Plant volatile organic compounds as chemical markers to identify resistance in sweet potato against weevil *Cylas formicarius*

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Headspace volatiles from intact plants, plants with flowers and storage roots of sweet potato were collected using dynamic headspace trapping system. Sweet potato weevils (SPWs), *Cylas formicarius* (F.), were bioassayed against these volatiles using dual-choice olfactometer. The volatile organic compounds (VOCs) produced by leaves attracted more number of male weevils, and those by flowers and storage roots were found to attract more female weevils. The flower and storage root VOCs elicited significant electrophysiological responses in female SPWs. Among the genotypes tested, 'Howrah', 'SB IB 400/22' and 'BX 86' were least preferred by SPWs, whereas 'Kishan' was found most preferred. Gas chromatography–mass spectrometry analyses revealed differences in expression of volatile compounds in genotypes resistant and susceptible to *C. formicarius*. A compound, 2-(2-butyl-cyclopropyl)-cyclopropanenonanoic acid methyl ester eluted from flower headspace volatiles of 'Howrah' genotype emitted as 9,12-(*Z*,*Z*)-octadecadienoic acid from the storage root periderm. 9,12-(*Z*,*Z*)-octadecadienoic acid is a precursor for production of several short-chain aromatic compounds through lipoxygenase (LOX) pathway, and is believed to operate in the storage roots. These compounds playing a role in SPW resistance in 'Howrah' and 'BX 86' genotypes, were absent in SPW susceptible-genotype 'Kishan'. We propose these esters, i.e. cyclopropane fatty acid esters as a 'diagnostic chemical marker' to identify sweet potato weevil resistance in genotypes of sweet potato.

Keywords: *Cylas formicarius*, host plant resistance, sweet potato, volatile organic compounds.

SWEET potato weevil (SPW) *Cylas formicarius* (F.) is the single most devastating pest of sweet potato (*Ipomoea batatas* (L.) Lam.) worldwide. Management of insect pests through integrated pest management (IPM) relies mainly on the use of host plant resistance (HPR) coupled with behaviour-modifying semiochemicals as they are ecosystem-friendly. Development of insect-resistant cultivars has been suggested as an essential component in IPM of SPW[1].

Finding a high-yielding and pest-resistant genotype is crucial for development of any variety. The general methods of screening of germplasm or a large group of plants against insect resistance are normally done in two ways. The first being destructive sampling, in which crop plants at frequent intervals will be uprooted from the field and inspected for insect infestation as in the case of tuber crops. This method is not always reliable, as in certain geographic locations, the pest is not found and researchers often categorize the plant as resistant. But when the same plant declared pest resistant, if tested in the hot spot areas of the pest, it would become susceptible to insect attack. Under these circumstances, such declaration of resistance in a particular genotype will be misleading. This method cannot be used to find the 'absolute resistance' (AR) factor in plants to pest attack, irrespective of the occurrence of the pest in an area. In addition to these drawbacks, it is not possible for adopting destructive analysis when there are few plants available for screening, as in the case of plant quarantine laboratories.

The second method is through molecular marker-assisted selection (MAS) which can tag only resistant source, but it will not reveal the mechanism governing resistance. DNA-based molecular markers have been developed for a variety of traits in many crops. But this method is time-consuming and expensive, as hundreds of markers are required to screen plants for insect resistance. Moreover, the DNA-based technique, i.e. random amplified polymorphic DNA has the disadvantage of lack of reproducibility over time[2,3]. Attempts to develop HPR have been moderately successful due in part to deficiencies in parent and progeny selection methods[4]. Conventional breeding for resistance also suffers from a serious limitation; reproductive barriers between species prevent

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the introduction of resistant genes into a crop from any plant, except closely related wild relatives[5].

