Differential volatile emission from sweet potato plant: mechanism of resistance in sweet potato for weevil *Cylas formicarius* (Fab.)

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Sweet potato (*Ipomoea batatas* L.) plays a significant role as host plant, in the olfactory-directed behaviour of weevil *Cylas formicarius* (Fab.). *C. formicarius* infestation pattern of sweet potato genotypes, viz. Goutam, Sourin, Gouri and CIP-6 showed that CIP-6 was the most susceptible. Electroantennogram studies with *C. formicarius* revealed that the electrophysiological response of female antenna to the volatile extracts of aerial plant parts and roots was higher than the male antenna. In olfactometer studies, the headspace volatiles of genotype CIP-6 attracted more number of female *C. formicarius* weevils than volatiles of Gouri, Goutam and Sourin. Variation in the preference of sweet potato genotypes to *C. formicarius* is attributed to differential emission of volatiles from the aerial parts and roots. This mechanism is helpful in the identification of resistant sweet potato genotypes to pests based on the volatiles they release, and the process of selection of such genotypes can be called 'volatile assisted selection'.

Keywords: *Cylas formicarius*, electroantennogram, genotypes, headspace volatiles, sweet potato.

SWEET POTATO weevil (SPW) *Cylas formicarius* (Fab.) is a serious insect pest of sweet potato grown in Asia, Africa and Latin America. Efforts towards the development of a resistant sweet potato (*Ipomoea batatas* L.) plant to weevil *C. formicarius* Fab. have not been successful in recent times. Development of a weevil-resistant sweet potato plant through conventional breeding procedures involves a lot of time and space. Conventional breeding of resistance also suffers from a serious limitation: reproductive barriers between species prevent the introduction of resistance genes into a crop from any plant, except closely related wild relatives[1].

Identification of a potential source of resistance to *C. formicarius* in sweet potato or its wild relatives is of paramount importance for successful development of insect-resistant plants. Recently, molecular marker-assisted selection (MAS) has been a widely adapted practice to identify and tag resistance sources. MAS has been suggested as a major approach to supplement conventional plant breeding for augmenting yield and quality in all major crops. For this purpose, DNA-based molecular markers have been developed for a variety of traits through marker-trait association studies in all major crops. These procedures involve high costs and are also time-consuming, and sometimes the results are not repetitive. For example, random amplified polymorphic DNA (RAPD) provided a significant advance in molecular marker studies[2]. However, the RAPD technique has the disadvantage of lack of reproducibility over time[3,4].

Another approach for successful identification of resistance in germplasm resources is through use of plant volatiles they emitted in space, which help in segregating them into different categories of insect resistance. Biologically active volatile phytochemicals represent an important means of communication within plants, and between plants and other organisms in their environment. Utilization of host-plant volatiles as attractants, repellents and other behaviour-modifying chemicals for insect pests and their natural enemies is gaining momentum, since such use of volatiles does not pose any threat to the ecosystem, either directly or indirectly. Initial work on volatile phytochemicals emanating from sweet potato stemmed from the findings that SPW could readily orient in the dark to the sweet potato plant parts placed in its vicinity[5,6]. The females are attracted by the volatiles emanating from leaves and storage roots. In contrast, males are attracted by leaf volatiles, but not by those emitting from the storage roots[5]. This suggested that there are qualitative and/or quantitative differences in the volatile compounds between the aerial plant parts and the storage roots. In addition to within-plant differences, the degree of attraction is known to vary among cultivars[5] and species of *Ipomoea* L.[6].

Qualitative and quantitative differences in volatiles released from host plants throw light on the chemical cues used by the female SPW for decision-making processes. Thus this approach is multi-faceted and has potential applications in the identification of *C. formicarius* resistance source in the germplasm, identification of attractant and/or repellant volatile in plants to weevils, and development of a sweet potato plant devoid of a particular volatile that governs the reproductive behaviour of the weevil. To test this hypothesis, we studied the olfactory behaviour of *C. formicarius* to headspace volatiles collected from different sweet potato genotypes.

Sweet potato genotypes, viz. Gouri, Goutam, Sourin and CIP-6 were planted in the farm at the Regional Centre, Central Tuber Crops Research Institute, Bhubaneswar, Orissa, India during 2008–09. *C. formicarius* infestation in the field on these genotypes was recorded at 45 and 90 days after planting (DAP). Destructive sampling was done for the estimation of weevil damage. In

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each genotype, ten plants were selected at random and whole plants were dug out along with the roots (tubers). The stem and roots were cut open longitudinally to count the number of *C. formicarius* grubs and adults. Paired *t*-test was used to test the level of significance ($P = 0.05$) of weevil infestation at 45 days and 90 days in stems and roots in different genotypes.

