Plant Stress 12 (2024) 100456

Contents lists available at ScienceDirect

Plant Stress

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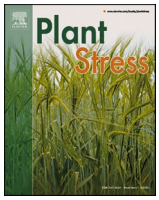

Eurydema oleracea-induced volatile organic compounds modulate Arabidopsis response to *Botrytis cinerea* infection

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ARTICLE INFO

Keywords:
Arabidopsis
Botrytis cinerea
Eurydema oleracea
Jasmonate defence pathways
Volatile organic compounds

ABSTRACT

Volatile organic compounds (VOCs) are secondary metabolites through which plants interact with the environment. Upon interaction with a stressor, a blend of plant VOC changes for an increasing amount of constitutive phytochemicals and/or for phytochemicals synthesized *de novo*. These latter compounds are referred as induced-VOCs. We previously showed that, on *Arabidopsis thaliana* Col-0 plants, VOCs blend induced via jasmonic acid-related pathway by pre-inoculation with the necrotrophic fungal pathogen *Botrytis cinerea* significantly reduced both plant choice and feeding damage related to the piercing sucking insect *Eurydema oleracea* infestation. Continuing to study the same tripartite combination, we here demonstrated that VOCs released by Arabidopsis immediately after *E. oleracea* infestation directly inhibited the *in vitro* growth of *B. cinerea* and indirectly reduced leaf damage caused by the pathogen, triggering the jasmonic acid-mediated pathway. Insect priming of defence against *B. cinerea* was short-term. Together with our previous findings, this study contributes to the knowledge on the tripartite plant-microbe-insect interaction and on the role of VOCs in the communication between plants and the other organisms of a natural community.

1. Introduction

Volatile organic compounds (VOCs), mainly consisting of terpenoids, phenylpropanoid/benzenoid, fatty acid- and amino acid derivatives and C6 green-leaf volatiles (GLVs), are produced by secondary metabolism of plants, through which they interact with the surrounding environment (Dudareva et al., 2004 and 2013; Lawo et al., 2011; Rahnamaie--Tajadod et al., 2019).

The role of VOCs has been demonstrated mainly in plant response to biotic and abiotic stress and, to a lesser extent, in plant-plant communication as modulators of defence, growth and reproduction (Holopalainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Brosset and Blande, 2022). Upon interaction with a stressor, plant VOC blend changes for an increasing amount of constitutive phytochemicals emitted and/or for one or more phytochemicals synthesized *de novo* (Quaglia et al., 2012). These latter compounds are known as induced-VOCs (IVOCs) (Quaglia et al., 2012). IVOCs include herbivore-induced plant volatiles (HIPVs), a blend of volatile compounds released by plants after herbivore attack (Lucas-Barbosa et al., 2011). The blend composition depends on the plant and insect species and on the insect feeding behaviour (chewing or sucking) (Kutty and Mishra, 2023), with more than 2000 volatiles identified from a thousand of plant families (War et al., 2011). In particular, blend induced by chewing herbivores includes GLVs, terpene and, sometimes, methyl-salicilate; blend induced by mesophyll-feeding stylet (sucking) feeders, via jasmonic acid (JA) signalling, includes mainly terpenoids and fatty acid derivatives and blend induced by phloem-feeding stylet (sucking) feeders includes also phenylpropanoid/benzenoid derivatives of the salicylic acid (SA) signalling pathway (Kutty and Mishra, 2023).

HIPVs can show both direct (toxicity, feeding and/or oviposition deterrent) and indirect (attraction of natural enemies) effects on the herbivore and can modulate its immunity to parasitoids, bacterial and viral pathogens (Zhang et al., 2019; Hu et al., 2021). Moreover, in plants attacked by herbivores but also in the unattacked neighbouring plants, HIPVs can induce or prime plant resistance responses (Zhang et al., 2019; Hu et al., 2021): in the first case the defence responses are immediately expressed, in the second case plant enter in an alert state leading to a quicker and stronger expression of defence response upon interaction with a subsequent stress factor. The amount of the emitted volatiles is negatively correlated with constitutive defence level and a trade-off between constitutive defences and HIPVs primed defences has been reported (Rasmann et al., 2014; Zhang et al., 2019). Both inducing

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https://doi.org/10.1016/j.stress.2024.100456
Received 12 December 2023; Received in revised form 4 March 2024; Accepted 1 April 2024
Available online 2 April 2024

or priming activities are related to HIPVs concentration and time of plant exposure (Girón-Calva et al., 2012). Thus, also at low concentrations, an inducing volatile can positively modulate the plant defence responses if the exposure time is long enough, indicating that the effect is related to volatile accumulation on receiver plant (Girón-Calva et al., 2012).

A series of studies have elucidated the mechanisms of volatile perception by plants, showing that volatiles are perceived by plasma membrane (Maffei et al., 2004) and that the received volatiles can be converted by plants in another volatiles, as demonstrated in tomato where the perceived (*Z*)−3-hexanol is transformed and accumulated in its glycosylated derivative (*Z*)−3-vicianoside (Sugimoto et al., 2014; Brosset and Blande, 2022). The early response to HIPVs perception is the production of reactive oxygen species (ROS), that can act as systemic signal in receiver plants, where several metabolic pathways are affected, such as mainly the JA-related but sometimes also the SA-related (Brosset and Blande, 2022).

The induced signalling pathways have a different role in response to different stressors, with SA pathway mainly involved in plant interaction with phloem-feeding (sucking) insects, biotrophic fungi and viruses and JA pathway in plant interaction with chewing- and mining- herbivores, some phloem-feeding insects, necrotrophic pathogens and nematodes (Quaglia et al., 2012; Moreira et al., 2018; Xu et al., 2019).

In the same plant, the two different defence signalling pathways can be induced simultaneously by different attackers and can interact with each other, both negatively or positively (Moreira et al., 2018; Ederli et al., 2021). This cross-talk between SA- and JA- signalling pathways affects the host response depending on the attacker and a plant mediated cross-effect between different attackers has been documented (Xu et al., 2019). Thus, an attack by a stressor can affect the attack ability and the performance of another stress factor and also affect the composition of the attacker community (Ederli et al., 2017; Moreira et al., 2018).

