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Attraction of Three Mirid Predators to Tomato Infested by Both the Tomato Leaf Mining Moth *Tuta absoluta* and the Whitefly *Bemisia tabaci*

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Abstract

Plants emit volatile compounds in response to insect herbivory, which may play multiple roles as defensive compounds and mediators of interactions with other plants, microorganisms and animals. Herbivore-induced plant volatiles (HIPVs) may act as indirect plant defenses by attracting natural enemies of the attacking herbivore. We report here the first evidence of the attraction of three Neotropical mirid predators (*Macrolophus basicornis*, *Engytatus varians* and *Campyloneuropsis infumatus*) toward plants emitting volatiles induced upon feeding by two tomato pests, the leaf miner *Tuta absoluta* and the phloem feeder *Bemisia tabaci*, in olfactometer bioassays. Subsequently, we compared the composition of volatile blends emitted by insect-infested tomato plants by collecting headspace samples and analyzing them with GC-FID and GC-MS. Egg deposition by *T. absoluta* did not make tomato plants more attractive to the mirid predators than uninfested tomato plants. *Macrolophus basicornis* is attracted to tomato plants infested with either *T. absoluta* larvae or by a mixture of *B. tabaci* eggs, nymphs and adults. *Engytatus varians* and *C. infumatus* responded to volatile blends released by tomato plants infested with *T. absoluta* larvae over uninfested plants. Also, multiple herbivory by *T. absoluta* and *B. tabaci* did not increase the attraction of the mirids compared to infestation with *T. absoluta* alone. Terpenoids represented the most important class of compounds in the volatile blends and there were significant differences between the volatile blends emitted by tomato plants in response to attack by *T. absoluta, B. tabaci,* or by both insects. We, therefore, conclude that all three mirids use tomato plant volatiles to find *T. absoluta* larvae. Multiple herbivory did neither increase, nor decrease attraction of *C. infumatus*, *E. varians* and *M. basicornis*. By breeding for higher rates of emission of selected terpenes, increased attractiveness of tomato plants to natural enemies may improve the effectiveness of biological control.

Keywords Multiple herbivory · Predator foraging behavior · Tomato pests · *Campyloneuropsis infumatus* · *Engytatus varians* · *Macrolophus basicornis*

Introduction

Plants under attack by herbivorous arthropods emit complex blends of compounds called herbivore-induced plant volatiles (HIPVs), which are exploited by predators and parasitoids during searching for their prey/host (Dicke and Sabelis 1988; Turlings et al. 1990; Geervliet et al. 1997; Dicke and Baldwin 2010). Understanding the role of HIPVs in prey/host habitat location (Pels and Sabelis 2000) and in reducing searching time by natural enemies is important for improving the use of these beneficial organisms in biological control programs (Turlings et al. 1991; Vet and Dicke 1992; Ninkovic et al. 2001). The outcome of plant-mediated interactions between herbivores and natural enemies depends on many factors, including plant species and genotype (Bukovinszky et al. 2005; Gencer et al. 2009), herbivore species (De Moraes et al. 1998) and developmental stage (Takabayashi et al. 1995).

Plants are usually colonized by more than one species of arthropod herbivore (Dicke et al. 2009; Ponzio et al. 2013).

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Nevertheless, most of the literature on tritrophic interactions mediated by HIPVs is based on the attack by a single herbivore species. These studies have yielded important insights, but we also need to understand multitrophic interactions mediated by HIPVs in response to attack by more than one species of herbivore (i.e., multiple herbivory). Multiple herbivory can lead to a different composition of HIPV blends than single-species herbivory and, consequently, can affect attraction of natural enemies (Shiojiri et al. 2001; Cardoza et al. 2002; Moayeri et al. 2007; Rasmann and Turlings 2007; de Boer et al. 2008; Gosset et al. 2009; Errard et al. 2015; Pangesti et al. 2015). The effect of multiple infestation on HIPV emission and natural enemy attraction appears difficult to predict and is highly variable, especially when multiple herbivory is inflicted by different feeding guilds such as leaf chewers and phloem suckers (Moayeri et al. 2007; Zhang et al. 2009; Dicke et al. 2009), which each induce a different defense signal transduction pathway: the jasmonic acid (JA) and salicylic acid (SA) pathways respectively. Interaction between the JA and SA signaling pathways commonly known as cross-talk may affect the HIPV composition and can result in positive (Cardoza et al. 2003; Rodriguez-Saona et al. 2005; Cusumano et al. 2015), negative (Zhang et al. 2009; Schwartzberg et al. 2011) or neutral (Erb et al. 2010) responses by natural enemies.

Tomato (*Solanum lycopersicon* L.) is one of the most consumed vegetables around the world (FAOSTAT 2015). Tomato plants are attacked by many pests (Errard et al. 2015), among which the tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), and the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) cause major yield reductions (Oliveira et al. 2001; Desneux et al. 2010, 2011). Currently, the major pests of tomato can successfully be controlled by augmentative releases of natural enemies of arthropod herbivores (Calvo et al. 2009, 2011, 2012; van Lenteren 2012). In Europe, the predatory mirid bugs *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* Rambour (Hemiptera: Miridae) proved effective in controlling *B. tabaci* and *T. absoluta* (Perdikis et al. 2008; Urbaneja et al. 2009, 2012; Calvo et al. 2012; Mollá et al. 2014). Both *N. tenuis* and *M. pygmaeus* (Ingegno et al. 2013; Lins et al. 2014; de Backer et al. 2015) exploit HIPVs emitted by tomato plants infested by either *T. absoluta* or *B. tabaci* to locate their prey.

