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ORIGINAL PAPER

Exogenous systemin peptide treatment in olive alters *Bactrocera oleae* oviposition preference

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Abstract

The olive fruit fly, *Bactrocera oleae* (Rossi), is a key pest of the olive crop, whose control relies mostly on the use of insecticides. Plant peptides may represent a more environmentally-friendly tool to manage olive fly, due to their recognized role to activate and/or prime plant defence responses against pests. In this work, behavioural experiments (no-choice and two-choice) and analysis of volatile compounds were carried out to evaluate the impact of the exogenous application of the peptide systemin to olive tree on olive fly infestation, and to elucidate its mode of action to prime plant defence. The treatment of olive branches with 10 nM systemin showed to confer protection against olive fly, by reducing significantly the ovipositions (up to 3.0-fold) and the number of infested fruits (up to 2.9-fold) when compared to non-treated branches. This protective effect was even detected in neighbouring non-treated branches, suggesting the ability of systemin to trigger plant-to-plant communication. The deterrent activity of the primed olives was associated with the emission of the volatiles 2-ethyl-1-hexanol, 4-tert-butylcyclohexyl acetate and 1, 2, 3-trimethyl-benzene, which were negatively correlated with oviposition and fly infestation. Systemin has also showed to trigger the biosynthesis of specific volatiles (esters) in olives in response to fly attacks. Overall, the observed protection conferred by systemin against olive fly is likely due to the emission of specific volatiles that can act as a defence and/or as signalling molecules to upregulate the plant defence response. Thus, systemin represents a novel and useful tool to manage olive fruit fly.

Keywords *Olea europaea* L. · Olive fruit fly · Priming defence · Infestation · Volatiles

Key message

- The olive fruit fly, *Bactrocera oleae*, is a key pest of the olive crop
- The application of the peptide systemin in olive branches protects fruits from this pest
- Systemin also seems to protect neighbouring non-treated olives
- The protection conferred by systemin is likely due to the release of specific volatile compounds
- Systemin represents a novel and useful tool to manage olive fruit fly

Introduction

The olive tree (*Olea europaea* L.) is one of the most important and traditional crop species of the Southern European countries, with more than 95% of the world olive oil production (Fraga et al. 2021). In Portugal, this perennial evergreen tree has similarly great socioeconomic importance, where it is distributed throughout the entire country, especially in Trás-os-Montes region (Peres et al. 2011). Nevertheless, the cultivation and sustainability of olive worldwide face many challenges, mainly related to the attack of pests and pathogens. The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is considered the major damaging pest of the olive tree, by reducing the quality of olive oil and making

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table olives unsuitable for the market (Valenčič et al. 2021). This pest damages the fruits by laying eggs inside them, which hatch and the subsequent larvae feed on the flesh, leading to negative changes in chemical fruit composition and premature fruit drop (Lantero et al. 2022; Jesu et al. 2022). Moreover, ovipositions provide entry points for bacteria and fungi that cause the fruit to rot (Podgornik et al. 2013). Strategies for the control of the olive fruit fly have been relied mostly on the application of chemical pesticides, especially organophosphate insecticides (Kampouraki et al. 2018). However, the recognized harmful effects of these insecticides on human health, environment, and beneficial insects as well as the appearance of insecticide-resistant populations (Araújo et al. 2023), have been promoting the exploitation of other more sustainable pest management tools. These include, for instances, mass trapping and attract and kill (Daane and Johnson 2010), but also the application of kaolin that act as physical repellent against female flies (Saour and Makee 2004) or of biocontrol agents, such as entomopathogenic fungi (Yousef et al. 2013) and *Bacillus thuringiensis* (Ilias et al. 2013). Another promising approach for the biological control of pests that could be an additional sustainable tool to manage olive fruit fly, is the application of plant resistance inducers (PRIs) to induce host plant resistance. The exogenous application of PRIs has the potential of activating the plant defence system, leading to an induced or primed state, that makes the plant respond more quickly and effectively to a subsequent biotic or abiotic stress (Conrath 2011). PRIs can be either chemical compounds (Bektas and Eulgem 2015) or biological stimulators (Wiesel et al. 2014). Recent studies have been identifying a number of peptides with a defence signalling function against several biotic stressors in different plant species, such as tomato, potato, *Arabidopsis* and soybean (Pastor-Fernández et al. 2023). These peptides are released upon pest or pathogen attack, and they trigger a cascade of plant defences, increasing plant immune response (Pastor-Fernández et al. 2020). Systemin (Sys), a peptide comprising 18 amino acids, is one of the most known peptide reported to play an important role in regulating the resistance of plants against herbivorous pests (Coppola et al. 2015, 2017, 2019) and phytopathogens (Coppola et al. 2015; Molisso et al. 2021; Pastor-Fernández et al. 2022). Systemin has been also reported to prime indirect responses in plants by inducing the emission of volatiles that attract pest natural enemies and alert neighbouring plants, priming their own defence (Corrado et al. 2007; Degenhardt et al. 2010; Coppola et al. 2017). This peptide is a part of 200 aa precursor protein, Prosystemin (ProSys), that is synthetized by plants upon tissue damage and in response to other stimuli (Degenhardt et al. 2010). Once released from its precursor, systemin binds to its membrane receptor triggering a cascade of signalling events and defence responses, that include the transport of long-distance signalling molecules, such as jasmonic acid and volatile organic compounds, for the elicitation of systemic immunity (Zhang et al. 2020). Due to its recognized role in inducing a primed state in plants, the application of systemin may be an important component of ecologically-based pest management program. In fact, some few studies have been already showed that the exogenous application of systemin enhances resistance to the pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina* in different plant species (Molisso et al. 2021; Pastor-Fernández et al. 2022) and to the noctuid moth *Spodoptera littoralis* (Boisduval, 1833) and the aphid *Macrosiphum euphorbiae* (Thomas, 1878) in tomato (Coppola et al. 2015, 2017, 2019). As far as we known, the effect of systemin in enhancing the olive tree ability to resist olive fruit fly attacks has never been tested.

Hence, this work aims to evaluated if the exogenous application of systemin in olive tree is able to prime defence responses via the induction of volatile organic compounds (VOCs) against the attack of the olive fruit fly *Bactrocera oleae*. Specifically, we wanted to address the following questions: (1) Can systemin affect both the number of egg laying and pupae of *B. oleae*?; (2) Does systemin primes plant defence against *B. oleae* by inducing changes in VOCs production?

