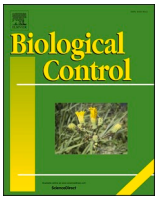
Biological Control 188 (2023) 105384

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon



Insect oviposition in herbaceous plants attracts egg parasitoids despite fungal phytopathogen infection

Mahmut Mete Karaca [a,b,1], Tuğcan Alınç [a,1], Antonino Cusumano [a,d,*], Giulia Mirabile [a], Livio Torta [a], Salvatore Guarino [c], Ezio Peri [a,d], Stefano Colazza [a,d]

[a] Department of Agricultural, Food and Forest Sciences, University of Palermo, Viale delle Scienze, Building 5, 90128 Palermo, Italy
[b] Agricultural Faculty, Department of Plant Protection, Cukurova University, 01330 Adana, Turkey
[c] Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy (CNR), Via Ugo La Malfa 153, 90146 Palermo, Italy
[d] Interuniversity Center for Studies on Bioinspired Agro-Environmental Technology (BATCenter), University of Napoli Federico II, 80055 Portici, Italy

HIGHLIGHTS

- Egg parasitoids exploit oviposition-induced plant volatiles (OIPVs) to locate hosts.
- Plants commonly suffer multiple biotic stresses in nature.
- OIPV emission is altered when plants are attacked by herbivores and pathogens.
- Egg parasitoid attraction towards OIPVs is not disrupted by phytopathogen infection.

ARTICLE INFO

Keywords:
Parasitoid host location
Oviposition-induced plant volatiles
Indirect plant defenses
Trissolcus basalis
Nezara viridula

ABSTRACT

Egg parasitoids are important natural enemies of several insect pests. The ability to kill the pest before it can inflict damage to the plant makes egg parasitoids ideal candidates for biological control. Several studies have shown that egg parasitoids exploit oviposition-induced plant volatiles (OIPVs) to locate host eggs laid on plant organs. Yet such studies have often overlooked that, in nature, plants frequently suffer concurrent attack by insect herbivores and phytopathogens. These dual attacks can modify the emission of induced plant volatiles, which may potentially interfere with the host location abilities of egg parasitoids. We investigated this research question using the following study organisms: the broad bean *Vicia faba*, the plant pathogen *Stemphylium* sp., the southern green stink bug *Nezara viridula* and its associated egg parasitoid *Trissolcus basalis*. We showed that *T. basalis* is able to exploit OPIVs in order to locate *N. viridula* egg masses even when *V. faba* plants were previously infected by *Stemphylium* sp. Chemical analyses indicate that the egg parasitoid ability to exploit OIPVs persists despite significant alterations of the volatile blends emitted by plants suffering multiple biotic stresses. This study highlights the importance of incorporating the complexity of multiple biotic stresses when studying parasitoid foraging behavior, in order to comprehend how to enhance the effectiveness of natural enemies in crop protection.

1. Introduction

Insect parasitoids are third trophic level organisms that play an important role in regulating populations of agricultural and forest pests (Heimpel and Mills, 2017). In particular, egg parasitoids are specialized to develop only on host eggs (Conti and Colazza, 2012; Fatouros et al., 2020). This capability makes them highly desirable candidates in biological pest control, as egg parasitoids can effectively eliminate the pest before the crop feeding stage has been reached, thus minimizing crop damage (Tamiru et al., 2015). When foraging, egg parasitoids face several challenges as their hosts are rather inconspicuous and available in the environment for a limited time due to fast embryo development (Vinson, 1998, Wajnberg and Colazza, 2013). Furthermore, the suitability of the host eggs decreases with egg age, so egg parasitoids are

* Corresponding author.
E-mail address: antonino.cusumano@unipa.it (A. Cusumano).
[1] Shared first authors.

https://doi.org/10.1016/j.biocontrol.2023.105384
Received 17 July 2023; Received in revised form 6 October 2023; Accepted 24 October 2023
Available online 26 October 2023

under strong selective pressure to locate patches with freshly laid eggs (Meiners and Peri, 2013; Greenberg et al., 2023).

In order to forage efficiently for hosts, egg parasitoids have evolved the ability to exploit oviposition-induced plant volatiles (OIPVs) which represent reliable cues associated with presence of host eggs laid on plant tissues (Hilker and Fatouros, 2015; Bertea et al., 2020; Hilker et al., 2023). OIPVs are considered a form of indirect plant defense as such plant volatiles attract natural enemies that eventually kill the herbivore eggs (Hilker and Fatouros, 2015). Several studies have indeed shown that OIPV emission is widespread in the plant kingdom, occurring in both annuals or perennials, monocotyledons or dicotyledons, gymnosperms and angiosperms (Fatouros et al., 2016). Depending on the nature and specificity of plant-herbivore interactions, OIPVs can be emitted as a result of physical damage inflicted before or during egg deposition as found in the elm beetle *Xanthogaleruca luteola* Mull. (Meiners and Hilker, 2000) and in the pine sawflies *Diprion pini* L. and *Neodiprion sertifer* Geoffr. (Hilker et al., 2002; Mumm et al., 2003), respectively. However, OIPVs can also be emitted without any apparent physical damage, as shown for butterflies and moths (Tamiru et al., 2011; Fatouros et al., 2012; Salerno et al., 2013). In stink bug species such as *Nezara viridula* L., females frequently feed on the plants where they lay eggs and a combination of feeding and oviposition is required to produce OIPVs that attract egg parasitoids (Colazza et al., 2004a; Moujahed et al., 2014; Frati et al., 2017).

