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Phytophagy of *Nesidiocoris tenuis* triggers the response of *Trichogramma achaeae* to tomato plants infested by *Tuta absoluta*

Pasquale Cascone[1] · Fatemeh Tabebordbar[2] · Gabriele Cencetti[3] · Marco Michelozzi[3] · Parviz Shishehbor[2] · Emilio Guerrieri[1] · Massimo Giorgini[1]

Abstract
The generalist predator *Nesidiocoris tenuis*, an effective biological control agent of numerous pests of the tomato crops, can also trigger plant defence mechanisms (direct and indirect) due to its phytophagous behaviour. In southern Europe, *Nesidiocoris tenuis* is frequently released in tomato greenhouses to control the invasive pest *Tuta absoluta*, sometimes combined with another biocontrol agent, the egg parasitoid *Trichogramma achaeae*. In this study, using olfactometer bioassays, we showed that the modification of the volatile chemical profile induced by the plant feeding activity of the mirid made tomato plants more attractive to *T. achaeae*, both in the absence and in the presence of *T. absoluta* eggs or larvae. This result was discussed in relation to difference observed among the chemical profiles of the volatile organic compounds released by tomato plants punctured by *N. tenuis*, without or in combination with *T. absoluta* infestation. We found that a few compounds (*e.g. β*-pinene and myrcene) could play a role in the foraging behaviour of *T. achaeae* in this scenario. Overall, our data support the emerging idea of an ecological role of *N. tenuis*, in addition to its predatory activity, in making tomato crops more resilient against the attack of pests, including *T. absoluta*.

Keywords Biological control · Volatile Organic Compounds · Parasitoid behaviour · Olfactometer

Introduction

In recent years, predatory mirid bugs (Hemiptera: Miridae) have been increasingly used in tomato crops as biocontrol agents against a wide range of pests (*i.e.* aphids, whiteflies and lepidopterans) (van Lenteren 2012; Madeira et al. 2019; Pérez-Hedo et al. 2021; Ingegno et al. 2021). One of the

✉ Emilio Guerrieri
emilio.guerrieri@ipsp.cnr.it

[1] Institute for Sustainable Plant Protection (IPSP), National Research Council of Italy, Piazzale Enrico Fermi 1, 80055 Portici, Na, Italy

[2] Department of Plant Protection, Shahid Chamran University of Ahvaz, Ahvaz, Iran

[3] Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy, Via Madonna del Piano, 10 -Polo Scientifico CNR, 50019 Sesto Fiorentino, FI, Italy

most successful applications of these biocontrol agents is that of *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) released against the invasive South American tomato pinworm *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) (Chang and Metz 2021) in southern Europe (Urbaneja et al. 2012; Pérez-Hedo et al. 2017). In invaded regions, *T. absoluta* represents one of the most destructive pests of tomato and frequently requires repeated applications of synthetic insecticides to prevent economic damage (Giorgini et al. 2019; Desneux et al. 2022). Larvae of *T. absoluta* dig mines in tomato leaves and stems and burrow in fruits, causing injuries that lead to production losses of up to 80–100% (López 1991; Picanço et al. 1998). To date, more than 100 countries outside the native area of South America are facing this pest (EPPO 2022). Several biological and ecological aspects of this species have contributed to its spread and high impact on tomato crops (Sylla et al. 2017; Campos et al. 2021; de Campos et al. 2021; Ponti et al. 2021). These aspects include the high reproductive potential with multiple overlapping generations per year and high resistance to most used insecticides (Mansour et al. 2018; Cherif et al. 2019; Guedes et al. 2019). Therefore, several research

projects focused on using biological control agents and biotechniques to reduce the synthetic inputs needed to preserve tomato production (Giorgini et al. 2019; Desneux et al. 2022). The use of *N. tenuis* has become a standard approach to control *T. absoluta* in wide areas of protected crops in southern Europe (Pérez-Hedo et al. 2017). However, other than feeding on arthropods, *N. tenuis* nymphs and adults can behave as herbivores by feeding on different plant parts (stem, sprouts, fruits and flower peduncles), causing reduced vegetative growth, necrotic rings and subsequent abortion of flowers and small fruits (Castañé et al. 2011; Calvo et al. 2012; Chinchilla-Ramírez et al. 2021; Pérez-Hedo et al. 2021). Consequently, when the mirid population is high and there is a shortage of arthropods to prey on, *N. tenuis* can become a pest (Arnó et al. 2010). The predator release must be properly managed, and sometimes low-impact insecticide sprayings are needed to regulate its populations and avoid economic losses (Pérez-Hedo and Urbaneja 2016; Pérez-Hedo et al. 2021). Currently, the omnivore *N. tenuis*, marketed in Europe, North Africa and Asia (Desneux et al. 2022), is mostly released in areas with warmer climates (*i.e.* Spain and South Italy) rather than in temperate zones (Pérez-Hedo and Urbaneja 2016). In southern Europe, the potential risk of tomato damage by *N. tenuis* is generally offset by its ability to control multiple key pests (*e.g.* whiteflies and *T. absoluta*) and to help reduce the populations of other pests (*e.g.* aphids, moths and thrips). Moreover, over the past decade, the mirids' phytophagous behaviour has become an attractive added value for sustainable pest management programmes (Urbaneja et al. 2022). The feeding activity of *N. tenuis* on tomato plants induces direct and indirect defence mechanisms similar to the response exerted by these plants to harmful herbivores (Pérez-Hedo et al. 2022). In tomato plants, the injection of saliva by both nymphs and adults of *N. tenuis* (Naselli et al. 2016) triggers a cascade of events regulated by the activation of the jasmonic, abscisic and salicylic acid metabolic pathways (Pérez-Hedo et al. 2018a). Therefore, secondary metabolites, including VOCs and proteins, are specifically produced in the plant punctured by *N. tenuis*. These compounds have a role in direct plant defence being toxic, repellent and/or acting as antifeedants against herbivores, as well as in indirect defence through the attraction of natural enemies of herbivores (Pérez-Hedo et al. 2018b). For example the profile of VOCs emitted by tomato plants injured by *N. tenuis* includes three green leaf volatiles (GLVs) that are attractive to *Encarsia formosa* Gahan, a parasitoid of whitefly pests (Pérez-Hedo et al. 2018b). This evidence motivated us to investigate whether *N. tenuis*, by modifying the tomato plant VOC emissions, could have an impact on a biocontrol agent of *T. absoluta*, namely *Trichogramma achaeae* Nagaraja and Nagarkatti (Hymenoptera: Trichogrammatidae), an egg parasitoid of Lepidoptera (Polaszek et al. 2012). Inundative releases of

