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Long-chain alkanes and fatty acids from *Ludwigia octovalvis* weed leaf surface waxes as short-range attractant and ovipositional stimulant to *Altica cyanea* (Weber) (Coleoptera: Chrysomelidae)

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Abstract

The importance of leaf surface wax compounds from the rice-field weed *Ludwigia octovalvis* (Jacq.) Raven (Onagraceae) was determined in the flea beetle *Altica cyanea* (Weber) (Coleoptera: Chrysomelidae). Extraction, thin layer chromatography and GC-MS and GC-FID analyses of surface waxes of young, mature and senescent leaves revealed 20, 19 and 19 *n*-alkanes between *n*-C_{15} and *n*-C_{35}, respectively; whereas 14, 14 and 12 free fatty acids between C12:0 and C22:0 fatty acids were identified in young, mature and senescent leaves, respectively. Tricosane was predominant *n*-alkane in young and mature leaves, whilst eicosane predominated in senescent leaves. Heneicosanoic acid, palmitic acid and docosanoic acid were the most abundant free fatty acids in young, mature and senescent leaves, respectively. *A. cyanea* females showed attraction to 0.25 mature leaf equivalent surface waxes compared with young or senescent leaves in a short glass Y-tube olfactometer bioassay. The insects were attracted to a synthetic blend of 0.90, 1.86, 1.83, 1.95, 0.50 and 0.18 µg ml^{-1} petroleum ether of hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid, respectively, comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes. *A. cyanea* also laid eggs on a filter paper moistened with 0.25 mature leaf equivalent surface waxes or a synthetic blend of 0.90, 1.86, 1.83, 1.95, 0.50 and 0.18 µg ml^{-1} petroleum ether of hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid, respectively. This finding could provide a basis for monitoring of the potential biocontrol agent in the field.

Keywords: *Altica cyanea*, Coleoptera, *Ludwigia octovalvis*, leaves, alkanes and fatty acids, Y-tube olfactometer bioassay, oviposition assay

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Introduction

Ludwigia octovalvis (Jacq.) Raven (Onagraceae) is considered as a noxious weed of rice in India, Bangladesh, Sri Lanka, Thailand, Vietnam, Indonesia, Malaysia and Thailand (Alam & Karim, 1980; Raju & Reddy, 1986; Moody, 1989). The weed competes with rice for nutrition, and heavy infestation by this weed results in reduced rice production. Control of this weed is very challenging due to its high reproductive growth rate. Farmers rely on the application of herbicides (thiobencarb, propanil, fenoprop, butachlor, propanil, MCPA, 2,4-D, quinclorac and bensulfuron) as a primary strategy for control of this weed (Raju & Reddy, 1986; Kandasamy & Palaniappan, 1990; Imeokparia *et al.*, 1992; Imeokparia, 1994). Application of herbicides is costly and is known to destroy or reduce the natural enemies of the weed species. Furthermore, intensive use of synthetic herbicides for control of weeds occurring on rice-filed is of great concern to agricultural communities due to its

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negative impact on health and environment. For this, use of natural enemies might be encouraged for control of *L. octovalvis* weed, which might be included in the biocontrol programme.

The flea beetle, *Altica cyanea* (Weber) (Coleoptera: Chrysomelidae) has been recognized as the potential biocontrol agent of the weeds belonging to Onagraceae family occurring in irrigated rice of tropical and subtropical countries (Maulik, 1936; Dubey, 1981; Nayek & Banerjee, 1987; Xiao-Shui, 1990; Naples & Kessler, 2005). *A. cyanea* causes widespread damage of the rice-field weed *Ludwigia* spp., and no damage has been recorded on rice by this insect (Nayek & Banerjee, 1987; Xiao-Shui, 1990; Naples & Kessler, 2005). The black larvae of *A. cyanea* gregariously consume leaves for 29–33 days to complete larval development. After pupation (3–4 days), adults feed on leaves for 7–8 weeks until death (Nayek & Banerjee, 1987).

Females of herbivorous insects lay eggs on the underside of leaves, implicating that females use sensory cues from the surface waxes of host plants to search for suitable host plant as oviposition site. Therefore, an understanding of the insect oviposition behavior and identification of the surface wax compounds responsible for such a behavior could help to develop future biocontrol programme (Padovan *et al.*, 2010; Piesik *et al.*, 2012; Smith & Beck, 2013; Wheeler & Schaffner, 2013). The amount and composition of leaf surface wax compounds such as alkanes, fatty acids, alcohols and esters vary not only in different species but also throughout the different stages of leaf development within a species (Baker & Hunt, 1981; Baker, 1982; Jetter *et al.*, 2000). Long-chain alkanes and fatty acids, major components of epicuticular wax, play important role as short-range volatile cues for the herbivorous insects to find their host in its microhabitat (Eigenbrode & Espelie, 1995; Müller & Hilker, 2001; Schoonhoven *et al.*, 2005; Müller, 2006; Malik & Barik, 2015; Sarkar & Barik, 2015). Long-chain *n*-alkanes and free fatty acids from the surface waxes of the Japanese knotweed *Fallopia japonica* (Houtt.) Ronse Decr. were recorded as ovipositional stimulant of the European corn borer *Ostrinia nubilalis* (Hübner) (Li & Ishikawa, 2006). In the Y-tube olfactometer bioassay, female *Galerucella placida* Baly were attracted toward individual synthetic lauric, myristic, pentadecanoic, palmitoleic, heptadecanoic, nonadecanoic and docosanoic acids, and their blend like *Polygonum orientale* L. leaf surface waxes (Malik & Barik, 2015). Thus, it is of considerable interest whether alkanes and free fatty acids present in surface waxes of *L. octovalvis* leaves can provide clues for short-range attraction and oviposition of *A. cyanea*. *A. cyanea* prefers to lay eggs on mature leaves than young and senescent leaves of *L. octovalvis*. Hence, identification of the surface wax compounds throughout the developmental stages of *L. octovalvis* leaves might provide a basis why females prefer to lay eggs on the mature leaves than young and senescent leaves. If the alkanes and free fatty acids present in surface waxes of young, mature and senescent leaves are used by the insect in host finding for egg laying, then these compounds might be helpful in developing a lure to monitor field population.

