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Microbe-mediated organic fertilization increases insect predator attraction upon fruit damage in olive trees

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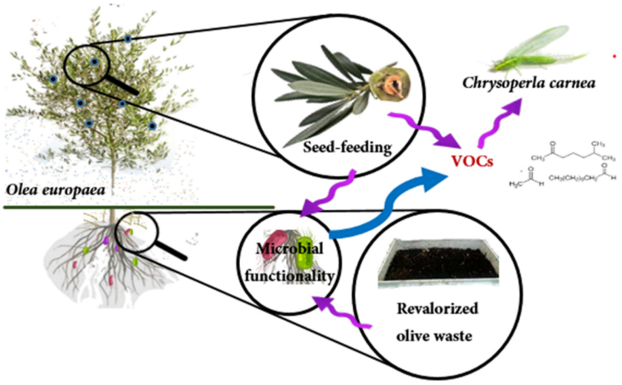
ABSTRACT

- Revalorized olive waste impacts root microbiome.
- Root microbiome modulates plant-induced defense.
- Insect's exudate simulates the pest attack.

The objective of this study was to investigate the combined effect of soil amendments and pest attack on plant-induced defense and their impact on a biological control agent's behavior. The effects of olive mill wastes revalorized through vermicomposting on the aboveground tri-trophic interactions among olive trees (*Olea europaea*), the olive seed-feeder, *Prays oleae*, and its natural predator, *Chrysoperla carnea*, were evaluated. The findings demonstrate that soil nitrogen and organic carbon levels, in conjunction with fungal diversity and functionality within olive roots, exert a significant influence on the volatile compounds emitted by the plant under attack that are most appealing to *C. carnea*. Moreover, the attractiveness of aerial volatiles was found to correlate with soil organic carbon content and the taxonomic and functional diversity of both bacteria and fungi in the olive root system. It is worthy of note that three particular volatile compounds, namely 5-hepten-2-one-6-methyl, acetic acid and nonanal, were consistently observed to attract *C. carnea*. These findings highlight the potential of soil amendments to enhance biological control strategies. Future research should prioritise the validation the greenhouse findings through large-scale field trials and the assessment of the practical applications of soil amendments in pest management programmes.



Keywords *Chrysoperla carnea*, HIPVs, *Olea europaea*, *Prays oleae*, roots microbiome, soil amendments

1 Introduction

For centuries, the olive tree has been a cornerstone of Mediterranean agriculture, significantly influencing the region's economy, culture, and dietary habits. Recently, there has been a growing global interest in olive farming (FAOSTAT, 2019). Nevertheless, olive trees face significant threats from pests, which can severely impact their vitality and productivity (Espinoza Vidaurre et al., 2023). Moreover, plants are part of a complex ecosystem where a multitude of factors influence their growth and health. In this context, current research emphasizes the pivotal role of the soil rhizosphere and root microbiome in the overall condition of olive plants (Ruano-Rosa et al., 2017; Fernández-González et al., 2020). Furthermore, in the pursuit of a 'zero waste' agricultural paradigm that optimizes the utilization of all by-products in the olive ecosystem, previous research has highlighted the beneficial effects of using revalorized olive waste as soil amendments on plant health (Sciubba et al., 2020). It has been demonstrated that these amendments influence the rhizosphere microbiome, which is associated with the enhancement of aboveground induced plant defenses following pest attacks. In particular, a shift in the emission of herbivore-induced plant volatiles (HIPVs) has been observed (Benítez et al., 2017), which play an indirect defensive role by revealing the plant's location to the pests'

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natural enemies (Dicke and Baldwin, 2010; Rani and Sulakshana, 2017).

In response to the increasing global demand for sustainable agricultural practices, it is essential to acknowledge the strategic role of predators and parasitoids in controlling pest populations. Biological control agents are particularly significant as they naturally suppress pest populations, thereby reducing the dependence on chemical pesticides. The olive moth (*Prays oleae* Bern.) (Lepidoptera: Praydidae), one of the most destructive pests of olive trees in the Mediterranean region (Tzanakakis, 2003), has been effectively managed through the use of specialist parasitoids and generalist predators within olive ecosystems (Pappalardo et al., 2021). Among these biological control agents, *Chrysoperla carnea* (Steph.) (Neuroptera: Chrysopidae) stands out as one of the most effective predators of the olive moth (Paredes et al., 2015; Villa et al., 2016). Nevertheless, and despite the abundance of information regarding the potential of *C. carnea* as a biological control agent for *P. oleae*, there is a noticeable gap in the research concerning the influence of root-associated microbiomes on the binomio *Prays-Chrysoperla*.

To further understand the defense responses of olive trees triggered by phytophagous insects, the use of simulated herbivory has been proposed (Aguirrebengoa et al., 2024). This method allows precise control over the timing and location of inflicted damage and provides a standard measure of damage, thereby eliminating the variable effects caused by different feeding behaviors (Waterman et al., 2019). Although there are certain limitations associated with the use of simulated herbivory in the study of tri-trophic interactions involving plants, herbivorous insects and their natural enemies (Hjältén, 2008), recent studies have demonstrated the efficacy of this approach using chemical or mechanical stimuli (Yang et al., 2023; Lidoy et al., 2023). Nevertheless, research on simulated carpophagy with a focus on plant defense is scarce, despite the significant impact of carpophagous pests in olive farming. Particularly, the carpophagous stage of *P. oleae* is one of the most destructive pests of olive trees in the Mediterranean region (Tzanakakis, 2003). This stage leads to considerable fruit drop when larvae enter the fruits in spring and also when larvae emerge from the fruits to pupate in early autumn.

Plant defenses are well known to be activated by insect-derived elicitors, while insects secrete effectors to mitigate these defenses (Wang et al., 2023). A diverse range of biomolecules, primarily found in exudates consisting of insect saliva, regurgitate, and frass, have been identified as elicitors or effectors (Jones et al., 2022). However, the complexity of the defense response elicited within tri-trophic interactions has been documented for certain elicitors but remains unexplored for others.

The utilization of organic amendments in sustainable pest control methodologies has been demonstrated to beneficially impact soil microbial activity and community structure. This, in turn, results in substantial enhancements in microbial functionality, which are crucial for maintaining plant health and fortifying resilience against various environmental stresses (Akanmu et al., 2021; Liu et al., 2023).

Interactions between plant roots and their associated microbiome can alter the plant's metabolic pathways, influencing the types and amounts of volatile organic compounds (VOCs) emitted from leaves and stems (Escobar-Bravo et al., 2023). These volatile compounds act as signaling molecules in plant-microbe interactions, affecting not only the root environment but also the aboveground parts of the plant. This can lead to systemic alterations in VOC emissions, enabling the plant to communicate more effectively with its surrounding environment (Zhang et al., 2020).