Materials and methods

Plant material

The effect of systemin in olive tree defence response was tested in detached olive tree branches. The branches were collected in an integrated pest management (IPM)-managed olive grove located in Suçães (41° 29′ 34.900″ N; 7° 15′ 39.222″ W), Trás-os-Montes region (Northeast of Portugal), in December 2022. In the IPM, the application of pesticides is based on the economic threshold and using products authorized for integrated pests' management (Malavolta and Perdikis 2018). However, during the study year, no treatments against olive fruit fly were performed. In this grove, 20 trees of the cultivar Cobrançosa with an age of more than 60 years, were randomly selected to collected branches. In each tree, 10 branches with approximately 40 cm length bearing 10 to 15 healthy and undamaged olives at maturation stage 4 [black epidermis and white pulp; (Hermoso et al. 2001)] were collected with sterilized shears, placed into plastic bags and transported to the laboratory under refrigerated conditions (at 4 ± 1ºC). Upon arrival to the laboratory, the branches were immediately placed in vessels containing approximately 25 mL of tap water, and maintained during 24 h under controlled conditions, at temperature of 26 ± 1 °C, relative humidity of 70 ± 10%, and photoperiod 16:8 h light: dark, prior to further treatments below.

Treatment with systemin

The systemin peptide with the sequence (N to C) AVQSKPP-SKRDPPKMQTD and HPLC purity > 90%, used in this study was synthetized by Biomatik Corporation (Canada). The lyophilized systemin was solubilized in double-distilled water to prepare a stock solution at concentration of 500 nM. The systemin used in the assays was prepared from this stock solution, by diluting the peptide in double-distilled water to obtain the final concentration of 10 nM. The branches preserved in tap water for 24 h were transferred to vessels containing 25 mL of 10 nM systemin or double-distilled water (non-treated control). The branches' cut ends were immersed in these solutions (systemin or double distilled water) for 24 h, and maintained under controlled environmental conditions as detailed earlier. The olives from these branches were offered to *B. oleae* 24 h after these treatments, in the oviposition assays.

Bactrocera oleae collection and rearing

Adults of *B. oleae* used in the oviposition experiments were obtained from olives collected from the same olive grove as described in the section "Plant material." Olives with signs of olive fly infestation were collected from cv. Cobrançosa trees, transported to the laboratory, and placed in humidified trays. These trays were checked daily for larvae and pupae, and when present, they were transferred to rearing cages (10 cm of diameter and 15 cm of height) for adult emergence. Once hatched from pupae, adult females and males were placed daily (for age control purposes) in separate cages. Adults were fed ad libitum with a honey aqueous solution (10% w/v), artificial diet [sucrose and yeast extract at a ratio of 4:1, w/w; (Malheiro et al. 2018)] and water, being the diet changed every 2 days. Groups of adult males and females with 10 days old (i.e., when attained sexual maturity) were then transferred to a new rearing cage, and left to crossed during five days, in order to ensure that all females were gravid and available to oviposit. These insects were further used in the oviposition assays. All these procedures were performed in the insectarium, with larvae, pupae, and adults being maintained under 26 ± 1 °C, 70 ± 10% of relative humidity, and a 16:8 h light:dark photoperiod.

Oviposition assays

Both no-choice and two-choice tests were used in oviposition assays to evaluate the oviposition preferences of *B. oleae* females towards systemin-treated and non-treated olives. All the oviposition assays were carried out in PET plastic cages (50 × 30 × 33 cm) covered with fine netting, in the insectarium at controlled conditions of temperature (26 ± 1 °C), humidity (70 ± 10%) and light (16:8 h light:dark photoperiod).

In the no-choice oviposition experiments, groups of 10 *B. oleae* adults (five gravid females and five males), were introduced into the cages, which contained five olives either from systemin-treated branches (systemin) or from non-treated branches (control). Olives were offered in 9-cm diameter petri plates borosilicate crystallizing glasses (Pyrex®), 24 h after treatment with systemin or water (control). The healthy status of the olives was previously confirmed in a binocular stereomicroscope (Leica EZ4). All the fruits used were at the same maturity stage [(4—black epidermis and white pulp; (Hermoso et al. 2001)], with similar shape, size and colour. Olives were removed from the cages and replaced by new ones after 24-h interval, for three days. Three replicates (i.e., cages) were performed for each treatment (5 olives × 3 cages × 3 days) and were repeated twice (i.e., 90 olives in total that were offered to 60 *B. oleae* adults—30 females and 30 males) (Fig. S1).

In the two-choice oviposition experiments, groups of 20 *B. oleae* adults (ten gravid females and ten males), were introduced into the cages, which contained five olives from systemin-treated branches and five olives from non-treated branches (control), placed in different Petri plates. As in the no-choice oviposition experiments, only healthy olives at maturity stage 4 were used. Olives were removed from the cages and replaced by new ones after 24-h interval, for three days. Three replicates (i.e., cages) were performed [10 olives (5 systemin-treated and 5 non-treated) × 3 cages × 3 days], and the experiment was repeated twice (i.e., 180 olives in total were offered to 120 *B. oleae* adults—60 females and 60 males) (Fig. S1).

In both experiments, the number of olives recovered with and without ovipositions and the number of ovipositions per infested assayed olive, was counted after each 24-h interval, under a stereomicroscope (Leica EZ4). Immediately after 24-h of offering the olives and recording ovipositions, both infested and non-infested olives from the two experiments were taken to analyse the composition of volatile compounds. In the end, after volatiles composition analysis, the olives were placed on humidified trays for pupae estimation.