Recently, three mirid species have been found in the field in Brazil, *Macrolophus basicornis* (Stal), *Engytatus varians* (Distant) and *Campyloneuropsis, infumatus* (Carvalho), which are able to consume large numbers of *T. absoluta* and other pests occurring on tomato in laboratory experiments and seem to be promising biological control agents (Bueno et al. 2013; Silva et al. 2016). Their capacity to search for and localize main tomato pests and the role HIPVs in prey searching has not yet been studied.

We investigated the olfactory responses of *M. basicornis*, *E. varians* and *C. infumatus* to HIPVs released by tomato plants infested with *T. absoluta* (eggs or larvae) or *B. tabaci* (eggs, nymphs and adults), in olfactometer assays. Also, we examined whether infestation of tomato plants with both *B. tabaci* and *T. absoluta* affects the olfactory response of the mirids to HIPVs. Furthermore, we chemically characterized the volatile compounds emitted by single- and double-infested tomato plants to assess how changes in HIPV blend might affect the behavior of the three mirid species. As feeding by *B. tabaci* suppresses JA-regulated defenses by inducing the SA-signaling pathway to manipulate the plant's immune system (Zarate et al. 2007; Estrada-Hernández et al. 2009; Zhang et al. 2009, 2013), we predicted that *B. tabaci* co-occurring with the chewing herbivore *T. absoluta* in tomato plants would release lower amounts of volatiles than plants infested with *T. absoluta* singly, negatively affecting the attraction of mirid predators to dual-infested tomato plants.

Methods and Materials

Plants and Insects Tomato plants, *Solanum lycopersicon* L. cv. Santa Clara, were grown in pots (volume 3 L) containing formulated substrate mixed with 200 g NPK 4–14-8 complex fertilizer and placed in a greenhouse. Plants that were 30–35 days old, 20–25 cm high with 5–6 expanded leaves were used in the experiments.

Mirid predators were collected in tobacco (*Nicotiana tabacum* L.) fields located in the municipalities of Ribeirão Vermelho and Lavras (MG, Brazil, 21°08.596′S and 045°03.466′W, 808 m of altitude). Nymphs and adults were identified based on the family- specific dichotomous key of Ferreira and Enry (2011). Identification of the three mirids found in the field were confirmed by P.S.F Ferreira (Federal University of Viçosa, Viçosa-MG, Brazil) as *Campyloneuropsis infumatus*, *Engytatus varians* and *Macrolophus basicornis*. Stock colonies of the three mirid species were kept under laboratory conditions following the methodology of Bueno et al. (2013). Female adults were individually maintained in acrylic cages (60 × 30 × 30 cm) containing eggs of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) for *ad libitum* feeding, and tobacco plants (*N. tabacum* L. cv. TNN) as oviposition substrate and water source. We used tobacco for maintaining the mirids to avoid conditioning effects to volatiles released by tomato plants. After seven days, tobacco plants containing eggs were transferred to new cages where nymphs hatched and fed on *E. kuehniella* eggs until reaching the adult stage. Females of 1–7 days old from the second generation in the laboratory were used in behavioral assays.

Eggs, larvae and pupae of *T. absoluta* were collected in a tomato field in an experimental area at the Federal University

of Lavras (Lavras, MG, Brazil, 21°14′S 45°00′W, 918 m of altitude). Newly-formed pupae in the laboratory were sexed (Coelho and França 1987) and placed in cages (60 × 30 × 30 cm) containing tomato plants (cv. Santa Clara). Larvae and adults were kept separately in cages (60 × 30 × 30 cm) covered with fine mesh and regularly supplied with tomato plants for feeding and oviposition.

Whiteflies, *B. tabaci* biotype B, were provided from the rearing maintained at the Agronomic Institute of Campinas - AIC (Campinas, SP, Brazil). Colonies were kept in fine mesh cages (60 × 40 × 40 cm) with cabbage plants (*Brassica oleracea* L. var. acephala DC. cv. Manteiga) for feeding and oviposition.

All insects and plants were maintained at the Department of Entomology and Acarology, at ESALQ/ USP (Piracicaba, SP, Brazil). Colonies of the three mirids and *T. absoluta* were maintained under laboratory conditions at 25 ± 2 °C, RH 70 ± 10% and 12 L: 12D. The tomato plants and the whitefly colony were kept at ambient conditions (light, temperature and humidity) in different greenhouses.

Olfactometer Assays Responses of female mirids to HIPVs were assessed in a glass Y-tube olfactometer (3.0 cm diameter, main arm 20 cm long, side arms 23 cm long, 70° angle between the side arms). The olfactometer device was vertically positioned, following the methodology used with other mirids (Moayeri et al. 2006; Ingegno et al. 2011, 2013; Lins et al. 2014) and connected to a volatile collection system (Analytical Research Systems, Gainesville, FL, USA). Each olfactometer side arm was connected to a 15-L glass vessel with a single tomato plant. Plastic pots in which the tomato plants were growing were wrapped with aluminum foil. Inlet air flow was adjusted to 0.8 L.min^{-1} for each side arm. The glass vessels were kept behind a black panel to prevent insects from visually detecting the plants.

Predator females used for the assays were considered naïve, *i.e.* insects that had not been exposed to tomato volatiles, nor had preyed on *B. tabaci* or *T. absoluta* before tests. A single naïve mirid female 1–7 days old since the adult moult was introduced in the main arm of the olfactometer and observed for up to 10 min. Females were considered to have made a choice when they crossed a line drawn 13 cm from the branching point of the Y-tube. Females not choosing a side arm within 10 min were considered as non-responsive and were excluded from data analysis. Each female was tested only once. Thirty replicates (responses) in total were performed for each treatment and each mirid species, using at least three pairs of tomato plants on three experimental days. Every two replicates, the olfactometer side arms were switched to minimize positional bias. After testing ten females, the Y-tube and glass vessels were washed with neutral soap and ethanol (70%) and dried. Bioassays were carried out

in a climatized room at 24 ± 1 °C and 70 ± 10% RH between 10 and 12 am and 2–4 pm.