T. achaeae have been considered an effective strategy to support *T. absoluta* management in tomato greenhouses of Mediterranean Countries (Giorgini et al. 2019; Desneux et al. 2022), either when the parasitoid is released alone (El-Arnaouty et al. 2014; Kortam et al. 2017) or in combination with predatory mirids (Chailleux et al. 2013; Cabello et al. 2015).

Our work starts from two recent findings: (i) feeding by *N. tenuis* adults and nymphs triggers volatile organic compounds (VOCs) by the injured plant that influences the behaviour of phytophagous pests and their natural enemies (Pérez-Hedo and Urbaneja 2016; Bouagga et al. 2018b; Pérez-Hedo et al. 2018b, 2021; Esmaeily et al. 2021); (ii) the parasitoid *T. achaeae* exploits VOCs emitted by tomato plants to locate its host (Gontijo et al. 2019). In this study, we try to answer the following questions: (i) does the phytophagy of *N. tenuis* influence the foraging behaviour of *T. achaeae*? (ii) does the phytophagy of *N. tenuis* alter the VOC blend released by un-infested or *T. absoluta*-infested tomato plants? and (iii) is the foraging behaviour of *T. achaeae* influenced by the contemporary presence of *N. tenuis* and *T. absoluta* on tomato plants?

Materials and methods

Plants and insects

The tomato plants (cultivar "San Marzano nano") were grown in a glasshouse at 24 ± 2 °C, with relative humidity (RH) of $65 \pm 5\%$ and a photoperiod of 16L:8D h. *Tuta absoluta* was reared on caged tomato plants in the same conditions starting from adult moths collected during the summer of 2019 in tomato greenhouses in Battipaglia (Salerno, Italy). Plants and *T. absoluta* colonies were maintained at the Istituto per la Protezione Sostenibile delle Piante, Portici, Italy.

The parasitoid *T. achaeae* was purchased biweekly from a commercial supplier (Agrobio, Almeria, Spain) as pupae developed in the eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). Upon arrival, they were placed under the same climatic conditions described above. The identity of the *Trichogramma* species was confirmed by COI and ITS2 gene amplification and sequencing performed on ten individuals randomly selected, as described by Cascone et al. (2015). Female parasitoids used in olfactometer tests were 24-h old, mated, fed (1:1 water/honey solution) and naïve, *i.e.* with no previous oviposition experience or contact with plants or host instars. Adult parasitoids were placed individually in micro-glass vials and sexed under a stereoscope 1–2 h prior to olfactometer tests.

Adults of the predator *N. tenuis* were purchased biweekly from a commercial supplier (Agrimpol, Montecorvino

Pugliano, Italy). They were reared on tomato plants in $35 \times 35 \times 60$-cm mesh cages, in a climate-controlled glasshouse at 24 ± 2 °C, $65 \pm 5\%$ RH and 16:8 (L:D) h photoperiod. Adult mirids were fed with eggs of *E. kuehniella* (purchased from Bioplanet, Cesena, Italy) *ad libitum* and ~100 aphids (*Macrosiphum euphorbiae* L.) infesting the tomato plants.

Olfactometer bioassay

The tomato plants used in the behavioural assay were 5 weeks old, with 4–6 fully expanded leaves and 18–20-cm tall. The behavioural responses of *T. achaeae* females to plant volatiles were measured and investigated in a Y-tube olfactometer. The latter consisted of a 1-cm diameter Y-shaped glass tube with a 9-cm long base and two 8-cm long arms connected to two glass jars as described in detail by Cascone et al. (2019). Tomato plants to be tested were placed individually inside one of the 20-L glass jars. The jars were closed tightly for 15–20 min prior to the beginning of each choice test to allow the sufficient diffusion of odour plumes and the achievement of a pressure balance inside the system. Each of the two glass jars was independently connected to the two arms of the Y-tube, and the airflow was adjusted to 100 ml/min for each arm. All tests were conducted between 10:00 and 14:00 h in a laboratory evenly illuminated by fluorescent lights (PPFD of 700 µmol m^{-2}s^{-1}) at 24 ± 2 °C. Olfactometer bioassays were performed by testing multiple female wasps simultaneously (Gols et al. 2012; Cusumano et al. 2015). A preliminary bioassay was done to exclude the possibility of a grouping effect. Ten groups of ten female wasps each were tested in an olfactometer two-choice bioassay towards the same odour source (a tomato plant infested by *T. absoluta* eggs) to verify whether the tested females would affect each other's choice when released in a group. The wasps' distribution in the two arms of the Y-tube olfactometer (46 versus 54) did not significantly differ from 50:50 (two-sided binomial test, $P = 0.484$). Thus, all subsequent experiments were performed releasing wasps in groups. Ten wasp females were released into the system simultaneously, and two groups of females, representing a single experimental replicate for each plant pair tested, were observed per tested plant pair (Cascone et al. 2019; Gontijo et al. 2019). The position of the plants was swapped after having tested the first group of ten females to avoid any position bias. The choice between the two odour sources (tested plants) was recorded within 30 min from the parasitoid release, with a choice considered to be made when wasps were found inside the trapping bulbs, located near the ends of the Y-tube arms. Wasps that did not respond (overall average $22.6 \pm 1.2\%$ SE) within the observation period were scored as non-responding and were excluded from the statistical analysis. Female parasitoids were tested