In light of the aforementioned premises, it is proposed that the application of vermicomposted olive waste as an organic soil amendment serves to enhance the induced plant defences in olive trees against the carpophagous generation of *P. oleae*. It is further proposed that these effects are mediated by changes in the root microbiome and aerial volatile emissions. To test this hypothesis, an attack on olive fruits was simulated using the exudates of *P. oleae*. Subsequently, the effects of the soil amendment on the root microbiome and the aerial volatile compounds released by olive trees under attack were investigated. Additionally, the influence of induced defense mechanisms in olive trees on the natural predator of the olive moth, *C. carnea*, was examined.

2 Materials and methods

2.1 Plants and potting media

We obtained *Olea europaea* L. var. Arbequina trees, aged two years, from a commercial nursery (Viveros Minerva, Cuevas de Almanzora, Almería, Spain), and subsequently transplanted into new 8-litre black pots. The pots were filled with a tyndallized mixture of soil and vermiculite (v:v 2:1) and cultivated under controlled greenhouse conditions (25−30 °C, 60%−80% relative humidity, 16:8 h light:dark cycle) at the Estación Experimental del Zaidín (EEZ-CSIC) in Granada, Spain. The soil used was a Eutric Regosol collected from the top 20 cm of a typical agricultural field in Granada, Spain. To ensure uniformity, the soil was sieved using a 10 mm sieve to remove any large stones. The soil had the following characteristics: 0.87 g kg^{-1} soil organic carbon, 1.58 g kg^{-1} total nitrogen, and a pH of 7.4.

2.2 Biological control agent

The larvae of *C. carnea* were obtained from Agrobio

(Almería, Spain). Each predator larva was individually reared in a Petri dish and fed on eggs of *Ephestia kuehniella* Zell. (Lepidoptera: Pyralidae), obtained from Bioline Agrosciences (El Ejido, Almería, Spain). Upon emergence, *C. carnea* adults were collected daily and housed in boxes (28 cm in diameter, 15 cm high) equipped with an oviposition surface. The adults were provided with a diet consisting of equal volumes of honey and pollen, along with mineral water. They were maintained in a regulated environmental chamber at a temperature of 25 °C, a relative humidity ranging from 50% to 60%, and a light:dark cycle of 16:8 hours over a period of 2 to 3 days. The light:dark cycle was calibrated such that their nocturnal activity, which is at its peak, commenced at 9:00 am. The sex of adult *C. carnea* was determined by examining the ventral surface of the abdominal tip. Only females were selected for use in bioassays. The selection of females *C. carnea* for bioassays involving plant volatiles is primarily due to their behavioural and physiological responses. It is typical for females to be more responsive to plant volatiles, as they utilise these cues to identify suitable oviposition sites for their eggs (Palomares-Pérez et al., 2019). This renders them more pertinent for studies aimed at elucidating the manner in which these insects interact with plant volatiles in their natural habitat. Furthermore, female insects frequently display more uniform and quantifiable responses in bioassays when compared to males, which can result in more dependable and reproducible outcomes (Mahzoum et al., 2020).

2.3 Volatile organic compounds

The volatile organic compounds (VOCs) emitted by olive trees were collected using SPME fibres (50/30 μm DVB/CAR/PDMS Stableflex 23Ga, Autosampler, 3pk, SUPELCO, Bellefonte, PA, USA) preconditioned prior to analysis at 250 °C for 1 h. Following the equilibration phase, these fibres were exposed to the headspace within each chamber. Upon the completion of the sampling process, the fibre was retracted into the needle and then introduced into the gas chromatography-mass spectrometry (GC-MS) system injection port in splitless injection mode at an injector temperature of 250 °C during 1 min. GC analyses and peak identification were performed under reported conditions (Aguirrebengoa et al., 2024).

2.4 Experimental setup: simulation of fruit damage

In September 2022, more than 500 olives were collected from various organic orchards in the provinces of Almería and Granada, Spain, where the fruits exhibited clear signs of *P. oleae* infestation. The almonds were removed from each fruit, and if it was found to be infested by the *Prays* larva, it was individually isolated (L), as well as the product of its digestion, a mix of seed-frass (D). "L" extract was obtained from 0.5 g (fresh weight) of *Prays* larvae, crushed with Milli-Q water and centrifuged at 2800 rpm to remove cell debris. The resulting supernatant was recovered and diluted with Milli-Q water to a concentration of 120 ppm. For the "D" extract, 20 g (dry weight) of seed-frass was squashed with Milli-Q water and centrifuged at 2800 rpm to remove large particles. The supernatant was then retrieved and diluted with Milli-Q water to a concentration of 4800 ppm. The control "A" was obtained from 20 g (dry weight) of seed from non-attacked olives and the extract was prepared in the same way as for "D".

We undertook chemical characterization of the three extracts with the objective of identifying any differentially present products between them. To achieve this objective, the extracts were subjected to LC/HRMS analysis on an Agilent 1200 RR HPLC coupled to a Bruker maXis QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) following the conditions previously reported by our research group (Martín et al., 2014). Subsequently, an evaluation was conducted on healthy olives grown under the aforementioned controlled greenhouse conditions. This evaluation involved measuring volatile emissions and observing of the behavior of female lacewings.

In order to replicate the chemical and biological elicitors/effectors released during pest feeding, ten fruits (approximately one third of the total olives per tree) on three trees were treated with substance D and another ten on three trees with substance L. Using a syringe, we injected 10 μL of either substance L or D into five olives per tree 24 hours prior to the experiment, ensuring the seeds were touched, and into another 5 olives pre tree one hour prior to the collection of volatile compounds and the observation of *C. carnea*'s choice. Our objective was to closely replicate the natural infestation process by fruit-eating larvae and to investigate the resultant effects within a controlled environment, capturing both slowly and rapidly synthesised HIPVs (Arimura et al., 2009; Peñaflor and Bento, 2013). To verify the reliability of the method, we performed the experiment twice over three different weeks.

Closed glass chambers (40 cm×40 cm×140 cm), sufficiently large to accommodate the plants and completely separated from the pots containing potting media were connected from the top to the glass tubing of a three-armed olfactometer through transparent polytetrafluoroethylene tubes. The attraction of *C. carnea* to volatiles emitted by olive trees was observed 24 hours after first simulated damage in the olfactometer connected to an EthoVision XT integrated video tracking system (Noldus Information Technology, Wageningen, The Netherlands). The EthoVision software automatically identifies the insect's location in the arena and computes several movement parameters based on positional changes. Behavioral experiments were conducted under artificial light from 09:00 to 18:00 at a temperature of 28±2 °C, with 10−12 female individuals per pair of plants. A white circular paperboard arena was set up around the olfactometer to eliminate visual disturbances. Adults of *C. carnea* females were introduced into one branch of the olfactometer and allowed to choose among the other two branches of the device (each linked to a different treatment), with a maximum observation period of 5 minutes. We established that the tested individuals made an effective choice when they entered and remained for a period of more than five seconds in designated areas (a radius of two centimetres at each branch). Insects that did not make choices were excluded from the subsequent data analysis. All insects were used only once and then released. To account for diurnal variation, behavioral tests were carried out over five consecutive days for each comparison (one pair of plant per comparison per day).