This work reports the identification and quantification of *n*-alkane and free fatty acid compositions present in surface waxes of *L. octovalvis* leaves throughout the developmental stages [young (≤1 week old), mature (2–3 weeks old) and senescent (4–5 weeks old)], and whether differences in alkane and free fatty acid concentrations throughout the developmental stages of leaves can act as short-range olfactory cues to attract *A. cyanea* was studied through a Y-shaped glass tube olfactometer under laboratory conditions. The role of individual synthetic compounds and blends of synthetic alkanes and fatty acids comparable with the surface waxes of young, mature and senescent leaves were studied as an olfactory cue for *A. cyanea*. We further studied whether the most attractive synthetic blend (showing highest attraction in the Y-shaped glass tube olfcatometer bioassay) comparable with mature leaf surface wax alkanes and fatty acids can act as ovipositional stimulant of *A. cyanea*.

Materials and methods

Insects

The test insect, *A. cyanea* were collected by light trap from the weed, *L. adscendens* L. growing in rice-fields adjacent to this University, and maintained in 1 liter glass jars, containing *L. adscendens* leaves covered with fine-mesh nylon nets at 27 ± 1°C, 65 ± 10% relative humidity (RH) and 12 L:12 D photoperiod in a 'BOD' incubator. Mated F_2 females of 5–7 days old (1–2 days after initial mating) were used for olfactometer and ovipositional bioassays.

Plant materials

L. octovalvis leaves were collected from the rice-fields adjacent to this University during July–August 2015. Different ages of leaves were classified as young (≤1 week old), mature (2–3 weeks old) and senescent (4–5 weeks old) (Supplementary material S1).

Extraction of leaf surface waxes

Seventy-five grams of each fresh leaf type were separately collected for three times, and were separately dipped in 1 liter *n*-hexane for 5 min at room temperature for extraction of surface waxes from the leaves (Sarkar *et al.*, 2013, 2014; Sarkar & Barik, 2015). Each dried crude extract from 75 g leaves of each leaf type was then dissolved in 30 ml *n*-hexane and divided into three equal crude fractions [each 10 ml crude fraction was equivalent to ~25 g of leaves; number of leaves for 25 g leaves for young (525 ± 7), mature (300 ± 5) and senescent (475 ± 6) (mean ± standard error; three replicate of each type of leaf)]. The first, second and third fractions (each fraction was equivalent to ~25 g of leaves) of each crude extract from each type of 75 g leaves were used for (i) olfactometer bioassay, (ii) identification and quantification of alkanes and (iii) identification and quantification of free fatty acids, respectively.

Identification and quantification of alkanes

The second fraction of the each crude extract (equivalent to ~25 g of leaves) from each leaf type was passed through a column of aluminum oxide (Alcoa, Frankfurt, Germany: F-20 grade) and eluted with petroleum ether. Rest of the procedure was followed by Sarkar *et al.* (2013) (Supplementary material S2).

Identification and quantification of free fatty acids

The third fraction of the each crude extract (equivalent to ~25 g of leaves) from each leaf type was mixed with diethyl ether and filtered through Whatman No. 41 filter paper, and rest of the procedure was followed by Sarkar & Barik (2015) (Supplementary material S3).

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Olfactometer bioassays

Mated *A. cyanea* females (5–7 days old) were provisioned with water and starved for 10 h prior to use in olfactometer bioassays. Females were used in bioassays as they are guided by olfactory cues for both adult feeding and egg laying. The behavioral responses of 90 adult *A. cyanea* females were investigated in a horizontal Y-shaped glass tube olfactometer, which had one common stem and two lateral arms at an angle of 45°. The stem and two arms were each 5 cm long, all with an internal radius of 0.6 cm. Each arm of the olfactometer was connected to a glass-made micro kit adapter fitted into a glass vial (1 cm radius × 3 cm long). Each adapter contained two entrances: one air inlet tube for pushing air into the glass vial and another one as outlet tube connecting the glass vial to one arm of the olfactometer. One glass vial contained a piece (2×2 cm^2) of Whatman No. 41 filter paper moistened with 1 ml of the test compounds, whilst the other glass vial contained a filter paper of same size moistened with 1 ml of the control solvent (petroleum ether). Charcoal-filtered air was pushed into the system at 250 ml min^{-1}. All the connections between different parts of the set-up consisted of silicon tubing.

The effectiveness of alkanes and fatty acids as attractant was evaluated in the following manner in the laboratory at 27 ± 1°C, 70 ± 3% RH and light intensity 150 lux. A 1 ml of the sample and the control solvent were applied to the filter paper pieces and allowed to evaporate the solvent in open space under laboratory condition, and these filter papers were introduced into the glass vials before the first insect was released into olfactometer, for each experiment. One adult female *A. cyanea* was introduced into the porous glass vial (1 cm radius × 3 cm long), which was then attached with the stem of the olfactometer 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 consisting of alkanes and fatty acids compounds) in another glass vial. The behavior of each female was observed for 2 min such as wandering in the Y-tube. A female was considered to have made a choice in case of reaching the end of one arm, the insect was removed from the Y-tube and the choice of the insect was recorded as a positive or negative response, respectively. In contrast, a female was discarded not having made a choice within 2 min (non-responding) and was replaced by a new one (Mukherjee *et al*., 2015*a*; Sarkar *et al*., 2015). Each experiment with one test sample was conducted until a total of 90 naïve female insects had responded; and after testing five insects the olfactometer set-up was cleaned with petroleum ether followed by acetone, and the position of the two arms was systematically changed in order to avoid positional bias.

Dual choice bioassays with female A. cyanea

Bioassay 1: Surface waxes (crude extract) from different aged leaves tested against the control solvent

The behavioral responses of *A. cyanea* females to 0.25 leaf equivalent surface waxes from three types of leaves (A: young, B: mature and C: senescent), respectively, were tested against the control solvent (petroleum ether) (Supplementary material table 1). The cause of selecting 0.25 leaf equivalent surface waxes was presented in Supplementary material S4.

