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Plant defense responses triggered by phytoseiid predatory mites (Mesostigmata: Phytoseiidae) are species-specific, depend on plant genotype and may not be related to direct plant feeding

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Abstract Zoophytophagous arthropods can elicit plant defense responses affecting potential prey beyond predation. Phytophagy prevails as the main trigger for these responses, as in the case of *Euseius stipulatus* (Athias-Henriot) (Mesostigmata: Phytoseiidae), a predator occurring in citrus. Because other triggers cannot be excluded, our aim was to examine whether other phytoseiids co-occurring with *E. stipulatus* but not engaged in plant feeding [*Neoseiulus californicus* (McGregor) and *Phytoseiulus persimilis* Athias-Henriot] could induce similar responses (in terms of herbivore induced plant volatiles, HIPVs, and main defensive pathways), and how these affected the behavior of conspecifics and the shared prey, *Tetranychus urticae* Koch (Prostigmata: Tetranychidae). *N. californicus* triggered plant genotype-specific defense responses, including the production of different HIPVs compared to clean plants. However, we could not observe these effects for *P. persimilis*. *T. urticae* avoided better protected plants, because of stronger direct or indirect defense. As plants with weaker direct defense levels should offer higher prey densities, and those harboring conspecific predators represent higher risk of cannibalism, predators were expected to behave similarly. However, they did not. Our results demonstrate that plant defense triggered by phytoseiids is species-specific, depend on plant genotype and can be triggered by non-feeding activities. As *N. californicus* is a highly efficient predator used worldwide, further studies with this species are needed. Likewise, cineol, one of the volatiles identified in the blends triggered by this phytoseiid, could be used to manipulate the prey. These studies could pave the way for a more efficient use of phytoseiids in agroecosystems.

Keywords HIPV · Induced plant defense · *Neoseiulus californicus* · *Phytoseiulus persimilis* · *Tetranychus urticae* · Zoophytophagy

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Introduction

Zoophytophagous insects can trigger plant defense responses which may affect their prey beyond predation (De Puysseleyr et al. 2011; Messelink et al. 2015; Pappas et al. 2015; Perdikis et al. 2011). Phytophagy is considered the most common trigger for these responses. However, other triggers including oviposition, excretion and walking have been described (Hilker and Fatouros 2015; Hilker and Meiners 2010; Karban 2019; Schuman and Baldwin 2016; Wu and Baldwin 2010). Cruz-Miralles et al. (2019) demonstrated that similar to zoophytophagous insects, a phytoseiid mite can induce this type of responses. The omnivorous predator *Euseius stipulatus* (Athias-Henriot) (Acari: Mesostigmata), can elicit genotype-dependent defense responses in *Citrus* spp. The jasmonic acid (JA), the salicylic acid (SA), and the flavonoids defense pathways were upregulated in sour orange (SO), *Citrus aurantium* L., while the JA- and the flavonoids-dependent signaling were upregulated and downregulated, respectively, in Cleopatra mandarin (CM), *C. reshni* hort. ex Tan., when infested by this phytoseiid (Cruz-Miralles et al. 2019). These two *Citrus* species had been chosen because of their extreme resistance and susceptibility to the herbivorous mite *Tetranychus urticae* Koch (Acari: Prostigmata), respectively (Agut et al. 2014; Bruessow et al. 2010), a potential prey for *E. stipulatus* (Ferragut et al. 1988; Pérez-Sayas et al. 2015). Different volatile blends (herbivore induced plant volatiles, HIPVs) were also induced in these *Citrus* species when exposed to *E. stipulatus*. These blends were exploited by this phytoseiid to select less defended plants, where higher prey densities could be expected, and did not inhibit *T. urticae* from choosing *E. stipulatus*-infested plants. Remarkably, in the same study the odors of *E. stipulatus* alone proved repellent to *T. urticae*.

Phytophagy prevails as the most likely cause for the observed plant responses to *E. stipulatus* as this is a zoophytophagous mite (Cruz-Miralles et al. 2021). However, as mentioned earlier other potential triggers cannot be excluded. *E. stipulatus* co-occurs in Spanish citrus orchards with other phytoseiids preying on *T. urticae* as well (Aguilar-Fenollosa et al. 2011; Pérez-Sayas et al. 2015; Vela et al. 2017); among them, *Neoseiulus californicus* (McGregor), which can also feed on both prey and plant-derived food (i.e., pollen) (McMurtry and Croft 1997; McMurtry et al. 2013), and the *Tetranychus* sp.-specialist *Phytoseiulus persimilis* Athias-Henriot. None of these species, though, can directly feed on SO and CM plants (Cruz-Miralles et al. 2021). These differences offer the opportunity to check whether the plant responses to *E. stipulatus* are widespread among phytoseiids associated with *Citrus* spp., and, therefore, could be triggered not only by herbivory. Moreover, this system also allows checking whether *N. californicus* and *P. persimilis* may select the two aforementioned *Citrus* species in a similar way to *E. stipulatus*. Although, as pointed out earlier, predators would benefit from choosing less defended plants as indicative of higher prey densities, this was not always the case when testing herbivore-free plants. When the three phytoseiids were offered uninfested plants, they preferred better protected SO to CM plants, and this was attributed to predators interpreting higher basal defense (JA and SA) in SO as a sign of infestation (Cabedo-López et al. 2019). Interestingly, CM was less attractive to *T. urticae* following HIPVs-induced resistance (Agut et al. 2015), whereas the phytoseiid *P. persimilis* did not exhibit any preference for induced plants (Cabedo-López et al. 2019) and the other two phytoseiids preferred again better protected plants (i.e., induced rather than clean CM plants). These results highlight the complex interplay between plant and herbivore-derived scents on phytoseiid olfactory choices.

Our initial hypotheses are that (1) neither *N. californicus* nor *P. persimilis* will trigger defense responses in citrus as they do not directly feed on plants and (2) to avoid predation/cannibalism risk, both prey and predators will prefer clean versus phytoseiid-infested plants. Should our first hypothesis prove correct, phytophagy would stand as the most likely cause for the observed responses in *E. stipulatus*. To challenge these hypotheses, we have characterized the behavior of *T. urticae*, *N. californicus*, and *P. persimilis* in different Y-tube olfactory tests. We have further characterized the volatile blends produced by these plants when exposed to phytoseiids, as well as the genetic changes in their main defensive pathways.

