Effect of Sequential Induction by *Mamestra brassicae* L. and *Tetranychus urticae* Koch on Lima Bean Plant Indirect Defense

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Abstract Attack by multiple herbivores often leads to modification of induced plant defenses compared to single herbivory, yet little is known about the effects on induced indirect plant defense. Here, we investigated the effect of sequential induction of plant defense by *Mamestra brassicae* caterpillar oral secretion and an infestation by *Tetranychus urticae* spider mites on the expression of indirect plant defense in Lima bean plants. The effect on indirect defense was assessed using behavior assays with the specialist predatory mite *Phytoseiulus persimilis* in an olfactometer, headspace analysis of 11 major herbivore-induced plant volatiles (HIPVs) including (*E*)-β-ocimene, and transcript levels of the corresponding gene *Phaseolus lunatus* (*E*)-β-*ocimene synthase* (*PlOS*). Predatory mites were found to distinguish between plants induced by spider mites and caterpillar oral secretion but not between plants with single spider mite infestation and plants induced by caterpillar oral secretion prior to spider mite infestation. Indeed, the volatile blends emitted by plants induced by spider mites only and the sequential induction treatment of caterpillar oral secretion followed by spider mite infestation, were similar. Our results suggest that plant indirect defense is not affected by previous treatment with oral secretion of *M. brassicae* caterpillars.

Introduction

Plants that are under attack by herbivores produce and release complex mixtures of volatiles, known as herbivore-induced plant volatiles (HIPVs). Natural enemies of herbivorous arthropods can use these HIPVs to locate their herbivorous prey or host [reviewed by Mumm and Dicke (2010)]. The phenomenon of recruitment of natural enemies via HIPV release is known as indirect plant defense. Phytohormones, such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) are involved in generating and modulating induced plant defense responses to herbivory. The phytohormone JA and its volatile derivative methyl jasmonate (MeJA) are the main signalling molecules for induction of plant defense against herbivores, and they play an important role in indirect plant defense [e.g., Dicke et al. (1999); Kessler and Baldwin (2002)]. However, other phytohormones, such as salicylic acid (SA) and ethylene (ET) are known to play modulating roles via pathway cross-talk (Ament et al. 2004; Koornneef and Pieterse 2008; Ozawa et al. 2000; Van Poecke and Dicke 2002). Depending on the signalling pathways induced by an herbivore, the composition of HIPV blends can differ significantly (Zhang et al. 2013), allowing natural enemies to distinguish between plants infested by prey and non-prey herbivores (De Boer et al. 2004; Erb et al. 2010).

In nature, prey and non-prey herbivores can simultaneously feed on the same plant and thereby differentially modulate defense pathways and alter plant defense responses. Multiple

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herbivory is subject to an increasing number of studies (Stam et al. 2014). However, most of these studies focus on the effect on direct plant defenses and plant-mediated interactions amongst the herbivores (Brunissen et al. 2009; Erb et al. 2011; Kaplan and Denno 2007; Mathur et al. 2013; Rodriguez-Saona et al. 2010). The few studies on indirect plant defense mechanisms (Dicke et al. 2009) show that volatile blends of multiple herbivore-attacked plants can differ quantitatively or qualitatively compared to single induction (Delphia et al. 2007; De Boer et al. 2008; Erb et al. 2010; Rodriguez-Saona et al. 2003; Schwartzberg et al. 2011; Shiojiri et al. 2001; Zhang et al. 2009, 2013) . Multiple herbivory can influence indirect plant defense in a positive, neutral, or negative manner. In Lima bean plants, for example, simultaneous feeding of non-prey caterpillars, *Spodoptera exigua* Hübner, and prey, the spider mite *Tetranychus urticae* Koch, results in a synergistic increase in volatile emission and an increased attraction of a natural enemy of the spider mite, i.e., the predatory mite *Phytoseiulus persimilis* Athias-Henriot (De Boer et al. 2008). However, simultaneous feeding of the cicadellid *Euscelidius variegatus* Kirshbaum and *Spodoptera littoralis* Boisduval caterpillars on maize plants did not alter volatile emission differently from *S. littoralis*-induced volatile emission and did not affect behavior of a parasitoid of *S. littoralis* (Erb et al. 2010). Whitefly infestation has a negative effect on predator attraction to *T. urticae*-infested Lima bean plants (Zhang et al. 2009). The underlying mechanism for the differences in the effect of multiple herbivory is likely phytohormone cross-talk (Zhang et al. 2009, 2013).

