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Behavioural mechanism of *Tuta absoluta* towards conspecific-heterospecific infested tomato plants in response to leaf volatiles

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Abstract
Tomato is one of the major vegetable grown in Andhra Pradesh especially in Chittoor district. This crop is grown all-round the year. In nature, usually a crop (Tomato) is infested by various pests in which South American Tomato leaf miner, *Tuta absoluta* is the major one. Along with this the other insect which we find usually is the Serpentine Leaf miner, *Liriomyza trifolii*. An experiment was conducted to characterize the behavioural mechanism of *T. absoluta* females by choice and no-choice oviposition assays, olfactometer studies towards the conspecific (*T. absoluta* infested), heterospecific (*L. trifolii* infested) and healthy tomato plants. Odour of tomato leaves elicited upwind orientation flight of mated females followed by landing as well as egg laying. In olfactometer, no-choice and choice oviposition experiments, *T. absoluta* females significantly preferred healthy tomato plants over conspecific and heterospecific infested plants. This clearly indicated that the leaf volatiles released by the healthy as well as infested plants varied and provided information on the suitability of plants as oviposition sites and larval host.

Keywords: *Tuta absoluta, Liriomyza trifolii*, tomato, leaf volatiles, behavioural mechanism

Introduction
Insects and plants have co-existed for at least 100 million years and evolved a variety of beneficial and deleterious interactions. 'Certainly insects cannot think, but they can react'- chemical signals/cues used by insects to survive and reproduce on their host [13]. Plant emit volatile organic compounds (VOC's) that play multiple roles in plant interactions, they are important cues for insects to locate an appropriate host plant, or mating and oviposition sites [3]. Additionally, upon herbivory attack, plants emit herbivore-induced plant volatiles (HIPV's) or oviposition induced plant volatiles (OIPV's) that could be perceived by natural enemies to locate their host [6,7,10,11]. This reliance of insects on chemical cues offers a number of opportunities for insect control.

Tuta absoluta (Lepidoptera: Gelechiidae) is a key pest of Tomato. Larva is the damaging stage. This pest may cause yield loss upto 100%. The damaging symptoms are blotch like mines on leaves and pin size holes on fruits, in severe cases the entire leaf or the plants get dried. This pest infests leaves, fruits and even stem also. Along with this pest we commonly find the serpentine leaf miner, *Liriomyza trifolii* (Diptera: Agromyzidae). Larva makes certain serpentine like mines on leaves. Though the damage caused by serpentine leaf miner is minimal but this is one of the most common pest associated with Tomato. Upon herbivore attack plant releases semiochemicals which may attract or repel their conspecifics, heterospecifics and their associated natural enemies. Hence, an experiment was conducted at ICAR-NBAIR during 2018-2019 to study the response of gravid *T. absoluta* females towards healthy, conspecific and heterospecific infested tomato plants through No-choice, choice oviposition assays and olfactometer studies.

Materials and Methods
Insects and plants
Maintenance of host plants
Tomato seeds (Sahoo TO-3251, Syngenta India Limited) were sown in seedling trays and were placed in net cages to avoid pest infestations. The host plants were maintained in polybags

after transplanting following standard agronomic practices without any pesticide application. To avoid pest infestation, regular water sprays were given at frequent intervals and maintained in net cages. The selected host plants *viz.*, Tomato (*Solanum lycopersicum*) healthy (uninfested plants), *Tuta absoluta* infested plants, *Liriomyza trifolii* infested plants were maintained in the poly bags (6"dia) at polyhouse.

Maintenance of *Tuta absoluta* cultures

Tomato leaf miner, *T. absoluta* (along with infested leaves) were collected from the polyhouse at ICAR-NBAIR, Yelahanka campus, Bengaluru and also from farmer's fields around Malur and Kolar districts of Karnataka. The freshly collected infested leaves of tomato along with *T. absoluta* larvae were placed in polythene covers (45 cm length * 30.5 cm breadth), secured with rubber bands and brought to the laboratory. In the laboratory, the larvae along with infested leaves were placed in plastic containers lined with tissue papers to avoid moisture accumulation. The containers were covered with muslin cloth and tied with rubber bands to avoid larval escape. The containers were kept in ambient conditions (27±1°C, 75±2% RH and 14L:10D h photoperiod) until emergence of adults.