Volatile characterization

Volatile organic compounds (VOCs) were analysed in infested and non-infested olives from the no-choice experiments in order to elucidate if systemin primes plant defence against *B. oleae* by inducing changes on volatile production. In this analysis was also included infested olives treated and non-treated with systemin from the two-choice experiments, in order to elucidate if systemin can prime neighbouring olives for an enhanced defence response via VOCs production. In the no-choice experiments, one infested-olive and one non-infested olive retrieved per cage, from a total of three cages, were analysed. In the two-choice experiments, one infested olives treated and another one non-treated with systemin per cage, from a total of three cages, were analysed. VOCs emitted by these olives were analysed by headspace solid phase microextraction gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS), following a similar procedure used by (Malheiro et al. 2018). Briefly, single olives with approximately 5 g of fresh weight was placed in 50 ml vials, that was further sealed with a polypropylene cap with silicon septum. The vial was placed in a water bath at 40 °C for 5 min to release volatile compounds. Then, under the same conditions of temperature and agitation, the SPME fibre (divinylbenzene/carbonex/polydimethylsiloxane) (DVB/CAR/PDMS 50/30 µm) (Supelco, Bellefonte, PA, USA) was exposed for 30 min for adsorption of the volatile compounds in the headspace. Volatile compounds were removed from the fibre by thermal desorption (220 °C) for 1 min in the chromatograph injection port. The fibre was kept in the injection port for 10 min for cleaning and conditioning for further analysis. The gas chromatograph used was a Shimadzu GC-2010 Plus equipped with a Shimadzu GC/MS-QP2010 SE mass spectrometer detector. A TRB-5MS column (30 m × 0.25 mm × 0.25 × m) (Teknokroma, Spain) was used. The injector was set at a temperature of 220 °C, and the manual injection was performed in spitless mode. The mobile phase consisted of helium 5.0 (Linde, Portugal), at a linear velocity of 30 cm/s and a 24.4 mL/min flow rate. The oven temperature was 40 °C for 1 min, followed by an increase of 2 °C /min until reaching 220 °C. The ionization source was maintained at 250 °C with an energy of 70 eV and a current of 0.1 kV. All mass spectra were obtained by electronic ionization in the m/z range 35–500. Compounds were identified by comparing the mass spectra and through the Kovats index using databases such as NIST 69, PubChem and ChemSpider. Retention indices were obtained using a commercial n-alkane series, C7–C30 (Sigma-Aldrich, St. Louis, MS, USA), by direct spitless liquid injection (1 µL), while all further conditions of GC and MS were settled for the volatile analysis. Retention indices were calculated according to the Kovats index. The identified volatile compounds were expressed based on the areas determined by TIC (total ion chromatogram) integration.

Data analysis

The results of oviposition assays and volatiles compounds are presented as the mean of each parameter accompanied by the respective standard deviation (SD). The application of Shapiro–Wilk test showed that the data of oviposition assays significantly deviate from a normal distribution ($p \leq 0.05$). Therefore, the Kruskal–Wallis nonparametric test was used to determine if there are significant differences between systemin-treated and not-treated olives in the two oviposition assays. If a significant difference was found, the Mann–Whitney U test was used to determine which specific groups differed from each other in terms of median values. These analyses were conducted using PAST v4.12b software.

Principal component analysis (PCA) was performed to identify the volatiles compounds that best discriminate the different treatments (systemin-treated and not-treated) on infested and non-infested olives. This analysis was performed in RStudio software v. 2023.03.0 + 386 (R Core Team 2021) using the function pca from the "FactoMineR" package (Lê et al. 2008). The biplot of the PCA was drawn using the fviz_pca_biplot function from the "factoextra" package (Kassambara and Mundt 2020). PCA arrows represent the contribution of each volatile compound to the two components (length of the arrow), and the specific gradient colour denotes their contribution to the explanation of the greatest variance in the dataset.

Pearson correlation analysis was performed to measure the correlation between the VOCs and the parameters analysed in the oviposition bioassay (i.e., number of ovipositions, number of infested and non-infested olives, number of ovipositions per olive, number of ovipositions per infested olive, and collected pupae). This analysis was performed in RStudio software v. 2023.03.0 + 386 (R Core Team 2021) using the function cor_mat and corrplot from the "corrplot" package (Wei and Simko 2021). In this analysis was used only the VOCs with greater importance on *B. oleae* oviposition. The importance of these VOCs was measured by a Gini coefficient value, with a higher Gini coefficient value representing a greater importance (Cutler et al. 2007). These VOCs were identified by conducting a Random Forest (RF) analysis, using the data from the infested fruits from the systemin-treated and not-treated treatments. This analysis was performed in RStudio software v. 2023.03.0 + 386 (R Core Team 2021) using the function randomForest and varImPlot from the "randomForest" package (Liaw and Wiener 2002). The Gini coefficient indicates the contribution (importance) of each VOC for *B. oleae* oviposition.

Results

Oviposition assays

Results of oviposition preference of *B. oleae* towards olives from branches treated and non-treated with systemin are shown in Table 1. In the no-choice assays, systemin-treated olives showed significantly ($p < 0.05$) lower average number of ovipositions (up to 3.0-fold) as well as ovipositions per olive

Table 1 Parameters evaluated in the no-choice and two-choice assays

Average number of:	No-choice assays		Two-choice assays	
	Systemin	Control	Systemin	Control
Ovipositions	0.50 ± 0.51b	1.50 ± 0.85a	0.72 ± 0.67b	0.77 ± 0.81b
Infested olives	0.50 ± 0.51b	1.44 ± 0.85a	0.66 ± 0.60b	0.77 ± 0.81b
Non-infested olives	4.50 ± 0.51b	3.55 ± 0.85a	4.33 ± 0.60b	4.22 ± 0.81b
Oviposition per olive	0.10 ± 0.10b	0.30 ± 0.17a	0.14 ± 0.13b	0.15 ± 0.16b
Oviposition per infested olive	0.50 ± 0.51b	0.88 ± 0.47a	0.66 ± 0.60ab	0.5 ± 0.5ab
Collected pupae	0.22 ± 0.43a	0.55 ± 0.85a	0.38 ± 0.61a	0.22 ± 0.55a

Systemin-treated and not-treated olive branches are indicated as systemin and control, respectively. In the same line, mean values with different letters differ significantly ($p < 0.05$; $n = 18$)

(up to 3.0-fold) and per infested olive (up to 1.8-fold) when compared to control. In this assay, the treatment with systemin was also showed to reduce significantly ($p < 0.05$) up to 2.9-fold the average number of infested olives when compared to control.

In the two-choice assays, no significant differences were found between systemin-treated and control olives for all the parameters evaluated (i.e., average number of ovipositions and levels of infestation). Interestingly, the results from two-choice assays when compared to no-choice assays were very similar to the ones observed in systemin-treated olives but significantly different from the control. Thus, it is likely that the volatiles compounds released by systemin-treated olives in the two-choice assays are able to prime neighbouring control olives, thereby enhancing their defence against *B. oleae*.

In both assays, no significant differences were found on the average number of pupae between systemin-treated and non-treated olive branches.

Volatile compounds emitted by olives

Volatile composition of infested and non-infested olives from no-choice assays and of infested olives from two-choice assays were assessed by HS-SPME-GC/MS. The detailed relative volatile composition is reported in Table 2. Overall, a total of 47 compounds were detected and identified by comparison to the NIST library mass spectra. The identified compounds are distributed by ten different chemical classes, being the most diversified the alkanes (19 VOCs), alcohols (8) and aromatic hydrocarbons (7), followed by sesquiterpenes (3), terpenes (3), esters (3), aldehydes (2), ketones (1), phenols (1) and phenylmethylamine (1) (Table S1). Both alkanes and alcohols, were the most abundant, accounting 41% and 26% of the total abundance of VOCs, respectively (Fig. S2).

