**SUPPLEMETRAY MATERIAL**

**The role of *Lathyrus sativus* flower surface wax in short-range attraction and stimulant for nymph laying by an adult viviparous aphid**

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*Alkanes – identification and quantification*

The second fraction of the 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 Mitra *et al.*(2017). 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*-C12 and *n*-C36. The extraction process was repeated for three times each for BIO L 212 Ratan and Nirmal B-1, and purified alkanes were isolated from each crude extract using TLC plates. A total of 6 purified alkane samples [as 3 × 60 g BIO L 212 Ratan and 3 × 60 g Nirmal B-1 flower samples were collected for separation of the surface waxes (each crude extract from 60 g flower samples was divided in to 3 equal fractions, i.e., ca. 20 g flowers; first, second and third fraction for alkane, fatty acids and bioassays, respectively), 3 alkane samples were prepared from 3 × ca. 20 g flower material each from BIO L 212 Ratan and Nirmal B-1] were prepared for gas chromatography-mass spectrometry (GC-MS) and GC-flame ionization detection (GC-FID) for identification and quantification, respectively. Half portion of each sample was used for GC-MS and the remainder for GC-FID. All solvents used were purchased from E. Merck, India Pvt. Ltd.

For identification of alkanes, 1 µl sample was analyzed with a Clarus 690 GC coupled to a SQ8C Mass Selective Detector using a SE-30 column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness). The temperature of injector was 280°C, and 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 (Mitra *et al.*, 2017). Helium was the carrier gas and flow rate was 1 ml/min. The MS parameters were: 280°C at the interface, ionization energy 70 eV, scan rate 5 scans/sec and scanned over the mass range 40–600 mass units. The identity of compounds was confirmed by injections of a mixture of synthetic *n*-alkanes (*n*-C12 to *n*-C36). 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 samples of either BIO L 212 Ratan or Nirmal B-1 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, but there is no interface. The carrier gas was nitrogen with a total flow rate of 18.5 ml/min and column flow rate of 2.3 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*-C12 through *n*-C36, and the areas of all peaks were converted into quantities of *n*-alkanes based on internal standard heneicosane (*n*-C21). All *n*-alkanes (>99% purity) between *n*-C12 and *n*-C36 were purchased from Sigma Aldrich.

*Free fatty acids − identification and quantification*

The third fraction of the each crude extract of either BIO L 212 Ratan or Nirmal B-1 was mixed with diethyl ether and filtered through Whatman No. 41 filter paper (Sarkar & Barik, 2015). 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), and we followed rest of the procedure adapted from Mitra *et al.*(2017). 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 twice in a separating funnel. The aqueous layer of each sample was discarded and the hexane fraction was passed through 50 g anhydrous Na2SO4 twice. Half portion of each esterified sample (hexane fraction) was used for GC-MS and another for GC-FID. The extraction of free fatty acids from each crude extract was separately repeated thrice followed by esterification, and a total of 6 samples [as 3 × 60 g BIO L 212 Ratan and 3 × 60 g Nirmal B-1 flower samples were collected for separation of the surface waxes (each crude extract from 60 g flower samples was divided into 3 equal fractions, i.e., ca. 20 g flowers; first, second and third fraction for alkane, fatty acids and bioassays, respectively), 3 fatty acid samples were prepared from 3 × ca. 20 g flower materials each from BIO L 212 Ratan and Nirmal B-1] were prepared.

One portion of the esterified fatty acids was analyzed with a Clarus 690 GC coupled to a SQ8C Mass Selective Detector with a SE-30 column (Agilent, USA; length: 30 m × 0.32 mm × 0.25-μm film thickness). One µl sample was injected. The temperature of injector was 280°C, and 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 and flow rate was 1 ml/min. The MS temperature parameters were: 280°C at the interface, ionization energy 70 eV, scan rate 5 scans/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 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 linolenate (C18:3), methyl nonadecanoate (C19:0), methyl arachidate (C20:0), methyl heneicosanoate (C21:0) and methyl docosanoate (C22:0)]. All standard esterified fatty acids (fatty acid methyl esters, purity ≥99%) were purchased from Sigma-Aldrich, Germany.