Both fungal-induced plant responses against herbivores and herbivore-induced plant responses against fungi have been reported (Rostás et al., 2003). Fungal-induced responses can affect both herbivore behaviour and performance (Rostás et al., 2003). For instance, pre-infection of the host plant *Rumex* (L.) ssp. by the biotrophic rust fungus *Uromyces rumicis* (Schumach.) G. Winter had a negative effect on the oviposition and feeding behaviour of the leaf beetle *Gastrophysa viridula* (De Geer) (Coleoptera: Chrysomelidae); in addition, the growth rate of larvae and adult fecundity decreased when they fed themselves on infected *Rumex* leaves (Hatcher et al., 1994a). Similarly, pre-infestation on the *Rumex* spp. leaves by the *G. viridula* larvae significantly reduced the severity of *U. rumicis* attack, both at the leaf part damaged by herbivores but also on undamaged leaf parts (Hatcher et al., 1994b).

In this context fit our research, dealing with the role of VOCs in the multiple interactions of the host plant *Arabidopsis thaliana* with the necrotrophic fungal pathogen *Botrytis cinerea* (Pers. ex Fr.) and the sucking herbivore insect *Eurydema oleracea* (L.) (Hemiptera: Pentatomidae). *B. cinerea* is one of the most destructive fungal pathogens worldwide, attacking over 200 crops mainly dicotyledonous, including species within the *Brassicaceae* family, amongst those *A. thaliana* (Williamson et al., 2007). Also, *E. olearacea* is reported as very harmful to *A. thaliana* and other *Brassicaceae* (Bohinc and Trdan, 2012; Ederli et al., 2020 and 2021). We previously showed that pre-inoculation with *B. cinerea* affects the *E. oleracea* feeding behaviour, significantly reducing herbivore feeding leaf damage; the protection conferred by the fungus was connected to the activation of the JA- defence pathway and the related VOCs emission by infected plants, since treatment with VOCs emitted by *B. cinerea* inoculated plants inhibited herbivore host plant choice and feeding damage (Ederli et al., 2021). Continuing to study the same tripartite combination, we here investigated the effect of pre-infestations with *E. oleracea* on the progress of *B. cinerea* infections, with particular attention to the direct and indirect (plant-mediated) role of HIPVs against the fungus. This could provide new insights into molecular ecology by helping to clarify the role of VOCs in shaping interaction between plant and other organisms of a natural community.

2. Materials and methods

2.1. Plant material, insect and fungal culture

Seeds of *A. thaliana* wild-type (WT) Columbia (Col-0) ecotype obtained from the Nottingham Arabidopsis Stock centre (NASC) were used in this study. Surface sterilization of seeds was carried out by soaking them in 70% (v/v) ethanol for 1 min and 15% (w/v) bleach for 5 min, in a 2 ml Eppendorf tube. After discarding the bleach, seeds were washed three times in sterile distilled water, resuspended in 500 µl sterile distilled water, and vernalized for 48 h at 4 °C, in the dark. Seeds were sown in sterile soil mix (Patzer Einheitserde, Manna Italia, Bolzano, Italy) into individual pots (5.5 cm diameter) and grown at 23 ± 2 °C and 60–75% relative humidity, with 12 h light (with variable intensity throughout the day within the range 80–200 µmol m^{-2} s^{-1})/12 h dark cycles for three weeks.

E. oleracea adults collected in a field close to Perugia (Umbria, Italy) on *Eruca vesicaria* (L.) Cav., were reared as a colony in isolation cages (30 × 30 cm) (Vermandel, Sluis, The Netherlands) at 24 ± 2 °C temperature, 70% relative humidity, and 16/8 h light/dark. The insects were continuously fed with fresh cabbage leaves. Adult unmated females were used in all experiments.

Necrotrophic pathogen *B. cinerea* isolate as reported by Ederli et al. (2015 and 2021) was used. The pathogen was grown on Potato Dextrose Agar (PDA, Biolife Italiana s.r.l.) pH 5.7, at 21 ± 2 °C, in the dark. Conidial suspension was obtained by washing 10 days old colonies with a sterile aqueous solution of 10 mM sucrose and 10 mM KH_2PO_4 added with 0.04% (v/v) of Tween20® (Sigma-Aldrich Inc., St. Louis, USA), filtering the washing liquid through cheesecloth, counting the numbers of conidia with a hemocytometer and adjusting the final concentration at 1 × 10^5 conidia/ml.

2.2. Eurydema oleracea infestation and Botrytis cinerea inoculation on Arabidopsis

Adult unmated females of *E. oleracea* starved for 24 h were used for Arabidopsis infestation. Each insect was placed on the leaf (1 leaf per plant) within a clip-cage made as described by Ederli et al. (2021). In detail, a plastic Petri dish (3.8 cm diameter; 1 cm high) with a mesh-covered hole (3 cm diameter) and with the rim covered by a sponge ring to prevent leaf damage was used. The insects were allowed to feed for 24 h on the leaf and then removed. Immediately after removal (T_{0h}), or 24 h after removal (T_{24h}), drop-inoculation of the *B. cinerea* conidial suspension (1 × 10^5 conidia/ml) was carried out on the leaves where the insect had fed. Fungal inoculation was performed also on uninfested (control) leaves, in which empty clip-cages were kept (1 cage per leaf) for 24 h, immediately or 24 h after removal of the empty cages. For each leaf, 2 drops of 5 µl each of the *B. cinerea* conidial suspension were applied. Inoculated plants were maintained in the growth chamber, at the conditions described above for plant grown, with the only difference that, for the first 24 h after inoculation, the relative humidity was set to 100%. In order to assess the effect of *E. oleracea* infestation on Arabidopsis susceptibility to *B. cinerea*, the quantification of the leaf damage produced by the pathogen was carried out at 1, 2, and 5 days post-inoculation (dpi) by measuring the diameter of leaf lesions from digital images using the open-source image-processing program ImageJ (Schneider et al., 2012).