Comparison between systemin-treated and non-treated olives

The produced VOCs by the olives were quali- and semi-quantitatively different between systemin-treated and non-treated olives, but with variable results according to the level of fruit infestation (Table 2; Figs. S2and S3). In the no-choice assays, the differences found on the volatile profile between treatments were greater in infested olives when compared to the non-infested. Indeed, most of the identified compounds in non-infested olives were common to the two treatments (23 out of 33 VOCs), with 1, 3-bis (1, 1-dimethylethyl)- benzene and D-limonene being amongst the most abundant VOCs detected in both systemin-treated olives and control. Nevertheless, the alcohols 1-hexanol and (Z)-3-hexen-1-ol were detected in high abundance in non-infested control olives while in systemin-treated olives these VOCs were absent or present at low abundance, respectively. Moreover, some VOCs classes were exclusively detected either on systemin-treated (phenylmethylamine) or in control (ketones) olives. In infested olives, only 18 out of 32 VOCs were common to both treatments. The most abundant VOCs emitted by infested olives were also different between systemin-treated and non-treated experiments. Although, 1,3-bis(1,1-dimethylethyl)-benzene was found as one of the most abundant VOCs emitted in both treatments, other VOCs were abundantly produced either in systemin-treated olives (2-ethyl-1-hexanol) or in the control (1-hexanol, (Z)-3-hexen-1-ol and 3,7-dimethyl-decane) (Table 2; Fig. S3). Moreover, in infested olives was also detected a high number of VOCs that were exclusively produced either in systemin-treated (9 VOCs) or control (5 VOCs) olives. These differences were inclusively observed at class VOCs level, with esters being uniquely emitted by infested olives from systemin-treated experiments.

In the two-choice assays, the infested olives from both systemin-treated and non-treated experiments showed a similar volatile profile (Table 2; Figs. S2and S3). Indeed, the same classes of VOCs were detected in both treatments (Fig. S2). Moreover, both treatments showed a volatile profile with prevalence of (Z)-3-hexen-1-ol and 1-hexanol, accounting together for 25.4% and 21.7% of the total VOCs abundance in systemin-treated and not-treated olives, respectively (Table 2).

Table 2 Volatile profile of olives infested (I) and non-infested (NI) by *Bactrocera oleae* from systemin-treated (Sys) and non-treated (Cont) olive branches assessed in no-choice and two-choices assays

Nº	RT (min)	RI cal[a]	RI Llit[b]	Volatile compounds	No-choice experiment				Two-choice experiment	
					Sys_NI	Cont_NI	Sys_I	Cont_I	Sys_I	Cont_I
1	6,70	832	788	Toluene	–	–	–	–	16.8 ± 0.073	–
2	7,85	848	832	2,3-dimethyl-1-Pentanol	–	–	–	–	–	5.1 ± 0.019
3	10,39	908	873	(Z)-3-Hexen-1-ol	4.1 ± 0.011	14.0 ± 0.058	4.9 ± 0.035	10.4 ± 0.058	21.9 ± 0.053	13.4 ± 0.043
4	10,82	914	881	4-methyl-Octane	6.9 ± 0.058	4.5 ± 0.027	5.3 ± 0.026	6.3 ± 0.041	–	3.5 ± 0.010
5	11,09	918	881	1-Hexanol	–	16.3 ± 0.083	–	12.7 ± 0.048	12.7 ± 0.002	13.4 ± 0.057
6	12,83	953	909	Heptanal	4.7 ± 0.046	–	–	–	–	2.8 ± 0.017
7	14,83	982	953	α -Pinene	–	1.4 ± 0.007	–	–	–	–
8	16,82	1008	978.3	2-methyl-Nonane	4.0 ± 0.004	2.4 ± 0.007	3.0 ± 0.006	2.9 ± 0.012	–	2.3 ± 0.003
9	17,66	1017	986	2,5-dimethyl-Nonane	3.8 ± 0.008	1.6 ± 0.004	–	3.5 ± 0.018	–	–
10	18,29	1024	987	6-methyl-5-Hepten-2-one	–	1.2 ± 0.007	–	–	–	–
11	18,65	1028	1020.6	1,2,3-trimethyl-Benzene	3.6 ± 0.007	2.6 ± 0.004	2.6 ± 0.002	–	2.4 ± 0.012	2.6 ± 0.013
12	19,23	1048	1015	Decane	–	5.8 ± 0.019	6.9 ± 0.003	6.1 ± 0.030	7.4 ± 0.018	5.4 ± 0.018
13	20,23	1061	1057	5-methyl-Decane	–	–	–	4.5 ± 0.022	4.3 ± 0.001	2.9 ± 0.0003
14	20,79	1070	1061	4-methyl-Decane	6.4 ± 0.002	3.2 ± 0.010	6.1 ± 0.008	6.1 ± 0.025	4.9 ± 0.003	3.3 ± 0.004
15	21,14	1074	1044	D-Limonene	10.8 ± 0.041	10.6 ± 0.106	–	–	–	–
16	21,22	1075	1048	2-ethyl-1-Hexanol	–	–	13.2 ± 0.022	–	9.1 ± 0.005	8.9 ± 0.076
17	21,86	1083	1042	N,N-dimethyl-Benzenemethanamine	6.9 ± 0.047	–	–	–	–	–
18	22,58	1092	1054	β -Ocimene	1.5 ± 0.06	0.9 ± 0.002	–	–	–	–
19	23,26	1100	1086	3,7-dimethyl-Decane	10.5 ± 0.015	6.0 ± 0.014	9.7 ± 0.016	11.6 ± 0.027	8.3 ± 0.030	6.6 ± 0.013
20	23,46	1103	1064	2,3-dimethyl-Nonane	–	1.2 ± 0.004	1.6 ± 0.002	1.5 ± 0.004	1.5 ± 0.002	–
21	23,64	1105	1086	3,4,5,6-tetramethyl-Octane	2.6 ± 0.004	1.6 ± 0.004	2.5 ± 0.005	2.9 ± 0.006	–	1.7 ± 0.003
22	24,21	1111	1074	2-methyl-6-methylene- 2-Octanol	–	–	–	–	–	1.7 ± 0.0004
23	24,59	1116	1104	4-ethyl-1,2-dimethyl-Benzene	–	2.5 ± 0.003	2.5 ± 0.006	–	–	1.8 ± 0.008
24	26,25	1146	1115	Undecane	1.9 ± 0.008	1.1 ± 0.001	1.6 ± 0.009	1.2 ± 0.004	2.5 ± 0.017	0.9 ± 0.003
25	26,45	1149	1130	Nonanal	8.3 ± 0.024	5.4 ± 0.011	6.7 ± 0.028	6.5 ± 0.014	7.7 ± 0.022	7.0 ± 0.024
26	26,81	1154	1154	5-methyl-Undecane	1.3 ± 0.002	0.7 ± 0.001	–	1.1 ± 0.001	1.1 ± 0.003	0.7 ± 0.004
27	27,21	1159	1150.4	1,2,3,4-tetramethyl-Benzene	–	1.5 ± 0.001	1.5 ± 0.004	–	–	1.4 ± 0.010
28	27,52	1163	1147	1,2,4,5-tetramethyl-Benzene	–	–	–	–	–	2.2 ± 0.014
29	30,79	1205	1166	2-methyl-Undecane	0.7 ± 0.002	0.5 ± 0.002	–	–	–	–
30	31,33	1212	1180.78	Levomenthol	–	–	0.4 ± 0.002	–	–	0.7 ± 0.0003
31	33,35	1247	1214	Dodecane	3.9 ± 0.005	2.4 ± 0.008	3.1 ± 0.003	2.6 ± 0.008	2.7 ± 0.009	2.0 ± 0.006
32	33,69	1251	1251	(*E*)-2-Decen-1-ol	1.7 ± 0.003	1.1 ± 0.003	–	–	1.6 ± 0.004	1.5 ± 0.005
33	34,33	1260	1253	6-methyl-Dodecane	1.9 ± 0.005	1.3 ± 0.003	2.0 ± 0.001	1.9 ± 0.004	1.8 ± 0.007	–
34	34,88	1268	1259	4-methyl-Dodecane	–	0.5 ± 0.002	–	0.7 ± 0.001	–	–
35	37,06	1298	1249	1,3-bis(1,1-dimethylethyl)-Benzene	14.5 ± 0.067	9.8 ± 0.031	15.2 ± 0.025	16.6 ± 0.067	9.0 ± 0.041	7.7 ± 0.027

