Garlic and peppermint essential oils elicit plant defensive responses in sweet peppers

Michele Ricupero[1,2], Antonio Biondi[1], Orlando Campolo[3], Miquel Alonso-Valiente[2], Carolina Gallego[2], Lucia Zappalà[1], Alberto Urbaneja[2], and Meritxell Pérez-Hedo[2,*]

[1] University of Catania, Department of Agriculture Food and Environment, via S. Sofia 100, 95123 Catania, Italy

[2] Instituto Valenciano de Investigaciones Agrarias (IVIA). Centro de Protección Vegetal y Biotecnología, Carretera CV-315, Km 10'7. 46113 Moncada, Valencia, Spain

[3] Department of Agriculture, University "Mediterranea" of Reggio Calabria, Loc. Feo Di Vito, 89122 Reggio Calabria, Italy

* Corresponding author: perez_merhed@gva.es

Abstract: Exploiting plant defense mechanisms is a promising tool for pest management in modern agriculture. Plant Essential Oils (EOs) are used for the sustainable control of agricultural pests; however, their impact on plant defense has been scarcely investigated. In this work, we study for the first time whether the spraying of EOs can activate plant defense mechanisms in sweet pepper. The olfactory capacity of *Encarsia formosa* (Hymenoptera: Aphelinidae) to respond to defense-activated plants was used to select garlic and peppermint EOs among nine EOs sprayed on sweet peppers. The expression level of defense-related genes in plant tissues and the phytotoxicity were measured in response to EO foliar applications. Moreover, the olfactory responses of the herbivores, *Bemisia tabaci* (Hemiptera: Aleyrodidae) and *Frankliniella occidentalis* (Thysanoptera: Thripidae), and their natural enemies, *Orius laevigatus* (Hemiptera: Anthocoridae) and *Nesidiocoris tenuis* (Hemiptera: Miridae), to EO induced plant volatiles were also investigated. The gene expression analysis revealed activated jasmonic and salicylic acid defense signaling pathways in EO-sprayed sweet pepper plants and a negligible phytotoxic effect was recorded. Choice tests revealed varying behavioral responses in selected insect models when plants were treated with garlic and peppermint EOs in different concentrations. Our results suggest that garlic and peppermint EO spray applications can enhance the defense mechanisms of sweet peppers and have a cascading bottom-up effects on the associated food chain. These initial findings provide a foundation for the future development of Integrated Pest Management strategies to protect solanaceous crops.

Keywords: Bell pepper; biological control; botanicals; IPM; plant defense; nanomaterial

1 Introduction

Current agricultural policies require a significant reduction in dependence on synthetic pesticides based on sustainable control practices to guarantee food and feed security and preserve natural resources worldwide (Johnson et al. 2020). Meeting this challenge implies identifying and implementing novel strategies in Integrated Pest Management (IPM) programs (Nicolopoulou-Stamati et al. 2016), such as exploiting the plant defense mechanisms.

Over the past thirty years, a wealth of knowledge has been produced on plant defense mechanisms. Some of the mechanisms that plants orchestrate against attacks by herbivores and diseases trigger the emission of Volatile Organic Compounds (VOCs), which fulfill a plethora of eco-physiological functions (Hare 2011). VOCs drive multitrophic interactions between plants, herbivores, and their natural enemies, as well as plant-to-plant communication (Heil & Ton 2008). Plants can recognize VOCs from other plants and activate their defenses in anticipation of future biotic attacks (Dicke & Baldwin 2010). Therefore, volatile signals that mediate tritrophic interactions can be exploited for agricultural pest control strategies, including possible applications such as odor dispensers to attract natural enemies, plant elicitors and enhanced crop emissions (Turlings & Erb 2018). One recent example is the use of polymeric dispensers releasing a constant amount of HIPVs which elicit plant defenses in tomato and sweet pepper crops (Pérez-Hedo et al. 2021, Rihai et al. 2022).

Plant essential oils (EOs) are secondary metabolites of plants that are widely used in many industrial applications, including cosmetics, food production, pharmacology, and

pesticide formulation. The success of EOs for pest control is documented by a large body of studies that show promising results with limited non-target risks (Isman 2020). The interest in EOs for controlling agricultural pests offers growing perspectives thanks to nanotechnologies that help mitigate EO constitutive drawbacks, such as phytotoxicity (Athanassiou et al. 2018). Despite the promising evidence as sustainable control tools, the effect of EOs on plant defense and their related trophic networks has yet to be merely investigated. Previous studies assessed the efficacy of EOs in controlling pests and plant pathogens by eliciting plant defenses (Vergenes et al. 2014, Ben-Jabeur et al. 2015, Banani et al. 2018, Ben-Abdallah et al. 2023). To the best of our knowledge, no previous research investigated the role of EOs in eliciting plant defenses in sweet pepper plants and their bottom-up effects on the upper trophic levels, including herbivores and beneficial arthropods (Han et al. 2022).

Here, we hypothesized that EO foliar applications could elicit plant defenses in sweet peppers. To test this, first, we used the parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae), which has been demonstrated to be attracted to defensive activated solanaceous plants (Pérez-Hedo et al. 2015a) to study its behavioral response to volatiles emitted by sweet pepper plants, *Capsicum annuum* L. (Solanaceae) sprayed with nine nanoemulsioned-EOs which we developed previously (Campolo et al. 2020a, Ricupero et al. 2022). We also assessed the transcriptomic of related genes of the main defensive signaling pathways and the phytotoxic effect in EO-treated plants. Moreover, the olfactory responses of two herbivores, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), and two natural enemies used for their biological control, *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) and *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae), to EO-activated host plants were investigated. The outcomes of this work can pave the way for the potential use of EOs as plant defense elicitors from an applied perspective in sweet-pepper cropping systems.

2   Materials and methods

2.1   Biological materials

Seedlings of sweet pepper plants, cv 'Lipari F-1' (Semillas Batlle S.A., Barcelona, Spain), were grown in a climatic chamber at constant environmental conditions ($25 \pm 1°C$, $60 \pm 10\%$ R.H. and 16L:8D h photoperiod). Six-week-old plants with seven to eight fully expanded leaves (BBCH 13.103) were used in the experiments.

Individuals of *E. formosa, N. tenuis*, and *O. laevigatus* were supplied by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). *Bemisia tabaci* and *F. occidentalis* adults were obtained from laboratory colonies established at Instituto Valenciano de Investigaciones Agrarias (IVIA) and originally collected from Campo de Cartagena (Murcia, Spain). Whiteflies and thrips were reared on tomato (*Lycopersicon esculentum* Mill., Solanaceae) and common bean (*Phaseolus vulgaris* L., Fabaceae), respectively. Laboratory insect colonies and supplied species were kept in a climatic chamber at $25 \pm 2°C$, $65 \pm 10\%$ RH, and 14L:10D h photoperiod. All tested individuals were females less than 2 days old.