The remaining portion of the esterified fatty acids (three separate samples of either BIO L 212 Ratan or Nirmal B-1) 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 mentioned in GC-MS analysis, but there is no interface. The injector port temperature was 280°C. The carrier gas was nitrogen with a total flow rate of 20 ml/ min and column flow rate of 2.5 ml/min (Mukherjee *et al*., 2014; Sarkar & Barik, 2014). 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 esterified fatty acids. The amount of individual free fatty acids was computed from the GC peak areas and the areas of all peaks 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). For choice experiments and viviparity bioassays, all standard fatty acids (purity ≥99%) were purchased from Sigma-Aldrich, Germany.

**Supplementary Table 1** One flower equivalent surface wax of BIO L 212 Ratan and Nirmal B-1 cultivars of *Lathyrus sativus* used for olfactory bioassay

|  |  |  |
| --- | --- | --- |
| Cultivar | One flower equivalent | |
| Amount (µg) |
| BIO L 212 Ratan | 24.32a  35.20b |
| Nirmal B-1 |

a: 20 g BIO L 212 Ratan flowers equivalent to 433 flowers, and 20 g flowers yielded 10.53 mg surface waxes

b: 20 g Nirmal B-1 flowers equivalent to 392 flowers, and 20 g flowers yielded 13.80 mg surface waxes

**Supplementary Table 2a** Individual synthetic alkane compounds used for choice experiments [as present in one flower equivalent surface wax of two cultivars (BIO L 212 Ratan and Nirmal B-1) of *Lathyrus sativus*]

|  |  |  |
| --- | --- | --- |
| Alkane | Amount (µg) | |
|  | BIO L 212 Ratan | Nirmal B-1 |
| Dodecane (*n*-C12) | 0.71a | 1.15b |
| Tetradecane (*n*-C14) | 1.14 | 1.60 |
| Pentadecane (*n*-C15) | 2.37 | 2.81 |
| Hexadecane (*n*-C16) | 1.14 | 1.61 |
| Heptadecane (*n*-C17) | 0.74 | 1.19 |
| Octadecane (*n*-C18) | 1.34 | 1.77 |
| Eicosane (*n*-C20) | 0.68 | 0.60 |
| Docosane (*n*-C22) | 0.52 | 0.50 |
| Tricosane (*n*-C23) | 0.24 | 0.30 |
| Tetracosane (*n*-C24) | 0.24 | 0.24 |
| Pentacosane (*n*-C25) | 0.12 | 0.09 |
| Hexacosane (*n*-C26) | 0.11 | 0.13 |
| Heptacosane (*n*-C27) | 0.25 | 0.20 |
| Octacosane (*n*-C28) | 0.13 | 0.09 |
| Nonacosane (*n*-C29) | 1.08 | 0.93 |
| Triacontane (*n*-C30) | 0.13 | 0.11 |
| Hentriacontane (*n*-C31) | 1.02 | 1.43 |
| Dotriacontane (*n*-C32) | 0.07 | 0.08 |
| Tritriacontane (*n*-C33) | 0.18 | 0.23 |
| Tetratriacontane (*n*-C34) | **-** | 0.13 |
| Pentatriacontane (*n*-C35) | 0.30 | 0.59 |
| Hexatriacontane (*n*-C36) | 0.30 | 0.48 |

a: For BIO L 212 Ratan – 20 g flowers or 433 flowers: 10.53 mg or 10530 µg surface waxes indicated presence of 5549.67 µg total identified alkane, and subsequently, 308.32 µg dodecane was detected. Hence, 308.32/433 = 0.71 µg dodecane is present in one flower equivalent surface wax.

b: For Nirmal B-1 flowers – 20 g flowers or 392 flowers: 13.80 mg or 13800 µg surface waxes indicated presence of 6368.67 µg total identified alkane, and subsequently, 450.41 µg dodecane was detected. Hence, 450.41/392 = 1.15 µg dodecane is present in one flower equivalent surface wax.