against the following odour sources: clean air (A), healthy tomato plants (H), *N. tenuis* punctured plants (N), *N. tenuis* punctured plants + *T. absoluta* eggs (N + TE) and *N. tenuis* punctured plants + *T. absoluta* larvae (N + TL). The odour sources were tested in nine combinations: (1) N + TE versus N + TL, (2) N versus N + TL, (3) N versus N + TE, (4) H versus N + TL, (5) A versus N + TL, (6) H versus N + TE, (7) A versus N + TE, (8) H versus N and (9) A versus N. Each combination was replicated ten times over 10 different days using different plants. Overall, 200 T. *achaeae* females were tested for each bioassay combination. The total number of *T. achaeae* attracted to one of the two odour sources (attractiveness), and the total number of wasps that made a choice (responsiveness) were subjected to a binomial test to assess if the observed distribution differed significantly from an equal distribution (1:1) between the two odour sources. Attractiveness and responsiveness were used as "number of successes" while wasps making a choice and total wasps tested as "number of trials", respectively. Analyses were made using the function "binom.test" within the R statistics programming environment 3.3.3 (R Core Team 2022). Olfactometer bioassays were run at the Istituto per la Protezione Sostenibile delle Piante, Portici, Italy.
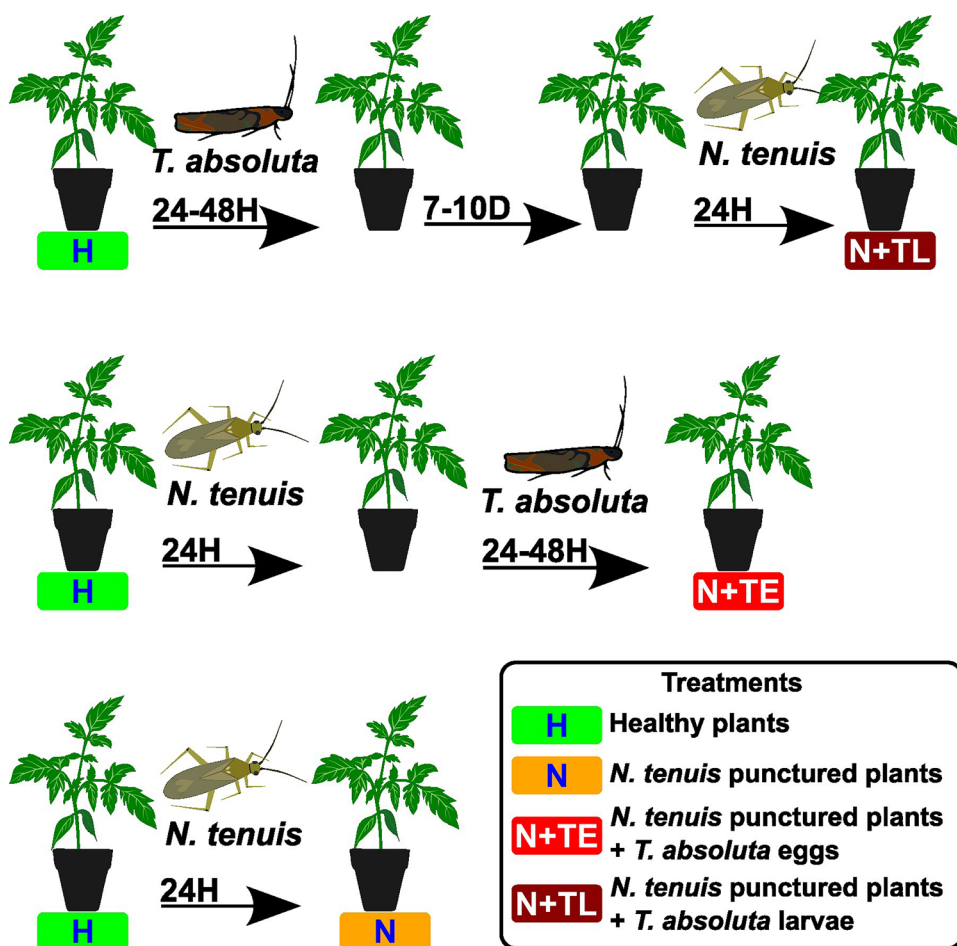
Plant treatments

All experimental treatments are summarized in Fig. 1.

Nesidiocoris tenuis punctured plants (N) were obtained by allowing 100 mirids to feed on four healthy plants (in average 25 mirids per plant) placed in a cage for 24 h, after that the insects were removed, and the plant stored in a new cage prior to test it in olfactometer. For the N + TE treatment, *N. tenuis* punctured plants (as described above) were immediately subjected to *T. absoluta* oviposition for 48–72 h prior to olfactometer tests. This exposure time resulted in N + TE plants with an average of 26.50 ± 3.00 (mean $\pm$ SE) *T. absoluta* eggs. For the N + TL treatment, plants were first exposed to *T. absoluta* ovipositing females for 24–48 h, then kept within cages for 7–10 days to allow the eggs hatching into larvae. Plants bearing an average of 10.30 ± 1.30 (mean $\pm$ SE) *T. absoluta* first instar larvae were produced. Finally, these plants were exposed to *N. tenuis* adults for 24 h as described above for the N treatment.

VOCs collection and analysis

Soon after the olfactometer bioassay, glass jars containing the tomato plants were connected to an airtight entrainment system consisting of a circulating pump (closed-loop) whose flow was adjusted to 200 cm^3 min^{-1} for the collection of VOCs from the headspace. Before re-entering the pump, the air passed through an adsorbent cartridge made of a narrow glass tube filled with a biphasic phase of 30 mg of Tenax