The recent approach, i.e. 'volatile assisted selection'[6] has been used for identification of *C. formicarius* resistance in sweet potato through differential volatile emissions from the sweet potato plants and storage roots. Sweet potato produces different types of volatile organic compounds (VOCs). Differences in volatile chemistry among sweet potato clones may relate to differences in susceptibility or resistance to SPW[4]. Host-plant chemistry can potentially modify any stage in the behaviour of the weevil from host finding (volatiles) through feeding and oviposition (surface chemicals) to larval development in the roots (internal root chemistry)[4]. Any one or a mixture of these VOCs can be used to change the behaviour of SPW. Both, a single plant attractant[7] and a mixture of compounds with synergistic effects[8] have been tested as lures for traps to monitor or manage certain insects. Starr *et al*.[9] reported that adult male and female *C. formicarius* were not equally attracted to root and leaf volatiles. Females were strongly attracted to both leaf and root volatiles, whereas males were attracted only to leaf volatiles[9]. Such variation is indicative of existence of qualitative and quantitative differences in production of VOCs by different parts of sweet potato. Resistance in sweet potato to weevil is mainly through the production of sesquiterpenes which differently express in leaves and storage roots as repellents or deterrents[4]. This information is useful to understand further the insect behaviour and to help in using such VOCs for categorizing HPR. It can further be used to deter physically SPW in the crop. These studies are useful in identification of insect resistance and its operational mechanism, identification of attractant/repellent compounds, identification of genes responsible for production of a particular VOC and production of sweet potato that does not produce a specific VOC. To test these hypotheses, we have studied the electrophysiological responses of SPW to headspace volatiles from different genotypes of sweet potato and identified VOCs governing resistance and proposed a biosynthetic pathway to understand more clearly the plant resistance mechanism.

Materials and methods

Plants

Nine sweet potato genotypes, viz. Howrah, BX 86, SB IB 400/22, 90/235, SB 72/7, BP-2, 90/693, S-643 and Kishan were planted in the farm of the Regional Centre, Central Tuber Crops Research Institute (CTCRI), Bhubaneswar, Odisha during 2009–2011. Weevil infestation in the field on these genotypes was recorded at 90 and 150 days after planting (DAP). Destructive sampling was done for the estimation of weevil infestation in vines and storage roots. In each genotype, 10 plants were randomly selected and the entire plant was dug along with storage roots. The vines (stems) and storage roots were cut open for counting grubs and adults. Analysis of variance (ANOVA) and *F*-test were used to test the level of significance ($P = 0.05$) between genotypes.

Dynamic headspace volatile collection

The resistant lines (Howrah and BX 86) and the susceptible check (Kishan and Kalinga) were selected for headspace volatile collection and further electrophysiological analyses. One sweet potato plant per genotype was planted in a plastic container (15 × 7 cm) filled with autoclaved sand and frequent watering was done. A volatile collection chamber (VCU) was used for collection of headspace volatiles. The plastic container was wrapped with aluminium foil to prevent plastic-related and soil-related volatiles. The whole plant was kept inside a 2.5 litre glass jar. Air (100 ml/min) was pumped into the jar through activated charcoal. Headspace volatiles from the sweet potato plant in the glass jar were collected onto adsorption tubes (Gerstel TDS 2/A; outside diameter 6.0 mm) packed with 100 mg (80/100 mesh) Tenax$^{(R)}$ (Alltech Associates, Inc., Deerfield, USA). Both sides of the tube were fitted with steel mesh grids to prevent the adsorbent falling apart. Headspace volatiles were trapped for 2 h and stopped for half an hour and again collected for another 2 h. The pumping was stopped to prevent accumulation of moisture inside the VCU and to prevent excess heat generated in the motor of the vacuum pump used for pumping air. Each time a new adsorption tube was used for collection of head space volatiles. The adsorbed volatiles were eluted immediately with 20 ml *n*-hexane. The adsorption tubes were cleaned with 5 ml of acetone (two times) and dried at 240°C for 30 min. Then the adsorption tubes were wrapped with aluminium foil again on both the ends to prevent outside air from entering into the tubes. The cleaned tubes were re-used for volatile collection. The storage roots @ 500 g per genotype were harvested, cleaned with tap water, dried for one day in shade and kept inside a glass jar for two days for volatile collection.