Phytohormone crosstalk also may be involved in interactions between herbivores that attack a plant in temporally spaced events (Kessler and Baldwin 2004; Poelman et al. 2008). Sequential herbivory can have long-lasting effects on plant defenses (Poelman et al. 2008), whereby prior feeding by one herbivore can result in a kind of "vaccination" that can affect direct and indirect plant defenses against a later-arriving second herbivore (Kessler and Baldwin 2004). Voelckel and Baldwin (2004) suggest that the order/identity of arrival is crucial because some herbivore-induced stress effects on plant defense seem to be more stable than others. This also is likely dependent on the intensity and timing of subsequent defense inductions. In *Zea mays* seedlings, for example, a low dose of exogenously applied SA increases endogenous JA levels and volatile production upon a second induction by an insect-derived elicitor (Engelberth et al. 2011). However, higher doses resulted in reduced JA responses due to negative cross-talk. Moreover, shorter incubation times than 15 h with the phytohormone did not result in accumulation of JA or enhanced volatile production.

Volatile induction by leaf-chewing lepidopterans, and certain cell-content feeding herbivores, such as the spider mite *Tetranychus urticae*, is regulated primarily by the jasmonic acid pathway, and therefore antagonistic effects on plant

defense are not expected between the two (Ozawa et al. 2000). Here, we investigated the effect of sequential induction of plant defense by *M. brassicae* caterpillar oral secretion and an infestation by *T. urticae* spider mites, which were temporally separated by a period of 48 h. We hypothesized that sequential induction of JA-induced plant defenses would result in increased attraction of *P. persimilis* through changes in the HIPV blend. Moreover, we investigated whether transcription levels of the JA-responsive gene *Phaseolus lunatus Ocimene Synthase* (*PlOS*), coding for a rate-limiting step in the biosynthesis of the spider-mite inducible plant volatile (*E*)-β-ocimene, would increase accordingly (Ament et al. 2004). The HIPV (*E*)-β-ocimene is known to be highly attractive to *P. persimilis* (Dicke et al. 1990).

Methods and Materials

Plants Lima bean plants (*Phaseolus lunatus* L., cv. Wonderbush, De Bruyn Seed Company, Michigan, USA) were cultivated in a greenhouse compartment at 23 ± 2 °C, 60 ± 10 % relative humidity (RH) and a 16:8 h L:D photoregime. Plants were grown in 5×5 cm plastic pots for gene transcription experiments or 11×11 cm for headspace volatile collection and behavioral experiments, respectively. After 12–14 d, plants with two expanded primary leaves were transferred to a climate chamber and incubated at 25 ± 1 °C, 60 ± 10 % R.H. and 16:8 h L:D. In the climate chamber, plants of different treatments were kept separate in plastic cages ($90\times90\times60$ cm) that were connected to house vacuum to prevent volatile transfer between plants of different treatments.

Herbivores and Predatory Mites A colony of two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), was maintained on Lima bean plants in a greenhouse compartment at 25 ± 5 °C, 50–70 % R.H., 16:8 h L:D. Adult female spider mites for experiments were selected randomly from the colony.

Cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) caterpillars were reared on cabbage plants (*B. oleracea* var. *gemmifera* cv. Cyrus, Syngenta seeds BV, Enkhuizen, The Netherlands) at 22 ± 1 °C and 50–70 % R.H., under the same photoregime as for plants and mites. Oral secretions were collected from 18–20 caterpillars in the 5th instar, which were randomly selected from the colony.

Predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), were reared in Petri dishes (diam 9 cm) on detached Lima bean leaves that were heavily infested with *T. urticae* under the same conditions as the *T. urticae* colony. Only gravid female predatory mites were used for behavioral experiments, ca. 1–2 d after their final molt. For behavioral experiments, the females were individually confined in 0.5 ml Eppendorf tubes containing a piece of moist cotton wool to avoid dehydration. Predatory mites were

starved for 24 h and left in the experimental room prior to the experiment in order to acclimatise.

Plant Treatments

Sequential induction experiments Plant treatments were: i) control, ii) infestation with 20 spider mites (48 h incubation), iii) caterpillar oral secretion (48 h incubation), iv) caterpillar oral secretion (48 h incubation) followed by infestation with 20 mites (48 h incubation), v) caterpillar oral secretion (96 h incubation). Plants treated with caterpillar oral secretion were first artificially damaged with a pattern wheel by drawing 6 lines of ca. 7 cm length on each primary leaf. Then, the damaged leaves were treated with 10 µl per leaf of diluted caterpillar oral secretion using a fine paint brush for application. Oral secretion consisted of a pooled stock, freshly collected from 18 to 20 5th instar *M. brassicae* caterpillars using a glass Pasteur pipette. The stock was diluted 1:1 with tap water and kept on ice prior to use to avoid degradation of compounds. Upon the application with oral secretion, plants were incubated for 48 h. Control plants and plants receiving single infestation by spider mites did not receive mechanical damage or oral secretion. Plants were kept in a climate chamber and incubated in groups separated by treatment in the cages described above. After 48 h, plants to be analyzed for single induction by oral secretion were sampled for transcriptional, volatile, or behavioral analysis. Plants with dual induction treatment or single infestation by *T. urticae* received 20 mites per plant, followed by another 48 h of incubation before sample collection.