Table 2 (continued)

Nº	RT (min)	RI cal[a]	RI Llit[b]	Volatile compounds	No-choice experiment				Two-choice experiment	
					Sys_NI	Cont_NI	Sys_I	Cont_I	Sys_I	Cont_I
36	37,78	1308	1288	2-methyl-Dodecane	1.3 ± 0.003	0.9 ± 0.002	1.2 ± 0.003	1.4 ± 0.007	1.1 ± 0.004	0.9 ± 0.003
37	38,9	1322	1320	2,6,11-trimethyl-Dodecane	1.9 ± 0.005	1.3 ± 0.003	2.1 ± 0.001	2.9 ± 0.019	1.9 ± 0.004	1.6 ± 0.007
38	39,75	1332	1322	4-tert-Butylcyclohexyl acetate	–	–	1.9 ± 0.007	–	1.6 ± 0.006	1.9 ± 0.004
39	44,94	1416	1415	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester	–	–	2.4 ± 0.022	–	1.0 ± 0.004	1.1 ± 0.003
40	45,23	1420	1407	α-Copaene	0.4 ± 0.002	0.2 ± 0.001	–	–	0.5 ± 0.004	0.4 ± 0.004
41	46,74	1441	1413	Tetradecane	2.1 ± 0.005	1.2 ± 0.003	1.4 ± 0.003	1.3 ± 0.005	1.7 ± 0.005	1.3 ± 0.008
42	48,01	1470	1467	Caryophyllene	–	–	–	–	–	0.2 ± 0.001
43	51,36	1521	1488	1-Dodecanol	–	–	–	–	–	0.6 ± 0.006
44	52,72	1541	1512	Pentadecane	–	–	0.7 ± 0.001	–	–	–
45	53,47	1561	1533	α -Farnesene	–	–	0.4 ± 0.0004	0.4 ± 0.002	–	–
46	53,73	1566	1555	3,5-bis(1,1-dimethylethyl)-Phenol	3.0 ± 0.018	1.8 ± 0.014	4.5 ± 0.013	2.9 ± 0.025	4.8 ± 0.002	4.6 ± 0.029
47	58,68	1644	1605	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	–	–	2.0 ± 0.019	–	–	–

Results are expressed in relative percentage of the total chromatogram area (mean ± standard deviation, $n = 3$)

“- “ – not det

[a] RIcalc: retention indices calculated from C8 to C20 n-linear alkanes with TRB-5MS capillary column. [b] RIlit: retention indices reported in the literature for TRB-5MS capillary column or equivalent. Identification method: Compounds were identified by comparing the MS fragmentation pattern with those of STD and mass spectra database performed using NIST Standard Reference Database 69, PubChem Compound Summary and ChemSpider database. Considering fit and retrofit values > 80%

The comparison of VOCs results between no-choice and two-choice assays, showed differences on the volatile profile emitted by infested olives (Table 2). Overall, the total number of VOCs emitted by the infested olives was higher in the two-choice (36 VOCs) than in the no-choice (32 VOCs) assays. Interestingly, most of the additional VOCs detected in the two-choice assays were exclusively produced in this experiment in relation to no-choice, either on systemin-treated and/or non-treated olives (VOCs 1, 2, 22, 28, 33, 42 and 43 of Table 2).

Comparison between infested and non-infested olives

The volatile profile between infested and non-infested olives was compared, irrespective to the treatment with systemin and type of assay. Overall, the results showed that infested olives emitted a greater number of VOCs (41) than non-infested olives (31) (Table 2). Moreover, only 27 out of 47 VOCs were common to infested- and non-infested olives, with some VOCs classes being detected uniquely in infested-olives (esters) or in non-infested olives (terpenes) across the whole treatments (Fig. S2). The aromatic hydrocarbon 1, 3-bis (1,1-dimethylethyl)-benzene and the alcohol (Z)-3-hexen-1-ol were amongst the most abundant VOCs emitted by both non-infested and infested olives. However, infested olives emitted abundantly also 1-hexanol, while in non-infested olives the terpene D-limonene was the second most prevalent VOC (Table 2).

