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Effects of leaf epicuticular wax compounds from *Solena amplexicaulis* (Lam.) Gandhi on olfactory responses of a generalist insect herbivore

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ABSTRACT

The TLC, GC-MS and GC-FID analyses of surface waxes of young, mature and senescent leaves of *Solena amplexicaulis* plants revealed 19, 18 and 21 *n*-alkanes between *n*-C_{15} and *n*-C_{36}, respectively and 14 free fatty acids between C12:0 and C22:0 fatty acids. In glass Y-tube olfactometer bioassay under laboratory condition the *A. foveicollis* females were attracted to surface waxes of young, mature and senescent leaves from *S. amplexicaulis* plants at the minimum concentrations of 6, 6 and 8 µg/ml, respectively. The insect showed highest attraction to 10 µg/ml epicuticular waxes from mature leaves than the same amount of epicuticular waxes from young and senescent leaves. The insect was attracted to individual synthetic pentadecane, heptacosane, nonacosane, palmitic acid, stearic acid and alpha-linolenic acid at 0.60, 0.50, 0.80, 4, 3 and 0.30 µg/ml, respectively in a dose response bioassay. The insect displayed highest attraction to a synthetic mixture of 0.93, 0.26, 3.09, 1.52 and 0.29 µg/ml of pentadecane, nonacosane, palmitic acid, stearic acid and alpha-linolenic acid, respectively and hence, this combination might be used for insect pest management programme such as baited traps.

Key words: *Aulacophora foveicollis*, alkanes and fatty acids, Chrysomelidae, Coleoptera, creeping cucumber, insect, herbivore, leaves, *Solena amplexicaulis*, Y-tube olfactometer bioassay.

INTRODUCTION

Solena amplexicaulis (Lam.) Gandhi (syn: *Melothria amplexicaulis*), commonly known as creeping cucumber (Cucurbitaceae family) are widely distributed in India, Srilanka, China and Taiwan (35). Its young leaves and fruits are consumed as vegetable in developing countries (34). The whole plant is a potential source of antioxidants and help in diabetes treatment including some other pharmacological activities such as antibacterial, hepatotoxicity, anti-inflammatory, etc (21,22,23,35,54). Its tubers, leaves and seeds are used extensively in traditional medina system to cure various ailments [hepatosplenomegaly, spermatorrhoea, thermogenic, appetizer, cardio tonic, diuretics and haemorrhoids (19, 22)].

Aulacophora foveicollis Lucas (Coleoptera: Chrysomelidae) is an important generalist herbivore pest on pumpkin, bottle gourd, sponge gourd, *Momordica cochin-*

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chinensis, etc in India, Bangladesh and Vietnam (24,31,32,37,49). Larvae of *A. foveicollis* pass through four instars on young and healthy roots of this plant to complete larval development within 12-13 days and adults voraciously consume leaves for 8-9 weeks and finally the plant turns brown (49). Presence of the insect in large numbers reduces the crop yields. A tachinid fly *Medinodexia morgani* (Hardy), mite *Histiostoma* sp. and reduviid bug *Rhynocoris fuscipes* (Fabricius) are recorded as natural enemies of this insect pest (9,56), but biocontrol programme using these natural enemies are not yet successful to control outbreaks of this insect pest. Hence it is controlled by chemical insecticide (Carbofuran, Diazinon-60EC) (37,50), which impacts the non-target organisms and human health. Such concerns result in development of alternate control strategies such as baited traps, which might be included into integrated pest management (IPM) schemes for this pest.

The epicuticular wax consists of alkanes, fatty acids, alkyl esters, primary and secondary alcohols, etc (2,48). They make the leaf surface waxy and shiny and the chemicals in the epicuticular wax layer act as low volatile cues in finding their host in its microhabitat (2,14,38,41,42,43,44). Alkanes and free fatty acids, major components of epicuticular wax, play important role in plant-insect interactions studies (5,26,33,41,42,43,44,48). Long-chain *n*-alkanes from labellum extracts of an orchid flower, *Ophrys sphegodes* Miller, showed attraction of the solitary bee, *Andrena nigroaenea* (Kirby) (47). A synthetic blend of palmitic, stearic, oleic, linoleic and α-linolenic acids mimicking the proportions as present in mature bitter gourd (*Momordica charantia* L.) leaves attracted the *Epilachna dodecastigma* (Wied.) (Coleoptera: Coccinellidae) in olfactometric bioassay (44). Further, individual synthetic lauric, palmitic, stearic and oleic acids and their blend like clover root extract attracted female clover root borer, *Hylastinus obscurus* Marsham (Coleoptera: Curculionidae) in olfactometric bioassays (30). However, to date no studies have established whether alkanes and free fatty acids present in the epicuticular waxes of *S. amplexicaulis* leaves can provide clues for attraction of *A. foveicollis*. If the alkanes and free fatty acids present in young, mature and senescent leaves are used by the insect in host finding, these compounds may contribute to sustainable pest management strategies such as baited traps. Further, the amount and composition of alkanes and free fatty acids in leaf surface waxes of plants vary among species and with the different stages of leaf development (2,20,43,44). To address this, the *n*-alkane and free fatty acid profile present throughout the different ages of *S. amplexicaulis* leaves were identified and quantified and whether these differences in alkane and free fatty acid concentrations can act as olfactory cues to attract *A. foveicollis* was studied through Y-shaped glass tube olfactometer bioassay under laboratory conditions. We also studied the role of individual synthetic compounds and mixtures of synthetic alkanes and fatty acids mimicking cuticular waxes of young, mature and senescent leaves as an olfactory cue for *A. foveicollis*.

MATERIALS AND METHODS

I. Insects

A. foveicollis insects were collected by light trap from bottle gourd [*Lagenaria siceraria* (Molina) Standl.] plants growing in our Crop Research Farm, University of

Burdwan (23°16′ N, 87°54′ E, an elevation of 31 m above sea level), West Bengal, India and separately maintained in 1 L glass jars, containing bottle gourd leaves covered with fine-mesh nylon nets at 27±1°C temperature, 65±10% relative humidity and 12 L: 12 D photoperiod in a 'BOD' incubator (ADS instruments and Tech., Calcutta, India). To maintain natural condition of leaves, a moist piece of cotton was placed around cut ends of bottle gourd leaves followed by wrapping with aluminium foil to prevent moisture loss and fresh leaves were given daily by replacing the previous ones.

II. Plant materials

S. amplexicaulis seeds were germinated on filter paper and each seed with cotyledon was planted in pots containing ~ 1500 cm^3 of soil [organic matter 5.3%, pH 7.7] collected from our Research Farm and grown in natural conditions during June-September, 2014 under a photoperiod of 13L:11D at 30-35°C. Plants were provided with water on alternate days. *S. amplexicaulis* leaves [young (1≤ week old), mature (2-4 weeks old) and senescent (5-7 weeks old)] were collected from the fields of our Farm in August 2014. Different ages of leaves were classified mainly with the developmental time following leaf emergence through continuous monitoring of plants in the field and size [i.e., young leaf: length = 3.7 ± 0.3 cm and breadth = 3.6 ± 0.3 cm, mature leaf: length = 8.16 ± 0.5 cm and breadth = 8.3 ± 0.4 cm and senescent leaf: length = 7.3 ± 0.4 cm and breadth = 7.2 ± 0.3 cm (mean ± standard error; 10-replicates of one leaf per each age)], colour (i.e., young leaf: light green, mature leaf: dark green and senescent leaf: yellowish green) and texture of the leaves were also considered during collection of different ages of leaves. Three hundred g fresh leaves of each type were harvested from 50 plants. Leaves were initially rinsed with distilled water and paper toweled for drying.

