The olfactive responses of *Tetranychus urticae* natural enemies in citrus depend on plant genotype, prey presence, and their diet specialization

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ABSTRACT

Sour orange, *Citrus aurantium*, displays higher constitutive and earlier inducible direct defenses against the two-spotted spider mite, *Tetranychus urticae*, than Cleopatra mandarin, *Citrus reshni*. Moreover, herbivore induced plant volatiles (HIPVs) produced by sour orange upon infestation can induce resistance in Cleopatra mandarin but not vice-versa. Because the role of these HIPVs in indirect resistance remains ignored, we have carried out a series of behavioral assays with three predatory mites with different levels of specialization on this herbivore, from strict entomophagy to omnivory. We have further characterized the volatile blend associated with *T. urticae*, which interestingly includes the HIPV methyl salycilate, as well as that produced by induced Cleopatra mandarin plants. Although a preference for less defended plants with presumably higher prey densities (i.e., *C. reshni*) was expected, this was not always the case. Because predators' responses changed with diet width, with omnivore predators responding to both HIPVs and prey-related odors and specialized ones mostly to prey, our results reveal that these responses depend on plant genotype, prey presence, and predator diet specialization. As the different volatile blends produced by infested sour orange, induced Cleopatra mandarin and *T. urticae* itself are attractive to *T. urticae* natural enemies but not to the herbivore, they may provide clues to develop new more sustainable tools to manipulate these agriculturally relevant species.

Key words: sour orange; Cleopatra mandarin; *Phytoseiulus persimilis*; *Neoseiulus californicus*; *Euseius stipulatus*; HIPV.

Key message:

- The role of herbivore induced plant volatiles (HIPVs) produced by citrus upon infestation by *T. urticae* in indirect resistance remains ignored.
- A higher attraction of phytoseiids for plants exhibiting relatively lower direct defense was expected.
- Omnivorous predators responded to both HIPVs and prey-related odors whereas specialized ones responded mostly to prey.
- Volatile blends attractive to *T. urticae* natural enemies but not to the herbivore may offer new opportunities to manage this system in a more sustainable way.

INTRODUCTION

Spider mites (Acari: Tetranychidae) comprise more than one thousand plant-feeding species worldwide (Migeon and Dorkeld 2006-2017). One of these species is the two-spotted spider mite, *Tetranychus urticae* Koch, a highly polyphagous and cosmopolitan species (Migeon and Dorkeld 2006-2017). The pest status of this herbivore changed from minor to key pest of many food and ornamental crops after World War II (Hoy 2011; Pérez-Sayas et al. 2015). The disruption of existing top-down regulation mechanisms (i.e., natural enemies) by pesticide abuse during the second half of the XX century is recognized as one of the main causes for that change (Huffaker et al. 1970). More recently, the implication of bottom-up regulation mechanisms by replacement of traditional resistant crops by more susceptible genotypes has been also highlighted (Bruessow et al. 2010; Agut et al. 2014). These studies focused on citrus, one of the many crops where *T. urticae* is considered a pest (Jacas and Urbaneja 2010). Indeed, in the case of clementine mandarins (*Citrus clementina* Hort. ex Tan.), *T. urticae* can achieve the status of key pest (Pascual-Ruiz et al. 2014; Gómez-Martínez et al. 2018).

Commercial citrus plants are regularly propagated vegetatively by bud-grafting onto a seedling rootstock. Sour orange, *Citrus aurantium* L. (Sapindales: Rutaceae), was the most widespread rootstock until the 1950s, when the emergence of the citrus quick decline disease caused by the Citrus Tristeza Virus (CTV, *Closteroviridae*) proved lethal for this rootstock. This triggered its massive replacement around the world (Cambra et al. 2000). Sour orange, though, is highly resistant to *T. urticae*, while one of the alternative CTV-tolerant rootstocks, Cleopatra mandarin, *Citrus reshni* Hort. ex Tan., is highly susceptible to this mite (Bruessow et al. 2010). Agut et al. (2016) provided evidence that resistance in sour orange was systemically transmitted from the roots to the shoots of the grafted cultivar. Both the jasmonic acid (JA) and the salicylic acid (SA) pathways were upregulated in sour orange plants upon mite attack, while these pathways remained unchanged in infested Cleopatra mandarin. However, the SA pathway proved irrelevant for the enhanced direct defense of sour orange (Agut et al. 2014). Further studies (Agut et al. 2015) showed that the release of *T. urticae* HIPVs (herbivore induced plant volatiles) from sour orange [namely, the terpenes α-ocimene, α-farnesene, pinene and D-limonene, and the green leaf volatile (GLV) 4-hydroxy-4-methyl-2-pentanone] had a marked repellent effect on conspecific mites and induced resistance in Cleopatra mandarin plants. Oviposition rates decreased while both the JA and the SA pathways were stimulated in this rootstock. Contrarily, Cleopatra mandarin HIPVs [namely, (2-

butoxyethoxy) ethanol, benzaldehyde, and methyl salicylate, MeSA] had a marked attractant effect on conspecific mites and did not induce any resistant response in uninfested Cleopatra mandarins. However, the potential role of these induced volatiles in indirect defense, i.e., the attraction of the natural enemies of the herbivore (Aljbory and Chen 2018; Cortés et al. 2016), remains unknown. Therefore, this system offers a good opportunity to study the possible effect of plant genotype on the behavior of *T. urticae* natural enemies. Because for a predator, directing its food search toward HIPVs emitted by well-defended plants may reduce its fitness, as its chances of finding abundant and well-nourished prey are lower, we would expect a higher attraction of clean Cleopatra mandarin relative to induced Cleopatra plants and clean sour orange.

