SUPPLEMATRAY MATERIAL

**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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**Supplementary material S1** (*Plant materials*)

Different ages of leaves were classified mainly with the developmental time following leaf emergence through continuous monitoring of weed plants in rice-field, and size [i.e., young leaf: length = 3.1 ± 0.2 cm and breadth = 0.9 ± 0.1 cm, mature leaf: length = 7.2 ± 0.3 cm and breadth = 2.1 ± 0.2 cm, and senescent leaf: length = 6.8 ± 0.2 cm and breadth = 2 ± 0.1 cm (mean ± standard error; ten replicate of one leaf per each age)], and color (i.e., young leaf: light green, mature leaf: dark green, and senescent leaf: yellowish with reddish) were considered during collection of leaves.

**Supplementary material S2** (*Identification and quantification of alkanes*)

The eluent 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. A faint yellowish band appeared on the TLC plate, and the plate was air-dried under laboratory conditions. The plate was then placed in an iodine chamber for 1 min, which produced a deep yellowish band with R*f* (Retardation factor) value of 0.86. The R*f* value (0.86) was compared with the R*f* value of a mixture of synthetic alkanes between *n*-C15 and *n*-C35. The single hydrocarbon band produced in each TLC plate was eluted from the silica gel layer with chloroform, which showed no absorption of detectable functional groups by IR spectroscopy. A total of 9 purified alkane samples (3 alkane samples from each type of leaf) were produced for gas chromatography-mass spectrometry (GC-MS) and GC-FID for identification and quantification, respectively. One portion of each sample was used for identification by GC-MS and the remainder for quantification of alkane compounds by GC-FID. All solvents used were of GR grade and purchased from E. Merck, India Pvt. Ltd.

For identification of alkane compounds, the extracts were analyzed with an Agilent 6890 GC coupled to a 5973 Mass Selective Detector using a SE-30 column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness). The oven temperature programme was initially 170°C held for 1 min, then raised at 4 °C/min to 300 °C and finally held for 15 min (Mukherjee *et al.,* 2013; Sarkar *et al.,* 2013). Helium was the carrier gas. The MS parameters were 280°C at the interface, ionization energy 70 eV, scan speed approximately 1 sec, and scanned over the mass range 40–600 mass units. The identity of the compounds was confirmed by injections of mixture of synthetic *n*-alkanes (*n*-C14 to *n*-C35). Alkanes were verified by comparison of the diagnostic ions and GC retention times with those of respective authentic standards.

For quantification of compounds, three separate extracts of each leaf type (i.e., young, mature and senescent) were analyzed by a Techcomp GC (Em Macau, Rua De Pequim, Nos. 202A-246, Centro Financeiro F7, Hong Kong) model 7900 fitted with a SE-30 capillary column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness) and a flame ionization detector which was run under same temperature conditions as mentioned in GC-MS analysis The carrier gas was nitrogen with a flow rate of 18.5 ml/min. The volume of the sample injected was 1 µl with a split ratio of 1:5. The peaks were identified by comparison of their retention times with those of standard *n*-alkanes from *n*-C15 through *n*-C35, and the areas of each peak were converted into quantities of *n*-alkanes based on internal standard heneicosane (*n*-C21). All *n*-alkanes (>99% purity) between *n*-C15 and *n*-C35 were purchased from Sigma Aldrich.

**Supplementary material S3** (*Identification and quantification of free fatty acids*)

The extract was purified by TLC on silica gel G layers (thickness 0.5 mm), which had been prepared using a Unoplan (Shandon, London) coating apparatus, 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 & Barik, 2015). The band was eluted from the silica gel layer with diethyl ether, and diethyl ether was removed under reduced pressure to get purified free fatty acids. The purified free fatty acids were esterified with 3 ml BF3-Methanol followed by warming for 5 min in a hot water bath at 50–60°C temperature, and cooled. Hexane (30 ml) was added to this mixture followed by washing with saturated NaCl for twice in a separating funnel. The aqueous layer of each sample was discarded and the hexane fraction was passed through 50 g anhydrous Na2SO4 for twice. One portion of each esterified sample (hexane fraction) was used for GC-MS and another for GC-FID. The extraction of free fatty acids from the each crude extract was separately repeated thrice followed by esterification, and a total of 9 samples were prepared.

