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Prey and Non-prey Arthropods Sharing a Host Plant: Effects on Induced Volatile Emission and Predator Attraction

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Abstract It is well established that plants infested with a single herbivore species can attract specific natural enemies through the emission of herbivore-induced volatiles. However, it is less clear what happens when plants are simultaneously attacked by more than one species. We analyzed volatile emissions of lima bean and cucumber plants upon multi-species herbivory by spider mites (*Tetranychus urticae*) and caterpillars (*Spodoptera exigua*) in comparison to single-species herbivory. Upon herbivory by single or multiple species, lima bean and cucumber plants emitted volatile blends that comprised mostly the same compounds. To detect additive, synergistic, or antagonistic effects, we compared the multi-species herbivory volatile blend with the sum of the volatile blends induced by each of the herbivore species feeding alone. In lima bean, the majority of

compounds were more strongly induced by multi-species herbivory than expected based on the sum of volatile emissions by each of the herbivores separately, potentially caused by synergistic effects. In contrast, in cucumber, two compounds were suppressed by multi-species herbivory, suggesting the potential for antagonistic effects. We also studied the behavioral responses of the predatory mite *Phytoseiulus persimilis*, a specialized natural enemy of spider mites. Olfactometer experiments showed that *P. persimilis* preferred volatiles induced by multi-species herbivory to volatiles induced by *S. exigua* alone or by prey mites alone. We conclude that both lima bean and cucumber plants effectively attract predatory mites upon multi-species herbivory, but the underlying mechanisms appear different between these species.

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Introduction

Plants can employ arthropod natural enemies and defend themselves against herbivorous insects and mites. Such arthropod natural enemies can have an impact on local herbivore populations and thereby also on plant fitness (Sabelis and van der Meer 1986; Dicke and Sabelis 1989; Pels and Sabelis 1999; van Loon et al. 2000; Fritzsche Hoballah and Turlings 2001; Kessler and Baldwin 2001). Plants can promote the effectiveness of natural enemies by providing alternative food, shelter, or volatiles (Price et al. 1980; Dicke and Sabelis 1988). The emission of herbivore-induced plant volatiles (HIPV) has been demonstrated in several families (e.g., Dicke et al. 1990a; Turlings et al.

1990; van den Boom et al. 2004; van Poecke and Dicke 2004). Many parasitoid wasps, predatory mites, and predatory insects use HIPV to locate their prey or hosts (reviewed in Dicke 1999; Dicke and Vet 1999; Sabelis et al. 1999). HIPV are thus thought to have an important influence on the interactions between plants, herbivores, and natural enemies in food webs (Turlings et al. 1995; Dicke and Vet 1999).

To date, studies on the production of HIPV by plants and the responses of natural enemies to these have been carried out mostly for systems with one species of plant, herbivore, and natural enemy. Yet, in the field, most plants are likely to be attacked by several herbivore species, or by herbivores and pathogens, at the same time. It is difficult to predict whether or not changes in HIPV blends upon multi-species herbivory affect attraction of natural enemies, and in what direction. Several studies have attempted to fill this gap by analyzing the chemical composition of volatile blends upon multi-species infestation of plants (Shiojiri et al. 2001; Cardoza et al. 2002; Rodriguez-Saona et al. 2003; Rostás et al. 2006; Moayeri et al. 2007; Rasmann and Turlings 2007; Soler et al. 2007). For instance, Shiojiri et al. (2000) showed that the parasitoid wasp *Cotesia plutellae* prefers the HIPV blend induced by its host *Plutella xylostella* to the blend induced by multi-species herbivory by its host and the non-host caterpillar *Pieris rapae* although volatile blends are similar (Shiojiri et al. 2001). *Cotesia glomerata*, on the other hand, prefers HIPV blends induced by its host *P. rapae* and non-host caterpillars to blends induced by one caterpillar species feeding alone (Shiojiri et al. 2000; Vos et al. 2001). *Cotesia marginiventris* and *Microplitis rufiventris* do not discriminate between the multi-species-induced HIPV blend of plants infested with their host (beet armyworm caterpillars) and a plant pathogen or plants infested with their host alone, although the pathogen significantly suppresses volatile induction (Rostás et al. 2006). Similarly, *C. glomerata* does not discriminate between mustard plants infested with a root herbivore and its host, or plants infested with its host alone (Soler et al. 2007). The parasitoid *C. marginiventris* and the entomopathogenic nematode *Heterorhabditis megidis* exhibited a reduced attraction to volatiles from maize plants that were infested by a non-host in addition to their host, although the non-host was feeding on different tissues from the host, i.e., leaves vs. roots (Rasmann and Turlings 2007).

In this study, we investigated the effects of multi-species herbivory by spider mites (*Tetranychus urticae*) and beet armyworm caterpillars (*Spodoptera exigua*) on volatile emissions by two different plant species: lima bean (*Phaseolus lunatus*) and cucumber (*Cucumis sativus*), and evaluated the responses of *Phytoseiulus persimilis*, a specialist natural enemy of spider mites. Both herbivore species are known to induce volatile emission in several

plant species, and remarkable differences in HIPV blend composition have been found in lima bean infested by either of these herbivores (e.g., Ozawa et al. 2000; Schmelz et al. 2003; de Boer et al. 2004). We statistically compared volatile emission by multi-species-infested plants with the sum of volatile emissions of plants infested with either herbivore species alone to evaluate the presence of potentially antagonistic or synergistic effects among signal transduction pathways (e.g., Engelberth et al. 2001). Plant responses to multi-species herbivory may depend on feeding modes of the herbivore species involved (Walling 2000), but whether or not plant species differ in these responses is unknown. Therefore, we compared two plant species in their relative responses to multi-species vs. single-species herbivory by using the same two herbivore species to attack both plant species. We also studied the olfactory responses of predatory mites (*P. persimilis*). In nature, plants are commonly attacked by more than one herbivore. Knowledge on natural enemy responses to volatile blends induced by multi-species vs. single-species herbivory is essential to understand whether differences in volatile composition are relevant in an ecological context.