The main natural enemies of *T. urticae* are predatory mites of the family Phytoseiidae (Acari: Mesostigmata). *Euseius stipulatus* (Athias-Henriot), *Neoseiulus californicus* (McGregor) and *Phytoseiulus persimilis* (Athias-Henriot) are the most common phytoseiids naturally associated with *T. urticae* in the canopy of Spanish citrus orchards (Abad-Moyano et al. 2009; Aguilar-Fenollosa et al. 2011). These predators have different diet specializations, ranging from selective predators of *Tetranychus* spp., as *P. persimilis*, to extreme diet generalists, omnivores feeding on both animal and plant derived food, as *E. stipulatus*, for which plant cell-sap feeding is suspected (Adar et al. 2012). The Tetranychidae specialist *N. californicus* would occupy an intermediate position feeding on both prey and plant derived food (i.e., pollen) (McMurtry and Croft 1997; McMurtry et al. 2013). However, same as *P. persimilis*, *N. californicus* is not considered a plant cell-sap feeding phytoseiid (Adar et al. 2012). These diet specializations may also have consequences on the behavior of predators and affect their choices. Although, as pointed out earlier, predators would benefit from choosing less defended plants, plant cell-sap-feeding, which would allow this type of omnivorous predators to switch to plant feeding when prey is scarce could result in a stronger attraction for these plants, which could be missing in strict entomophagous predators (i.e., *P. persimilis*).

Here, we present a study of the effects of plant genotype and predator diet specialization on the indirect plant defense responses triggered by *T. urticae* in citrus. To achieve this goal, we have carried out a series of Y-tube olfactory choice assays (Bruin et al. 1992) using the two extreme citrus genotypes partly characterized in terms of their response to *T. urticae* herbivory (defensive pathways and HIPV profiles): sour orange and Cleopatra

mandarin (Agut et al. 2014, 2015, 2016). We have also characterized the volatile blends produced by induced Cleopatra mandarin and *T. urticae*.

MATERIALS AND METHODS

Plant material

Sour orange, Cleopatra mandarin, clementine mandarin (*C. clementina* cv. Clementina de Nules grafted on citrange Carrizo rootstock) and bean (*Phaseolus vulgaris* L. cv. Buenos Aires roja) plants were used in our assays. These plants were grown on vermiculite and peat (1:3; v:v). No pesticides were applied to these plants, which were watered every 3 days with approximately 30 ml of a 1:100 (vol:vol) modified Hoagland's solution (Bañuls et al. 1997). Bean plants were used for rearing purposes only (see below).

Three-month-old plants of sour orange and Cleopatra mandarin were used in the behavioral assays (see below). They were maintained in a climatic chamber at 22 ± 2.5°C and 60 ± 10% relative humidity (RH) under a 16:8 h L:D (Light:Dark) photoperiod. Two-year-old clementine mandarin plants maintained in a greenhouse at 25 ± 10 °C, 75 ± 30% RH, under natural photoperiod and lemon (*Citrus limon* (L.) Burm f.) fruit obtained from a pesticide-free orchard at Universitat Jaume I Riu Sec Campus (UJI; 30º59'38''N; 0º03'59''W, 30 m alt.), the same location, were used to maintain *T. urticae* stock colonies. Finally, pesticide-free bean leaves obtained from plants grown at UJI greenhouses were used to maintain *E. stipulatus* and *P. persimilis* colonies.

Spider mite stock colony

The colony of *T. urticae* used in the assays was initiated with specimens collected in clementine mandarin orchards in the region of La Plana (Castelló, Spain) in 2011. Mites were maintained on lemons kept in a climatic chamber (22 ± 2.5°C and 75 ± 5% RH and 16:8 h L:D photoperiod). Colonies consisted of 8–10 lemons, which were replaced weekly in groups of four. Adult females (5-6 day-old) obtained from these stock colonies were used in the behavioral assays (see below), either directly to infest citrus plants, or subjected to a previous 24-h starvation period, before measuring their preferences. For the characterization of *T. urticae* associated volatiles, we used individuals from these colonies but also from an additional colony maintained on detached clementine mandarin leaves. These leaves were placed upside down on top of sponges (14 × 14 × 4 cm) covered

with cotton in water-containing trays (35 × 20 × 7 cm) that served both as a water source for leaves and mites and as a barrier against mite dispersal.

Phytoseiidae mite stock colony

Three different phytoseiid mite species were used in our studies: *E. stipulatus*, *N. californicus* and *P. persimilis*. Colonies of *P. persimilis* and *E. stipulatus* were initiated with specimens collected in clementine mandarin orchards in the region of La Plana (Castelló, Spain) whereas *N. californicus* was obtained from Koppert Biological Systems (SPICAL®) and these specimens were directly used in our choice tests. The colonies of *P. persimilis* and *E. stipulatus* were maintained on detached leaves of bean plants in a climatic chamber at the same conditions as above. The rearing took place on units consisting of a single bean leaf placed upside down on moistened cotton, placed on top of a water-saturated sponge in water-containing trays as before. Moist cotton was folded over the edges of the leaves to prevent mites from escaping. A mix of different stages of *T. urticae* was provided twice a week to *P. persimilis*, whereas *E. stipulatus* was supplied *Typha* L. spp. (Typhaceae) pollen, only. 5-6 day-old phytoseiid adult females obtained from these stock colonies were used in the behavioral assays (see below).

Y-tube olfactory choice assays

Olfactory choice assays were conducted using a Y-tube olfactometer according to Bruin et al. (1992). This assay involves the use of a 4-cm-diameter Y-shaped glass tube with a 13 cm base and two 13 cm arms containing a Y-shaped 1-mm diameter metal wire of the same dimensions, which occupies the core of the olfactometer. The two short 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 (mite odors, plant odors or a combination of both, see Figure 1-4). Each vessel was connected to an air pump that produced a unidirectional airflow of 1.5 l h^{-1} (measured with a flowmeter) from the arms to the base of the tube. The air was purified with a granular activated charcoal filter (Sigma-Aldrich). The environmental conditions inside the Y-tube were 23 ± 2°C and 60 ± 10% RH. Adult females offered water only during the 24 h before the assay, were individually deposited at the beginning of the basal arm of the wire using a soft-bristle paintbrush. Females were allowed to make a choice within 10 min. As soon as a mite reached the end of one of the two arms of the Y-tube, the mite was removed from the set-up and discarded. Mites failing to reach either end of the two arms within the allocated time were scored as ‘no choice’. Each combination was evaluated four times at different

dates (i.e., four replicates). Each replicate included 10 responding mites which meant that up to 13 mites per combination per date were tested as the non-choice rate ranged from 0 to 3. The glass vessels were switched after five females had been tested. After every 10 females had been tested, the plants were replaced and the whole system was rinsed with ethanol (70%), followed by air drying. The glass vessels were switched to reduce the effects of spatial influence on choice. To exclude any bias from the set-up, before the beginning of the assays, 10 mites were exposed to clean air in both arms.