Time Series Experiment Plant treatments included i) control, ii) infestation with 20 spider mites, and iii) caterpillar oral secretion. Plant treatments were executed the same as in the previous section. Plants were kept in a climate chamber and incubated separately according to treatment in cages. Samples for transcriptional analysis were taken 6, 20, 26, or 46 h following treatment.

Y-tube Olfactometer Responses of predatory mites were tested in a Y-tube olfactometer (Takabayashi and Dicke [1992](#)). A Y-shaped metal wire was located in the center of a glass Y-tube, and each arm was connected to a 5-L glass jar. Glass jars containing plants were connected to air inlets providing a 2 L/min charcoal-filtered air influx to carry volatiles into the two arms of the Y-tube. For behavior experiments, plant pots and loose soil were gently removed, and roots with soil were carefully wrapped in aluminium foil. Three plants of a treatment were placed in a glass jar as odor source, and the system was purged for 30–60 min without closing the vessels. Afterwards, glass jars were sealed with viton-lined glass lids, and the whole Y-tube olfactometer setup was flushed with air for 7–10 min before commencing the behavior experiment. Individual predatory mites were placed downwind on the Y-shaped wire and their choice for either odor source was

recorded when they passed a line located halfway up one of the two olfactometer arms, or no-choice was recorded when they had not passed the line within 5 min. Sides of treatments were alternated after every five predatory mites to avoid positional bias. Plants were replaced after every 20 predatory mites or approximately 90 min after the first mite was tested, whichever came first. Each comparison was tested on two different days with 40–60 predatory mites per day.

Dynamic Headspace Collection of Plant Volatiles Plants were prepared for volatile collection by gently removing pots and loose soil, and wrapping roots with soil in aluminium foil. Two plants of each treatment were transferred to a 5-L glass jar. Glass jars were sealed with viton-lined glass lids equipped with an air inlet and outlet. The setup was flushed with 100 ml/min synthetic air (Linde Gas Benelux B.V., The Netherlands) filtered by passing through charcoal before entering the glass jar. Glass jars with plant samples were flushed with air for 30–45 min. A stainless steel tube filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) was connected to the outlet of each glass jar, and volatile collection was done by sucking air out of the jars at 100 ml/min for 2 h. A total of eight replicates of each treatment were sampled over 2 d. Fresh weight of above-ground plants tissue was determined immediately after volatile collection using an analytical balance (NewClassic ML, Mettler Toledo, Greifensee, Switzerland).

Chemical Analysis of Plant Volatiles A Trace Ultra gas chromatograph (GC) coupled with Trace DSQ quadrupole mass spectrometer (MS) both from Thermo (Thermo Fisher Scientific, Waltham, MA, USA) were used for separation and identification of plant volatiles was as described previously (Menzel et al. [2014](#)).

Standards of (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, acetate, (*Z*)-3-hexen-1-ol, butanoate, (*Z*)-3-hexen-1-ol, isovalerate, linalool, methyl salicylate, indole, (*E*)-β-ocimene, as well as allocimene were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Additional standards (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT] and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E-E*)-TMTT] were kindly provided by Prof. W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany). For quantification, calibration lines were constructed for each compound using seven data points at different concentrations and two replicates of each data point.

RNA Extraction and cDNA Synthesis Spider mites, eggs, feces, and webbing were gently removed from plants of the respective treatments using a soft paint brush. Plant material was obtained by cutting 4 leaf discs out of one primary leaf per plant with a cork borer (diam 2 cm). Leaf discs obtained from the primary leaf of 3 plants were pooled to yield one biological replicate. All samples were shock-frozen in liquid nitrogen immediately after collection and then stored at −80 °C until further processing. Frozen leaf material was homogenized

with a mortar and pestle while avoiding thawing. Total RNA was isolated and purified using the Qiagen (Hilden, Germany) RNeasy Plant Mini kit with integrated DNAse treatment, according to manufacturer's instructions. RNA quality was assessed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Labchip® kit (all from Agilent Technologies, Santa Clara, CA, USA). RNA quantifications were done using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only RNA samples with 260/280 wavelength ratio >2 and a RIN value >7 were used for cDNA synthesis. cDNA was generated from total RNA by using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