Materials and methods

Plant material

Three-month-old pesticide-free SO and CM plants (about ten true leaves present) were used in our assays. Plants were grown from seed on vermiculite and peat in 320 ml pots in a climatic chamber at 22 ± 5 °C, 60 ± 10% RH and L:D 16:8 photoperiod (same environmental conditions as for mite rearing and experiments below). Pesticide-free lemons and bean plants (*Phaseolus vulgaris* L. cv. Buenos Aires roja) were used to maintain *T. urticae* and phytoseiid colonies, respectively. *Typha* sp. pollen was used to feed phytoseiids.

Spider mite stock colony

This colony was initiated with specimens collected in clementine orchards close to our campus in 2001. To avoid maternal effects that could render the offspring phenotype better suited to its future host (Freinschlag and Schausberger 2016), spider mites used in the olfactory test were reared on lemons following Cruz-Miralles et al. (2019). In short, between eight and ten lemons were set on top of a wooden structure placed in an open plastic box (40 × 30 × 8 cm) half-filled with water. The wooden structure maintained the lemons above the water, which prevented mites escaping from the rearing. Lemons were replaced weekly in groups of four.

Phytoseiid stock colonies

Phytoseiulus persimilis was originally collected in 2012 in a citrus orchard close to our campus. Since then, colonies of this species have been maintained on rearing units using standard protocols (Pina et al. 2012). Basically, they consist of bean leaflets placed on a water-saturated sponge in a plastic tray with water. A mix of different stages of *T. urticae* was provided twice a week as food. *N. californicus* was regularly obtained from Koppert Biological Systems (SPICAL®) and a small colony was established on bean leaflets following the same procedure as for *P. persimilis*. For this phytoseiid, *Typha* sp. pollen was also provided twice a week.

Y-tube olfactory choice assays

Different two-choice experiments involving *T. urticae*, *N. californicus* and *P. persimilis*, which were exposed to the body odors of the two phytoseiids and those of SO and CM plants in different combinations (see Figs. 1, 2) were performed using a Y-tube olfactometer (Bruin et al. 1992) as in previous work (Agut et al. 2014; Cruz-Miralles et al. 2019; Cabedo-López et al. 2019). Two of the Y-tube arms were directly connected via a plastic pipeline to the outlets of two identical 5 l glass vessels (Duran, Mainz, Germany) containing different odor sources (i.e., nothing, a mesh bag containing 25 gravid phytoseiid females, or a citrus plant either clean or infested with 25 gravid phytoseiid females). Each vessel was connected to an air pump that produced a unidirectional airflow of 1.5 l h^{-1}. The air was purified with a granular activated charcoal filter (Sigma-Aldrich). To remove any traces of food or carrier from the bodies of the gravid females allowed to make a choice, they were moved from the original substrate (the stock colonies for *T. urticae* and *P. persimilis* and the commercial vials for *N. californicus*) with a soft-bristle paintbrush to an arena consisting of a thin black plastic board (9.5 cm diameter) placed on top of a water-saturated foam cube (3–4 cm thick) in an open plastic box (20 × 15 × 4 cm) half-filled with water to prevent mites escaping from the arena. Then, females were further moved into 50 ml plastic vials (eight females per vial) containing a water-soaked cotton ball as water supply, where they were starved for 24 h. Subsequently, they were individually deposited at the beginning of the base of the Y-wire using a soft-bristle paintbrush. They were allowed to make a choice between the two odors sources. Mites failing to reach either end of the arms within 10 min were scored as 'no choice'. After five females had been tested, the glass vessels were switched and after every ten females had been tested, the odor sources (i.e., the mesh bag or the plant) were replaced and the whole system was rinsed with ethanol (70%), followed by air drying. Four sets of ten responding mites per species and choice combination were considered. To avoid pseudoreplication, each set was run at different dates. Plants and mites were discarded after use. To exclude any bias from the set-up, before the beginning of the assays, ten mites were exposed to clean air in both arms. A random response was expected and