A solid-phase microextraction (SPME) fibre was inserted into each arm of the olfactometer connected to the different treatments for volatile collection. Using air pressure, a synthetic pure air at an airflow rate of 1.5 L min^{-1} per channel was drawn into the bottom of the chambers. The collection of volatiles and the observation of *C. carnea* behavior were concurrently conducted over a span of one hour.

2.5 Experimental setup

Following the confirmation of the efficacy of insect exudate inoculation in activating the induced defense of olive trees, a greenhouse experiment was conducted to investigate the impact of soil amendments on above- and below-ground trophic levels. This was done in order to ascertain whether the inoculation could be used as a means of enhancing the natural defenses of olive trees.

We utilized a vermicompost derived from olive-mill waste, produced at the EEZ-CSIC facility in Granada, Spain, as detailed in Vivas et al. (2009). To achieve a soil organic carbon content of 30 g kg^{-1}, considered a critical threshold for maintaining healthy soil functions (Food and Agriculture Organization of the United Nations, 2019), we mixed 7 000 g of the potting media with vermicompost at a rate of 50 g kg^{-1} (VC treatment). As controls, we used soil alone (C1) and soil amended with an equivalent amount of tyndallized vermicompost, that is, sterilized for three consecutive days at 95 °C for 45 minutes (C2). The soil moisture content was initially set to approximately 70%−80% of field capacity and maintained at this level throughout the experiment by irrigating with sterilized deionized water. The experiment was conducted under the controlled conditions described above and the pots were arranged in randomized blocks. As one of the trees was injured, there were 19 replicates for C1 and 20 replicates for C2 or VC at the end of a three-month growth period.

Treatments was evaluated in pairs, with and without herbivory, under identical conditions and a design identical to that employed in the aforementioned experiment. In the choice tests with *C. carnea*, the percentage preference for each plant over its partner was calculated considering the individuals of *C. carnea* that showed a preference for one or the other. Using five plant pairs for each comparison (4 in the case of under attack C1 *vs.* C2 comparison, 29 pairs of plants tested in total), we analysed the preference of *C. carnea* in each tested comparison by means of general linear mixed models with plant pair as random effect. Volatile samples were collected and analysed also in accordance with the methodology outlined in the preceding section.

Post volatile trapping, the olive trees were removed from the glass chamber and the root system was isolated from the bulk soil by shaking and subsequent washing with distilled water, cut into 2−5 mm pieces and immediately frozen in liquid nitrogen and preserved at −80 ºC for imminent analyses.

2.6 Molecular analyses of root microbiome

DNA of root surface-bound (ectophytic, i.e., rhizoplane) and inside-root (endophytic) was extracted together from two replicates of 0.1 g for each homogenised root sample using the TissueLyzer II method with the aid of a DNeasy Plant Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. For each root sample, the two extracted replicates were pooled and concentrated at 35 ºC to a final volume of 50 uL using a Savant Speedvac® concentrator (Fisher Scientific, Madrid, Spain). The ProV3V4 primers (5′-CCTACGGGNBGCASCAG-3′ and 5′-GACTACNVGGGTATCTAATCC-3′) (Lundberg et al., 2013; Takahashi et al., 2014) to amplify the V3-V4 hypervariable regions of the bacterial 16S rRNA gene and the primers ITS4-ITS2_fITS7 (5′-TCCTCCGCTTATTGATATGC-3′ and 5′-GTGARTCATCGAATCTTTG-3 ′) (Ihrmark et al., 2012) to amplify the fungal ITS2 region were used to characterize the microbial communities from the samples using the Illumina MiSeq platform (2 × 300 nucleotide paired-end protocol) at the genomic facilities of the López-Neyra Institute of Parasitology and Biomedicine (IPBLN-CSIC). Blockers were used to minimize amplification of mitochondria and chloroplasts (Lundberg et al., 2013). Illumina reads were deposited in the Sequence Read Archive (SRA) service of the European Bioinformatics Institute (EBI) database under BioProject ID: PRJNA1103158 (Biosamples accession numbers SAMN41043216-SAMN41043227).

All raw paired-reads from 16S rRNA gene and ITS2 region were analysed in R version 4.2.2 (R Core Team, 2022). The DADA2 v1.24.0 pipeline (Callahan et al., 2016) was used to process raw sequences and construct an amplicon sequence variant (ASV) table. ASV taxonomic assignment was achieved by implementing the *assignTaxonomy* function (based on naïve Bayesian classifier method) against the SILVA v138.1 (Quast et al., 2013) and UNITE v10.0 (Abarenkov et al., 2024) databases for bacteria and fungi, respectively. An ASV×matrix was generated using the Marker Data Profiling module on the MicrobiomeAnalyst web platform (Dhariwal et al., 2017; Chong et al., 2020). ASVs were filtered according to minimum prevalence in samples of 10% and a minimum variance of 10% and all samples reached a plateau based on the rarefaction curves generated by the MicrobiomeAnalyst tool.

2.7 Functional diversity metrics

Functional diversity of bacteria and fungi was determined through metagenomics analysis by characterising their traits. In the case of bacteria, the functional parameter employed was enzyme richness, extracted from the BacDive database (Reimer et al., 2022) at the genus/species level. The presence or absence of each enzyme was assigned for each taxon, as determined by the metagenomic analysis. The BacDive database provided data on enzymes for 848 of the 1320 bacterial taxa, enabling the assignment of the presence/absence of a total of 74 different enzymes (Supplementary Table S1). From these data, the functional diversity metric Rao's Quadratic Entropy Index (RaoQ), which is a measure of diversity that quantifies functional heterogeneity in a community, was calculated for each single sample. RaoQ is based on the total number of functional types present in the community (sample), the relative abundances of these types, and the functional distance between them. We used RaoQ as a measure of alpha-diversity, with higher index values indicating greater functional diversity within the community (Botta-Dukát, 2005). In turn, we calculated the community weighted mean (CWM), which represents the weighted average value of each enzyme in each sample. The CWM was calculated based on the presence/absence of each enzyme in each taxon, the abundance of each taxon in each sample, and the total number of taxa in the community. We used the CWM of each enzyme in each sample as a beta-diversity metric. In the case of fungi, the functional parameter used was lifestyle, extracted from the FungalTraits database (Põlme et al., 2020). Each taxon was assigned a potential lifestyle based on the metagenomics analysis. The FungalTraits database provided us with data for 104 of the 107 fungal taxa, and we were able to assign one or more of a total of 16 potential lifestyles to each taxon (Supplementary Table S1). From these data, and as for bacteria, we used the RaoQ of fungal lifestyles as a measure of alpha-diversity, and the CWM of fungal lifestyles as a measure of beta-diversity.

2.8 Statistical analyses

The impact of soil and simulated damage treatments on soil nutrients, plant volatiles, bacterial and fungal taxonomic alpha-diversity (Shannon index) and functional diversity (RaoQ metric) was analysed using analysis of variance (ANOVA). To analyse the taxonomic and functional beta-diversity of bacteria and fungi, as well as the volatile blends as a whole, we used permuted multivariate analysis of variance (PERMANOVA). The clustering patterns of volatile blends were visualized using discriminant analysis of principal components (DAPC).