Bioassay 2: Surface waxes (crude extract) from different aged leaves tested against each other

The behavioral responses of *A. cyanea* females to 0.25 leaf equivalent surface waxes were tested as: (1) mature vs. young; (2) mature vs. senescent; (3) young vs. senescent to find out the most attractive leaf equivalent surface wax.

Bioassay 3: Individual synthetic compounds or synthetic blends (comparable with the proportions as present in 0.25 leaf equivalent surface waxes of three types of leaves) tested against the control solvent

Individual synthetic compounds (alkanes and fatty acids) present in 0.25 leaf equivalent surface waxes of each leaf type were dissolved in 1 ml petroleum ether and was tested against the control solvent to find behavioral response of the insect (Supplementary table 2a and 2b). Females showed behavioral responses to five individual compounds (hexadecane, octadecane, eicosane, tricosane and palmitic acid), ten individual compounds (hexadecane, octadecane, eicosane, docosane, tricosane, tetracosane, hexacosane, octacosane, palmitic acid and alpha-linolenic acid) and only one compound (palmitic acid) comparable with the proportions as present in 0.25 leaf equivalent surface waxes of young, mature and senescent leaves, respectively. The standard synthetic fatty acids identified in this study, and *n*-alkanes between *n*-C_{15} and *n*-C_{35} were all purchased from Sigma Aldrich, Germany.

The insect showed behavioral response to those individual synthetic compounds were combined comparable with the proportions as present in 0.25 leaf equivalent surface waxes of young, mature (Supplementary table 3) and senescent leaves (0.24 µg palmitic acid), and were assayed against the control solvent. As the insect showed response only to palmitic acid comparable with the proportions as present in 0.25 senescent leaf equivalent surface waxes, so no synthetic blend was tested. Furthermore, a synthetic blend comprising hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes (the insect did not show a clear attraction to docosane, tetracosane, hexacosane and octacosane, and for this, four compounds were not mixed) was tested against the control solvent.

Bioassay 4: Surface waxes (crude extract) from the most attractive leaf vs. individual synthetic compounds or synthetic blends

The behavioral responses of *A. cyanea* females to 0.25 leaf equivalent surface waxes from mature leaves were tested against ten individual compounds (hexadecane, octadecane, eicosane, docosane, tricosane, tetracosane, hexacosane, octacosane, palmitic acid and alpha-linolenic acid) or synthetic blends (a synthetic blend of above ten compounds or a synthetic blend subtracting docosane, tetracosane, hexacosane and octacosane) comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes.

Bioassay 5: Dose response of synthetic compounds against the control solvent

The insect displayed behavioral responses to those ten individual compounds were tested at different doses (hexadecane: 0.80, 1.60 and 3.20 µg ml^{-1} petroleum ether; octadecane: 0.75, 1.50 and 3 µg ml^{-1} petroleum ether; eicosane: 1, 2, 4 and

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8 µg ml^{-1} petroleum ether; docosane: 1.50, 3, 6 and 12 µg ml^{-1} petroleum ether; tricosane: 1, 2 and 4 µg ml^{-1} petroleum ether; tetracosane: 1, 2, 4 and 8 µg ml^{-1} petroleum ether; hexacosane: 1.5, 3 and 6 µg ml^{-1} petroleum ether; and octacosane: 1, 2, 4 and 8 µg ml^{-1} petroleum ether; palmitic acid: 0.15, 0.30 and 0.60 µg ml^{-1} petroleum ether, and alpha-linolenic acid: 0.10, 0.20, 0.40 and 0.80 µg ml^{-1} petroleum ether).

Oviposition assay

A. cyanea males and females mate 4–5 days after emergence, and females start to lay eggs 2–3 days after their initial mating (Nayek & Banerjee, 1987). One *A. cyanea* female during its life time deposited 145.66 ± 2.8 eggs in masses on 8–18 weed leaves in the laboratory (Nayek & Banerjee, 1987). An egg-mass contained 4–18 eggs, and a mated female laid between 10 and 22 masses of eggs on weed leaves (Nayek & Banerjee, 1987).

Adults of *A. cyanea* were collected from *L. adscendens* weeds occurring in rice-field. Larvae of *A. cyanea* were reared on *L. adscendens* leaves in the laboratory. Newly emerged F_2 males and females were also fed on *L. adscendens* leaves and were kept on this leaves for 40 hr after mating. After that, mated females were used for oviposition assay. Fifteen square glass chambers (15 cm^2) were used for ovoposition assay, and coarse grade emery papers were used along the sides of the glass jars to prevent egg laying on the wall and floor of the glass jar. During oviposition assay, females were without leaves, but a cotton piece soaked with sucrose solution was provided in a petridish (3 cm diameter). Filter papers (Whatman No. 41) of 2 × 2 cm^2 sizes were used for ovoposition assay. Initially females did not indicate any bias for egg laying on the filter paper moistened with control solvent (petroleum ether). A 1 ml of the test sample and the control solvent were applied to separate filter paper pieces and allowed to evaporate (2 min) the solvent in open space under laboratory condition, and these filter papers were separately placed in two round petridishes (each petridish 3 cm diameter). One filter paper containing the test sample in a petridish and the other filter paper containing the control solvent in another petridish were placed with a gap of 8 cm inside an experimental glass jar (15 cm^2), and a mated female was released in the experimental glass jar. Fifteen mated females were separately used for each experiment, and each mated female was used to lay eggs (one egg mass) in a square glass jar (15 cm^2) and then discarded. The following combinations were tested during oviposition assay.