Olfactory responses of the three mirid predators were assessed to volatiles emitted from: (*i*) uninfested tomato plants ('uninfested'), (*ii*) plants on which *T. absoluta* deposited eggs ('*T. absoluta* eggs'), (*iii*) *T. absoluta* larvae-infested plants ('*T. absoluta* larvae'), (*iv*) *B. tabaci* egg-nymph-adult-infested plants ('*B. tabaci*'), and (*v*) *T. absoluta* larvae + *B. tabaci* (double infestation – '*T. absoluta* + *B. tabaci*').

Plants were transferred from the greenhouse to the olfactometer room just before the beginning of each assay series. To obtain tomato plants on which *T. absoluta* deposited eggs, plants were covered with organza bags containing five 1–3-day-old *T. absoluta* couples. Females were allowed to lay eggs for 48 h, and then the adults were removed. According to Silva et al. (2015), five *T. absoluta* females lay on average 125 eggs/day, resulting in an estimated 250 eggs after 48 h. Tomato plants with eggs (24 h after oviposition period) were used for the olfactometer tests.

Plants infested with *T. absoluta* larvae were obtained by hatching of larvae from eggs deposited by females on the plants. Egg survival at 25 °C is 98%, thus we obtained an estimated 245 first instar larvae (Silva et al. 2015), which were allowed to feed for 72 h (Lins et al. 2014).

Fifty *B. tabaci* adults (females and males) were released in a cage (60 × 30 × 40 cm) with one tomato plant for 10 days, in order to obtain tomato plants infested by mixed stages (i.e., eggs, nymphs and adults) of *B. tabaci* that were used in the tests (Lins et al. 2014). Tomato plants were also infested with both pests. We studied the effect of dual infestation by first infesting *B. tabaci* for 7 days and then introducing 30 first and second instar *T. absoluta* larvae on the same plants for another 3 days as a standard procedure. We chose this sequence of infestation (first *B. tabaci* then the chewer herbivore *T. absoluta*) to test the supposed negative cross-talk between SA and JA-signaling pathways elicited by *B. tabaci* feeding (Zarate et al. 2007).

Headspace Collection and Analysis of Plant Volatiles Volatiles from different groups of uninfested and herbivore-infested tomato plants (treatments *ii* − *v* described above) and pots filled with soil (blank) were collected under laboratory conditions at 24 ± 1 °C, 70 ± 10% RH between 10 and 12 am and 2–4 pm, in a push-pull volatile collection system (ARS, Gainesville, FL, USA).

Prior to volatile collection, plant pots were carefully wrapped with aluminum foil to avoid trapping volatiles from plastic and soil and individually enclosed in a 15-L glass vessel. The volatile collections were randomly distributed between treatments. Six plants per treatment were sampled for 2 h (flow rate 0.8 L min^{-1}) using a trap filled with 30 mg of HayeSep® (Supelco, Bellefonte, PA, USA). Volatile traps were immediately eluted with 150 μl dichloromethane

(Merck, Kenilworth, NJ, USA) mixed with 30 μl of nonyl acetate solution (Sigma-Aldrich, Sto Louis, MO, USA) at 10 ng/μL used as internal standard. All extracts were stored at −80 °C until analyses. Immediately after the collection of volatiles, plant shoot fresh weight was determined.

The headspace analyses were performed by gas chromatography (Shimadzu, GC-2010 Gas Chromatograph) with flame ionization detection GC-FID operated at 280 °C. Quantification was based on comparison of area under the GC-FID peak with the internal standard and standardized per unit fresh shoot biomass (g) of each replicate. Briefly, a 2-μl aliquot of each sample was injected in the pulsed splitless mode into a HP-1 capillary column (Agilent J&W GC Columns, Santa Clara, CA, USA - 30 m, 0.25 mm ID, 0.25 μm film thickness). The carrier gas was high purity helium with a flow rate of 0.9 mL/min. The oven was programmed with an initial temperature of 40 °C for 5 min, increasing at 5 °C/min to 150 °C, and then held for 1 min subsequently to 200 °C at 20 °C/min followed by a post-run of 5 min at 250 °C. GCsolution (version 2.32.00, Shimadzu) was used for signal acquisition and peak integration.

The most representative sample of each treatment, selected based on the mean of quantity of compounds and the one which presented less contaminants compared to blank samples were also analyzed by a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer. The GC-MS operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230 °C, source 230 °C, ionization potential 70 eV, scan range 33–280 amu). Briefly, a 2-μl aliquot of each sample was injected in the pulsed splitless mode into a HP-1 capillary column (Alltech Associates, Deerfield, IL, USA - 30 m, 0.25 mm ID, 0.25 μm film thickness). Helium (0.9 mL/min) was used as carrier gas. GC oven temperature was initially held at 40 °C for 3 min, raised to 100 °C at 8 °C/min and subsequently to 200 °C at 5 °C/min followed by a post-run of 5 min at 250 °C. Detected volatiles were identified by comparing their mass spectra with those of the NIST 11 library and with published retention times (López et al. 2012; Anastasaki et al. 2015; De Backer et al. 2015). In addition, the injection of authentic standards (except for carene, δ-elemene and β-elemene) and calculation of the linear retention index (LRI) of each compound were used as an additional criterion for the identification of the compounds.