One portion of the esterified fatty acids was analyzed with an Agilent 6890 GC coupled to a 5973 Mass Selective Detector with a SE-30 column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness). The oven temperature program was initially held at 160°C for 2 min, then raised at the rate of 3°C/ min to 220°C and finally held at 220°C for 18 min (Mukherjee *et al.,* 2014; Sarkar & Barik, 2015). Helium was the carrier gas. The MS temperature parameter was 280°C at the interface, ionization energy 70 eV, scan speed approximately 1 sec, and scanned over the mass range 40–600 mass units. Fatty acids were verified by comparison of the diagnostic ions and GC retention times with those of respective standard esterified fatty acids [methyl laurate (C12:0), methyl tridecanoate (C13:0), methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl heptadecanoate (C17:0), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), methyl *α*-linolenate (C18:3), methyl nonadecanoate (C19:0), methyl arachidate (C20:0), methyl heneicosanoate (C21:0), methyl docosanoate (C22:0) and methyl tricosanoate (C23:0)]. All standard esterified fatty acids (fatty acid methyl esters) were purchased from Sigma-Aldrich, Germany.

The remaining portion of the esterified fatty acids (three separate samples from each type of leaf) were analyzed using a Techcomp Gas Chromatograph model 7900 fitted with a SE-30 capillary column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness) and a flame ionization detector which was run under same temperature conditions as described for GC-MS analysis. The injector port temperature was 280°C. The carrier gas was nitrogen with a flow rate of 20 ml/ min (Mukherjee *et al.,* 2014; Sarkar & Barik, 2015). The volume of the sample injected was 1 micro liter with a split ratio of 1:5. The peaks were identified by comparison of their retention times with those of standard esterified fatty acids. The amount of individual free fatty acids was computed from the GC peak areas and the areas of each peak were converted into quantities of fatty acids based on reference standard methyl tricosanoate (C23:0). All solvents used were of analytical grade and purchased from E. Merck (Mumbai, India).

**Supplementary material S4**

Preliminarily, the behavior of 90 females to 1 leaf equivalent mature leaf surface waxes (63.47 µg) was tested against the control solvent assuming that surface waxes from one single mature leaf would be sufficient to attract the insect, and subsequently, the insect showed highest (*P* < 0.0001) attraction to this dose (Supplementary material table 1). So, we tried to find out the dose (i.e., 0.5 and 0.25 leaf equivalent mature leaf surface waxes) where the insect showed similar attraction to that of 1 leaf equivalent mature leaf surface waxes (Supplementary material table 1). The insect displayed similar attraction response (*P* < 0.0001) to 0.25 leaf equivalent surface waxes (15.87µg) like 1 leaf equivalent mature leaf surface waxes.

**Supplementary Table 1** Leaf equivalent surface waxes of three types of *L. octovalvis* leaves used for olfactory bioassay.

|  |  |  |  |
| --- | --- | --- | --- |
| Leaf | Leaf equivalent | | |
| 0.25 | | 0.5 | 1 |
| Amount (µg) | | |
| Young | 7.41 | 14.82 | 29.64a |
| Mature | 15.87 | 31.74 | 63.47b |
| Senescent | 8.16 | 16.32 | 32.63c |

a: 25 g young leaves equivalent to 525 leaves, and 25 g leaf yielded 15.56 mg surface waxes

b: 25 g mature leaves equivalent to 300 leaves, and 25 g leaf yielded 19.04 mg surface waxes

c: 25 g senescent leaves equivalent to 475 leaves, and 25 g leaf yielded 15.5 mg surface waxes