To analyse the relationships between the abiotic and biotic soil conditions of the various treatments, the volatile emissions and *C. carnea* preference, we employed a dimensionality reduction approach (Nguyen and Holmes, 2019; Armstrong et al., 2022). We utilised Shannon and RaoQ metrics for bacteria and fungi, and conducted discriminant functions to obtain a single variable for volatile emission. We performed discriminant functions on the set of volatiles, and on the subset of volatiles that best explained *C. carnea* preference. Specific subsets of volatiles were identified by selecting the optimal subset of predictor variables for a linear mixed regression model (*C. carnea* preference as response variable, discriminant function of optimal subset of volatiles as predictor, plant pair as random effect). All possible combinations of predictor variables were iterated over using permutations until the combination with the lowest Akaike information criterion (AIC) was obtained. This approach enabled the relationship between plant volatiles and *C. carnea* preference to be elucidated through linear regression, while the potential influence of abiotic and biotic soil variables on volatile emissions was determined through random forest classification.

Analyses were conducted in R (R Core Team, 2022). Taxonomic diversity metrics were calculated using *vegan* (Oksanen et al., 2022, 2024), and RaoQ functional diversity metric using *FD* (Laliberté and Legendre, 2010; Laliberté et al., 2014). ANOVAs were performed using *nlme* (Pinheiro et al., 2022), linear mixed regression models using *lme4* (Bates et al., 2015), PERMANOVAs using *vegan* (Oksanen et al., 2022, 2024), DAPC using *adegenet* (Jombart, 2008; Jombart and Ahmed, 2011), and random forest using *ranger* (Wright and Ziegler, 2017).

3 Results

3.1 Mimicking carpophagy

Figure 1 illustrates the volatile emissions from olives that were inoculated with either D or L (Fig. 1A), as well as the preference of *C. carnea* females for different volatile blends (Fig. 1B). The relative values of the emissions are presented

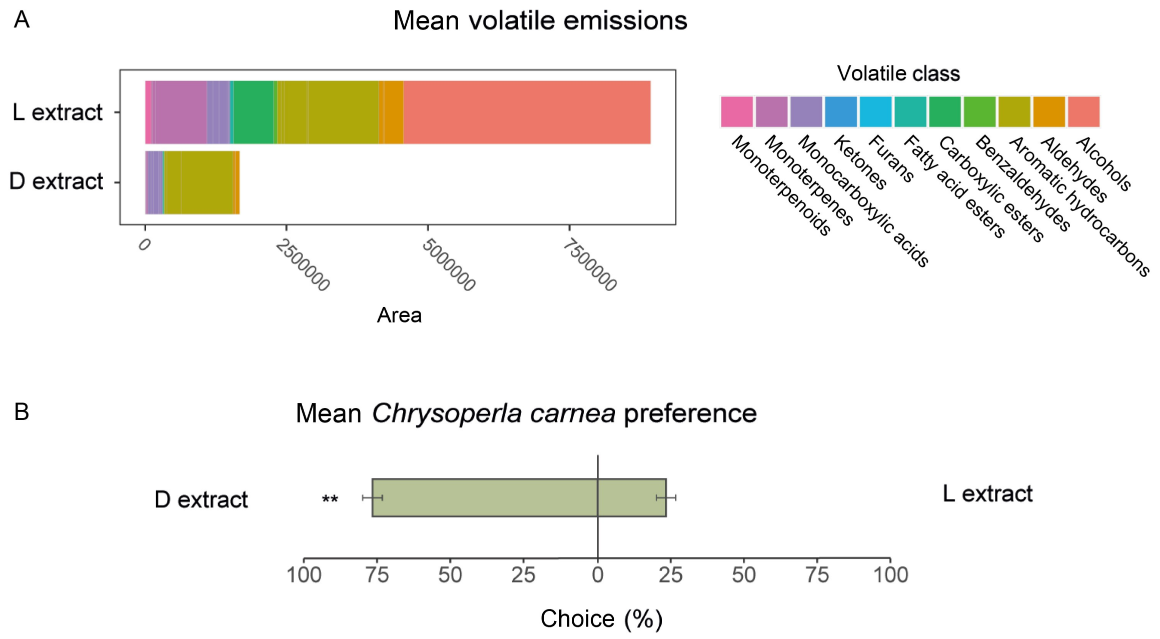

Fig. 1 (A) average emission area of each volatile compound in L extract plants (*Prays oleae* larva) and D extract plants (product of *P. oleae* digestion), grouped by volatile class; (B) mean preference of *Chrysoperla carnea* in the D extract *vs*. L extract choice test. ** denotes a significant difference (P<0.01).

in Supplementary Fig. S1. Interestingly, over 75% of *C. carnea* were drawn to the volatile blends produced by inoculating D extract over L extract (P<0.01). Even though this blend was emitted in significantly smaller quantities compared to L, and did not contain alcohols, benzaldehydes, and monoterpenes, it was still the preferred choice for the majority of lacewings. In our analysis, we identified two compounds almost exclusive to extract D with molecular formulae of $C_9H_{12}O_3$ and $C_{10}H_{16}O_5$ and nine compounds present in both extracts D and the control A (Supplementary Table S2). As expected, most of the components detected in both samples transitioned from being minor constituents in the extract A from non-attacked seeds to becoming the primary components in the D extract. This fact is likely attributable to the digestion/metabolism-mediated elimination of accompanying compounds, resulting in an enrichment of undigested compounds in D. The higher levels of jaspolyoleoside B/C in A compared to the extract D might be attributed to a degradation/metabolization of the compound by the action of *P. oleae*.

3.2 Effect of treatment on soil nutrients and the root microbiome

Table 1 shows the ANOVA results of soil treatments, simulated fruit damage and their interaction on soil nutrients and taxonomic and functional diversity of roots' bacteria and fungi. The addition of the organic amendment to soil exerted a considerable influence on soil total and organic C, N and P, as well as bacterial and fungal diversity within olive roots. Fruit damage, when considered alone, did not exert any discernible influence on these parameters. However, an effect could be observed when soil treatment was interacted with, resulting in a notable impact on fungal diversity within olive roots. The use of both natural and tyndallized vermicompost resulted in a marked enhancement of soil nutrient content, especially in the concentrations of total and organic C, and total N and P, when compared to unamended soil (Fig. 2A−2D). The alpha-diversity of bacterial taxa was influenced by the type of soil treatment, although the functional diversity of bacteria remained unaffected (Table 1, Fig. 2E−2F).

Table 1 ANOVA results of soil and simulated fruit damage treatments and their interaction on soil nutrients and taxonomic/functional diversity of bacteria and fungi.