Gas-chromatography coupled electroantennogram detection

Antennal responses of male and female SPWs to the collected extracts and headspace volatiles from sweet potato plants, flowers and storage roots were analysed by gas-chromatography coupled electroantennographic detector (GC–EAD). The GC is of Agilent GC 7890A series, Singapore, Capillary S/Sl inlet with EPC, equipped with a cold on-column injector. The GC is also equipped with an Alltech 30 m AT-5 (5% phenyl, 95% polydimethylsiloxane) fused silica column, 0.25 mm ID and 0.5 μm

film thickness. Conditions were: carrier gas, helium (constant flow 2.5 ml/min); temperature programming: 80°C (0.8 min hold) to 280°C (10 min hold) at 10°C/min; detector temperature: 250°C; temperature of the transfer line (Syntech Laboratories, Germany) between the GC and EAD followed the oven temperature. The injected sample (1.0 µl) was equally split between a flame ionization detector (FID) and the EAD. The EAD plus peripheral equipment were manufactured by Syntech®, Germany. Weevils used for this study were kept in a glass vial with a loosely tightened cap and placed in a refrigerator for 30 sec to make them immobile, in order to cut the antenna later. One-fourth of the distal segment of the antenna was cut with a razor-steel blade. Antenna was mounted between two glass electrodes filled with Ringer solution (6.4 mM KCl, 12 mM $MgCl_2 \cdot 6H_2O$, 9.6 mM KOH, 12 mM NaCl, 20 mM KH_2PO_4, 1 mM $CaCl_2$ and 354 mM glucose in deionized water). Over the antenna, a flow of purified and humidified air was maintained at a flow rate of 80 cm/s. To distinguish EAD responses from noise, 5–6 GC–EAD runs were carried out with each sample. Each GC–EAD run was followed by two consecutive cleaning runs (5 µl *n*-hexane injected). Honey (10%), 10 µl, was applied on a piece of filter paper strip (5 cm length and 4 mm width), and after drying or being completely absorbed by the filter paper, it was inserted into a Pasteur pipette fitted with an air bulb. This Pasteur pipette was inserted into a small hole embedded in the glass tube (that allows the gaseous extract comes from GC) and puffed once, soon after the hexane is eluted from the sample, to test the longevity of the antenna. Puffing was repeated again at the end of the temperature programme to ascertain the longevity of the antenna. GC–EAD is an advanced method of electroantennography (EAG) extensively used along with other detector systems in chemical ecology for the detection of volatiles that bind the antennal olfactory receptors. EAG is only indicative of the presence of electrophysiologically active compounds, whereas GC–EAD indicates the compounds that are responsible for exciting olfactory receptors in the antenna. However, both the methods are based on recording of small voltage fluctuations between the tip and base of an insect antenna during stimulation with volatile compounds. Voltage fluctuation is caused by electrical depolarization of many olfactory neurons in the antenna of the insects. The amplitude of an antenna response increases with increasing stimulus concentration, until a saturation level is reached. The amplitude is further dependent on the nature of the stimulus, insect species, its sex and several unknown factors[10].

Gas chromatography–mass spectrometry

Hexane extracts and headspace volatile extracts analyses were carried out on a Hewlett Packard 5973 mass selective detector (70 eV) coupled to a Hewlett Packard 6890 gas chromatograph equipped with a 10 : 1 split injector. The gas chromatograph was equipped with an HP5 MS 30 m fused silica capillary column having 0.25 mm ID and 0.25 µm film thickness run in constant flow mode (1.0 ml/min helium). Oven temperature programming: 60°C (1 min hold) to 100°C at 5°C/min rate (1 min hold), then to 220°C at 10°C/min rate (5 min hold) and then to 240°C at 50°C/min rate (8 min hold). Injector temperature was set at 275°C. One microlitre of the extract or concentrated headspace volatiles was injected manually into the gas chromatography–mass spectrometry (GC–MS) system for analysis. Injections were done in split 10 : 1 mode. MSD Productivity Chem-Station software was used for the analysis of compounds in the extracts and headspace volatiles. The compounds of interest were identified using standard NBS75K mass spectral library.