2.3. Collection of VOCs from Arabidopsis infested by Eurydema oleracea

From Arabidopsis plants unifested (CNT) or infested for 24 h by *E. oleracea*, VOCs collection was carried out immediately (T_{0h}) or at 24 h (T_{24h}) and 48 h (T_{48h}) after insect removal. Five plants about three

weeks old, selected for their homogeneity in number (8) and extension of leaves, with the pot wrapped by food aluminium foil, were placed in a cylindrical glass chamber (5 l volume) (Fig. S1). An air stream purified by passing through a charcoal filter was pumped through the chamber at 500 ml/min. A glass cartridge (10 × 0.5 cm) filled with 100 mg of Porapak Q (80–100 mesh; Sigma-Aldrich) was connected to a glass chamber to collect the VOCs. The air was pumped out from the trap by an additional pump positioned at the end of the device. After collecting for 24 h, at 25 °C, the traps were eluted in a glass vials with 800 µl of hexane and concentrated to 200 µl under a gentle nitrogen stream. The extracts from 3 traps (15 plants) for each sample were mixed. Extracts were stored in a freezer at − 18 °C in glass vials with Teflon cap liners, until use. Three independent experiments were carried out to obtain three biological replications per each treatment and the control. All replicates were carried out under controlled conditions (22 ± 2 °C, 50 ± 10% RH, and photoperiod 12L:12D). After each collection, the chambers were washed with water and fragrance-free detergent, rinsed with hexane and acetone, and baked overnight at 150 °C.

2.4. Effect of VOCs blend emitted by Eurydema oleracea-infested plants on Botrytis cinerea and its interaction with Arabidopsis

Initially, the direct effect of VOC blend emitted by *E. oleracea* infested plants on *B. cinerea in vitro* growth was studied. For this purpose, sterilized filter paper strips (15 mm × 20 mm, Whatman No. 1), used as odour cartridges, were impregnated with 30 µl of VOC extract collected at different times (T_{0h}, $T_{24h,}$ and T_{48h}) after infestation (as described above), and applied to the internal face of the Petri dishes (9 cm diameter) covers. As controls, impregnation with 30 µl of VOC extract collected from uninfested Arabidopsis plants (CNT) or with 30 µL of hexane, the solvent used for eluting VOCs, or no treatment (NT) were applied. For each treatment, four plates were set up and the experiment was repeated three times. A 0.5 cm-diameter mycelial plug of *B. cinerea* taken from the margin of 10 days old colonies grown as above described was placed at the centre of each Petri dish, on PDA. Plates were incubated at 21 ± 2 °C, in the dark. At 72 h of incubation, *B. cinerea* colonies area was quantified on digital photo captured through a white light transilluminator (Uvitec Ltd., Cambridge, United Kingdom) and related UVITEC1D image acquisition software, using the image analysis software ImageJ (Schneider et al., 2012).

The indirect effect of VOCs on the susceptibility of Arabidopsis to *B. cinerea* was assessed on detached Arabidopsis leaves, taken from plants grown as above reported, placed on 1.2% water-agar (Agar Bacteriological, Biolife Italiana, S.r.l., Milan, Italy) in 9 cm diameter Petri dishes, and exposed to VOCs or control treatments, as described for the direct effect. After 24 h of exposure, each leaf was inoculated with 2 drops of 5 µl each of the *B. cinerea* conidial suspension at a concentration of 1×10^5 conidia/ml, as described above for the experiments in *planta*. Quantification of *B. cinerea* symptoms was carried out at 5 dpi by measuring the diameter of leaf lesions. In addition, after 24 h of VOCs exposure, two leaves per plate were harvested, frozen in liquid nitrogen and stored at −80 °C until RNA extraction and transcript analysis. For each treatment, three plates containing six leaves taken from three Arabidopsis plants (one plate per plant) were set up. Three independent experiments were carried out.

2.5. RNA extraction and transcript analysis by RT-qPCR

Total RNA was extracted from leaf tissue (100 mg) using PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions. Total RNA was processed with TURBO DNase kit (Thermo Fisher Scientific) to eliminate potential DNA contamination and purified RNA (500 ng) was used to prepare cDNA using PrimeScript™ RT-PCR Kit (Takara, Shiga, Japan), according to the manufacturer's protocol. Real-time quantitative PCR (RT-qPCR) analyses were performed with 50 ng cDNA in a final volume of 25 µL using the SYBR Premix Ex Taq II reagent (Takara) following the manufacturer's instructions. The RT-qPCR was carried out using the CFX96 detection system (Bio-Rad, Hercules, CA, USA). Cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. All RT-qPCR experiments were performed using three biological and three technical replicates. Relative amounts of the transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using the Arabidopsis Elongation factor-1α (EF-1α; At1g07940) as internal standard. Primer sequences were shown in Table 1.

2.6. Chemical analysis of VOCs from Eurydema oleracea-infested Arabidopsis

To verify qualitative differences in emitted VOCs, chemical analyses were conducted by injecting one µL of each of the samples obtained by VOC collection from plants (CNT, T_{0h}, $T_{24h,}$ and T_{48h}) in an Agilent 6890 GC system equipped with a DB5-MS column and interfaced with an MS5973 quadruple mass spectrometer. The GC–MS was configured in a non-split mode with helium as the carrier gas. Injector and detector temperatures were set at 260 °C and 280 °C, respectively. The GC oven temperature was initiated at 40 °C for 5 min and then increased at a rate of 5 °C/min to reach 250 °C. Electron impact ionization spectra were generated at 70 eV, recording mass spectra ranging from 40 to 550 amu. To quantify, the ChemStation software was employed to compute the peak area of each detected compound. Compounds were putatively identified through a comparison of mass spectra with references in the literature (Adams, 2007; www.pherobase.com) and the NIST 2011 and Wiley 17 libraries. Confirmation of these identifications was further supported by matching with published mass spectra and linear retention indices (LRIs). LRIs were calculated using a range of n-alkanes (C8-C40) that were previously injected at the same GC–MS conditions at a concentration of 10 ppm. Additionally, authentic standards (Sigma-Aldrich, Milan, Italy) were used for identification when available. Periodic black headspace collections were conducted to eliminate potential contaminants from the analysis.

