane, and nonacosane is attractive to *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) (Renou et al. 1992). Parr et al. (1998) demonstrated that fatty acids play an important role in oviposition of *C. maculatus* on chickpea and mung bean seed coat waxes, but females show better oviposition on mung bean seeds compared to chickpea seeds due to higher amounts of fatty acids in mung bean seed coat waxes, and concluded that an appropriate mixture of fatty acids in seed coat waxes stimulates oviposition in females. The current study supports the hypothesis that majority of insects respond to the specific ratio of compounds as stimulant for nymph or egg laying, and the olfactory host plant recognition for nymph or egg laying by the insect can fade away when the relative ratio of key compounds are replaced (Udayagiri and Mason 1997; Parr et al. 1998; Grant et al. 2000; Li and Ishikawa 2006; Mitra et al. 2017).

This study concludes that a BIO blend 3 (2.75, 1.34, and 1.18 µg/ml of pentadecane, tridecanoic acid, and linoleic acid, respectively) or a NIR blend 6 (5.70, 3.73, 0.43, 0.42, 0.17, and 0.72 µg/ml of pentadecane, docosane, pentacosane, heptacosane, tritriacontane, and linoleic acid, respectively) stimulated nymph laying in *A. craccivora*. However, NIR blend 6 stimulated *A. craccivora* females to lay more nymphs compared to BIO blend 3. This study suggested that once volatile organic compounds (VOCs) from leaves of *L. sativus* causing long-range attraction of *A. craccivora* females have been identified, and then a NIR blend 6 along with the VOCs of leaves could be used as lures to developing baited traps in integrated pest management programme (IPM). Further, this information could be used in genetic engineering of *L. sativus* crop cultivars with wax phenotypes designed to limit damage by *A. craccivora* (Eigenbrode and Espelie 1995). The extraction of plant cuticular waxes by dipping in organic solvents has long been employed (Jetter et al. 2006). It has been demonstrated that solvent molecules rapidly enter into the deeper layers of the cuticle and release a mixture of both epi- and intracuticular waxes (Jetter et al. 2000). Alternatively, the gum arabic method could be employed for extraction of epicuticular waxes. Triterpenoids are more abundant or dominant in the intracuticular wax, whereas linear long-chain aliphatic compounds occur in both the epi-and intracuticular wax fractions (Buschhaus et al. 2007). Further investigations on the extraction of leaf waxes by the gum arabic method from both cultivars of *L. sativus* followed by identification and quantification of these compounds could help to consolidate whether there are differences in compositions and quantities of long-chain alkanes and free fatty acids determined in the present study.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by PM and SD. The first draft of the manuscript was written by AB and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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