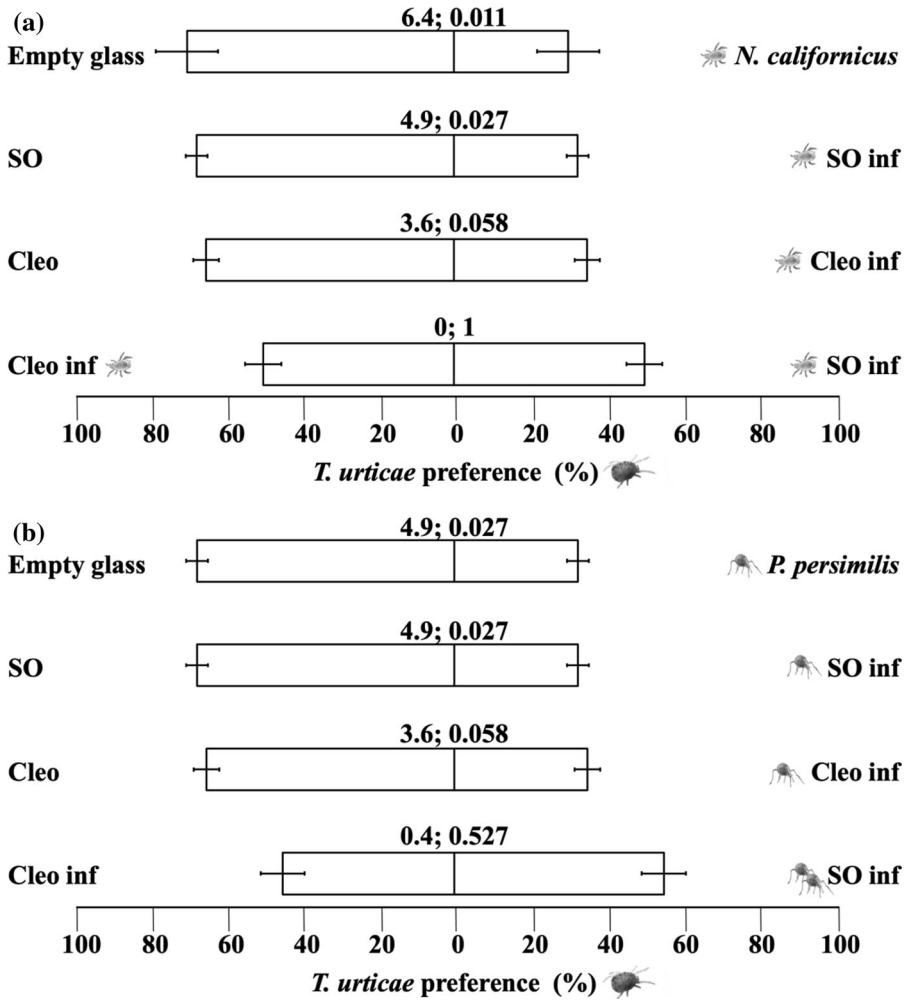


Fig. 1 Olfactory responses (mean ± SE) of *T. urticae* gravid females to **a** *N. californicus* and **b** *P. persimilis*. For each phytoseiid species, *T. urticae* had to choose between two odor sources. Four sets of ten females per choice combination were tested. From top to bottom these combinations were: empty glass versus the phytoseiid, sour orange (SO) versus SO-infested plants (SO inf), Cleopatra mandarin (Cleo) versus Cleo-infested plants (Cleo inf), and Cleo inf versus SO inf. Infested plants had been exposed to 25 phytoseiid gravid females for 48 h before the onset of the assay. Results were pooled and subjected to χ^2 test for a 1:1 distribution (χ^2 and *P*-values for each treatment are shown in the figure; df were always one)

confirmed. To obtain the mesh bags containing 25 females, we followed the same procedure as above. However, females were moved from the black plastic board into the bag (10 × 5 mm), which was closed with a magnet, and immediately used as an odor source. When plants infested by phytoseiids were needed, 25 females collected on the black plastic board were regularly distributed on the leaves of the plant. Plants remained in a climatic chamber for 48 h before use. To prevent ambulatory mite movement between plants, pots were isolated from each other by singly setting them in a tray (14 × 14 × 7 cm), placed inside a larger tray filled with water. Plants grouped by genotype and infestation status, were kept isolated to avoid any exposure to plant volatiles from other treatments (Agut et al. 2015).

To assess the number of phytoseiids that remained on the plants during our assays, we carried out a separate experiment where we infested six plants of each genotype with either *N. californicus* or *P. persimilis* as before. Half of these plants were subjected to a destructive sampling 24 h after infestation, and the remaining half 24 h later. Plants were cut in pieces and individually placed in a beaker with

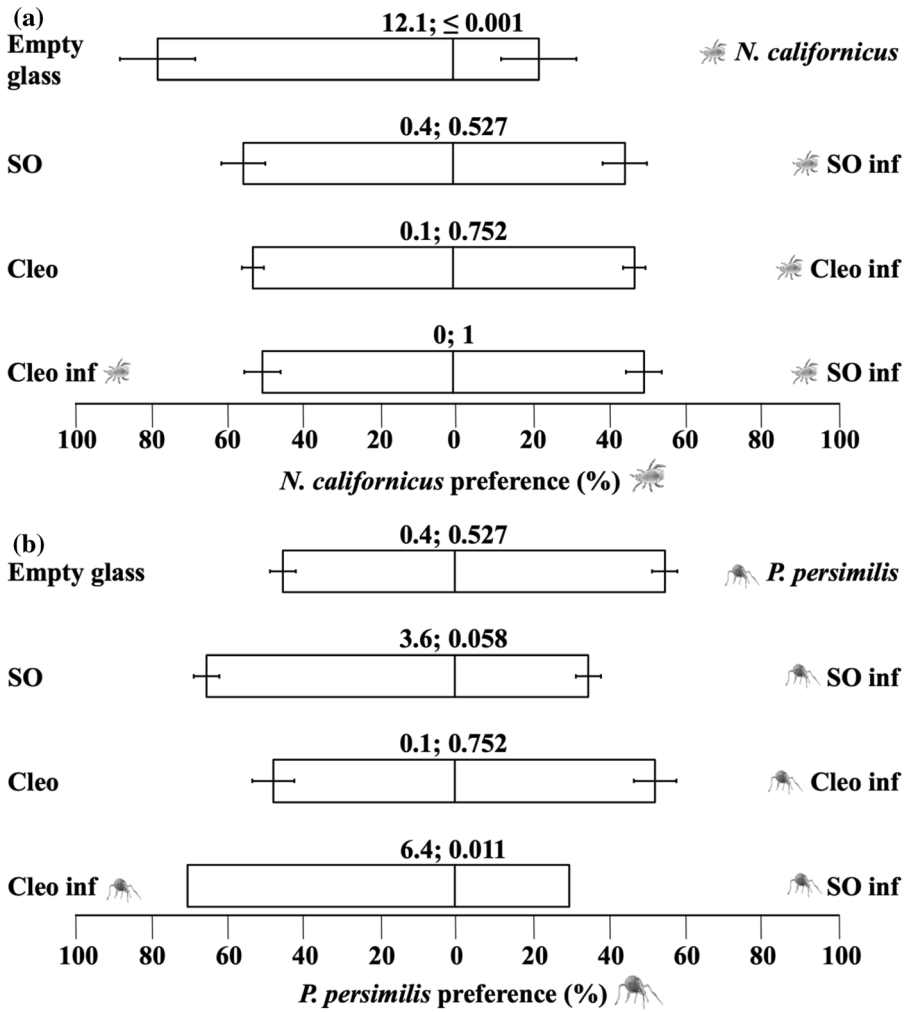


Fig. 2 Olfactory responses (mean ± SE) of **a** *N. californicus* and **b** *P. persimilis* gravid females to conspecific odors. For each phytoseiid species, four different combinations, in which the phytoseiid had to choose between two odor sources, were tested. Four sets of ten females per choice combination were tested. From top to bottom these combinations were: empty glass versus the phytoseiid, sour orange (SO) versus SO-infested plants (SO inf), Cleopatra mandarin (Cleo) versus Cleo-infested plants (Cleo inf), and Cleo inf versus SO inf. Infested plants had been exposed to 25 phytoseiid gravid females for 48 h before the onset of the assay. Results were pooled and subjected to χ^2test for a 1:1 distribution (χ^2 and *P*-values for each treatment are shown in the figure; df were always one)

500 ml of 70% ethanol and stirred for 10 min with a glass stirring rod. Subsequently, the suspension was poured onto a cellulose nitrate filter with a pore size of 0.45 µm (Sartorius Stedim Biotech; Barcelona, Spain) fitted to a filtration unit PSF 500/500 ml (Thermo Fisher Scientific Inc.; Sant Cugat del Vallès, Spain). Phytoseiids (all stages) retained on the filter were counted under a binocular microscope.