Effect of HIPVs on neighboring plants

To determine the effect of the volatiles released by Cleopatra mandarin plants previously exposed to *T. urticae*-infested sour orange on mite behavior, an olfactory choice assay was performed. First, sour orange plants were infested with 25 adult *T. urticae* females per plant. After 24 h, one infested sour orange plant was placed in a tray (65 × 50 × 30 cm) containing five untreated Cleopatra mandarin plants. Subsequently, the tray was covered with a transparent lid. To avoid mite ambulatory dispersal, the tray was filled with water. After 72 h, one Cleopatra mandarin and one sour orange plants were defoliated. Detached leaves were immediately frozen at -80°C for further analysis (mRNA expression). The remaining four presumably-induced Cleopatra mandarin plants were used in an olfactory choice assay together with control plants where the preferences of *T. urticae, E. stipulatus, N. californicus* and *P. persimilis* were studied following the same procedure as above.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

RNA was extracted using a plant RNA protocol with trizol (Kiefer et al. 2000). For qRT-PCR experiments, 1 µg of total RNA was digested with 0.7 µg of DNase (RNase-free DNase I) in 0.7 µl of DNase buffer and Milli-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, 10X 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 Milli-Q sterile water (Maxima SYBR Green/ROX qPCR, Thermofisher Scientific Inc.).

qPCR was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA, USA) sequence detector with standard PCR conditions (95ºC-10 min; 40×(95ºC-10 sec; 55ºC-10 sec; 72ºC-20 sec); 60ºC-10 sec; 95ºC-15 sec). qRT-PCR analysis was replicated three times. The primer of lipoxygenase2 (*LOX2*) and pathogenesis-related protein 5 (*PR5*) was determined. Relative expression was compared with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Table 1 Suppl.).

Characterization of Cleopatra-mandarin volatiles induced by exposure to sour orange HIPVs

Volatiles emitted by Cleopatra mandarin plants previously exposed to *T. urticae*-infested sour orange (see above) and Cleopatra mandarin control plants were collected using a headspace collection system similar to that described by Bruinsma et al. (2010). Open glass vials containing 300 mg of Porapak (Sigma-Aldrich, Barcelona, Spain) were used as volatile retention filters. They were connected to the air outlet hole at the top of 5-l glass vessels described above. This system was ventilated with carbon-filtered pressure-air at 1.5 l/h. The system (glass vessels and Porapak filters) was cleaned with acetone and dried in an oven 1 hour prior to the assay. Plants were set individually inside these glass vessels. Volatile compounds were collected in 1 ml of ethyl acetate. This collection took place in a climatic chamber at 22 ± 2.5°C and 60 ± 10% relative humidity (RH) under a 16:8 h L:D photoperiod during 24 hours. An Agilent 6890N 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 to characterize the volatiles. A fused silica DB-5MS capillary column of 30 m length, 0.25 mm internal diameter and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA) was used to the 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 1 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 c. 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. Volatiles were identified by matching to the National Institute of Standards and Technology library (NIST\EPA\NIH Mass Spectral Library, version 2.0, build 4/2005) using match values of at least >80% as a threshold for identification, as described by Wallis et al. (2008). Finally, for each volatile identified the TOF-MS-derived peak areas were calculated.

Characterization of *Tetranychus urticae* associated volatiles

Groups of 1000-2000 spider mite individuals (mixed instars and sexes) were placed in 20-ml closed screw-cap headspace vials by carefully brushing the rearing substrate. Volatiles were collected in static conditions by solid-phase microextraction (SPME) using Supelco SPME holders equipped with a polydimethylsiloxane/divinylbenzene fiber (PDMS/ DVB), film thickness = 100 μm (Supelco Inc., Bellefonte, PA, USA). SPME fibers were conditioned before volatile sampling in a GC injector at 250°C for 10 min under a 20 ml min^{-1} helium flow rate. SPME needles were inserted through the polytetrafluoroethylene (PTFE)-silicone septa, and fibers were exposed to each sample for 24 h at 23 ± 2°C, under a 16:8 h L:D photoperiod. This sampling period was chosen in order to achieve maximum sensitivity (Alfaro et al. 2011). Then, fibers were removed and inserted into the GC injection port to desorb volatiles. Nine replicates were carried out with different groups of *T. urticae* individuals, six of them obtained from the colony maintained on lemons, and three from the colony on clementine mandarin leaves. SPME fibers were thermally desorbed into the GC injection port, set at 250°C for 1 min, and operated in the splitless mode. The extracted volatiles were analyzed by GC-MS using a Clarus 600 GC-MS (PerkinElmer Inc., Wellesley, MA, USA). The column used was a 30 m × 0.25 mm i.d., 0.25 μm film thickness, ZB-5MS fused silica capillary column (Phenomenex Inc., Torrance, CA, USA). The oven was held at 40°C for 2 min and then programmed at 5°C min^{-1} to 180°C; when reached, temperature was raised to 280°C at 10°C min^{-1} and maintained at 280°C for 1 min (total analysis run of 41 min). Helium was used as the carrier gas with a flow rate of 1.2 ml min^{-1}. Detection was performed in the EI mode (ionization energy, 70 eV; source temperature, 180 °C), and spectra acquisition was done in the scanning mode (mass range m/z 35−400). Chromatograms and spectra were recorded with GC-MS Turbomass software version 5.4 (PerkinElmer Inc.). Volatiles were identified by either comparing their retention times and mass spectra with those of pure standards (Sigma-Aldrich) or, same as before, by matching to the National Institute of Standards and Technology library (NIST\EPA\NIH Mass Spectral Library, version 2.0, build 4/2005) using match values of at least >80% as a threshold for

identification, as described by Wallis et al. (2008). For each rearing substrate, the different peak areas in the chromatogram corresponding to these compounds were calculated and used to estimate their relative abundance in the blend.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 23. The results of the two-choice assays were initially subjected to logistic regression to check for the effect of replicate (n = 4) on mite preference. Depending on whether this effect was significant or not ($P > 0.05$), either each single replicate or the combination of the four, respectively, were subjected to chi-square analysis to test whether they departed from a 1:1 distribution. Student *t*-tests were used to compare the results of genetic expression results. The TOF-MS-derived peak areas were checked for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). As these assumptions were fulfilled, the area values were subjected to analysis of variance (ANOVA; $P < 0.05$).