2.7. Statistics

All data were submitted to one- or two-way analysis of variance (ANOVA), using the extension for Excel ® DSAASTA version 1.514 (Onofri and Pannacci, 2014), followed by Tukey's HSD test. Experimental designs are described in the figure legends.

3. Results

3.1. Infestation by Eurydema oleracea altered the susceptibility of Arabidopsis to Botrytis cinerea

The infestation of Arabidopsis by the herbivore insect *E. oleracea* resulted in a modified plant susceptibility to the necrotrophic fungal pathogen *B. cinerea*. Indeed, with respect to the uninfested control plants, a significant reduction (20.5% on average) in leaf lesion size was observed at 1 and 2 dpi both when the pathogen was inoculated immediately after insect removal, and 24 h after insect removal (Fig. 1). Instead, at 5 dpi, the leaf lesions diameter was similar in plants not previously infested, used as control, than in plants previously infested with *E. oleracea* (Fig. 1).

3.2. VOCs emitted by Eurydema oleracea-infested plants inhibit the in vitro growth of Botrytis cinerea and Arabidopsis susceptibility to the pathogen

In previous work (Ederli et al., 2021) we showed that pre-treatment of Arabidopsis Col-0 with VOCs emitted by *B. cinerea* inoculated plants significantly reduced both *E. oleracea* plant choice and feeding damage. Continuing to dissect the role of VOCs in plant defence under

Table 1
Sequences (5'-3') of the primers used for RT-qPCR analysis.

Gene	Forward sequence	Reverse sequence
Elongation factor 1-α (EF-1α; At1g07940)	AGTGGTCGTACAACCGGTATTGT	TGGTGGTCTCGAACTTCCAG
Phenylalanine ammonia lyase 1 (PAL1; At2g37040)	ATGGAGATTAACGGGGCACAC	GGCGGTGATTGTACCACGGAG
Pathogenesis-related protein 1a (PR1a; At2g14610)	CGAAAGCTCAAGATAGCCCAC	AAACTCCATTGCACGTGTTCG
Allene oxide synthase (AOS; At5g42650)	GCGACGAGAGATCCGAAGA	CTCGCCACCAAAACAACAAA
Plant defensin 1.2 (PDF1.2; At5g44420)	TTTGCTGCTTTCGACGCAC	TAACATGGGACGTAACAGATA
Vegetative storage protein 2 (VSP2; At5g24770)	CTTGATCACTTCCAAACAGTACC	GGGTATCCTCAACCAAATCAG

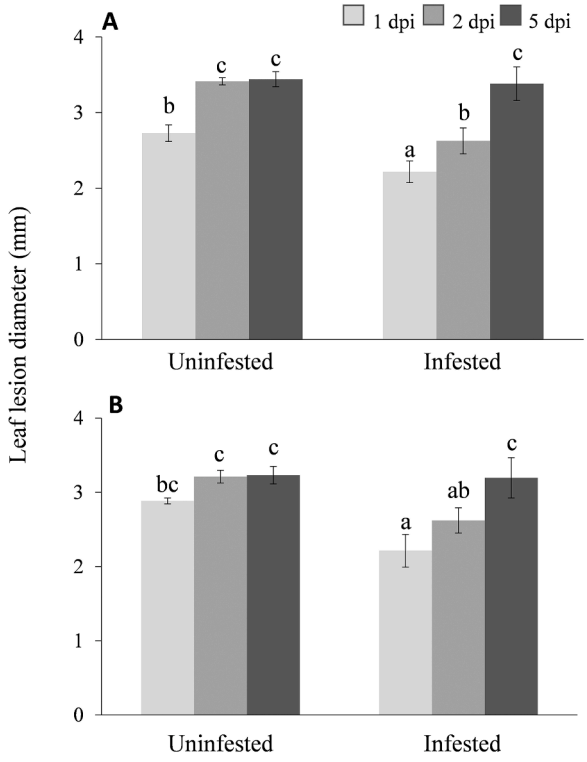

Fig. 1. Susceptibility of *Arabidopsis thaliana* Col-0 plants to the necrotrophic fungal pathogen *Botrytis cinerea* after *Eurydema oleracea* infestation. Measurement of leaf lesion size (diameter) produced by the pathogen was performed at 1, 2 and 5 days post-inoculation (dpi), both when the pathogen was inoculated immediately after insect removal (A), and 24 h after herbivore removal (B). Each column represents the mean ± SE of three independent experiments (n = 6). Columns with different letters are statistically different [$p \leq 0.05$; ANOVA two-way (time x treatment), Tukey's HSD tests].

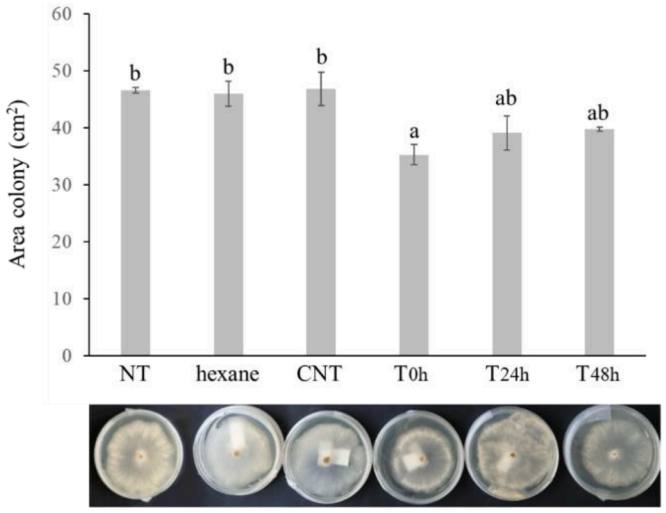

Fig. 2. Effect of *Eurydema oleracea*-induced volatile organic compounds (VOCs) collected from Arabidopsis Col-0 plants immediately after infestation (T_{0h}) or at 24 h (T_{24h}) and 48 h (T_{48h}) after insect removal on *in vitro* growth of *Botrytis cinerea* on potato dextrose agar, expressed as colony area (in cm^2) measured after 72 h of cultivation. CNT=VOC_S collected from uninfested Arabidopsis Col-0 plants; NT= no treatment; Hexane = solvent used to elute VOCs. Each column represents the mean ± SE of three independent experiments (n = 4). Columns with different letters are statistically different ($p \leq 0.05$; ANOVA one-way; Tukey's HSD tests). A representative image of the four replicated plates for each treatment is shown below the histogram.