The procedure described by Korada and Griepink[7] was used for collection of headspace volatiles from sweet potato. One sweet potato plant per genotype was planted in a plastic container (10 × 5 cm) filled with sand and frequent watering was done. The plant with the plastic container was kept in a 2.5 l glass jar. Air (100 ml/min) was passed into the jars through activated charcoal. The 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) filled with 100 mg (80/100 mesh) Tenax (Alltech Associates, Inc., Deerfield). Both sides of the tube were fitted with quartz wool (Interscience BV, Breda, The Netherlands). Headspace volatiles were collected at 24 h intervals for two consecutive days. Adsorption tubes were changed every day in the morning at 11.00 am and the absorbed volatiles were extracted immediately with 5 ml *n*-hexane. Extracts were pooled and concentrated in a rotary flash evaporator. The adsorption tubes were cleaned with 5 ml of acetone (two times) and dried at 240°C for 30 min. The cleaned tubes were reused for volatile collection. Similarly, root portion from one plant per genotype was cut and kept inside a glass jar for two days for volatile collection.

Electroantennography (EAG) is a bioassay widely used along with other detector systems in chemical ecology[7] for the detection of volatiles perceived by the antennal olfactory apparatus of insects. The method is based on the recording of small voltage fluctuations between the tip and base of an insect antenna during stimulation with pheromones[8]. Voltage fluctuation is caused by electrical depolarization of many olfactory neurons in the antenna of the insects. The amplitude of an EAG 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[8]. The weevils used for EAG analyses were kept in a glass vial with a loosely tightened cap and placed in a refrigerator for 30 s to make the weevils immobile. The glass vial was taken out and the antenna of the weevil (male or female) was cut with surgical scissors and mounted in an electrically conducive gel connected between the electrode probes. Next, 10 µl extract of the sample was applied to a piece of filter paper strip (5 cm length and 4 mm width). After allowing for solvent evaporation, the impregnated filter-paper strip was inserted into a glass Pasteur pipette. The control stimulus was a similar pipette containing a filter-paper strip impregnated with a 10 µl aliquot of hexane. The tip of the pipette was placed about 3 mm into a small hole in the wall of a glass tube (13 cm long, 8 mm diameter), oriented towards the antennal preparation (~0.5 cm away from the preparation). In this way, the stimuli were provided as 0.2 s puffs of air into a continuous humidified air stream at 1000 ml/min generated by an air stimulus controller (Syntech®, The Netherlands). Puffing of air or sample extract was carried out five times with the same antenna and was repeated five times. The amplitude (mV) of the antenna was measured with the EAG software (Syntech®, The Netherlands). Cis-3-hexene-1-ol was used to ascertain the longevity of the antenna during EAG analyses. Data from EAG were analysed using two-way analysis of variance (ANOVA), and Duncan's Multiple Range Test (DMRT) was conducted to compare EAG responses to different volatile extracts.

Ten-day-old adult weevils used for the behavioural study were not provided food (honey and water) for one day before the start of the bioassay. The behavioural assays were conducted in a customized Y-tube olfactometer made of glass (left arm 20 cm, right arm 20 cm, and central arm 20 cm in length and internal diameter in 8 mm). The procedures described in the literature[9–11] were modified for our experiments. The Y-tube was fabricated at a local glass-blowing unit and contains a steel wire (0.5 mm diameter) that runs through the tube, which facilitates walking of the weevils in the Y-tube. The olfactometer consisted of a release chamber (20 ml capacity) at one end and two test chambers (20 ml capacity). An aquarium air pump was used to generate an air current (20 ml/s). A silicon tube was connected to the pump to pass air through an air inlet chamber fitted with a charcoal filter into left arm of the Y-tube. The right arm of the tube was used as a control. A similar air pump was used to generate air current from the bottom of the central arm (insect release chamber). The release chamber and the test chambers were provided with removable glass lid. The movement of the test insect in the test chamber was restricted using a net. The net was provided in the test chamber in such a way that the released males were not prevented from entering into the test chamber. The experiment was carried out during 1600–1800 h in a chamber fitted with 5 W fluorescent bulb. A black cloth was laid below the Y-tube olfactometer to prevent the weevils from being disturbed with visual cues.