III. SEM study of leaf

The leaf surface of each type of leaf sample (young, mature and senescent) was mounted on aluminium holders (stabs) and coated with gold-palladium (2 nm thickness) using Hitachi made Scanning Electron Microscope (Model: S 530 with IB 2 ion cotter, Japan) to observe the surface pattern resulting from superficial hydrocarbon depositions.

IV. Extraction of leaf surface wax alkanes and fatty acids

Three hundred g fresh leaves of each type were collected thrice [i.e., young (number of leaves : 953 ± 9), mature (number of leaves: 400 ± 10) and senescent (number of leaves : 700 ± 26) (mean ± standard error; three replicate of one leaf sample of each age)] and were separately dipped in 4L *n*-hexane for 5 min at room temperature for extraction of surface waxes from the leaves, which yielded a straw coloured extract without trace of chlorophyll (4,41,43). A total of nine crude extracts were collected from three types of leaves. Each total crude extract was then passed through Whatman (Maidstone, UK) No. 41 filter paper and the solvent was removed under reduced pressure. Each crude extract was divided into three equal portions (equivalent to 100 g leaves), which were used for (i) olfactory bioassay, (ii) identification and quantification of alkanes and (iii) identification and quantification of free fatty acids, respectively.

Identification and quantification of alkanes

Extract was passed through a column of aluminium oxide (Alcoa, Frankfurt, Germany: F-20 grade) and eluted with petroleum ether. 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*-C_{15} and *n*-C_{36}. 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. Total nine purified alkane samples were produced for gas chromatography-mass spectrometry (GC-MS) and GC-FID for identification and quantification, respectively. First portion of each sample was used for identification by GC-MS and the second portion 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 an HP-5 column. 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 (4, 31, 43). 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*-C_{15} to *n*-C_{36}). 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 type of leaf (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 an HP-5 column (Agilent, USA; length: 30 m × 0.25 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 1:10. The peaks were identified by comparison of their retention times with those of standard *n*-alkanes from *n*-C_{15} through *n*-C_{36} and the areas of each peak were converted into quantities of *n*-alkanes based on internal standard tricosane (*n*-C_{23}) [preliminary study of three types of leaf extracts by GC-MS study did not indicate presence of *n*-C_{23}, so *n*-C_{23} was used as internal standard]. All *n*-alkanes (>99% purity) between *n*-C_{15} and *n*-C_{36} were purchased from Sigma Aldrich.

Identification and quantification of free fatty acids

Extract was mixed with diethyl ether and filtered through Whatman No. 41 filter paper. The extract was purified by TLC on silica gel G layers (thickness 0.5 mm), 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 (32,42). 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 BF_3-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 twice through 50 g anhydrous Na_2SO_4. First 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 repeated thrice separately, followed by esterification and a total of nine samples were prepared.

First portion of the esterified fatty acids was analyzed with an Agilent 6890 GC coupled to a 5973 Mass Selective Detector with an HP-5 column. 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 (40, 42, 44). Helium was the carrier gas. The MS temperature parameter was 180°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 decanoate (C10:0), 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), methyl tricosanoate (C23:0) and methyl tetracosanoate (C24:0)]. All standard esterified fatty acids (fatty acid methyl esters) were purchased from Sigma-Aldrich, Germany.

Second 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 an HP-5 column (Agilent, USA; length: 30 m × 0.25 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 (40, 43). The volume of the sample injected was 1 micro liter with a split ratio of 1: 10. The peaks were identified by comparison of their retention times with those of standard esterified fatty acids. The percentage composition of 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) [preliminary study of three types of leaf extracts by GC-MS study did not indicate presence of methyl tricosanoate, so methyl tricosanoate was used as internal standard). All solvents used were of analytical grade and purchased from E. Merck (Mumbai, India).

V. Olfactometer bioassays

A. foveicollis females of different ages were provided with water and starved for 10 h prior to use in olfactory bioassays. Age is not considered during olfactory bioassays as the adult females consume leaves of *S. amplexicaulis* plant voraciously for 8-9 weeks until death (49). The behaviour of 90 females or males to 4, 6, 8 and 10 µg/ml surface waxes of mature leaves against a control solvent was observed for 2 min in preliminary assays and it was observed that olfactory responses of males or females to leaf surface waxes odor were the same. Females were used in bioassays because they are guided by

olfactory cues for both adult feeding and location of suitable larval hosts. The behavioural responses of adult female *A. foveicollis* were investigated in a Y-shaped glass tube olfactometer (7 cm stem and arms long, 0.6 cm radius, 45° Y angle). The stem of the olfactometer was connected to a porous glass vial (1 cm radius × 3 cm long) in which test insects were released. Each arm of the olfactometer was connected to a glass-made micro kit adapter fitted into a (1 cm radius × 3 cm long) glass vial. One glass vial contained a piece (2 × 2 cm^2) of Whatman No. 41 filter paper moistened with 1 ml of volatiles, 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 300 ml min^{-1}. All the connections between different parts of the set-up consisted of silicon tubing.

The effectiveness of volatiles as attractant was evaluated in the following manner in the laboratory at 27±1ºC, 70±3 % relative humidity (RH) and light intensity 150 lux. One adult female *A. foveicollis* was introduced into the porous glass vial which was then attached with the stem of the olfactometer and exposed to a particular odor, consisting of 1 ml of the control solvent (0.5 ml of petroleum ether was first applied on the filter paper followed by evaporation at open space under laboratory condition for 30 sec and again 0.5 ml of petroleum ether was again applied on the same filter paper) in one glass vial and 1 ml of one of the different odors (leaf surface waxes, individual synthetic alkanes and fatty acids or blend of synthetic alkanes and fatty acids compounds: 0.5 ml of one sample was first applied on the filter paper followed by evaporation at open space under laboratory condition for 30 sec and again 0.5 ml was again applied on the same filter paper) in another glass vial. This insect was not attracted by the control solvent (petroleum ether) in preliminary assays. The behavior of each female was observed for 3 min such as wandering in the Y-tube. The olfactory response of the insect was recorded as one of the three categories choosing between petroleum ether or the treatment volatiles or 'nonresponding' (individuals remained in the common arm of the Y-tube by the end of the observation period) (1, 28, 29). Each experiment with one volatile sample was conducted until a total of 90 female insects had responded. After testing 5 insects, the olfactometer set-up was cleaned with petroleum ether followed by acetone and the olfactometer was dried in a hot-air oven before further use. Further, the position of the two arms was systematically changed after testing 5 insects in order to avoid positional bias.