simultaneous attack of pathogen and insect, we here assessed the direct effect of VOCs emitted by herbivore *E. oleracea*-infested Arabidopsis Col-0 plants on *in vitro* growth of *B. cinerea* and explored the possibility that pre-treatment with VOCs emitted by herbivore-infested Arabidopsis Col-0 plants may affect the plant response to the pathogen.

The effect of VOCs on fungal growth *in vitro*, expressed as colony area, was evaluated after 72 h of *B. cinerea* cultivation on PDA. Concerning unexposed fungal colonies (NT), after 72 h of exposure, only treatment with VOCs collected from *E. oleracea*-infested plants immediately after infestation (T_{0h}) caused a significant reduction (−23,8% compared to control) of the *B. cinerea* growth (Fig. 2). Differently, treatment with VOCs produced from *E. oleracea*-infested plants at 24 h (T_{24h}) and 48 h (T_{48h}) after herbivore removal only determined a slight and not significant reduction of the colony's growth (Fig. 2). Hexane, the solvent used to elute the VOCs, did not affect the growth of the pathogen (Fig. 2).

Compared to unexposed leaves, only exposure of Arabidopsis leaves within 24 h before pathogen inoculation to VOCs collected from Arabidopsis Col-0 plants immediately after herbivore infestation (T_{0h}) caused a significant reduction in the leaf lesions size produced by *B. cinerea* at 5 dpi (−65,21% compared to control; Fig. 3). Instead, exposure to VOCs collected at 24 h (T_{24h}) and 48 h (T_{48h}) after the insect removal or on uninfested control plants as well as exposure to hexane did not significantly affect lesion diameter (Fig. 3).

3.3. VOCs emitted by Eurydema oleracea-infested plants induced JA-related defence pathway, but not the SA-dependant one

To elucidate the molecular mechanism through which the *E. oleracea*-induced VOCs affect Arabidopsis susceptibility to *B. cinerea*, we examined the expression of marker genes of the SA- and JA- plant defence pathways. In particular, the transcripts of the SA biosynthetic *PAL1* gene and the SA-marker *PR1a* gene, as well as the JA biosynthetic *AOS* gene and the JA-marker *PDF1.2* and *VSP2* genes were quantified by RT-qPCR. The results clearly showed that VOCs released after *E. oleracea* infestation significantly affected only the JA-related, but not the SA-dependant defence pathway (Fig. 4). Indeed, with respect to unexposed leaves, there was no statistical difference in the transcriptional profile of *PAL1* and *PR1a* genes in Arabidopsis leaves exposed to VOCs collected from both uninfested and infested Arabidopsis plants at 0, 24 and 48 h post-infestation (T_{0h}, T_{24h} and T_{48h}) (Fig. 4). On the contrary, VOCs emitted immediately after herbivore infestation (T_{0h}) strongly induced the expression of the *AOS, PDF1.2* and *VSP2* genes (Fig. 4). The

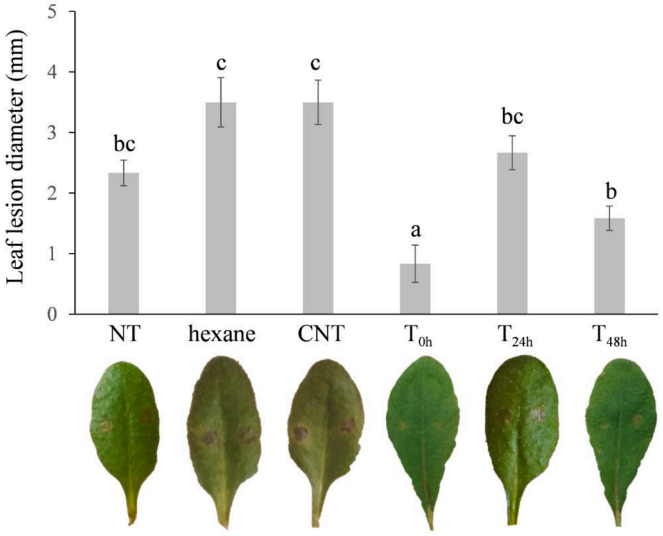

Fig. 3. Effect of Arabidopsis Col-0 leaves exposition for 24 h to *Eurydema oleracea*-induced volatile organic compounds (VOCs) collected from Arabidopsis plants immediately after infestation (T_{0h}) or at 24 h (T_{24h}) and 48 h (T_{48h}) after insect removal on the infection by the *Botrytis cinerea*. CNT=VOC_S collected from uninfested Arabidopsis Col-0 plants; NT=no treatment; Hexane = solvent used to elute VOCs. Quantification of *B. cinerea* symptoms was carried out at 5 dpi, as lesion diameter (in mm). Each column represents the media ± SE of three independent experiments ($n = 6$). Columns with different letters are statistically different ($p \leq 0.01$; ANOVA one-way, Tukey's HSD tests). For each treatment, a representative leaf is shown below the histogram.

VOCs released at later times (24 h and 48 h) after the insect removal had no impact on the expression of all the examined genes, except for the VOCs collected at T_{48h}, which significantly induced the expression of AOS gene, involved in JA biosynthesis (Fig. 4).