Quantitative RT-PCR A real-time quantitative RT-PCR was used to quantify gene transcript levels of *P. lunatus Ocimene synthase* (*PlOS*; GenBank accession EU194553), acidic pathogenesis-related protein 4 (PR-4), and the two reference genes *P. lunatus Actin1* (*PlACT1*; GenBank accession DQ159907) and *P. lunatus Nuclear matrix protein 1* (*PlNMP1*; GenBank accession AF289260.1). Quantitative RT-PCR was performed in a Rotor-Gene 6000 machine (Corbett Research) with a 72-well rotor; for a detailed description see Menzel et al. (2014). *PlOS* primers were F-*PlOS* 5′-TGCATGGGTCTCAGTCTCTG-3′ and R-*PlOS* 5′-TGCT GCTTCCCCTCTCTCTA-3′, *PlPR*-4 were F-*PlPR*-4 5′-ACGCTTTCCTCAGTGCTCTC-3′ and R-*PlPR*-4 5′-TCCT CGTCGTCGCAGTAATCCTT-3′, *PlACT1* primers were F-*PlACT1* 5′-CCAAGGCTAACCGTGAAAAG-3′ and R-*PlACT1* 5′-AGCCAGATCAAGACGAAGGA-3′, and *PlNMP1* primers F-*PlNMP1* 5′-CCGGAATGGAGTGTTG ACGAGCA-3′ and *R-PlNMP1* 5′-CCAGCT CAGAAACA TCTGGCAATGG5-3′.

Statistical Analysis Log transformation was applied to data from gene transcription experiments and volatiles in order to meet assumptions of normality and homogeneity of variances. Data were analyzed using one-way ANOVA or generalized linear model (GLM) followed by Tukey's Honestly Significant Difference (HSD) *post-hoc* tests for pair-wise comparisons between treatments in the statistical software SPSS version 19 (SPSS Inc., Chicago, IL, USA). Data that violated assumptions on normality and equal variance after log transformation were analyzed by Kruskal-Wallis tests followed by Mann–Whitney *U* tests applying the Bonferroni correction for multiple comparisons.

Predator choices in the Y-tube olfactometer experiments were analyzed using a binomial test to examine whether the choice distribution significantly differed from 50:50.

Volatile profiles of plants exposed to different treatments were analyzed using multivariate data analysis. Data were expressed per unit of plant fresh weight, log-transformed, and univariate-scaled. Then, an Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) was performed using the software SIMCA P + version 12 (Umetrics, Umeå, Sweden). Pairwise comparisons for individual volatiles among treatments were executed using Mann–Whitney *U* tests.

Results

Response of Predatory Mites to Single or Multiple Herbivore Infestation The attraction of predatory mites to Lima bean plants exposed to different treatments was tested in a two-choice behavioral assay (Fig. 1). Feeding by *T. urticae*, with and without prior treatment with *M. brassicae* oral secretion, was preferred over control plants (binomial test, *P*<0.001 in both comparisons). However, predators did not distinguish between control plants and plants treated with *M. brassicae* oral secretion alone (binomial test, *P*>0.05). Moreover, predatory mites showed a preference for odors from plants infested by their prey compared to odors from plants induced by caterpillar oral secretion (binomial test, *P*<0.001). The volatile blend of plants induced by the combination of non-prey oral secretion and prey herbivores was more attractive than the volatile blend of plants induced by oral secretion of *M. brassicae* (binomial test, *P*<0.001). Predators did not display a significant preference when they were offered plants infested by prey herbivores vs. volatiles from plants induced by the combination of non-prey oral secretion and prey herbivores (binomial test, *P*>0.05).

Volatile Analysis Spider mite feeding resulted in higher emission rates of several compounds compared to treatment with *M. brassicae* oral secretion (Suppl. Information, Figs. S1 and S2). Comparison of volatile profiles consisting of the 11 major HIPV compounds emitted in response to the treatments showed that volatile blends were significantly different between treatments: OPLS-DA resulted in a model with three significant principal components (Fig. 2). Figure 2b shows that the first distinction between treatments was made for treatments including exposure to feeding by *T. urticae* vs. treatments without *T. urticae*, which were separated by the first principal component. The second component separated treatments with caterpillar oral secretion from treatments that did not include oral secretion. The volatile blend of the combination treatment of caterpillar oral secretion and *T. urticae* was most similar to the volatile blend from plants treated with *T. urticae* infestation alone. Together with the third principal component, 89 % of the total variability of the data could be explained. Moreover, the model showed that four volatile compounds, namely (*E,E*)-TMTT, (*E*)-β-ocimene, (*Z*)-3-hexen-1-ol acetate, and alloocimene, had variable importance in the projection (VIP) values higher than 1, thus contributing most to discrimination between treatments. Pairwise comparison between the two treatments (1) 20 *T. urticae*

(*T.u.*) and (2) *M. brassicae* oral secretion followed by infestation with 20 *T. urticae* (*M.b.* + *T.u.*), did not yield significant principal components and thus were not significantly different.