**Supplementary Table 2a** Individual synthetic alkane compounds present in 0.25 leaf equivalent surface waxes of three types of leaves used for olfactory bioassay

|  |  |  |  |
| --- | --- | --- | --- |
| Alkanes | Young | Mature | Senescent |
|  | Amount (µg) |  |  |
| Pentadecane (*n*-C15) | 0.38a | 0.18b | 0.04c |
| Hexadecane (*n*-C16) | 0.69 | 0.90 | 0.29 |
| Heptadecane (*n*-C17) | 0.00 | 0.00 | 0.01 |
| Octadecane (*n*-C18) | 0.81 | 1.86 | 0.67 |
| Nonadecane (*n*-C19) | 0.02 | 0.08 | 0.02 |
| Eicosane (*n*-C20) | 0.83 | 1.83 | 0.72 |
| Docosane (*n*-C22) | 0.62 | 1.41 | 0.60 |
| Tricosane (*n*-C23) | 0.98 | 1.95 | 0.32 |
| Tetracosane (*n*-C24) | 0.44 | 1.04 | 0.43 |
| Pentacosane (*n*-C25) | 0.03 | 0.19 | 0.05 |
| Hexacosane (*n*-C26) | 0.24 | 0.71 | 0.33 |
| Heptacosane (*n*-C27) | 0.02 | 0.07 | 0.05 |
| Octacosane (*n*-C28) | 0.13 | 0.45 | 0.22 |
| Nonacosane (*n*-C29) | 0.02 | 0.16 | 0.09 |
| Triacontane (*n*-C30) | 0.10 | 0.28 | 0.15 |
| Hentriacontane (*n*-C31) | 0.02 | 0.15 | 0.20 |
| Dotriacontane (*n*-C32) | 0.05 | 0.17 | 0.12 |
| Tritriacontane (*n*-C33) | 0.01 | 0.10 | 0.14 |
| Tetratriacontane (*n*-C34) | 0.02 | 0.07 | 0.06 |
| Pentatriacontane (*n*-C35) | 0.01 | 0.07 | - |

a: For young leaves: 15.56 mg or 15560 µg surface waxes indicated presence of 11442 µg total identified alkane, and subsequently, 798.82 µg pentadecane was detected. 0.25 leaf equivalent surface waxes is equivalent to 7.41 µg surface waxes (See Supplementary Table 1). 7.41 µg surface wax yielded 11442 × 7.41 / 15560 = 5.449 µg alkane. Hence, 5.449 µg alkane indicates presence of pentadecane = 798.82 × 5.449 / 11442 = 0.3804 µg or 0.38 µg. For hexadecane: 1454.10 × 5.449 / 11442 = 0.6924 µg or 0.69 µg.

b: For mature leaves: 19.04 mg or 19040 µg surface waxes indicated presence of 14190 µg total alkane, and subsequently, 221.82 µg pentadecane was detected. 0.25 leaf equivalent surface waxes is equivalent to 15.87 µg surface waxes (See Supplementary Table 1). 15.87 µg surface wax yielded 14190 × 15.87 / 19040 = 11.827 µg alkane. Hence, 11.657 µg alkane indicates presence of pentadecane = 221.82 × 11.827 / 14190 = 0.18 µg. For hexadecane: 1075.11 × 11.827 / 14190 = 0.8961 µg or 0.90 µg.

b: For senescent leaves: 15.5 mg or 15500 µg surface waxes indicated presence of 8720 µg total identified alkane, and subsequently, 69.36 µg pentadecane was detected. 0.25 leaf equivalent surface waxes is equivalent to 8.16 µg surface waxes (See Supplementary Table 1). 8.16 µg surface wax yielded 8720 × 8.16 / 15500 = 4.590 µg alkane. Hence, 4.590 µg alkane indicates presence of pentadecane = 69.36 × 4.590 / 8720 = 0.0365 µg or 0.04 µg. For hexadecane: 543.68 × 4.590 / 8720 = 0.2862 µg or 0.29 µg.