	Soil treatment		Simulated fruit damage		Soil×Simulated fruit damage		
	F	*P*	*F*	*P*	*F*	*P*	R^2
C	**8.90**	**0.0004**	0.00	0.93	0.16	0.85	0.258
Organic C	**7.53**	**0.001**	0.10	0.74	0.08	0.91	0.228
N	**5.81**	**0.005**	0.03	0.84	0.59	0.55	0.198
P	**15.06**	**<0.0001**	0.12	0.73	0.83	0.44	0.380
Shannon index bacteria	**3.38**	**0.041**	1.48	0.22	0.31	0.72	0.144
RaoQ enzymes bacteria	1.90	0.15	0.62	0.43	1.45	0.24	0.124
Shannon index fungi	0.93	0.40	0.52	0.47	**3.21**	**0.048**	0.144
RaoQ lifestyle fungi	**4.06**	**0.022**	3.69	0.060	0.60	0.54	0.202

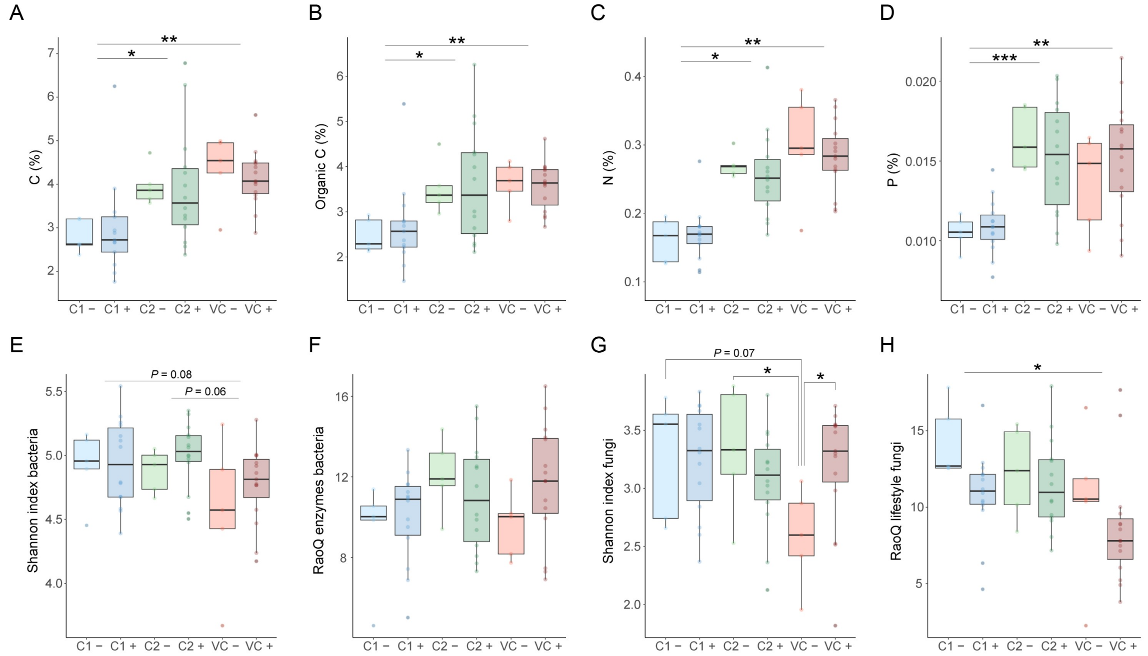

Fig. 2 Comparative analysis of soil nutrients and root-microbial diversity under soil treatments. The above panel (plots A−D) illustrates the variation in soil nutrient concentrations, while the panel below (plots E−H) depicts the alterations in bacterial and fungal diversity. C1=soil with no vermicompost, C2=soil with tyndallized vermicompost, VC=soil with vermicompost. The symbols (+) and (-) denote the presence and absence of simulated fruit damage, respectively. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

The application of vermicompost led to a decrease in the variety of bacterial ASVs compared to both control groups (Fig. 2E). The alpha-diversity of fungal taxa was affected by the treatments in an interactive manner; a reduction was observed in the VC treatment, but this was only in scenarios where simulated seed-feeding was absent (Fig. 2G). The functional alpha-diversity of fungi was affected by the soil treatment, with less diversity observed in the VC treatment compared to the C1 control. Additionally, the simulated fruit damage treatment had a marginally significant effect in reducing it (Table 1, Fig. 2H).

As a result, a strong correlation was observed between root microorganisms and soil nutrients (Supplementary Fig. S2). This correlation implies that an augmentation in soil nutrients is inversely proportional to the diversity of fungal ASVs and their functional capabilities, with a similar but less pronounced trend observed in bacteria. A direct correlation was identified between the taxonomic and functional diversities of fungi, meaning that an enhancement in fungal ASV diversity is concomitant with an increase in the diversity of their functional roles. Conversely, an inverse relationship was observed in bacteria, where an increase in bacterial ASV diversity corresponded to a reduction in the diversity of their functional roles. Lastly, bacterial functional diversity showed a positive correlation with fungal taxonomic diversity. However, significant level > 0.05 from Supplementary Table S3 demonstrated that neither vermicompost application nor simulated carpophagy significantly impacted the taxonomic and functional beta-diversity of bacteria and fungi.

3.3 Plant volatiles emission

A total of twenty-eight different VOCs were identified as being emitted by the plants under study, which fell into ten distinct classes (Fig. 3A). The relative values of the emissions are shown in Supplementary Figure S1, and the pairwise Spearman rank correlation coefficients between the different VOCs are shown in Supplementary Figure S3. Considering the total emissions, no significant differences were observed in the profiles of volatile compounds between the soil treatments and the simulated fruit damage treatments (Permanova test, Soil treatment: F=0.78, P=0.66; Simulated fruit damage: F=1.44, P=0.17; Soil treatment × Simulated fruit damage: F=0.30, P=0.99). However, in the absence of artificial fruit damage, there was a discernible trend for the volatile blends to cluster based on the specific soil treatment applied (Fig. 3B). Specifically, the application of vermicompost amendments led to a significant increase in the emission of the ketone 6-methyl-5-hepten-2-one (F=15.06; P< 0.0001), and a marginally significant effect on the aldehyde octanal (F=2.94; P=0.061) emissions when considering individual volatile compounds. Conversely, the introduction of simulated damage resulted in more homogenous volatile blends. The artificial damage resulted in a reduction in the emissions of the monocarboxylic butanoic (F=4.77;

P=0.033) and pentanoic acids (*F*=8.84; *P*=0.004), monoterpene thujene (*F*=4.48; *P*=0.038), and the aromatic hydrocarbon o-cymene (*F*=6.45; *P*=0.014), and a marginal reduction in the emission of the fatty acid ester hexadecenoic acid methyl ester (*F*=3.93; *P*=0.052).

3.4 Relationship between microbial diversity, volatiles and *C. carnea* behaviour

Out of the 317 female lacewings utilized across 29 experimental plant dyads, 194 (representing 61% of the total) exhibited a predilection towards one plant over the other. Figure 4 shows the impact of various treatments on the preferences of *C. carnea* in the context of simulated seed-feeding. There were no observable differences in the preference of *C. carnea* between the control groups, as well as between the treatments involving tyndallized and regular vermicompost. However, female lacewings exhibited in the VC treatment a significant preference for plants that were attacked over those that had not been attacked. Furthermore, when we compared soil-to-soil preferences for attacked plants, there was a notable preference for the VC treatment as opposed to the unamended soil treatment C1.