RESULTS

In order to understand the role of HIPVs in direct and indirect defense we first confirmed that sour orange strongly reacts to *T. urticae* infestation by triggering expression of both *LOX2* and *PR5* marker genes of the JA and the SA-signaling pathways, respectively (Figures 1A and 2A Suppl.). Likewise, Cleopatra mandarin could be stimulated by sour orange HIPVs that triggered an upregulation of *LOX2* and *PR5* gene expression (Figures 1B and 2B Suppl.).

Preferences of adult *T. urticae* females when exposed to the odors of clean and infested plants, which had already been recorded in our previous work (Agut et al. 2015), were studied again. In addition, we also checked the responses to conspecific mites alone, and to induced Cleopatra mandarin. As the effect of the factor 'replicate' was not significant in any case, for each 2-choice experiment, the results of the four replicates were pooled and subjected to chi-square test. Preferences are shown in Figure 1. Without plant, adult females did not respond to the blend of volatiles associated to conspecifics. However, when plants were considered, Cleopatra mandarin was always preferred to sour orange, irrespective of the infestation status. Moreover, when comparing the same genotype, clean versus infested plants, infested sour orange became repellent, whereas infested

Cleopatra mandarin became attractive, which correlates the level of direct response with the infestation observed in both genotypes (Figure 1 Suppl.), and confirms our previous observations (Agut et al. 2015). Remarkably, Cleopatra mandarin plants induced by sour orange HIPVs became repellent as well. This result correlates not only with the enhanced expression of SA and JA markers in induced Cleopatra (Figure 1 and 2 Suppl.) but also with a specific volatile profile. From the eight volatiles reported in Table 1, the production of the GLV 2-ethyl-1-hexanol increased in induced Cleopatra, whereas that of two aromatic derivatives and two additional GLVs decreased. These results confirm that Cleopatra mandarin is sensitive to the VOCs-induced direct resistance producing an antixenotic response, which is likely based on the production of a specific blend of volatiles.

The preferences of the three phytoseiids when exposed to the odors of *T. urticae*, plants, and the combination of these two are shown in Figures 2, 3 and 4. Contrary to what was observed for *T. urticae*, the three predators always preferred the odor of its prey, *T. urticae*, to clean air. This clearly suggests that these predators can effectively smell the herbivore. The characterization of *T. urticae* volatile profile allowed the identification of twelve compounds that were consistently detected regardless of the mite rearing substrate (Table 2). Seven of them were confirmed with commercial standards and include six GLVs: three simple isoprenoid alcohols, two short-chain aldehydes, and hexanoic acid. The last confirmed volatile in the blend is the HIPV MeSA. Four additional volatiles were tentatively identified as the structurally related lilac ketone and lilac aldehyde isomers. In the experiments where both clean genotypes (no previous mite infestation) were contrasted, all three predators preferred sour orange independently of their degree of specialization (Figures 2 to 4). This behavior changed when the phytoseiids had to choose between *T. urticae*-infested plants. The generalist *E. stipulatus*, same as its prey, preferred Cleopatra mandarin whereas the other two phytoseiids showed no preference for any of them. When comparing the same plant genotype, either infested or not, predators always preferred infested plants. Despite these interesting observations, in the experiments where we studied the VOCs-induced indirect defense, we observed that both *E. stipulatus* and *N. californicus* preferred Cleopatra mandarin-induced plants while *P. persimilis* remained neutral. These diverging results may be related predator diet specialization.

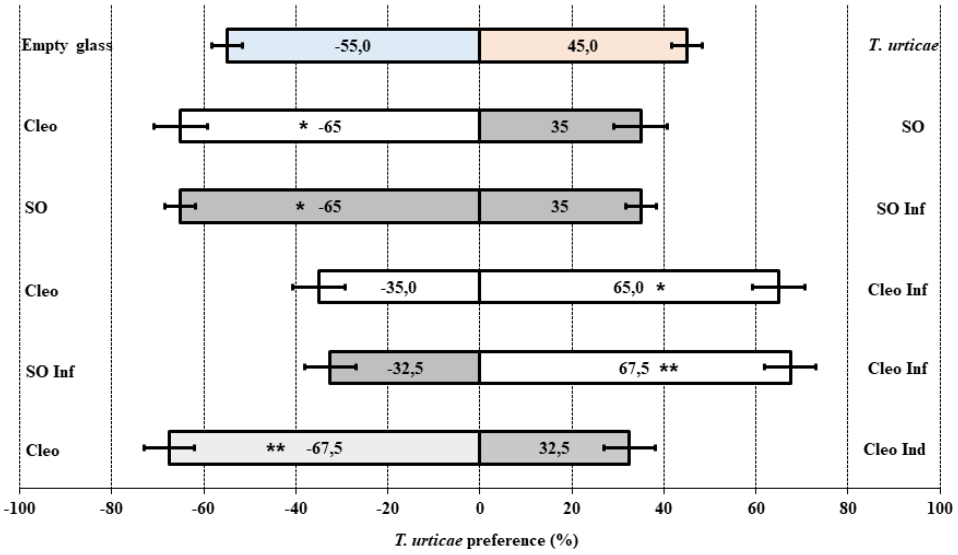

Figure 1. Olfactory response of *T. urticae* to conspecific mites either with or without plant substrate. Six different combinations, in which *T. urticae* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus conspecifics, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