2.2   Essential oils

The following EOs were used: anise [*Pimpinella anisum* (Apiaceae)], artemisia [*Artemisia vulgaris* (Asteraceae)], fennel [*Foeniculum vulgare* (Apiaceae)], garlic [*A. sativum* (Liliaceae)], lavender [*Lavandula latifolia* (Lamiaceae)], peppermint [*Mentha x piperita* (Lamiaceae)], rosemary [*Rosmarinus officinalis* (Lamiaceae)], sage [*Salvia officinalis* (Lamiaceae)] and oregano [*Origanum vulgare* (Lamiaceae)]. The EO characterization, formulation, and solution preparation were carried out as described in Campolo et al. (2020a) and Ricupero et al. (2022).

Sweet pepper plants were sprayed at the initial concentrations of 1.9% (w/v) per each EO because preliminary trials revealed that higher concentrations cause high levels of phytotoxicity (data not shown). Moreover, based on the observed attraction of *E. formosa* towards plant-emitted volatiles, a different range of lower concentrations (i.e., 1% for garlic EO and 0.5 and 0.15% for peppermint EO) was also tested. This was done because one of IPM's aims claims that using chemicals or other forms of intervention should be kept only to economically and ecologically justified levels of minimizing risk to human health and the environment (Nicolopoulou-Stamati et al. 2016). Plants were sprayed until runoff with a handle sprayer (2L Dea, Volpi, Italy) and dried outdoors. Control plants were sprayed with distilled water. Once dried, EO-sprayed plants were isolated in 60 × 60 × 60 cm screened cages (BugDorm-2, MegaView Science Co., Ltd.; Taichung, Taiwan) and kept at the greenhouse condition of $25 \pm 2°C$, $65 \pm 10\%$ RH, and a natural photoperiod (approximately 14L:10D h). Control plants were maintained at the same conditions but in separated glasshouses to exclude any potential plant defense activation by volatiles emitted by activated plants nearby (Pérez-Hedo et al. 2015a). Sprayed and control plants were used 24 h later for the experiments.

2.3   Phytotoxicity assessment

The toxic effect of the tested EO-based nanoformulations on sweet pepper plants was evaluated by the Phytotoxicity index (*Pi*) as proposed by Campolo et al. (2017). The *Pi* is calculated with this formula:

$$Pi = \sum_{j=0}^{n} \left(\frac{DLj}{TL} \times \frac{DC}{n-1} \right)$$

where *DL* is the number of damaged leaves for each damage severity class *j*, *TL* is the total number of leaves sprayed, *DC* is the damage severity class, and *n* is the number of damage severity classes. The damage severity was classified as: 0 (no damage), 1 (partially damaged leaf surface, with chlorosis and without necroses), 2 (leaves with evident necroses), and 3 (dead leaves). For this, five other sweet pepper plants were sprayed with 1.9% for all the 9 tested EOs and, 1% for garlic EO, 0.5 and 0.15% for peppermint EO, following the same methodology described above. A control treatment with distilled water was also included. Sprayed plants were kept in 60 × 60 × 60 cm insect-proof cages (BugDorm-2, MegaView Science Co., Ltd.; Taichung, Taiwan) in greenhouse conditions described above. Sweet pepper plants were inspected 1, 3, 7, and 14 days after the treatments, recording the number of damaged leaves and the severity of the damage.

2.4 Insect olfactory responses

The parasitoid *E. formosa* which is generally attracted by plant volatiles emitted by elicited plants was initially used in dual choice experiments to test whether foliar applications of EOs could induce plant defenses in sweet pepper plants.

The behavioral responses of *E. formosa* females to plant volatiles were investigated in a Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) consisting of a 2.4-cm-diameter Y-shaped glass tube with a 13.5-cm-long base and two 5.75-cm-long arms. The bottom of the Y-tube was connected to an air pump that produced a unidirectional airflow at 150 ml/min from the arms to the base of the tube. The arms were connected via plastic tubes to two identical glass jars (5-l volume) containing a test odor source. Each odor source vial was connected to a flow meter and a water filter. Four 60-cm-long fluorescent tubes (OSRAM, L18 W/765, OSRAM GmbH, Germany) were positioned 40 cm above the arms. Each female was observed until it had walked at least 3 cm up one of the side arms or until 10 min had elapsed and time laps for each choice was recorded. Females that did not choose a side arm within 10 min were considered 'non-responders' and were not considered for statistical analysis. After five individual females had been tested, the olfactometer arms were flipped around (180°) to minimize the spatial effect on arm choice. The olfactometer setup was thoroughly cleaned after ten female trials using a soap and water rinse, followed by a grade acetone purification and air drying. Both plants (treated and untreated) were used only once to test the response of 10 females and then were replaced with new plants. For each odor source pair, 35 and 40 responder females were observed. Each individual was considered as a single replicate. The environmental conditions in the Y-tube experiments were 23 ± 2°C and 60 ± 10% R.H.

Since *E. formosa* was attracted by sweet pepper plants sprayed with garlic and peppermint EOs (see 3.1 Insect olfactory responses) and the activation of defense pathways in sweet pepper plants was proved (see 3.3. Plant gene expression), the olfactory responses of *B. tabaci*, *F. occidentalis*, and *N. tenuis* were also assayed as described above.

To corroborate the initial hypothesis (i.e., garlic and peppermint EOs activate plant defense in sweet pepper plants) and exclude the potential attraction of the tested species by EO as unique odor source, the dual choice test was replicated by posing the EO on an inert substrate. Only the treatments that resulted attractive for *E. formosa*, *B. tabaci*, and *O. laevigatus* when sprayed on sweet pepper plants (see Results 3.1 Insect olfactory responses) were tested on an inert substrate. For this, 10 µl of EO was released on a piece of circle filter paper (3 cm in diameter) that was placed into the jar. The EO was used at the same concentration that caused a significant olfactory response when sprayed on plants. The control odor source consisted of 10µl of distilled water released on the filter paper. Per each tested species, at least 35–40 valid replicates (i.e., responder females) were also conducted per each odor pair.

2.5 Plant gene expression in response to garlic and peppermint EOs

To assess changes in gene expression of significant genes involved in plant defense signaling pathways, the apical regions of EO-sprayed and untreated sweet pepper plants were analyzed to determine the following target gene expression levels: (1) *PIN2* (wound-induced proteinase inhibitor II precursor), a marker gene for jasmonic acid (JA), and (2) *PR1* (basic PR-1 protein precursor) a marker gene for salicylic acid (SA) signaling pathway.