3.4. Qualitative variations in VOCs bouquet emitted by Arabidopsis thaliana after infestation with Eurydema oleracea

The GC–MS analysis findings of the *A. thaliana* VOCs are presented in Table 2 and Fig. S2. Through chemical analysis, the presence of a total of fourteen compounds was detected, with twelve being identified while two remain unknown.

Qualitative differences emerged in the composition of VOCs blend collected from plants uninfested (CNT) or in the different time intervals after insect infestation (T_{0h}, T_{24h} and T_{48h}). In the latter, under our experimental conditions (capture method and GC–MS analysis), most of the VOCs were below the detectable limit. About infested plants, (*Z*)−3-hexenol, 1-hexanol, heptanal, 4-ethylacetophenone, tetradecanal and methyl jasmonate (MeJA) were detected only in VOCs blend collected at T_{0h}, while myrcene and 6-methyl-5-hepten-2-one only in VOC blend collected at T_{48h}.

4. Discussion

In a previous paper (Ederli et al., 2021), we had already pointed out that the infection produced by the necrotrophic fungal pathogen *B. cinerea* influenced the Arabidospsis response to the subsequent infestation by the herbivore *E. oleracea*, and part of the defence mechanism was attributable to the plant VOCs emissions after inoculation with the pathogen. Similarly, in this study, we demonstrated that the susceptibility of Arabidopsis to *B. cinerea* could be influenced by prior infestation with *E. oleracea*, and this effect persisted over time. In fact, a significant reduction of leaf damage caused by the pathogen was evident not only when *B. cinerea* inoculation was carried out immediately after the insect infestation, but also when it was performed 24 h after the insect removal. In addition, we elucidate that the mitigation of the foliar injury was short-term and precisely limited to the first two dpi, while at

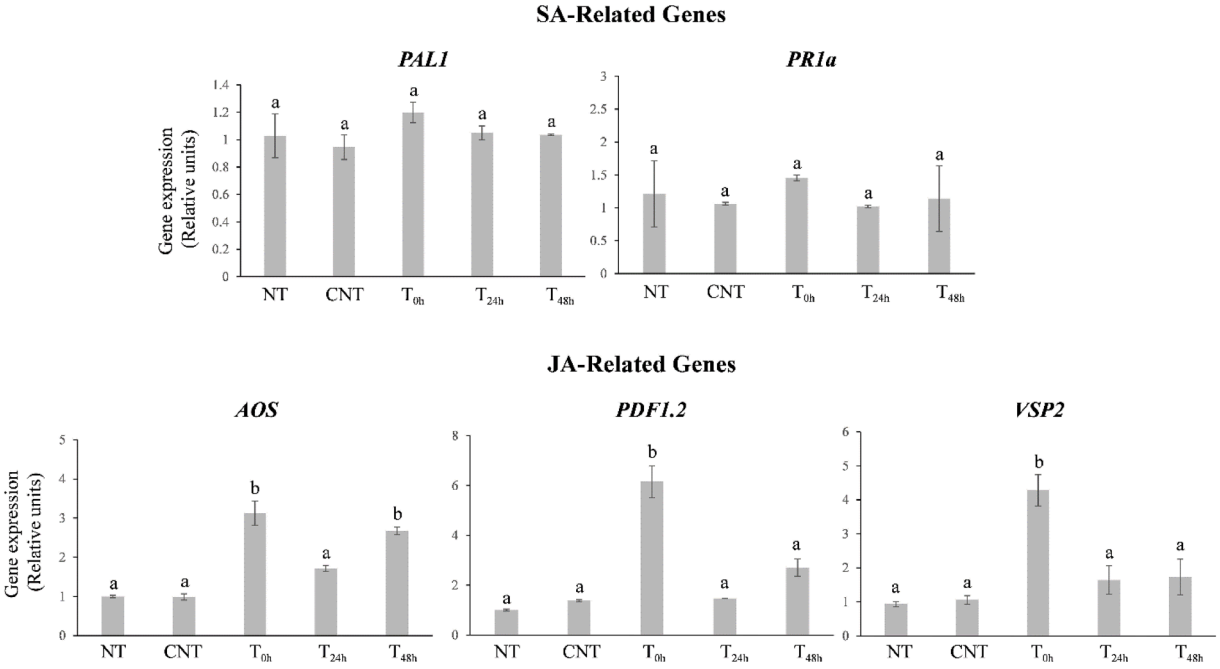

Fig. 4. Expression profiling by Reverse Trascription-qPCR analysis of SA- and JA-dependant genes after Arabidopsis leaves exposure to *Eurydema oleracea*-induced volatile organic compounds (VOCs) collected from Arabidopsis Col-0 plants immediately after infestation (T_{0h}) or at 24 h (T_{24h}) and 48 h (T_{48h}) after infestation. NT=no treatment; CNT=VOC_S collected from uninfested Arabidopsis Col-0 plants. Each column represents the mean ± SE of relative expression levels of SA-biosynthetic *PAL1* gene, SA-responsive *PR1a* gene, JA-biosynthetic *AOS* gene and JA-responsive *PDF1.2* and *VSP2* genes from three independent experiments. In each experiment, three biological replicates per treatment, each obtained from eight individual plants, and three technical replicates were analysed. Columns with different letters are statistically different ($p \leq 0.01$; ANOVA one-way, Tukey's HSD tests).

Table 2
Composition of VOCs blend collected from infested *Arabidopsis thaliana* Col-0 plants immediately after *Eurydema oleracea* infestation (T_{0h}), or 24 h (T_{24h}) and 48 h (T_{48h}) after insect removal. Volatile emissions are given in mean peak area (counts per *sec*) divided by 10^3. RT: retention time; LRI: linear retention index calculated by a range of n-alkanes (C8-C40); *=Chemical compounds identified using synthetic standards.