(1) 0.25 mature leaf equivalent surface waxes from *L. octovalvis* vs. the control solvent (petroleum ether).
(2) A synthetic blend of six compounds comparable with 0.25 mature leaf equivalent surface waxes of *L. octovalvis* (0.90 µg hexadecane + 1.86 µg octadecane + 1.83 µg eicosane + 1.95 µg tricosane + 0.50 µg palmitic acid + 0.18 µg alpha-linolenic acid, as the insect showed highest attraction to this blend in the Y-tube olfactometer bioassay) vs. the control solvent.
(3) 0.25 mature leaf equivalent surface waxes from *L. octovalvis* vs. a synthetic blend (0.90 µg hexadecane + 1.86 µg octadecane + 1.83 µg eicosane + 1.95 µg tricosane + 0.50 µg palmitic acid + 0.18 µg alpha-linolenic acid).

Statistical analyses

The data on total amounts of alkanes and fatty acids, and amounts of individual alkanes and fatty acids from three types of *L. octovalvis* leaves were subjected to Levene's test for homogeneity of variance with respect to treatments (Supplementary tables 4 and 5). Following this, one-way analysis of variance (ANOVA) were conducted to compare the effects on total and individual alkanes and fatty acids. In case of significant *F*-values of one-way ANOVA, the data were subjected to post hoc Tukey test using SPSS software (SPSS 16.0; SPSS Inc., Chicago, IL, USA). The data obtained on behavioral responses of *A. cyanea* to the test samples were analyzed by a Chi-square test (Adhikary *et al.*, 2015; Sarkar *et al.*, 2015). Insects that did not respond by selection either arm of the olfactometer were excluded from the analyses.

Results

Leaf surface wax in different ages of leaves

The *n*-hexane extracts of 25 g of young, mature and senescent *L. octovalvis* leaves yielded 15.56 ± 0.08, 19.04 ± 0.07 and 15.5 ± 0.04 mg leaf surface waxes, respectively. Among the total amounts of leaf surface waxes, alkanes represented 11.44 ± 0.14, 14.19 ± 0.14 and 8.72 ± 0.06 mg in young, mature and senescent leaves, respectively, whilst free fatty acids accounted for 1.97 ± 0.03, 2.46 ± 0.04 and 2.16 ± 0.01 mg in young, mature and senescent leaves, respectively, with the balance consisting of unidentified surface wax compounds.

Alkanes in different ages of leaves

The identified hydrocarbons of the mature and senescent leaves represented 13.99 ± 0.15 and 8.52 ± 0.06 mg *n*-alkanes, with the balance consisting of unidentified branched-chain alkanes, respectively (table 1); whereas no branched-chain alkane was detected in young leaves. The total amounts of alkanes varied significantly with treatments through one way ANOVA, and the Tukey multiple pair wise comparisons test revealed that total alkanes were higher in mature leaves followed by young leaves and senescent leaves (table 1). Twenty, 19 and 19 *n*-alkanes were identified between *n*-C_{15} and *n*-C_{35} alkanes (table 1). Heptadecane (*n*-C_{17}) and pentatriacontane (*n*-C_{35}) were absent in mature and senescent leaves, respectively. Tricosane (*n*-C_{23}) predominated in young and mature leaves; whilst eicosane (*n*-C_{20}) predominated in senescent leaves. Heptadecane (*n*-C_{17}) was detected in the least amount in young and senescent leaves; whereas *n*-C_{35} was at the lowest level in mature leaves. Rest of the alkanes displayed different patterns in three types of leaves.

Free fatty acids in different ages of leaves

The total amounts of free fatty acids varied significantly with treatments through one way ANOVA, and the Tukey multiple pair wise comparisons test revealed that total free fatty acids were higher in mature leaves followed by senescent leaves and young leaves (table 2). Fourteen, 14 and 12 free fatty acids between C12:0 and C22:0 fatty acids were detected in surface waxes of young, mature and senescent *L. octovalvis* leaves, respectively (table 2). Heneicosanoic acid (C21:0), palmitic acid (C16:0) and docosanoic acid (C22:0) were predominant fatty acids in young, mature and senescent leaves, respectively (table 2). Lauric acid (C12:0) was the least abundant fatty acid in young and mature leaves, whilst tridecanoic acid (C13:0) was the least abundant in senescent leaves. Palmitoleic acid (C16:1), linoleic acid (C18:2) and arachidic

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Table 1. Composition of alkanes (μg 25 g^{-1} leaf) in different ages of *L. octovalvis* leaves.

Alkanes	Amount (μg) (Mean ± SE, $N = 3$)			$F_{2, 6}$
	Young	Mature	Senescent	
Pentadecane (n-C_{15})	798.82 ± 19.66[a]	221.82 ± 5.60[b]	69.36 ± 3.06[c]	544.82
Hexadecane (n-C_{16})	1454.10 ± 29.09[a]	1075.11 ± 17.23[b]	543.68 ± 12.92[c]	175.64
Heptadecane (n-C_{17})	6.52 ± 0.49[a]	–	18.56 ± 0.67[b]	19.67
Octadecane (n-C_{18})	1704.64 ± 42.70[a]	2226.97 ± 48.42[b]	1271.96 ± 33.02[c]	602.71
Nonadecane (n-C_{19})	46.31 ± 2.41[a]	90.32 ± 4.72[b]	38.38 ± 2.68[a]	66.59
Eicosane (n-C_{20})	1746.08 ± 38.85[a]	2196.47 ± 39.13[b]	1372.11 ± 35.98[c]	168.22
Docosane (n-C_{22})	1310.81 ± 37.45[a]	1692.82 ± 46.46[b]	1135.40 ± 28.03[c]	183.27
Tricosane (n-C_{23})	2057.97 ± 64.84[a]	2336.85 ± 76.29[b]	602.34 ± 10.79[c]	256.67
Tetracosane (n-C_{24})	929.79 ± 13.07[a]	1242.45 ± 23.20[b]	810.09 ± 14.77[c]	302.78
Pentacosane (n-C_{25})	64.69 ± 1.93[a]	225.59 ± 6.85[b]	89.39 ± 2.18[c]	253.51
Hexacosane (n-C_{26})	511.07 ± 12.94[a]	849.17 ± 15.91[b]	635.07 ± 13.71[c]	187.29
Heptacosane (n-C_{27})	37.08 ± 0.94[a]	86.08 ± 1.22[b]	88.91 ± 1.51[b]	549.29
Octacosane (n-C_{28})	271.33 ± 4.87[a]	537.77 ± 11.70[b]	422.27 ± 11.24[c]	284.27
Nonacosane (n-C_{29})	52.33 ± 1.37[a]	190.58 ± 2.58[b]	167.23 ± 5.54[c]	114.22
Triacontane (n-C_{30})	206.37 ± 5.15[a]	340.54 ± 9.88[b]	289.74 ± 6.16[c]	106.96
Hentriacontane (n-C_{31})	45.59 ± 1.04[a]	177.17 ± 3.69[b]	370.87 ± 7.98[c]	1025.59
Dotriacontane (n-C_{32})	99.27 ± 2.19[a]	199.03 ± 5.83[b]	220.47 ± 5.24[b]	66.51
Tritriacontane (n-C_{33})	30.87 ± 2.51[a]	125.61 ± 4.60[b]	270.16 ± 6.22[c]	329.92
Tetratriacontane (n-C_{34})	48.50 ± 2.45[a]	87.67 ± 1.44[b]	105.20 ± 2.29[c]	189.19
Pentatriacontane (n-C_{35})	20.20 ± 0.83[a]	83.50 ± 2.88[b]	–	445.42
Total	11442.34 ± 140.50[a]	13985.53 ± 146.03[b]	8521.19 ± 60.51[c]	597.26