Adult moths emerging from the containers were collected and released into net cages (1m*1m*1m). The freshly emerged moths were provided with honey solution (0.5%) on cotton wads *ad libitum*. For supporting egg laying, healthy tomato plants (Sahoo TO-3251, Syngenta India Limited) of 40-50days age grown in polybags (6"dia) were placed in cage on regular basis. From these infested plants the prepupae were collected and placed in insect culture cage (90*50*50 cm) for further adult emergence under laboratory conditions as mentioned above.

The newly emerged moths were provided with food as mentioned earlier and allowed to mate. Mated pairs were collected during the first photo-phase and used for choice and no-choice assays. Mated gravid females were collected separately and used for EAG/GC-EAD studies as well as olfactometer assays.

Maintenance of *Liriomyza trifolii* cultures

As mentioned similar to *T. absoluta*, serpentine leaf miner (*L. trifolii*) infested tomato leaves were also collected from experimental fields and farmer's field, maintained in the laboratory. The infested leaves were placed in plastic containers lined with blotting paper to avoid moisture accumulation. These containers were kept at ambient conditions until adult emergence (27±1°C, 75±2% RH and 14L:10D h photoperiod). The freshly emerged leaf miner adults were provided with honey solution (0.5%) on cotton wads *ad libitum*. The same adults were used for experimentation.

Ovipositional preference of gravid female moth *T. absoluta* among conspecific, heterospecific and healthy tomato plants

The host plants (Tomato) were infested by both target insects, *T. absoluta* and also *L. trifolii* separately. Healthy tomato seedlings were infested with larvae of *T. absoluta* and *L. trifolii*. The infested plants were given in choice/ no-choice assays to study the oviposition preference of gravid female moths of *T. absoluta*. For both the assays, the selected host plants were arranged randomly (Completely Randomized Design) in net cages and were replicated five times. This study provides us clues about gravid female *T. absoluta* choice towards conspecifics and heterospecifics infested host plants in relation to control (healthy plants).

No-Choice assay

In case of no-choice assay, each treatment was arranged randomly in net cages and were replicated five times, then the plants were exposed to gravid females of *T. absoluta* @ 10 gravid females/replication.

Choice-assay

Choice assay can provide a better estimate of insect performance. In the free choice assay, a single plant from each treatment was arranged randomly in net cages. Then the plants were exposed to ten gravid females of *T. absoluta*/replication. These assays were repeated five times. Observations were recorded on the number of eggs laid, number of larvae emerged. Number of eggs was recorded 5 days after exposure and number of larvae were recorded 10 days after exposure.

Plant volatile collection

HIPV's were provoked by releasing ten *T. absoluta* larvae, ten *L. trifolii* larvae into cages containing healthy tomato plants separately. Healthy plants were used as control. Five plants were used in each treatment. Volatiles were collected from healthy (uninfested), conspecific (ten *T. absoluta* larvae infested) and heterospecific (ten *L. trifolii* larvae infested) tomato plants. A single potted tomato plant was placed inside an autoclave bag where the soil part was covered with aluminium foil to avoid volatiles from roots and soil. Gentle stream of air was blown through activated charcoal cartridge @ 30 mL/min. It was allowed to pass over the samples held in an autoclave bag. The odour laden air was trapped in glass tube containing Porapak-Q adsorbent (Supelco) 30 mg (50-80 mesh) with glass wool on either side as stoppers. The collection was done after 6 hours. The trapped volatiles in the adsorbent were eluted with HPLC grade Hexane (400 µL) and condensed to 200 µL by passing gentle stream of ultra-high pure nitrogen. Sample vials with extracts were stored in -20 °C until analysis by Gas chromatography coupled mass spectrometry (GC-MS). In each volatile collection, the system was cleaned to prevent contamination. The absorbent vial was first cleaned 5 times with DCM, then cleaned 5 times with methanol and finally 3 times again with Hexane. To ensure the system free of contaminants before volatile collection, blank-run was obtained each time. The blank collection was eluted as mentioned above and tested in GC-MS.

Chemical identification of volatiles

Collected volatile samples were chemically identified using GC-MS at Chemical Ecology laboratory, ICAR-NBAIR, Bengaluru. GC-MS analyses were performed on a 7890A Gas Chromatograph system (GC) (Agilent technologies) interfaced with a 5975C Mass Spectrometer Detector (MSD) (Agilent technologies) with triple axis detector (electron impact ionization, 70 eV) through a HP-5MS phenyl siloxane capillary column (inner diameter 30 m x 0.25 mm) (J&W Scientific, Folsom, California). The temperature of column and oven were maintained at 40 °C for 1 min. and then increased @ 20 °C/min to 280 °C and held at 300 °c for 10 min. The injector and column temperatures were 250 °C. The total run was for 36 min. The mass spectrometry data library was NIST.17 and MS search 2.0 software was used in the

analysis. Internal standard Bromodecane 100ng was used. One micro litre of concentrated volatile was injected in to the machine using Hamilton micro syringe.