Characterization of plant volatiles

Volatiles from SO and CM plants, including clean and phytoseiid-infested plants (same procedure as above), were collected using a headspace collection system (Agut et al. 2015; Bruinsma et al. 2010; Cruz-Miralles et al. 2019). The same 5 l glass vessels and ventilation system used in the Y-tube tests were used. Pasteur pipettes with 300 mg of Porapak (Sigma-Aldrich, Barcelona, Spain) were used as a volatile retention filter. The system was cleaned with acetone and dried

in an oven 1 h prior to the assay. Plants, either infested or not, were individually introduced into the glass vessels. Volatiles were collected in 1 ml of ethyl acetate during the following 24 h. Three plants per genotype and infestation status were considered in three different replicates.

An Agilent 6890N Gas Chromatography (GC) system (Palo-Alto, CA, USA), equipped with an Agilent 7683 autosampler, coupled to a time-of-flight mass spectrometer (TOF–MS), GCT (Waters Corp., Manchester, UK), operating in electron ionization (EI) mode was used. A fused silica DB-5MS capillary column of 30 m length, 0.25 mm internal diameter and a film thickness of 0.25 m (J&W Scientific, Folson, CA, USA) were used for GC separation. The temperature program for this process was the following: 50 °C (1 min), 5 °C min^{-1} to 210 °C (1 min), 20 °C min^{-1} to 300 °C (2 min). This resulted in a total analysis run of 40.50 min. Splitless injections were carried out. Helium was used as carrier gas at 1 ml min^{-1}. The interface and source temperatures were both set to 250 °C and a solvent delay of 3 min was selected. The TOF–MS was operated at one spectrum s^{-1} acquiring the mass range *m/z* 50–650 and using a multi-channel plate voltage of 2800 V. The TOF–MS resolution was ca. 8500 (full width at half-maximum, FWHM) at *m/z* 614. Heptacose, used for the daily mass calibration as well as lock mass, was injected via syringe into the reference reservoir at 30 °C. The *m/z* ion monitored was 218.9856. The application manager ChromaLynx, a module of MassLynx software, was used to investigate the presence of non-target compounds in the samples. Volatile compounds were tentatively identified using GC–MS and matching to the National Institute of Standards and Technology (NIST\EPA\NIH Mass Spectral Library, version 2.0, build 4/2005) using match values of at least 850 as a threshold for identification, as described by Wallis et al. (2008). Furthermore, for each HIPV identified the TOF–MS-derived peak areas were calculated and used to estimate their relative concentration.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT- PCR) analysis in plants infested by phytoseiids

Different replicates including six plants per citrus genotype were considered for each phytoseiid species/rearing (three for the commercial rearing and two for the laboratory colony) combination. Three plants were infested with 25 females, whereas the other three remained phytoseiid-free and were used as control. 48 h later, leaves were cut and immediately introduced into 50 ml Falcon vials, which were immersed in liquid nitrogen and stored at − 80 °C until extraction. Leaves from the same plant were pulled together in the same vial. RNA was extracted using a Plant RNA protocol with TRIzol (Kiefer et al. 2000) and further processed as in previous studies (Agut et al. 2014; Cabedo-López et al. 2019; Cruz-Miralles et al. 2019). For qRT-PCR experiements, 1 µg of total RNA was digested with 0.7 µg of DNase (RNase-free DNase I) in 0.7 µl for DNase buffer and Mili-Q water up to 4.9 µl and incubated for 30 min at 37 °C. After incubation, 0.7 µl of EDTA was added and incubated again at 65 °C for 10 min to inactivate DNase (Thermofisher Scientific Inc.). The RT reaction was performed by adding 7 µl of DNase reaction, 2 µl of PrimeScript buffer and 0.5 µl of PrimeScript RT and Oligo-dT respectively (PrimeScript RT Reagent Kit, Takara Bio Inc.). The reaction mixture was incubated at 37 °C for 15 min. Complementary DNA from the RT reaction, 10× diluted, was used for qPCR. Forward and reverse primers (0.3 µM) were added to 5 µl of Maxima SYBR Green qPCR Master Mix, 1 µl of cDNA and 3 µl Mili-Q sterile water (Maxima SYBR Green/ROX qPCR, Thermofisher Scientific Inc.). qPCR was carried out using a StepOne Instrument (Applied Biosystems) sequence detector with standard PCR conditions (95 °C − 10 min; 40 × (95 °C − 10 s; 55 °C − 10 s; 72 °C − 20 s); 60 °C − 10 s; 95 °C − 15 s). qRT-PCR analysis was replicated three times. The expression of lipoxygenase 2 (*LOX2*; accession Cit.16756.1.S1_sat; forward primer: 5′ → 3′ GAACCATATTGCCACTTTCG; reverse primer 5′ → 3′: CGTCATCAATGACTTGACCA), pathogenesis-related protein 5 (*PR5*; accession BAI63297.1; forward primer: 5′ → 3′ CATCAAGCTTCACAGTGCTTAG; reverse primer 5′ → 3′: CCACAACGTACAGACTGATGAC) and chalcone synthase (*CHS*; accession CF417078; forward primer: 5′ → 3′: AGACGATCCTCCCTGACTCT; reverse primer 5′ → 3′: CTCCACTTGGTCCAGAATTG) genes was determined. Relative expression was compared with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; accession Cit.122.1; forward

primer: 5′ → 3′: GGAAGGTCAAGATCGGAATCAA; reverse primer 5′ → 3′: CGTCCCTCTGCAAGATGACTCT).