The discriminant function, when applied to the whole blend of volatile compounds, showed no significant correlation with the average preference of *C. carnea* across the 29 plant pairs tested (Fig. 5). However, a significant correlation was found when *C. carnea* showed a clear preference. According to the random forest classification, the diversity of fungal ASVs and, to a lesser extent, diversity of fungal functions were the most influential factors in determining the mixture of volatiles preferred by female lacewings.

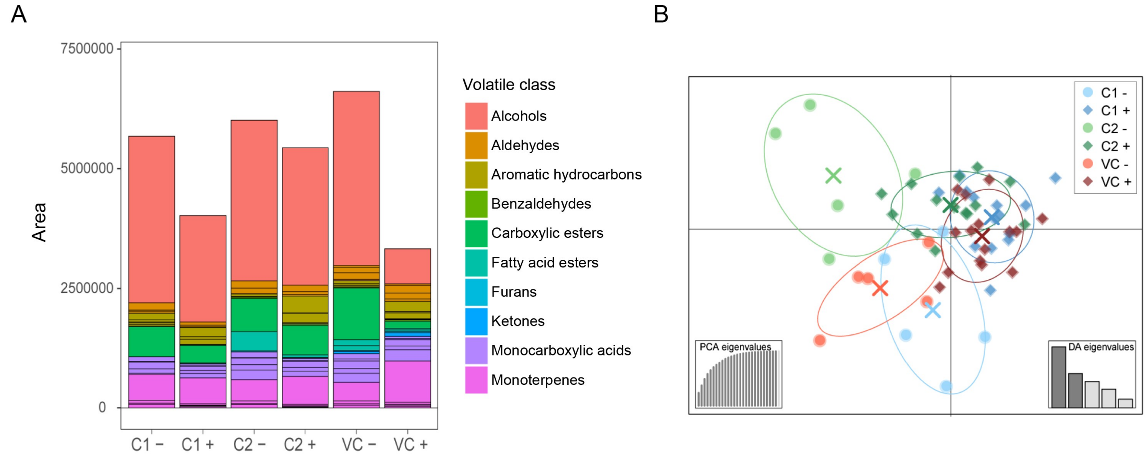

Fig. 3 (A) average emission area of each volatile compound in the different soil treatments (C1, C2, VC) with (+) or without (-) simulated fruit damage, grouped by volatile class; (B) clustering patterns of volatile emissions based on discriminant analysis of principal components (DAPC) according to soil treatments with (diamonds) or without (circles) simulated fruit damage. The PC eigenvalues represent the amount of variance explained by each principal component, and the number of eigenvalues used in the discriminant analysis are highlighted in dark. The DA eigenvalues, highlighted in dark, represent the amount of variance explained by the first two DAs plotted. The data have been subjected to centring and scaling.

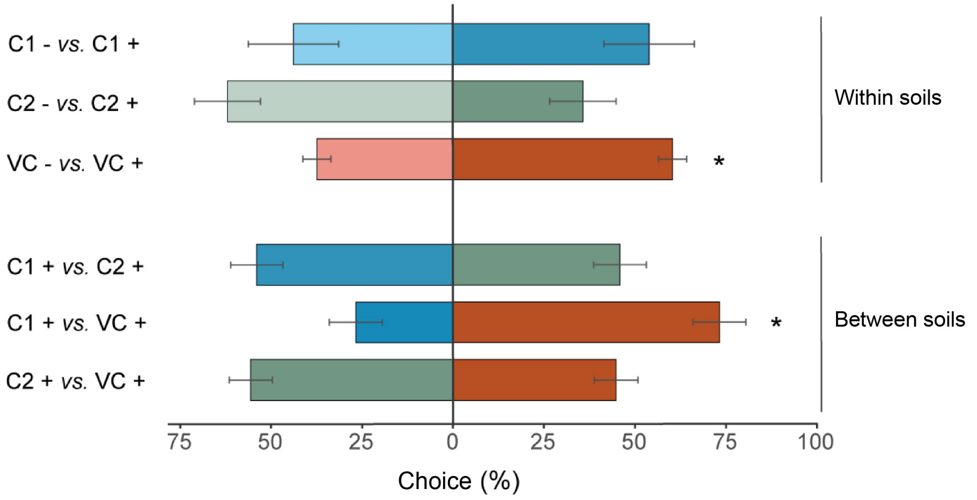

Fig. 4 Mean preference of *C. carnea* in the different choice tests performed between soil (C1, C2, VC) and with (+) and without (-) simulated fruit damage treatments. * denotes a significant difference (*P*<0.05).

We then identified the discriminant function by examining volatile compound subsets. These subsets showed the strongest correlation with *C. carnea* preferences for two scenarios: VC-attacked versus non-attacked plants, and C1-attacked versus VC-attacked plants (Fig. 6). Remarkably, the optimal subset of volatiles consisted of six compounds, with three compounds being shared between both cases. These were identified as 6-methyl-5-hepten-2-one, acetic acid, and nonanal. Alongside fungal diversity, bacterial diversity and/or specific soil nutrients appeared to contribute to the emission of these volatile subsets. The relationships for the remaining comparisons can be found in Supplementary Table S4.

4 Discussion

This research aimed to shed light on the intricate relationships between soil organic amendments, plant defense, above-ground pests and their natural enemies. Our primary objective was to assess the feasibility of the plant response to potential elicitors/effectors from the olive pest *Prays oleae*, by comparing the preferences of the predator *Chrysoperla carnea*. It is widely recognized that the oral secretions of chewing insects, which include regurgitant and saliva, contain bioactive molecules that significantly influence plant defense responses, eliciting reactions that are distinct from those triggered by sole mechanical damage (Snoeck et al., 2022; Wang et al., 2023). A particular focus was placed on two potential groups of elicitors/effectors, with the objective of mimicking *P. oleae* damage within olive fruits. The distinction is that one included not only the insect's saliva and regurgitate but also the frass from insect digestion as well as damaged plant tissue. In addition, unlike the other, it did not include the insect tissue. In our experimental design, female *C. carnea* demonstrated a clear preference for olive trees bearing fruits treated with a mixture of frass, saliva, and regurgitate. Despite the significant disparity in chemical composition between this extract and the insect itself, no definitive conclusions can be drawn due to the pivotal role of