Fig. 1 Representation of the time sequence of plant infestation events for each of the three experimental treatments. *Nesidiocoris tenuis* punctured (N) tomato plants were obtained by exposing them to *N. tenuis* adults for 24 h. *Nesidiocoris tenuis* punctured + *Tuta absoluta* eggs (N + TE) plants were obtained by exposing them to mirids and after to *T. absoluta* adults for 24–48 h. *Nesidiocoris tenuis* punctured + *T. absoluta* larvae (N + TL) plants were exposed first to *T. absoluta* adults for 24–48 h, then reared for 7–10 days to allow the eggs hatching into larvae and eventually exposed to mirids adults for 24 h

and 30 mg of Carboxen. Ten tomato plants for each treatment were sampled individually for 3 h to collect volatiles. Volatile cartridges were analysed by CIS4–TDU–GC/MS. Gerstel TDU heated at 300 °C for 7 min under a helium stripping flow of 30 ml min^{-1}. The TDU unit was directly assembled to the PTV injector (CIS4 Gerstel, Germany) with a liner-in-liner coupling, eliminating the carry-over effect and analyte loss. During this process, the CIS4 was cooled to −20 °C by computer-controlled liquid CO2 pulsed flow. After cryo-trapping on a Tenax packing liner, the PTV was quickly ramped to 260 °C for desorption, and the analyte was transferred to CIS4. Agilent® 7890 GC equipped with an Agilent ® 5975 MSD (Palo Alto, USA) was used for the analysis with helium chosen as carrier gas kept at a constant flow of 1.2 ml min $^{-1}$. The chromatographic settings were as follows: injector in splitless mode set at 260 °C, J&W Innowax column (50 m, 0.20 mm i.d., 0.4 um df); oven temperature programme: initial temperature 40 °C for 1 min, then 10 °C min^{-1} increase until 130 °C, then 5 °C min^{-1} increase until 210 °C, then 20 °C min^{-1} increase until 260 °C, hold time 3 min. The mass spectrometer operated with an electron ionization of 70 eV, in scan mode in the m/z range 29–330, at three scans sec^{-1}. The deconvoluted peaks

spectra obtained by Agilent MassHunter® software were matched against the NIST 11 spectral library for tentative identification. Kovats' retention indices were calculated for further compound confirmation and compared with those reported in the literature for the chromatographic column used (Kovat 1965). Authentic standards were also injected into the system to confirm the tentative identity of the compounds. The volatile emission patterns were standardized by leaf surface measured by Easy Leaf Area software (Easlon and Bloom 2014) on all leaves from each tested plant. A one-way ANOVA analysis was run to assess if the quantity of each VOC emitted by tomato plants differed among the four treatments. To verify if data met the ANOVA assumptions, the Shapiro–Wilk's test and Q–Q plots were used to check for normality and the Levene's test for homogeneity of variances of each individual VOC. When ANOVA assumptions were not satisfied, log transformation achieved normality and homogeneity of variances for the dependent variable. When the ANOVA test was significant, Tukey's HSD *post hoc* test at the confidence level of 0.95 was conducted to test which means are different in pairwise comparisons of plant treatment. When the assumption of equal variances was violated, even after data transformation, comparisons

were made using the non-parametric Kruskal–Wallis test followed by the Fisher's least significant difference test using the R package "Agricolae" (de Mendiburu 2021). Multivariate analysis of variance (MANOVA) and linear discriminant analysis (LDA) were then applied to explore the possibility of differentiating VOC profiles according to mirids phytophagy and *T. absoluta* infestation using the R package "MASS" (Venables and Ripley 2002). Chemical analyses were run at the Portici and Firenze units of the Istituto per la Protezione Sostenibile delle Piante.

Results

Nesidiocoris tenuis feeding triggers *Trichogramma achaeae* host searching

Plants punctured by *N. tenuis* (N, N + TE and N + TL) were more attractive towards *T. achaeae* females than clean air or healthy (H) plants (Fig. 2a).

The combination of *N. tenuis* punctures and *T. absoluta* larval feeding (N + TL) was more attractive to *T. achaeae* than both *N* treatment (tomato plants punctured by *N. tenuis*) and N + TE treatment (tomato plants with *N. tenuis* punctures in combination with *T. absoluta* eggs) (Fig. 2a). Parasitoid females did not make a choice between N and N + TE treatments (Fig. 2a). Overall, the attractiveness was high and statistically significant in all olfactometer bioassays with an average of 76.3% ± 1.3% SE of wasps making a choice (Fig. 2b).

Nesidiocoris tenuis feeding modifies VOC emission by tomato plants

Nesidiocoris tenuis punctures alone (N) and in combination with *T. absoluta* oviposition (N + TE) or larval feeding (N + TL) significantly enhanced the total emissions of VOCs by tomato plants (MANOVA, *Pillai's trace* = 2.320, $F_{39,78} = 3.563$, $P < 0.001$). LDA on plants from the different treatments succeeded in splitting them into appropriate groups, with the first two functions explaining 98% of
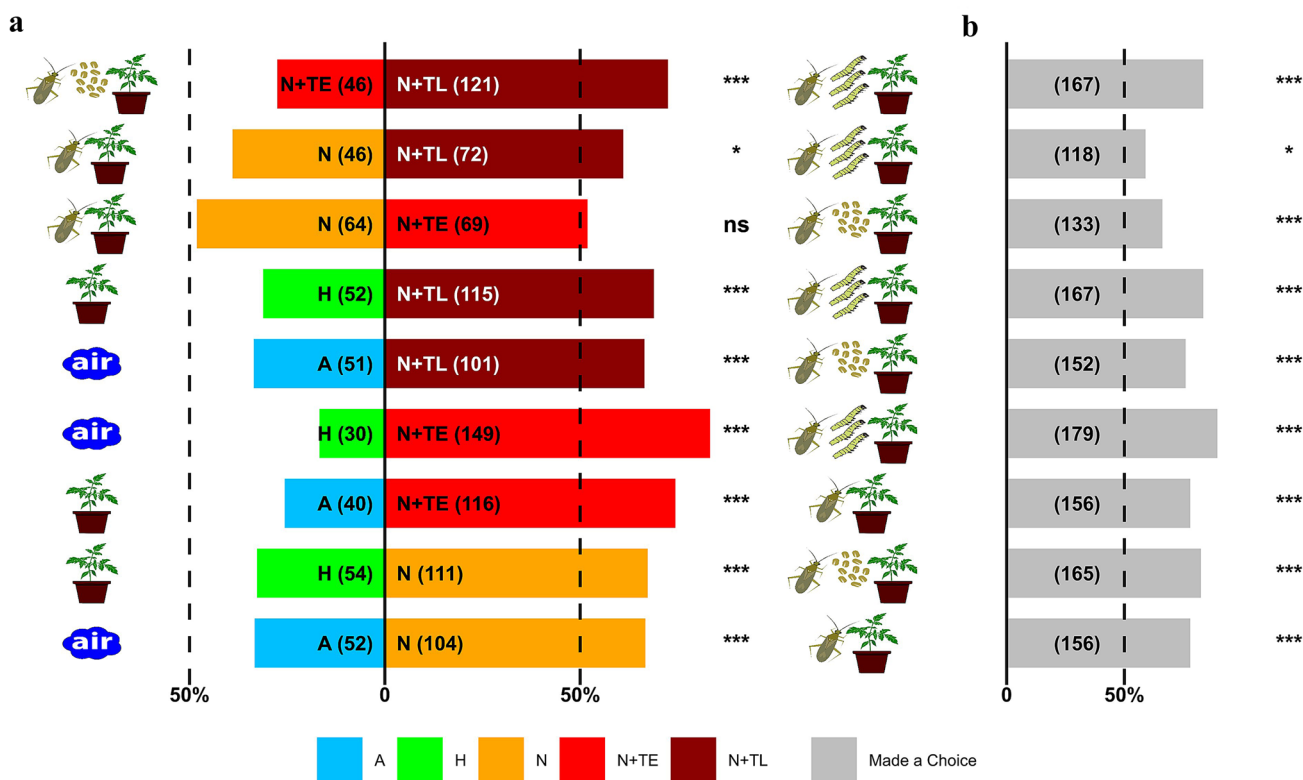

