Research Article

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Two in one: the neotropical mirid predator *Macrolophus basicornis* increases pest control by feeding on plants

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Abstract

BACKGROUND: Plant defenses activated by European zoophytophagous predators trigger behavioral responses in arthropods, benefiting pest management. However, repellence or attraction of pests and beneficial insects seems to be species-specific. In the neotropical region, the mirid predator *Macrolophus basicornis* has proved to be a promising biological control agent of important tomato pests; nevertheless, the benefits of its phytophagous behavior have never been explored. Therefore, we investigated if *M. basicornis* phytophagy activates tomato plant defenses and the consequences for herbivores and natural enemies.

RESULTS: Regardless of the induction period of *M. basicornis* on tomato plants, *Tuta absoluta* females showed no preference for the odors emitted by induced or control plants. However, *Tuta absoluta* oviposited less on plants induced by *M. basicornis* for 72 h than on control plants. In contrast, induced plants repelled *Bemisia tabaci* females, and the number of eggs laid was reduced. Although females of *Trichogramma pretiosum* showed no preference between mirid-induced or control plants, we observed high attraction of the parasitoid *Encarsia inaron* and conspecifics to plants induced by *M. basicornis*. While the mirid-induced plants down-regulated the expression of genes involving the salicylic acid (SA) pathway over time, the genes related to the jasmonic acid (JA) pathway were up-regulated, increasing emissions of fatty-acid derivatives and terpenes, which might have influenced the arthropods' host/prey choices.

CONCLUSION: Based on both the molecular and behavioral findings, our results indicated that in addition to predation, *M. basicornis* benefits tomato plant resistance indirectly through its phytophagy. This study is a starting point to pave the way for a novel and sustainable pest-management strategy in the neotropical region.
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Keywords: *Macrolophus basicornis*; herbivore-induced plant volatiles; biological control; zoophytophagous predators; plant defenses

1 INTRODUCTION

Zoophytophagous predatory bugs (Hemiptera: Heteroptera) have been used successfully in biological control programs and have been commercially available since the 1990s.[1–3] The families Anthocoridae and Miridae contain species that efficiently regulate herbivore population densities and reduce plant damage caused by a wide range of agricultural and forest pests.[3, 4] Mirid predators such as *Nesidiocoris tenuis* (Reuter), *Dicyphus hesperus* Knight, and *Macrolophus pygmaeus* Rambur, for instance, are used in both applied and conservative biological control programs in European countries and the United States to control mites, thrips, and two important tomato pests, the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) and the leafminer *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae).[5–7]

Studies on zoophytophagous mirid predators as potential biological control agents in the neotropical region have increased in the last 10 years.[8–12] Among the neotropical species, *Macrolophus basicornis* Stal (Hemiptera: Miridae) is the most promising predator for the major tomato pests, including *Bemisia tabaci* and *Tuta absoluta*.[13–15] *Macrolophus basicornis* has also been shown to be attracted to tomato plant volatiles[16] and does not cause economic damage to tomato plants due to phytophagy,[13] which makes this species a potentially safer candidate than the widely used European species *Nesidiocoris tenuis*, which can become a serious pest in certain circumstances.[3] Mirid phytophagy has the advantages of facilitating its establishment in the crop when preys are scarce.[17]

When attacked by herbivores, plants induce specific defenses. Induced plant defenses can directly (e.g. producing toxins and

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deterrents) and indirectly (e.g. releasing volatile compounds) affect the performance and behavior of herbivores.[18] Insect behavior is mainly affected by herbivory-induced plant volatiles (HIPVs), a complex blend of secondary metabolites released into the air by plants after being attacked.[19] Synthesis of these HIPVs is mediated by complex chemical pathways, most notably the jasmonic acid (JA) and salicylic acid (SA) pathways, and the resulting blend of compounds can be used as cues by both herbivores and natural enemies, potentially affecting the local community of arthropods.[19,20] The ways that arthropods respond to these HIPVs have significant ecological consequences for species distribution and diversity,[21] as well as evolutionary consequences.[20] Knowledge of these responses can be applied to pest management, mainly biological control programs.[22–25]

Tritrophic interactions mediated by HIPVs occurs in a complex, species-specific way.[26] Numerous recent studies have shown that this is also the case for interactions involving European zoophytophagous mirids in biological control programs (reviewed by Pérez-Hedo *et al.*).[3] For example, in tomato plants, *Nesidiocoris tenuis* induced the JA, SA, and ABA pathways, repelling *Bemisia tabaci* and *Tuta absoluta* while attracting the parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae).[27] In contrast, phytophagy by *D. bolivary* and *M. pygmaeus* induced only the JA pathway, and the volatiles released attracted the main tomato pest, the leafminer *Tuta absoluta*, while *Bemisia tabaci* was indifferent.[28] Similar studies conducted with sweet pepper plants had different results, with *Nesidiocoris tenuis* and *M. pygmaeus* HIPVs repellent to both *Bemisia tabaci* and *Tuta absoluta*, and attractive to *Encarsia formosa*.[29] Different responses of herbivores and natural enemies to induced plant defenses help to highlight the importance of species-specific studies to guide biological control programs using zoophytophagous mirid predators. Whether phytophagy by the neotropical mirid predator *M. basicornis* mediates defense-related signaling pathways in tomato and thus increases resilience to damage from its pests has not been investigated.

Given how herbivores and parasitoids were found to respond to plants exposed to mirids,[3] we hypothesized that herbivorous pests would avoid tomato plants exposed to *M. basicornis*, while natural enemies would be attracted. Therefore, we initially tested if (1) the feeding activity of *M. basicornis* on tomato plants changed the behavioral response of two herbivorous species, *Bemisia tabaci* and *Tuta absoluta*, and their natural enemies: one predator, *M. basicornis*; and two parasitoids, *Encarsia inaron* and *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), (2) we then investigated whether mirid phytophagy could induce defensive responses in tomato plants using gene-expression analysis; and (3) we measured whether tomato plants exposed to *M. basicornis* changed the profile of their volatile compounds. The results of our study may provide insight into the complex plant–zoophytophage systems and further optimize its capacity as a potential biological control agent of key pests on tomato.