The sweet pepper apical region samples were collected individually from 1-day-sprayed plants using a sterile scalpel and immediately stored in liquid nitrogen at -80°C. Total RNA was extracted from the homogenized samples using TRizol reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The extracted RNA (3 µg/µl) was treated with a TURBO DNA-free kit (Invitrogen) to remove any genomic DNA contamination. Reverse transcription was performed using PrimeScript RT reagent kit (TAKARA BIO INC., Japan) starting from 1 µg/µl of RNA-DNA free template. The forward and reverse primers (10 mM, 0.5 µl each) were added to 5 µl of Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix and 1 µl of cDNA, resulting in a total reaction volume of 10 µl. Quantitative PCR was conducted using the LightCycler 480 Instrument II (Roche Molecular Systems, Inc., USA) with the following PCR conditions: 1 cycle at 95°C for 13 minutes, 40 cycles at 95°C for 15 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and a final cycle at 95°C for 5 seconds and 60°C for 1 minute. The relative fold gene expression of the samples was calculated using the $2^{-\Delta\Delta CT}$ method. The qPCR amplification reaction was performed three times, with three biological replicates evaluated in total. The gene expression of EF-1 (Elongation Factor-1) was used as an internal control gene for normal-

ization. The gene-specific primer nucleotide sequences were the same as proposed by Bouagga et al. (2018a), as follows: EF1(forward 5'-CCTGGACAGATTGGAAATGG-3'; reverse 5'-GACCACCTGTCGATCTTGGT-3'), PIN2 (forward 5'-CTTGCCCCAAGAATTGTGAT-3'; reverse 5'-GCCCTAGCGTATTACGGAGA-3') and PR1 (forward 5'-ACGTCTTGGTTGTGCTAGGG-3'; reverse 5'-CCATACGGACGTTGTCCTCT-3').

2.6   Data analysis

Raw datasets were tested for normality and homogeneity of the variance through Kolmogorov-Smirnov D-test and Levene's test, respectively. Chi-squared tests were conducted to highlight the deviation from the null hypothesis, which considers equal frequency distribution of side-arm choices between pairs of odors. Significant differences ($P < 0.05$) were determined with a one-tailed Student's test performed in a pairwise manner for the concentration of each EO. One-way ANOVA followed by a comparison of means (Tukey's post hoc test) was conducted to highlight differences in the transcriptional responses of the *PR1* and *PIN2* genes in the apical parts of the sweet pepper plant induced by EOs. The *Pi* was calculated according to the formula described above and subjected to univariate variance analysis, with EO and time after the treatment as fixed factors and application rates as covariate. Statistics were conducted on IBM® SPSS® Statistics for Macintosh, v. 23 (IBM Corp. Released 2015. Armonk, NY, USA).

3   Results

3.1   Phytotoxicity assessment

The phytotoxicity observed in sweet pepper plants was significantly affected by the type of EO ($F_{9, 280} = 7.826$; $P < 0.001$) and its interaction with the application rates and time elapsed after the treatment ($F_{108, 280} = 3.757$; $P < 0.001$). Amongst the tested nine EOs, only oregano caused the highest phytotoxicity ($Pi = 1$) on sweet pepper plants, and the damages were remarkably evident after 1 day after the treatment (Fig. S1). At the 1.9% concentration, negligible phytotoxicity was observed in plants sprayed with peppermint and garlic EOs. Similarly, no phytotoxic effect was recorded in plants sprayed with garlic EO at 1% and peppermint EO at 0.5 and 0.15% that were used in dual choice tests (Fig. S2).

3.2   Insect olfactory responses

3.2.1   *Encarsia formosa*

Encarsia formosa females were significantly attracted by the odor emitted by sweet pepper plants that were sprayed with the nanoemulsions of garlic ($\chi^2 = 6.40$; $P = 0.011$) and peppermint ($\chi^2 = 10.0$; $P = 0.002$) EOs at the concentration of 1.9%, in comparison to the untreated control. Conversely, parasitoid females showed no significant preference between the odors emitted by plants sprayed with the other tested

Table 1. Olfactory response of *Encarsia formosa* (*E.f*), *Bemisia tabaci* (*B.t*), and *Orius laevigatus* (*O.l*) females in the dual choice test when exposed to different EOs concentrations (previously triggering significant response in tested species when sprayed on sweet pepper plants) as unique odor source and clean air (chi-square test, *P* < 0.05). "n" is total number of responder females and "nc" is number of individuals that made no choice.

	EOs	Rate (%)	Choice (%) EO	Air	nc	X²	P
E.f	Garlic	1.9	40.0	60.0	1	1.60	0.206
		1	47.5	52.5	11	0.10	0.752
	Peppermint	1.9	47.5	52.5	2	0.10	0.752
		0.5	42.5	57.5	10	0.90	0.343
		0.15	42.5	57.5	14	0.90	0.343
B.t	Garlic	1.9	45.7	54.3	0	2.07	0.150
	Peppermint	1.9	40.0	60.0	5	1.60	0.206
O.l	Garlic	1.9	52.5	47.5	0	0.10	0.752

seven EOs at the concentration of 1.9% and the control plants (Fig. 1).

The same significant attraction of *E. formosa* by EO-sprayed sweet pepper plants was encountered at lower concentrations of garlic EO at 1% and peppermint EO at 0.5% and 0.15% ($\chi^2 = 6.40$, $P = 0.011$; $\chi^2 = 8.10$, $P = 0.004$; $\chi^2 = 6.40$; $P = 0.011$, respectively) (Fig. 2).

Interestingly, when *E. formosa* had to choose between the odor source of the EO directly placed on a piece of filter paper and its control treatment (distilled water on the piece of filter paper), no difference in preference was observed (Table 1).

3.2.2   Herbivores

Females of *B. tabaci* were highly repelled by the odor emitted by sweet pepper plants previously sprayed with 1.9% concentration of either garlic and peppermint EOs ($\chi^2 = 4.90$, $P = 0.027$ and $\chi^2 = 10$; $P = 0.002$, respectively) (Figs. 2 and 3). However, none of these treatments caused a significant response to the whiteflies on the filter paper bioassay (Table 1).

Contrary to that, female *F. occidentalis* showed no preference between the odors from intact plants and those from plants induced by essential oils (EOs). This trend was consistent across all EO concentrations tested (Figs. 2 and 3). Since none of the EOs at different concentrations elicited a response in *F. occidentalis*, the dual choice test using filter paper was not performed.