Statistical analysis

Results of each olfactometer test were initially subjected to logistic regression with a logit link function to check for the effect of the set of mites used on each date on mite preference. Lack of significance ($P > 0.05$) was a prerequisite to pool the four sets, which were then subjected to χ^2 analysis to test whether they departed from a 1:1 distribution. The TOF–MS-derived peak areas were subjected to ANOVA considering the factors plant genotype, infestation status and their interaction. When necessary, we used Bonferroni post-hoc test for mean separation. The relative expression of JA, SA, and flavonoid signaling pathways homologous marker genes *LOX2*, *PR5* and *CHS*, respectively, were analyzed in phytoseiid-infested and clean plants and compared using Student *t*-test. IBM SPSS Statistics 23 was used.

Results

The presence of either *N. californicus* or *P. persimilis* on citrus plants modifies the behavior of conspecifics and their potential prey *T. urticae*

More than 87% of the mites used in the olfactometer responded to the tested odors (Supplementary Fig. S1 and S2). Maximum rates of response were observed for *T. urticae* (92.8 ± 1.3%; mean ± SE), followed by *P. persimilis* (88.9 ± 2.4%) and *N. californicus* (87.0 ± 2.4%). To check whether our initial hypothesis that a preeminence of phytoseiid odors would result in the three mite species preferring clean versus phytoseiid-exposed plants, we first tested for each choice test the effect of the set of mites used on each date, which was not significant (Supplemetary Table S1). As a consequence, the results of the four replicates per choice test were pooled and subjected to χ^2 tests (Figs. 1, 2). In agreement with our hypothesis, *T. urticae* gravid females were similarly repelled by *N. californicus* (Fig. 1a) and *P. persimilis* (Fig. 1b) regardless of whether they were exposed solely to the body odors of the predators ($P < 0.027$) or to those of phytoseiid-exposed plants. Although infested SO proved repellent ($P = 0.027$) and infested CM triggered a similar but non-significant effect ($P = 0.058$), *T. urticae* showed no preference for any of these citrus genotypes when both of them had been exposed to these phytoseiids ($P \geq 0.527$). Remarkably, we were unable to recover any specimen of *P. persimilis* from the plants exposed to this phytoseiid when the choice-tests took place, 48 h after infestation. However, 10–12 adults per plant and no eggs could be recovered 24 h earlier. Therefore, the observed preferences for *P. persimilis* should be attributed to the traces (e.g., feces) left by this phytoseiid on the plant. In the case of *N. californicus*, 11–15 adults and 0–2 eggs per plant were found when the choice experiments were performed. These figures were higher (18–20 adults and 0–2 eggs per plant) 24 h earlier.

None of the phytoseiids was attracted to conspecific body odors. While *N. californicus* preferred clean air to conspecific body odors (Fig. 2a; $P < 0.001$), *P. persimilis* did not show any preference (Fig. 2b; $P = 0.527$). No preeminence of conspecific body odors, though, was observed for *N. californicus*, which did not show any preference when exposed to the three combinations including conspecific-infested plants (Fig. 2a; $P > 0.527$). On the contrary, when *P. persimilis* had to choose between clean and conspecific-exposed plants, choice depended on plant genotype, with a preference for CM over SO plants (Fig. 2b; $P = 0.011$). These contrasting choices highlight the importance of the interaction between plant and mite-associated odors for triggering ambulatory responses in phytoseiids.

N. californicus but not *P. persimilis* triggers the production of volatiles in citrus plants

When the volatile metabolome of phytoseiid-exposed relative to clean plants was characterized, we found no differences for *P. persimilis* whereas *N. californicus* generated different blends depending on the citrus species considered. This result may be related to the escape of *P. persimilis* from infested plants, as reported in the Y-tube assays. Keep in mind though that volatile collection took place during the 24 h after infestation, when 10–12 adult *P. persimilis* were still present on the plant. From the ten compounds differentially produced upon exposure to *N. californicus* (Table 1, Fig. 3), seven were observed in one

Citrus species only. Two of them appeared in CM plants only and did not change with infestation: 2-methyl-3-heptanone and bezaldehyde. Likewise, 6-benzoyloxy-3,4-dimethyl-coumarin and 1-ethyl-3-(1-methylethyl)-benzene were detected in SO plants only and did not change with infestation. Contrarily, 1,4-diethyl-benzene and 1,15-pentadecanedioic acid appeared in SO only and increased with infestation, and 1,2-benzisothiazole decreased with infestation in this genotype only. The remaining three compounds: cineole, 1-phenyl-1-hexanone, and 3,4-dimethylbezamide were higher in SO and increased with infestation.

N. californicus triggers defensive responses in citrus

The different volatile blends observed for *N. californicus* could be related to the activation of different defensive pathways in SO and CM plants upon infestation. Remarkably, the same patterns were observed irrespective of the immediate origin of the tested mites (commercial and laboratory colonies) (Table 2). Both the JA marker *LOX2* and the flavonoids marker *CHS* genes were downregulated in SO with infestation while the SA marker *PR5* did not change (Table 2). Contrarily, the JA marker *LOX2* gene was upregulated in CM with infestation while *PR5* and *CHS* genes remained unchanged (Table 2). None of these genes was induced by *P. persimilis*. This result is coherent with the lack of differences observed in the volatile metabolome of clean relative to *P. persimilis*-exposed citrus plants.

Discussion

Plant defense against herbivores has been mostly attributed to either mechanical feeding damage or herbivory-derived elicitors found in the oral secretions of the herbivore (Hilker and Meiners 2010; Schuman and Baldwin 2016), to both of them, or to other herbivory-related secretions (i.e., aphid honeydew; Schwartzberg and Tumlinson 2014). Although *N. californicus* does not engage in direct plant-feeding (Cruz-Miralles et al. 2021), our results show that this species interacts with plant defense in a plant-genotype specific manner (Table 2). Therefore, triggers different from plant feeding occur in *N. californicus*. As, contrary to *N. californicus*, it was not possible to maintain *P. persimilis* on plants during the whole study period (i.e., 48 h for the genetic analyses), whether this species may be able to elicit this type of responses remains an unsolved question. Because the only way to force this specialist predator to stay on plants would require previous infestation with *T. urticae*, it will be extremely difficult to address this question. Our results, though, prove that plant defense triggered by phytoseiids (1) may be related to activities different from direct plant feeding, (2) is species-specific and (3) depends on plant genotype. These issues are discussed below.