Data Analysis To investigate whether mirid female's preference differed when various combinations of plant treatments were offered, data were analyzed by Generalized Linear Models with a binomial distribution and a logit-link function. The response variable was the proportion of insects responding to one of the volatile sources. In experiments of single infestation and double infestations, the effect of day was included in the GLM model. For all experiments, we fitted a separate binomial GLM to estimate the proportional response of each predator to test whether their choice was significantly different from a 50% distribution. The significance of the response was tested using a χ^2 test. Volatile emission (relative amounts of individual compounds standardized per unit plant fresh weight) was tested for normality and homogeneity of variances using the Shapiro-Wilk and Bartlett tests, respectively. As distributions, even after log-transformation, did not meet the assumptions for parametric tests volatile emission data were analyzed by the non-parametric *Kruskal-Wallis* test followed by Bonferroni posthoc analysis ($P < 0.05$ and $P < 0.01$). The total emission of volatiles was submitted to one-way analysis of variance (ANOVA) and when significant differences among averages were found, *Tukey's HSD* test at $P < 0.05$ level of significance was applied. Principal component analysis (PCA) was also applied. This projection method determines whether samples collected from different treatment groups can be separated by quantitative and/or qualitative differences in their volatile blends. All statistical analyses were performed using R statistical software (R Core Team 2014).

Results

### Response of *C. infumatus*, *E. varians* and *M. basicornis* to Tomato Volatiles
No influence of experimental day was found on the response of either predator in any of the treatments (GLM, $P > 0.005$). *Macrolophus basicornis*, *E. varians* and *C. infumatus* preferred volatiles from uninfested plants, plants carrying *T. absoluta* eggs, *T. absoluta* larvae-infested, *B. tabaci* infested and *T. absoluta* larvae + *B. tabaci* infested tomato over clean air (Fig. 1, Table 1). In addition, all three mirid predators oriented preferentially to volatiles emitted by *T. absoluta* larvae-infested tomato over uninfested tomato plants (Fig. 1, Table 1), but they did not discriminate between volatiles from plants carrying *T. absoluta* eggs and volatiles from uninfested plants (Fig. 1, Table 1).

Females of *M. basicornis* preferred odors from *B. tabaci*-infested plants over uninfested plants, but *E. varians* and *C. infumatus* did not discriminate between these two treatments (Fig. 1, Table 1). None of the three mirid species discriminated between volatiles from *T. absoluta* larvae + *B. tabaci* infested over *T. absoluta* larvae-infested tomato plants (Fig. 1, Table 1). However, *M. basicornis*, *E. varians* and *C. infumatus* preferred volatiles emitted from plants with double infestation (*T. absoluta* larvae + *B. tabaci*) over the volatile blend from *B. tabaci* infested plants (Fig. 1, Table 1).

Chemical Composition of the Headspace Volatile Blend of Uninfested and Infested Tomato Plants
Egg deposition by *T. absoluta* on tomato induced higher emission levels of eight terpenes: α-pinene, α-terpinene, β-phellandrene, β-ocimene, γ-terpinene, terpinolene, β-caryophyllene and α-humulene

Fig. 1 Responses of *Macrolophus basicornis* (**a**), *Engytatus varians* (**b**) and *Campyloneuropsis infumatus* (**c**) females (*n* = 30/species) to volatiles from uninfested tomato plants (UP), plants carrying *T. absoluta* eggs (TE) or larvae (TL) of *Tuta absoluta* or *Bemisia tabaci* (BT) or double infestation (BT + TL) in a Y-tube olfactometer. The horizontal axis represents the number of predators that moved towards the volatile sources in the corresponding choice situations indicated on the left. NC indicates the number of tested individuals that did not make a choice. ** *P* < 0.01, * *P* < 0.05, ns *P* > 0.05 (GLM, binomial test)
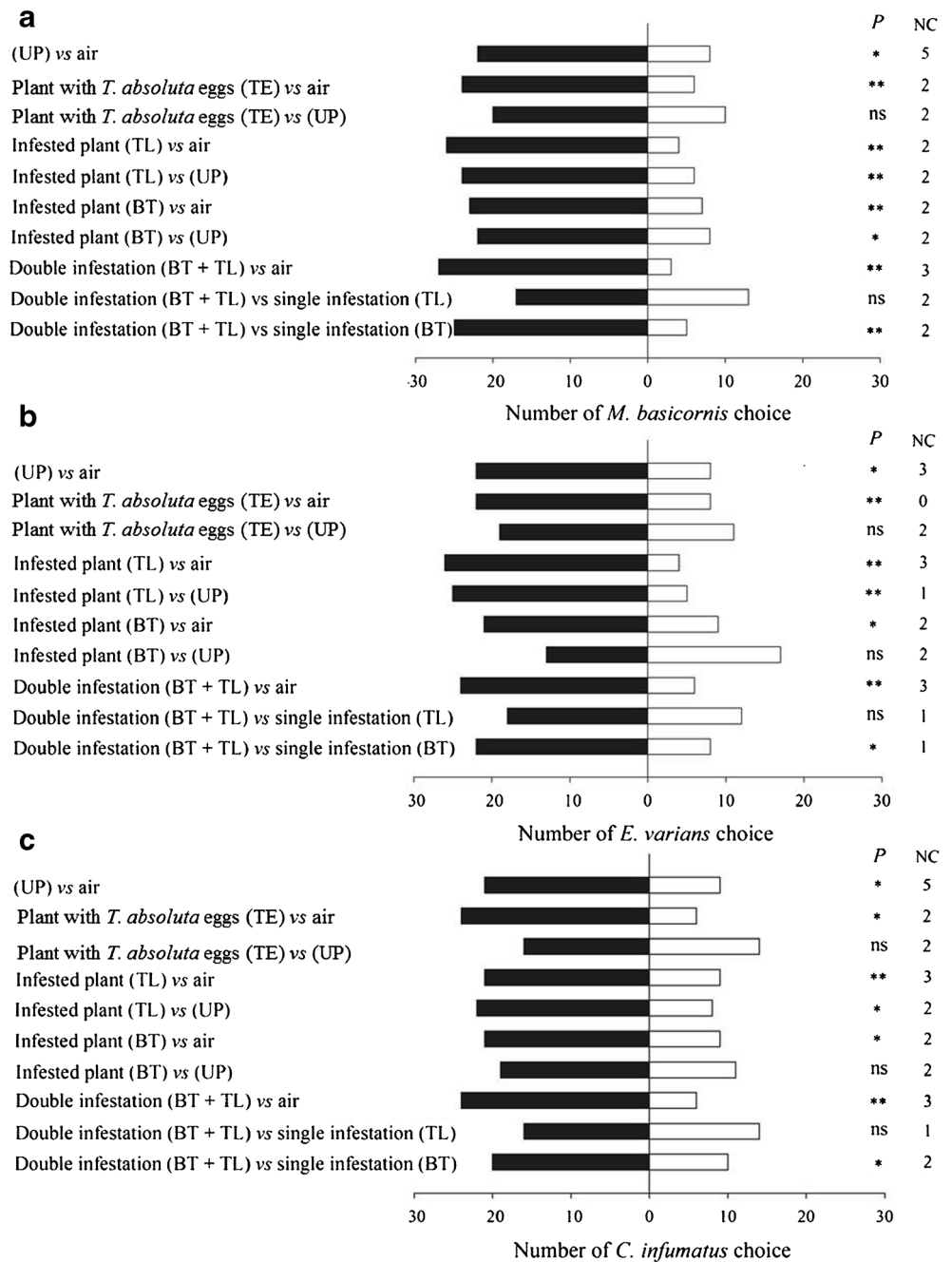
compared to uninfested plants (Table 2, Bonferroni, *P* < 0.05). Leaf feeding by *T. absoluta* larvae, in turn, promoted elevated amounts of volatile emissions, including green leaf volatiles and 12 terpenes when compared to uninfested plants (Table 2, Bonferroni, *P* < 0.05).