The majority of the studies that have investigated parasitoid attraction towards OIPVs have been carried out in simple tritrophic systems consisting of one species of plant, herbivore and parasitoid. However, in nature, it is common that plants suffer multiple biotic stresses and, in particular, concurrent attack by herbivores and phytopathogens is widespread (Tack and Dicke, 2013). The release of induced plant volatiles in response to multiple biotic stresses may vary in a specific manner depending on factors such as the identity of the attackers, the plant organ targeted, the order in which biotic stresses occur and the time interval between their arrival (de Rijk et al., 2013; Ponzio et al., 2013). Parasitoids therefore need to cope with variation in plant volatile emission in order to maximize their chances to find suitable hosts on plants suffering multiple biotic stresses (Meiners, 2015). In the case of larval parasitoids, which exploit herbivore-induced plant volatiles (HIPVs), contrasting responses towards plants attacked by herbivore hosts in the presence of fungal or bacterial infection have been found (Rostás et al., 2006; Ponzio et al., 2014, Desurmont et al., 2016; Peñaflor and Bento, 2019). For example, in *Zea mays* L. plants challenged by both *Setosphaeria turcica* Leonard fungus and *Spodoptera littoralis* Boisduval caterpillars, the emission rate of induced plant volatiles was reduced by almost 50 % compared to herbivory alone; however, the foraging behavior of larval parasitoids remained unaffected (Rostás et al., 2006). Similarly, *Cotesia glomerata* L. parasitoids were able to exploit HIPVs and locate *Pieris brassicae* L. caterpillars feeding on *Brassica nigra* L. plants in the presence of the pathogenic bacterium *Xanthomonas campestris* Pammel (Ponzio et al., 2014). However, phytopathogen infection can also negatively affect the foraging behavior of larval parasitoids as found for powdery mildew *Erysiphe cruciferarum* Opiz ex. L. Junel which interferes with the host location of *C. glomerata* (Desurmont et al., 2016) and for red-rot *Fusarium verticillioides* (Sacc.) Nirenberg which attenuates the attractiveness of sugarcane borer-induced plant volatiles to *Cotesia flavipes* Cameron (Penaflor and Bento, 2019). To date whether egg parasitoid attraction towards OIPVs is disrupted by plant pathogen infection remains yet to be explored.

We investigated this research question using the following study organisms: the broad bean *Vicia faba* L., the plant pathogen *Stemphylium* sp., the southern green stink bug *N. viridula* and its associated egg parasitoid *Trissolcus basalis* (Wollaston). The stink bug *N. viridula* is a cosmopolitan pest which has been shown to cause damage particularly on legumes (Todd, 1989; Esquivel et al., 2018). The egg parasitoid *T. basalis* is considered the main natural enemy of *N. viridula* and has been employed in several biological control programs worldwide (Jones, 1988; Corrêa-Ferreira and Moscardi, 1996). The necrotrophic fungal pathogen *Stemphylium* spp. induce leaf blight in various crops including legumes such as broad bean (Vaghefi et al., 2020) and lentil (Kant et al., 2017). The foliar symptoms of *Stemphylium* sp. generally consist of large and greyblack lesions expanding from the edge of leaves as the disease progresses (Vaghefi et al., 2020). For instance, the infection of *Stemphylium vesicarium* on broad bean plants causes necrotic spots that become visible after 7 days of induction (Dell'Olmo et al., 2023). Previous studies have shown that *T. basalis* females locate *N. viridula* host eggs by exploiting several chemical cues including volatile and contact kairomones released by adults (Colazza et al., 1999, Peri et al., 2006, Abram et al., 2015) as well as OIPVs emitted by *V. faba* L. plants in response to oviposition and feeding activity of *N. viridula* females (Colazza et al., 2004a, 2004b; Moujahed et al., 2014; Frati et al., 2017). In southern Italy it is common to find *V. faba* plants concurrently challenged by *N. viridula* eggs and *Stemphylium* sp. (Cusumano, personal observations), so it is relevant to investigate whether the fungal pathogen interferes with the host location behavior of *T. basalis* as such foraging scenario may be frequently encountered by the egg parasitoid.

In this study we carried out olfactory bioassays under laboratory conditions to investigate whether the egg parasitoid *T. basalis* is able to exploit OIPVs induced by *N. viridula* on *V. faba* plants infected with the fungal pathogen *Stemphylium* sp. We also used gas-chromatography coupled with mass spectrometry (GC–MS) in order to study the chemical composition of induced plant volatile blends in the attempt to link plant volatile composition with parasitoid behavioral responses.

2. Materials and methods

2.1. Plants and insects

Broad bean (*V. faba* cv. Superaguadulce) seeds were sown individually in plastic pots (9 × 9 × 13 cm) filled with a mixture of agriperlite (Superlite, GyprocSaint-Gobain, PPC Italia, Italy), vermiculite (Silver, GyprocSaint-Gobain, PPC Italia, Italy), and sand (1:1:1). The plants were grown under controlled conditions of 25 ± 2 °C and 50 ± 10 % relative humidity. For both olfactometer bioassays and headspace collections, 14–15-day-old broad bean plants with approximately six fully expanded leaves were used.