Behavioural assays

A dual choice olfactometer[11] was used to compare responses of SPW to leaf, flower and storage root volatiles collected from both the resistant and susceptible genotypes. The olfactometer was modified in our study, in which plastic containers were used instead of glass chambers and air was pumped into the chambers using small aquarium pumps. The dual-choice olfactometer consists of three chambers: main chamber, treatment chamber and control chamber. The latter two chambers lay one on each side of the main chamber, that was kept 30 cm above the two chambers. Two holes were made on the lid of the side chambers (treatment and control) and fitted with Tygon® tubings (6.4 mm ID, 9.6 OD and 15 cm length). One tube was connected to the aquarium pump and another to the main chamber. For tests of leaf and flower volatiles, five numbers of either leaves or flowers were kept in the treatment chamber. For testing root volatiles, a single washed and shade-dried storage root weighing 50 g was kept in the treatment chamber. Then 30 weevils (starved for 2 h; male or female) were released into the main chamber and air was pumped into the treatment chambers for 2 h. After 2 h, the number of weevils in the treatment chamber and control chamber was counted. Weevils found in the tubes that connected to the side chambers were regarded as having made a choice and were counted along with those in their respective side chambers. The experiment was repeated four times. The data were analysed with two-sample (two-tailed) Student's *t*-test ($P < 0.01$) to assess whether there is significant difference between the response of male and female SPWs to volatiles from leaves, flowers and storage roots of a genotype. ANOVA was also performed to know the differential response of male or female SPWs to different volatile extracts; and the means were compared with Duncan's Multiple Range Test (DMRT).

Results and discussion

Search for plant resistance in crop plants to insect pests is an important consideration for the breeders to develop high-yielding and pest-resistant varieties by adopting various conventional and biotechnological methods. As indicated earlier, molecular MAS of crop plants for pest resistance has its own drawbacks, in the sense that the molecular markers do not explain the resistance mechanism in the plant. Recently, Korada *et al.*[6] reported that HPR is governed by differential volatile emissions from the sweet potato plant. They named the process of screening or selecting sweet potato genotypes for *C. formicarius* resistance based on volatile compounds as volatile-assisted selection (VAS). Further, in the present study we have found some of the volatile compounds released by sweet potato, which can be used as 'chemical markers' to identify resistance in sweet potato to SPW.

Of the several genotypes evaluated for SPW infestation in the field (Table 1), Howrah, BX 86 and SB IB 400/22 recorded significantly lower percentage of storage root infestation ($P < 0.01$) than the remaining varieties at 90 and 150 DAP, indicating that these genotypes are highly resistant to SPW even when the storage roots are exposed to the pest for prolonged periods. The control genotype Kishan had recorded the highest SPW infestation ($P < 0.01$). The normal harvesting time for sweet potato is 90–100 DAP and after harvest, heavy populations of SPW is present in the field. With this high weevil population load, the three genotypes listed above have shown very low infestation of weevils, suggesting that these genotypes contain antifeedants or repellents against *C. formicarius.*

Headspace volatiles collected from SPW-resistant genotypes were bioassayed to evaluate the potential of the extracts to attract male and female SPWs (Table 2). The three genotypes tested generally attracted significantly lesser number of SPWs than the control genotype Kishan. In general, the data indicated that flower and storage root volatiles attracted more number of female weevils than the male weevils. In case of sweet potato leaf volatiles, the males were found to be attracted more to the three SPW-resistant genotypes than females, whereas the volatiles from Kishan attracted more number of female weevils than males (t stat $6df = 15.49$, $P < 0.01$). Male SPW did not show any significant preference to any of the volatiles from the flower (Table 2, fifth column) or storage root (Table 2, eighth column), irrespective of the resistance or susceptibility of the genotypes. Differences in infestation pattern and preference to volatile extracts from different plant parts may relate to variation in the susceptibility or resistance among genotypes. Wang and Kays[4], and Korada *et al.*[6] reported that the volatile extracts from storage roots were found to be more attractive to females than those from plant volatiles.