Infestation of tomato with *B. tabaci* induced a blend composed of higher concentrations of nine terpenes and the green leaf volatile (*Z*)-3-hexen-1-ol relative to the control (Table 2, Bonferroni, *P* < 0.05). The volatile blend from *B. tabaci* infested plants had six terpenes at lower concentrations, but one at a higher concentration (terpinolene), compared to the

blend emitted by *T. absoluta* larvae-infested plants. δ – Elemene, which was present in the blend of *T. absoluta* larvae-infested plants, was not found in the volatile emission from *B. tabaci* infested plants.

Double infestation (*T. absoluta* + *B. tabaci*) of tomato resulted in augmented emissions of all terpenes found in the tomato HIPV blend, and of (*Z*)-3-hexen-1-ol, relative to the blend from uninfested plants (Table 2, Bonferroni, *P* < 0.05). The blend emitted by double infested plants contained higher concentrations of β-myrcene, limonene, γ-terpinene, terpinolene and β-elemene, but lower levels of α-pinene, compared to the blend

Table 1 Statistical data of dual choice olfactometer assays of *Macrolophus basicornis*, *Engytatus varians* and *Campyloneuropsis infumatus* analyzed by Generalized Linear Models (GLM) with a binomial distribution and a logit-link function

Y-tube olfactometer assay	*M. basicornis*		*E. varians*		*C. infumatus*	
	χ	*P*	χ	*P*	χ	*P*
Uninfested plant (UP) *vs* Air	6.69	< 0.01	8.22	< 0.01	8.93	< 0.01
Plant with *T. absoluta* eggs (TE) *vs* Air	4.01	< 0.05	4.02	< 0.05	9.63	< 0.01
Plant with *T. absoluta* eggs (TE) *vs* UP	0.84	> 0.05	0.55	> 0.05	0.93	> 0.05
Infested plant (TL) *vs* Air	4.21	< 0.05	5.01	< 0.05	4.22	< 0.05
Infested plant (TL) *vs* UP	4.69	< 0.05	4.11	< 0.05	7.69	< 0.01
Infested plant (BT) *vs* Air	4.09	< 0.05	9.33	< 0.01	8.03	< 0.01
Infested plant (BT) *vs* UP	10.22	< 0.01	1.03	> 0.05	0.88	> 0.05
Double infestation (TL + BT) *vs* Air	4.81	< 0.05	3.98	< 0.05	3.63	< 0.05
Double infestation (TL + BT) *vs* Single Infestation (TL)	0.28	> 0.05	0.09	> 0.05	0.29	> 0.05
Double infestation (TL + BT) *vs* Single Infestation (BT)	3.55	< 0.05	11.41	< 0.01	9.82	< 0.01

P values significant differences according to chi-square test

from *T. absoluta* larvae-infested plants (Table 2, Bonferroni, $P < 0.05$). Besides β-myrcene, limonene, γ-terpinene and β-elemene, the emissions from *T. absoluta* + *B. tabaci*-infested plants also contained higher amounts of α-terpinene, β-phellandrene, β-ocimene, δ-elemene and α-humulene than the *B. tabaci*-infested plant blend (Table 2, Bonferroni, $P < 0.05$).

Tuta absoluta larvae-infested and *T. absoluta* + *B. tabaci*-infested plants emitted higher total amounts of volatiles compared to either uninfested plants (up to 32-fold difference) or plants carrying *T. absoluta* eggs (up to 6-fold difference) or *B. tabaci* (up to *ca.* 3-fold difference) (Table 2, Tukey HSD, $P < 0.05$).