**Supplementary Table 2b** Individual synthetic fatty acid compounds present in 0.25 leaf equivalent surface waxes of three types of leaves used for olfactory bioassay

|  |  |  |  |
| --- | --- | --- | --- |
| Fatty acids | Young | Mature | Senescent |
| Amount (µg) |  |  |
| Lauric acid (C12:0) | 0.01a | 0.02b | 0.02c |
| Tridecanoic acid (C13:0) | 0.02 | 0.02 | 0.00 |
| Myristic acid (C14:0) | 0.02 | 0.05 | 0.03 |
| Pentadecanoic acid (C15:0) | 0.05 | 0.08 | 0.05 |
| Palmitic acid (C16:0) | 0.13 | 0.50 | 0.24 |
| Palmitoleic acid (C16:1) | 0.10 | 0.04 | - |
| Heptadecanoic acid (C17:0) | 0.07 | 0.07 | 0.06 |
| Stearic acid (C18:0) | 0.12 | 0.18 | 0.07 |
| Oleic acid (C18:1) | - | - | 0.03 |
| Linoleic acid (C18:2) | 0.04 | 0.05 | 0.03 |
| Alpha-linolenic acid (C18:3) | 0.04 | 0.18 | - |
| Nonadecanoic acid (C19:0) | 0.06 | 0.13 | 0.04 |
| Arachidic acid (C20:0) | 0.03 | 0.11 | - |
| Heneicosanoic acid (C21:0) | 0.13 | 0.18 | 0.05 |
| Docosanoic acid (C22:0) | 0.12 | 0.43 | 0.54 |

a: For young leaves: 15.56 mg or 15560 µg surface waxes indicated presence of 1967 µg total free fatty acids, and subsequently, 14.98 µg lauric acid was detected. 0.25 leaf equivalent surface waxes is equivalent to 7.41 µg surface waxes (See Supplementary Table 1). 7.41 µg surface waxes yielded 1967 × 7.41 / 15560 = 0.9369 µg free fatty acid. Hence, 0.9369 µg free fatty acid indicates presence of lauric acid = 14.98 × 0.9369 / 1967 = 0.0071 µg or 0.01 µg.

b: For mature leaves: 19.04 mg or 19040 µg surface waxes indicated presence of 2461 µg total free fatty acid, and subsequently, 20.33 µg lauric acid was detected. 0.25 leaf equivalent surface waxes is equivalent to 15.87 µg surface waxes (See Supplementary Table 1). 15.87 µg surface waxes yielded 2461 × 15.87 / 19040 = 2.051 µg free fatty acid. Hence, 2.051 µg free fatty acid indicates presence of lauric acid = 20.33 × 2.051 / 2461 = 0.0169 µg or 0.02 µg.

b: For senescent leaves: 15.5 mg or 15500 µg surface waxes indicated presence of 2156 µg total free fatty acid, and subsequently, 37.01 µg lauric acid was detected. 0.25 leaf equivalent surface waxes is equivalent to 8.16 µg surface waxes (See Supplementary Table 1). 8.16 µg surface waxes yielded 2156 × 8.16 / 15500 = 1.134 µg free fatty acid. Hence, 1.134 µg free fatty acids indicates presence of lauric acid = 37.01× 1.134 / 2156 = 0.0195 µg or 0.02 µg.

**Supplementary Table 3** Combinations of synthetic compounds (comparable to 0.25 leaf equivalent surface waxes of young and mature *L. octovalvis*leaves) prepared for *A. cyanea* olfactory bioassay.

|  |  |
| --- | --- |
| Young leaves | Mature leaves |
| Hexadecane + Octadecane + Eicosane + | Hexadecane + Octadecane + Eicosane + Docosane + Tricosane + Tetracosane + |
| Tricosane + Palmitic acid (µg/ml) | Hexacosane + Octacosane + Palmitic acid + alpha-Linolenic acid (µg/ml) |
| 0.69 + 0.81 + 0.83 + 0.98 + 0.13 | 0.90 + 1.86 + 1.83 + 1.41 + 1.95 + 1.04 + 0.71 + 0.45 + 0.50 + 0.18 |