Peak	RT	LRI	Chemicals	CNT	T0	T24	T48
1	6.85	757	1-pentenal	15.24 ± 3.49	16.79 ± 8.66	21.02 ± 8.22	107.84 ± 6.62
2	7.01	774	3-penten-2-ol	12.63 ± 4.45	10.03 ± 3.68	19.72 ± 6.49	120.13 ± 18.68
3	7.40	801	hexan-2-ol	35.26 ± 9.35	60.17 ± 13.15	70.76 ± 15.41	66.15 ± 9.47
4	9.67	860	(*Z*)−3-hexenol	0	72.68 ± 12.23	0	0
5	10.01	869	1-hexanol	0	26.39 ± 8.12	0	0
6	11.22	900	heptanal	0	33.72 ± 10.13	0	0
7	14.19	981	myrcene*	0	0	0	34.80 ± 7.34
8	14.36	985	6-methyl-5-hepten-2-one	0	0	0	11.85 ± 3.15
9	18.51	1107	nonanal*	26.95 ± 6.31	47.66 ± 11.13	0	27.82 ± 8.27
10	22.71	1244	unknown 1	0	48.90 ± 9.43	0	0
11	23.23	1262	4-ethylacetophenone	0	289.50 ± 65.63	0	0
12	23.85	1285	unknown 2	0	148.19 ± 39.68	0	0
13	32.74	1610	tetradecanal	0	21.19 ± 6.33	0	0
14	33.74	1645	methyl jasmonate	0	493.31 ± 82.42	0	0

5 dpi this effect was no longer observed, indicating the establishment of a short-term priming memory.

Priming of defence by herbivore insects is well documented in the literature, especially against attack by other harmful pests and much is correlated to the impact of plant-derived VOCs following the first stress (Engelberth et al., 2004; Ton et al., 2007; Frost et al., 2008; Guarino et al., 2017). Indeed, it is known that VOCs can act directly as repellent or toxic molecules (Howe and Schaller, 2008), but also indirectly by attracting the natural enemies of insect herbivores (Arimura et al., 2000; Dicke, 2009; Heil, 2015) or by inducing defence pathways in the host plant that prepare it for the next pest attack (Zhou and Jander, 2022). In fact, plants regulate the production of volatiles through signalling molecules and, in particular, by activating hormonal pathways such as those SA and JA/ET-dependant (Erb et al., 2012). The release of terpenoid compounds from cotton even after only surface contact with insects has been associated with increased resistance to other both chewing and piercing-sucking herbivores (Wu et al., 2009; Song et al., 2021). Some studies have shown that plant HIPVs could suppress the growth of secondary pests, as well as negatively affect their feeding behaviour and oviposition (Wang et al., 2008; Sugimoto et al., 2014). However, also experiments in which plant volatiles released after insects attack induced metabolic changes in the host plant and increased the performance of other herbivores have been reported (Frago et al., 2022). Thus, while the contrasting roles of insect-induced plant VOCs in the host response to other pests is well documented, much less is known about their action on other biotic stressor such as pathogenic microorganisms, including fungi. In Arabidopsis, induced emission of the sesquiterpene β-caryophyllene after caterpillar chewing feeding (Body et al., 2019) and its ability to trigger a JA-mediated response to pathogenic attack (Frank et al., 2021) have been reported.

After initially proved a priming effect of *E. oleracea* infestation and its protective effect against the necrotrophic fungus *B. cinerea*, we investigated whether this protection was related to plant VOCs emitted after infestation. In particular, we explored both the direct antimicrobial effect of VOCs and their indirect effect as inducers of plant defence pathways. Our results showed that VOCs released by Arabidopsis Col-0 plants immediately after infestation by *E. oleracea* (T_{0h}) directly inhibited the *in vitro* growth of *B. cinerea* colonies and indirectly reduced leaf damage caused by the pathogen. The indirect effect was linked to the specific activation of the JA-dependant, but not the SA-related, defence pathway. Indeed, plant VOCs are often related to the induction of JA-mediated signalling resulting in the induction of effective defence responses, especially against necrotrophic fungi such as *B. cinerea* (Engelberth et al., 2013; Escobar-Bravo et al., 2017; Hammerbacher et al., 2019), but also against the biotrophic ones such as *Golovinomyces cichoracearum* (DC.) V.P. Heluta, (Quaglia et al., 2012). In addition, applications of jasmonoyl-isoleucine (JA-Ile), active regulator of the responses jasmonate signalling resulted in increased protection of Arabidopsis against *B. cinerea* (Li et al., 2021).

After *E. oleracea* removal, the protective effect was not long-lasting, indicating that only VOCs emitted during the *E. oleracea* infestation were effective in inducing the defence response and therefore the interaction with the insect was required for VOCs-induced priming. On the other hand, evidence that previous insect infestations can induce short- and long-term priming against subsequent herbivore attacks has already been described (Mertens et al., 2021; Valsamakis et al., 2022; Schott et al., 2023). Our study showed that an initial infestation of plants with insects could confer short-term protection against the phytopathogenic fungus *B. cinerea* at least partly mediated by VOCs triggering the induction of JA pathway. In the priming action exerted by Arabidopsis infestation with *E. oleracea*, the role of SA-dependant pathway cannot also be excluded. Indeed, in our previous paper (Ederli et al., 2021) we observed that during the presence of the herbivore on the plant, SA-mediated genes were induced early, but transiently, together with JA-responsive genes whose activation persisted even after the removal of the insect.