Relationship between volatile profile and oviposition preference of *B. oleae*

To identify which VOCs are characteristic of each treatment, a principal component analysis (PCA) was performed with VOCs emitted from infested and non-infested olives of systemin-treated and non-treated branches in the two assays (no-choice or two-choice) (Fig. 1). The PCA showed a variance of 41.4% considering the two principal components (Dim1 and Dim2), with samples grouping according to the treatment and infestation level. In the no-choice assays, infested olives treated with systemin were distinct from the other treatments due to the emission of pentadecane (VOC 44 of Table 2) and pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester (VOC 47), while the infested olives from the control were mostly characterized by the 6-methyl-dodecane (VOC 33) and 1,3-bis(1,1-dimethylethyl)-benzene (VOC 35). Non-infested olives treated with systemin were distinguished from the other samples mostly due to the emission of both β–ocimene (VOC 18) and 2-methyl-undecane (VOC 29), whereas non-infested control olives were characterized by the release of nonanal (VOC 25), α-pinene (VOC 7), 6-methyl-5-hepten-2-one (VOC 10) and tetradecane (VOC 41). In the two-choice assays, infested olives treated with systemin were characterized by VOCs toluene (VOC 1), 5-methyl-decane (VOC 13), (Z)-3-hexen-1-ol (VOC 3), 1-hexanol (VOC 5), 4-ethyl-1,2-dimethyl-benzene (VOC 23) and 3,5-bis(1,1-dimethylethyl)-phenol (VOC 46), while control infested olives were characterized by the 2-methyl-6-methylene-2-octanol (VOC 22), 2,3-dimethyl-1-pentanol (VOC 2), 1,2,4,5-tetramethyl-benzene (VOC 28) and caryophyllene (VOC 42).

One of the main goals of this study was to elucidate whether systemin induced a defence response on olives via the release of specific VOCs. Therefore, a Pearson correlation analysis was conducted to identify the VOCs that were positively or negatively associated with oviposition preference of *B. oleae*. In this analysis was only used the VOCs emitted from attacked olives, since they are more likely to reflect the defence response, and preselected by the random forest analysis. This analysis ranks the contribution of each VOCs in the discrimination of the treatments (i.e., systemin-treated and non-treated). Seventeen VOCs were identified as the most important (mean Gini values > 0.23) for discriminate systemin-treated from non-treated olives (Fig. S4). These VOCs were then used to perform Pearson correlations with oviposition data (Fig. 2). The results revealed that the 4-methyl-dodecane (VOC 14 of Table 2) exhibited significant ($p < 0.05$) positive correlations with oviposition and infested olives. In contrast, the alcohol 2-ethyl-1-hexanol (VOC 16), the aromatic hydrocarbon 1, 2, 3-trimethyl-benzene (VOC 11), and the ester 4-tert-butylcyclohexyl (VOC 38) were significantly ($p < 0.05$) negative correlated with oviposition and infested olives.

Discussion

The use of resistance inducers in plant priming (PRIs) is increasingly recognized as an important approach in sustainable agriculture, owing to their potential to reduce environmental impact and minimize metabolic costs for plants (Perazzolli et al. 2022). Peptides, such as systemin, are one of these PRIs that are gaining more attention due to their recognized role as defence signalling molecules, with ability to amplify the immune response of the plant against invading attackers (Pastor-Fernández et al. 2023). However, such role of systemin has been mostly reported in solanaceous species, such as tomato, and under natural environmental conditions (Pastor-Fernández et al. 2023). Works focussing on its potential for induced plant resistance against pests and pathogens when applied exogenously are scarce, but with promising results. For example, the exogenous application of systemin in tomato showed to induce the emission of volatiles that attract pest natural enemies and, on the other hand, alert neighbouring

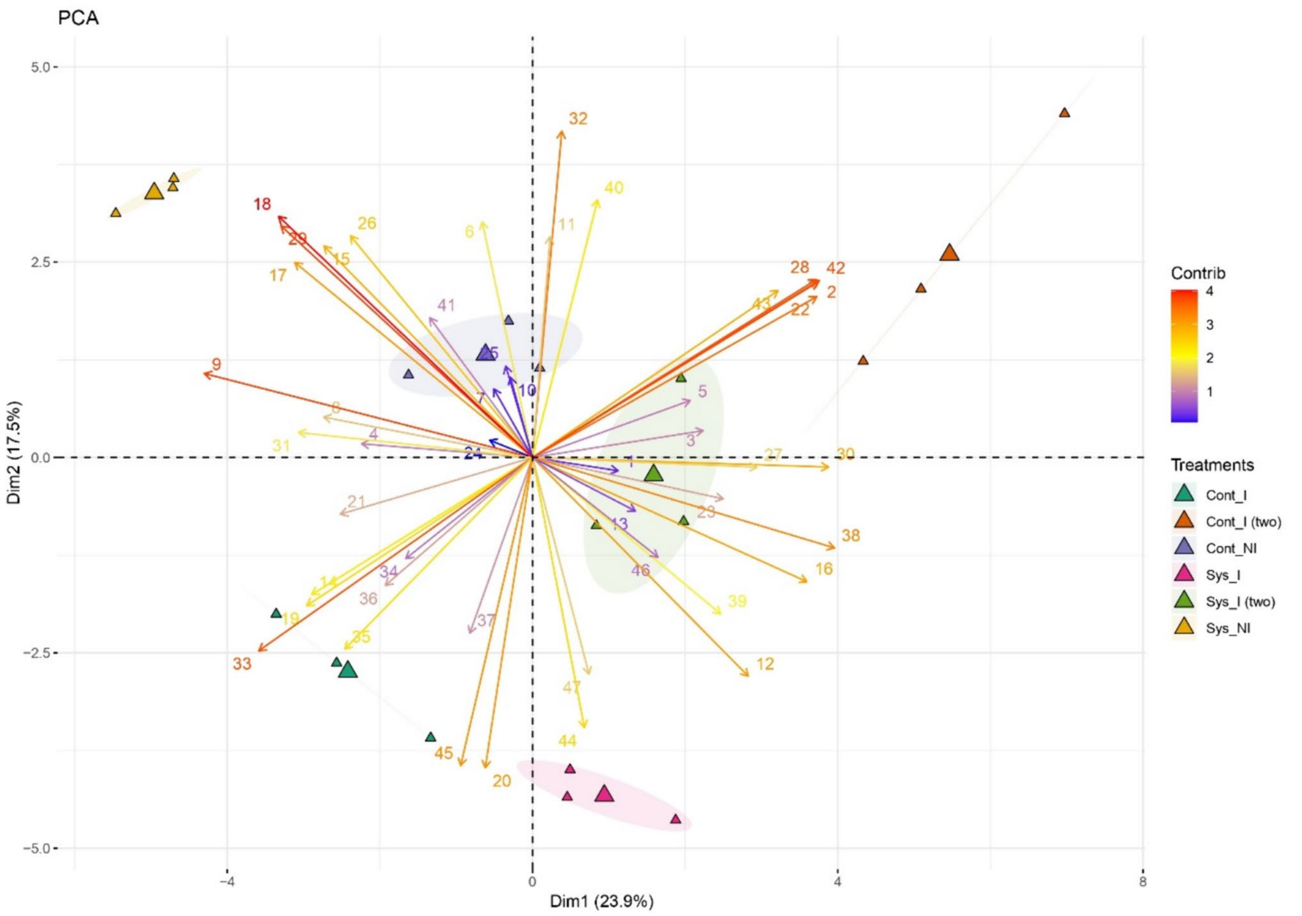

Fig. 1 Principal Component Analysis score plots obtained from the volatile profile of olives infested (I) and non-infested (NI) by *Bactrocera oleae* from systemin-treated (Sys) and non-treated (Cont) olive branches assessed in no-choice and two-choices assays (two). Each number corresponds to a particular volatile compound, as indicated in Table 2. The gradient colour represents the contribution of each variable (i.e., volatile compound) to the explanation of the greatest variance in the dataset. Each triangle represents treatments, being the ones with the greater size representing the average