Within the row means followed by different letters, i.e., a, b and c indicate that means are significantly different by Tukey test with $P < 0.05$.

Table 2. Composition of free fatty acids (μg 25 g^{-1} leaf) in different ages of *L. octovalvis* leaves.

Fatty acids	Amount (μg) (Mean ± SE, $N = 3$)			$F_{2, 6}$
	Young	Mature	Senescent	
Lauric acid (C12:0)	14.98 ± 0.55[a]	20.33 ± 0.72[b]	37.01 ± 1.38[c]	93.889
Tridecanoic acid (C13:0)	38.21 ± 1.24[a]	29.33 ± 1.86[b]	10.90 ± 0.39[c]	45.254
Myristic acid (C14:0)	37.99 ± 1.02[a]	55.88 ± 0.71[b]	54.58 ± 0.76[b]	140.393
Pentadecanoic acid (C15:0)	101.00 ± 1.24[a]	99.00 ± 1.72[a]	85.70 ± 1.46[b]	31.260
Palmitic acid (C16:0)	270.64 ± 4.54[a]	603.51 ± 13.10[b]	435.21 ± 8.36[c]	264.299
Palmitoleic acid (C16:1)	199.70 ± 2.95[a]	45.68 ± 3.64[b]	–	385.412
Heptadecanoic acid (C17:0)	154.86 ± 2.74[a]	81.16 ± 1.76[b]	112.33 ± 3.20[c]	196.810
Stearic acid (C18:0)	248.67 ± 4.73[a]	215.38 ± 4.06[b]	129.45 ± 2.38[c]	32.040
Oleic acid (C18:1)	–	–	60.20 ± 0.69	
Linoleic acid (C18:2)	86.04 ± 2.73[a]	62.77 ± 2.29[b]	–	42.588
Alpha-linolenic acid (C18:3)	80.02 ± 2.57[a]	220.13 ± 3.85[b]	55.55 ± 1.55[c]	367.272
Nonadecanoic acid (C19:0)	130.33 ± 3.68[a]	158.67 ± 4.07[b]	96.07 ± 1.52[c]	122.933
Arachidic acid (C20:0)	63.65 ± 1.81[a]	138.59 ± 3.14[b]	–	420.377
Heneicosanoic acid (C21:0)	281.01 ± 4.65[a]	216.33 ± 2.33[b]	91.20 ± 1.44[c]	290.363
Docosanoic acid (C22:0)	260.32 ± 3.92[a]	514.67 ± 8.11[b]	987.33 ± 9.98[c]	2261.213
Total	1967.41 ± 33.05[a]	2461.01 ± 40.53[b]	2155.52 ± 14.98[c]	65.178

Within the row means followed by different letters, i.e., a, b and c indicate that means are significantly different by Tukey test with $P < 0.05$.

acid (C20:0) were absent in senescent leaves, whilst oleic acid (C18:1) was present only in senescent leaves.

Dual choice bioassays with female A. cyanea

Bioassay 1: The insect displayed attraction toward 0.25 leaf equivalent surface waxes from the mature leaves against the control solvent, but the insect did not show a significant preference to 0.25 leaf equivalent surface waxes from the young leaves or senescent leaves against the control solvent (table 3).

Bioassay 2: A. cyanea females displayed attraction to 0.25 leaf equivalent surface waxes from the mature leaves against young leaves or senescent leaves (fig. 1). But, females did not show a significant preference to 0.25 leaf equivalent surface waxes from the young leaves against senescent leaves (fig. 1). The results indicated that *A. cyanea* showed highest attraction to 0.25 leaf equivalent surface waxes from the mature leaves compared with young and senescent leaves.

Bioassay 3: The insect did not show a significant preference to hexadecane or octadecane or eicosane or tricosane or palmitic acid comparable with the proportions as present in 0.25 young leaf equivalent surface waxes against the control solvent (table 3). The insect did not show a significant response to a synthetic blend of five compounds (hexadecane, octadecane, eicosane, tricosane and palmitic acid) against the control solvent (table 3).

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Table 3. Behavioral responses of *A. cyanea* females to 0.25 leaf equivalent surface waxes from *L. octovalvis* leaves and individual compounds or synthetic blends comparable with the proportions as present in 0.25 leaf equivalent surface waxes vs. the control solvent (petroleum ether) ($N = 90$ in each bioassay).