2 MATERIALS AND METHODS

2.1 Plant and insects

Seeds of tomato (*Solanum lycopersicon* L. cv. Santa Clara) were sown on sterilized substrate (Tropstrato HA Hortaliças, Vida Verde, Mogi Mirim, Brazil) and irrigated daily. Seedlings 1 cm high were transplanted to 1.8-L plastic pots, each containing that substrate mixed with 20 g NPK 4-14-8 Vitaplant® fertilizer (Nutriplan, Cascavel, Brazil). Tomato plants were maintained in a glasshouse [25 ± 2 °C, 70 ± 10% relative humidity (RH), 12 h:12 h light (L)/dark (D)] and irrigated daily. Plants 25 days old, 20 cm tall, and with five expanded leaves were used in the experiments.

The population of *Tuta absoluta* was provided from the stock colony maintained at the Insect Biology Laboratory, ESALQ/USP (Piracicaba, Brazil). Colonies were maintained in cages (60 cm × 30 cm × 30 cm) containing tomato plants. Larvae and adults were kept separately in cages (60 cm × 30 cm × 30 cm) covered with fine mesh and regularly supplied with tomato plants for feeding and oviposition. Newly emerged females (3–5 days old) were placed in individual glass vials (Ø 6 cm × height 6 cm) and starved for 2 h prior to the experiments.

Whiteflies, *Bemisia tabaci* biotype B, were provided from the stock colony maintained at the Agronomic Institute of Campinas – AIC (Campinas, Brazil). Colonies were maintained in the glasshouse (conditions as earlier) in insect-proof cages (60 cm × 40 cm × 40 cm) on cabbage plants (*Brassica oleracea* L. var. *acephala* DC. cv. Manteiga). Adults were removed with a manual aspirator, and females 3–5 days old were placed in individual glass vials (Ø 6 cm × height 6 cm) and starved for 2 h prior to the experiments.

The population of *Encarsia inaron* was obtained from the rearing stock maintained in nymphs of *Bemisia tabaci*. Colonies were maintained in the glasshouse (conditions as earlier) in insect-proof cages (60 cm × 40 cm × 40 cm) at the Insect Biology Laboratory, ESALQ/USP. Newly emerged females (1–3 days old) of *Encarsia inaron* were separated based on antenna structure,[30] placed in individual glass vials (Ø 6 cm × height 6 cm), and starved for 2 h prior to the experiments.

The population of *Trichogramma pretiosum* used in the experiment was obtained from the rearing stock maintained in eggs of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) at the Insect Biology Laboratory, ESALQ/USP. Newly emerged females (1–3 days old) of *Trichogramma pretiosum* were separated based on antenna structure, placed in individual glass vials (Ø 6 cm × height 6 cm), and starved for 2 h prior to the experiments.

The stock colony of *M. basicornis* was kept at the Insect Biology Laboratory, ESALQ/USP following the methodology of Bueno *et al.*[31] Briefly, the predator was maintained in acrylic cages (60 cm × 30 cm × 30 cm) containing eggs of *Ephestia kuehniella* for *ad libitum* feeding and tobacco plants (*Nicotiana tabacum* L. cv. TNN) as an oviposition substrate and water source. After 7 days, tobacco plants containing eggs were transferred to new cages where nymphs hatched and fed on *Ephestia kuehniella* eggs until reaching the adult stage. Females 1–7 days old were placed in individual glass vials (Ø 6 cm × height 6 cm) and starved for 24 h prior to the experiments.

2.2 Treatments

To test whether exposure of tomato plants to *M. basicornis* can affect its herbivores and natural enemies, plants were individually placed in acrylic cages (60 cm × 30 cm × 30 cm) which contained 25 adults, all less than 4 days old (sex ratio 1:1). The omnivores could freely move, walk, feed, and lay eggs on the plants.

The predators were allowed to feed on the plants for: (i) 24 h or (ii) 72 h. They were removed prior to the experiment. (iii) Control plants were placed inside cages without predators. All treatments were kept in a different room during the induction periods.

2.3 Olfactometer assays

The responses of herbivores and natural enemies to HIPVs were assessed in glass Y-tube olfactometers. The olfactometers were positioned horizontally and connected to an ARS volatile collection system [Analytical Research Systems (ARS), Gainesville, FL, USA]. Each olfactometer side arm was connected to a 15-L glass vessel containing a single tomato plant. Plastic pots (1.8 L) in which the tomato plants were growing were wrapped with aluminum foil. The glass vessels were kept behind a black panel to prevent the insects from visually detecting the plants. Four supplemental 120-cm lights (GreenPower LED DR/W 18 W, Philips, Amsterdam, the Netherlands) were positioned 50 cm above the olfactometer to provide uniform light (total light intensity of 40 μmoles m^{-2} s^{-1}) during the test period. The environmental conditions in the Y-tube experiments were 25 ± 2 °C and 70 ± 10% RH.

To evaluate the behavioral responses of *Tuta absoluta*, *Bemisia tabaci*, and *M. basicornis* females, a glass Y-tube olfactometer (4.0 cm in diameter, main arm 9 cm long, side arms 10 cm long, 70° angle between side arms) was used, and the inlet air flow was adjusted to 0.8 L min^{-1} for each side arm. The responses of *Trichogramma pretiosum* and *Encarsia inaron* females were assessed in a glass Y-tube olfactometer (5 mm in diameter, main arm 5 cm long, side arms 4.5 cm long, 70° angle between side arms) and the inlet air flow was adjusted to 0.2 L min^{-1} for each side arm.

Each female was introduced into the main arm of the olfactometer and observed for up to 10 min. Herbivores and natural enemies were considered to have made a choice when they crossed a line drawn 3 or 8 cm (depending on the species) from the branching point of the Y-tube. Females that did not choose a side arm within 10 min were considered to be non-responsive and were excluded from the data analysis. Each female was tested only once. After every second individual tested, the odor sources were interchanged to avoid positional bias. After ten females were tested, the Y-tube and glass vessels were washed with neutral soap and ethanol (70%) and dried. A total of 30 replicates (responses) were performed for each treatment and arthropod species, using at least three pairs of tomato plants on three different days. Each day was treated as a block in a randomized complete block design. The bioassays of both arthropod species were carried out in a climate-controlled room at 25 ± 2 °C and 70 ± 10% RH, between 10:00 and 12:00 h and again between 14:00 and 16:00 h.

The following combinations were tested: (i) intact plants that were undisturbed and isolated from arthropods until use, (ii) plants exposed to *M. basicornis* for 24 h, and (iii) plants exposed to *M. basicornis* for 72 h.