Table 1. Sweet potato weevil infestation in different sweet potato genotypes at 90 and 150 days after planting

Sweet potato genotype	Sweet potato weevil infested storage roots (%)	
	90 days	150 days
Howrah	0.87 (7.73)a	6.67 (14.91)b
SB IB 400/22	2.80 (11.19)bc	4.00 (11.43)a
84X1	4.73 (13.85)d	32.13 (34.51)g
BX 86	0.00 (5.74)a	8.20 (16.51)c
Baster 45	0.13 (6.11)a	12.47 (20.64)d
SB BPLR 48	1.73 (9.50)b	9.07 (17.44)c
SB 21/57	2.93 (11.43)c	30.53 (33.53)f
S-783	0.07 (5.94)a	17.80 (24.89)e
Kishan	57.50 (50.07)e	90.00 (73.13)h
F-test$_{8,3df}$	$P < 0.01$	$P < 0.01$

Figures in parentheses are arcsin transformed values. Values in the column not followed by the same letters are significantly different ($P < 0.01$) by Duncan's Multiple Range Test.

Electrophysiological studies indicated that the female SPWs showed a high depolarization of 0.2 mV to a compound emitted from Howrah flower extracts at 11.9 min, whereas the male antenna did not respond to the same extract with the same GC–EAD programme (Figure 1), thus providing evidence that female SPWs could detect specific compounds, even when they are present/emitted in very minute quantities. Olfactory response of the female SPW is important for development of behaviour modifying chemicals for its management, because female insects not only damage the storage root but also reproduce and survive for longer periods. GC–MS analyses of volatiles of Howrah, BX 86 and Kishan genotypes indicated presence of strong repellents present in the headspace volatile extracts collected from flowers and storage roots. A compound, 2-(2-butylcyclopropyl)-cyclopropanenonanoic acid methyl ester (a cyclopropane fatty acid ester) emitted at 33.63 min (± 0.01) from flowers of Howrah and BX 86 genotypes (Figure 2) got released from the periderm of the storage root as 9,12-(*Z*,*Z*)-octadecadienoic acid (C18:2) (linoleic acid) at 25.31 min (Figure 3). We propose the biosynthetic transformation of the cyclopropanes to C18:2 (Figure 4), since C18:2 is the precursor for production of several short-chain aromatic compounds through the lipoxygenase (LOX) pathway[12], presumed to be operated in the storage roots of the SPW-resistant genotypes, i.e. Howrah, BX 86, SB IB 400/22 and not in the SPW-susceptible genotype Kishan. When the storage roots become infested by SPW or by any mechanical impact, these resistant genotypes breakdown the C18:2 into C_6–C_8 compounds. These short chain compounds have enormous role in plant defence against herbivory[13,14]. The first *in vivo* evidence of C_6-aldehyde involved in plant defence was provided by Croft *et al.*[13]. These investigators noted that the inoculation of *Phaseolus vulgaris* L. leaves with an avirulent strain of *Pseudomonas syringae* pv. *phaseolicola* Burkholder resulted in a burst of hexenals and hexenols. However, a virulent, compatible strain of *P. syringae* pv. *phaseolicola* did not

Table 2. Behavioural response of sweet potato weevil *Cylas formicarius* (F.) to volatiles from leaves, flowers and storage roots in a dual-choice olfactometer

	Response of weevils (%) to leaf volatiles			Response of weevils (%) to flower volatiles			Response of weevils (%) to storage root volatiles		
Sweet potato genotype	Male	Female	*t* stat 6df[@]	Male	Female	*t* stat 6df[@]	Male	Female	*t* stat 6df[@]
Howrah	4.00a	2.00a	6.65**	6.77	8.75a	4.53**	4.00a	4.25a	4.68**
BX 86	5.66ab	2.67ab	7.97**	6.62	12.50b	3.21*	5.75a	4.50a	2.54*
SBIB 400/22	7.70c	4.14b	7.61**	5.95	13.50b	0.89[NS]	6.75a	12.82b	7.29**
Kishan	21.0d	35.0c	15.49**	7.05	61.25c	8.11**	7.50a	53.75c	4.62**
F-stat$_{3,3df}$[$]	33.4**	95.8**		0.3[NS]	79.8**		2.46[NS]	85.06**	