**Supplementary Table 2b** Individual synthetic fatty acid compounds used for choice experiments [as present in one flower equivalent surface wax of two cultivars (BIO L 212 Ratan and Nirmal B-1) of *Lathyrus sativus*]

|  |  |  |
| --- | --- | --- |
| Fatty acids | Amount (µg) | |
|  | BIO L 212 Ratan | Nirmal B-1 |
| Lauric acid (C12:0) | 0.23a | 0.46b |
| Tridecanoic acid (C13:0) | 1.56 | 2.10 |
| Pentadecanoic acid (C15:0) | 0.47 | 0.94 |
| Palmitoleic acid (C16:1) | 0.83 | 1.40 |
| Palmitic acid (C16:0) | 0.06 | 0.13 |
| Heptadecanoic acid (C17:0) | 0.22 | 0.77 |
| Linolenic acid (C18:3) | 0.18 | 0.34 |
| Linoleic acid (C18:2) | 0.98 | 1.42 |
| Oleic acid (C18:1) | 0.07 | 0.11 |
| Stearic acid (C18:0) | 0.02 | 0.06 |
| Nonadecanoic acid (C19:0) | 0.36 | 0.72 |
| Arachidic acid (C20:0) | 0.37 | 0.73 |

a: For BIO L 212 Ratan – 20 g flowers or 433 flowers: 10.53 mg or 10530 µg surface waxes indicated presence of 2321.67 µg total free fatty acids, and subsequently, 98.19 µg lauric acid was detected. Hence, 98.19/433 = 0.23 µg lauric acid is present in one flower equivalent surface wax.

b: For Nirmal B-1 flowers – 20 g flowers or 392 flowers: 13.80 mg or 13800 µg surface waxes indicated presence of 3599.33 µg total free fatty acids, and subsequently, 181.06 µg lauric acid was detected. Hence, 181.06/392 = 0.46 µg lauric acid is present in one flower equivalent surface wax.

Table 3. Experimental design to observe behavioural responses of adult viviparous *Aphis craccivora* females towards individual synthetic compounds or synthetic blends comparable to the amounts as present in one flower equivalent surface wax of BIO L 212 Ratan or Nirmal B-1 of *Lathyrus sativus* vs. control solvent (Petroleum ether) in Y-tube choice experiments..

|  |  |
| --- | --- |
| Comparison | |
| T1 | T2 |
| Synthetic compounds comparable to one flower | Control solvent |
| equivalent surface wax of BIO L 212 Ratan (µg/ml) |  |
| b. Tetradecane (1.14) |  |
| c. Pentadecane (2.37) |  |
| d. Heptacosane (0.25) |  |
| e. Nonacosane (1.08) |  |
| f. Triacontane (0.13) |  |
| g. Tridecanoic acid (1.56) |  |
| h. Linoleic acid (0.98) |  |
| i. Nonadecanoic acid (0.36) |  |
| b + c + d + e + f + g + h + i |  |
| e + g + h |  |
| Synthetic compounds comparable to one flower |  |
| equivalent surface wax of Nirmal B-1(µg/ml) |  |
| a. Dodecane (1.15) |  |
| b. Tetradecane (1.60) |  |
| c. Pentadecane (2.81) |  |
| d. Heptacosane (0.20) |  |
| e. Nonacosane (0.93) |  |
| f. Triacontane (0.11) |  |
| g. Tridecanoic acid (2.10) |  |
| h. Linoleic acid (1.42) |  |
| i. Nonadecanoic acid (0.72) |  |
| a + b + c + d + e + f + g + h + i |  |
| b + c + e + g + h |  |

Table 4. Experimental design to observe behavioural responses of adult viviparous *Aphis craccivora* females to one flower equivalent surface wax from two cultivars (BIO L 212 Ratan and Nirmal B-1) of *Lathyrus sativus* vs. individual synthetic compounds or synthetic blends comparable to the amounts as present in one flower equivalent surface wax of BIO L 212 Ratan and Nirmal B-1.