2.4 Effect of tomato plants exposed to *M. basicornis* on herbivore oviposition

Oviposition of *Tuta absoluta* and *Bemisia tabaci* was evaluated for ten tomato plants exposed to *M. basicornis* for 24 h or 72 h, and for intact control plants. Using a mouth aspirator, six whitefly couples were transferred to an acrylic cage (60 cm × 30 cm × 30 cm) containing one tomato plant that previously received one of the earlier treatments. Three *Tuta absoluta* couples were also placed in acrylic cages (60 cm × 30 cm × 30 cm) containing one tomato plant. All treatment plants were left undisturbed for 24 h. After this period, the tomato plants were inspected for the number of eggs laid. This experiment was repeated in two blocks at a time, with five plants per treatment in each block. The cages were kept in a climate-controlled room at 25 ± 2 °C and 70 ± 10% RH with 12 h:12 h L/D.

2.5 Plant gene-expression analysis

The apical part of the tomato plants (cv. Santa Clara), defined as the first 5 cm of the plant formed by the apical developing stem and leaves, was subjected to targeted gene-expression analysis to detect: (1) *PR1* (basic PR1 protein precursor) and PAL (phenylalanine ammonia-lyase) marker genes for the SA signaling, and (2) *PIN2* (wound-induced proteinase inhibitor II precursor) and LoxD (lipoxygenase D), marker genes for the JA pathway. *EF1* (elongation factor-1) was used as a standard control gene for normalization. The nucleotide sequences of the gene-specific primers are described in Table 1. Five samples of the apical part of the tomato plant, as defined earlier, were collected from tomato plants exposed to *M. basicornis* for 24 h, tomato plants exposed to *M. basicornis* for 72 h, and control plants, and were removed and immediately ground in liquid nitrogen or stored at −80 °C. For each leaf sample, 100 mg was used to extract the total RNA, with minor modifications to increase yield. The total RNA from tomato leaf tissue was isolated with the RNeasy Plant mini kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. The residual DNA was removed by treatment with one unit of RNase-free DNase I (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA samples were quantified using a NanoDrop 2000 (Thermo Fisher Scientific), and their quality was assessed by agarose gel electrophoresis. Complementary DNA (cDNA) was synthetized from 1 μg of total RNA by the ImProm-II Reverse Transcriptase (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Qualitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a StepOne Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The reference gene was re-validated under experimental conditions. The relative expression levels between the control *versus* treatment and gene-amplification efficiencies and statistical analyses were calculated according to Pfaffl.[34] Five biological replicates and two technical replicates were used.

2.6 Headspace collection and analysis of plant volatiles

Volatiles from the different groups of control and tomato plants exposed to *M. basicornis* [treatments (i)–(iii) described earlier] and pots filled with soil (blank) were collected under laboratory

Table 1. Forward and reverse primer sequences of marker genes and the constitutive gene *EF-1a*

Gene	Primer forward (5′ → 3′)	Primer reverse (5′ → 3′)	Reference
EF1	5-GATTGGTGGTATTGGAACTGTC-3	5-AGCTTCGTGGTGCATCTC-3	[32]
PAL	5- TTCGAGTTGCAGCCTAAGGAAGGA -3	5- ATAGCAGCAGCCTCAATCTGACCA-3	[101]
PR1	5-CCGTGCAATTGTGGGTGTC-3	5-GAGTTGCGCCAGACTACTTGAGT-3	[33]
LOXD	5-GTTCATGGCCGTGGTTGACACATT-3	5- TGGTAATACACCAGCACCACACCT -3	[101]
PIN2	5-GAAAATCGTTAATTTATCCCAC-3	5-ACATACAAACTTTCCATCTTTA-3	[32]

conditions at 24 ± 1 °C and 70 ± 10% RH, from 10:00 to 12:00 h and from 14:00 to 16:00 h, in a push-pull volatile collection system (ARS).

Before the volatiles were collected, the plant pots were carefully wrapped with aluminum foil to avoid trapping volatiles from the plastic and soil, and were enclosed individually in 15-L glass vessels. Plant 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 hexane (Merck, Kenilworth, NJ, USA) mixed with 30 μL of nonyl acetate solution (Sigma-Aldrich, St Louis, MO, USA) at 10 ng μL^{-1}, used as the internal standard. All extracts were stored at −80 °C for 3–5 days until analysis. Immediately after the volatiles were collected, the dry weight of the plant shoot was determined.

The headspace analysis was performed by gas chromatography (GC-2010 Gas Chromatograph; Shimadzu, Kyoto, Japan) with a flame ionization detector (GC-FID) operated at 280 °C. Quantification was based on comparing the area under the GC-FID peak with the internal standard, and standardized per unit of dry-shoot biomass (in grams) of each replicate. Briefly, a 2-μL aliquot of each sample was injected in the pulsed splitless mode into an HP-1 capillary column [Agilent J&W GC Columns, Santa Clara, CA, USA; 30 m, 0.25 mm inner diameter (ID), 0.25 μm film thickness]. The carrier gas was high-purity helium with a flow rate of 0.9 mL min^{-1}. The oven was programmed with an initial temperature of 40 °C for 5 min, raised at 5 °C min^{-1} to 150 °C, held for 1 min, and then raised to 200 °C at 20 °C min^{-1}, followed by a post-run of 5 min at 250 °C. GC solution (version 2.32.00, Shimadzu) was used for signal acquisition and peak integration.

A 2-μL aliquot of each sample was also run in a gas chromatograph coupled to a mass spectrometer (Agilent 6890 Series GC system G1530A). The gas chromatography–mass spectrometry (GC–MS) operated in electron impact mode (Agilent 5973 Network Mass Selective Detector, transfer line 230 °C, 230 °C sources, 70 eV ionization potential, 33–28 amu scan range). Each sample was injected in the pulsed splitless mode into an HP-1 capillary column (Alltech Associates, Deerfield, IL, USA; 30 m, 0.25 mm ID, 0.25 μm film thickness), using the same GC-FID method. The volatiles detected were identified by comparing their mass spectra with those of the NIST 11 library, when available [asterisks (*) on Table 2], to those of the synthetic standards and calculation of each compound's linear retention index (LRI) were used as supplemental criteria for identification.