3.2.3   Predators

Treated plants with 1.9% garlic essential oil attracted nearly 70% of tested *O. laevigatus* females ($\chi^2 = 4.9$; $P = 0.027$) (Fig. 4A). Conversely, the mirid *N. tenuis* showed no signifi-
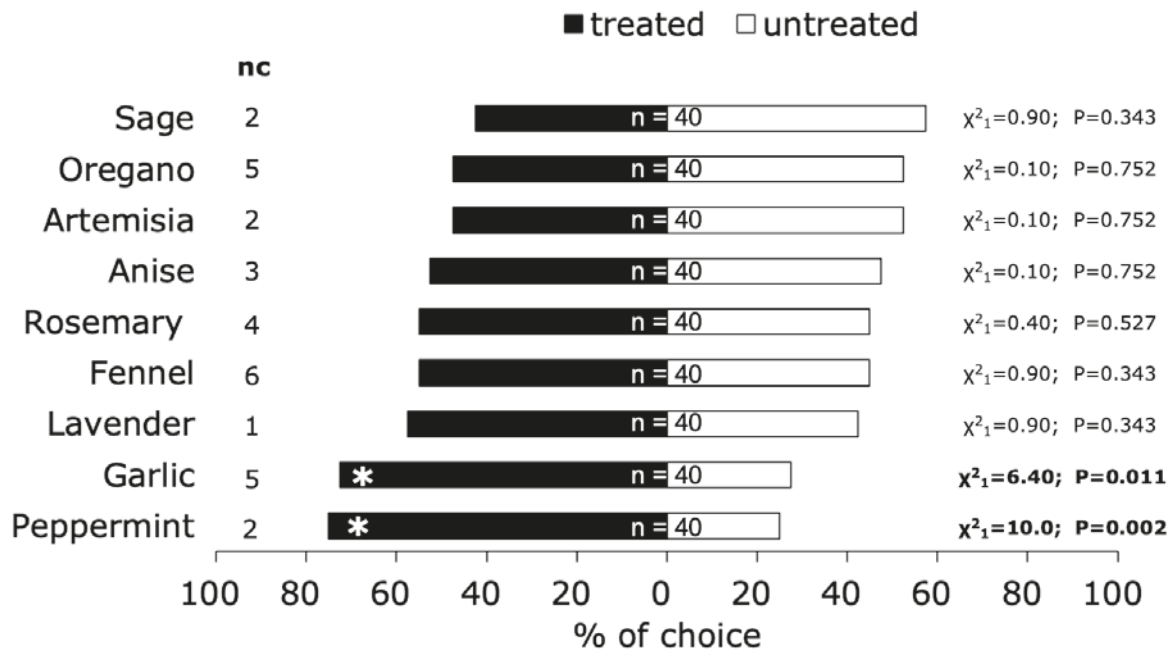
Fig. 1. Behavioral response of *Encarsia formosa* females in in dual choice test when exposed to sweet pepper plants sprayed 24h before with nine nanoemulsions at the concentration of 1.9% in EO; "n" is total number of responder females and "nc" is number of individuals that made no choice. Significant differences based on a chi-square test are marked with (*) ($P < 0.05$).
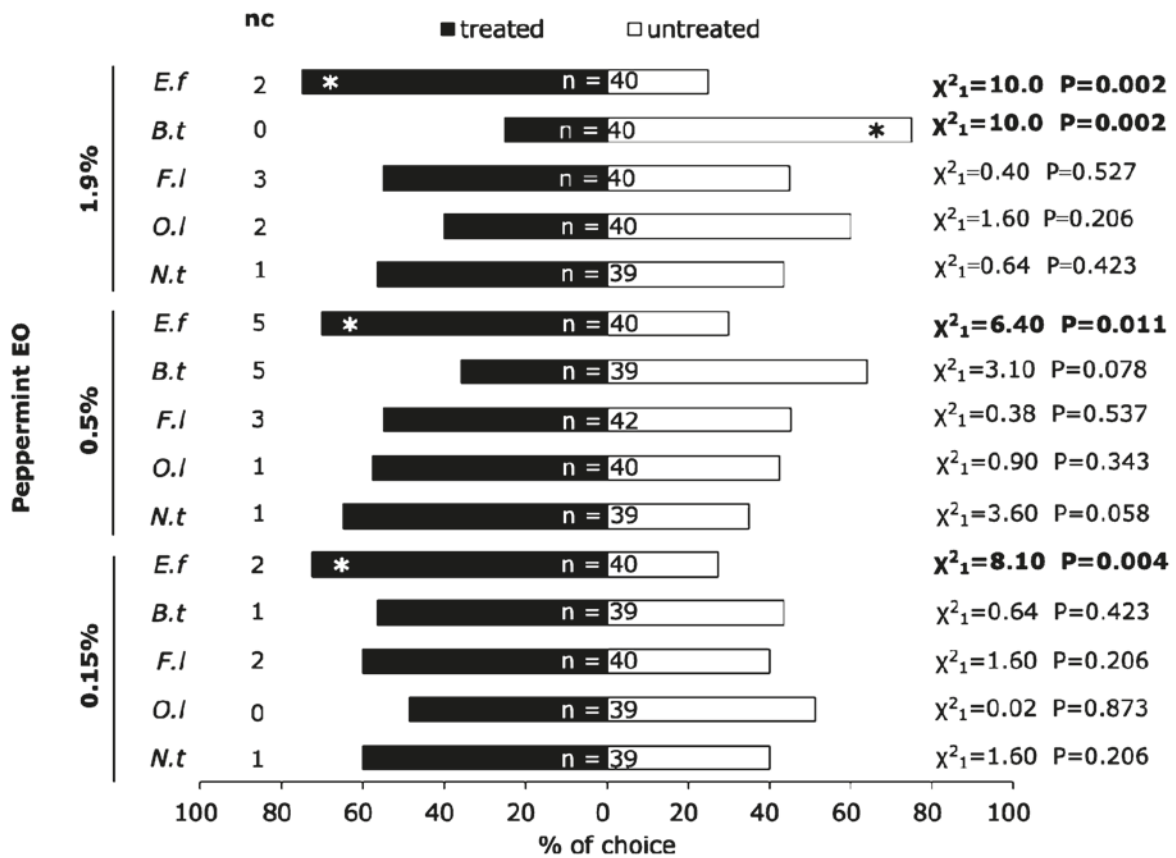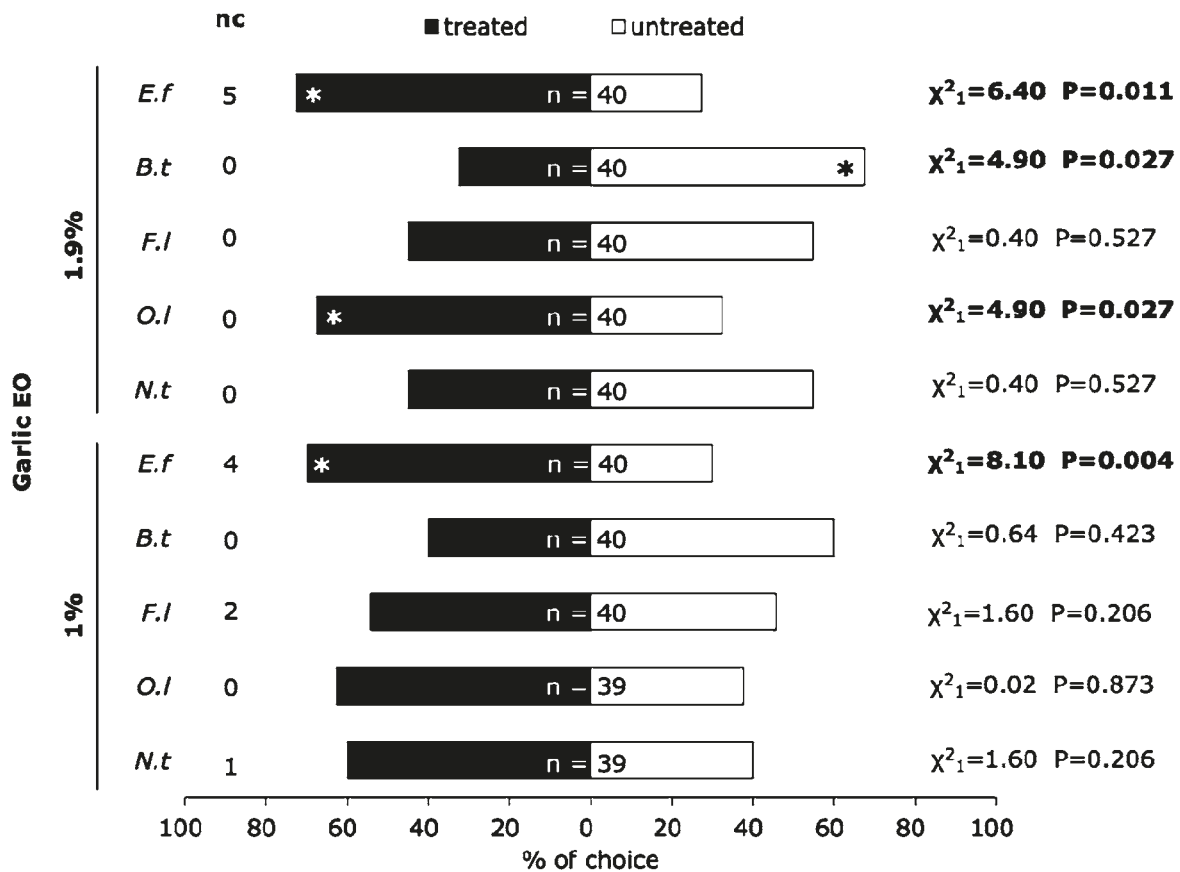