Fig. 2 Results of the two-choice olfactometer bioassays. (a) Preference (percentage) of *Trichogramma achaeae* female wasps in a two-choice setup in a Y-tube olfactometer. Different combinations of clean air (A), healthy (H), *Nesidiocoris tenuis* punctured (N), *N. tenuis* punctured + *Tuta absoluta* eggs (N + TE) and *N. tenuis* punctured + *T. absoluta* larvae (N + TL) tomato plants were tested. A number of female parasitoids that have chosen the two odour sources are reported in parentheses. (b) Percentage and number (in parenthesis) of *T. achaeae* female wasps who made a choice. Asterisks indicate significant statistical differences between the percentages of parasitoids choosing one of the two odour sources or between wasps making or not a choice (binomial test, *P < 0.05, ***P < 0.001). ns means no significant difference

Fig. 3 Linear discriminant analysis (LDA) of VOCs produced by tomato plants. Each point represents the multivariate VOC profile of a single plant in LDA space, belonging to one of the four treatments (H = healthy, N = *Nesidiocoris tenuis* punctured, N + TE = *N. tenuis* punctured + *Tuta absoluta* eggs and N + TL = *N. tenuis* punctured + *T. absoluta* larvae). Black dots represent the centroids. Dotted lines represent 95% confidence interval ellipses for each group
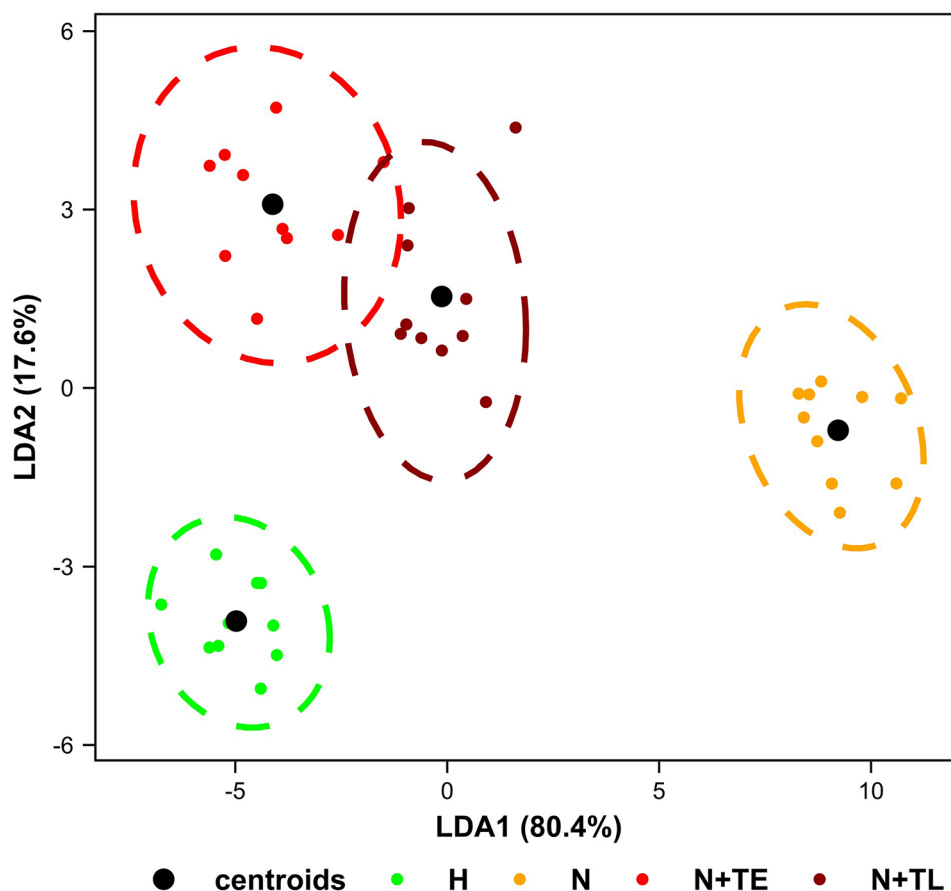
the total variance (Fig. 3). In total, 13 plant-related volatile compounds were identified from the headspace of all treatments (Fig. 4).