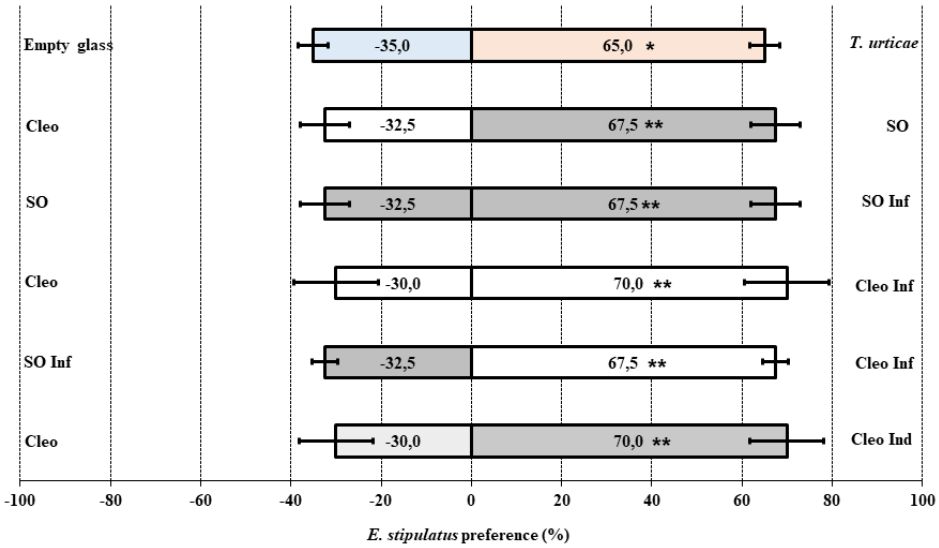

Figure 2. Olfactory response of *E. stipulatus* to *T. urticae* either with or without plant substrate. Six different combinations, in which *E. stipulatus* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus conspecifics, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

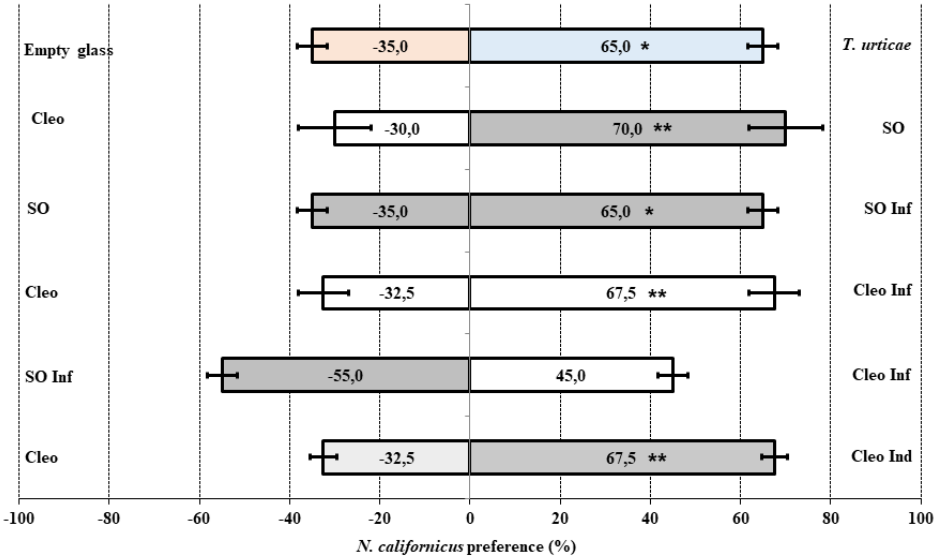

Figure 3. Olfactory response of *N. californicus* to *T. urticae* either with or without plant substrate. Six different combinations, in which *N. californicus* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus conspecifics, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

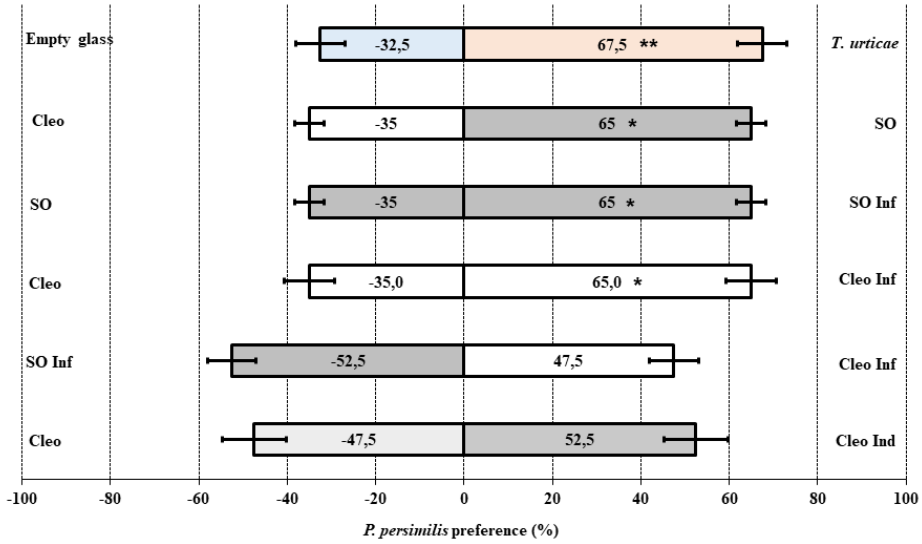

Figure 4. Olfactory response of *P. persimilis* to *T. urticae* either with or without plant substrate. Six different combinations, in which *P. persimilis* had to choose between two odor sources, were tested. A minimum of 40 adult females per choice combination was tested. These females were subjected to a starvation period of 24 h prior to the onset of the assay. From top to bottom these combinations were: empty glass versus conspecifics, Cleopatra mandarin untreated plants (Cleo) vs sour orange untreated plants (SO), SO vs SO-infested plants (SO Inf), Cleo vs Cleo-infested plants (Cleo Inf), SO Inf vs Cleo Inf, and Cleo vs Cleo-induced plants (Cleo Ind). Infested plants had been exposed to 25 adult females for 48 h before the onset of the assay. Induced plants had been exposed to sour orange infested plants for 72 hours. Asterisks indicate significant differences from a 1:1 distribution between treatments (chi-square test: $*P < 0.10$, $**P < 0.05$).

Table 1. Tentative identification[1] of the compounds detected in the headspace of Cleopatra mandarin (Cleo) plants without treatment (Cleo control) or induced by the HIPVs from *T. urticae* infested sour orange plants (Cleo induced) (mean TOF-MS-derived peak areas ± standard error). Different letters represent significant differences between treatments (analysis of variance, ANOVA, $P < 0.05$).