Table 2 Relative amounts of volatile emission (mean ± SE ng.g^{-1} shoot fresh weight) emitted by uninfested (UP), plants carrying *Tuta absoluta* eggs (TE), *Tuta absoluta* larvae-infested (TL), *Bemisia tabaci* infested (BT) and *T. absoluta* larvae + *B. tabaci* infested (double infestation - DI) tomato plants. Quantification was based on peak area relative to the internal standard

N	Compound	Chemical Class	Treatments[*]				
			UP (*N* = 6)	TE (*N* = 6)	TL (*N* = 6)	BT (*N* = 6)	DI (*N* = 6)
1	(*E*)-2-hexenal	GLV[***]	0.4 ± 0.1a	0.7 ± 0.1a	5.2 ± 1.1b	2.1 ± 0.9a	4.1 ± 1.5ab
2	(*Z*)-3-hexen-1-ol	GLV	0.4 ± 0.1a	0.4 ± 0.1a	2.5 ± 0.9b	1.7 ± 0.8b	3.3 ± 1.2b
3	α-pinene	Monoterpene	1.3 ± 0.4a	49.6 ± 12.0c	51.9 ± 26.9c	13.8 ± 4.9b	11.1 ± 1.8b
4	β-myrcene	Monoterpene	0.3 ± 0.1a	1.2 ± 0.3a	4.5 ± 2.2a	2.4 ± 0.4a	10.7 ± 3.0b
5	Carene	Monoterpene	13.3 ± 5.3a	92.8 ± 19.4ab	621.5 ± 333.0b	128.9 ± 56.1ab	519.4 ± 145.6b
6	α-terpinene	Monoterpene	4.6 ± 1.8a	15.4 ± 1.9b	125.7 ± 58.6c	12.5 ± 4.8b	85.2 ± 22.5c
7	Limonene	Monoterpene	0.7 ± 0.3a	2.9 ± 0.4a	53.6 ± 18.4b	3.7 ± 0.9a	94.7 ± 23.9c
8	β-phellandrene	Monoterpene	13.3 ± 5.3a	61.1 ± 7.7b	175.9 ± 69.1c	68.0 ± 23.5b	199.5 ± 19.5c
9	β-ocimene	Monoterpene	0.0 ± 0.0a	0.5 ± 0.3a	6.6 ± 2.6c	2.1 ± 0.8b	6.6 ± 1.7c
10	γ-terpinene	Monoterpene	1.4 ± 0.5a	3.1 ± 0.5b	4.2 ± 0.8b	5.0 ± 1.2b	20.2 ± 5.0c
11	Terpinolene	Monoterpene	1.8 ± 0.5a	5.6 ± 0.5b	9.2 ± 2.1b	72.9 ± 40.4c	75.4 ± 40.6c
12	δ-elemene	Sesquiterpene	1.8 ± 0.4a	0.0 ± 0.0a	4.4 ± 1.3b	0.0 ± 0.0a	5.0 ± 1.6b
13	β-elemene	Sesquiterpene	0.0 ± 0.0a	0.0 ± 0.0a	2.8 ± 1.2b	3.1 ± 0.9b	8.3 ± 1.0c
14	β-caryophyllene	Sesquiterpene	1.2 ± 0.3a	6.8 ± 1.4b	34.5 ± 19.6c	26.2 ± 7.2c	24.6 ± 6.7c
15	α-humulene	Sesquiterpene	0.0 ± 0.0a	1.1 ± 0.3a	10.2 ± 3.8c	3.8 ± 1.4b	8.2 ± 1.3c
	Average total peak area[**]	–	2.29 ± 0.95a	11.52 ± 4.26a	73.89 ± 20.56b	26.97 ± 8.23a	51.65 ± 8.45b

[*]Means followed by different letters in the same row indicate a significant difference between treatments according to Kruskal Wallis non-parametric test $P < 0.05$

[**]Means followed by different letters in the same row indicate a significant difference between treatments according to Tukey HSD test $P < 0.05$

[***]GLV = green leaf volatile

The principal component analysis, showed three significant principal components (PC) with the first two explaining 55% and 13% of the total variance, respectively. A plot based on these first and second PC axes revealed a clear separation of blends from uninfested and *T. absoluta* egg- carrying plants from *T. absoluta* + *B. tabaci*-infested plants (Fig. 2).

Discussion

The three mirid predators, *M. basicornis*, *E. varians* and *C. infumatus*, were attracted to tomato plants infested with *T. absoluta* larvae. Although *B. tabaci* is used as prey by these mirids (Van Lenteren et al. 2016), only *M. basicornis* discriminated between *B. tabaci*-infested and uninfested tomato plants. The mirids also did not distinguish double infested (*T. absoluta* + *B. tabaci*) plants from *T. absoluta* larvae-infested plants, but preferred double infested (*T. absoluta* + *B. tabaci*) over *B. tabaci*-infested plants. This demonstrates that the HIPV-profile of *T. absoluta* larvae-infested plants is preferred and that simultaneous infestation with the alternative prey *B. tabaci* does not interfere with attraction.