2.7 Data analysis

To investigate insect preferences when various combinations of volatile sources (olfactometer experiment) were offered, the data sets were analyzed with a chi-square (χ^2) goodness-of-fit test. The response variable was the proportion of insects responding to one of the odor sites, with the null hypothesis that the treatments chosen would show a 50% distribution. Prior to analysis, the raw data were tested for normality and homogeneity of variances, using the Shapiro–Wilk and Bartlett tests, respectively. One-way analysis of variance (ANOVA) followed by a comparison of means (Tukey test) was applied to compare the oviposition rates and to identify differences in the transcriptional responses of defense genes among the three treatments, a *t*-test was performed. The volatile emissions were initially tested for normality and homogeneity of variances, using the Shapiro–Wilk and Bartlett tests,

Table 2. Relative amounts of volatile emissions (mean ± standard error, ng g^{-1} shoot fresh weight) by *Macrolophus basicornis* induced tomato plants for 24 h, *M. basicornis* induced tomato plants for 72 h and control plants. Quantification was based on the peak area relative to the peak area of the internal standard. Plant headspaces are given separately and grouped according to their chemical type

		Treatments		
Compound number	Compounds	Control (*n* = 6)	*Macrolophus basicornis* 24 h (*n* = 6)	*Macrolophus basicornis* 72 h (*n* = 6)
	Fatty acid derivatives			
1	(*Z*)-3-Hexenol*	0.77 ± 0.17	1.82 ± 0.64	2.40 ± 0.65
2	(Z)-3-Hexenyl* propanoate	n.d.	0.64 ± 0.22	1.84 ± 0.08
3	Hexenyl acetate*	0.97 ± 0.22	0.94 ± 0.05	1.13 ± 0.24
	Total	**1.74 ± 0.39b**	**3.40 ± 0.91ab**	**5.37 ± 0.96a**
	Terpenes			
4	α-Pinene*	5.31 ± 2.07	6.76 ± 2.14	29.44 ± 7.69
5	Myrcene*	0.64 ± 0.23	1.73 ± 0.26	4.23 ± 1.17
6	Carene	24.39 ± 12.15	60.18 ± 23.17	291.76 ± 73.47
7	Terpinene*	4.61 ± 2.08	7.78 ± 1.26	16.81 ± 4.42
8	Cymene	2.15 ± 0.85	1.18 ± 0.29	5.10 ± 1.23
9	β-Phellandrene*	37.5 ± 21.22	160.5 ± 61.97	753.3 ± 180.37
10	Limonene*	5.53 ± 1.28	0.31 ± 0.24	0.38 ± 0.08
11	Terpinolene*	n.d.	0.61 ± 0.09	5.80 ± 1.81
12	Elemene	0.39 ± 0.16	0.56 ± 0.18	2.64 ± 0.71
13	Caryophyllene*	0.82 ± 0.48	1.79 ± 0.37	3.90 ± 0.86
14	Humulene	1.71 ± 0.74	4.20 ± 1.06	8.16 ± 2.09
	Total	**83.05 ± 41.26b**	**244.6 ± 91.08b**	**1101.02 ± 274,14a**
	Total production	**84.79 ± 42.01b**	**247,7 ± 91,94b**	**1106.39 ± 275.1a**

Means followed by different letters indicate a significant difference between treatments based on the non-parametric Kruskal–Wallis test ($P < 0.05$) and subsequent pairwise comparisons using the Monte Carlo method. *Compounds confirmed by synthetic standards. n.d., not detected.

respectively. As the distributions did not meet the assumptions for the parametric tests even after transformation, the data were analyzed using the non-parametric Kruskal–Wallis test followed by the average fit test of the Monte Carlo method. The quantity of each compound was considered to differ among treatments based on a non-overlap of 1.5 times each standard error around the means.[35, 36] A principal components analysis (PCA) was then performed to evaluate whether the treatment groups could be separated by quantitative and/or qualitative differences in their volatile blends. The composition of the volatiles was analyzed using R software version 3.1.1 (www.R-project.org).

3 RESULTS

3.1 Olfactometer assays

The leafminer *Tuta absoluta* showed no preference for the odor emitted by intact tomato plants compared to plants exposed to the mirid predator *M. basicornis* (Fig. 1(A); $\chi^2 = 1.2$; $P = 0.273$; $\chi^2 = 0.533$; $P = 0.465$). Also, differences in the period of exposure to *M. basicornis* (24 or 72 h) did not result in odor preferences (Fig. 1(A); $\chi^2 = 1.2$; $P = 0.273$). In contrast, the phloem-feeder *Bemisia tabaci* was significantly repelled by tomato plants exposed to *M. basicornis* (Fig. 1(B); $\chi^2 = 8.53$; $P = 0.003$; $\chi^2 = 4.8$; $P = 0.028$), but with no significant difference in repellence between plants exposed for 24 h and those exposed for 72 h (Fig. 1(B); $\chi^2 = 0.53$; $P = 0.465$).

The parasitoid *Trichogramma pretiosum* showed no preference for the odor emitted by intact tomato plants compared to plants exposed to *M. basicornis* (Fig. 2(A); $\chi^2 = 2.13$; $P = 0.144$; $\chi^2 = 0$; $P = 1$). Also, differences in the mirid exposure period (24 or 72 h) did not result in odor preferences (Fig. 2(A); $\chi^2 = 0$ $P = 1$). However, tomato plants exposed to *M. basicornis* were highly attractive to the parasitoid *Encarsia inaron* relative to intact tomato plants (Fig. 2(B); $\chi^2 = 10.8$; $P = 0.001$; $\chi^2 = 4.8$; $P = 0.028$). Conspecific attraction was observed only when *M. basicornis* fed on tomato plants for a longer period (72 h) compared to intact tomato plants (Fig. 2(C); $\chi^2 = 13.33$; $P = 0.001$). No mirid preference was reported when *M. basicornis* individuals fed on tomato plants for 24 h, over intact plants (Fig. 2(C); $\chi^2 = 2.13$; $P = 0.144$). Neither *Encarsia inaron* nor *M. basicornis* showed a preference between tomato plants exposed to *M. basicornis* for different periods (24 h *versus* 72 h) (Fig. 2(B); $\chi^2 = 0.032$; $P = 0.857$; Fig. 2 (C); $\chi^2 = 3.33$; $P = 0.067$, respectively).

3.2 Effect of tomato plants exposed to *M. basicornis* on herbivore oviposition

The numbers of *Tuta absoluta* and *Bemisia tabaci* eggs were significantly affected by exposure of tomato plants to *M. basicornis* ($F = 10.64$; df = 2; $P < 0.001$; $F = 15.31$; df = 2; $P < 0.001$ respectively). While the number of *Tuta absoluta* eggs decreased only on plants exposed for 72 h (Fig. 3(A); Tukey test: $P = 0.05$), *Bemisia tabaci* egg deposition was reduced after both periods (24 and 72 h) compared to intact tomato plants (Fig. 3(B), Tukey test: $P = 0.05$).