The volatile extract in hexane (10 µl) was impregnated on Whatman No. 1 filter paper and inserted into the test chamber of the Y-tube and at the other end a filter paper with hexane (control) was inserted. Fifty weevils of each sex were released one after the other from the release chamber (bottom arm). The initial choice of a weevil that responded by walking on the steel wire into one of the arms (choice chambers) and remained there at least 15 s, was recorded. If a weevil had not made a choice within 2 min of being released, it was removed and discarded. Weevils that did not walk into any of the arms were not counted. After 25 individual weevils had been tested, the

Table 1. Infestation of weevil *Cylas formicarius* in sweet potato genotypes

Genotype	Number of weevil grubs in stems of ten plants		Number of weevil grubs in roots of ten plants		Average number of weevil grubs
	45 days	90 days	45 days	90 days	
CIP-6	30	25	16	71	35.50
Gouri	20	11	0	19	12.50
Goutam	10	7	0	40	14.25
Sourin	18	24	0	25	16.75
Paired *t*-test	NS		Significant ($P < 0.05$)		

NS, Non-significant ($P > 0.05$).

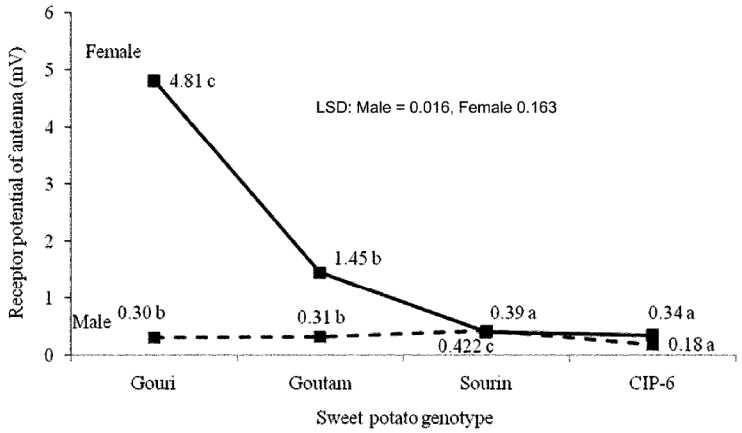

Figure 1. Electrophysiological response of *Cylas formicarius* antenna to root volatiles collected from different sweet potato genotypes. Values not followed by the same letters are significantly different (*F*-test, $P < 0.01$) by Duncan's Multiple Range Test (DMRT).

olfactometer arms were flipped around (180°) to minimize positional effect[11]. After 50 weevils had been bioassayed, they were discarded and replaced with a new set of weevils (either male or female). The olfactometer set-up was rinsed with soap water and hexane, and then air-dried whenever a new set of weevils was released. The experiment was repeated four times. The data were analysed using two-sample (two-tailed) Student's *t*-test ($P < 0.01$) to assess whether there is significant difference between male and female *C. formicarius* response to each volatile extract. ANOVA was also performed to know the differential response of male or female *C. formicarius* to different volatile extracts; and the means were compared with DMRT.

Stems and roots of four sweet potato genotypes, Gouri, Goutam, Sourin and CIP-6 showed different infestation levels at 45 and 90 DAP (Table 1). Significant difference ($P < 0.05$, $t_{3df} = 4.308$) in root infestation was recorded during both days of observation in four sweet potato genotypes. At 45 DAP, CIP-6 recorded the highest *C. formicarius* infestation of stem (30 weevil grubs per ten plants) followed by Gouri, Sourin and Goutam. The data indicated that more number of weevil grubs was found in stem than in the roots at 45 days. Later, when the roots were developed into tubers by 90 DAP, high weevil count was recorded in the tubers. At 45 days, coinciding with the tuber initiation stage, CIP-6 recorded 16 weevil grubs in roots of ten plants, whereas other varieties showed no degree of infestation of tuber. At the end of tuber formation, though all the genotypes showed severe infestation of tubers, CIP-6 recorded the highest infestation (71 grubs). On an average, Gouri recorded the lowest weevil infestation of 12.5 grubs, whereas CIP-6 recorded 35.5 grubs/ten plants, indicating that it was the most favourable genotype for the weevil throughout crop growth.