**Supplementary Table 4** Levene’s test for homogeneity of variance for individualalkane compounds from young, mature and senescent *L. octovalvis* leaves by SPSS software

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Levene Statistic | df1 | df2 | Sig. |
|  |  |  |  |  |
| Pentadecane (*n*-C15) | 4.726 | 2 | 6 | 0.059 |
| Hexadecane (*n*-C16) | 4.062 | 2 | 6 | 0.077 |
| Heptadecane (*n*-C17) | 4.882 | 2 | 6 | 0.055 |
| Octadecane (*n*-C18) | 1.232 | 2 | 6 | 0.356 |
| Nonadecane (*n*-C19) | 0.803 | 2 | 6 | 0.491 |
| Eicosane (*n*-C20) | 0.251 | 2 | 6 | 0.786 |
| Docosane (*n*-C22) | 0.393 | 2 | 6 | 0.691 |
| Tricosane (*n*-C23) | 4.809 | 2 | 6 | 0.057 |
| Tetracosane (*n*-C24) | 0.245 | 2 | 6 | 0.791 |
| Pentacosane (*n*-C25) | 2.937 | 2 | 6 | 0.129 |
| Hexacosane (*n*-C26) | 1.422 | 2 | 6 | 0.312 |
| Heptacosane (*n*-C27) | 0.606 | 2 | 6 | 0.576 |
| Octacosane (*n*-C28) | 3.481 | 2 | 6 | 0.099 |
| Nonacosane (*n*-C29) | 4.678 | 2 | 6 | 0.060 |
| Triacontane (*n*-C30) | 2.550 | 2 | 6 | 0.158 |
| Hentriacontane (*n*-C31) | 4.690 | 2 | 6 | 0.060 |
| Dotriacontane (*n*-C32) | 3.766 | 2 | 6 | 0.087 |
| Tritriacontane (*n*-C33) | 4.767 | 2 | 6 | 0.058 |
| Tetratriacontane (*n*-C34) | 1.081 | 2 | 6 | 0.397 |
| Pentatriacontane (*n*-C35) | 2.481 | 2 | 6 | 0.190 |
| Total alkanes | 0.940 | 2 | 6 | 0.442 |

**Supplementary Table 5** Levene’s test for homogeneity of variance for individualfree fatty acid compounds from young, mature and senescent *L. octovalvis* leaves by SPSS software

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Levene Statistic | df1 | df2 | Sig. |
| Lauric acid (C12:0) | 1.320 | 2 | 6 | 0.335 |
| Tridecanoic acid (C13:0) | 0.024 | 2 | 6 | 0.976 |
| Myristic acid (C14:0) | 0.143 | 2 | 6 | 0.869 |
| Pentadecanoic acid (C15:0) | 0.310 | 2 | 6 | 0.745 |
| Palmitic acid (C16:0) | 0.235 | 2 | 6 | 0.798 |
| Palmitoleic acid (C16:1) | 2.531 | 2 | 6 | 0.187 |
| Heptadecanoic acid (C17:0) | 0.723 | 2 | 6 | 0.523 |
| Stearic acid (C18:0) | 3.467 | 2 | 6 | 0.097 |
| Linoleic acid (C18:2) | 0.056 | 2 | 6 | 0.824 |
| Alpha-linolenic acid (C18:3) | 4.057 | 2 | 6 | 0.076 |
| Nonadecanoic acid (C19:0) | 0.941 | 2 | 6 | 0.441 |
| Arachidic acid (C20:0) | 1.178 | 2 | 6 | 0.339 |
| Heneicosanoic acid (C21:0) | 1.294 | 2 | 6 | 0.341 |
| Docosanoic acid (C22:0) | 1.572 | 2 | 6 | 0.283 |
| Total fatty acids | 1.750 | 2 | 6 | 0.252 |