Relative Gene Transcription Transcript levels for *PlOS*, the gene encoding for the enzyme that mediates the rate-limiting step in the biosynthesis of (*E*)-β-ocimene, which is a principal attractant for *P. persimilis* (Dicke et al. 1990; Zhang et al. 2009), were compared between treatments. Treatments significantly affected *PlOS* transcript levels (GLM, $P<0.001$; Fig. 3). Plants infested by *T. urticae*, with or without prior treatment with *M. brassicae* oral secretion, showed increased levels of *PlOS* compared to control plants and plants treated with *M. brassicae* oral secretion alone (*post hoc* Tukey's HSD, $P<0.01$). Oral secretion from *M. brassicae* did not increase *PlOS* transcript levels different from control (*post hoc* Tukey's HSD, $P>0.05$). Transcript levels of *PlPR*-4 also were affected by the treatments (ANOVA, $F_{4,15}=16.86$ $P<0.001$). Infestation by *T. urticae* and treatment with *M. brassicae* oral secretion both resulted in significantly increased *PlPR*-4 transcript levels compared to control plants (*post hoc* Tukey's HSD, $P<0.001$). Sequential treatment induced an increase in transcript levels compared to control plants, and transcript levels were even higher than in response to single treatments (*post hoc* Tukey's HSD, $P<0.001$).

Time Series for Relative PlOS Gene Transcription in Response to Single Treatments To investigate *PlOS* gene transcript levels for plants infested with *T. urticae* and plants with caterpillar oral secretion treatment, a time series experiment was conducted. The time series of *PlOS* gene transcription for the single treatment with 20 *T. urticae* or *M. brassicae* oral secretion showed clear differences in the gene transcription patterns between the two treatments (Fig. 4). Infestation by 20 *T. urticae* led to 14 times higher transcript levels than in control plants already after 6 h post treatment (hpt). At 20 hpt, expression levels of both the plants treated with *M. brassicae* oral secretion and plants exposed to *T. urticae* feeding were significantly higher than control levels (ANOVA, Tukey's HSD tests, $P<0.05$), whereas at 26 hpt these differences had disappeared. At 46 hpt, the same pattern as at 6 hpt was found, although the degree of upregulation of *PlOS* was lower. Treatment with caterpillar oral secretion also led to an increase of *PlOS* transcript levels compared to control levels but only at 20 hpt (ANOVA, Tukey's HSD tests, $P<0.05$). Before and after this time point transcript levels were not different from control levels.

Discussion

Plants frequently are attacked by multiple herbivores, which may arrive at different moments in time. The resulting sequential herbivory has long-lasting effects on plant resistance against the subsequent herbivores (Mathur et al. 2011; Poelman et al. 2008; Stam et al. 2014; Viswanathan et al. 2007). However, little is known about the effect of sequential herbivory on plant indirect defense (but see e.g., Erb et al. 2010; Zhang et al. 2009, 2013). Here, we investigated the effect of the sequential attack by different herbivore species on plant indirect defense against *T. urticae* spider mites through the attraction of the specialist predator *P. persimilis*. Previous studies suggest that plants can form "memories" after stressful events such as herbivory, which enables them to adjust their defense accordingly, thus responding in an enhanced manner to a second stress (Conrath 2009; Frost et al. 2008). Our results show that prior treatment of plants with oral secretions of the generalist caterpillar *M. brassicae*, as a mimic of caterpillar feeding, does not affect the attraction of *P. persimilis* to plants infested with its prey *T. urticae*. The modulating effects caused by interactions with two herbivore species may, thus, depend on several factors such as severity of initial damage or infestation, timing between attacks, identity of the herbivore, and the associated defense pathway induced (Dicke et al. 2009; Viswanathan et al. 2007; Voelckel and Baldwin 2004; Zhang et al. 2009). The defense pathway commonly induced by leaf-chewing insects such as *M. brassicae*, is the octadecanoid pathway with JA as signalling molecule
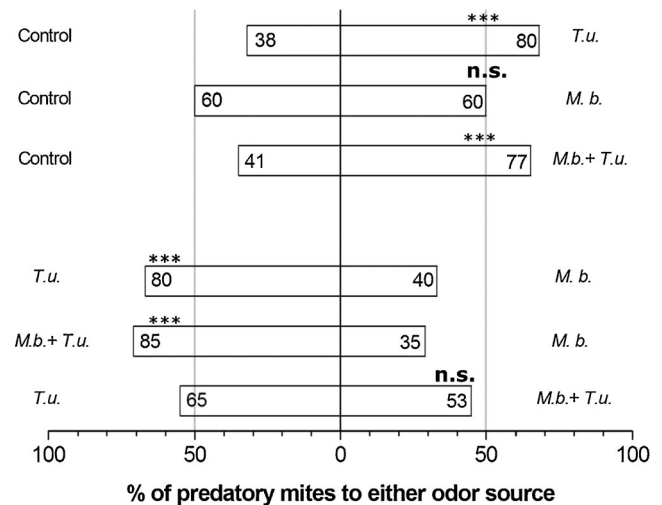