plants priming their defences (Corrado et al. 2007; Coppola et al. 2017). In another work, systemin-treated tomato plants showed to be more resistant to the pathogen *Botrytis cinerea* and to the noctuid moth *Spodoptera littoralis* (Boisduval, 1833), and to be more attractive towards insect natural enemies via the release of volatile compounds (Coppola et al. 2019). Similarly, the application of systemin in *Solanum melongena* and *Vitis vinifera* showed to confer protection against *B. cinerea*, by activating the defence and antioxidant machineries (Molisso et al. 2021). In our study was demonstrated, for the first time, that the exogenous application of systemin may also confer protection of olive tree against pests. Indeed, *B. oleae* females exhibited a significantly higher preference for olives non-treated with systemin, ovipositing more eggs on these olives as compared to olives treated with 10 nM of systemin. These results open a novel perspective on the use of systemin in olive crop protection.

In the present study, the volatile profile evaluated in infested olives showed to differ quali- and quantitatively between olives treated and non-treated with systemin. In fact, a number of VOCs were exclusively produced either in systemin-treated or in control, with esters being uniquely emitted by systemin-treated olives upon *B. oleae* infestation. Thus, it is likely that the release of VOCs triggered by systemin might be an important part of the plant's defence strategy against *B. oleae* attack. Indeed, the systemin pathway has been reported in the literature to play a role in the regulation of the production of volatile emissions. For example, tomato plants that overexpress the precursor of systemin, ProSys, produce a distinct profile of VOCs compared to wild-type plants, resulting in an increased production of bioactive volatile compounds and the activation of genes involved in volatile production (Corrado et al. 2007; Degenhardt et al. 2010). Furthermore, plants that lack the ability to induce direct defences mediated by the systemin

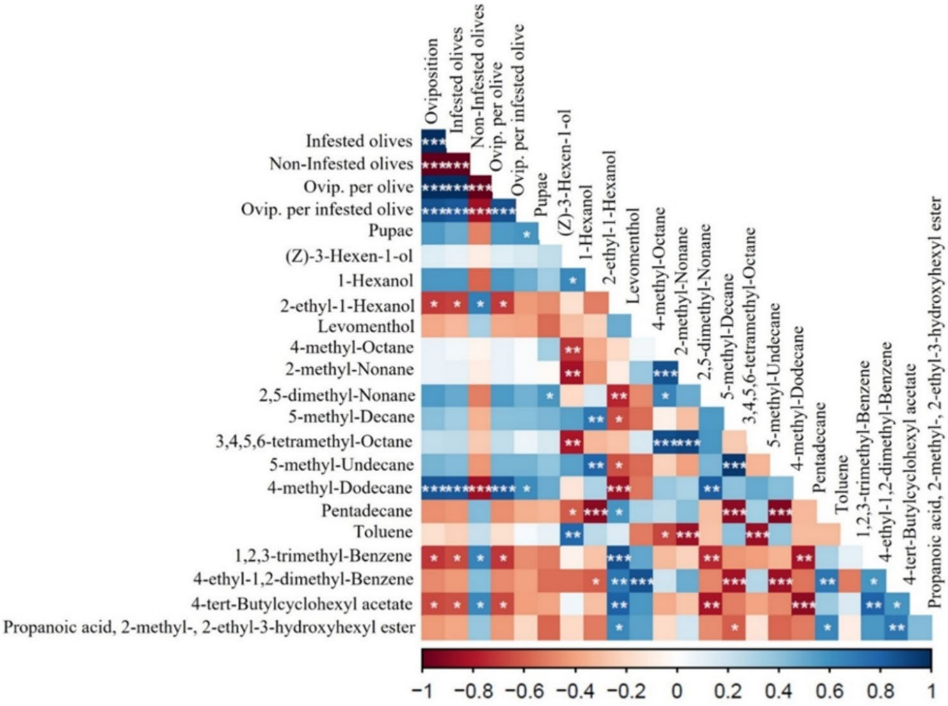

Fig. 2 Pearson correlation between oviposition preference of *Bactrocera oleae* and volatile organic compounds (VOCs) emitted from olives. These correlations were only performed with VOCs preselected by the random forest analysis. The oviposition preference was estimated through the total number of ovipositions, number of infested and non-infested olives, number of ovipositions per olive, number of ovipositions per infested olive, and number of pupae. Blue colour represents positive correlations (max = 1), while red colour represents negative correlations (min = − 1). Asterisks indicate statistically significant correlations at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