EAG studies with *C. formicarius* indicated that, in general, the electrophysiological response of female weevil antenna to the headspace volatiles collected from different genotypes was higher than the male antenna. Data indicated that except Gouri, the response of female antenna to the volatile extract collected from the roots was higher (Figure 1) compared to the volatile extract from plants (Figure 2; $P < 0.01$). Similarly, Wang and Kays[9] reported that the volatile extracts from storage roots were found to be more attractive than the foliage. In our studies, the female antenna showed the highest receptor potential (4.813 mV) (Figure 3) with Gouri root volatiles than its corresponding male antenna (0.305 mV). The results suggest that female antenna responds more efficiently and effectively to volatiles from different genotypes than the male antenna. This was clearly evident with the root headspace volatiles, wherein, the difference between CIP-6

Table 2. Behavioural response of male and female sweet potato weevil *Cylas formicarius* (Fab.) to volatiles collected from different genotypes in a Y-tube olfactometer

Sweet potato genotype	Attraction (%) of sweet potato weevil to plant volatiles			Attraction (%) of sweet potato weevil to root volatiles		
	Male weevil	Female weevil*	t_{6df} stat ($P < 0.01$)**	Male weevil	Female weevil*	t_{6df} stat ($P < 0.01$)**
Gouri	4.0	23.0 b	12.1***	3.2	25.4 a	17.4***
Goutam	3.8	22.0 b	22.8***	3.9	32.0 b	33.3***
Sourin	4.4	15.1 a	18.5***	3.4	37.1 b	20.2***
CIP-6	5.4	35.3 c	16.3***	3.8	57.2 c	33.8***
$F_{3,3df}$ stat†	3.1^{NS}	45.0***		1.9^{NS}	$9.4^{\S}$	

*Values in the column not followed by the same letters are significantly different ($P < 0.05$, $P < 0.01$) by Duncan's Multiple Range Test.
**Table t value at ***$P = 0.01$ is 3.71.
†F-test (3, 3 df) table value: $^{\S}P = 0.05 = 6.39$; ***$P = 0.01$ is 15.98; NS = non-significant.

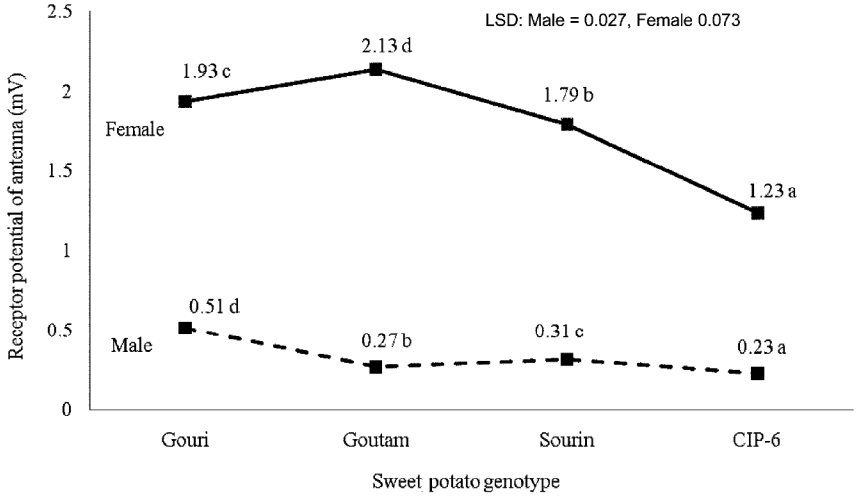

Figure 2. Electrophysiological response of *Cylas formicarius* antenna to plant volatiles collected from different sweet potato genotypes. Values not followed by the same letters are significantly different (F-test, $P < 0.01$) by DMRT.

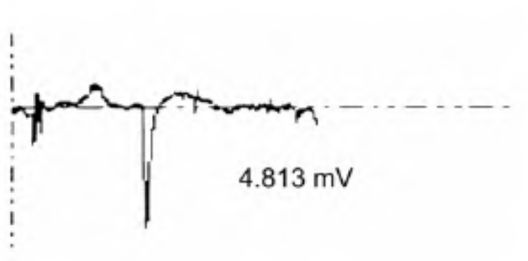

Figure 3. Electroantennogram response of female *Cylas formicarius* weevil to root volatile extracts of sweet potato genotype Gouri.

and Gouri genotypes to elicit electrophysiological response in female antenna was 13.87 times for the female and 1.62 times for the male antenna. Such a wide variation in the depolarization of the antenna indicated that the female antenna was highly sensitive to volatiles from roots than volatiles from plants. Similarly, wide variation in receptor potential of insect antenna to volatiles has been reported[7,12].