Fig. 1 Responses of *Phytoseiulus persimilis* in a Y-tube olfactometer to volatiles emitted by Lima bean plants induced by mechanical damage and oral secretion of non-prey (*Mamestra brassicae*; *M.b.*), prey (*Tetranychus urticae*; *T.u.*) infestation, or a combination of the two (*M.b.* + *T.u.*). Volatile sources consisted of three Lima bean plants per treatment, i) control plants, or ii) plants infested with 20 *T. urticae* for 48 h, iii) induction by mechanical damage and *M. brassicae* oral secretion incubated for 48 h, or iv) induction by mechanical damage and *M. brassicae* oral secretion incubated for 48 h followed by infestation by 20 *T. urticae* for 48 h. *Bars* represent the overall percentages of predators choosing either odor source. Numbers in *bars* correspond to the number of predators choosing either odor source. For each comparison, the number of mites that did not make a choice within 5 min ranged between 0 and 3. Asterisks indicate significance of predatory mite choices (binomial test; n.s. = not significant, *** $P<0.001$)

(Kessler and Baldwin 2002; McConn et al. 1997). The same defense pathway also is induced by feeding by cell-content feeders such as *T. urticae* (Dicke et al. 1999; Li et al. 2002; Ozawa et al. 2000). Multiple herbivory that occurs simultaneously by herbivores that induce the same pathway may lead to changes in volatile emission that results in increased attraction of predators (De Boer et al. 2008). In our study with sequential herbivory, predators did not distinguish between volatile blends from plants fed upon by *T. urticae* only and plants pre-treated with caterpillar oral secretions and subsequently exposed to feeding by *T. urticae*. Headspace analysis demonstrated that the volatile profiles of plants that had been exposed to these two treatments largely overlapped. It thus is possible that the initial defense induction was not strong enough to induce a memory effect or that the memory had decayed at the onset of the second attack by *T. urticae*. However, the volatile profile from plants with caterpillar oral secretion treatment was notably different compared to plants treated with *T. urticae*, and predators were significantly more attracted to plants infested with their prey. This is in accordance with data of De Boer and co-workers (2008) who found that the specialist predator *P. persimilis* can distinguish between volatiles induced by prey and non-prey herbivores. Nevertheless, De Boer et al. (2008) found an increased attraction of *P. persimilis* to dual-infested Lima bean plants, which were fed upon by *T. urticae* and *Spodoptera exigua* caterpillars. In their study, the dual infestation resulted in the emission

of increased amounts of a subset of the plant volatiles. In the study by De Boer et al. (2008), the plants were exposed to simultaneous infestation with spider mites and *S. exigua* caterpillars. Whether the differentiation by the predators in their study and not in ours was due to the different caterpillar species, simultaneous compared to sequential treatments, or to caterpillar feeding instead of the use of oral secretion, remains to be elucidated.

It has been shown that *P. persimilis* is attracted to five HIPVs, two of which, namely (*E,E*)-TMTT and (*E*)-β-ocimene, were indeed found to play a significant role in separating the volatile profiles of Lima bean plants that had received different treatments (De Boer and Dicke 2004; Dicke et al. 1990). Dicke et al. (1999) found that the emission rates of the two homoterpenes (*E,E*)-TMTT and (*E*)-DMNT, as well as the phenolic ester MeSA, were involved in the differentiation between JA-treated plants and *T. urticae*-treated plants, the latter being more attractive to *P. persimilis*. The two other compounds, namely the terpene alcohol linalool and the monoterpene (*E*)-β-ocimene also are known to play important roles in the attraction of *P. persimilis* to *T. urticae*-infested Lima bean plants (Dicke et al. 1990; Zhang et al. 2009). Arimura et al. (2008) found a close relationship between JA levels and transcription of *PlOS*, which is the enzyme that leads to the production of the principal predator attractant (*E*)-β-ocimene in Lima bean. Voelckel and Baldwin (2004) found that simultaneous and sequential herbivory can cause
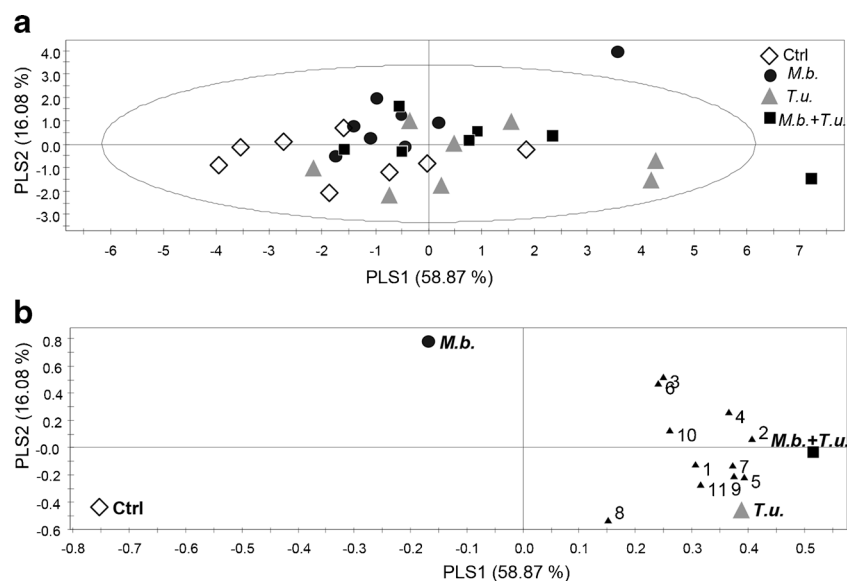