Fig. 2. Behavioral response of *Encarsia formosa* (*E.f*), *Bemisia tabaci* (*B.t*), *Frankliniella occidentalis* (*F.o*), *Orius laevigatus* (*O.l*), and *Nesidiocoris tenuis* (*N.t*) females in the dual choice test, when exposed to sweet pepper plants, sprayed 24h before with nanoemulsioned peppermint EOs at 1.9%, 0.5% and 0.15%; "n" is total number of responder females and "nc" is number of individuals that made no choice. Significant differences based on a chi-square test are marked with (*) ($P < 0.05$).

Fig. 3. Behavioral response of *Encarsia formosa* (*E.f*), *Bemisia tabaci* (*B.t*), *Frankliniella occidentalis* (*F.o*), *Orius laevigatus* (*O.l*), and *Nesidiocoris tenuis* (*N.t*) females in dual choice test when exposed to sweet pepper plants sprayed 24h before with selected nanoemulsioned garlic EOs at 1.9% and 1%; "n" is total number of responder females and "nc" is number of individuals that made no choice. Significant differences based on a chi-square test are marked with (*) (*P* < 0.05).

cant preference between EO-sprayed and intact sweet pepper plants (Fig. 4B).

3.3   Plant gene expression

The transcriptional analysis showed that the apical portion of treated plants significantly increased the expression levels of the *PIN2* gene (JA pathway) in sweet pepper plants sprayed with increasing concentrations of peppermint EO ($F_{3,3}$ = 53.821; $P < 0.001$) and garlic EO ($F_{2,3}$ = 33.933; $P = 0.001$) when compared to the control plants. Similarly, the *PR1* gene (SA pathway) was overexpressed in peppermint EO ($F_{3,3}$ =397.516; $P < 0.001$) and garlic EO ($F_{2,3}$ =1607.058; $P < 0.001$) treated plants in comparison to untreated plants.

4   Discussion

Relevant crop losses are due to damage caused by insect pests, and several international legislations urgently demand sustainable practices for their control. Over the past decades, EOs have been claimed as a potential pest control tool in

IPM programs (Pavela & Benelli 2016). Similarly, secondary metabolites such as plant volatiles that mediate tritrophic interactions can be successfully exploited in agriculture to repel insect pests and lure beneficial arthropods (Turlings & Erb 2018). Therefore, studying plant defense elicitors is important for the global food system's current challenges.