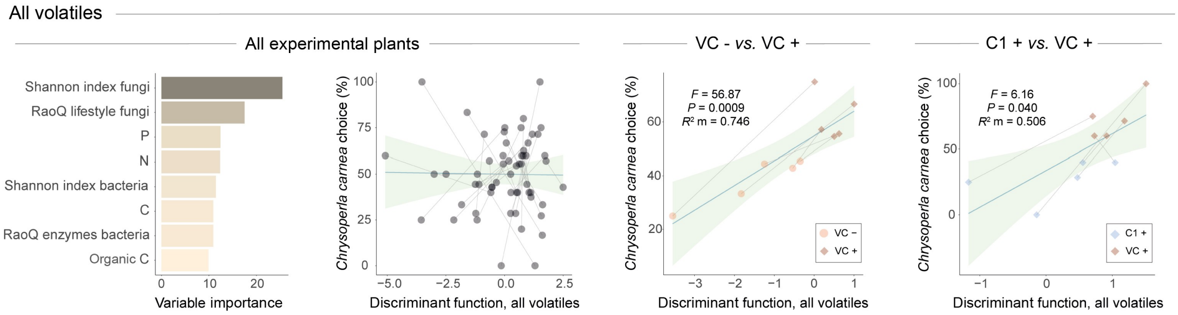

Fig. 5 Left: linear regressions between the discriminant function of the entire blend of volatile compounds and the average preference of *C. carnea* across all paired plant comparisons (each confronted pair of plants is linked by a grey line), showing the random forest ranking of the importance of soil nutrients and microbial diversity variables. Right: same linear regression but limited to comparisons between VC with (+) and without (-) simulated fruit damage, and VC + *vs.* C +. The statistics and R^2 (marginal explained variance) of the plots reflect the result of the following linear mixed regression model: *C. carnea* preference as response variable, discriminant function of volatiles as predictor, plant pair as random effect.

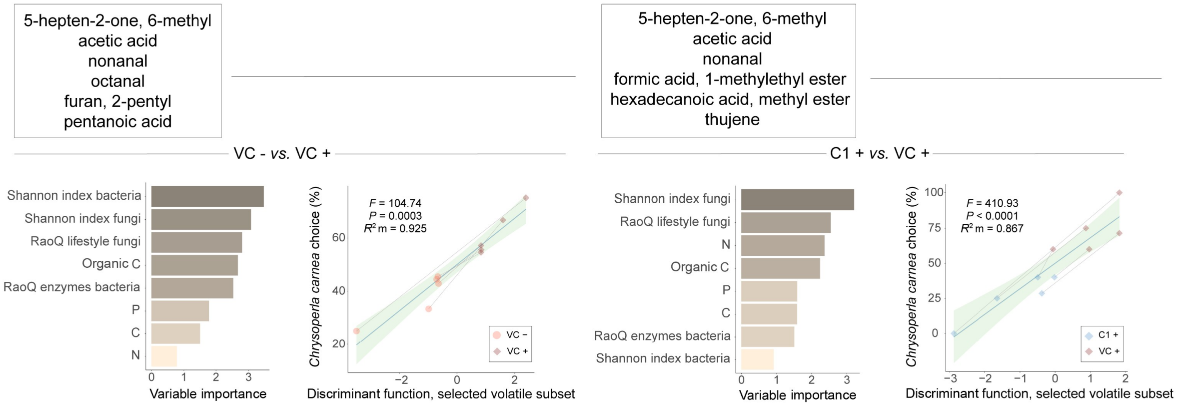

Fig. 6 Linear regressions between the discriminant function of the best subsets of volatiles explaining the mean preference of *C. carnea* in each VC and C1 comparison, with (+) or without (-) simulate fruit damage, and the random forest ranking of the importance of each soil nutrient and microbial diversity variable for each discriminant function of the best subsets of volatiles. The statistics and R^2 (marginal explained variance) of the plots reflect the result of the following linear mixed regression model: *C. carnea* preference as response variable, discriminant function of volatiles as predictor, plant pair as random effect.

microorganisms within the elicitors/effectors. Nevertheless, the preferences of the lacewings were unambiguous, with three potential volatile blends predominantly influencing their choices, accounting for a total of 96% of their selections. These blends shared various compounds previously related to plant response, such as ethyl alcohol+isopropyl alcohol, formic acid (1-methylethyl ester), hexadecanoic acid (methyl ester), hexanoic acid (methyl ester), nonanoic acid, pentanoic acid, and α-terpineol. These preliminary findings suggested that the presence or absence of a single compound does not determine the attraction of *C. carnea* to artificially attacked fruits in olive trees. Instead, it is likely the specific combination or ratio of compounds in each blend that influences the attraction of the natural enemy.

Upon establishing that one combination of elicitors/effectors was effective in attracting *C. carnea*, we opted to incorporate both inocula into our study to simulate a more realistic scenario.

Evaluating the experiment in its entirety, the introduction of either regular or tyndallized vermicompost to the soil did not significantly alter the composition of the volatile blend naturally released by the olive trees. Similarly, artificial fruit damage did not cause major changes in the blends emitted by plants grown under specific soil treatments. However, a pattern emerged where simulated seed-feeding seemed to cluster treatments together, regardless of soil treatment.

The simultaneous presence of both factors may exert an influence on the plant's chemical signalling pathways that differs from the influence of each factor when present alone (Allen et al., 2010). Furthermore, the combination of soil treatment and simulated seed-feeding may create unique conditions that amplify certain plant responses, which would not be as noticeable when each factor is applied separately. This suggests that the two factors have a synergistic effect (Sato et al., 2024). Furthermore, plants often possess complex stress response systems. The simultaneous occurrence of both factors could result in a more pronounced defensive response (Cui et al., 2022), leading to discernible alterations in the volatile blends.

We identified certain patterns suggesting that both the soil treatment and fruit attack can influence the VOCs released by olive trees, which can subsequently impact the attraction of lacewings. In this sense, two specific blends were the most effective in luring *C. carnea* under varying conditions. They have three common components: 5-hepten-2-one-6-methyl, acetic acid, and nonanal, which are potentially crucial in attracting lacewings. Plants are known to synthesize defensive compounds such as the ketone 6-methylheptenone and the aldehyde nonanal as an induced response to insect attacks (Shree et al., 2021). Acetic acid has also been proven to prime tomato defenses against chewing caterpillars (Chen et al., 2019). Specifically, it has been recognized as compounds that can draw lacewings. For example, research conducted on apple trees infested with two species of aphids revealed that *C. carnea* exhibited a strong response to a mixture of acetic acid and 2-phenylethanol, but only when combined, not when each volatile was present individually (Badra et al., 2021). Specifically, the blend also including octanal, 2-pentyl furan and pentanoic acid proved successful in luring lacewings in artificially damaged plants as opposed to non-attacked plants when both types of plants were cultivated in soil amended with vermicomposted olive wastes. This implies that seed-feeding might amplify the impact of these chemical components in attracting lacewings. Additionally, the blend that also included formic acid 1-methylethyl ester, hexadecanoic acid-methyl ester, and thujene was effective in drawing lacewings when comparing mimic seed-feeding in non-amended soil to vermicompost-amended soil. It might suggest that the additional components play a part in luring *C. carnea* in the presence of seed feeders due to the soil amendment. On the contrary, the specific chemical combinations in the blends related to the other soil treatments/seed-attack combinations failed to attract *C. carnea*. It is plausible that certain chemicals present in these blends may function as repellents, or their combinations do not appeal to lacewings, or they are equally attractive to lacewings.