Fig. 2 Multivariate data analysis by orthogonal PLS-DA (OPLS-DA) (*panel A*) and corresponding loading plot (*panel B*) of volatile blends of *Phytoseiulus. lunatus* plants with i) no treatment (Ctrl), or treated with ii) 20 *Tetranychus urticae* for 48 h (*T.u.*), iii) *Mamestra brassicae* oral secretion 48 h (*M.b.*), iv) *M. brassicae* oral secretion 48 h followed by infestation with 20 *T. urticae* for 48 h (*M.b. + T.u.*); 8 replicates for each treatment. The first two principal components are depicted (*panel A*) with

the percentage of variation explained in parentheses. Numbers in the loading plot (*panel B*) represent 1) (*Z*)-3-hexen-1-ol, 2) (*Z*)-3-hexen-1-ol, acetate, 3) (*E*)-β-ocimene, 4) linalool, 5) (*E*)-4,8-dimethylnona-1,3,7-triene [(*E*)-DMNT], 6) alloocimene, 7) (*Z*)-3-hexen-1-ol,butanoate, 8) methyl salicylate, 9) (*Z*)-3-hexen-1-ol,isovalerate, 10) indole, 11) (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E-E*)-TMTT]
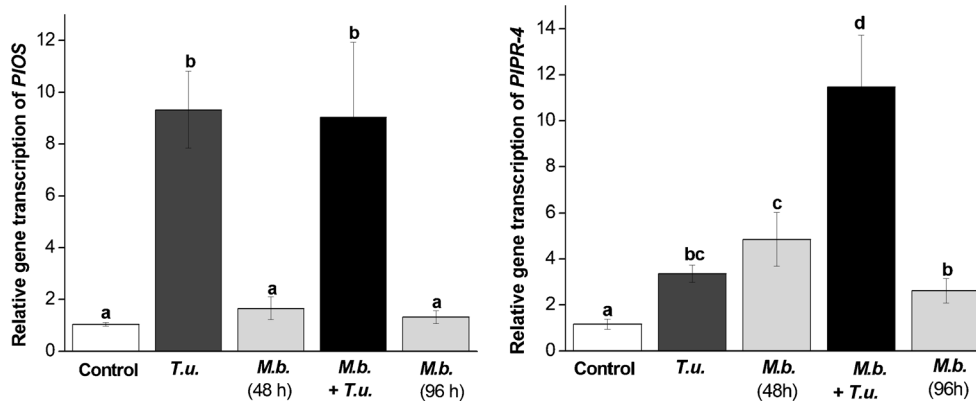
Fig. 3 Relative gene transcript levels of *Phytoseiulus lunatus* (*E*)-β-ocimene synthase (*PlOS*) and *P. lunatus* pathogenesis-related protein 4 (*PlPR-4*) of plants i) without treatment (control), or treated with ii) 20 *Tetranychus urticae* for 48 h (*T.u.*), iii) *Mamestra brassicae* oral secretion for 48 h [*M.b.* (48 h)], iv) *M. brassicae* oral secretion for 48 h followed by infestation with 20 *T. urticae* for 48 h (*M.b.* + *T.u.*), v) *M. brassicae* oral secretion 96 h [*M.b.* (96 h)]. Values are the mean (±SE) of 10 to 12 biological replicates, pooled from three replications of the same treatment. Different letters above *bars* indicate significant differences in transcript levels between treatments (Tukey's HSD tests, *P*<0.05). Gene transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *P. lunatus* Actin1 (*PlACT1*) and *P. lunatus* nuclear matrix protein 1 (*PlNMP1*) for each sample

different patterns of gene transcription when compared to individual feeding by the two different herbivores. In our study, treatments with the prey *T. urticae* and with the prey plus *M. brassicae* oral secretion resulted in increased *PlOS* transcript levels in Lima bean plants, but were not different from each other. Treatment with caterpillar oral secretion, which did not result in attraction of *P. persimilis*, showed *PlOS* transcript levels comparable to control levels. However, De Boer et al. (2008) found that Lima bean plants infested with *S. exigua* caterpillars or *T. urticae* spider mites do both emit large amounts of (*E*)-β-ocimene. Moreover, it is likely that a time lag exists between gene transcription and metabolite production (Stam et al. 2014). To investigate the temporal effect of caterpillar oral secretion on *PlOS* transcript