The results described here therefore indicated that the induction of the SA-dependant pathway was not mediated by VOCs, but by other factors such as for instance the feeding injury, or elicitors produced by the herbivore. In contrast, the production of plant-derived VOCs was crucial for the activation of the JA-dependant pathway and the mitigation of pathogen susceptibility, although in the short term, as the VOCs effective in protection were only those emitted during the infestation and not those produced in the days following the removal of the insect. As confirmation of this, significant differences in plant VOCs blend collected at various times were observed, with the greater variability of the compounds detected in the blend collected immediately after the infestation (T_{0h}). The characterized compounds detected only in this blend were MeJA, 4-ethylacetophenone, (*Z*)−3-hexenol, heptanal, 1-hexanol and tetradecanal. Jasmonates and their derivatives can induce different metabolic changes in plants, including the up-regulation of JA defence pathway and the production of secondary metabolites such as VOCs (Quaglia et al., 2012). That is, jasmonates, including MeJA, can both be stress-induced and induce defence responses to stress. Indeed, the role of MeJA application in plant protection against *B. cinerea* has been reported by several authors (*i.e.* Moline et al., 1997; Yu et al., 2009). The protective effect conferred by treatment was connected to an increased content of jasmonates and activity of defence-related enzymes such as lipoxygenase (Yu et al., 2009). Treatments with MeJA also showed to determined qualitative and quantitative changes in VOC blend emitted by tomato plants (Quaglia et al., 2012). This complex scenario, where the same molecule can be both induced and inducing, clearly indicates that MeJA included in VOC blend induced by *E. oleracea* can positively regulate Arabidopsis resistance to *B. cinerea* via

JA-related pathway. As showed by Yu et al. (2009), the protective effect of MeJA treatment against *B. cinerea* decreased over time and this may justify the lower protection here observed in Arabidopsis Col-0 plants infected at 24 h after *E. oleracea* removal compared to those inoculated immediately after the infestation. Moreover, a direct inhibitory effect of MeJA on *B. cinerea in vitro* growth has been reported in literature (Mahsa et al., 2014). Another component was the 4-ethylacetophenone which was found in VOC blends capable of attracting the biocontrol agent during early vegetative period of different weeds grown in association with wheat, mustard, potato and rice (Koner et al., 2022). In the sample of Arabidopsis volatiles captured immediately after the removal of *E. oleracea*, (*Z*)−3-hexenol was also present, a well known regulator of both plant defence and herbivore immunity. Indeed, (*Z*)−3-hexenol is reported as capable to induce a series of defence related genes in maize, including those involved in Ca^{2+} and lipid signalling (Farag et al., 2005; Engelberth et al., 2013) but also to increase the resistance of the *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) larvae to the biocontrol agent *Bacillus thuringensis* (Berliner), by increasing the polyphenol oxidase activity and hemocyte and phagocyte number (Ghosh and Venkatesan, 2019). Another here-detected VOC, 1-hexenol, has been previously detected in healthy chickpea (*Cicer arietinum* L.) plants and shown to inhibit the *in vitro* growth of the necrotrophic fungal pathogens *Fusarium avenaceum* (Fr.) Sacc. and *F. graminearum* Schwabe and to protect wheat seedlings against *F. graminearum*, suggesting the rotation with chickpea genotypes that constitutively release high levels of 1-hexanol from the roots in the wheat protection against *F. graminearum* infection (Cruz et al., 2012). Tetradecanal has been reported as a component of VOC blend emitted by *Polygus minus* Huds. leaves, whose emission significantly increased after MeJA treatment (Rahnamaie-Tajadod et al., 2019). Lastly, heptanal was detected as one of the main components of VOC blend in wheat cultivar resistant to heat stress. In those plants, high temperature not only induced an increase in heptanal emission but also an up-regulation of the lipoxygenase pathway (Farahbakhsh et al., 2023). Another interesting finding was that also VOC blend emitted at T_{48h} showed a protective effect against *B. cinerea* infection. However, the protection conferred by VOC blend emitted at T_{48h} was weak and not comparable to those conferred by treatment with T_{0h} VOC blend. Finally, the monoterpene myrcene has been identified as distinctive component of the T_{48h} VOC blend. This compound has been reported as component of VOC blend emitted by the biocontrol fungi *Trichoderma virens* and able to enhanced in Arabidopsis both development and defence responses against *B. cinerea* (Contreras-Cornejo et al., 2014).

5. Conclusions

This research work highlights that infestation by the herbivore insect *E. oleracea* alters the subsequent interaction of Arabidopsis plants with the necrotrophic fungal pathogen *B. cinerea*. In particular, *E. oleracea* infestation causes a slower development of the leaf damage produced by *B. cinerea* in Arabidopsis leaves and induces a modification in VOC blend emitted by bug-damaged plants. Against the pathogen, the VOC blend emitted by infested plants showed both a direct effect, due to its antimicrobial activity, and an indirect effect, due to its ability to trigger a JA-defence pathway. However, the protection conferred by infestation is not long-lasting, indicating that the interaction with the insect is required for VOCs-induced priming. Together with our previous finding (Ederli et al., 2021) on the role of pre-inoculation with *B. cinerea* on the Arabidopsis plant responses to infestation with *E. oleracea* and the effect of B. *cinerea* induced plant VOCs against the insect, these findings contribute to characterize plant defence responses, also mediated by VOCs, in their multiple interactions with herbivore insects and fungal pathogens. Indeed, the signals produced by plants, in particular the volatile ones, ensure communication not only with other plants but also with other living organisms of the ecological community, affecting their competition. Understanding the mechanisms by which plants, as host organisms, interact with multiple biotic stressors, provides insight into how they can adapt themselves to different and constantly changing conditions, thereby ensuring the stability of ecosystems and the maintenance of biodiversity. This knowledge is useful both for the development of innovative and eco-friendly mitigation strategies and to understand the plasticity of plant responses, necessary in the era of climate change, which is upsetting both natural and artificial ecosystems.

Founding sources

This work was supported by the "Fondo di Ateneo per la Ricerca di Base 2019″ financed by the University of Perugia.

Funding

Open access funding provided by Università degli Studi di Perugia within the CRUI-CARE Agreement.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

CRediT authorship contribution statement

Mara Quaglia: Writing – original draft, Methodology, Investigation, Conceptualization. **Gianandrea Salerno:** Methodology, Investigation, Conceptualization, Writing – review & editing. **Valerio Saitta:** Investigation, Writing – review & editing. **Salvatore Guarino:** Investigation, Writing – review & editing. **Luisa Ederli:** Writing – original draft, Conceptualization, Investigation, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100456.

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