Volatile Compounds	Cleo control	Cleo induced
(1-methylethyl)-Benzene	8,413.0 ± 455.9 b	15,407.5 ± 1,485.6 a
1-ethyl-2-methyl-Benzene	30,487.5 ± 6,152.8 b	43,507.5 ± 3,093.2 a
2-ethyl-1-Hexanol	15,468.7 ± 3,909.6 b	50,200.3 ± 9,780.5 a
3-ethyl-3-methyl-Pentane	88,573.0 ± 8,009.3 a	44,584.7 ± 870.6 b
2-butoxyethyl Acetate	20,543.8 ± 7,199.3 b	38,083.7 ± 3,746.1 a
3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester Benzenepropanoic acid	2,550.8 ± 289.9 a	1,717.7 ± 513.9 a
4-hydroxy-4-methyl-2-Pentanone,	28,166.5 ± 4,526.2 a	24,584.8 ± 1,477.6 a
1R-α-Pinene	60,245.0 ± 21,100.1 a	47,417.2 ± 6,888.6 a

[1]Tentative identification of the compounds with spectra and high probability matches (>80%) according to NIST mass spectral database (Wallis et al., 2008).

Table 2. Compounds detected in volatile collections of *T. urticae* (relative mean ± standard error[1] percentage considering the total chromatogram area of the detected compounds) reared on either lemon fruits or clementine mandarin leaves.

Compound	id.[4]	Rearing substrate	
		Lemon fruits	Clementine leaves
2-methyl-3-buten-2-ol	C	18.34 ± 5.05	0.51 ± 0.37
3-methyl-3-buten-1-ol	C	6.44 ± 2.00	6.31 ± 4.06
3-methyl-2-buten-1-ol	C	18.08 ± 9.43	2.22 ± 1.00
Hexanal	C	3.07 ± 1.13	10.21 ± 8.92
Hexanoic acid	C	10.73 ± 4.41	50.91 ± 20.81
5-ethenyldihydro-5-methyl-2(3H)-furanone[2]	T	3.07 ± 1.17	5.29 ± 2.33
Nonanal	C	28.48 ± 10.04	15.27 ± 6.48
5-ethenyltetrahydro-α,5-dimethyl-2-Furanacetaldehyde[3] isomer	T	4.33 ± 2.54	2.28 ± 0.48
Lilac aldehyde isomer	T	7.44 ± 4.11	3.23 ± 0.87
Lilac aldehyde isomer	T	2.39 ± 1.39	0.73 ± 0.26
Methyl salicylate	C	5.23 ± 3.26	3.20 ± 2.62

[1]Means of six replicates for volatile samplings of individuals of the stock colony maintained on lemons and three replicates for samplings of individuals from a colony maintained on clementine mandarin leaves
[2] lilac lactone
[3] lilac aldehyde
[4] Identification of the compound: C, confirmed with commercial standard; T, tentative with spectra and high probability matches (>80%) according to NIST mass spectral database (Wallis et al., 2008).

DISCUSSION

Predators are not always attracted to less defended plants

Sour orange plants display higher constitutive and faster inducible direct defense against *T. urticae* compared with Cleopatra mandarins, which eventually results in the latter supporting higher *T. urticae* densities and increased plant damage (Bruessow et al. 2010; Agut et al. 2014, 2015). Therefore, according to our initial hypothesis, infested Cleopatra mandarins were expected to be more attractive for phytoseiids than infested and well-defended sour orange plants. However, in our experimental conditions only the omnivorous predator *E. stipulatus*, same as the herbivore, preferred Cleopatra mandarin when the two infested genotypes were simultaneously offered (Figures 1 and 2). The other two predators showed no preference for these infested genotypes (Figures 3 and 4). Following the same rationale, induced Cleopatra mandarin plants, which exhibit

enhanced expression of *LOX2* and *PR5* genes (Figures 1B and 2B Suppl.), should not have been chosen by predators when simultaneously offered with clean Cleopatra mandarin plants. Indeed, this is what the herbivore did. However, both *E. stipulatus* and *N. californicus* preferred the better-protected and void-of-prey induced plants, whereas *Tetranychus* spp.-specialist *P. persimilis* did not show any preference. Consequently, these results provide evidence that predator responses depend on plant genotype and diet specialization. Interestingly, predators are not always attracted to the less defended plants. For omnivores, plant defense induction could be a general clue of *T. urticae* presence in the area.

The well-known negative crosstalk between JA- and SA- defense pathways may be missing in citrus

Although some trade-offs between direct and indirect defenses have been suggested in specific plant-arthropod interactions (Koricheva et al. 2004), there are also reports in which both sorts of defense function synergistically (Rasmann et al. 2011; Pellissier et al. 2016). This could be the case for citrus as well, as evidenced by our observations in sour orange and induced Cleopatra mandarin plants (Figures 1B and 2B Suppl.). Indeed, sour orange appears to be a jack-of-all-trades, as it seems to have maximized different types of defense against this mite. A clear observation in the absence of infestation is that all predators are more attracted to sour orange, contrary to what was observed for the herbivore. Furthermore, the volatile profile of infested sour orange and induced Cleopatra mandarin changed relative to clean plants. Remarkably, the VOC profiles described in infested sour orange (Agut et al. 2015) and those found in induced Cleopatra mandarin are different and just share the monoterpene pinene. It is very likely that these defense responses are responsible for the repellence of *T. urticae* as well as the attractiveness of phytoseiids. Therefore, the three volatile blends identified so far (those corresponding to infested sour orange, induced Cleopatra mandarin, and *T. urticae*) are triggering similar behavioral responses in the four mite species studied: attraction of natural enemies but not of the herbivore. These blends deserve further studies, as they may provide new tools to manage these mites in crops.

Plant feeding by spider mites can activate both JA- and SA-related signaling pathways (Kant et al. 2004; Kawazu et al. 2012). However, the decreased performance of these mites (i.e., direct defense) has been associated with the induction of JA-related defenses and the accumulation of additional secondary metabolites such as glucosinolates (Kant et al. 2008; Agut et al. 2014, 2016; Zhurov et al. 2014). Therefore, the simultaneous

upregulation of both defensive pathways in infested sour orange (Figures 1A and 2A Suppl.; Agut et al. 2014) and in induced Cleopatra mandarin (Figures 1B and 2B Suppl.) indicates that the well-known negative crosstalk between JA- and SA- defense pathways (i.e., the antagonistic interaction between the SA- and the JA-response pathways) (Pieterse et al. 2009; Robert-Seilaniantz et al. 2011) may be missing in citrus.