All compounds except camphene, β-pinene and sabinene were released at a significant higher rate by plants exposed to the feeding activity of *N. tenuis* (N) in respect to healthy plants (H) (Fig. 4). Similarly, both eggs oviposition (N + TE) and larval feeding by *T. absoluta* (N + TL) triggered a significant increase in the emission of almost all compounds in respect to healthy plants (H). Exceptions are represented by α-terpinene, β-phellandrene and carvacrol, which were not influenced by N + TE treatment in respect to healthy plants (H). The emission of α-terpinene was not influenced by either N + TE or N + TL treatments in respect to control (H) plants. The activity of *T. absoluta* (eggs or larvae) combined to mirids phytophagy (N + TE or N + TL) resulted in the significant lower emission of 2-carene, myrcene, α-phellandrene, α-terpinene, limonene, β-phellandrene, p-cymene and carvacrol in respect to only punctured (N) plants. On the contrary, the release of α-pinene, camphene, β-pinene, sabinene and methyl salicylate was significantly higher in plants infested by *T. absoluta* (N + TE or N + TL) in respect to only punctured plants (N). Methyl salicylate was the most abundant compound found in the VOC blends

of all treatments (Fig. 4). Camphene, β-pinene and sabinene were mostly emitted by tomato plants infested by *T. absoluta* eggs or larvae, while p-cymene and methyl salicylate were mostly emitted by tomato plants exposed to mirids, with or without *T. absoluta* (Fig. 4).

Discussion

The omnivore *N. tenuis*, marketed in Europe, North Africa and Asia (Desneux et al. 2022), is commonly released into greenhouses to control the invasive pest *T. absoluta* infesting tomato crops. In addition to its direct activity as predator of insect pests, seasonal releases of *N. tenuis* are now conceived as a mean to increase the resilience of greenhouse crops (Urbaneja et al. 2022). Indeed, when *N. tenuis* feeds on host plants, it induces the activation of direct and indirect defence mechanisms similar to the response exerted by these plants to harmful herbivores (Pérez-Hedo et al. 2022), thus increasing the ability of the host plants to deal with pest attack. For example sweet pepper and tomato plants eaten upon by *N. tenuis*, can repel *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae)*, Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) and *Tetranychus urticae* Koch (Acari:
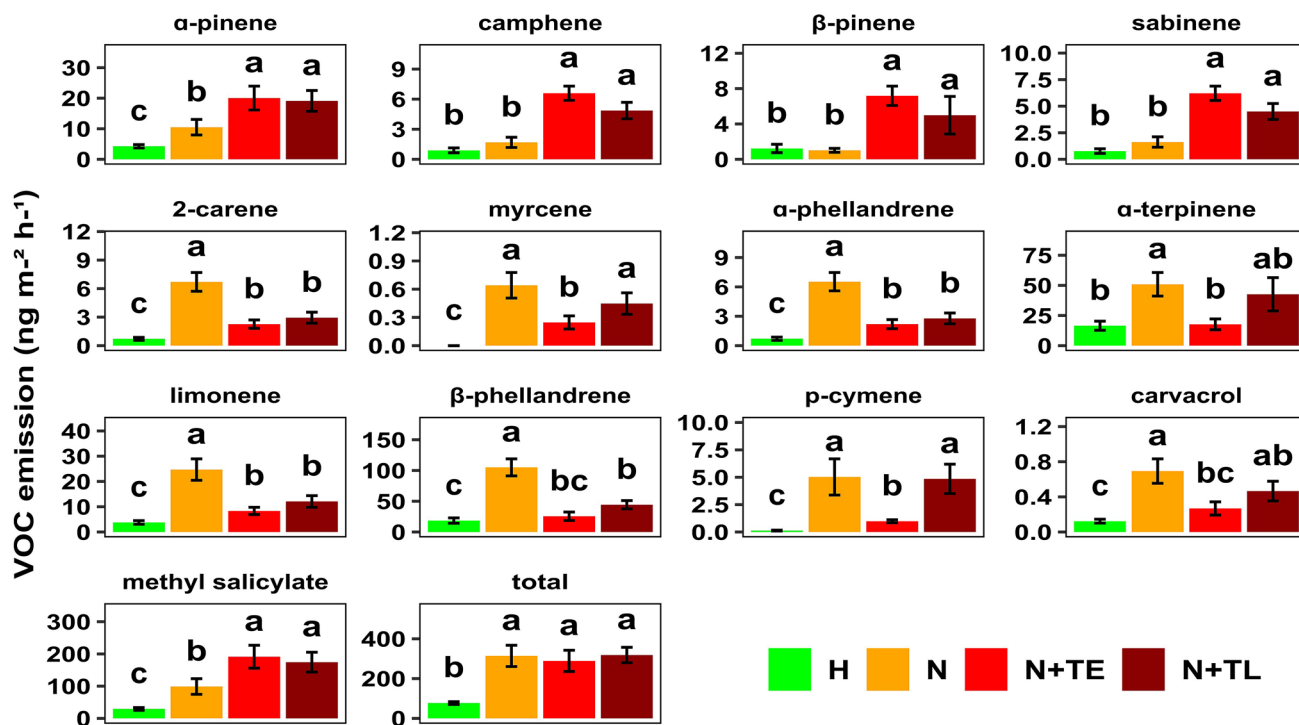
Fig. 4 VOCs emitted by tomato plants. Histograms represent the mean amounts and the bars the standard error of the VOCs emitted by healthy (H), punctured by *N. tenuis* (N), punctured by *N. tenuis* and infested by *T. absoluta* eggs (N + TE) and punctured by *N. tenuis* and infested by *T. absoluta* larvae (N + TL) tomato plants. For each VOC, different letters mean a significant difference in pairwise comparisons (Tukey's HDS *post hoc* test or Kruskal–Wallis test, $P < 0.05$)