Dual choice bioassays with female *A. foveicollis*

1) *Epicuticular waxes from different aged leaves tested against solvent control*

Four different concentrations (4, 6, 8 and 10 µg/ml) of surface waxes were prepared for olfactory bioassays. The insect did not show any responses below 4 µg/ml concentration, whereas the insect showed highest attraction ($P < 0.0001$) at 10 µg/ml concentration of waxes from mature leaves. Responses of female *A. foveicollis* to different concentrations of epicuticular waxes (4, 6, 8 and 10 µg/ml) collected from three types of leaves (A: young, B: mature, C: senescent), respectively, were tested against control solvent (petroleum ether).

2) *Epicuticular waxes from mature leaves tested against young or senescent leaves and young leaves against senescent leaves*

The responses of female *A. foveicollis* to 10 µg/ml epicuticular waxes were tested in the following combinations: 1) mature vs. young; 2) mature vs. senescent; 3) young vs. senescent to find out more attractive leaf.

3) *Individual synthetic compounds or synthetic blends of volatile compounds (equivalent to the proportions of epicuticular compounds of three types of leaves) tested against solvent control*

Individual synthetic compounds (alkanes and fatty acids) present in 10 µg surface waxes of each type of leaf were dissolved in l ml petroleum ether and was tested against 1 ml control solvent to find response of the insect (Table 1a and 1b). The standard synthetic fatty acids [i.e., lauric (C12:0), tridecanoic (C13:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), palmitoleic (C16:1), heptadecanoic (C17:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), alpha-linolenic (C18:3), nonadecanoic (C19:0), arachidic acid (C20:0), heneicosanoic acid (C21:0) and docosanoic acid (C22:0)] and *n*-alkanes between *n*-C_{15} and *n*-C_{36}, that were identified in natural leaves, were all purchased from Sigma Aldrich, Germany.

The individual synthetic compounds (the amount present in 10 µg epicuticular waxes of each type of leaf) that produced response to the insect were combined mimicking each type of leaf and combinations of synthetic compounds were also assayed against solvent control [young: 0.85 µg pentadecane + 3.45 µg palmitic acid + 0.27 µg alpha-linolenic acid were dissolved in 1 ml petroleum ether (equivalent to 10 µg epicuticular waxes) or mature: 0.93 µg pentadecane + 0.26 µg nonacosane + 3.09 µg palmitic acid + 1.52 µg stearic acid + 0.29 µg alpha-linolenic acid were dissolved in 1 ml petroleum ether (equivalent to 10 µg epicuticular waxes) or senescent: 0.37 µg pentadecane + 0.37 µg heptacosane + 0.59 µg nonacosane + 0.24 µg alpha-linolenic acid were dissolved in 1 ml petroleum ether (equivalent to 10 µg epicuticular waxes).

4) *Epicuticular waxes from most attractive leaves (10 µg/ml epicuticular waxes from mature leaves) vs. individual synthetic compounds or synthetic blend of compounds*

Responses of female *A. foveicollis* to 10 µg/ml waxes extracted from mature leaves were compared with individual compounds or combination of five synthetic compounds (equivalent to the proportions of 10 µg epicuticular waxes of mature leaves).

5) *Dose response of synthetic volatile compounds against control solvent*

Six individual compounds that displayed responses to the insect were also tested at different doses (pentadecane : 0.30, 0.60 and 1.20 µg were separately dissolved in 1 ml petroleum ether, respectively; heptacosane: 0.25, 0.50 and 1 µg were separately dissolved in 1 ml petroleum ether, respectively; and nonacosane: 0.20, 0.40, 0.80 and 1.60 µg were separately dissolved in 1 ml petroleum ether, respectively; palmitic acid: 2, 4 and 8 µg were separately dissolved in 1 ml petroleum ether, respectively; stearic acid: 1.5, 3 and 6 µg were separately dissolved in 1 ml petroleum ether, respectively; alpha-linolenic acid: 0.15, 0.30 and 0.60 µg were separately dissolved in 1 ml petroleum ether, respectively).

Statistical Analyses

The data on total amounts of individual alknaes and fatty acids from three types

Table 1a. Individual synthetic alkane compounds present in 10 µg surface waxes of three types of leaves used for olfactory bioassay

Alkane	Young	Mature	Senescent
	Amount (µg)		
Pentadecane (*n*-C_{15})	0.85	0.93	0.37
Hexadecane (*n*-C_{16})	0.51	0.70	0.10
Heptadecane (*n*-C_{17})	1.57	1.50	1.47
Octadecane (*n*-C_{18})	1.19	1.08	0.91
Nonadecane (*n*-C_{19})	0.12	-	0.10
Eicosane (*n*-C_{20})	1.46	1.42	1.42
Heneicosane (*n*-C_{21})	-	-	0.10
Docosane (*n*-C_{22})	1.30	1.10	1.14
Tetracosane (*n*-C_{24})	0.89	0.84	0.81
Pentacosane (*n*-C_{25})	0.03	0.02	0.03
Hexacosane (*n*-C_{26})	0.55	0.50	0.54
Heptacosane (*n*-C_{27})	0.04	0.20	0.37
Octacosane (*n*-C_{28})	0.35	0.34	0.39
Nonacosane (*n*-C_{29})	0.08	0.26	0.59
Triacontane (*n*-C_{30})	0.20	0.20	0.26
Hentriacontane (*n*-C_{31})	0.09	0.17	0.40
Dotriacontane (*n*-C_{32})	0.12	0.11	0.14
Tritriacontane (*n*-C_{33})	0.05	0.03	0.06
Tetratriacontane (n-C_{34})	0.06	0.05	0.06
Pentatriacontane (n-C_{35})	0.03	-	0.01
Hexatriacontane (*n*-C_{36})	-	0.03	0.03

Table 1b. Individual synthetic fatty acid compounds present in 10 µg surface waxes of three types of leaves used for olfactory bioassay

Fatty acid	Young	Mature	Senescent
	Amount (µg)		
Lauric acid (C12:0)	0.20	0.30	0.35
Tridecanoic acid (C13:0)	0.37	0.28	1.03
Myristic acid (C14:0)	0.18	0.26	0.56
Pentadecanoic acid (C15:0)	0.65	0.49	1.89
Palmitic acid (C16:0)	3.45	3.09	1.43
Palmitoleic acid (C16:1)	0.36	0.19	-
Heptadecanoic acid (C17:0)	0.84	1.10	1.33
Stearic acid (C18:0)	0.50	1.52	0.04
Oleic acid (C18:1)	-	0.27	0.15
Linoleic acid (C18:2)	0.33	0.43	0.18
Alpha-linolenic acid (C18:3)	0.27	0.29	0.24
Nonadecanoic acid (C19:0)	0.65	-	0.75
Arachidic acid (C20:0)	0.53	0.34	0.43
Heneicosanoic acid (C21:0)	0.91	0.74	1.08
Docosanoic acid (C22:0)	0.76	0.69	0.54

of *S. amplexicaulis* leaves were subjected to Levene's test for homogeneity of variance with respect to treatments. Following this, one-way 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 responses of *A. foveicollis* to volatiles were analyzed by a Chi-square test (28, 45). Insects that did not respond by selection either arm of the olfactometer were excluded from the analyses (28, 45).