***Tetranychus urticae*-associated volatiles include MeSA**

Interestingly, our results have shown that *T. urticae* associated odors include MeSA (Table 2), a volatile that had been previously identified in Cleopatra mandarin and sour orange HIPVs (Agut et al. 2015). However, we suspect that the amount of MeSA produced by the mite is orders of magnitude below what plants can produce, as we have been unable to detect this compound in infested lemons using the method described above for induced Cleopatra mandarin HIPVs. MeSA had been also found in the blend of volatiles produced by *T. urticae* female teliochrysalis and adult males (both stages were likely present in the mixed pool of mites used to characterize *T. urticae* associated volatiles) together with three additional volatiles, including methyl *cis*-dihydrojasmonate (Oku et al. 2015). In their study, this blend was shown to mediate male discrimination between male-guarded and solitary female teliochrysalis. Although different butterfly species of the genus *Pieris* Schrank (Lepidoptera: Pieridae) can use the amino acid phenylalanine as a precursor to MeSA (Andersson et al. 2000, 2003), *T. urticae* most probably obtains this volatile from its host plants (Oku et al. 2015). Because SA has been widely recognized as a key factor for predator recruitment by infested plants (i.e., indirect defense) (Rodríguez-Saona et al. 2011; Kaplan 2012; Mallinger et al. 2011; Rowen et al. 2017; Salamanca et al. 2017), the question of why a plant volatile exploited by natural enemies as a kairomone is not immobilized/degraded by its potential prey, deserves further investigations.

Blends rather than single compounds matter

Importantly, it is often the whole blend rather than single volatiles what predatory mites exploit to communicate (Clavijo-McCormick et al. 2012). Indeed, in their study Oku et al. (2015) could not attribute the behavioral differences observed in male *T. urticae* to a single compound but to the whole blend. Moreover, van Wijk et al. (2008, 2011), showed that although MeSA alone, which was produced by *T. urticae*-injured lima bean plants, was attractive to *P. persimilis*, attraction increased when MeSA was part of the natural HIPV blend produced by the plant. Interestingly, one of the volatiles in that blend, the

GLV (Z)-3-hexenyl acetate, was repellent to *P. persimilis* when tested alone. Likewise, in our case, attraction to the three phytoseiids tested could be attributed to the blend in Table 2 rather than to a single volatile. Most of these compounds have been reported as aggregation pheromones in several bark beetles (Bakke et al. 1977; Stoakley et al. 1978; Bowers et al. 1991). Lilac related compounds have been described as volatile constituents of plant essential oils (Jerković et al. 2017; Peron et al. 2017). Moreover, lilac aldehyde stereoisomers have been identified in the flower scent of many plant species, with an important role for the attraction of pollinators (Dötterl and Jürgens 2005; Dötterl et al. 2006). Although the role of *T. urticae* associated volatiles needs further investigations, their origin, same as MeSA, is likely the host plant (Castro-Vázquez et al. 2009), from where they may have been acquired either directly or as precursors (Reddy and Guerrero 2004).

Diet specialization may partly explain phytoseiid choices

As pointed out earlier, the SA-dependent signaling pathway is considered key for indirect defense. Actually, MeSA has been shown to attract phytoseiid mites (de Boer and Dicke 2004; van Wijk et al. 2008, 2011; Shimoda 2010). Therefore, plants with relatively enhanced activation of the SA signaling pathway were expected to be selected by phytoseiids in our two choice-tests. However, this was not always the case. For most of these exceptions, an over-ruling of prey-related odors, which interestingly include MeSA (Table 2), can explain the results. This is the case of *N. californicus* and *P. persimilis*, which showed no preference when offered the two infested genotypes (when a preference for infested Cleopatra mandarin was anticipated as MeSA levels are higher in this genotype, Agut et al. 2015). Nevertheless, this prey over-ruling hypothesis does not explain the preferences of *E. stipulatus* and *N. californicus* for induced Cleopatra mandarin over clean Cleopatra plants (where no preference was expected as MeSA was not differentially produced in these genotypes; Table 1). These differences among predators may be partly due to their different diet specializations (McMurtry and Croft 1997; McMurtry et al. 2013), which may affect the interpretation of the meaning of the different volatile blends.

The high polyphagy of *T. urticae* (Migeon and Dorkeld 2006-2017) results in the induction of quantitatively and qualitatively different HIPVs in different host plants (Van den Boom et al. 2004) and this might hamper prey location by its natural enemies. *P. persimilis* can locate their prey from a distance using volatiles, including MeSA, emitted by plants infested with spider mites (Sabelis and van de Baan 1983; Sabelis et al. 1984;

Dicke et al. 1990). However, this phytoseiid selected volatiles from prey-infested leaves, *T. urticae*, rather than leaves infested with a non-prey close relative, *Panonychus ulmi* (Koch) (Acari: Tetranychidae) (Sabelis and van de Baan 1983). For specialist predators (i.e., *P. persimilis*), the density of its main prey on the infested plant has to be enough as a reward as this is their only suitable food for complete development and successful reproduction. Therefore, it is not surprising that in our experiments *P. persimilis* responded mainly to the blend of *T. urticae*-associated volatiles (Figure 4). Although it detected and reacted to the upregulation of SA-signaling *PR5* gene in clean sour orange when offered together with clean Cleopatra mandarin, the lower levels in induced Cleopatra mandarin (Figure 2B Suppl.) did not trigger the same behavior when the predator had to choose between induced and clean Cleopatra mandarin plants. Indeed, this predator is known to respond to MeSA, which was induced in both sour orange and Cleopatra mandarin by *T. urticae* (Agut et al. 2015), in a dose-dependent manner (de Boer and Dicke 2004). However, for extreme omnivorous predators, including zoophytophagous species, which can obtain their food from different prey species and even from the host plant, both prey-specific chemical cues and HIPVs may be equally important to select patches with enough prey diversity and abundance but also with minimal plant direct defense. *E. stipulatus* is the only predator from the three species included in this study that most probably belongs to the group of phytoseiids that may complement their nutrition requirements by feeding on leaf epidermal cells (Adar et al. 2012; McMurtry et al. 2013). Therefore, *E. stipulatus* may benefit from choosing the plant genotype showing the weakest defense when infested by *T. urticae* (Agut et al. 2014). By preferring Cleopatra mandarin to sour orange when both genotypes were infested (Figure 2), *E. stipulatus* also selects the host likely offering higher densities of the prey and this would eventually benefit the plant as well, as this omnivorous predator may choose to feed preferentially on the prey and not on the plant. As MeSA was not differentially produced in the blend of volatiles produced by Cleopatra mandarin upon induction by sour orange HIPVs (Table 1), other volatiles must have a more important role in governing *E. stipulatus* choices and this should be partly true for *N. californicus* as it exhibited a behavior in between this generalist and the specialist *P. persimilis*.