Tetranychidae) (Pérez-Hedo et al. 2015, 2018a, b; Bouagga et al. 2018b, a), while being attractive to the whitefly parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) (Pérez-Hedo et al. 2015; Bouagga et al. 2018a). In this paper, we have deepened the knowledge on the role played by *N. tenuis* in the regulation of multitrophic interactions of tomato plants by focusing on the impact of the predator, alone or in combination with the pest *T. absoluta*, on the foraging behaviour of the egg parasitoid *T. achaeae*. The interest about this parasitoid species is motivated by its use as a biological control agent of *T. absoluta* by inundative releases into tomato greenhouses, even in combination with predatory mirids (Chailleux et al. 2013; Cabello et al. 2015; El-Arnaouty et al. 2014; Kortam et al. 2017). It must be considered, however, that the inundative release of *T. achaeae* is in general an expensive strategy (Desneux et al. 2022), not always economically sustainable for the tomato crops. To face this problem, recent research aimed to exploit olfactory cues related to the *T. absoluta*—tomato plant complex to increase the searching behaviour and the parasitization efficiency of *T. achaeae* (Gonthier et al. 2022). However, both Gonthier et al. (2022) and Milonas et al. (2019) did not find any attraction of the parasitoid to tomato plants infested by *T. absoluta* eggs in olfactometer bioassay. Conversely, our group (Gontijo 2019) highlighted the innate capacity

of *T. achaeae* to exploit VOCs emitted by tomato plants, regardless of the presence of *T. absoluta* larvae and eggs, to locate its host. These conflicting results can be explained by the different tomato cultivars used in the three studies and by the fact that in Gonthier et al. (2022), the olfactometer bioassays were conducted with leaves excised by the plant and using a different experimental setup. For example it has been demonstrated that leaf cutting induces the emission of VOCs and activates a wound-eliciting mechanism (Rasulov et al. 2019 and reference therein). Hence, using cut leaves can affect the characterization of VOCs released upon herbivore attack and their subsequent effect on the natural enemies' behaviour. In the present study, performed using the same tomato cultivar tested in our previous works, the plants eaten by *N. tenuis* (N), even when infested by *T. absoluta* eggs (N + TE) or larvae (N + TL), resulted more attractive to *T. achaeae* than healthy plants (H). In addition, the fact that in this study, the egg parasitoid was significantly more attracted to plants injured by *N. tenuis* than to clean air further confirms the results obtained by Gontijo et al. (2019). Here, we showed that *N. tenuis* feeding activity helped the egg parasitoid in locating its host more efficiently. This result was supported by the different VOC profiles produced by tomato plants punctured by *N. tenuis* compared to healthy plants. All compounds, with the exception of carvacrol,

are emitted in greater amounts by the plants infested by *T. absoluta* (N + TE and N + TL) (Fig. 4), which is in agreement with the results of the previous studies on tomato plant infested by *T. absoluta* eggs (Anastasaki et al. 2015; Naselli et al. 2017; Gontijo et al. 2019; Milonas et al. 2019) or larvae (De Backer et al. 2015; Naselli et al. 2017; Silva et al. 2017; Anastasaki et al. 2018; Gontijo et al. 2019; Abdollahipour et al. 2020; Ayelo et al. 2021, 2022; Maneesha et al. 2021; Agbessenou et al. 2022). However, the VOCs emitted by *N. tenuis* punctured plants in this study only partially overlap with those found by Pérez-Hedo et al. (2018b). The latter showed that the VOC profile of tomato plants injured by *N. tenuis* differed from that of healthy plants by significantly greater quantities of methyl salicylate and three green leaf volatiles ((Z)-3-hexenyl butanoate, (Z)-3-hexenyl propanoate and (Z)-3-hexenol). In *N. tenuis* punctured plants, we detected larger amounts only of two of these compounds, namely methyl salicylate (Fig. 4) and trace amounts of 3-hexenol (data not shown). The differences between the two studies can be explained by the major differences in the methods used to collect the volatiles. The comparison between SPME (used by Pérez-Hedo et al. 2018b) and the adsorption on Tenax followed by thermal desorption (here adopted) revealed dramatic variations in the volatile profile obtained by tomato plants (see Rambla et al. 2015). These two techniques differ in their ability to capture VOCs with different vapour pressures (Rambla et al. 2015). SPME fibres are effective for analysing VOCs with low volatility, whereas Tenax cartridges are better suited for VOCs with high volatility. This is in line with the results of our study where compounds such as α-pinene and sabinene, which have high volatility (vapour pressures of 4.75 and 2.6 mmHg at 25 °C (PubChem), respectively), were detected using Tenax cartridges, while compounds such as octyl acetate and hexyl butanoate, which have lower volatility (vapour pressures of 0.4 and 0.2 mmHg at 25 °C (PubChem), respectively), were not detected. The latter compounds were instead recorded by Pérez-Hedo et al. 2018b using SPME fibres. Moreover, even the tomato cultivar used could be responsible for the differences recorded. Here, we tested the cultivar "San Marzano nano", characterized by a determinate growth habit while other authors used the cultivar "Optima", characterized by an indeterminate growth habit. The genetic background, specific for each cultivar, regulates both the quantity and quality of volatiles emitted (Raghava et al. 2010). However, since we detected VOCs emitted by tomato plants in response to *N. tenuis* that were not detected by Pérez-Hedo et al. (2018b), the combined results of the two studies help to better understand the chemical response of tomato plants to *N. tenuis*.

Methyl salicylate was found to be emitted by plants in response to both *T. absoluta* infestation (Silva et al. 2017; Anastasaki et al. 2018; Gontijo et al. 2019; Milonas et al.

2019; Ayelo et al. 2021, 2022; Agbessenou et al. 2022) and *N. tenuis* punctures (Pérez-Hedo et al. 2018b). Here, we recorded an additive effect due to *T. absoluta* and *N. tenuis* with significant increases in N + TE and N + TL treatments compared to the control N treatment (Fig. 4). However, it is reasonable to hypothesize that other VOCs can be induced specifically by *N. tenuis* damage in tomato plants. For example carvacrol has never been identified in the VOC profiles released by tomato plants infested by *T. absoluta* eggs (Anastasaki et al. 2015; Naselli et al. 2017; Gontijo et al. 2019; Milonas et al. 2019) or larvae (De Backer et al. 2015; Naselli et al. 2017; Silva et al. 2017; Anastasaki et al. 2018; Gontijo et al. 2019; Abdollahipour et al. 2020; Ayelo et al. 2021, 2022; Maneesha et al. 2021; Agbessenou et al. 2022). Here, we recorded the presence of carvacrol in all *N. tenuis* treatments (N, N + TE and N + TL). The compound myrcene also seems to be produced by tomato plants in response to *N. tenuis* feeding. Indeed, it was not found to be associated with *T. absoluta* oviposition alone (Anastasaki et al. 2015; Naselli et al. 2017; Gontijo et al. 2019; Milonas et al. 2019), and here, we reported a consistent release in all *N. tenuis* treatments (N, N + TE and N + TL).