RESULTS AND DISCUSSION

SEM study

The surface of young leaf showed immurations of variable thickness forming reticulum of variable mesh size and undulating lamellations forming inconspicuous areolae and secretary trichomes (Figure 1a). In mature leaf surface, conspicuous annulus (rings) with numerous rays of variable thickness, areolae with thick borders and granular depositions were observed (Figure 1b). The surface of senescent leaf indicated conspicuous immurations cohering characteristically to form annulus with radiating rays and thin foggy coating, areolae of different shapes, granules of different shapes and surface pattern with punctae becoming more conspicuous (Figure 1c).

The first physical contact between an insect and plant occurs on the leaf surface. Therefore, the epicuticular wax layer with spines, setae and trichomes are the first line of defence against insect herbivores (16, 17, 51). In the present study, some structural traits such as radiating rays, trichomes and incorporation of granular materials were observed throughout the developmental stages of *S. amplexicaulis* plant leaves surface, indicating that all these structures play an important role in plant defence against insect herbivory.

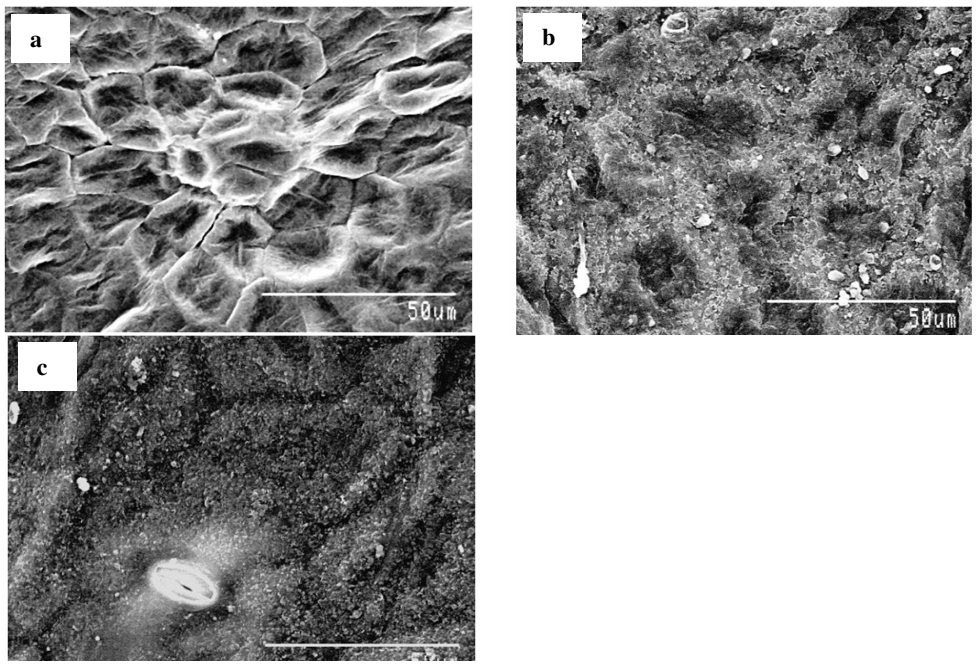

Figure 1. Scanning electron micrographs of (a) young, (b) mature and (c) senescent *S. amplexicaulis* leaf documenting cuticular wax layer from adaxial surface.

Alkanes in different ages of leaves

The *n*-hexane extracts of 100 g of the young, mature and senescent leaves represented 7.57 ± 0.38, 12.25 ± 0.18 and 9.90 ± 0.30 mg alkanes, respectively. The identified hydrocarbons of the young, mature and senescent leaves represented 7.18 ± 0.38, 11.61 ± 0.30 and 9.24 ± 0.27 mg *n*-alkanes, with the balance consisting of unidentified branched-chain alkanes, respectively (Table 2). Nineteen, 18 and 21 *n*-alkanes were identified between *n*-C_{15} and *n*-C_{36} alkanes in young, mature and senescent leaves, respectively. Heptadecane (*n*-C_{17}) predominated in young, mature and senescent leaves, accounting for 1188.46 ± 71.59, 1842.84 ± 69.09 and 1453.34 ± 31.16 µg, respectively. Pentacosane (*n*-C_{25}) was detected in the lowest amount in young (19.26 ± 0.11 µg) and mature (23.25 ± 1.45 µg) leaves; whereas pentatriacontane (*n*-C_{35}) was at the lowest level in senescent leaves (12.39 ± 1.33 µg), which was absent in young and mature leaves. Eicosane (*n*-C_{20}) was the second most abundant alkane in young, mature and senescent leaves, accounting for 1101.15 ± 28.37, 1736.14 ± 58.21 and 1408.59 ± 30.63 µg, respectively. Heneicosane (*n*-C_{21}) was absent in young and mature leaves; whereas nonadecane (*n*-C_{19}) was absent in mature leaves only. Rest of the alkanes displayed different patterns in three types of leaves.

Table 2. Amount of alkanes (µg/100 g leaf) in different ages of *S. amplexicaulis* leaves

Alkane	Amount (µg) (Mean ± SE, N =3)			$F_{2, 6}$
	Young	Mature	Senescent	
Pentadecane (*n*-C_{15})	645.03 ± 29.63^{a}	1135.19 ± 27.57^{b}	367.36 ± 16.77^{c}	236.23
Hexadecane (*n*-C_{16})	385.02 ± 14.58^{a}	853.23 ± 6.75^{b}	103.04 ± 1.55^{c}	1795.72
Heptadecane (*n*-C_{17})	1188.46 ± 71.59^{a}	1842.84 ± 69.09^{b}	1453.34 ± 31.16^{c}	63.93
Octadecane (*n*-C_{18})	901.24 ± 65.23^{a}	1327.43 ± 24.64^{b}	896.59 ± 32.31^{a}	31.09
Nonadecane (*n*-C_{19})	93.84 ± 2.88	-	102.82 ± 6.48	
Eicosane (*n*-C_{20})	1101.15 ± 28.37^{a}	1736.14 ± 58.21^{b}	1408.59 ± 30.63^{c}	23.73
Heneicosane (*n*-C_{21})	-	-	103.72 ± 7.72	
Docosane (*n*-C_{22})	980.94 ± 19.23^{a}	1351.68 ± 22.63^{b}	1132.64 ± 31.21^{c}	56.12
Tetracosane (*n*-C_{24})	672.24 ± 41.60^{a}	1030.28 ± 13.61^{b}	802.42 ± 16.44^{ab}	45.07
Pentacosane (*n*-C_{25})	19.26 ± 0.11^{a}	23.25 ± 1.45^{a}	31.84 ± 1.71^{b}	20.09
Hexacosane (*n*-C_{26})	418.06 ± 22.44^{a}	606.59 ± 16.99^{b}	537.18 ± 14.51^{c}	27.20
Heptacosane (*n*-C_{27})	31.06 ± 2.66^{a}	248.02 ± 2.35^{b}	367.18 ± 12.54^{c}	512.67
Octacosane (*n*-C_{28})	265.31 ± 15.43^{a}	411.43 ± 5.81^{b}	386.20 ± 15.56^{b}	35.61
Nonacosane (*n*-C_{29})	61.77 ± 4.27^{a}	316.53 ± 13.03^{b}	588.85 ± 17.96^{c}	408.24
Triacontane (*n*-C_{30})	151.92 ± 10.78^{a}	250.33 ± 5.34^{b}	257.42 ± 12.05^{b}	35.97
Hentriacontane (*n*-C_{31})	66.35 ± 2.51^{a}	208.66 ± 5.01^{b}	399.51 ± 4.37^{c}	1658.58
Dotriacontane (*n*-C_{32})	92.12 ± 6.30^{a}	130.01 ± 2.85^{b}	137.21 ± 3.70^{b}	28.63
Tritriacontane (*n*-C_{33})	34.64 ± 3.03^{a}	38.36 ± 12.49^{a}	63.60 ± 2.12^{b}	21.89
Tetratriacontane (n-C_{34})	46.76 ± 1.41^{a}	61.43 ± 2.84^{b}	62.69 ± 4.71^{b}	7.28
Pentatriacontane (n-C_{35})	-	-	12.39 ± 1.33	
Hexatriacontane (*n*-C_{36})	25.30 ± 0.92^{a}	35.60 ± 1.44^{bc}	29.95 ± 1.61^{ac}	14.40
Total	7180.49 ± 380.84^{a}	11606.98 ± 296.18^{b}	9244.54 ± 268.84^{c}	77.64