Behavioural assays of *C. formicarius* to different volatiles collected from sweet potato genotypes and statistical analyses with two-sample (two-tailed) t-test indicated the existence of significantly higher response of female to both plant and root volatiles ($P < 0.01$) compared to male *C. formicarius* (Table 2). The results of ANOVA also suggested that plant and root volatiles of sweet potato genotypes elicited significant behavioural reactions (% attraction) only in female *C. formicarius* ($F_{3,3df}$ cal. value 45.04**, $P < 0.01$ and 10.61*, $P < 0.05$ respectively) and not in male *C. formicarius* ($F_{3,3df}$ cal. value 3.14^{NS}, $P > 0.05$ and 1.90^{NS}, $P > 0.05$ respectively). In case of plant volatiles, CIP-6 attracted significantly highest number of females (35.3%, $P < 0.05$) and Sourin attracted significantly lowest number of females (15.1%, $P < 0.05$). In case of root volatiles, the highest female attraction was recorded with CIP-6 (57.2%, $P < 0.05$) and the lowest with Gouri (25.4%, $P < 0.05$; Table 2). Differences in infestation pattern (Table 1) and behavioural responses (Table 2) of *C. formicarius* to four sweet potato genotypes may relate to differences in susceptibility or resistance. Wang and Kays[9] also reported that volatile extracts from storage roots and aerial plant parts were attractive to female SPW, the former being substantially greater. Differences in relative attraction among four

sweet potato cultivars to female SPW were inversely correlated with the composite concentration of headspace sesquiterpenes[9].

An overview of the results suggests that the plants of genotype Gouri release volatiles that might not be preferred by female SPW, as evidenced by the lower infestation levels recorded (Table 1). The genotype CIP-6 was found to be more attractive to female SPW, as evident by the high infestation (Table 1). The root volatile extract CIP-6 was found to be more attractive to *C. formicarius* than the corresponding plant/leaf volatiles (Table 2). In other words, CIP-6 stem/roots emit volatiles favourable for decision-making of female weevil, thus making it more susceptible than all the other varieties. A negative significant correlation ($r = -0.722^{**}$, $df = 10$, $P < 0.01$) was recorded between EAG data of female weevil and Y-tube bioassay for female (root volatiles); and for the plant volatiles the relationship was also found to be significantly negative ($r = -0.732^{**}$, $df = 10$, $P < 0.01$). This correlation indicates an inverse relationship between attraction (%) and EAG response, thus pointing to the possibility that the most favourable genotype CIP-6 (susceptible) emits attractant compounds (or less repellent compounds), whereas Gouri (resistant) may be releasing repellent substances (lesser attractants) to female *C. formicarius*. The relationship between male *C. formicarius* and plant volatiles ($r = -0.511^{*}$, $df = 10$, $P < 0.01$), as well as root volatiles ($r = -0.401^{NS}$, $df = 10$, $P > 0.05$) indicates a substantially lower negative correlation. It was reported that some repellant compounds produced low EAGs (citral), whereas other repellents produced high EAGs (nonanal) in a polyphagous apple moth *Epiphyas postvittana*[13]. It has been reported that volatile compounds which elicited EAG responses also show significant behavioural responses in insects[7,12–16]. Sweet potatoes emanate significant amounts of terpenes in volatile form, which are attractive to *C. formicarius* and that the roots produce quantitatively lower amounts of composite terpenes than the aerial parts[9]. The aerial plant parts emanated a higher composite concentration of sesquiterpenes than the storage roots, which acted as repellents to *C. formicarius*[9]. Thus, studies assessing the alternation in volatiles, that the plant releases through its different parts, help in the identification of the most resistant genotypes and also to develop plants devoid of a particular volatile, which is attractive to *C. formicarius*. Thus this method of identification and selection of resistant lines to insect pests, based on the volatiles they emit can be called 'volatile assisted selection' (VAS), which can be used for screening a large number of germplasm resources. VAS can also be used for the development of attractants or repellents for successful management of *C. formicarius*. Further work is required to identify the volatile compounds that govern the olfactory-directed behaviour of *C. formicarius*, as it is important to formulate strategies for developing host-plant resistance.

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Received 11 December 2009; revised accepted 15 October 2010