Concluding remarks

To sum up, our results provide evidence that the response of the four mite species included in this study is plant genotype dependent and is modulated by their feeding habits, as well as by the presence of the herbivore on the plant. Some of these behavioral

responses in *T. urticae* had already been described by our group (Agut el al. 2015). Interestingly, the discrimination by *T. urticae* between Cleopatra mandarin plants either clean or induced with HIPVs from *T. urticae*-infested sour orange, and the fact that this mite did not show any preference when exposed to volatiles emitted by conspecifics, confirms that this behavior is triggered by plant HIPVs only. Further research focused on the three volatile blends that have been identified in this study as attractive for *T. urticae* natural enemies but not for the herbivore could provide new more sustainable tools with clear applications in crop protection (i.e., use of volatile dispensers for predator recruitment and plant defense enhancement). Furthermore, the accumulation of MeSA in *T. urticae*, which, on the one hand, may have a direct impact on plant defense (i.e., priming) and, on the other, on recruiting natural enemies, should be also further studied.

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Compliance with Ethical Standards

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Supplementary material

Table 1 suppl. Primers used in qRT-PCR reactions.

Description	Accession	Forward primer 5'→3'	Reverse primer 5'→3'
LOX2	Cit.16756.1.S1_s_at	GAACCATATTGCCACTTTCG	CGTCATCAATGACTTGACCA
PR5	BAI63297.1	CATCAAGCTTCACAGTGCTTAG	CCACAACGTACAGACTGATGAC
GAPDH	Cit.122.1	GGAAGGTCAAGATCGGAATCAA	CGTCCCTCTGCAAGATGACTCT

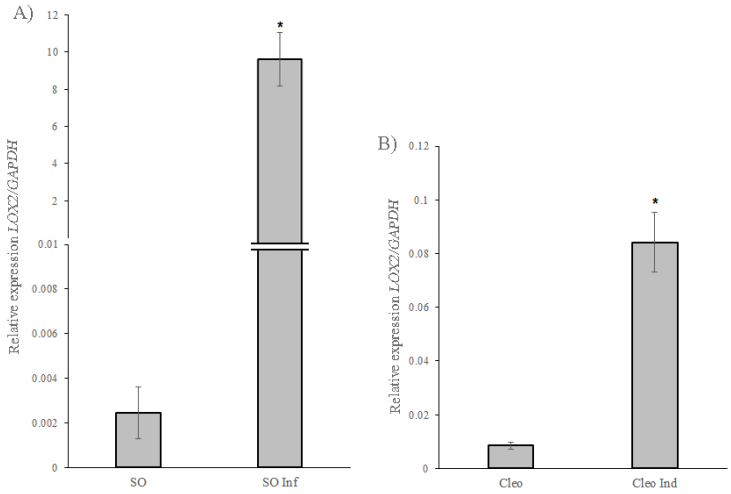

Figure 1 suppl. Induction of defensive pathways in Cleopatra mandarin by exposure to HIPVs produced by neighboring sour orange plants infested with *T. urticae*. Lipoxygenase2 gene (*LOX2*) induction following different treatments; A) *LOX2* expression in untreated sour orange plants and 72 h post-infested sour orange plants with *T. urticae*. B) *LOX2* expression in untreated Cleopatra mandarin plants and at 72 h post-exposure to sour orange herbivore-induced plant volatiles (HIPVs). The *LOX2* transcript levels were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured in the same sample. The data are presented with a representative figure for four independent experiments of the analysis behavior through the olfactometer of the mites studied in the present work, in Cleopatra mandarin induced plants. Significant differences in the relative transcript levels between different treatments were estimated using a *t*-test. The asterisk indicates significant difference to different treatments (*t*-test; $P < 0.05$).

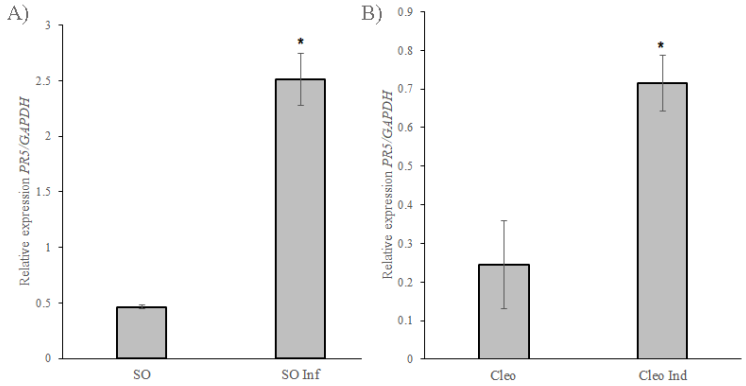

Figure 2 suppl. Induction of defensive pathways in Cleopatra mandarin by exposure to HIPVs produced by neighboring sour orange plants infested with *T. urticae*. Pathogenesis-related protein 5 (*PR5*) induction following different treatments; A) *PR5* expression in untreated sour orange plants and 72 h post-infested sour orange plants with *T. urticae*. B) *PR5* expression in untreated Cleopatra mandarin plants and at 72 h post-exposure to sour orange herbivore-induced plant volatiles (HIPVs). The *PR5* transcript levels were normalized to the the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) measured in the same sample. The data are presented with a representative figure for the four independent experiments of the analysis behavior through the olfactometer of the mites studied in the present work, in Cleopatra mandarin induced plants. Significant differences in the relative transcript levels between different treatments were estimated using a *t*-test. The asterisk indicates significant difference to different treatments (*t*-test; $P < 0.05$).