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

Free fatty acids in different ages of leaves

The *n*-hexane extracts of 100 g of the young, mature and senescent leaves represented 3.73 ± 0.15, 5.32 ± 0.14 and 3.93 ± 0.15 mg fatty acids, respectively. Table 3 shows 14 free fatty acids between C12:0 and C22:0 fatty acids in the surface waxes of young, mature and senescent *S. amplexicaulis* leaves, respectively. Palmitic acid (C16:0) was predominant fatty acid in young and mature leaves, accounting for 1287.78 ± 40.54 and 1643.09 ± 24.99 µg, respectively; whereas pentadecanoic acid (C15:0) was predominant in senescent leaves (744.95 ± 10.35 µg). Myristic acid (C12:0), palmitoleic acid (C16:1) and stearic acid (C18:0) were the least abundant fatty acids, accounting for 67.36 ± 1.49, 99.50 ± 3.94 and 16.0 ± 0.66 µg in young, mature and senescent leaves, respectively. Heneicosanoic acid (C21:0), stearic acid and palmitic acid were the second most abundant fatty acid representing 341.29 ± 17.55, 810.57 ± 23.71 and 563.93 ± 31.90 µg in young, mature and senescent leaves, respectively. Oleic acid (C18:1), nonadecanoic acid (C19:0) and palmitoleic acid were absent in young, mature and senescent leaves, respectively.

Table 3. Amount of free fatty acids (µg/100 g leaf) in different ages of *S. amplexicaulis* leaves

Fatty acid	Amount (µg) (Mean ± SE, $N = 3$)			$F_{2, 6}$
	Young	Mature	Senescent	
Lauric acid (C12:0)	73.17 ± 4.91[a]	162.06 ± 1.62[b]	136.65 ± 8.41[c]	64.50
Tridecanoic acid (C13:0)	137.77 ± 16.98[a]	147.06 ± 2.12[a]	404.55 ± 20.29[b]	97.91
Myristic acid (C14:0)	67.36 ± 1.49[a]	139.89 ± 2.99[b]	221.13 ± 5.99[c]	377.46
Pentadecanoic acid (C15:0)	242.56 ± 5.52[a]	262.04 ± 8.85[a]	744.95 ± 10.35[b]	1125.46
Palmitic acid (C16:0)	1287.78 ± 40.54[a]	1643.09 ± 24.99[b]	563.93 ± 31.90[c]	276.14
Palmitoleic acid (C16:1)	133.43 ± 9.14	99.50 ± 3.94	-	
Heptadecanoic acid (C17:0)	315.36 ± 10.91[a]	582.49 ± 14.61[b]	522.53 ± 10.37[b]	133.92
Stearic acid (C18:0)	186.25 ± 30.54[a]	810.57 ± 23.71[b]	16.0 ± 0.66[c]	351.09
Oleic acid (C18:1)	-	144.59 ± 7.56	60.75 ± 10.06	
Linoleic acid (C18:2)	124.55 ± 3.08[a]	230.57 ± 11.65[b]	70.29 ± 5.47[c]	113.82
Alpha-linolenic acid (C18:3)	98.96 ± 4.56[a]	154.03 ± 14.88[b]	95.08 ± 12.01[a]	8.44
Nonadecanoic acid (C19:0)	241.97 ± 2.13	-	294.53 ± 19.35	
Arachidic acid (C20:0)	199.58 ± 1.58[a]	180.04 ± 1.51[b]	168.67 ± 2.68[b]	66.54
Heneicosanoic acid (C21:0)	341.29 ± 7.55[a]	393.10 ± 5.62[b]	423.60 ± 7.62[b]	13.06
Docosanoic acid (C22:0)	283.28 ± 21.57[a]	367.54 ± 7.32[b]	210.67 ± 12.55[c]	27.33
Total	3733.33 ± 180.36[a]	5316.67 ± 179.88[b]	3933.33 ± 208.03[a]	34.76

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

Dual choice bioassays with female *A. foveicollis*

1) *Epicuticular waxes from different aged leaves tested against solvent control*

The insect displayed attraction to young leaf surface waxes significantly at 6 ($\chi^2 = 4.44$, $df = 1$, $P = 0.03502$), 8 ($\chi^2 = 6.4$, $df = 1$, $P = 0.01141$) and 10 ($\chi^2 = 11.38$, $df = 1$, $P = 0.00074$) µg/ml concentrations, but no clear positive or negative responses were observed at 4 µg/ml ($\chi^2 = 0.71$, $df = 1$, $P = 0.39908$) concentration (Figure 2a). The insect responded positively to mature leaf surface waxes at 6 ($\chi^2 = 6.4$, $df = 1$, $P = 0.01141$), 8 ($\chi^2 = 12.84$, $df = 1$, $P = 0.00034$) and 10 ($\chi^2 = 25.6$, $df = 1$, $P < 0.0001$) µg/ml concentrations, but no clear

positive or negative responses were observed at 4 µg/ml (χ^2 = 2.84 *df* =1; *P* = 0.09169) concentration (Figure 2b). Epicuticular waxes from senescent leaf were attractive to the insect at 8 (χ^2 = 4.44, *df* =1, *P* = 0.03502) and 10 (χ^2 = 6.4, *df* =1, *P* = 0.01141) µg/ml concentrations, but the insect did not indicate any clear positive or negative responses at 4 (χ^2 = 0.4, *df* =1, *P* = 0.52709) and 6 (χ^2 = 1.6; *df* =1, *P* = 0.20590) µg/ml concentrations (Figure 2c).