Comparison		Insects responded				
T1	T2	T1	T2	Non-responders	$\chi 2$ ($df = 1$)	*P* values of insect responded
0.25 leaf equivalent surface waxes from *L. octovalvis*	Control solvent					
Young		52	38	8	2.18	0.14002
Mature		70	20	3	27.78	<0.0001
Senescent		50	40	11	1.11	0.29184
Synthetic compounds mimicking 0.25 young leaf equivalent surface waxes						
a. Hexadecane (0.69 µg ml^{-1})		48	42	10	0.4	0.52709
b. Octadecane (0.81 µg ml^{-1})		48	42	9	0.4	0.52709
c. Eicosane (0.83 µg ml^{-1})		46	44	9	0.04	0.83385
d. Tricosane (0.98 µg ml^{-1})		48	42	10	0.4	0.52709
e. Palmitic acid (0.13 µg ml^{-1})		46	44	8	0.04	0.83385
a + b + c + d + e		52	38	7	2.18	0.14002
Synthetic compounds mimicking 0.25 mature leaf equivalent surface waxes						
a. Hexadecane (0.90 µg ml^{-1})		55	35	6	4.44	0.03502
b. Octadecane (1.86 µg ml^{-1})		62	28	4	12.84	0.00034
c. Eicosane (1.83 µg ml^{-1})		57	33	4	6.4	0.01141
d. Docosane (1.41 µg ml^{-1})		53	37	8	2.84	0.09169
e. Tricosane (1.95 µg ml^{-1})		57	33	4	6.4	0.01141
f. Tetracosane (1.04 µg ml^{-1})		51	39	7	1.6	0.20590
g. Hexacosane (0.71 µg ml^{-1})		50	40	7	1.11	0.29184
h. Octacosane (0.45 µg ml^{-1})		47	43	11	0.18	0.67327
i. Palmitic acid (0.50 µg ml^{-1})		60	30	4	10	0.00157
j. Alpha-linolenic acid (0.18 µg ml^{-1})		55	35	5	4.44	0.03502
a + b + c + d + e + f + g + h + i + j		68	22	4	23.51	<0.0001
a + b + c + e + i + j		66	24	4	19.6	<0.0001
Synthetic compounds mimicking 0.25 senescent leaf equivalent surface waxes						
Palmitic acid (0.24 µg ml^{-1})		48	42	10	0.4	0.52709

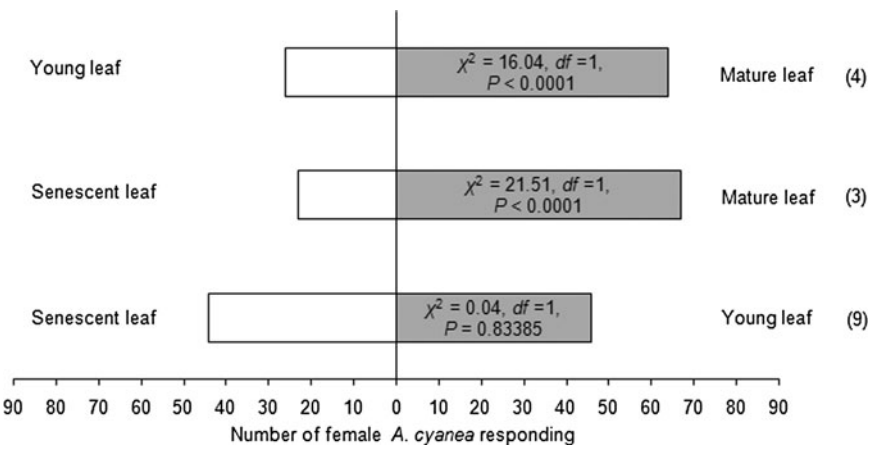

Fig. 1. Behavioral responses of *A. cyanea* females to 0.25 leaf equivalent surface waxes of mature vs. young, mature vs. senescent and young vs. senescent *L. octovalvis* leaves in the Y-tube olfactometer bioassay. Numbers in brackets are the number of insects that did not respond to either treatment.

The insect displayed attraction to hexadecane or octadecane or eicosane or tricosane or palmitic acid or alpha-linolenic acid comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes against the control solvent (table 3). The insect did not indicate a significant preference to docosane or tetracosane or hexacosane or octacosane against the control solvent (table 3). The insects were attracted to a synthetic blend of above ten compounds against the control solvent; whereas the insects were also attracted by a synthetic blend without docosane, tetracosane, hexacosane and octacosane against the control solvent (table 3).

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Table 4. Behavioral responses of *A. cyanea* females to 0.25 mature leaf equivalent surface waxes from *L. octovalvis* leaves vs. individual synthetic compounds or synthetic blends comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes ($N = 90$ in each bioassay).

Comparison		Insects responded				*P* values of insect responded
T1	T2	T1	T2	Non-responders	$\chi 2$ ($df = 1$)	
0.25 mature leaf equivalent surface waxes from *L. octovalvis*	Synthetic compounds or blends					
	a. Hexadecane (0.90 µg ml^{-1})	61	29	4	11.38	0.00074
	b. Octadecane (1.86 µg ml^{-1})	56	34	5	5.38	0.02037
	c. Eicosane (1.83 µg ml^{-1})	60	30	3	10	0.00157
	d. Docosane (1.41 µg ml^{-1})	67	23	3	21.51	0.0001
	e. Tricosane (1.95 µg ml^{-1})	59	31	4	8.71	0.00316
	f. Tetracosane (1.04 µg ml^{-1})	68	22	3	23.51	0.0001
	g. Hexacosane (0.71 µg ml^{-1})	69	21	2	25.6	0.0001
	h. Octacosane (0.45 µg ml^{-1})	70	20	2	27.78	0.0001
	i. Palmitic acid (0.50 µg ml^{-1})	57	33	4	6.4	0.01141
	j. Alpha-linolenic acid (0.18 µg ml^{-1})	61	29	4	11.38	0.00074
	a + b + c + d + e + f + g + h + i + j	46	44	3	0.04	0.83385
	a + b + c + e + i + j	49	41	3	0.71	0.39908

The insect did not indicate a clear positive or negative responses to palmitic acid comparable with the proportion as present in 0.25 senescent leaf equivalent surface waxes against the control solvent (table 3).