Values in the column not followed by the same letters are significantly different ($P < 0.05$) by Duncan's Multiple Range Test.
[$]*F*-test (3,3df) table value: Probablity, $P = 0.05 = 9.28$ denoted by *, $P = 0.01 = 29.50$ denoted by **.
[@]*t*-test (6df) table value for arcsin transformed values: Probability, $P = 0.05 = 2.45$ denoted by *, $P = 0.01 = 3.71$ denoted by **; NS, Non-significant.

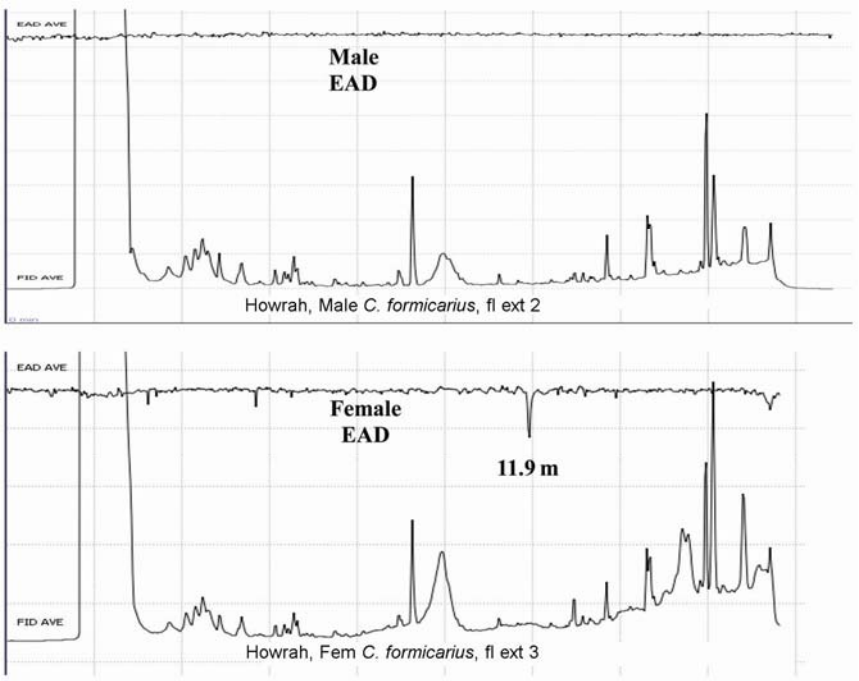

Figure 1. Gas chromatography–electroantennogram responses of male and female sweet potato weevils *Cylas formicarius* to hexane extracts of sweet potato genotype Howrah.

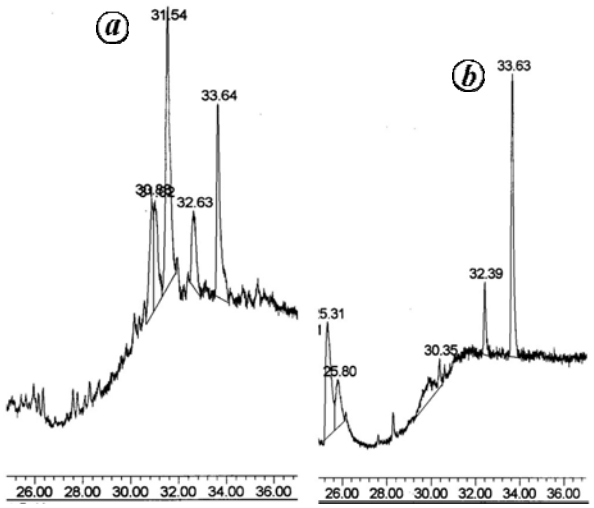

Figure 2. Gas chromatography–mass spectrometry analysis of sweet potato weevil-resistant genotype Howrah releasing 2-(2-butylcyclopropyl)-cyclopropanenonanoic acid methyl ester at 33.63 (± 0.01) min: (***a***) flower headspace volatiles and (***b***) periderm extracts.