Y-tube olfactometer studies

Olfactometer studies were conducted to test the attraction of *T. absoluta* gravid females to selected host plants. Female adults were subjected to the following tests (Dual choice assays) in Y-arm olfactometer: Healthy plants *vs* Conspecific infested plants; Healthy plants *vs* Heterospecific infested plants; Conspecific infested plants *vs* Heterospecific infested plants. Olfactometer with a 1-cm internal diameter, 15-cm main length, side arms 15 cm long was used. Air pumped through activated charcoal filter and humidified by passing through it connected to a glass flask with distilled water before being directed into the two arms of the olfactometer. The airflow was adjusted to 30mL/min. The olfactory stimuli were obtained by impregnating 10µl of volatiles on to separate filter papers strips (Whatmann No.1, 5 cm length × 0.75 cm width). The solvent was allowed to evaporate for 1 min before placing the filter papers inside the arms of olfactometer. *T. absoluta* adults (30) were released at the entrance of the main arm and left for five minutes to make a choice. In all bioassays, after each run, the olfactometer was rotated by 90º to avoid any directional bias. The whole set up was covered with a muslin cloth to avoid light. After three replicates, the olfactometer was thoroughly washed with methanol and oven dried at 120 °C.

Statistical analysis

The data of no-choice were subjected to one-way ANOVA with multiple comparisons and for choice assay repeated measure one-way ANOVA with multiple comparisons were followed. Chi-Square test was used for the analysis of Y-tube olfactometer test using SPSS version 25.

Results

Oviposition preference of *T. absoluta* gravid females on selected host plants

Choice and No-choice oviposition assays were conducted to study the oviposition preference of *T. absoluta* among selected host plants under both no-choice and choice conditions.

No- choice assays

Under no-choice assays, healthy plants, conspecific infested tomato plants and heterospecific infested plants were exposed to gravid *T. absoluta* females and the mean number of eggs laid on each treatment were recorded (Table 1 and Table 2). Results indicated that there was significant difference between number of eggs laid on selected host plants [F=13.98; p = 0.001]. Highest number of eggs were laid significantly on Healthy plants [27.60 ± 2.44; Mean number of eggs ± S.E.] followed by conspecific infested plants [20.00 ± 2.43; Mean number of eggs ± S.E.]. Heterospecific infested plants has minimal number of eggs [12.00 ± 1.09; Mean number of eggs ± S.E.].

Further multiple comparisons of selected host plants were subjected to post hoc Tukey's test. Results revealed that there was no significant difference in the number of eggs laid by gravid female moths between healthy plants and conspecific infested plants in no-choice assays (Table 2). Under no-choice conditions *T. absoluta* female moths preferred healthy and conspecific infested plants over heterospecific infested plants for oviposition.

Choice assays

Under choice assays, all the selected host plants were provided at a time to gravid females of *T. absoluta* for oviposition. Though the number of eggs laid on each treatment varied, Similar trend of results were obtained as of No-choice assays.

Tomato leaf miner adults preferentially oviposited on healthy plants [50.00 ± 3.39; Mean number of eggs ± S.E.] followed by Conspecific infested plants [19.20 ± 3.44; Mean number of eggs ± S.E.] and heterospecific infested plants [9.60 ± 1.47; Mean number of eggs ± S.E.] (Table 1). Results revealed that there was significant difference in the number of eggs laid on selected host plants [F= 52.41; p = 0.001]

Pair wise comparisons among different treatments indicated that there was no significant difference in the number of eggs laid by female moths between conspecific infested and heterospecific infested plants though the number of eggs laid was different.