Bioassay 4: The insects were attracted toward 0.25 mature leaf equivalent surface waxes against ten individual synthetic compounds (hexadecane, octadecane, eicosane, docosane, tricosane, tetracosane, hexacosane, octacosane, palmitic acid and alpha-linolenic acid) comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes (table 4). The insect did not show a significant preference to 0.25 mature leaf equivalent surface waxes against a synthetic blend of ten compounds (table 4). Furthermore, the insect did not display a significant preference to 0.25 mature leaf equivalent surface waxes against a synthetic blend of six compounds (hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid) (table 4).

Bioassay 5: In the Y-tube olfactometer bioassays, *A. cyanea* females showed attraction to hexadecane, octadecane, eicosane, docosane, tricosane, tetracosane, hexacosane, octacosane, palmitic acid and alpha-linolenic acid against the control solvent (table 5). Females were attracted to hexadecane at the minimal concentration of 1.60 µg ml^{-1} and subsequently, showed highest attraction at 3.20 µg ml^{-1} (table 5). Octadecane was attractive to the females between 1.50 and 3 µg ml^{-1} (table 5). Females displayed gradual increase in attraction to eicosane from 2 to 8 µg ml^{-1} (table 5). Females showed gradual increase in attraction to docosane from 3 to 12 µg ml^{-1} (table 5). Females were attracted to tricosane at the lowest concentration of 2 µg ml^{-1} and subsequently, showed highest attraction at 4 µg ml^{-1} (table 5). Tetracosane was attractive to the females between 2 and 8 µg ml^{-1} (table 5). Hexacosane was attractive to the females between 3 and 6 µg ml^{-1}; whilst octacosane was attractive between 4 and 8 µg ml^{-1} (table 5). Females were attracted to palmitic acid at the lowest concentration of 0.30 µg ml^{-1} and showed highest attraction at 0.60 µg ml^{-1} (table 5). Females displayed gradual increase in attraction to alpha-linolenic acid from 0.20 to 0.80 µg ml^{-1} (table 5).

Oviposition assay

Among 15 mated females, 12 females laid 10.0 ± 0.78 eggs on the filter papers containing 0.25 mature leaf equivalent surface waxes when tested against the filter papers containing the control solvent. Three females laid 10.67 ± 0.88 eggs on the filter papers containing the control solvent. This study revealed that females significantly laid more eggs on the surface waxes ($\chi^2 = 50.95$, $df = 1$, $P < 0.05$) than the control solvent.

Among 15 mated females, ten females laid 9.8 ± 0.44 eggs on the filter papers containing the synthetic blend of six compounds (hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid) comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes when tested against the control solvent. Five females laid 9.8 ± 0.86 eggs on the filter papers containing the control solvent. This study indicated that females significantly laid more eggs on the filter papers containing the synthetic blend ($\chi^2 = 16.33$, $df = 1$, $P < 0.05$) than the control solvent.

In two choice assays, eight mated females laid 10.13 ± 0.8 eggs on the filter papers containing 0.25 mature leaf equivalent surface waxes, whilst seven females laid 10.00 ± 0.8 eggs on the filter papers containing the synthetic blend of six compounds (hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid) comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes. The study indicates that the insect did not show a significant egg laying behavior on 0.25 mature leaf equivalent surface waxes ($\chi^2 = 0.801$, $df = 1$, $P > 0.05$) against a synthetic blend of six compounds.

Discussion

The present study demonstrated that the total amounts of *n*-alkanes and free fatty acids decreased after maturity in the surface waxes of *L. octovalvis* leaves. Twenty, 19 and 19 *n*-alkanes were identified between *n*-C_{15} and *n*-C_{35} alkanes in young, mature and senescent *L. octovalvis* leaves, respectively; whereas 14, 14 and 12 free fatty acids were recorded

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Table 5. Behavioral responses of *A. cyanea* females to individual synthetic compound vs. the control solvent (petroleum ether) in the Y-tube olfactometer bioassay ($N = 90$ in each concentration bioassay).

Synthetic compounds	Concentration (μg ml^{-1})	χ^2 ($df = 1$)	P values of insect responded
Hexadecane	0.80	1.11	0.29184
	1.60	10	0.00157
	3.20	19.6	<0.0001
Octadecane	0.75	0.4	0.52709
	1.50	7.51	0.00613
	3	19.6	<0.0001
Eicosane	1	1.11	0.29184
	2	4.44	0.3502
	4	12.84	0.00034
	8	17.78	<0.0001
Docosane	1.50	1.6	0.2059
	3	5.38	0.02037
	6	10	0.00157
	12	19.6	<0.0001
Tricosane	1	1.11	0.29184
	2	6.4	0.01141
	4	16.04	<0.0001
Tetracosane	1	1.6	0.2059
	2	5.38	0.02037
	4	12.84	0.00034
	8	16.04	<0.0001
Hexacosane	1.50	2.18	0.14002
	3	7.51	0.00613
	6	16.04	<0.0001
Octacosane	1	0.4	0.52709
	2	2.84	0.09169
	4	7.51	0.00613
	8	12.84	0.00034
Palmitic acid	0.15	0.04	0.83385
	0.30	4.44	0.03502
	0.60	16.04	<0.0001
Alpha-linolenic acid	0.10	0.4	0.52709
	0.20	4.44	0.03502
	0.40	7.51	0.00613
	0.80	19.6	<0.0001