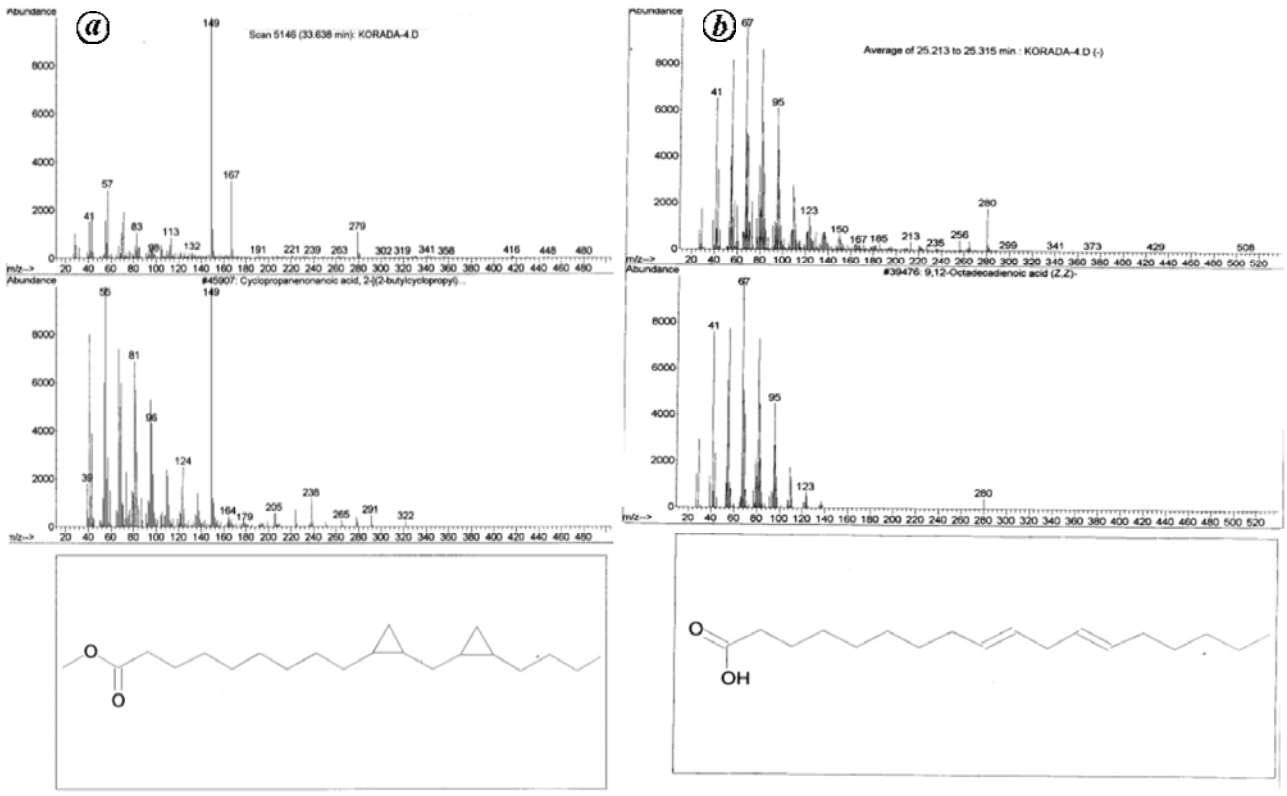

Figure 3. Gas chromatography–mass spectrometry analysis of sweet potato weevil resistant 'BX 86' genotype releasing 2-(2-butylcyclopropyl)-cyclopropanenonanoic acid methyl ester at 33.63 (± 0.01) min from flowers (***a***) and transformed into (*Z*,*Z*)-9,12-octadecadienoic acid in tuber periderm extract (***b***).