3.3 Plant gene-expression analysis

The relative gene expression levels of SA-related and JA-related marker genes were analyzed. PAL transcript levels increased after 24 h of *M. basicornis* tomato-plant exposure, and decreased at 72 h (Fig. 4(a)). The PR1 gene was down-regulated in comparison to intact control tomato plants and showed no difference

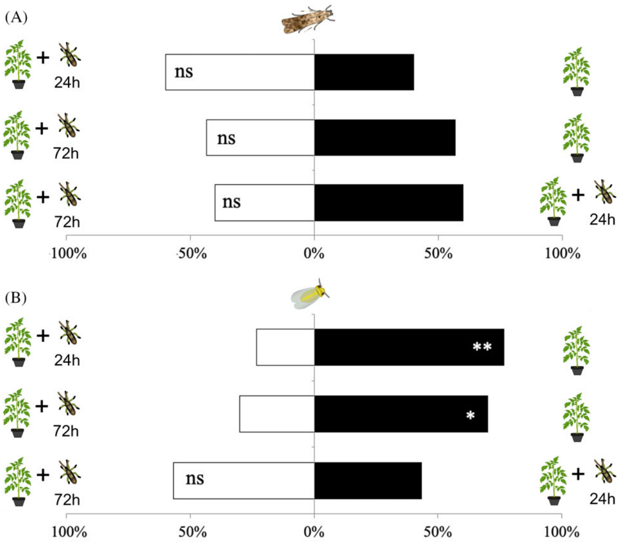

Figure 1. Responses of (A) *Tuta absoluta* females ($n = 30$) and (B) *Bemisia tabaci* females ($n = 30$) to volatiles from intact tomato plants, tomato plants exposed to *Macrolophus basicornis* for 24 h, and tomato plants exposed to *M. basicornis* for 72 h, in a Y-tube olfactometer. The horizontal axis represents the number of herbivores that moved toward the volatile sources. Chi-square (χ^2) test: ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$.

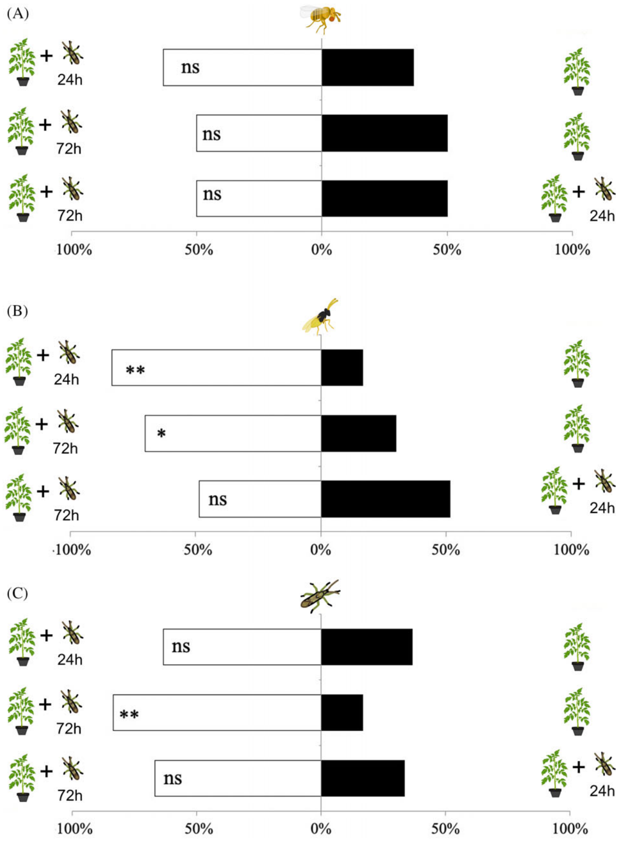

Figure 2. Responses of (A) *Trichogramma pretiosum* females ($n = 30$), (B) *Encarsia inaron* females ($n = 30$), and (C) *Macrolophus basicornis* females ($n = 30$) to volatiles from intact tomato plants, tomato plants exposed to *M. basicornis* for 24 h, and tomato plants exposed to *M. basicornis* for 72 h, in a Y-tube olfactometer. The horizontal axis represents the number of natural enemies that moved toward the volatile sources. Chi-square (χ^2) test: ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$.

between treatments (Fig. 4(b)). The JA-related gene LoxD showed down-regulation compared to the control after 24 h of induction and was up-regulated after 72 h (Fig. 4(c)), while the PIN2 gene was up-regulated after 24 h compared to the control, with an increase in gene expression after 72 h of induction (Fig. 4(d)).

3.4 Headspace collection and analysis of plant volatiles

The herbivore-induced volatile profiles of *M. basicornis*-exposed and intact control tomato plants differed both quantitatively and qualitatively. The induction time also affected the profiles. Overall, analysis of the volatiles emitted by the tomato plants showed a blend consisting of 14 major compounds, of which 12 were common to all treatments but in different proportions (Table 2). Qualitative differences were found for two volatile organic compounds (VOCs) ((*Z*)-3-hexenyl propanoate and terpinolene), which were up-regulated in samples from the mirid-exposed tomato plants. Major quantitative differences were found for many VOCs from plants exposed to *M. basicornis* for 72 h, which emitted more total fatty-acid derivatives (FADs) compared to control plants (Kruskal–Wallis, df = 2, $P = 0.00015$) and terpenes compared to mirid-exposed plants for 24 h and control plants (Kruskal–Wallis, df = 2, $P = 0.0001$).

The total production of volatiles (FADs and terpenes) was affected by the changes in individual compounds, and was higher in plants exposed to *M. basicornis* for 72 h. No differences were observed between *M. basicornis*-exposed plants for 24 h and control plants (Kruskal–Wallis, df = 2, $P = 0.0039$; Table 2).