Table 1: Oviposition preference of gravid females *T. absoluta* on selected host plants under No-choice and choice assays

Host plants	Mean number of eggs			
	No-choice assay		Choice assay	
	Mean	S.E.	Mean	S.E.
Healthy plants	27.60	2.44	50.00	3.39
Conspecific infested plants	20.00	2.43	19.20	3.44
Heterospecific infested plants	12.00	1.09	9.60	1.47
$F_{cal\ (2,12)}$	13.98		52.41	
P value	0.001		<0.001	

Table 2: Tukey's multiple comparison test: Oviposition preference of gravid *T. absoluta* females on selected host plants under No-choice and choice assays

Host plants	No-choice		Choice	
	Mean difference	p value	Mean difference	p value
Healthy plants vs Conspecific infested plants	7.60	0.059	30.80	<0.001
Healthy plants vs Heterospecific infested plants	15.60	0.001	40.40	<0.001
Conspecific infested plants vs Heterospecific infested plants	8.00	0.046	9.60	0.900

Emergence of *T. absoluta* larvae on selected host plants under no-choice and choice assays:

After oviposition by gravid female moths, observations were taken on number of larvae emerged after 10 days of experimental setup. Under No-choice and choice assays, larval count results were similar to oviposition preference assays. There was a significant difference in the mean larval count among selected host plants [F= 13.16, p= 0.001; F= 44.15, P= ,0.001], respectively. Significantly, highest mean larvae were observed on healthy plants followed by conspecific infested plants followed by heterospecific infested plants in no-choice and choice assays (Table 3).

Table 3: *T. absoluta* larval count on selected host plants under No-choice and choice assays

Host plants	Mean number of larvae emerged			
	No-choice assay		Choice assay	
	Mean	S.E.	Mean	S.E.
Healthy plants	21.40	2.30	41.00	2.14
Conspecific infested plants	14.80	2.22	14.00	3.97
Heterospecific infested plants	7.80	0.58	7.00	1.22
$F_{cal\ (2,12)}$	13.165		44.155	
P value	0.001		<0.001	

Identification of compounds present in plant volatiles among selected host plants:

Volatile organic compounds present in healthy tomato plants, conspecific infested and heterospecific infested plants were identified using Gas chromatography couples Mass Spectrometry.

There was lot of variation in the chemical composition of selected host plant volatiles. Compounds present in Healthy, conspecific infested and heterospecific infested plants were tabulated in (Table 4, Table 5 and Table 6), respectively. Mass peaks of the compounds of Healthy, conspecific infested and heterospecific infested plants were represented in (Fig. 1, Fig. 2 and Fig. 3), respectively. Results from GC-MS analysis revealed that healthy plants and conspecific infested plants have 3- Hexanol, p-Xylene, (1R)-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene, Dodecane, Pentadecane, 3-Hexanone, (+)-4-Carene, alpha-Phellandrene in common. Compounds that were unique to healthy plants were 4-methyl-Undecane, 2-methyl-Tetradecane, 2,4-bis(1,1-dimethylethyl)-Phenol. o-Cymene, Caryophyllene, Heneicosane, beta-Myrcene, Tetradecane were found only in conspecific infested plants. volatile profile of heterospecific infested plants were dissimilar to the profile of healthy plants and conspecific infested plants comparatively except for p-Xylene.

Table 4: List of compounds found in healthy tomato plants

Sl. No.	Retention time	Name of the compound
1.	4.249	2-Pyrrolidinone
2.	7.547	3-Hexanone
3.	8.238	3-Hexanol
4.	9.505	p-Xylene
5.	11.243	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
6.	13.149	(+)-4-Carene
7.	13.253	alpha-Phellandrene
8.	14.949	Dodecane
9.	16.489	4-methyl-Undecane
10.	21.106	Pentadecane
11.	22.341	2-methyl-Tetradecane
12.	32.141	2,6,10,14-tetramethyl-Hexadecane
13.	33.931	Pentacosane
14.	35.994	2,4-bis(1,1-dimethylethyl)-Phenol