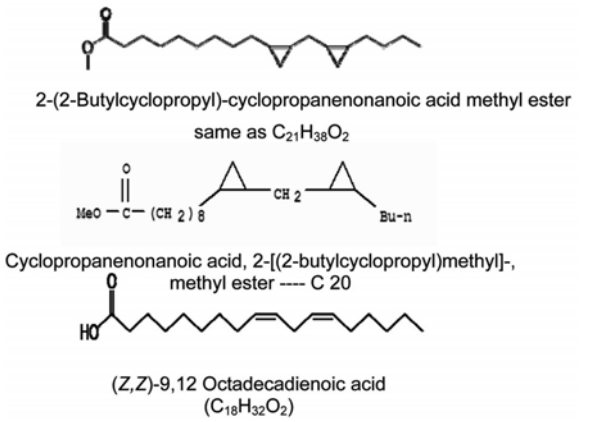

Figure 4. Biosynthetic transformations of 2-(2-butylcyclopropyl)-cyclopropanenonanoic acid methyl ester to 9,12-(*Z*,*Z*)-octadecadienoic acid (C18:2, linoleic acid).

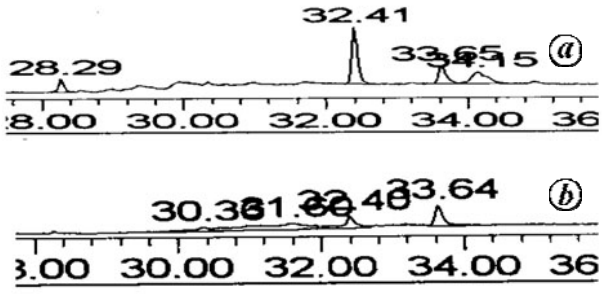

Figure 5. Gas chromatography–mass spectrometry analysis of flower headspace volatiles of sweet potato weevil susceptible genotype showing very less expression of 2-(2-butylcyclopropyl)-cyclopropanenonanoic acid methyl ester at 33.63 (± 0.01) min in (***a***) Kishan and (***b***) Kalinga.

elicit the burst. The burst occurred after 15 to 24 h, which corresponds to the time of the hypersensitive response of plant tissues to pest invasion. Hence, we assume in all probability that C18:2 is formed on the periderm of storage roots, and when these come in contact with SPW, the compounds break down into short-chain compounds that repel/deter or inhibit the feeding of SPW. At close distances, or once pest makes a contact with host, interactions between them are influenced by the detection of toxic/antifeedant compounds by the herbivore[15]. Considerable LOX activity is detected in various plant organs, such as seeds, stems, leaves, fruits, tuber and florets[16,17]. Cyclopropane fatty acids (CPA) interfere with maturation and reproduction of some insect species suggesting that in addition to their traditional role as storage lipids, they can also contribute to the protection of plants from herbivory[18].

The C_6 volatiles are formed from polyunsaturated octadecanoic fatty acids (*Z*,*Z*)-9,12-octadecadienoic acid or (*Z*,*Z*,*Z*) 9,12,15-octadecatrienoic acid (linolenic acid). The straight-chain fatty acids, especially C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 have broad repelling and deterring effects on oviposition[19]. The SPW-susceptible genotypes, var Kishan and var Kalinga expressed very minute quantities of 2-(2-butylcyclopropyl)-cyclopropanenonanoic acid methyl ester compared to the SPW-resistant genotypes (Figure 5). The relative concentration of these esters in the sweet potato storage roots influences the feeding by SPW. It was reported that the plants expressing the CPA ester synthase gene (*cfa* gene) produce CPAs leading to production of specialty crops[20]. These CPAs can be used as 'chemical markers' to screen sweet potato plants for resistance against *C. formicarius*. In order to develop SPW-resistant crops, further work is required to identify the whole set of plant volatiles and enzymes responsible for the production of these CPAs in sweet potato for better understanding of insect–plant interactions and for utilization of these interactions for better pest management.

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