Our findings suggest that incorporating vermicompost enhances *C. carnea* attraction to olive plants under attack. However, despite including specific volatiles associated with regular vermicompost, attacked plants cultivated in soils enriched with sterilized vermicompost were not more appealing to *C. carnea* compared to those grown in non-amended soil. Therefore, it can be postulated that the introduction of regular vermicompost into the soil contributes certain additional elements that could account for this observed behavior. Given this context, our experimental framework revealed that the incorporation of vermicomposted olive residues, irrespective of whether they were sterilized or not, uniformly enhanced the nutrient content in the soil. However, the influence on the root microbiome exhibited variability. Specifically, regular vermicompost reduced the functional diversity of fungi compared to unamended soil and reduced the taxonomic diversity of fungi compared to both unamended soil and soil amended with sterilized vermicompost. The effect on root bacteria was less evident. It is well-established that the diversity of bacteria and fungi and their functions are closely linked with each other and with the amount of nutrients in the soil. However, increased diversity is not always associated with greater nutrient availability. In fact, it's often the opposite. This could be due to diverse microbial communities utilizing more nutrients, or because nutrient-rich conditions favor a few dominant species over a diverse community (Niu et al., 2021; Yamamichi et al., 2022). Interestingly, a surge in the species diversity and a decline in functional diversity of root fungi were observed 24 hours after the aboveground attack, exclusively in plants cultivated in regular vermicompost. The alteration in the root microbiome following an aerial herbivore assault has been previously ascribed to a multitude of factors, including modifications in plant metabolites, availability of resources, mechanisms of plant defense, HIPVs, and feedback from the soil community. In other words, the initial microbial community in the soil can also dictate the transformation of the community in response to herbivory (Ourry et al., 2018). These elements can shape the microbial community in the olive roots, resulting in an escalation in the variety of species and a reduction in the functional diversity, particularly in reaction to seed-feeding in plants cultivated in standard vermicompost.

The influence of the root microbiome on plant health and development has been the subject of extensive documentation. Firstly, the root-associated microbial communities, including bacteria and fungi, facilitate the availability and uptake of nutrients. Furthermore, the presence of beneficial microbes has been demonstrated to induce systemic resistance in plants, thereby enhancing their resilience to biotic and abiotic stresses. Additionally, specific microbes are capable of producing phytohormones, including auxins, cytokinins, and gibberellins, which facilitate root and shoot growth. Moreover, they are capable of regulating plant hormone levels, thereby promoting overall plant development. Moreover, the root microbiome is involved in the formation and maintenance of soil structure and fertility. Microbial activity, in particular, facilitates the decomposition of organic matter, thereby recycling nutrients and enhancing soil quality. Furthermore, plants and microbes engage in a form of chemical communication. Specifically, the secretion of root exudates by plant roots attracts beneficial microbes and facilitates the establishment of symbiotic relationships, which are of vital importance for the maintenance of a healthy root microbiome (Song et al., 2020; Molefe et al., 2023; Chauhan et al., 2023). Additionally, it has been demonstrated that VOCs produced by fungi can instigate signalling cascades that modulate plant secondary metabolism, resulting in alterations in plant volatile emission. Furthermore, intra-community interactions among bacteria are pivotal in suppressing biotic stresses and enhancing plant growth (Mendes et al., 2011; Pascale et al., 2020).

Although the explicit function of the olive root microbiome in luring lacewings remains under-researched, there exists prior evidence supporting the notion that microbes in the olive roots trigger the production of aerial VOCs which have the potential to repel herbivores or draw in their predators (Aguirrebengoa et al., 2024). Given that VOCs serve as pivotal elements in the tripartite interactions among plants, microbes, and insects, comprehending the intricate manipulation and response among these three partners within the framework of plant defense systems emerges as a significant challenge, as underscored by Noman et al. (2021). In this scenario, we observed a change in volatile emission following the simulated attack of the seed-feeder *P. oleae* in olive trees grown in soil amended with vermicomposted olive mill waste. The emitted blend was generally more attractive to the generalist predator *C. carnea*. The emission of VOCs by olive tree leaves was found to be significantly influenced by the nitrogen levels present in the soil, the organic carbon content, and the diversity of fungi present in the soil. These soil factors could facilitate the establishment of a rich and diverse microbial environment around the roots, which in turn affects the plant's metabolic processes and the types of VOCs it produces (Benítez et al., 2017; Chauhan et al., 2023; Wei et al., 2024). Three specific VOCs–5-hepten-2-one-6-methyl, acetic acid, and nonanal–were identified as particularly effective in attracting the lacewing *C. carnea*. This suggests that these compounds play a pivotal role in the plant's defence strategy by attracting natural enemies of pests, thereby contributing to biological pest control. The attractiveness of these VOCs to female lacewings was found to be particularly linked to the soil's organic carbon content and the diversity of both bacterial and fungal communities. This indicates that the development of healthier and more diverse soil ecosystems may enhance the plant's capacity to attract natural pest predators through the production of specific VOCs.

Prior research has demonstrated the efficacy of certain volatile compounds as pest control agents in controlled environments, such as commercial greenhouses (Pérez-Hedo et al., 2021). Other studies adopt a more holistic approach, emphasising soil health as a crucial factor in the control of pests in agroecosystems (Farooqi et al., 2023). Our perspective is aligned with the latter, particularly in view of the lack of control over environmental conditions in extensive crops such as olives. It has been demonstrated that a mixture of volatiles can attract natural enemies of pests, and that this mixture is emitted more intensely in olive trees grown in soils amended with vermicompost. It is therefore proposed that this organic amendment be used in olive farming. The application of vermicompost not only improves soil health, thereby indirectly benefiting the plant, but also directly enhances the plant's induced defences. This finding is particularly noteworthy given that the organic amendment is derived from phytotoxic waste produced in large quantities during the olive oil extraction process. The integration of this practice into the circular economy could facilitate its utilisation for the advancement of sustainable agriculture.

5 Conclusions

In our investigation of the reuse of organic waste as soil fertilisers within a circular economy, we highlight the crucial role of belowground ecology in plant defense. Our study reveals that vermicomposted olive wastes as soil amendment and the simulation of *Prays oleae* attack have an impact on volatiles compounds emitted by *Olea europaea* and influence the attraction of *Chrysoperla carnea*, a key pest predator. It is noteworthy that soil nitrogen, organic carbon content, and belowground fungal diversity exert a significant influence on the composition of aboveground plant volatiles. Furthermore, the appeal of aerial volatiles correlates with soil organic carbon content and the diversity of both bacteria and fungi. Notably, three specific volatile compounds–5-hepten-2-one-6-methyl, acetic acid, and nonanal–consistently attract the lacewing *C. carnea*. These findings highlight the importance of continuously monitoring soil amendments to understand their impact on plant-induced defense and their potential to improve biological control strategies.

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Data availability statement

All raw Illumina sequence data were deposited in the Sequence Read Archive (SRA) service of the NCBI database (https://www.ncbi.nlm.nih.gov/) (BioProject ID: PRJNA1103158; Biosamples accession numbers SAMN41043216-SAMN41043227).

Electronic supplementary material

Supplementary material is available in the online version of this article at https://doi.org/10.1007/s42832-024-0281-z and is accessible for authorized users.

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