Plant defense triggered by phytoseiids may be related to activities different from plant feeding

As phytoseiids lack a specialized ovipositor, they cannot insert their eggs into the plant tissue. Therefore, touch and touch-associated secretions, like walking and oviposition, are the most likely triggers of the responses observed. Although the nature of the secretions that phytoseiids produce when walking and ovipositing remains largely ignored, in our experiments successful oviposition was observed in plants exposed to *N. californcus*. Therefore, eggs could be the trigger for the responses observed. However, as not all plants infested with *N. californicus* showed eggs (the number of eggs per plant ranged from zero to two), further research is needed to confirm this hypothesis. Because in our assays *P. persimilis* gradually abandoned the plant during the assays, another possible explanation for our results could be related to the conspicuous differences in the morphology and size of the legs of *N. californicus* and *E. stipulatus* compared to *P. persimilis* (Athias-Henriot 1960; Beaulieu and Beard 2018; Croft et al. 1999; Okassa et al. 2010). These differences, together with species-specific chetotaxy, could explain why, contrary to *E. stipulatus* (Cabedo-López et al. 2019) and *N. californicus*, *P. persimilis* did not trigger plant defense. Same as before, though, further research is needed.

Plant defense triggered by phytoseiids is species-specific and depends on plant genotype

Landing, walking and oviposition by an herbivorous arthropod on a host plant is a reliable indicator for an upcoming herbivory (Bandoly and Steppuhn 2016).

Therefore, plants using these activities as either a trigger for induced defense (Hilker and Fatouros 2015; Wu and Baldwin 2010) or a priming signal to boost particular feeding-induced defense traits (Conrath 2011) could be expected. The reactions observed in CM plants to *N. californicus* could, therefore, be related to this genotype mistakenly identifying the predator as a potential threat or as an indication of the presence of herbivores, which pose a risk to plants (Helms et al. 2019). The upregulation of *LOX2* in CM by *N. californicus* was one order of magnitude lower than that elicited by *T. urticae* in SO (Agut et al. 2014) and similar to that triggered by *E. stipulatus* in SO and CM plants (Cabedo-López et al. 2019). *E. stipulatus*-infested plants, though, were attractive to *T. urticae* (Cruz-Miralles et al. 2019). *T. urticae* avoidance of plants exposed to predators has been repeatedly documented (Fernández-Ferrari and Schausberger 2013; Grostal and Dicke 1999, 2000; Hackl and Schausberger 2014; Pallini et al. 1999; Škaloudová et al. 2007). Accordingly, citrus plants either infested by *N. californicus* or previously exposed to *P. persimilis* proved repellent for *T. urticae* (Fig. 1).

Cineole may play a crucial role for in *T. urticae* plant choices

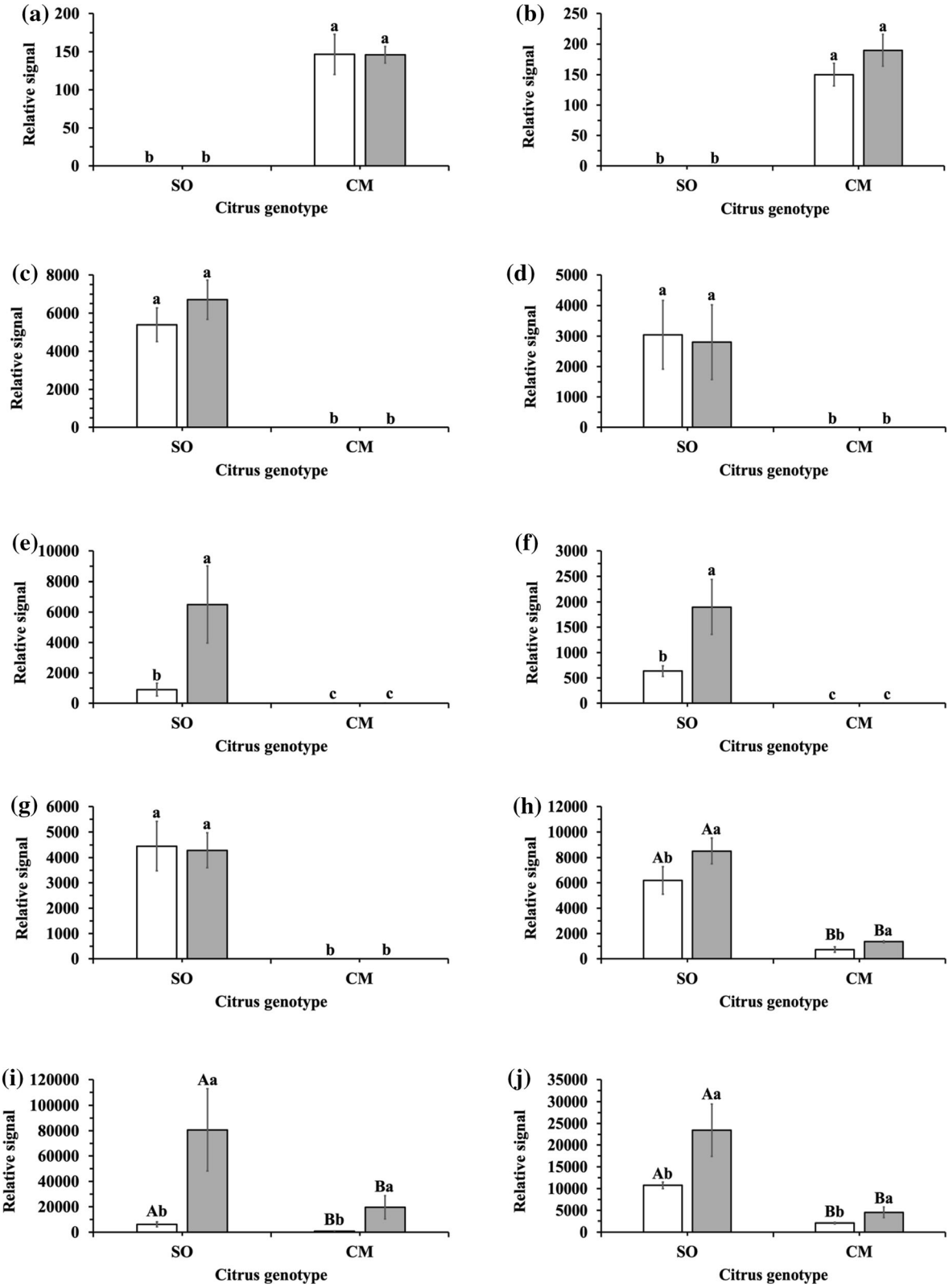
Only one compound out of the six volatiles differentially produced by SO and CM plants when exposed to *N. californicus*, namely cineole (Table 2, Fig. 3h), was also found when examining the response of the same *Citrus* spp. to *E. stipulatus* (Cruz-Miralles et al. 2019). However, contrary to *N. californicus*, infestation by *E. stipulatus* decreased the emission of this compound. Therefore, this terpenoid may play a crucial role in *T. urticae* plant choices and could explain attraction to *E. stipulatus*-infested plants (Cruz-Miralles et al. 2019) but repellence to *N. californicus*-infested plants (Fig. 1a). Consequently, cineole deserves further studies as it could prove useful to manipulate *T. urticae* populations. As two additional volatiles showed the same trend as cineole upon *N. californicus* infestation (1-phenyl-1-hexanone and 3,4-dimethyl-bezamide), their involvement in the observed results cannot be excluded (Gregg et al. 2018). It has to be noted that this type of results may change depending on the context (Fernández Ferrari and Schausberger 2013; Pallini et al. 1999; Zhang and Sanderson 1992).