Chemical analysis of volatile blends emitted by herbivore-infested plants and intact plants demonstrated qualitative and/ or quantitative changes in the emission of infochemicals in

Fig. 2 Principal component analysis (PCA) on volatile composition emitted by uninfested tomato plants (UP), tomato plants infested with: *Tuta absoluta* eggs (TE), *T. absoluta* larvae (TL), *B. tabaci* (BT) or double infestation (DI). Vector numbers correspond with compound numbers in Table 1

several plant-herbivore complexes (Dicke et al. 2009; Fatouros et al. 2012; Poelman et al. 2012; Weldegergis et al. 2015; Silva et al. 2017). Oviposition on tomato plants by *T. absoluta* triggered increased emission of eight terpenes (Table 1). Discrete differences between volatile blends of oviposited and uninfested plants can be perceived by egg parasitoids (Wei et al. 2007; Fatouros et al. 2012; Hilker and Fatouros 2015). However, the generalist mirid predators *C. infumatus*, *E. varians* and *M. basicornis* did not distinguish *T. absoluta* egg-carrying from uninfested plants. Other mirid species such *N. tenuis*, *M. pygmaeus* and *Dicyphus errans* Knight (Lins et al. 2014; Ingegno et al. 2013) were also not attracted to plants carrying *T. absoluta* eggs, suggesting that the lack of attraction of the mirid predators to such plants may be due to a low level of volatile emission. Mollá (2013) found *N. tenuis* attraction towards plants carrying *T. absoluta* eggs, but egg numbers per plant was about four times higher than in our experiments and in those reported by Lins et al. (2014).

Herbivore density and duration of herbivore induction might also result in different HIPV blends (López et al. 2012; Bawin et al. 2014). Tomato plants infested with 15 first instar *T. absoluta* larvae for 24 h emitted increased amounts of several terpenoids such as: α-phellandrene, isoterpinolene, α-cubebene, TMTT and (Z)-nerolidol and the aromatic compound methyl salicylate (Strapasson et al. 2014). In contrast, in the present study the same tomato variety infested with about 245 first instar *T. absoluta* larvae for 72 h did not emit those compounds, but increased the emission of 14 from 15 found compounds (Table 1). Tomato cultivar is another factor that can differentiate the quality and/or quantity of the compounds. For instance, tomato plants (cv. Moneymaker) infested with *T. absoluta* and/or *B. tabaci* released 80 organic compounds (Silva et al. 2017), nine of which were also found in the headspace of the cv. Santa Clara, but in lower quantities. Moreover, six terpenes, α-pinene, carene, β-phellandrene, γ-terpinene, δ-elemene and α-humulene found in the headspace of cv. Santa Clara were not found in the headspace of cv. Moneymaker (Silva et al. 2017). Interestingly, Megido et al. (2014) did not report the mono- and sesquiterpenes γ-terpinene and δ-elemene in the emissions of tomato cv. Moneymaker, but similar to our findings, they found α-pinene, carene, β-phellandrene and α-humulene. HIPVs and changes in the amounts of constitutive VOCs can also be observed in headspace of other tomato cultivars infested with *T. absoluta* or *B. tabaci* (López et al. 2012; Fang et al. 2013; Strapasson et al. 2014).

Immediately upon attack by herbivores, tomato plants enhanced the emission of fatty acid-derived volatile compounds, which are the result of the breakdown of lipids through the lipoxygenase pathway (Shen et al. 2014). Breakdown of plant cell membranes gives rise to free linoleic and/or linolenic acid, both of which are acted upon by lipoxygenase to form C_5 volatile compounds and the C_6 green leaf volatiles (Croft
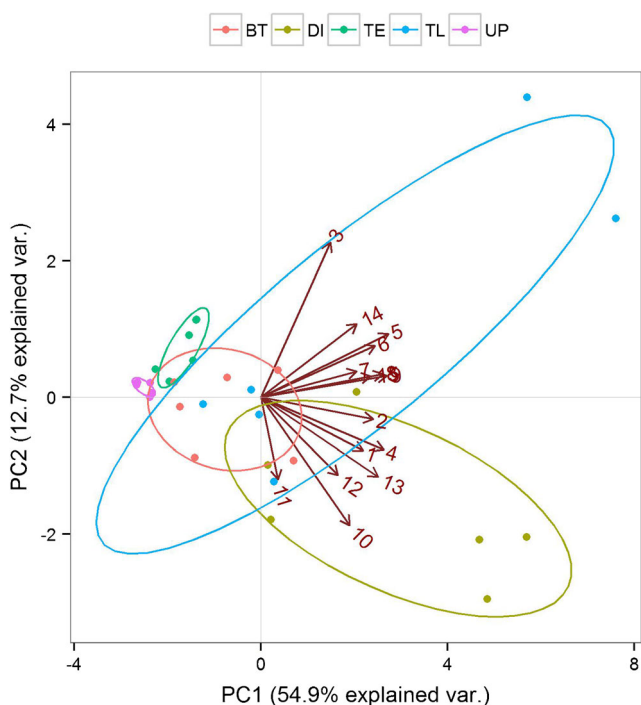
et al. 1993; McCormick et al. 2012; Shen et al. 2014). When released by the plant, these compounds can decrease herbivore feeding rates on tomato (Hildebrand et al. 1993) and can also trigger responses of natural enemies (Dicke et al. 2009).

Tomato infested with *B. tabaci* released also augmented amounts of several terpenes compared to uninfested plants. However, compared to the blend released by *T. absoluta*-larvae-infested-plants, the *B. tabaci*-infested plant blend emitted lower concentrations of six terpenes and a higher concentration of terpinolene. Differences in induction of plant volatiles can be the result of different insect feeding modes, where biting-chewing tomato borers induced higher amounts and higher numbers of compounds than phloem sucking whiteflies (Silva et al. 2017), which might influence mirid choices (Lins et al. 2014).