2) *Epicuticular waxes from mature leaves tested against young or senescent leaves and young leaves against senescent leaves*

Female *A. foveicollis* displayed attraction to 10 µg/ml epicuticular waxes from mature *S. amplexicaulis* leaves (χ^2 = 5.38, *df* =1, *P* = 0.02037) against 10 µg/ml epicuticular waxes from young leaves, whereas the insect showed attraction to 10 µg/ml epicuticular waxes from mature *S. amplexicaulis* leaves (χ^2 = 7.51, *df* =1, *P* = 0.00613) against 10 µg/ml epicuticular waxes from senescent leaves (Figure 3). But females did not indicate any positive or negative reactions to 10 µg/ml epicuticular waxes from young *S. amplexicaulis* leaves (χ^2 = 0.18, *df* =1, *P* = 0.67327) against 10 µg/ml epicuticular waxes from senescent *S. amplexicaulis* leaves. The results indicated that 10 µg/ml epicuticular wax compounds from mature leaves caused higher attraction of *A. foveicollis* than the same amount of epicuticular waxes either from young or senescent leaves.

3) *Individual synthetic compounds or synthetic blends of volatile compounds (equivalent to the proportions of epicuticular compounds of three types of leaves) tested against solvent control*

A. foveicollis produced responses to three components (pentadecane, palmitic acid and alpha-linolenic acid) of young leaves, five components (pentadecane, nonacosane, palmitic acid, stearic acid and alpha-linolenic acid) of mature leaves and four components (pentadecane, heptacosane, nonacosane and alpha-linolenic acid) of senescent leaves present in the proportions of 10 µg epicuticular waxes of *S. amplexicaulis* leaves; whereas rest of the identified compounds present in the proportions of 10 µg epicuticular waxes did not provoke any response to the test insect.

The insect indicated clear positive reactions to pentadecane (*n*-C_{15}) (χ^2 = 7.51, *df* = 1, *P* = 0.00613) and alpha-linolenic acid (C18:3) (χ^2 = 4.44, *df* = 1, *P* = 0.03502) at the proportions equivalent to 10 µg epicuticular waxes of young leaves against solvent control, respectively; whereas the insect did not indicate clear positive and negative responses to palmitic acid (χ^2 = 2.84, *df* = 1, *P* = 0.09169) against solvent control (Figure 4a). The insects were attracted to a synthetic blend of three synthetic components equivalent to the proportions of three components present in 10 µg epicuticular waxes from young leaves against solvent control (χ^2 = 10, *df* =1, *P* = 0.00157) (Figure 4a).

The insect displayed attraction to pentadecane (χ^2 = 10, *df* = 1, *P* = 0.00157) and alpha-linolenlic acid (χ^2 = 4.44, *df* = 1, *P* = 0.03502) at the proportion equivalent to 10 µg epicuticular waxes from mature leaves against solvent control; whereas the insect did not indicate clear positive and negative reactions to nonacosane (*n*-C_{29}) (χ^2 = 1.11, *df* = 1, *P* = 0.29184), palmitic acid (χ^2 = 1.6, *df* = 1, *P* = 0.20590) and stearic acid (χ^2 = 0.71, *df* = 1, *P* = 0.39908) against solvent control (Figure 4b). The insects were attracted to a synthetic

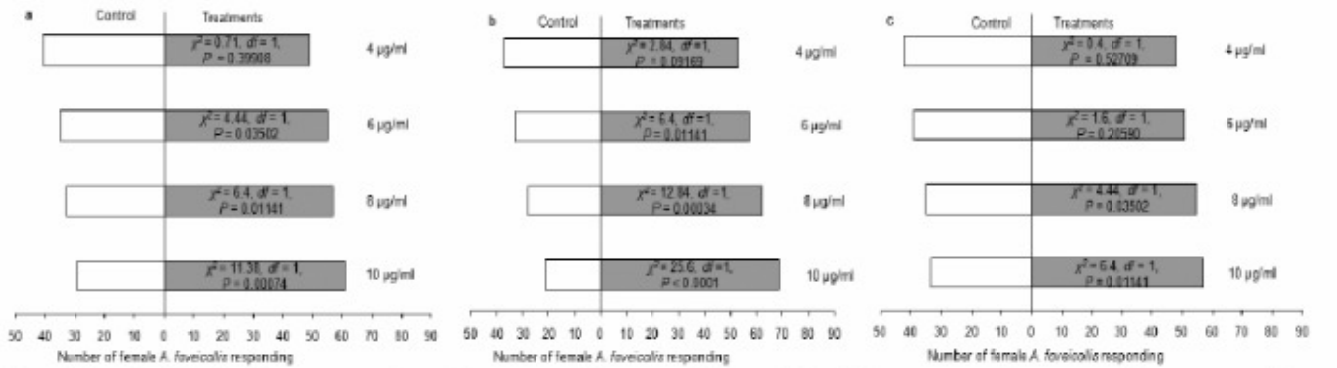

Figure 2. Female *A. foveicollis* responses to the surface waxes of three types of *S. amplexicaulis* leaves: young (a) or mature (b) or senescent (c) vs. solvent (petroleum ether) control in Y-tube olfactometer bioassay.

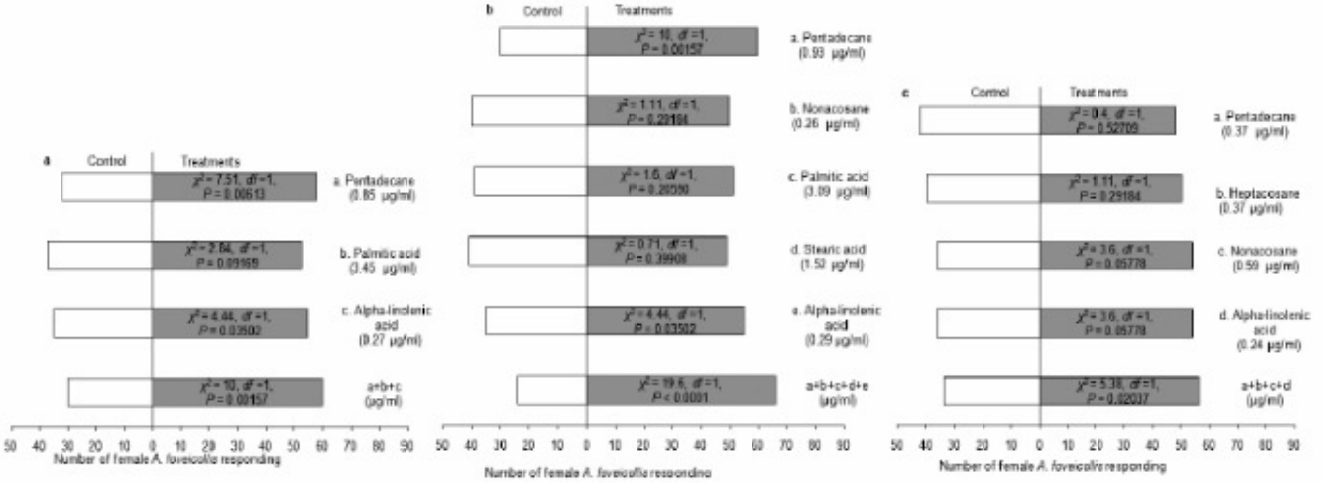

Figure 4. Female *A. foveicollis* responses to individual synthetic compounds and their combinations equivalent to the proportions of 10 µg epicuticular waxes: young (a) or mature (b) or senescent (c) vs. solvent (petroleum ether) control in Y-tube olfactometer bioassay