Phytoseiid-related odors modulate host selection by *T. urticae*

As pointed out earlier, *T. urticae* responded to the different odor sources used in our behavioral assays as expected (i.e., attraction to less defended plants and repellence for phytoseiid body odors). Remarkably, as CM was preferred over SO when both plants were clean (Cabedo-López et al. 2019) but no preference was observed when they were infested by phytoseiids (Fig. 1), these results can be taken as evidence of a preeminence of phytoseiid-related odors for host selection by *T. urticae*. Similar results had been observed in previous studies involving *T. urticae* (Agut et al. 2015) and *E. stipulatus* (Cruz-Miralles et al. 2019). The upregulation of *LOX2* in CM observed upon *N. californicus* infestation (Table 2) may have reinforced the preference for clean plants of this genotype, which were relatively less defended than infested ones (Fig. 2a). However, the opposite did not occur for SO although both *LOX2* and *CHS* were downregulated (and therefore these plants became less defended in terms of direct defense) upon *N. californicus* infestation (Table 2). This result highlights the important effect of the odors related to the presence, either actual or previous, of these two phytoseiids for *T. urticae*. This effect would also explain the behavior of *T. urticae* when exposed to *P. persimilis* (Fig. 1b) and this is not surprising as these volatiles should be reliable indicators of predator presence (i.e., indirect defense) and, therefore, of an imminent predation risk (Fernández Ferrari and Schausberger 2013; Pallini et al. 1999; Zhang and Sanderson 1992).

The interaction between plant and phytoseiid-related odors are key for phytoseiid ambulatory responses

In the case of phytoseiids, some of our initial hypotheses had to be rejected. When *N. californicus* and *P. persimilis* responded to conspecific-infested plants, choices did not follow the rationale of choosing less defended plants to avoid cannibalism. The strong repellence observed in *N. californicus* for conspecific body odors disappeared when this phytoseiid was present in citrus (Fig. 2a), whereas the neutral role played by *P. persimilis* body odors when offered alone affected choice when combined with citrus odors


(a)
Relative signal
200
150
100
50
0
a
a
b
b
SO
CM
Citrus genotype
(b)
Relative signal
250
200
150
100
50
0
a
a
b
b
SO
CM
Citrus genotype
(c)
Relative signal
8000
6000
4000
2000
0
a
a
b
b
SO
CM
Citrus genotype
(d)
Relative signal
5000
4000
3000
2000
1000
0
a
a
b
b
SO
CM
Citrus genotype
(e)
Relative signal
10000
8000
6000
4000
2000
0
a
b
c
c
SO
CM
Citrus genotype
(f)
Relative signal
3000
2500
2000
1500
1000
500
0
a
b
c
c
SO
CM
Citrus genotype
(g)
Relative signal
6000
5000
4000
3000
2000
1000
0
a
a
b
b
SO
CM
Citrus genotype
(h)
Relative signal
12000
10000
8000
6000
4000
2000
0
Aa
Ab
Bb
Ba
SO
CM
Citrus genotype
(i)
Relative signal
120000
100000
80000
60000
40000
20000
0
Aa
Ab
Bb
Ba
SO
CM
Citrus genotype
(j)
Relative signal
35000
30000
25000
20000
15000
10000
5000
0
Aa
Ab
Bb
Ba
SO
CM
Citrus genotype

◀**Fig. 3** Relative signal (TOF–MS-derived peak areas; mean ± SE) of the volatiles differentially produced by infested (grey bars) and clean (white bars) sour orange (SO) and Cleopatra mandarin (CM) plants during the first 24 h of infestation with 25 *N. californicus* gravid females. **a** 2-methyl-3-heptanone; **b** benzaldehyde; **c** 6-benzyloxy-3,4-dihydro-4,4-dimethyl-coumarin; **d** 1-ethyl-3-(1-methylethyl)-benzene; **e** 1,4-diethyl-benzene; **f** 1,15-pentadecanedioic acid; **g** 1,2-benzisothiazole; **h** cineole; **i** 1-phenyl-1-hexanone; **j** 3,4-dimethylbenzamide. For each figure, bars with the same letter(s) are not significantly different (ANOVA, $P > 0.05$). When both plant genotype and infestation status were significant but their interaction was not (Table 1), upper-case letters refer to genotype and lower-case to infestation