Multivariate analyses (PCA), which evaluated the effect of each compound in the blend, showed different patterns of treatment grouping, where the blends from mirid-exposed tomato plants for 72 h differed from the mirid-exposed plants for 24 h and control plants, which contributed ~73.4% of the variance (Fig. 5). The separation was influenced mainly by the exposure treatment, where the two green-leaf volatiles (GLVs) (*Z*)-3-hexenol and (*Z*)-3-hexenyl propanoate were correlated with both mirid-induced

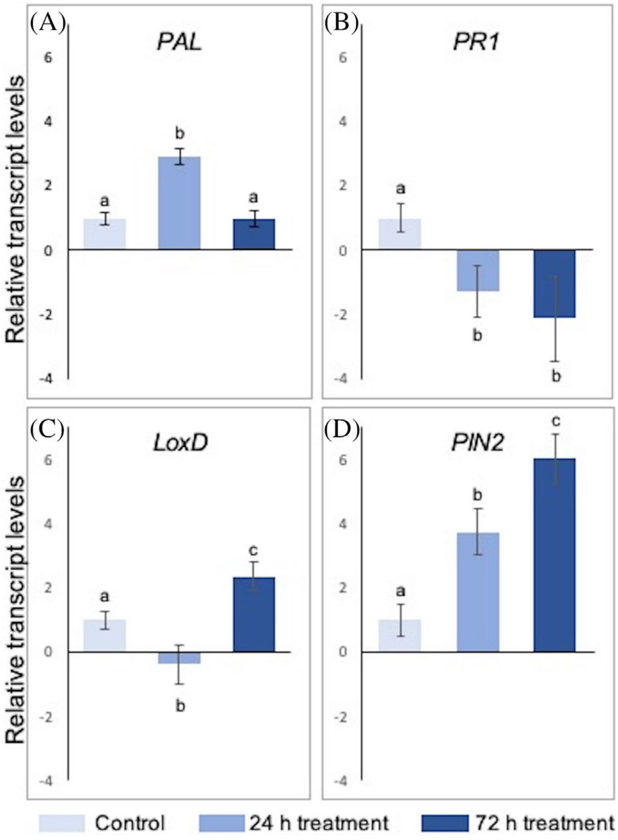

Figure 4. Effect of exposure to *Macrolophus basicornis* on salicylic acid (SA)- and jasmonic acid (JA)-mediated defenses of tomato plants. The relative transcript levels (log2-fold changes) of SA-related (a and b) and JA-related (c and d) genes were measured in plants exposed to *M. basicornis* for 24 h and 72 h compared to intact control plants. Values represent mean ± standard error ($n = 5$). Different letters indicate significant differences between treatments (*t*-test, $P < 0.05$).

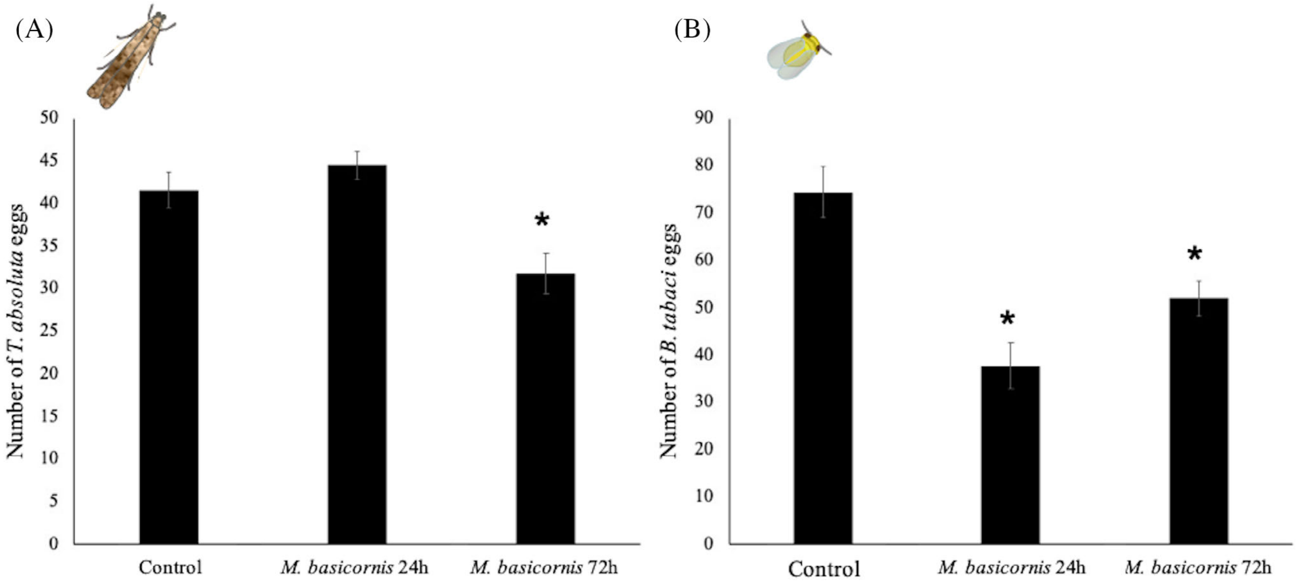

Figure 3. Oviposition (eggs/female ± standard error) of (A) *Tuta absoluta* females and (B) *Bemisia tabaci* females on tomato plants previously subject to one of the following treatments: intact tomato plants, tomato plants exposed to *Macrolophus basicornis* for 24 h, and tomato plants exposed to *M. basicornis* for 72 h. Means in columns with an asterisk (*) are significantly different between treatments, based on ANOVA, Tukey's test $P < 0.05$.

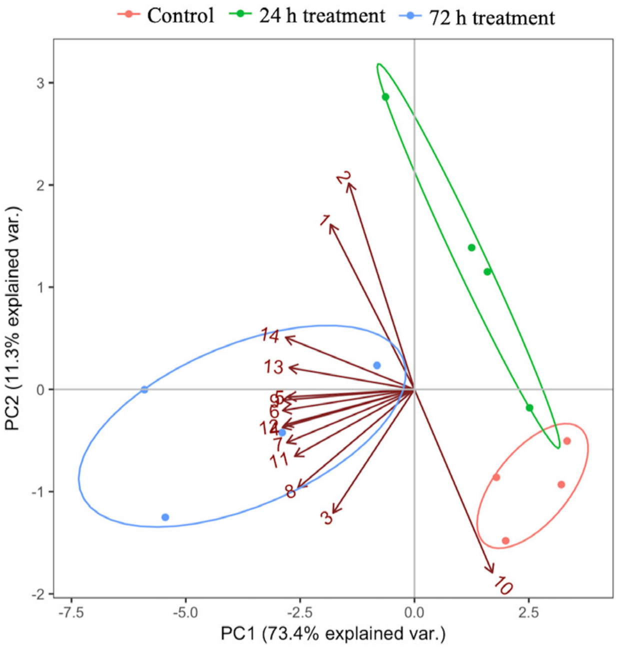

Figure 5. Principal components analysis (PCA) of the composition of volatiles emitted by intact tomato plants, tomato plants exposed to *Macrolophus basicornis* for 24 h, and tomato plants exposed to *M. basicornis* for 72 h. Vector numbers correspond to compound numbers in Table 2.

treatments, and most of the terpenoid compounds were highly correlated with mirid-exposed plants for 72 h. However, the terpene limonene was highly correlated with control plants.