pathway have also been shown to be deficient in volatile emissions in response to wounding, when compared to plants that overexpress ProSys (Degenhardt et al. 2010). Therefore, the protection conferred by systemin in olive tree against olive fruit fly observed in our study is likely due, at least in part, to the release of specific VOCs. Indeed, the level of infestation and ovipositions by *B. oleae* was found to be negatively correlated with the VOCs 2-ethyl-1-hexanol, 4-tert-butylcyclohexyl acetate and 1, 2, 3-trimethylbenzene. The alcohol 2-ethyl-1-hexanol and the ester 4-tert-butylcyclohexyl acetate were amongst the most abundant or were exclusively detected in infested systemin-treated olives, suggesting being part of the plant defence response. Interestingly, 2-ethyl-1-hexanol has been used as an inert ingredient in pesticide formulation (Dougnon and Ito 2022) and reported in having a repellence effect against pests. For example, this volatile was suggested to increase the resistance of apple tree to the aphid *Aphis citricola* (van der Goot, 1912) via chemical repulsion (Song et al. 2017) and to repel the weevil *Curculio chinensis* (Chevrolat, 1978) attack in the host oil plant *Camellia oleifera* (Qiu et al. 2022). Nevertheless, this volatile is also reported to be able to attract pest insects, such as the sweetpotato whitefly *Bemisia tabaci* (Gennadius, 1889) (Chen et al. 2017) and the weevil *Callosobruchus maculatus* (Fabricius, 1775) on legumes seeds (Ajayi et al. 2015). Therefore, this volatile compound might elicit varied behavioural reactions among different insects and plant species. In our study was also identified one VOC that was positively correlated with olive infestation. The alkane 4-methyl- octane exhibited significant positive correlation with oviposition and infested olives. Moreover, in the PCA cluster analysis, infested olives from the control in the no-choice assays were mostly characterized by another alkane (6-methyl-dodecane). Alkanes are biosynthesized from fatty acid intermediates through reduction to fatty aldehydes, followed by decarboxylation via aldehyde decarboxylase enzymes that have been found in plants (Bernard et al. 2012). Unbranched saturated hydrocarbons (n-alkanes) are important constituents of the cuticular waxy layer of plants, which has a critical primary function of protection against water loss, UV light, pathogens, and pests (Samuels et al. 2008; Blomquist et al. 2020). Thus, the increase in the emission of volatiles belonging to this class could be a defensive response of the plant to subsequent ovipositions, by reinforcing olive cuticle.

When plants are exposed to abiotic or biotic stress, serial events can be induced, including the emission of volatiles from the vegetative parts (Dong et al. 2016). In accordance with, an induction of volatile emission was observed in infested olives in relation to non-infested olives, irrespective the exogenous application of systemin. Previous studies reported changes in VOCs emission by *B. oleae*-infested olive fruits. For instance, Giunti et al. (2016) reported that the production of the terpene (*E*)-*β*-ocimene and the ketone 2-methyl-6-methylene-1,7-octadien-3-one were increased by *B. oleae* infestation and four terpenes were decreased, namely *α*-pinene, *β*-pinene, limonene, and *β*-elemene, being characteristic of uninfested fruits. Similarly, in a more recent study, Bongka et al. (2023) detected compounds that were only found in the volatile blend of larval *B. oleae* -infested fruits, namely *n*-nonanal, *n*-octane, heptanal, 1-nonanol, 2-nonanone, and (*E*)-*β*-ocimene. In the present work, specific volatile compounds were uniquely detected in infested-olives (esters) and in non-infested olives (terpenes). Esters have been described as having the potential to repel or attract and kill pests, such as *Acyrthosiphon pisum* (Harris, 1776) (Hemiptera: Aphididae) and *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) (Giner et al. 2013). Several esters compounds showed to cause rapid toxic effects in a range of different agricultural insect pests, at low concentrations (Feng et al. 2018), and therefore have potentials for use in pest control. Terpenes are also known to mediate plant–insect interactions, and thus playing significant properties in the context of chemical ecology (Gershenzon and Dudareva 2007). Apart from their effect to attract natural enemies of pests and beneficial insects, some terpenes exhibited toxicity against many insect pests (Boncan et al. 2020; Ninkuu et al. 2021). For example, the terpenes *α* –pinene, limonene and ocimene detected in our work, were previously reported to be effective against several insect pests (Ninkuu et al. 2021). Similarly, the presence of different profiles of terpenes, particularly of monoterpenes, emitted from *Citrus sinensis* plants grafted on different rootstock showed to influence the aphid *Aphis gossypii* (Glover, 1877) preference behaviour towards a specific scion/rootstock combination (Guarino et al. 2022). Furthermore, our results suggest that, in addition to confer protection within systemin-treated olives, systemin seems to protect neighbouring non-treated olives against *B. oleae* attack. This hypothesis is based on the lack of *B. oleae* preference to lay eggs either in systemin-treated or in control olives in the two-choice assays, with both treatments exhibiting a repellence effect. In previous studies conducted on tomato plants, was observed that the application of systemin resulted in an altered expression pattern of defence genes in neighbouring unchallenged plants, ultimately enhancing their resistance to *Spodoptera littoralis* (Boisduval 1833) larvae attack (Coppola et al. 2017). According to the same authors, this communication between plants was probably controlled by volatile signal molecules produced via systemin-dependent metabolic pathways (Coppola et al. 2017). In our work, the treatment of olive tree branches with systemin seems similarly to triggers priming responses in neighbouring olives via the released of VOCs. In fact, upon infestation, the olives from two-choice assays showed to emitted more number of different VOCs when compared to one-choice assays. It is possibly that upon *B. oleae* attack, the systemin induces the emission of VOCs that in turns are perceived by neighbouring not-treated systemin olives. These olives, when infested, are likely to produced more and new volatile compounds enhancing their resistance to the pest. This hypothesis is reinforced by the higher number of new and unique volatile compounds produced in control olives in relation to systemin-treated olives in the two-choice assays. However, further investigation is needed to confirm the potential role play by systemin in triggering a priming response in neighbouring olives.

Currently, peptides are recognized as important signalling molecules in various plant processes, and our study extends the understanding of significant effects of systemin on olives against *B. oleae* infestation. From a practical perspective, this peptide could be very promising to mitigate infestation and reduce losses caused by olive fruit fly in olive crop. The beneficial effect of systemin observed through the method employed in this study, which involved immersing the cut ends of branches carrying fruits in a systemin solution, suggests its potential for application in olive crops via irrigation for olive fruit fly management. However, its effectiveness as a defence peptide will need to be validated through future field trials.

Conclusions

In our work, the exogenous application of the peptide systemin in olive branches showed to protect fruits from the attack of the olive fruit fly, *Bactrocera oleae*. In fact, systemin-treated olives were less attractive to oviposition than not-treated olives. This protective effect was positively correlated with the emission of a set of volatile compounds (in particular 2-ethyl-1-hexanol, 4-tert-butylcyclohexyl acetate and 1, 2, 3-trimethyl-benzene) that can probably act by priming or inducing a plant defence response. Besides protecting fruits from *B. oleae* attack, the treatment with systemin seems also protect neighbouring not-treated olives against the infestation via the emission of specific volatiles. Although preliminary, our results are very promising for the development of new tools to control olive fruit fly via the application of systemin. Its role on the control of olive fruit fly under more realistic conditions (e.g., field assays) as well as the elucidation of its mode of action should be considered in future research works.

Author contributions

P.B. and J.A.P. designed the experiments, supervised the study and revised the manuscript. L.S. performed most of the experiments, analysed the data and drafted the manuscript. A.E.C. assisted with data analysis and investigation. N.R. assisted with the analysis of volatile compounds. All authors have read and agreed to the published version of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest The authors declare no conflict of interest.

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