The *N. viridula* colony was established by collecting adults from both cultivated and uncultivated fields located around Palermo (Sicily, Italy). The insects were reared under controlled environmental conditions (25 ± 2 °C; 70 ± 5 % RH; 16 h:8h L:D) in rearing cages (47.5 × 47.5 × 47.5 cm, BugDorm-44545 MegaView Science Co. Ltd, Taichung, Taiwan). Seasonal organic vegetables, sunflower and soybean seeds were used as a food source which was refreshed every 2–3 days. Separate cages were used for nymphs and adults and paper towels were hung inside the cages containing adults as ovipositional substrates. Egg masses were collected daily to maintain colonies of both *N. viridula* and *T. basalis*.

The colony of *T. basalis* was established from wasps emerging from *N. viridula* egg masses found in wild and uncultivated fields around Palermo (Sicily, Italy). The wasps were reared on *N. viridula* egg masses glued on paper strips and maintained in 85 ml glass tubes under the same controlled environmental conditions of the host *N. viridula*. Adults were fed with a honey-water solution. After emergence, male and female wasps were kept together to allow mating. Females used in the experiments were isolated 24 h prior to the bioassays and used only once when they were naïve and 2–4 days old.

2.2. Fungal isolation, morphological identification and pathogenicity tests

Fungal isolation was carried out from symptomatic leaves originally collected from a *V. faba* field located in Palermo (Sicily, Italy) during spring 2022. Infected leaf tissues showing necrotic spots were washed in 5 % NaClO for 5 min and then rinsed in sterile distilled water. The symptomatic tissues were cut into small pieces (area = 2 mm^2) and

transferred in Petri plates containing Potato Dextrose Agar (PDA) (5 pieces per plate). The isolation was carried out in triplicate. Plates were incubated at 25 ± 1 °C and checked daily to monitor fungal growth. After the incubation period, fungal isolates were subcultured on new PDA plates to obtain pure colonies which were used for morphological identification following procedures described in (Mirabile et al., 2023; Torta et al., 2022). In brief, small portions of mycelial mass grown in PDA plates were treated with a drop of lactophenol solution (25 ml distilled water, 25 ml glycerin, 25 ml lactic acid, 25 g phenol crystals), added 0.01 % of methylene blue. Microscopic observations were conducted using a light microscope coupled to an AxioCam MRc5 (Zeiss, Germany) digital camera and images were captured using the software AxioVision 4.6 (Zeiss, Germany).

To satisfy Koch's postulates, pathogenicity tests were carried out. Pure colonies of the isolated fungi were grown on PDA for 7 days, until spore production. To prepare spore suspension, 10 ml of sterilized distilled water were poured onto the cultured agar plate and the spores were gently collected with a sterile spatula. Then the liquid media containing the spores was collected, filtered in sterilized gauze to eliminate mycelium residues and the obtained suspension was adjusted to a concentration of 10^5 spore/ml. Leaves of 5 asymptomatic plants of *V. faba* were punctured with sterilized needles to facilitate pathogen penetration inside leave tissues. We punctured 3 holes (≈ 0.5 mm^2) within an area of 2.5 cm in diameter to a fully expanded leaf and inoculated it with the spore suspension of the fungal pathogen. Other 5 asymptomatic plants were punctured in the same manner but treated with sterile water (controls). Finally, both inoculated and water-sprayed plants were observed daily until symptoms appeared and isolation tests were carried out to reisolate the inoculated pathogen.

2.3. Plant treatments and Y-tube olfactometer bioassays

This behavioral observation aimed to assess the response of *T. basalis* females in Y-tube olfactometer towards different plant treatments: plants subjected to *N. viridula* feeding and oviposition activity (F_O), plants infected with *Stemphylium* sp. (PAT), and plants exposed to both infection + feeding and oviposition activity of *N. viridula* (PAT + F_O). In addition, healthy plants not subjected to stink bug damage or pathogen infection we used as controls (CTRL). To obtain plants subjected to *N. viridula* feeding and oviposition, a gravid female of *N. viridula* was confined in a clip cage measuring 3.8 cm in diameter and 1 cm in height. The cage had a mesh-covered hole with a diameter of 3 cm, which was attached to a fully expanded leaf of the bean plant. This setup was maintained for 24 h before bioassays, allowing *N. viridula* to feed and deposit an egg mass on the plant. As *N. viridula* does not leave any visual marks on the leaves they fed on, the feeding activity was confirmed by visual observation, once the females inserted their stylet into leaf tissue. *Stemphylium* sp.-infected plants were inoculated with a spore suspension of the fungal pathogen 7 days before bioassay (when symptoms due to occurrence of necrotic spots were clearly visible), using the same protocol of pathogenicity tests. To obtain bean plants subjected to both *N. viridula* feeding and oviposition activity + *Stemphylium* sp. infection, plants were first inoculated with *Stemphylium* sp. and after 6 days, the infected plants were exposed to gravid females of *N. viridula* for additional 24 h, following the same procedure as described above. Preliminary assays indicated no significant differences between the size of the egg masses laid on pathogen-free *versus* pathogen-infected leaves (Karaca personal observations).