between C12:0 and C22:0 fatty acids in young, mature and senescent *L. octovalvis* leaves, respectively. The variation in the level of individual *n*-alkanes and free fatty acids in three types of *L. octovalvis* leaves is due to the rate of wax accumulation throughout leaf development stages (Baker & Hunt, 1981; Jetter *et al.*, 2000). Li & Ishikawa (2006) demonstrated *n*-C_{15} to *n*-C_{36} alkanes and C9 to C22 free fatty acids in surface waxes of *Fallopia japonica* (Hout.) R. Decr. leaves. Nineteen, 20 and 18 *n*-alkanes between *n*-C_{15} and *n*-C_{35} were detected in surface waxes of young, mature and senescent *Momordica cochinchinensis* Spreng leaves, whilst 13 free fatty acids between C12 and C20 were identified in surface waxes throughout the developmental stages of *M. cochinchinensis* leaves (Mukherjee *et al.*, 2014, 2015*b*). Nonacosane (*n*-C_{29}) and hexadecanoic acid were predominant *n*-alkanes and free fatty acids in surface waxes of *F. japonica* mature leaves, respectively (Li & Ishikawa, 2006). Pentatriacontane and palmitic acid were the most abundant alkane and free fatty acid in surface waxes of *M. cochinchinensis* leaves, respectively (Mukherjee *et al.*, 2014, 2015*b*). However in the present investigation, tricosane was predominant alkane in young and mature *L. octovalvis* leaves, whilst eicosane predominated in senescent leaves; whereas heneicosanoic acid (C21:0), palmitic acid (C16:0) and docosanoic acid (C22:0) were the most abundant free fatty acids in young, mature and senescent leaves, respectively. In literature, several studies indicated that different alkanes and free fatty acids predominated in leaf surface waxes of different species (Hellmann & Stoesser, 1992; Burdi *et al.*, 2007; Demir & Cakmak, 2007; Sato *et al.*, 2008; Van Maarseveen *et al.*, 2009; Sarkar *et al.*, 2014; Koukos *et al.*, 2015; Malik & Barik, 2015). The present study supports the hypothesis that the variation in the composition of surface wax compounds might occur between plant species as well as between different stages of leaf development within a species (Eigenbrode & Espelie, 1995; Jetter *et al.*, 2000; Piasentier *et al.*, 2000; Mukherjee *et al.*, 2014; Sarkar *et al.*, 2014; Dodoš *et al.*, 2015; Malik & Barik, 2015).

Alkanes and free fatty acids are the common constituents of plant leaf surface waxes (Baker, 1982; Jetter *et al.*, 2000) and play important roles in bitrophic herbivore–plant interactions such as attractants (Dutton *et al.*, 2000; Schoonhoven *et al.*, 2005; Roy & Barik 2012; Mukherjee *et al.*, 2013; Sarkar *et al.*, 2013; Sarkar & Barik, 2014, 2015) or oviposition stimulants (Eigenbrode & Espelie, 1995; Grant *et al.*, 2000; Li & Ishikawa, 2006). Long-chain alkanes and free fatty acids are low-volatile substances, which act as close range allelochemicals once an insect reaches to the plant. The navel orangeworm *Amyelois transitella* Walker (Lepidoptera: Pyralidae) displayed attraction to long-chain fatty acids such as palmitic, oleic and linoleic acids alone or blended with crude almond oil in wind tunnel bioassays (Youngman & Baker, 1989). The clover root borer *Hylastinus obscurus* females were attracted toward individual synthetic lauric, palmitic, stearic and oleic acids, and their blend like clover root extract in the Y-tube olfactometer bioassay (Manosalva *et al.*, 2011). Furthermore, *Epilachna dodecastigma* females were attracted to a synthetic blend of nonadecane, eicosane, heneicosane, pentacosane, heptacosane, octacosane, nonacosane, hentriacontane and tritriacontane mimicking the proportions as present in mature leaves of *Momordica charantia* L. in the Y-tube olfactometer bioassay (Sarkar *et al.*, 2013).

The present Y-tube olfactometer bioassay results clearly revealed that *A. cyanea* females could discriminate 0.25 leaf equivalent leaf surface waxes from mature leaves against young or senescent leaves. A synthetic blend of six compounds (hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid) comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes of *L. octovalvis* leaves acted as short-range attractant of *A. cyanea* in the Y-tube olfactometer bioassay and also acted as oviposition stimulant. In literature, several reports indicated the importance of alkanes and fatty acids as allelochemicals (Parr *et al.*, 1998; Schiestl *et al.*, 1999; Tasin *et al.*, 2005; Mukherjee *et al.*, 2013; Sarkar *et al.*, 2013; Sarkar & Barik, 2014; Adhikary *et al.*, 2014, 2016). *A. cyanea* showed attraction to hexadecane at 0.90 μg ml^{-1}, octadecane at 1.86 μg ml^{-1}, eicosane at 1.83 μg ml^{-1}, tricosane at 1.95 μg ml^{-1}, palmitic acid at 0.50 μg ml^{-1} and alpha-linolenic acid at 0.18 μg ml^{-1} in initial bioassays comparable with the proportions as present in 0.25 mature leaf equivalent surface waxes of *L. octovalvis*; whereas the insect did not show a significant preference to docosane at 1.41 μg ml^{-1}, tetracosane at 1.04 μg ml^{-1}, hexacosane at 0.71 μg ml^{-1} and octacosane at 0.45 μg ml^{-1}. However, the amount of hexadecane was higher in 25 g of young leaves than the same amount of mature leaves (see table 1), but number of leaves (300) representing 25 g mature leaves are lower than the number of leaves (525) representing same amount

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of young leaves (see Supplementary table 1). This documented that the amount of hexadecane was higher in 0.25 mature leaf equivalent surface waxes than 0.25 young leaf equivalent surface waxes (Supplementary table 2a). This observation indicate that the ratio of compounds present in 0.25 mature leaf equivalent surface waxes of *L. octovalvis* becomes vital components, which act as olfactory cues for oviposition of *A. cyanea*.

The present study summarizes that *A. cyanea* females were attracted to 0.25 mature leaf equivalent surface waxes of *L. octovalvis* weed compared with the same amount of leaf equivalent surface from young and senescent leaves. *A. cyanea* females were most attractive to a synthetic blend of 0.90, 1.86, 1.83, 1.95, 0.50 and 0.18 µg ml^{-1} petroleum ether of hexadecane, octadecane, eicosane, tricosane, palmitic acid and alpha-linolenic acid, respectively, which could offer valuable insights on the prospects of using these compounds for short-range attraction and oviposition stimulant of the biocontrol agent to target weed plants during early emergence in field. Oviposition by *A. cyanea* females during early emergence of the weed would help to defoliate the weeds by the larvae of the potential biocontrol agent in rice-field; however, it remains to be seen in the ecological context the interaction with other plants.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485316001012.

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