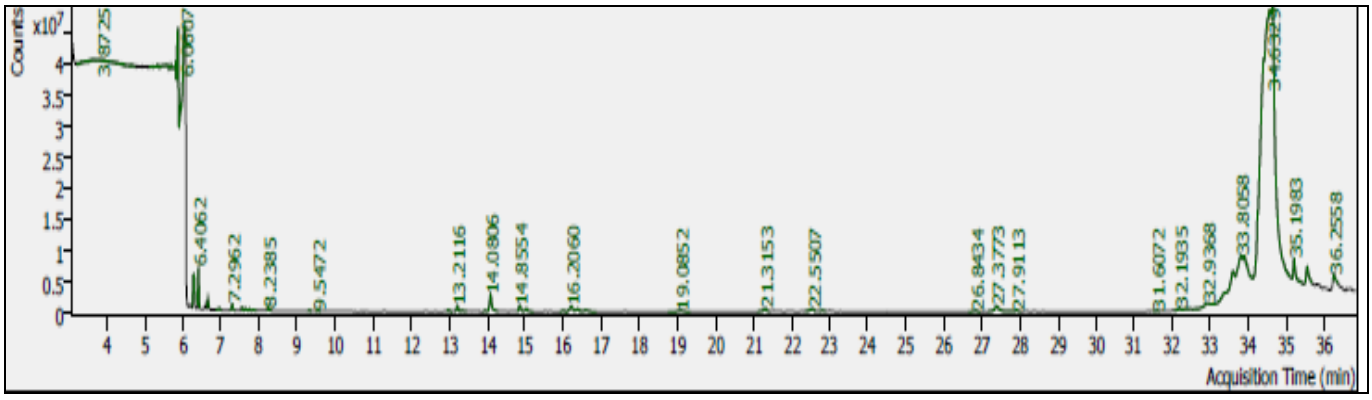

Fig 1: Chromatogram of healthy tomato plants

Table 5: List of compounds found in conspecific infested plants (24 hours after infestation)

Sl. No.	Retention time	Name of the compound
1.	6.364	Methyl-Cyclohexane
2.	7.076	Toluene
3.	7.380	3-Hexanone
4.	7.579	3-Hexanol
5.	9.421	p-Xylene
6.	11.212	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
7.	12.835	beta.-Myrcene
8.	13.159	(+)-4-Carene
9.	13.274	alpha-Phellandrene
10.	13.881	o-Cymene
11.	14.991	Dodecane
12.	18.719	Naphthalene
13.	19.137	Tetradecane
14.	22.739	Pentadecane
15.	25.147	Caryophyllene
16.	26.770	Heneicosane

Table 6: List of compounds found in heterospecific infested plants (24 hours after infestation)

Sl. No.	Retention time	Name of the compound
1.	4.689	2-Pyrrolidinone
2.	5.055	3-methyl- Hexane
3.	5.317	Heptane
4.	6.615	Toluene
5.	9.306	p-Xylene
6.	13.222	beta-ionone
7.	13.986	m- cymene
8.	18.897	Naphthalene
9.	25.231	cis-5,8,11,14,17-Eicosapentaenoic acid
10.	27.911	2,6,10,14-tetramethyl- Hexadecane
11.	31.607	5,8-diethyl- Dodecane
12.	33.366	bis (2)-1,2-Benzenedicarboxylic acid
13.	33.523	Eicosane
14.	35.020	Nonacosane