The present study focused on the ability of EOs to elicit plant defenses in sweet pepper. We proved for the first time that peppermint and garlic EOs could prime defenses on sweet pepper plants by foliar applications at different concentrations. Our hypothesis was confirmed by changes in defense gene expression in plants. These changes explained the observed attraction of *E. formosa* and *O. laevigatus* and the repellent effect on *B. tabaci* by sweet pepper plants sprayed with 1.9% garlic and peppermint EO. Although the 1.9% concentration did not cause phytotoxicity, lower concentrations of 0.5% peppermint EO triggered defensive signaling pathways, leading to the continued attraction of *E. formosa* by sweet pepper plants. This highlights the potential of garlic and particularly peppermint EO to act as elicitors of sweet pepper plant defenses.
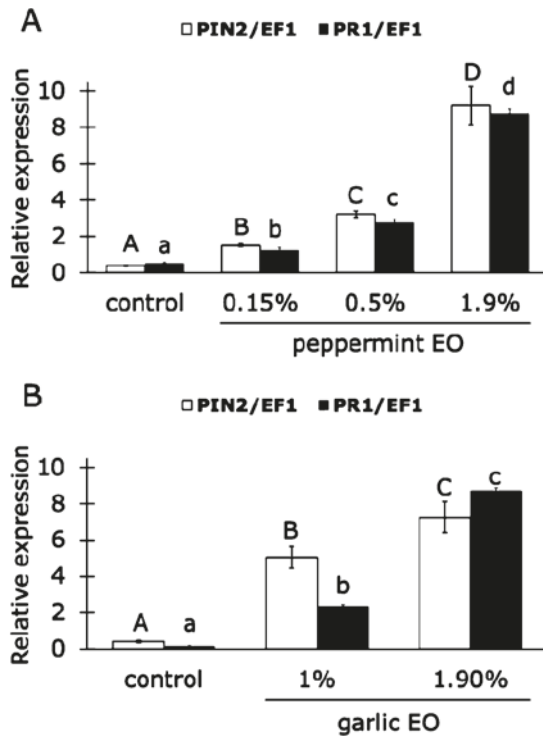
Fig. 4. *PIN2* and *PR1* transcriptional response, which are respectively jasmonic acid (JA) and salicylic acid (SA) responsive, in the apical part of control (water-sprayed) and EO-sprayed sweet pepper plants. Data are presented as the mean (± SE) of three independent analyses of transcript expression relative to housekeeping gene plants (n = 3). Different letters over the bars (upper case letters: within *PIN2* transcriptional response, lower case letters: within PR1 transcriptional response) indicate significant differences at $P < 0.05$ (ANOVA; Tukey HSD post hoc test for multiple comparisons).

Eliciting plant defenses through EOs have been emphasized to demonstrate the efficacy in controlling microbial plant pathogens when sprayed on fruits (Banani et al. 2018), aerial plant parts (Vergenes et al. 2014) or added with irrigation water (Ben-Jabeur et al. 2015). Noteworthy is the study by Kessler et al. (2006) in which *Artemisia tridentata* Nutt. (Asteraceae) plants via VOCs stimulated wild tobacco plants to get less damage by the caterpillar *Manduca sexta* (L.) by accelerating the production of trypsin proteinase inhibitors which cause anti-nutritional effects to the herbivore.

However, to the best of our knowledge, only one study has assessed the impact of essential oils (EOs) on plant-induced defenses and their higher trophic levels in an economically relevant crop such as tomato (Ben-Abdallah et al. 2023). In that study, Ben-Abdallah et al. (2023) demonstrated how treatments with EOs (yarrow and garlic) increased the expression of defense-related genes, but differently depending on the EO used. While the garlic treatment activated defensive pathways related to JA and ABA, the yarrow treatment activated the SA pathway. Consequently, this differen-

tial induction of defenses by both oils led to the emission of distinct volatile patterns.

The variety of plant responses to environmental factors is determined by different patterns in the expression of the genes involved in the defense. Therefore, the production of plant volatiles can be induced by herbivore injury (Dicke & Baldwin 2010) and abiotic factors that alter the levels of many different metabolites (Loreto et al. 2014). In general, stressors increase VOC emission, which has been recognized for temperature, light intensity, water, nutrients, air pollutants, and atmospheric gases (Holopainen & Gershenzon 2010). Our hypothesis was supported by the activation of SA and JA signaling pathways in sweet pepper plants treated with garlic and peppermint essential oils (EOs), which suggests that the use of these EOs as elicitors may enhance the accumulation of SA or JA and induce systemic acquired resistance (SAR) in the plant. SAR is important in preventing further infection or damage to the entire plant through volatile compounds that signal plant-plant communication and mediate tritrophic interactions, as seen in the behavioral changes of *E. formosa*, *B. tabaci*, and *O. laevigatus* between treated and untreated plants in our olfactory trials.

In our experiments, *E. formosa* and *O. laevigatus* significantly preferred EO-sprayed plants, which have higher JA and SA expression than intact plants. Recent studies revealed that *E. formosa* is attracted by emitted volatiles from whitefly-adult infested tomato plants and their synthetic components (e.g., α-phellandrene, β-myrcene, and β-caryophyllene) in the laboratory (Ayelo et al. 2021, Chen et al. 2021). Yang et al. (2020) found that Z)-3-hexenol application on *B. tabaci*-infested tomato plants activated JA and SA pathways and enhanced the attraction of *E. formosa*. Similarly, the attraction of *E. formosa* has been recorded multiple times when tomato and sweet-pepper defenses were activated by the puncturing of hemipteran predators (Pérez-Hedo 2015b, Bouagga et al. 2018b), and the application of synthetic green leaf volatiles (GLVs) and methyl salicylate (Pérez-Hedo 2018a, Pérez-Hedo 2021). Similarly, *O. laevigatus* strongly preferred sweet pepper plants, which were previously exposed to five selected VOCs (Riahi et al. 2022).

Interestingly, the whitefly *B. tabaci* was repelled by garlic and peppermint EO-activated plants. Shi et al. (2016) found that exogenous salicylic acid application on tomatoes increased the number and quantity of plant volatiles, such as methyl salicylate and δ-limonene, which had a repellent effect on *B. tabaci*. Consistent with these findings, Pérez-Hedo et al. (2018a) reported the repellency of *B. tabaci* when exposed to six synthetic GLVs and methyl salicylate identified in mirid punctured-tomato plant emissions.

By contrast, the lack of response to EO-sprayed sweet pepper plants by *F. occidentalis* and *N. tenuis* might be attributed to the specificity of emitted VOCs for these species or the activation of different defense metabolic routes. Bouagga et al. (2018a) observed the activation of JA and SA pathways and the consequent aversion of both *B. tabaci*

and *F. occidentalis* on *O. laevigatus*-punctured sweet pepper plants. Similar results were obtained for sweet pepper plants exposed to (Z)-3-hexenyl propanoate and methyl salicylate (Riahi et al. 2022). On the other hand, terpenes are the major constituents of EOs (Isman 2020); consequently, the potential effect of EO constitutive volatiles might also explain our preliminary findings. Nonetheless, our observations in the dual choice test in which EOs were the unique odor sources invalidated this counterhypothesis. None of the tested concentrations for garlic and peppermint EOs triggered a significant response towards the tested insect models when EO was the only odor provided, thus supporting our initial hypothesis. However, analyzing VOCs emitted by EO-activated plants by gas chromatography could validate our findings and unveil additional details (Tholl et al. 2006).

One major constraint of using EOs is phytotoxicity. Nevertheless, in recent years different nanotechnological solutions have been proposed to overcome this not negligible drawback (Campolo et al. 2017, 2020a). Among our tested EO-based nanoemulsions, only oregano EO caused a remarkable phytotoxicity on sweet pepper plants that was evident one day after the treatment. The observed effect might be due to carvacrol, its main constitutive compound that disrupts plant cell walls leading to their death, as consistently recorded in cucumber and tomato seedlings (Ibáñez et al. 2020). Conversely, we observed a negligible phytotoxic effect for the rest of the EOs tested during the time elapsed in our evaluation. In particular, a very low *Pi* occurred in plants sprayed with lower concentrations of garlic and peppermint EOs that were used for activating plant defenses. This result enables us to exploit multiple strategies in using EOs for crop protection with minimum impact on plants and the surrounding environment.