The Y-tube olfactometer bioassays were conducted as paired choices in which the parasitoids were offered the following combinations: (1) plant with *N. viridula* feeding and oviposition *versus* healthy plant (F_O vs CTRL); (2) *Stemphylium* sp.-infected plant *versus* healthy plant (PAT vs CTRL); (3) *Stemphylium* sp.-infected plant + *N. viridula* feeding and oviposition *versus* healthy plant (PAT + F_O vs CTRL); (4) *Stemphylium* sp.-infected plant *versus Stemphylium* sp.-infected plant + *N. viridula* feeding and oviposition (PAT vs PAT + F_O); (5) *Stemphylium* sp.-infected plant + *N. viridula* feeding and oviposition *versus* plant with *N. viridula* feeding and oviposition (PAT + F_O vs F_O). We did not consider the PAT vs. F_O comparison because the focus of the paper is to evaluate whether the concurrent presence of the pathogen on egg-induced plants (i.e. treatment PAT + F_O) would disrupt egg parasitoid attraction towards OIPVs.

In the pairwise comparison involving plants infected with *Stemphylium* sp. *versus* controls, a leaf from healthy plants used as controls was mechanically damaged with sterile pins and sprayed with distilled water 7 days before bioassays. In the pairwise comparison involving plants induced with *N. viridula* feeding and oviposition *versus* controls, an empty clip cage was attached to a leaf of healthy plants used as controls 24 h before bioassays. In the pairwise comparisons involving plants infected with *Stemphylium* + induction with *N. viridula* feeding and oviposition *versus* controls, a leaf from healthy plants used as controls was mechanically damaged with a sterile pin, sprayed with distilled water and after 6 days an empty clip cage was attached to a leaf for additional 24 h. All bioassays were carried out from 09:00 h to 13:00 h under controlled conditions (26 ± 1 °C, 50 ± 5 % RH).

The Y-tube olfactometer used in the bioassays is made of a polycarbonate body (stem 9 cm; arms 8 cm at 130° angle; ID 1.5 cm) sandwiched between two glass plates. The flow of clean, humidified air was regulated in each arm by a flowmeter at a rate of approximately 400 ml/min. The air stream was directed through cylindrical glass chambers, each containing a treated or control *V. faba* plant as odor sources. The entire olfactometer was reversed after testing every parasitoid female to prevent that leftover volatiles could still have been present from the previous tests in the arms. At the end of the day, the entire system was cleaned using alcohol and the glass components were dried in oven at 120 °C. Wasp females were introduced individually into the Y-tube olfactometer at the entrance of the stem and were given 600 s to explore the area freely. In the Y-tube olfactometer, *T. basalis* females have the tendency to explore first the whole area and then spend more time on the arm associated with the most attractive stimuli. Thus, parasitoid responses for this species are better predicted by analyzing residence time data rather than binary choices (Colazza et al., 2004a, Colazza et al., 2004b, Moujahed et al., 2014). To calculate the total time that a wasp spent in each olfactometer arm, we defined a hypothetical line in each arm, 5 mm distal to the central Y-junction. The wasp behavior was recorded using a monochrome CCD video camera (Sony SSCM370CE) equipped with a 12.5–75 mm/F1.8 zoom lens. An infrared pass filter (Kodak Wratten filter 87 Å) was placed over the camera lens to eliminate visible wavelengths. The analog video signals from the camera were digitized by a video frame grabber (Canopus® ADVC110, Grass Valley CA, USA). The digitized data obtained from the video recordings were analyzed using XBug video tracking and motion analysis software (Colazza et al., 1999). As odour source combinations, 8 different plant pairs were tested with 5 female wasps per plant pair combinations (total number of wasps tested = 40 per each treatment).

2.4. Headspace collection of plant volatiles

To collect headspace volatiles from the *V. faba* plants, cylindrical glass chambers (height 40 cm and inner diameter of 25 cm) were used. Prior to each collection, the glass chambers were washed with water, rinsed with acetone and baked at 120 °C overnight. Prior to volatile collections, the plant pots were wrapped into aluminum foil and then placed into glass chambers for collection. In addition, the volatiles collection from glass chambers containing only potted soil (wrapped in aluminum) were regularly carried out to identify possible pollutant chemicals and exclude them from the analysis. Charcoal-filtered air passed through chambers at a flow rate of 400 ml/min using a NMP 830 KNDC 12 V pump (KNF, Milan, Italy). Volatile organic compounds (VOCs) from the plants were collected for 24 h using adsorbent traps. The traps, positioned at the outlet of the chamber, were made up of glass tubes filled with PorapakQ (Sigma-Aldrich; 60 mg, 80–100 mesh). Traps

were pre-cleaned with hexane and then heat-conditioned in a nitrogen stream (100 ml/min) at 130 °C for at least 2 h. After each collection, the traps were eluted with 1 ml of hexane, and the extracts were concentrated using a gentle stream of nitrogen until total evaporation of the extract, which was then re-dissolved using 50 µl of hexane. Extracts were stored at −20 °C in glass vials with Teflon cap liners until they were used for gas chromatography-mass spectrometry (GC–MS) analysis. For each plant treatment, 6–8 replicates were sampled.