levels, a time series experiment was conducted. Results show that treatment with caterpillar oral secretion does increase *PlOS* transcript levels. Moreover, *PlOS* transcript levels of plants treated with caterpillar oral secretion peaked at a different time point from in plants infested with *T. urticae* infestation, and showed different transcriptional patterns. Compared to *T. urticae*-treated plants, *PlOS* transcription peaked quite late for plants treated with caterpillar oral secretion. This is in accordance with Arimura et al. (2008) who found that feeding by the generalist caterpillar *S. littoralis* induced *PlOS* transcript levels only after 24 hpt but not at 6 hpt, whereas JA treatment and wounding already induced *PlOS* after 6 h. Continuous infestation, and thus feeding by *T. urticae*, resulted in elevation of *PlOS* transcript levels in three out of four
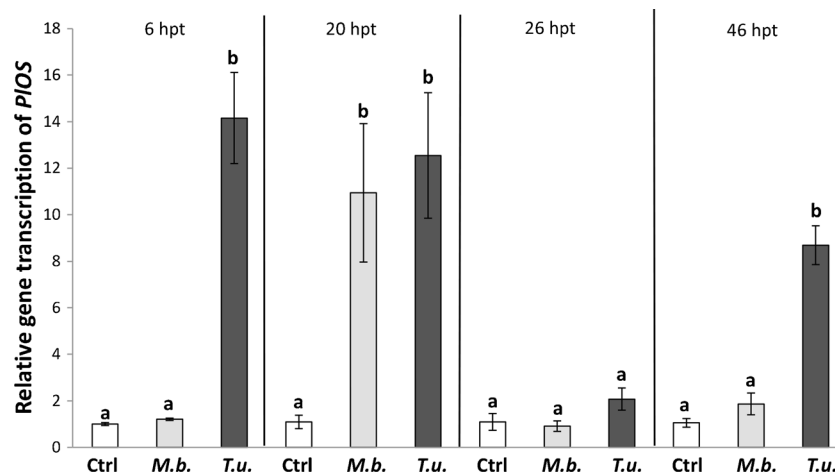

Fig. 4 Relative gene transcript levels of *PlOS* over four time points separated by hours post treatment (hpt). *Phytoseiulus lunatus* plants were treated with i) no treatment (control; Ctrl), ii) 20 *Tetranychus urticae* (*T.u.*), or iii) *Mamestra brassicae* oral secretion (*M.b.*). Values are the mean (±SE) of 4 biological replicates. Different letters above *bars* indicate significant differences in transcript levels between treatments (Tukey's HSD tests, *P*<0.05) within each time point. Gene transcript levels were normalized to the normalization factor obtained from geometrically averaging the Ct values of the two reference genes *P. lunatus* Actin1 (*PlACT1*) and *P. lunatus* nuclear matrix protein 1 (*PlNMP1*) for each sample

time points. Moreover, *PlOS* transcript levels of plants treated with caterpillar oral secretion declined to control levels within 6 h after the peak. Apart from an effect of *M. brassicae* oral secretion on transcription of *PlOS*, transcription of another gene investigated also was affected. Transcription levels of *PlPR*-4 were upregulated by *T. urticae* feeding as well as application of *M. brassicae* oral secretion, and combined application led to higher transcript levels than either single treatment. *PlPR*-4 is an acidic chitinase, which is MeSA-responsive (Arimura et al. 2000; Margis-Pinheiro et al. 1991). Chitinases commonly are involved in plant direct defense against plant pathogens and arthropods (Kramer and Muthukrishnan 1997; Schlumbaum et al. 1986). Consequently, other defense mechanisms apart from indirect defense could in fact be affected by the combination treatment of *T. urticae* and *M. brassicae* oral secretion.

In our study, predatory mite behavior was well reflected in volatile blend analysis. Four compounds, i.e., (*E*,*E*)-TMTT, (*E*)-β-ocimene, (*Z*)-3-hexen-1-ol acetate, and alloocimene, were found to have an important function in discrimination between treatments. The involvement of (*E*,*E*)-TMTT and (*E*)-β-ocimene in the attraction of *P. persimilis* to prey-infested Lima bean plants has been described previously (Dicke et al. 1990). Nevertheless, there are other well-known compounds that play an important role in predator attraction (De Boer et al. 2004; Dicke et al. 1990). In fact, volatile blends convey more information than a single compound e.g. on herbivore identity and herbivore developmental stage (De Moraes et al. 1998; Mumm and Dicke 2010; Takabayashi et al. 1995).

In summary, in our study, sequential herbivore treatment involving herbivores that induce the same plant defense pathway did not enhance or interfere with indirect defense against *T. urticae*. Yet, differences in volatile blends perceived by the predator play a role in distinguishing between prey and non-prey infested plants. Volatiles attractive to natural enemies are perceived in the context of other volatiles thus extracting specific information about the presence of prey. Furthermore, gene transcription is differently induced in terms of timing and magnitude by different herbivore species, and a considerable time lag exists between gene transcription of genes relevant in indirect defense and metabolite emission.

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