Since the efficacy of nanoformulation EOs to control glasshouse chewer and sap-sucking insect pests has been demonstrated (Campolo et al. 2017, Sciortino et al. 2020, Ricupero et al. 2022), the use of EOs to control pests and elicit plant defenses to repel herbivores and harbor biocontrol agents can be a promising and synergistic tool to develop. A long-standing ambition could be the inclusion of garlic and peppermint EOs in the IPM of sweet pepper cropping systems. For example, field applications of EOs by foliar spray, volatile dispensers, or added dip irrigation might give good insights. Notwithstanding, multi-level side effects studies on EO-based nanoemulsions (Campolo et al. 2020b, Giunti et al. 2022) are required to implement in high-tier risk assessment procedures. Although we have not observed any possible trade-off effect between defensive activation and plant yield in our work, this aspect should be carefully evaluated in future research related to this topic, especially under field conditions. In this sense, it will be necessary to distinguish between the positive impact of protection against pests and diseases provided by EOs on crop yield and the potential physiological effect of activating the plants' immune system (Campolo et al. 2020b).

Our discovery offers a powerful resource for investigating the role of EOs in tritrophic networks. Additional studies are warranted to determine the role of plant defense induction-related genes, such as proteinase inhibitor precursor or abscisic acid pathway. Moreover, other biological traits (e.g., fecundity, development time) of pest insects and their associated natural enemies over activated plants should be investigated (Pérez-Hedo et al. 2018b). Overall, our scientific evidence demonstrates that EO exposure can elicit a resistant response in sweet pepper via the induction of JA and SA signaling pathways. Thus, EOs can enhance plant resistance to herbivores and mediate susceptibility to natural enemies throughout their attraction. In conclusion, these findings open up new possibilities in using EOs as plant defense elicitors extending thus their versatile application in the complex framework of IPM.

References

Athanassiou, C. G., Kavallieratos, N. G., Benelli, G., Losic, D., Usha Rani, P., & Desneux, N. (2018). Nanoparticles for pest control: Current status and future perspectives. *Journal of Pest Science, 91*(1), 1–15. https://doi.org/10.1007/s10340-017-0898-0

Ayelo, P. M., Yusuf, A. A., Pirk, C. W., Mohamed, S. A., Chailleux, A., & Deletre, E. (2021). The role of *Trialeurodes vaporariorum*-infested tomato plant volatiles in the attraction of *Encarsia formosa* (Hymenoptera: Aphelinidae). *Journal of Chemical Ecology, 47*(2), 192–203. https://doi.org/10.1007/s10886-021-01245-2

Banani, H., Olivieri, L., Santoro, K., Garibaldi, A., Gullino, M. L., & Spadaro, D. (2018). Thyme and savory essential oil efficacy and induction of resistance against *Botrytis cinerea* through priming defense responses in apple. *Foods, 7*(2), 11. https://doi.org/10.3390/foods7020011

Ben Abdallah, S., Riahi, C., Vacas, S., Navarro-Llopis, V., Urbaneja, A., & Pérez-Hedo, M. (2023). The dual benefit of plant essential oils against *Tuta absoluta. Plants, 12*(5), 985. https://doi.org/10.3390/plants12050985

Ben-Jabeur, M., Ghabri, E., Myriam, M., & Hamada, W. (2015). Thyme essential oil as a defense inducer of tomato against gray mold and *Fusarium* wilt. *Plant Physiology and Biochemistry, 94*, 35–40. https://doi.org/10.1016/j.plaphy.2015.05.006

Bouagga, S., Urbaneja, A., Rambla, J. L., Granell, A., & Pérez-Hedo, M. (2018a). *Orius laevigatus* strengthens its role as a biological control agent by inducing plant defenses. *Journal of Pest Science, 91*(1), 55–64. https://doi.org/10.1007/s10340-017-0886-4

Bouagga, S., Urbaneja, A., Rambla, J. L., Flors, V., Granell, A., Jaques, J. A., & Pérez-Hedo, M. (2018b). Zoophytophagous mirids provide pest control by inducing direct defences, antixenosis and attraction to parasitoids in sweet pepper plants. *Pest Management Science, 74*(6), 1286–1296. https://doi.org/10.1002/ps.4838

Campolo, O., Cherif, A., Ricupero, M., Siscaro, G., Grissa-Lebdi, K., Russo, A., … Palmeri, V. (2017). Citrus peel essential oil nanoformulations to control the tomato borer, *Tuta absoluta*: Chemical properties and biological activity. *Scientific Reports, 7*(1), 13036. https://doi.org/10.1038/s41598-017-13413-0

Campolo, O., Giunti, G., Laigle, M., Michel, T., & Palmeri, V. (2020a). Essential oil-based nano-emulsions: Effect of different surfactants, sonication and plant species on physicochemical characteristics. *Industrial Crops and Products, 157*, 112935. https://doi.org/10.1016/j.indcrop.2020.112935

Campolo, O., Puglisi, I., Barbagallo, R. N., Cherif, A., Ricupero, M., Biondi, A., … Zappala, L. (2020b). Side effects of two citrus essential oil formulations on a generalist insect predator, plant and soil enzymatic activities. *Chemosphere, 257*, 127252. https://doi.org/10.1016/j.chemosphere.2020.127252

Chen, C. S., Zhao, C., Wu, Z. Y., Liu, G. F., Yu, X. P., & Zhang, P. J. (2021). Whitefly-induced tomato volatiles mediate host habitat location of the parasitic wasp *Encarsia formosa*, and enhance its efficacy as a bio-control agent. *Pest Management Science, 77*(2), 749–757. https://doi.org/10.1002/ps.6071

Dicke, M., & Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: Beyond the 'cry for help'. *Trends in Plant Science, 15*(3), 167–175. https://doi.org/10.1016/j.tplants.2009.12.002

Giunti, G., Benelli, G., Palmeri, V., Laudani, F., Ricupero, M., Ricciardi, R., … Campolo, O. (2022). Non-target effects of essential oil-based biopesticides for crop protection: Impact on natural enemies, pollinators, and soil invertebrates. *Biological Control, 176*, 105071. https://doi.org/10.1016/j.biocontrol.2022.105071