Chemical analyses were conducted using an Agilent 6890 GC system equipped with a DB5-MS column and interfaced with an MS5973 quadruple mass spectrometer. The GC–MS operated in splitless mode with helium as carrier gas. The injector and detector temperatures were set at 260 °C and 280 °C, respectively. The GC oven temperature was initially set at 40 °C and ramped up at a rate of 10 °C/min to 250 °C, with hold times of 5 min and 30 min at the beginning and end, respectively. Mass spectra were recorded from 40 to 550 amu through electron impact ionization at 70 eV. The peak area of each detected compound was calculated using the ChemStation software for quantification purposes. Tentative identification of compounds was carried out based on comparison of retention index and mass spectra with those reported in the literature (Adams, 2012), https://www.pherobase.com, and the NIST 2011 library. Authentic standards (Sigma-Aldrich, Milan, Italy) were injected for identification when available. Identification was confirmed when a good match of mass spectrum and retention index was obtained. Periodic blank headspace collections were conducted to identify and exclude the presence of possible contaminants from the analysis.

2.5. Statistical analysis

Residence time data were not significantly different from a normal distribution (Shapiro–Wilk test) and thus analyzed with parametric tests. A paired *t*-test for dependent samples was used to statistically compare the time spent by the wasp in each arm of the Y-tube olfactometer using the R statistical software version 4.2.2 (R Core Team, 2022). Projection to latent structures discriminant analysis (PLS-DA) was used to analyze peak areas of chemical compounds. The measured peak areas were divided by the fresh biomass of the plant, then log-transformed, mean-centered and scaled to unit variance before they were processed using the software MetaboAnalyst (Xia et al., 2009) The results of the analysis were visualized in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables to these components. The compounds that contribute the most in explaining statistical differences were identified based on the variable importance in the projection (VIP values) (Wold et al., 2001). Furthermore, we used a Kruskal-Wallis test to evaluate differences in measured peak areas of individual VOCs, as well as in the total amount of VOCs, among the various treatments and control plants. If significant differences were observed, the data were further analyzed by Dunn's post-hoc test.

3. Results

3.1. Fungal isolation, morphological identification and pathogenicity test

Colonies with light brown mycelium clearly appeared from symptomatic leaf tissues in Petri plates after six days from incubation. Macroscopic and microscopic observation of pure colonies allowed to identify the pathogen as *Stemphylium* sp., which is characterized by brown conidiophore, oblong or oval conidia 21–35 × 12–15 µm in size with 1–6 transverse septa and 1–3 longitudinal septa.

All plants (N = 5) inoculated with a spore suspension of *Stemphylium* sp. showed, after 7 days, necrotic spots (areas covered = 10–15 % of the leaf, Karaca personal observations) from which *Stemphylium* sp. colonies were reisolated satisfying Koch's postulates whereas control plants showed no symptom (N = 5).

3.2. Y-tube olfactometer bioassays

Trissolcus basalis females did not discriminate between volatiles of healthy plants and those emitted by plants infected only with *Stemphylium* sp. (PAT vs CTRL: $t = 0.19$, df = 38, $P = 0.848$) (Fig. 1). The wasps were significantly attracted to volatiles emitted by plants induced with *N. viridula* feeding and oviposition in comparison with healthy plants (F_O vs CTRL: $t = 4.24$, df = 39, $P < 0.001$). The wasps preferred volatiles emitted by plants infected by *Stemphylium* sp. and subsequently induced with *N. viridula* feeding and oviposition over volatiles of healthy plants (PAT + F_O vs CTRL: $t = 2.40$, df = 38, $P < 0.05$) or volatiles emitted by *Stemphylium* sp. infected plants (PAT + F_O vs PAT: $t = 3.57$, df = 39, $P < 0.001$). Inoculation of *Stemphylium* sp. did not alter the response of *T. basalis* females when wasps were given a choice between infected plants induced by *N. viridula* feeding and oviposition and non-infected plants induced only by *N. viridula* feeding and oviposition (PAT + F_O vs F_O: $t = -0.25$, df = 37, $P = 0.802$).

3.3. Headspace analysis of plant volatiles

A total of ten volatile organic compounds were detected in the headspace of *V. faba* plants across four treatments (Table 1). Overall, differently treated plants emitted the same compounds, but in different proportions. A Kruskal-Wallis test conducted on the quantity of each VOC emitted across various treatments and control plants revealed a significant difference solely in the levels of α-farnesene ($H_3 = 9.83$; $P < 0.05$) whereas no significant differences were found for the other compounds. Specifically, the amount of α-farnesene emitted from *Stemphylium*-infected plants exceeded that from control plants. The total amount of VOCs produced by the different treatment and the control plants was also significant ($H_3 = 10.95$; $P < 0.05$).

A comparison by PLS-DA among the four treatments resulted in a significant model indicating that the composition of the blend varied according to plant treatment (permutation test, $P < 0.05$) (Fig. 2). In the PLS-DA model, α-farnesene had a VIP value > 3.0 indicating that this compound strongly contributed to explaining the differences among treatments. Other influential volatiles (VIP value range: 1–0.5) were decanal, (*Z*)-3-hexenol and (*E,E*)-4,8,12-trimethyl-1,3,7,11- tridecatetraene (TMTT).

4. Discussion

In this paper we showed that *T. basalis* is able to exploit OPIVs to locate *N. viridula* egg masses laid on *V. faba* plants previously infected by *Stemphylium* sp. To the best of our knowledge, this is the first study that investigated egg-induced indirect plant defenses in the context of phytopathogen infection.