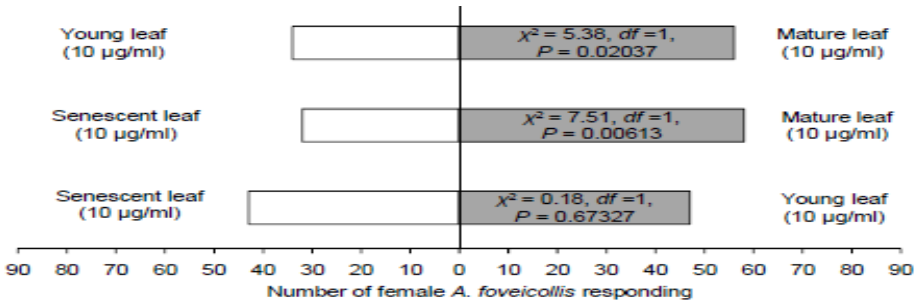

Figure 3. Female *A. foveicollis* responses among the surface waxes of three types of *S. amplexicaulis* leaves.

blend of five volatile components equivalent to the proportions of five components present in 10 µg epicuticular waxes from mature leaves against solvent control (χ^2 = 19.6, *df* =1, *P* < 0.0001) (Figure 4b).

The insect did not indicate clear positive and negative responses to pentadecane (χ^2 = 0.4, *df* = 1, *P* = 0.52709), heptacosane (*n*-C_{27}) (χ^2 = 1.11, *df* = 1, *P* = 0.29184), nonacosane (χ^2 = 3.6, *df* = 1, *P* = 0.05778) and alpha-linolenic acid (χ^2 = 3.6, *df* = 1, *P* = 0.05778) against solvent control (Figure 4c). The insects were attracted to a synthetic blend of four synthetic components equivalent to the proportions of four components present in 10 µg epicuticular waxes from senescent leaves against solvent control (χ^2 = 5.38, *df* =1, *P* = 0.02037) (Figure 4c).

4) *Epicuticular waxes from most attractive leaves (10 µg/ml epicuticular waxes from mature leaves) vs. individual synthetic compounds or synthetic blend of compounds*

The insects were attracted to 10 µg/ml epicuticular waxes extracted from mature leaves against five individual synthetic compounds in the proportions detected in 10 µg mature leaf epicuticular waxes [epicuticular waxes (χ^2 = 5.38, *df* =1, *P* = 0.02037) vs. pentadecane, epicuticular waxes (χ^2 = 23.51, *df* =1, *P* < 0.0001) vs. nonacosane, epicuticular waxes (χ^2 = 17.78, *df* =1, *P* < 0.0001) vs. palmitic acid, epicuticular waxes (χ^2 = 21.51, *df* =1, *P* < 0.0001) vs. stearic acid, epicuticular waxes (χ^2 = 11.38, *df* =1, *P* = 0.00074) vs. alpha-linolenic acid]; whereas the insect did not indicate clear positive and negative responses to the epicuticular waxes from mature leaves (χ^2 = 0.18, *df* =1, *P* = 0.67327) against a combination of five synthetic compounds equivalent to the proportions present in 10 µg mature leaf surface waxes (Figure 5).

Bioassay 5: Dose-dependent responses to six synthetic compounds

In Y-tube olfactory bioassays, the insect displayed attraction to pentadecane, heptacosane, nonacosane, palmitic acid, stearic acid and alpha-linolenic acid against solvent control (Table 4). The insect showed attraction to pentadecane at 0.60 µg/ml (χ^2 = 4.44, *df* =1, *P* = 0.03502) and 1.20 µg/ml (χ^2 = 23.51, *df* =1, *P* < 0.0001) (Table 4). The insects were attracted to heptacosane at 0.50 µg/ml (χ^2 = 4.44, *df* =1, *P* = 0.03502) and 1 µg/ml (χ^2 = 19.6, *df* =1, *P* < 0.0001) (Table 4). The insect produced attraction to

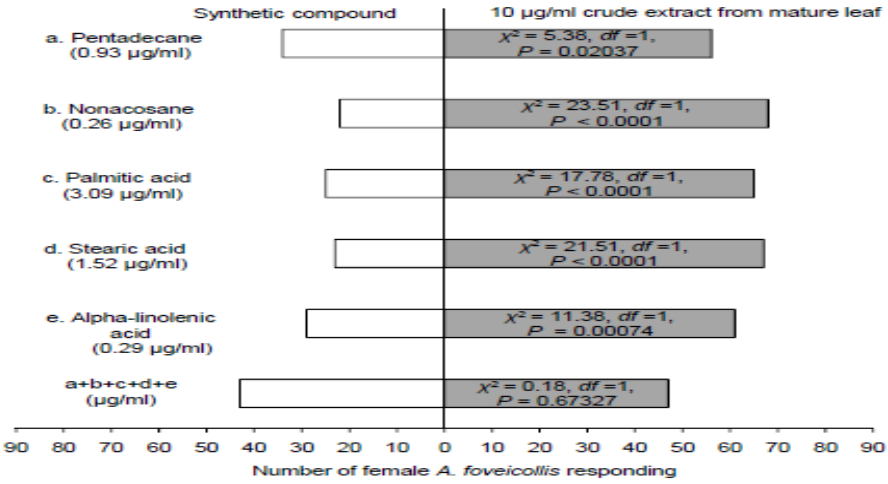

Figure 5. Female *A. foveicollis* response to 10 µg/ml epicuticular waxes extracted from mature *S. amplexicaulis* leaves vs. individual synthetic compound or a combination of synthetic compounds in the proportions present in mature *S. amplexicaulis* leaves.