4 DISCUSSION

Host plants play a central and active role in the outcomes of tritrophic interactions.[26] One example of this complexity can be found in systems involving zoophytophagous mirids, where host plants not only harbor prey, but also act as a substrate for egg-laying and food resources that prey animals cannot provide.[37,38] From the plant perspective, phytophagy by those groups of predators can also be advantageous since the predator can trigger plant defenses against herbivorous pest.[33,39,40] Here we report for the first time that a neotropical mirid predator can induce such plant defenses (Fig. 6). This is of particular interest, as the predator *M. basicornis* has been selected as the most promising neotropical mirid predator agent for horticultural pests.[9–11,16] The phytophagous behavior of *M. basicornis* induced plant-mediated adverse effects on the behavior of pests and attraction of natural enemies. Tomato plants responded to the phytophagy by this omnivorous predator by changing levels of genes involved in plant defense, and consequently augmented the release of certain HIPVs (Fig. 6).

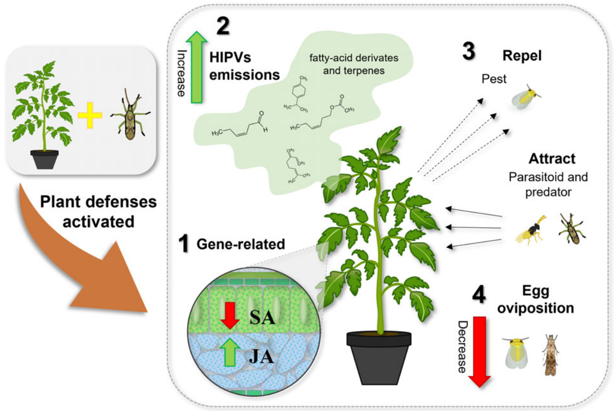

Figure 6. *Macrolophus basicornis* induces tomato resistance. On the top left of the flow chart, *M. basicornis* feeds on tomato plant which: (1) change the expression of genes related to the jasmonic acid (JA) and salicylic acid (SA) pathways, leading to (2) an increase of plant volatiles componunds emission that (3) repel the whitefly *Bemisia tabaci*, and attract natural enemies, the whitefly parasitoid *Encarsia inaron* and the predator *M. basicornis* and (4) decrease *Bemisia tabaci* and *Tuta absoluta* egg oviposition.

The 'two-in-one' characteristic highlighted for *M. basicornis* in this study is demonstrated by initially its high capacity for killing important lepidopteran pests like *Tuta absoluta*,[8–11,14] *Helicoverpa armigera* (Hubner; Lep.: Noctuidae) and *Phthorimaea operculella* (Zeller; Lep.: Gelechiidae), nymphs of *Bemisia tabaci*, aphids and mites.[14, 15] Beyond the rather wide prey spectrum of *M. basicornis*, it is attracted by volatiles produced by tomato plants after attack by tomato pests[16] and cause little injury to tomato plants and fruit as a result of phytophagy.[13] However, when feeding on plant tissue, zoophytophagous species activate plant defenses.[3] In some cases, this defense triggers an antagonistic effect against herbivore performance (antibiosis) and host choice (antixenosis), and attraction of natural enemies.[34,40] These consequences of mirid plant feeding, discussed from now on, is the second aspect for implementation of the 'two-in-one' strategy for pest management using *M. basicornis*.

Induction of plant defenses by zoophytophagous predators varies in different plant–insect systems.[33,39] Our results demonstrated that during host selection, *Bemisia tabaci* preferred clean plants over plants previously exposed to *Macrolophus basicornis*. Whitefly preference for intact plants was also observed for tomato plants induced by *Nesidiocoris tenuis* and sweet pepper plants induced by *Macrolophus pygmaeus*, although *Bemisia tabaci* did not show such a preference when tomato plants were colonized by *Dicyphus bolivary*.[28,29] In addition, mirid-exposed plants proved to be less attractive to key horticultural pests such as the two-spotted spider mite *Tetranychus urticae*, the glasshouse whitefly *Trialeurodes vaporariorum* (Westwood), and the western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae).[28,29,32,40,41] Overall, there are more reports of herbivore aversion behavior than of neutral behavior[40] or attraction, such as the leafminer *Tuta absoluta* for tomato plants exposed to *Macrolophus pygmaeus*.[28]

Interestingly, *M. basicornis*-exposed tomato plants did not affect the olfactory response of *Tuta absoluta*. However, exposure of plants for a longer period resulted in fewer eggs laid compared to intact plants. Several species of Lepidoptera use various cues related to plant quality to discriminate and select their host.[32] After landing, a short-distance decision, chemoreceptors on antennae, mouthparts, and ovipositors are used to detect chemical cues.[42] The reduced oviposition of both pests, *Bemisia tabaci* and *Tuta absoluta*, might be a consequence of direct defense induction, related to the elevated level of the JA-related gene observed in our study. A high content of JA is linked to changes in arthropod feeding behavior, and the non-volatile chemical compounds present on plant surfaces play a role in oviposition avoidance.[33,42,43]

Previous studies have demonstrated that zoophytophagous predators interfere with the JA-regulated and SA-regulated defense pathways by injecting saliva into a host plant. Transcriptomic and proteomic profiles have shown that the plants can discriminate among arthropod species and activate specific temporal responses.[44] For instance, the widely used biological control agent *Nesidiocoris tenuis* induces both the JA and SA pathways, while the closely related *M. pygmaeus* activates only the JA pathway.[40,41,45] The increased activation of the JA-related genes, PIN2 and LoxD, over time, and the similarly time-related suppression of the SA marker genes PAL and PR1 suggest that *M. basicornis* feeding led to a crosstalk between SA- and JA-dependent tomato defense responses. We hypothesize that the increased crosstalk over time may be a response to the continuous injury of epidermal cells. According to Chinchilla *et al.*,[46] mirid predators execute a large number of probes by continuously piercing the plant tissues in order to lacerate them and ingest the cell contents, and the enzyme-rich saliva thus exposed can be highly variable among mirid species.[47] The observed crosstalk among phytohormone-mediated signaling pathways is of particular interest in terms of insect resistance, since activation of different phytohormone pathways can cause changes in plant–herbivore–natural enemy interactions.[48,49] Our findings suggest that *M. basicornis* phytophagy had an effect on the level of SA, and this effect changed over time, however, to properly address this, further studies using mutant tomato plants are required.[28]