Parasitoids are under strong selection pressure to optimize their foraging efficiency, particularly when foraging in heterogeneous environments where host resources are scattered among non-resources (Aartsma et al., 2017, (Aartsma et al., 2019)). In this study, multiple lines of evidence indicate that *T. basalis* is well adapted to forage for *N. viridula* eggs in complex scenarios. In particular: (1) *T. basalis* does not respond to volatiles emitted by plants infected only by *Stemphylium* sp.; (2) *T. basalis* prefers volatiles emitted by plants concurrently induced by *N. viridula* eggs + *Stemphylium* sp. over volatiles of heathy plants; (3) *T. basalis* does not discriminate between volatiles emitted by plants induced with *N. viridula* eggs + *Stemphylium* sp. and those of plants induced by *N. viridula* eggs only. Taken together these results indicate that egg-induced plant volatiles can be effectively exploited by egg parasitoids even when plants are suffering multiple biotic stresses. Our results are consistent with some of the findings on larval parasitoids (Cardoza et al., 2003; Rostas et al., 2006; Ponzio et al., 2014) suggesting that OIPVs are robust signals that withstand disruption by fungal pathogen infection. However studies on larval parasitoids have shown that phytopathogens can also disrupt HIPV exploitation (Desurmont

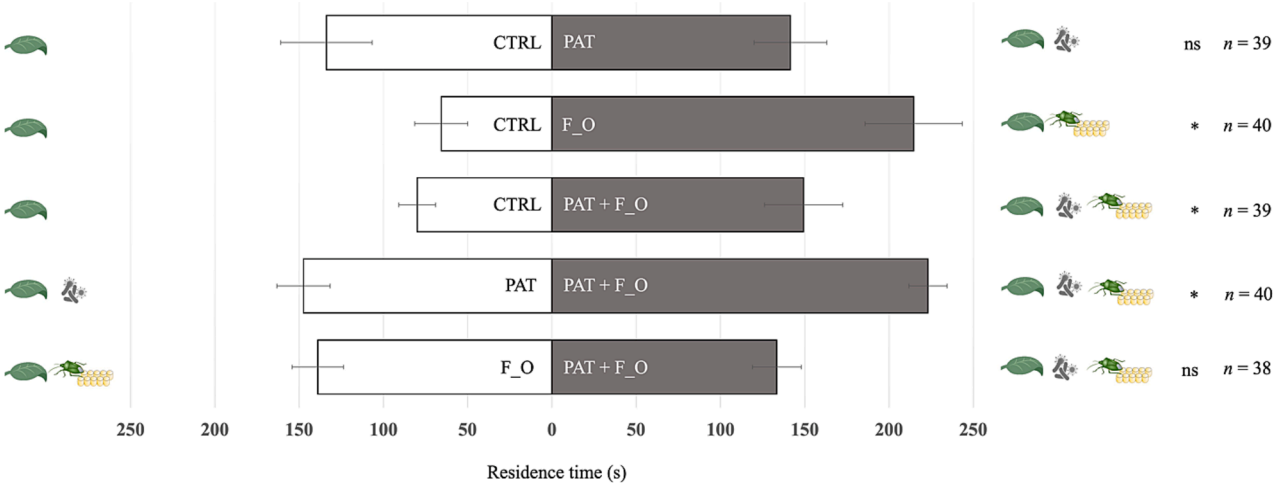


Fig. 1. Residence time of *T. basalis* females in a Y-tube olfactometer. *Vicia faba* plants were either left untreated (CTRL) or subjected to the following treatments: infection with *Stemphylium* sp. (PAT); induction by *N. viridula* feeding and oviposition (F_O); induction by *N. viridula* feeding and oviposition + infection with *Stemphylium* sp. (PAT + F_O). Bars indicate mean (±SE) of the time spent by wasp females in each arm over an observation period of 600 s. Asterisks indicate statistical significance; ns: not significant, n = number of replicates.

Table 1
Volatile organic compounds detected in the headspace of differently treated *Vicia faba* plants. Plants were either left untreated (CTRL) or subjected to the following treatments: infection with *Stemphylium* sp. (PAT); induction by *N. viridula* feeding and oviposition (F_O); induction by *N. viridula* feeding and oviposition + infection with *Stemphylium* sp. (PAT + F_O). * = identification carried out with authentic standard; Tr. = traces; TMTT = (*E,E*)-4,8,12-trimethyl-1,3,7,11- tridecatetraene. Unk1 and Unk2 indicate unknown compounds. Amounts are reported in mean peak area ± SE/g fresh weight of foliage divided by 10^3. No letter in common indicate significant differences for $P < 0.05$ (Kruskal-Wallis test).