|  |  |
| --- | --- |
| Comparison | |
| T1 | T2 |
| One flower equivalent surface | Synthetic compounds or blends |
| wax from BIO L 212 Ratan | comparable to one flower equivalent |
|  | surface wax of BIO L 212 Ratan (µg/ml) |
|  | b. Tetradecane (1.14) |
|  | c. Pentadecane (2.37) |
|  | d. Heptacosane (0.25) |
|  | e. Nonacosane (1.08) |
|  | f. Triacontane (0.13) |
|  | g. Tridecanoic acid (1.56) |
|  | h. Linoleic acid (0.98) |
|  | i. Nonadecanoic acid (0.36) |
|  | b + c + d + e + f + g + h + i |
|  | e + g + h |
| One flower equivalent surface | Synthetic compounds or blends |
| wax from Nirmal B-1 | comparable to one flower equivalent |
|  | surface wax of Nirmal B-1 (µg/ml) |
|  | a. Dodecane (1.15) |
|  | b. Tetradecane (1.60) |
|  | c. Pentadecane (2.81) |
|  | d. Heptacosane (0.20) |
|  | e. Nonacosane (0.93) |
|  | f. Triacontane (0.11) |
|  | g. Tridecanoic acid (2.10) |
|  | h. Linoleic acid (1.42) |
|  | i. Nonadecanoic acid (0.72) |
|  | a + b + c + d + e + f + g + h + i |
|  | b + c + e + g + h |