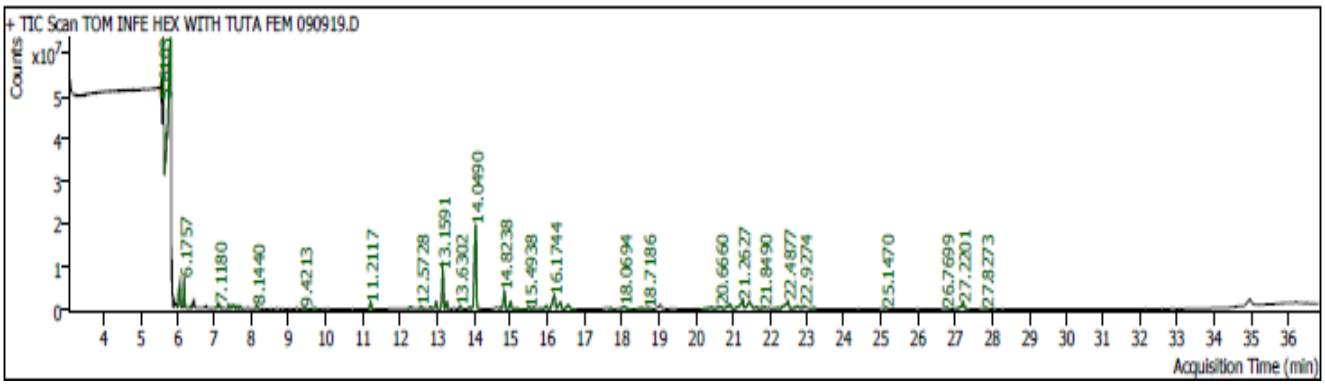

Fig 2: Chromatogram of conspecific infested plants

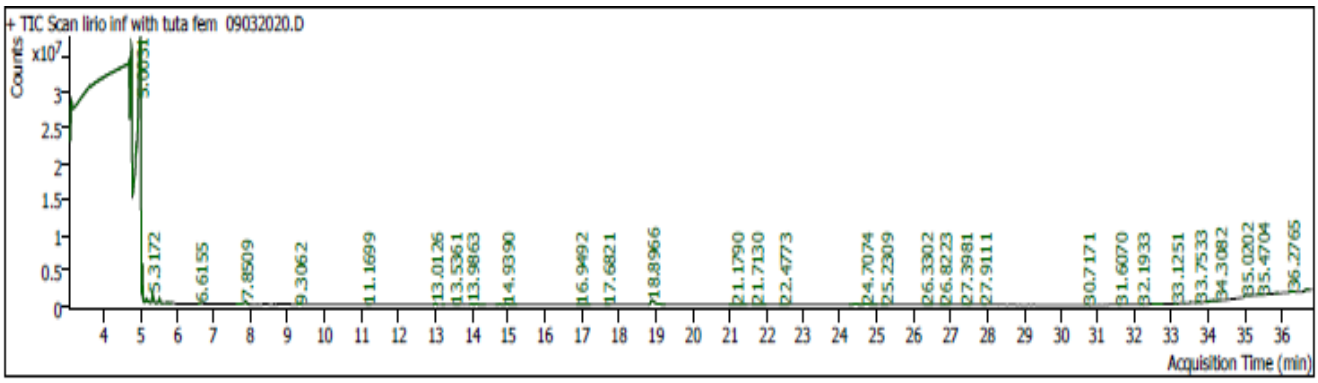

Fig 3: Chromatogram of heterospecific infested plants

Attraction of *T. absoluta* gravid female moths to selected host plant volatiles in olfactometer studies:

Dual choice assays were conducted in Y- tube olfactometer. Olfactometer studies of dual choice assays revealed that the mated females of *T. absoluta* were attractive to both Healthy and conspecific infested plants which was statistically not significant ($\chi2$=2.66, df =1, p= 0.100); significant difference was observed between Healthy and heterospecific infested plants ($\chi2$=6.76, df =1, p= 0.009). Females of *T. absoluta* has chosen conspecific infested plants over heterospecific infested plants and was significantly different ($\chi2$=9.86, df =1, p= 0.001).

Table 7: Behavioural response of *T. absoluta* mated females to plant volatiles in Y-tube olfactometer (Dual choice assays)

Sl. No.	Treatments	Respondents		Chi-Square value	Non-Respondents	p-value
		Arm -1	Arm- 2			
1.	Healthy *vs* Conspecific infested plants	16	8	2.666	6	0.102
2.	Healthy *vs* Heterospecific infested plants	19	6	6.760	5	0.009*
3.	Conspecific infested plants *vs* Heterospecific infested plants	13	3	9.866	13	0.001*

*****Mean response between Arm-1 and Arm -2 were significantly different at $p<0.05$; n=30

Discussion

In this study, we have studied the oviposition preference of gravid female moth's response to plant chemical cues, we found that the females were able to discriminate between healthy tomato plants and the plants infested by conspecifics and heterospecifics using their olfactory cues. We have characterized the volatile compounds released in the headspace volatiles of selected host plants. Results have clearly stated that a higher number of individuals has chosen healthy tomato plants. These findings were similar to [2].

Volatile profile of uninfested and infested plants have been extensively studied [1, 4, 8, 12, 15, 17]. The results were in contrast with [13], who reported that β-phellandrene and 2- carene as major compounds. Difference in variety, plant stage, size and structure of volatile collection chambers, environmental conditions may influence the volatile blend emitted by host plants [8]. Analysis by GC-MS has clearly indicated that healthy and infested tomato plants shared different volatile profiles observed after 24 hours of infestation. Plant react to the herbivore attack immediately within hours of attack [5, 16, 14].

Conclusion

This study substantiated that the gravid females of *T. absoluta* uses the chemical cues for host plant selection. The volatile profile provides support to the oviposition preference and attraction of *T. absoluta* by varying during infestation. Further, electrophysiological compounds can be determined, as either attractants or repellents of their conspecifics, heterospecific pests and their associated natural enemies by behavioural assays. These results may lay foundation in developing semiochemicals strategies that can be implemented in IPM strategies along with the existing practices like pheromone technology to control South American tomato leaf miner.

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