RT	chemical	RI	CTRL	PAT	F_O	PAT + F_O
9.52	(*Z*)-3-hexenol	856	57.52 (±34.39) [a]	67.46 (±45.39) [a]	261.75 (±262.53) [a]	tr. [a]
21.60	decanal*	1206	734.55 (±218.31) [a]	1410.70 (±605.31) [a]	1240.79 (±299.54) [a]	404.51 (±138.56) [a]
25.17	unk1	1330	0.10 (±0.10) [a]	58.26 (±0.36) [a]	25.94 (±14.30) [a]	3.74 (±30.09) [a]
25.42	unk2	1339	0.50 (±0.50) [a]	47.39 (±31.31) [a]	38.18 (±28.10) [a]	3.46 (±3.39) [a]
25.60	*δ*-elemene	1345	5.93 (±5.93) [a]	18.70 (±12.99) [a]	21.41 (±12.24) [a]	0.28 (±0.17)
26.06	cyclosativene	1362	tr. [a]	4.24 (±2.81) [a]	55.81 (±54.56) [a]	tr. [a]
27.73	*β*-caryophyllene*	1423	14.08 (±9.35) [a]	18.04 (±12.11) [a]	75.86 (±40.36) [a]	192.34 (±168.72) [a]
28.31	geranyl acetone	1445	150.76 (±73.83) [a]	558.47 (±280.71) [a]	233.40 (±97.24) [a]	112.59 (±61.16) [a]
29.89	*α*-farnesene*	1504	35.29 (±16.23) [b]	1691.29 (±594.00) [a]	155.89 (±80.03) [ab]	1001.42 (±400.35) [ab]
31.58	TMTT	1567	87.10 (±73.45) [a]	270.25 (±126.16) [a]	255.92 (±130.19) [a]	166.62 (±86.37) [a]
	TOTAL		1085.88 (±359.56) [b]	4144.84 (±1515.89) [a]	2364,99 (±472.00) [ab]	1885.00 (±321.43) [ab]

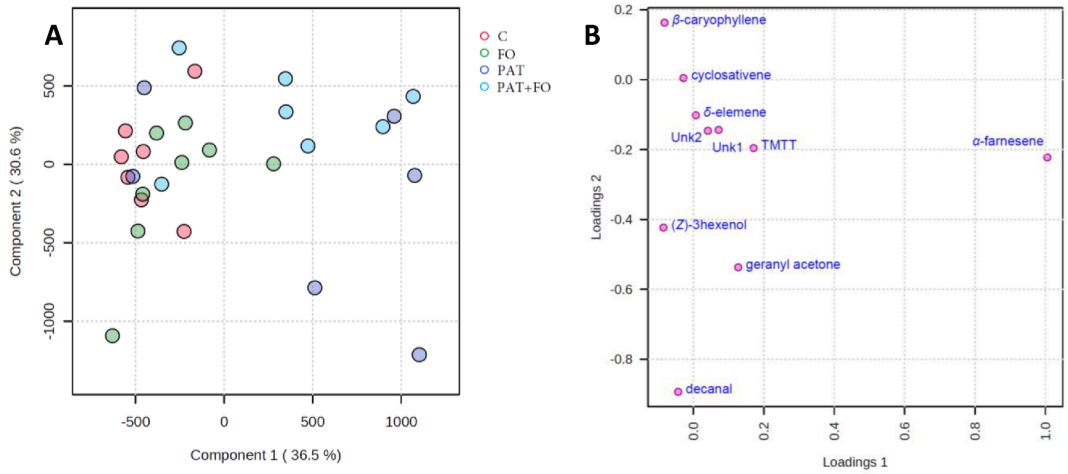


Fig. 2. Projection to latent structures discriminant analysis (PLS-DA) of the different plant treatments used in the olfactometer assays (C: $n = 7$; FO: $n = 8$; PAT: $n = 6$; PAT + FO: $n = 7$). A) Score plot visualizing the grouping pattern of the samples according to the first two principal components (PCs) with the explained variance in parenthesis. B) Loading plot of the first two PCs showing the contribution of each compound to the two PLS-DA components.

et al., 2016, Penaflor and Bento, 2019); thus, it is not possible to exclude that variations in our experimental conditions (such as an increase in the *Stemphylium* sp. pathogen load or in the duration of the infection before bioassay) might affect the strength of *V. faba* plant responses with consequences for the OIPV emission and attraction of *T. basalis* egg parasitoids.

Previous studies have investigated the foraging behavior of *T. basalis* in a multi-herbivore scenario. Moujahed et al. (2014) found that feeding by the non-host *Sitona lineatus* L. on *V. faba* plants infested with *N. viridula* eggs disrupted parasitoid attraction towards egg-induced plant volatiles, regardless if non-host feeding occurred above-ground (due to adults) or below-ground (due to larvae). The foraging behavior of the egg parasitoids *Trichogramma brassicae* Bezdenko and *T. evanescens* Westwood is not affected by the presence of the phloem-feeding aphid *Brevicoryne brassicae* L.; however, both *Trichogramma* species are negatively impacted by chewer insects such as *Spodoptera exigua* Hübner caterpillars, which disrupted egg parasitoid attraction towards *Pieris brassicae* L. egg-induced volatiles (Cusumano et al., 2015). Overall, more evidence is available on parasitoid foraging behaviour under multi-herbivore scenarios whereas studies that focused on dual attack with herbivores and plant pathogens have lagged behind (de Rijk et al., 2013; Ponzio et al., 2013). This is probably due to the fact that plant-herbivore and plant-pathogen interactions have been historically studied in isolation, and not because multiple herbivore attacks are more likely to occur than mixed attacks by herbivores and pathogens (Pieterse and Dicke, 2007).