Han, P., Lavoir, A. V., Rodriguez-Saona, C., & Desneux, N. (2022). Bottom-up forces in agroecosystems and their potential impact on arthropod pest management. *Annual Review of Entomology, 67*(1), 239–259. https://doi.org/10.1146/annurev-ento-060121-060505

Hare, J. D. (2011). Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annual Review of Entomology, 56*(1), 161–180. https://doi.org/10.1146/annurev-ento-120709-144753

Heil, M., & Ton, J. (2008). Long-distance signalling in plant defence. *Trends in Plant Science, 13*(6), 264–272. https://doi.org/10.1016/j.tplants.2008.03.005

Holopainen, J. K., & Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. *Trends in Plant Science, 15*(3), 176–184. https://doi.org/10.1016/j.tplants.2010.01.006

Ibáñez, M. D., & Blázquez, M. A. (2020). Phytotoxic effects of commercial essential oils on selected vegetable crops: Cucumber and tomato. *Sustainable Chemistry and Pharmacy, 15*, 100209. https://doi.org/10.1016/j.scp.2019.100209

Isman, M. B. (2020). Botanical insecticides in the twenty-first century–fulfilling their promise? *Annual Review of Entomology, 65*(1), 233–249. https://doi.org/10.1146/annurev-ento-011019-025010

Johnson, A. C., Jin, X., Nakada, N., & Sumpter, J. P. (2020). Learning from the past and considering the future of chemicals in the environment. *Science, 367*(6476), 384–387. https://doi.org/10.1126/science.aay6637

Kessler, A., Halitschke, R., Diezel, C., & Baldwin, I. T. (2006). Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuata*. *Oecologia, 148*(2), 280–292. https://doi.org/10.1007/s00442-006-0365-8

Loreto, F., Dicke, M., Schnitzler, J. P., & Turlings, T. C. (2014). Plant volatiles and the environment. *Plant, Cell & Environment, 37*(8), 1905–1908. https://doi.org/10.1111/pce.12369

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: The urgent need for a new concept in agriculture. *Frontiers in Public Health, 4*, 148. https://doi.org/10.3389/fpubh.2016.00148

Pavela, R., & Benelli, G. (2016). Essential oils as ecofriendly biopesticides? Challenges and constraints. *Trends in Plant Science, 21*(12), 1000–1007. https://doi.org/10.1016/j.tplants.2016.10.005

Pérez-Hedo, M., Urbaneja-Bernat, P., Jaques, J. A., Flors, V., & Urbaneja, A. (2015a). Defensive plant responses induced by *Nesidiocoris tenuis* (Hemiptera: Miridae) on tomato plants. *Journal of Pest Science, 88*(3), 543–554. https://doi.org/10.1007/s10340-014-0640-0

Pérez-Hedo, M., Bouagga, S., Jaques, J. A., Flors, V., & Urbaneja, A. (2015b). Tomato plant responses to feeding behavior of three zoophytophagous predators (Hemiptera: Miridae). *Biological Control, 86*, 46–51. https://doi.org/10.1016/j.biocontrol.2015.04.006

Pérez-Hedo, M., Arias-Sanguino, Á. M., & Urbaneja, A. (2018a). Induced tomato plant resistance against *Tetranychus urticae* triggered by the phytophagy of *Nesidiocoris tenuis*. *Frontiers in Plant Science, 9*, 1419. https://doi.org/10.3389/fpls.2018.01419

Pérez-Hedo, M., Rambla, J. L., Granell, A., & Urbaneja, A. (2018b). Biological activity and specificity of Miridae-induced plant volatiles. *BioControl, 63*(2), 203–213. https://doi.org/10.1007/s10526-017-9854-4

Pérez-Hedo, M., Alonso-Valiente, M., Vacas, S., Gallego, C., Rambla, J. L., Navarro-Llopis, V., … Urbaneja, A. (2021). Eliciting tomato plant defenses by exposure to herbivore induced plant volatiles. *Entomologia Generalis, 41*(3), 209–218. https://doi.org/10.1127/entomologia/2021/1196

Ricupero, M., Biondi, A., Cincotta, F., Condurso, C., Palmeri, V., Verzera, A., … Campolo, O. (2022). Bioactivity and physico-chemistry of garlic essential oil nanoemulsion in tomato. *Entomologia Generalis, 42*(6), 921–930. https://doi.org/10.1127/entomologia/2022/1553

Riahi, C., González-Rodríguez, J., Alonso-Valiente, M., Urbaneja, A., & Pérez-Hedo, M. (2022). Eliciting plant defenses through herbivore-induced plant volatiles' exposure in sweet peppers. *Frontiers in Ecology and Evolution, 9*, 776827. https://doi.org/10.3389/fevo.2021.776827

Sciortino, M., Scurria, A., Lino, C., Pagliaro, M., D'Agostino, F., Tortorici, S., … Ciriminna, R. (2021). Silica-microencapsulated orange oil for sustainable pest control. *Advanced*

Sustainable Systems, 5(4), 2000280. https://doi.org/10.1002/adsu.202000280

Shi, X., Chen, G., Tian, L., Peng, Z., Xie, W., Wu, Q., … Zhang, Y. (2016). The salicylic acid-mediated release of plant volatiles affects the host choice of *Bemisia tabaci. International Journal of Molecular Sciences, 17*(7), 1048. https://doi.org/10.3390/ijms17071048

Tholl, D., Boland, W., Hansel, A., Loreto, F., Röse, U. S., & Schnitzler, J. P. (2006). Practical approaches to plant volatile analysis. *The Plant Journal, 45*(4), 540–560. https://doi.org/10.1111/j.1365-313X.2005.02612.x

Turlings, T. C., & Erb, M. (2018). Tritrophic interactions mediated by herbivore-induced plant volatiles: Mechanisms, ecological relevance, and application potential. *Annual Review of Entomology, 63*(1), 433–452. https://doi.org/10.1146/annurev-ento-020117-043507

Vergnes, S., Ladouce, N., Fournier, S., Ferhout, H., Attia, F., & Dumas, B. (2014). Foliar treatments with *Gaultheria procumbens* essential oil induce defense responses and resistance against a fungal pathogen in *Arabidopsis. Frontiers in Plant Science, 5*, 477. https://doi.org/10.3389/fpls.2014.00477

Yang, F., Zhang, Q., Yao, Q., Chen, G., Tong, H., Zhang, J., … Zhang, Y. (2020). Direct and indirect plant defenses induced by (Z)-3-hexenol in tomato against whitefly attack. *Journal of Pest Science, 93*(4), 1243–1254. https://doi.org/10.1007/s10340-020-01234-6

The pdf version (Adobe JavaScript must be enabled) of this paper includes an electronic supplement:
Figures S1 and S2