The three mirid species might benefit more from orientation to the *T. absoluta*-larvae infested HIPV blend than by the blend emitted by *B. tabaci*-infested plants, since *T. absoluta* larvae are much larger prey items than whitefly eggs and nymphs. Several studies have shown the benefit to mirid bugs of preying on lepidopteran eggs and larvae (Devi et al. 2002; Urbaneja et al. 2009; Hamdi and Bonato 2014; Silva et al. 2016). An alternative explanation might be that we tested naïve adults of the three mirids and that *E. varians* and *C. infumatus* will react to *B. tabaci*-infested plant volatiles after an associative learning experience like the mirid predator *N. tenuis*, which was attracted by volatiles from *B. tabaci*-infested plants only after experience with this prey (Lins et al. 2014). Moreover, in our study, mirids were maintained on tobacco plants, not being exposed to any type of conditioning to tomato volatiles. According to a recent study (Rim et al. 2017), mirids, in laboratory rearing, likely associate the availability of *E. kuehniella* eggs with the volatiles emitted from the host plant, in a way they become conditioned to the plant species. Therefore, it is possible that tomato-reared mirids may have their ability to recognize infested tomato volatile blends enhanced and, unlike tobacco-reared insects, would discriminate volatiles of *B. tabaci*-infested from uninfested tomatoes.

To locate prey in a complex system that undergoes changes in plant-derived odour cues due to single or multiple pest infestation, mirid females could rely on learning abilities to enhance responses. Insect learning is a well-known and widely studied experience-based modification of behavior (Steidle and van Loon 2003; De Boer et al. 2005; Glinwood et al. 2011; Rim et al. 2015), in particular for parasitoids (Vet and Dicke 1992). Predatory mirid bugs have only recently been studied, and indications for associative learning have been reported (Lins et al. 2014). Also, some studies show that the attack of multiple herbivores species can lead to a different composition of HIPV blends, and consequently being more attractive to natural enemies than plants infested by a single herbivore species (Rodriguez-Saona et al. 2005; Moayeri et al. 2007; Cusumano et al. 2015). Furthermore, simultaneous

feeding by herbivores with different feeding modes (biting-chewing or piercing-sucking) may interfere with the attraction of natural enemies (Zhang et al. 2009; Schwartzberg et al. 2011). Results in this study do not confirm our initial hypothesis that the whitefly *B. tabaci* sharing the same host with the larvae of the tomato borer *T. absoluta* suppress plant volatile emission compared to the emission by plants infested with the tomato borer only. Indeed, infestation with *T. absoluta* + *B. tabaci* in tomato triggered an overall increase of terpene emissions compared to infestation by either of the two single herbivores. Although we infested tomato plants with 50 whitefly adults (or 8–10 adults/leaf), which fed and reproduced for 10 days on the same plants, the level and/or duration of whitefly infestation may still have not been enough for suppressing the JA-signaling pathway. Previous studies that detected the repression of JA-responsive genes by the activation of SA-signaling pathway infested plants with higher densities of *B. tabaci* adults (more than 13 adults/leaf) and for longer periods (12–14 days), coinciding with feeding by second- to third-instar nymphs (Estrada-Hernández et al. 2009; Zhang et al. 2009, 2013).

The three mirids did not distinguish between *T. absoluta* + *B. tabaci*-infested and *T. absoluta*-infested plant volatile blends, demonstrating that double infestation by *T. absoluta* + *B. tabaci* does not increase attraction to these mirid predators compared to plants infested with *T. absoluta* only when no previous experience with the plant-prey combinations had occurred. Similarly, two other mirid predators (*M. pygmaeus* and *N. tenuis*) did not distinguish double-infested (*T. absoluta* + *B. tabaci*) from single-infested (*T. absoluta* or *B. tabaci*) tomato plants, and this behavioral pattern did not change after experience (Lins et al. 2014).

The PCA analysis showed differences in blends of uninfested over double infested tomato plants, indicating an alteration of volatile blend after the oviposition and/or feeding by *T. absoluta* and by *B. tabaci*. The vector for β-myrcene, γ-terpinene, terpinolene, δ-elemene and β-elemene was correlated with the volatile samples from *T. absoluta* + *B. tabaci*-infested plants in the PCA plot (Fig. 2) which is in line with their higher quantities in headspace samples compared to the other treatments (Table 1). We suggest that higher amounts of those compounds on double infestation mostly contributed to the separation of these treatments in the PCA. In addition, the vector for α-pinene was correlated with the samples from *T. absoluta*-infested plants, and also presented higher quantities in headspace samples compared to the other treatments (Table 1). High amounts of this compound were also found in other studies (Degenhardt et al. 2010; Megido et al. 2014; Fang et al. 2013; Strapasson et al. 2014), but small quantities or absence of this compound have also been reported (López et al. 2012; Silva et al. 2017).

Due to the complexity of HIPV blends emitted by single- and double-infested tomatoes, it is difficult to select volatile

compounds that potentially play a role in mirid attraction. In this study, combining the information on the attraction of mirids to *T. absoluta* larvae-infested and *T. absoluta* + *B. tabaci*-infested plants, and contrasting it with the lack of attraction to plants carrying *T. absoluta* eggs and *B. tabaci*-infested plants over uninfested plants, suggests that α-terpinene, limonene, β-phellandrene and δ-elemene are important compounds for attraction of these mirids. This hypothesis should be addressed in future studies by manipulating the concentration of these single terpenes in the blend from an otherwise non-attractive plant in behavioral assays, and/or testing different mixtures and concentrations of single synthetic compounds in such assays.

The results of this study show that volatiles of tomato infested with *T. absoluta* larvae attract all three mirid species. *Engytatus varians* and *C. infumatus* did not respond innately to HIPVs from *B. tabaci*-infested tomato. Multiple herbivory by herbivores of two different feeding guilds – chewing (*T. absoluta*) and phloem-sucking insects (*B. tabaci*) – did neither increase, nor decrease attraction of *C. infumatus*, *E. varians* and *M. basicornis*. In addition, *M. basicornis* is likely more efficient in finding infested tomato plants for control of *B. tabaci*, unless the two other mirids (*C. infumatus* and *E. varians*) quickly learn to associate HIPVs emitted by *B. tabaci* infested tomato plants with prey availability.

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