The reason why *T. basalis* attraction towards egg-induced plant volatiles withstands interference by the fungal pathogen *Stemphylium* sp. whereas it is negatively affected by the chewer insect *S. lineatus* deserves to be further explored. It is well known that plants respond to insect herbivore and pathogen attacks by activating Jasmonic Acid (JA) and Salicylic Acid (SA) defense signaling pathways (Howe and Jander, 2008; Pieterse et al., 2012). The JA- and SA-pathways are also involved in induced emission of plant volatiles (Mumm and Dicke, 2010; Turlings and Erb, 2018). In particular chewer insects and necrotrophic pathogens activate JA-signaling pathway while piercing-sucking insects and biotrophic pathogens activate the SA-signaling pathway (Howe and Jander, 2008; Pieterse et al., 2012). Insect egg deposition has been shown to activate mostly SA- but also JA-induced defenses (Hilker and Fatouros, 2015; Rondoni et al., 2018). Whether disruption of egg-induced plant defenses by the chewer insect *S. lineatus* - and not by fungal pathogen *Stemphylium* sp. - is due to synergistic or antagonistic effects on plant defense signaling pathways should be investigated in future studies.

The emissions of volatile compounds from both treated and untreated plants exhibit qualitative similarities, but they also show quantitative variations. Specifically, differently treated plants emit compounds in distinct proportions, indicating that *T. basalis* likely employs odor recognition based on specific ratios in its host searching strategy (Bruce et al., 2005; McCormick et al., 2012). This strategy has also been shown in other egg parasitoids (*Trichogramma*) when foraging in multi-herbivore scenarios (Cusumano et al., 2015). Previous studies conducted by Colazza et al., (2004b) on the host searching behavior of *T. basalis* indicated that β-caryophyllene is a crucial compound released in the blend of OIPVs by *V. faba* plants and is likely to be employed by *T. basalis* to locate plants that are infested with *N. viridula* eggs. However, based on the present observations and analysis conducted using the PLS-DA model, it was found that β-caryophyllene was detected in the headspace of sampled plants but it did not emerge as a key compound responsible for chemical differences among treatments. Taken together these results suggest that β-caryophyllene is a key compound used by *T. basalis* when *V. faba* plants are only challenged by *N. viridula* feeding and oviposition, but not when plants suffer multiple biotic stresses. Under such circumstances plants alter the emission of volatile profiles, and it is possible to hypothesize that *T. basalis* might rely on other compounds when foraging for hosts to cope with variation of volatile blend compositions. The results from the multivariate statistical analysis suggest that α-farnesene could be an important compound for *T. basalis* to locate *N. viridula* eggs laid on *V. faba* plants under dual biotic stress conditions. The observation that α-farnesene emissions were at intermediate amounts in insect-damaged plants, peaking in *Stemphylium* infected-plants, and were lowest in control plants has implications for the host location of insect parasitoids. In fact, prior research has shown that natural enemy attraction to infochemicals is maximized at moderate concentrations, thanks to a dose–response relationship characterized by a hump-shaped curve (Whitman and Eller, 1992; de Boer and Dicke, 2004). This particular compound has previously been detected in legume plants attacked by the stink bug *Euschistus heros* (Fabricius) and has been found to induce attraction in its egg parasitoid *Telenomus podisi* Ashmead (Michereff et al., 2013). Similarly, α-farnesene has been described as a functional odorant in the oviposition behavior of *Anastatus japonicus* Ashmed, an egg parasitoid of the stink bug *Tessaratoma papillosa* Stål (Wang et al., 2017). Finally, such attracting role of α-farnesene was shown also in other species of egg parasitoid such as *Gonatocerus ashmeadi* Girault (Krugner et al., 2014).

Overall, our study provides novel insights into the complex interactions between plants, herbivores, pathogens, and egg parasitoids. Our results suggest that the indirect defenses induced by egg deposition are not disrupted by phytopathogen infection, but further studies are needed to confirm this pattern as indirect plant defenses under dual attack with herbivores and plant pathogens have been largely overlooked. In particular future research should be carried out to investigate whether compounds such as α-farnesene, which can potentially play a role when egg parasitoids forage in complex scenarios, can enhance parasitism levels in the field. In this perspective dispensers baited with different doses of α-farnesene should be implemented into crops in order to identify optimal delivery rates of infochemicals. To conclude, this study highlights the importance of incorporating multiple biotic stresses when studying parasitoid foraging behavior, in order to comprehend how to enhance the effectiveness of egg parasitoids in biological control programs by manipulating OIPVs.

CRediT authorship contribution statement

Mahmut Mete Karaca: Data curation, Formal analysis, Methodology, Visualization. **Tuğcan Alınç:** Data curation, Formal analysis, Methodology, Visualization. **Antonino Cusumano:** Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft. **Giulia Mirabile:** Methodology, Data curation. **Livio Torta:** Methodology, Data curation. **Salvatore Guarino:** Methodology, Data curation, Visualization, Writing – original draft. **Ezio Peri:** Writing – original draft, Writing – review & editing. **Stefano Colazza:** Writing – original draft, Writing – review & editing.

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.

Acknowledgements

We thank three anonymous reviewers for their useful suggestions that improved the quality of the manuscript. This research was partially funded by the PRIN project 2017 – Prot. 2017JLN833 – Plant multitROphic interactions for bioinspired Strategies of PEst ConTrol (PROSPECT). MMK acknowledges support from the European Union Erasmus + programme.

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