Table 4. Female *A. foveicollis* responses to individual synthetic compound vs. solvent (Petroleum ether) control in Y-tube olfactometer bioassay (N = 90 in each concentration bioassay)

Synthetic compounds	Concentration (µg/ml)	$\chi 2$ (df = 1)	P values of insect responded
Pentadecane	0.30	0.18	0.67327
	0.60	4.44	0.03502
	1.20	23.51	< 0.0001
Heptacosane	0.25	0.04	0.83385
	0.50	4.44	0.03502
	1	19.6	< 0.0001
Nonacosane	0.20	0.71	0.39908
	0.40	2.18	0.14002
	0.80	7.51	0.00613
	1.60	19.6	< 0.0001
Palmitic acid	2	0.71	0.39908
	4	6.4	0.01141
	8	21.51	< 0.0001
Stearic acid	1.50	0.71	0.39908
	3	5.38	0.02037
	6	23.51	< 0.0001
Alpha-linolenic acid	0.15	0.04	0.83385
	0.30	6.4	0.01141
	0.60	17.78	< 0.0001

nonacosane at 0.80 µg/ml (χ^2 = 7.51, df =1, P = 0.00613) and 1.60 µg/ml (χ^2 = 19.6, df =1, P < 0.0001) (Table 4). Palmitic acid was attractive at 4 µg/ml (χ^2 = 6.4, df =1, P = 0.01141) and 8 µg/ml (χ^2 = 21.51, df =1, P < 0.0001) (Table 4). The insects were attracted to stearic acid at 3 µg/ml (χ^2 = 5.38, df =1, P = 0.02037) and 6 µg/ml (χ^2 = 23.51, df =1, P < 0.0001)

(Table 4). The insect displayed attraction to alpha-linolenic acid at 0.30 µg/ml (χ^2 = 6.4, *df* =1, *P* = 0.01141) and 0.60 µg/ml (χ^2 = 17.78, *df* =1, *P* < 0.0001) (Table 4).

The changes in rate of wax accumulation throughout leaf development stages result in variation of *n*-alkane and free fatty acid levels in three types of *S. amplexicaulis* leaves (3,12,20,53). The *n*-alkane and free fatty acid profile in the three types of *S. amplexicaulis* leaves underwent substantial variations and a decline in total *n*-alkane concentrations and free fatty acids was observed after maturity. A progressive decline in total *n*-alkane concentrations with increasing leaf age was recorded in *Pennisetum glaucum* (L.) R. Br. (25) and *Malus domestica* Borkh. leaves (18). Nineteen, 18 and 21 *n*-alkanes and 14 free acids were recorded from *n*-C_{15} to *n*-C_{36} and C12:0 to C22:0 fatty acids in young, mature and senescent *S. amplexicaulis* leaves, respectively. *n*-Alkanes with chain lengths from *n*-C_{15} to *n*-C_{36} and *n*-C_{16} to *n*-C_{33} were present in leaf epicuticular waxes of *Fallopia japonica* (Hout.) R. Decr. (26) and *Momordica charantia* L. (43), respectively. Nonacosane (*n*-C_{29}) and hentriacontane (*n*-C_{31}) were the predominant *n*-alkanes in the epicuticular wax of mature leaves of *F. japonica* (26) and *M. charantia* L. (43), respectively. However in the present investigation, *n*-C_{17} was the predominant alkane in all three types of *S. amplexicaulis* leaves. Saturated fatty acids were higher in the *Abies pindrow* (8) and *M. cochinchinensis* (32) plants belonging to the Pineceae and Cucurbitaceae family, respectively. Palmitic acid was the major fatty acid in plants (10,27,46) and this study also indicated higher amount of palmitic acid in young and mature leaves, but pentadecanoic acid was the major fatty acid in senescent leaves.

Alkanes and free fatty acids, common constituents of plant leaf epicuticular waxes (2,20), serve an important role in bitrophic herbivore-plant interactions: as attractant (13,31,39,41,42,43,44,48) or as attractant for oviposition (14,15,26). Long-chain alkanes and fatty acids, which are low-volatile substances that might act as close range allelochemicals after arrival of the insect to the plant. Long-chain fatty acids such as palmitic, oleic and linoleic acids alone or blended with crude almond oil showed highest attraction of navel orangeworm, *Amyelois transitella* Walker (Lepidoptera: Pyralidae) in wind tunnel bioassays to black sticky traps compared to white sticky traps (57). Phelan et al. (36) demonstrated that oleic acid and linoleic acid showed orientation of the flight of gravid females of the navel orangeworm *A. transitella* toward the source of an odor in a wind tunnel. Further, a synthetic blend of lauric, myristic, pentadecanoic, palmitoleic, heptadecanoic, nonadecanoic and docosanoic acids mimicking the proportions as present in mature leaves of *Polygonum orientale* L. weed elicited attraction of *Galerucella placida* Baly (Coleoptera: Chrysomelidae) in Y-tube olfactometer bioassay (29). The olfactory responses of the test insect, *A. foveicollis* using the Y-tube olfactometer provide evidence for a directional movement towards the odor source by perceiving the difference between the odor-loaded air flow and control solvent-loaded air flow, although anemotactic responses cannot be excluded. Chemicals that act as repellants would serve to move away from the source (11), which provides a basis for oriented movement from the source (55). By this study, it was possible to observe the beetles at the Y-junction of the olfactometer where they had to decide to move towards the control solvent airflow and airflow loaded with odor. When, the odor was attractive, the beetle continued upwind movement toward the odor source. The olfactometric bioassay results clearly revealed that *A. foveicollis* could discriminate between 6-10 µg/ml epicuticular waxes extracted from young and mature leaves and 8-10 µg/ml from senescent leaves of *S. amplexicaulis* plants against

control solvent, and the insect preferentially respond to 10 µg/ml epicuticular waxes from mature leaves among the three types of leaves due to synergistic effect of pentadecane, nonacosane, palmitic acid, stearic acid and alpha-linolenic acid in the epicuticular waxes. The importance of alkanes and fatty acids as allelochemicals has been demonstrated in different insects (1,31,41, 43,44,47,52). Insects employ between the ranges of 3-10 compounds as host location cue (6). *A. foveicollis* showed attraction to pentadecane and alpha-linolenic acid at the amounts of 0.93 and 0.29 µg/ml in initial bioassays at the proportions detected in 10 µg mature leaf epicuticular waxes, respectively; whereas the insect did not produce any positive or negative responses to nonacosane, palmitic acid and stearic acid at 0.26, 3.09 and 1.52 µg/ml, respectively. Further, the insect did not indicate any positive and negative responses to heptacosane at 0.37 µg/ml present at 10 µg epicuticular waxes of senescent leaves. However, the insect showed attraction to heptacosane, nonacosane, palmitic acid and stearic acid at the minimal amounts of 0.50, 0.80, 4 and 3 µg/ml in dose response bioassays, respectively. Hence, the ratio of volatiles released by 10 µg waxes from mature leaves becomes vital components, which act as olfactory cue for *A. foveicollis* (6, 7). Visual cues from the *S. amplexicaulis* plants might also play a role in the attraction, but these cues were not identified in the olfactometer bioassay.

CONCLUSIONS

Our findings found that 10 µg/ml concentration of waxes from mature leaves were more attractive than young and senescent *S. amplexicaulis* leaves. *A. foveicollis* were most attracted to a synthetic blend of 0.93, 0.26, 3.09, 1.52 and 0.29 µg/ml of pentadecane, nonacosane, palmitic acid, stearic acid and alpha-linolenic acid, respectively, which might be used to develop much needed eco-friendly trapping tools for this insect pest. Bioassays in greenhouse to evaluate responses of *A. foveicollis* to the above combination of 5 synthetic compounds are necessary to authenticate the attractiveness tested in the present study.

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