(Fig. 2b). Interestingly, Janssen et al. (1997) had observed that *P. persimilis* uses volatiles to avoid prey patches with conspecifics although both conspecifics and prey alone on bean leaves were attractive. A similar situation was observed for *E. stipulatus* (Cruz-Miralles et al. 2019). Therefore, these results point at a highly relevant interaction between plant and phytoseiid own odors for phytoseiid choices. Furthermore, our results show that the interspecific variations in foraging responses of phytoseiids to prey- and

Table 1 Volatile profiling in the headspace of sour orange (SO) and Cleopatra mandarin (Cleo) plants either clean or infested (inf)

Volatile compound	Statistics (F; df; P)		
	Plant genotype (A)	Infestation status (B)	A × B
2-Methyl-3-heptanone	118.54; 1, 32; < 0.001 SO < Cleo	< 0.01; 1, 32; 0.980 Clean = inf	< 0.01; 1, 32; 0.980
Benzaldehyde	127.15; 1, 32; < 0.001 SO < Cleo	1.75; 1, 32; 0.195 Clean = inf	1.75; 1, 32; 0.195
6-Benzyloxy-3,4-dihydro-4,4-dimethyl-coumarin	88.91; 1, 32; < 0.001 SO > Cleo	1.05; 1, 32; 0.314 Clean = inf	1.05; 1, 32; 0.314
1-Ethyl-3-(1-methylethyl)-benzene	13.89; 1, 32; < 0.001 SO > Cleo	0.02; 1, 32; 0.877 Clean = inf	0.02; 1, 32; 0.877
1,4-Diethyl-benzene	9.40; 1, 32; 0.004 SO > Cleo	5.38; 1, 32; 0.027 Clean < inf	5.38; 1, 32; 0.027 SO inf > SO clean > Cleo inf = Cleo clean
1,15-Pentadecanedioic acid	23.79; 1, 32; < 0.001 SO > Cleo	5.92; 1, 32; 0.021 Clean < inf	5.92; 1, 32; 0.021 SO inf > SO clean > Cleo inf = Cleo clean
1,2-Benzisothiazole	60.34; 1, 32; < 0.001 SO > Cleo	0.02; 1, 32; 0.883 Clean = inf	0.02; 1, 32; 0.883
Cineole	80.94; 1, 28; < 0.001 SO > Cleo	4.42; 1, 28; 0.045 Clean < inf	1.48; 1, 28; 0.234
1-Phenyl-1-hexanone	4.39; 1, 32; 0.044 SO > Cleo	8.59; 1, 32; 0.006 Clean < inf	3.07; 1, 32; 0.089
3,4-Dimethylbenzamide	22.16; 1, 32; < 0.001 SO > Cleo	6.65; 1, 32; 0.015 Clean < inf	3.05; 1, 32; 0.090

For each volatile, TOF–MS-derived peak areas were compared using ANOVA considering the factors plant genotype, infestation status, and their interaction. Bonferroni procedure was used for mean separation when needed. Volatiles were tentatively identified by comparing to the National Institute of Standards and Technology (NIST) Library as described by Wallis et al. (2008)

Table 2 Relevance of lypoxygenase 2, *LOX2* (cit16759.1S1), pathogenesis-related protein 5, *PR5* (*BAI63287.1*), and chalcone synthase, *CHS* (CF417078) triggered by *N. californicus* in either sour orange or Cleopatra mandarin

Gene marker	Relative gene expression		Student *t*-test (*t*; df; *P*)
	Clean	Infested	
Sour orange			
Koppert Biological Systems			
LOX2	0.371 ± 0.106	0.133 ± 0.016	2.383; 8; 0.044
PR5	0.263 ± 0.074	0.209 ± 0.026	0.995; 8; 0.349
CHS	0.087 ± 0.019	0.034 ± 0.003	3.384; 8; 0.010
Laboratory			
LOX2	0.082 ± 0.006	0.065 ± 0.012	2.576; 5; 0.005
PR5	0.150 ± 0.022	0.110 ± 0.031	2.373; 5; 0.064
CHS	3.548 ± 0.137	1.799 ± 0.771	2.688; 5; 0.043
Cleopatra mandarin			
Koppert Biological Systems			
LOX2	0.756 ± 0.061	1.376 ± 0.133	3.850; 8; 0.005
PR5	0.136 ± 0.015	0.124 ± 0.047	0.317; 8; 0.759
CHS	0.198 ± 0.013	0.183 ± 0.056	0.268; 8, 0.795
Laboratory			
LOX2	0.015 ± 0.001	0.118 ± 0.037	2.969; 5; 0.031
PR5	0.026 ± 0.005	0.060 ± 0.025	1.270; 5; 0.260
CHS	1.760 ± 0.390	0.573 ± 0.271	2.078; 5; 0.093

Specimens used in these assays were originally obtained from Koppert Biological Systems and either directly used or reared for several generations on clementine leaves in our laboratory before use. Data are presented as a mean ± SE of transcript expression relative to the housekeeping gene *GAPDH* (*Cit.122.1*). Significant differences between infested and clean plants were estimated performing different Student *t*-tests for each gene and mite origin

predator-associated stimuli described by Zhang and Sanderson (1992), are also dependent on plant genotype.

To sum up, our results prove that the outcome of citrus-phytoseiid interactions is species-specific and affected by plant genotype. Whether these differences should be attributed to direct plant feeding (i.e., for *E. stipulatus*) or to other activities (i.e., *N. californicus*) deserves further research. A better understanding of the system could be used to refine current crop protection practices. By exploiting the semiochemicals involved, like cineole or those related to the traces left by *P. persimilis*, which seem to play a crucial role for *T. urticae* in citrus, the overall efficacy of biological control could be enhanced. Likewise, as *N. californicus* is one of the top species of biological control agents commercially produced and used worldwide in augmentative biological control (van Lenteren 2012), further studies aimed at determining whether the plant defense induction observed in citrus occurs in other crop plants and how this may affect prey beyond predation are needed.

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Data availability Raw data not provided in the supplementary online material deposited in UJI Public Digital Repository.

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest to disclose.

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