Table 5. Behavioral responses of adult viviparous *Aphis craccivora* females towards individual synthetic compounds comparable to the amounts as present in one flower equivalent surface wax of BIO L 212 Ratan or Nirmal B-1 vs. the control solvent (Petroleum ether) in Y-tube choice experiments. Behavioral responses of females in choice experiments were analyzed by a Chi-square test (H0: *P* = 50%). Each test was carried out with 60 adult viviparous females. N. R. means no response (insect remained in the common arm of the Y-tube olfactometer).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Comparison | | Insects responded | | *χ2* | *P* values |
| T1 | T2 | T1 | T2 | (*df* = 1) |  |
| Synthetic compounds comparable to one flower | Control solvent |  |  |  |  |
| equivalent surface wax of BIO L 212 Ratan (µg/ml) | |  |  |  |  |
| Dodecane (0.71) |  | N. R. | N. R. | - | - |
| Tetradecane (1.14) |  | 35 | 25 | 1.67 | 0.1967 |
| Pentadecane (2.37) |  | 35 | 25 | 1.67 | 0.1967 |
| Hexadecane (1.14) |  | N. R. | N. R. | - | - |
| Heptadecane (0.74) |  | N. R. | N. R. | - | - |
| Octadecane (1.34) |  | N. R. | N. R. | - | - |
| Eicosane (0.68) |  | N. R. | N. R. | - | - |
| Docosane (0.52) |  | N. R. | N. R. | - | - |
| Tricosane (0.24) |  | N. R. | N. R. | - | - |
| Tetracosane (0.24) |  | N. R. | N. R. | - | - |
| Pentacosane (0.12) |  | N. R. | N. R. | - | - |
| Hexacosane (0.11) |  | N. R. | N. R. | - | - |
| Heptacosane (0.25) |  | 34 | 26 | 1.07 | 0.3017 |
| Octacosane (0.13) |  | N. R. | N. R. | - | - |
| Nonacosane (1.08) |  | 38 | 22 | 4.27 | 0.0389 |
| Triacontane (0.13) |  | 35 | 25 | 1.67 | 0.1967 |
| Hentriacontane (1.02) |  | N. R. | N. R. | - | - |
| Dotriacontane (0.07) |  | N. R. | N. R. | - | - |
| Tritriacontane (0.18) |  | N. R. | N. R. | - | - |
| Tetratriacontane (*n*-C34) |  | N. R. | N. R. | - | - |
| Pentatriacontane (0.30) |  | N. R. | N. R. | - | - |
| Hexatriacontane (0.30) |  | N. R. | N. R. | - | - |
| Lauric acid (0.23) |  | N. R. | N. R. | - | - |
| Tridecanoic acid (1.56) |  | 38 | 22 | 4.27 | 0.0389 |
| Pentadecanoic acid (0.47) |  | N. R. | N. R. | - | - |
| Palmitoleic acid (0.83) |  | N. R. | N. R. | - | - |
| Palmitic acid (0.06) |  | N. R. | N. R. | - | - |
| Heptadecanoic acid (0.22) |  | N. R. | N. R. | - | - |
| Linolenic acid (0.18) |  | N. R. | N. R. | - | - |
| Linoleic acid (0.98) |  | 38 | 22 | 4.27 | 0.0389 |
| Oleic acid (0.07) |  | N. R. | N. R. | - | - |
| Stearic acid (0.02) |  | N. R. | N. R. | - | - |
| Nonadecanoic acid (0.36) |  | 32 | 28 | 0.27 | 0.6056 |
| Arachidic acid (0.37) |  | N. R. | N. R. | - | - |
| Synthetic compounds comparable to one flower | Control solvent |  |  |  |  |
| equivalent surface wax of Nirmal B-1 (µg/ml) |  |  |  |  |  |
| Dodecane (1.15) |  | 34 | 26 | 1.07 | 0.3017 |
| Tetradecane (1.60) |  | 38 | 22 | 4.27 | 0.0389 |
| Pentadecane (2.81) |  | 38 | 22 | 4.27 | 0.0389 |
| Hexadecane (1.61) |  | N.R. | N.R. | - | - |
| Heptadecane (1.19) |  | N.R. | N.R. | - | - |
| Octadecane (1.77) |  | N.R. | N.R. | - | - |
| Eicosane (0.60) |  | N.R. | N.R. | - | - |
| Docosane (0.50) |  | N.R. | N.R. | - | - |
| Tricosane (0.30) |  | N.R. | N.R. | - | - |
| Tetracosane (0.24) |  | N.R. | N.R. | - | - |
| Pentacosane (0.09) |  | N.R. | N.R. | - | - |
| Hexacosane (0.13) |  | N.R. | N.R. | - | - |
| Heptacosane (0.20) |  | 31 | 29 | 0.07 | 0.7963 |
| Octacosane (0.09) |  | N.R. | N.R. | - | - |
| Nonacosane (0.93) |  | 38 | 22 | 4.27 | 0.0389 |
| Triacontane (0.11) |  | 34 | 26 | 1.07 | 0.3017 |
| Hentriacontane (1.43) |  | N.R. | N.R. | - | - |
| Dotriacontane (0.08) |  | N.R. | N.R. | - | - |
| Tritriacontane (0.23) |  | N.R. | N.R. | - | - |
| Tetratriacontane (0.13) |  | N.R. | N.R. | - | - |
| Pentatriacontane (0.59) |  | N.R. | N.R. | - | - |
| Hexatriacontane (0.48) |  | N.R. | N.R. | - | - |
| Lauric acid (0.46) |  | N.R. | N.R. | - | - |
| Tridecanoic acid (2.10) |  | 40 | 20 | 6.67 | 0.0098 |
| Pentadecanoic acid (0.94) |  | N. R. | N. R. | - | - |
| Palmitoleic acid (1.40) |  | N. R. | N. R. | - | - |
| Palmitic acid (0.13) |  | N. R. | N. R. | - | - |
| Heptadecanoic acid (0.77) |  | N. R. | N. R. | - | - |
| Linolenic acid (0.34) |  | N. R. | N. R. | - | - |
| Linoleic acid (1.42) |  | 39 | 21 | 5.4 | 0.0201 |
| Oleic acid (0.11) |  | N.R. | N.R. | - | - |
| Stearic acid (0.06) |  | N.R. | N.R. | - | - |
| Nonadecanoic acid (0.72) |  | 35 | 25 | 1.67 | 0.1967 |
| Arachidic acid (0.73) |  | N. R. | N. R. | - | - |