This negative crosstalk between JA and SA has also been shown to regulate several natural enemy–pest interactions through HIPV releases. While herbivorous pests are repelled, parasitoids and predators are attracted.[27,29,50] JA-related HIPVs are important for attracting natural enemies.[16,51] The increased expression of JA-related genes agrees with the changed behavior of *Encarsia inaron* and *M. basicornis*. The volatile compounds emitted by tomato plants induced by *M. basicornis* were attractive to these two important natural enemies of *Bemisia tabaci* and *Tuta absoluta*. The slight and non-significant difference in the volatile blend released when the plant was exposed to *M. basicornis* for a short period (24 h) compared to control plants was enough to attract *Encarsia inaron*. Similar attractivity was reported for the whitefly parasitoid *Encarsia formosa* to the volatiles of sweet pepper and tomato plants induced by *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) and *M. pygmaeus*, respectively.[27,29] Parasitoids are more sensitive than generalist predators to differences in plant volatiles released.[52] Indeed, several studies have demonstrated that predator attraction could be related to quantitative differences in the volatile blend, and also to species-specific responses.[53,54] *Macrolophus basicornis* was significantly attracted only when its conspecific induced the tomato plant for 3 days, which triggered it to release more volatiles. The differences observed here in the volatile profiles of tomato plants for different periods of mirid induction, as shown by the PCA, support this. Green bean plants exposed to *M. pygmaeus* for a similar period also attracted the conspecific mirid, but a longer induction period (10 days) was necessary for the volatiles of tomato plant induced by *M. pygmaeus* and *Nesidiocoris tenuis* to change their respective conspecific behavior.[55,56] Similarly high releases of induced volatile blends were required to attract insect predators to sweet pepper and eggplants.[57,58]

The overall composition of major volatile compounds released by Solanaceae plants induced by different zoophytophagous predators are similar, although with possibly important quantitative differences.[59–61] These compounds were also found in the headspace of tomato plants infested with tomato pests, which are also attractive to *M. basicornis*.[16] As such, compounds shared by host plants may play a central role in determining the specificity or polyphagous behavior of natural enemies.[62,63] The HIPVs identified in this study were classified as GLVs, involving the fatty acid/lipoxygenase biosynthesis pathway, and terpenes, a product derived from the isoprenoid pathway. These two classes of compounds are constantly mentioned as important signals in plant–insect interactions, causing both repellence and attraction effects.[64] For instance, GLVs are immediately released after insect damage and the specific relationship between the plant and the inducer is reported to play a role in the attraction of parasitoids, providing valuable information about which organism is damaging a plant.[64] In agreement, we hypothesize that reason for the high attraction of *Encarsia inaron* to and lack of preference of *Trichogramma pretiosum* for plants exposed to *M. basicornis* over control plants might be that the volatile blend composition of mirid-exposed tomato plants is closer to the volatile blend from *Bemisia tabaci*-infested than the blend from *Tuta absoluta*-infested tomato plants, respectively (hosts of the respective earlier-mentioned parasitoids).[16,59] It is known that volatiles compounds of plants exposed to *Bemisia tabaci* attract conspecifics.[36] However, we believe that the reason for whitefly repellence in our study was due to the simultaneous induction of the earlier-mentioned JA signaling pathway, leading to release few different, but important whitefly repellent volatiles compounds such as (*Z*)-3-hexenyl propanoate (Silva *et al.*, in prep.) and α-pinene.[65]

Herbivores and natural enemies use a mixture of volatile compounds to locate their host/prey. However, major individual compounds can also determine their choice. The up-regulation of other terpenoids and fatty-acid compounds in plants exposed to *M. basicornis*, such as (*Z*)-3-hexenyl propanoate, *p*-cymene, terpinene, and α-phellandrene. These compounds are repellent to *Tuta absoluta* and *Bemisia tabaci* and attractive to natural enemies, including *M. basicornis*,[62,63,66–68] and application of this compound in field conditions increased recruitment of several natural enemies, including heteropteran predators and whitefly parasitoids (Silva *et al.*, in prep.). In addition, these compounds can indirectly promote pest control through inducing healthy plants to release volatile compounds, resulting in repellence of herbivores and attraction of natural enemies.[3] We have recently reported[63] that European mirid predators were attracted to the synthetic form of the same induced FADs such as (*Z*)-3-hexenyl propanoate. Thus, evaluation of whether *M. basicornis* and *Encarsia inaron* trigger chemosensory responses to each compound identified and the capacity of these compounds to activate defense mechanisms of Brazilian tomato varieties and other crops is important for exploring new and more sustainable strategies for pest management.[69,70]

Another application derived from this study would be exploration of the two-in-one function that *M. basicornis* exhibited. Plant vaccination[43,71] by the mirid (i.e. release of early stages for mirid feeding and establishment on the crop) will directly affect the further arrival of herbivores via predation and indirectly via induction of direct and indirect plant defense responses. Releases of *Nesidiocoris tenuis* on a nursery tomato crop reduced pest populations and increased resilience to pest damage.[3,8] As is well known, the presence of flowers on plants increases the survival rate of omnivorous predators[72,73]; however, Zhang *et al.*[40] recently reported that plants colonized by a mirid predator developed flowers faster and its fruits produced five times more seeds than intact plants. Moreover, omnivores can interact directly and indirectly with other organisms (e.g. plant viruses, soil microbes, and entomopathogens through plant-mediated effects), indicating that omnivores have multiple roles.[74,75] (Taking into account all the benefits from *M. basicornis*-plant feeding behavior, our study suggests that

M. basicornis can indirectly promote plant protection. This study is a starting point for a future *M. basicornis* augmentative or inoculative biological control program. Follow-up field trials evaluating the early release of *M. basicornis* and utilization of plant volatiles are needed for better understanding in more realistic scenarios. Nonetheless, the multiple benefits of zoophytophagous predators could result in a modern and effective tomato pest management strategy, enhancing their overall biocontrol efficacy, reducing environmental impacts of pesticide use, and increasing plant production.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name] at [DOI].

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