All VOCs identified in our study (with the exception of camphene, limonene and carvacrol) have been previously recorded in the headspace of tomato plants infested by *T. absoluta* and tested for their capacity to induce an electroantennographic response of *T. achaeae* (Milonas et al. 2019). The authors showed that parasitoid females responded mostly to minor compounds rather than major compounds emitted by *T. absoluta*-infested plants. Small qualitative differences and specific ratios are usually more important than obvious quantitative differences in volatiles that affect insect behaviour (Bruce et al. 2010). Coherently, terpenes such as β-pinene and myrcene, produced in small quantities by tomato plants (Fig. 4) and found to be electroantennographically active compounds for *T. achaeae* (Milonas et al. 2019), could explain our results. To better explain this concept, we have summarized behavioural bioassay results (Fig. 2) and β-pinene and myrcene emissions by tomato plants (Fig. 4) in a supplementary figure (Fig. S1). When we tested plants fed upon by *N. tenuis* (N) against healthy plants (H), the parasitoid showed a preference for N plants which were characterized by a higher release of myrcene (Fig. S1). Similarly, both N + TE and N + TL plants emitted greater amounts of myrcene and β-pinene in respect to the healthy ones (H) (Fig. S1). The two compounds could be responsible for the attractiveness of these treatments when tested against the healthy plants. The emission of these two compounds could also explain the attractiveness observed in the choice tests N versus N + TL, N + TE versus N + TL and N versus N + TE (Fig. S1). In fact, mirids activity in combination with larvae induced a significantly higher emission of myrcene and β-pinene and resulted more attractive

towards the parasitoid in respect to *N. tenuis* punctured plants (Fig. S1). The link between VOCs and attractiveness was registered also for myrcene in the bioassay N + TE versus N + TL (Fig. S1). On the contrary, the emission of myrcene and *β*-pinene had opposite trends for the bioassay N versus N + TE. In fact, mirids phytophagy and *T. absoluta* eggs infestation (N + TE) emitted lower amount of myrcene than *N. tenuis* punctured (N) plants (Fig. S1), while N + TE plants emitted a greater amount of *β*-pinene than N plants (Fig. 4). These contrasting values in VOC emissions can explain the non-preference of *T. achaeae* in the choice test N versus N + TE and support the idea that myrcene and *β*-pinene could have a pivotal role in the attraction of *T. achaeae* females. Moreover, in our experiment, the VOCs camphene, limonene and carvacrol showed emission patterns and significant differences between the plant treatments (H, N, N + TE and N + TL) similar to myrcene and *β*-pinene. Limonene and carvacrol resulted to be electrophysiologically active for the egg parasitoid *Trichogramma chilonis* Ishii (Sen et al. 2005). Measuring the EAD responses of *T. achaeae* females to camphene, limonene and carvacrol, which were not found and tested by Milonas et al. (2019), may help to better clarify the parasitoid behaviour observed in our bioassay. Finally, it should be pointed out that the differential attractiveness of the four plant treatments towards *T. achaeae* females was also supported by LDA analyses that separated the N, N + TE and N + TL tomato plants from the healthy plants (H) on the basis of their VOCs profiles.

The inundative release of the egg parasitoid *T. achaeae* in combination with the inoculative release of the predator *N. tenuis* has been suggested as a reliable strategy to increase the effectiveness of biological control of *T. absoluta* in southern Europe (Cabello et al. 2012, 2015). We found that the feeding activity of *N. tenuis* on tomato plants does not represent a factor hampering the attraction of *T. achaeae* to tomato plants. In fact, the egg parasitoid was preferentially attracted towards tomato plants injured by *N. tenuis*, either in the absence or in the presence of *T. absoluta* eggs or larvae, compared to healthy plants. Considering that *N. tenuis* is usually released in tomato greenhouses at the beginning of the cultivation cycle, even before *T. absoluta* arrives on the crop (Desneux et al. 2022), our results suggest that *T. achaeae* could be released both at the beginning of the infestation of *T. absoluta*, when the moth eggs are prevalent, and at medium–high levels of infestation during the crop cycle to improve the control activity of the predator alone (Cabello et al. 2015). The delayed release of *T. achaeae* should also avoid any negative effect, observed for a different *Trichogramma* species and associated with a shortage of moth eggs, consisting in the reduction of the efficacy of *N. tenuis* and in the higher number of plants injured by predator phytophagy (Mirhosseini et al. 2019). Even if more studies are needed to measure the effect of intraguild predation by

N. tenuis and *T. absoluta* eggs parasitized by *T. achaeae*, we have highlighted that the mirid enhances the parasitoids attractiveness. This suggests a complementary action of both species where the IPM strategy relies on conservation of both *N. tenuis* and *T. achaeae* populations (Desneux et al. 2022). In conclusion, our results further confirm: The possibility of integrating the inundative release of *T. achaeae* in a sustainable IPM strategy aimed at the maximum possible reduction of synthetic insecticides; the ecological role of *N. tenuis* in increasing the resilience of tomato plants to biotic stressors (Pérez-Hedo et al. 2022).

Together with the results of Pérez-Hedo et al. (2018a, b), our findings suggest that indirect defence mechanisms activated by *N. tenuis* can be exploited by multiple natural enemies of different pests benefiting IPM strategies that rely on the augmentation or conservation of the natural enemies.

Author contributions

MG, EG, PC and PS designed the experiments; FT and PC performed bioassays; PC and MG supervised the experimental work; GC and MM performed VOC analysis and PC performed the statistical analysis. PC wrote the manuscript; EG and MG revised the manuscript. All the authors contributed to the manuscript writing.

Declarations

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