Methods and Materials

Plants, Herbivores, and Predators Lima bean plants (*Phaseolus lunatus* L. cv Sieva) and cucumber plants (*C. sativus* L. cv Lange Groene Giganten) were grown in a greenhouse at 20–30°C, 50–70% R.H. and 16 hr of light. Bean plants were used when their primary leaves had unfolded (and were about 8–12 cm wide), which was 10–15 d after sowing. Cucumber plants were used when their first two leaves were about 8–12 cm wide, which was 15–35 d after sowing.

Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on lima bean plants in a greenhouse under the same conditions as uninfested plants. Eggs of the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from a colony reared on artificial diet in the laboratory of Virology, Wageningen University, The Netherlands (Smits et al. 1986). Two to three batches of eggs were placed in a Petri dish together with a lima bean or cucumber leaf in a growth chamber at 23±1°C. Larvae were used in experiments within 24 hr of hatching.

A colony of predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), was kept on spider mite-infested lima bean leaves in a growth chamber at 23±1°C, 50–70% R.H. and continuous light. To obtain females of the same age for olfactometer experiments, gravid females were allowed to oviposit on a spider mite-infested bean leaf for 2 d. The female offspring were used

in experiments 9 d after initiation of the colonies, i.e., 1–2 d after their final molt. Before the experiments, females were kept individually in 1.5-ml microcentrifuge tubes with a small droplet of water, for 24 ± 2 hr at $23 \pm 1^\circ\text{C}$.

Plant Treatments Treatments were applied to the two oldest leaves of lima bean or cucumber plants 3 d before an experiment. Plants were incubated in plastic cages in a climate-controlled chamber at $24 \pm 1^\circ\text{C}$, 50–70% R.H. and 16L:8D, each treatment in a separate cage. We used the following four treatments:

- (1) *T. urticae*: Adult female spider mites were transferred to experimental plants. Twenty mites per leaf were used on lima bean, and 100 mites per leaf were used on cucumber because the same number of spider mites leads to lower attraction of *P. persimilis* to cucumber than to lima bean plants (Dicke et al. 1990b).
- (2) *S. exigua*: Newly hatched *S. exigua* larvae were placed in clip cages to keep caterpillars on the leaves (clip cages were made of two plastic cylinders, 2.5 cm diam., and a hairpin; the upper side was covered with gauze and the underside with Parafilm). Two larvae in one clip cage were used per leaf. Clip cages were moved daily to a new position on the same leaf, and any missing larvae were replaced. The clip cages were supported by sticks to prevent the leaves from bending and incurring damage due to the weight of the cages.
- (3) Multi-species herbivory: Two newly hatched *S. exigua* larvae in a clip cage and 20 (lima bean) or 100 (cucumber) *T. urticae* females were placed on each leaf.
- (4) Uninfested: Plants without herbivores were treated in the same way as plants of the other treatments. This treatment was only used for volatile collections of cucumber. We did not analyze volatile emission from uninfested lima bean plants because it is documented that they emit low amounts of volatiles (e.g., de Boer et al. 2004).

When clip cages were used to keep caterpillars in place, *T. urticae*-infested or uninfested plants were treated in the same way with empty clip cages to avoid any cage effects.

Volatile Collections and Analyses Just before volatile collections, plants were cut above the soil line. Stems of individual plants were wrapped in moist cotton and aluminum foil to prevent them from wilting during volatile collection, which lasted at most 4 hr. All clip cages, caterpillars, and their products were removed. Five plants of one treatment were transferred to a 5-l glass vessel. A viton O-ring and a metal clamp were used to attach the glass lid to the vessel to make it airtight. Purified air (filtered

through silica, a molecular sieve, activated charcoal, and 90 mg Tenax) was split into two air streams of about 100 ml/min each and led into the vessels through teflon tubing. Volatiles from two treatments were collected simultaneously in parallel. The system was purged for 30 min before attaching a collection tube with Tenax (90 mg for bean samples, 200 mg for cucumber samples) to the air outlet in the lid of the vessel. Bean volatiles were collected for 25 min, cucumber volatiles were collected for 3 hr. For lima bean, volatile collection was repeated four times for each treatment, except for uninfested from which volatiles were not sampled. For cucumber, volatile collections were replicated seven times for the uninfested, six times for *T. urticae*, and five times for *S. exigua* and multi-species herbivory each. One sample of the *T. urticae* and one of the *S. exigua* treatments of cucumber were lost during the analysis.

Volatiles from lima bean and cucumber were analyzed on two different gas chromatography-mass spectrometry (GC-MS) systems.

Lima bean Volatiles were released from Tenax traps with a thermodesorption cold trap setup (Chrompack, Middelburg, The Netherlands) by heating at 250°C for 10 min, with a He-flow of 12 ml/min. Desorbed compounds were collected in the cold trap at -90°C . Volatiles were injected in splitless mode into the DB5 column (60 m \times 0.25 mm ID, 0.25 μm film thickness) by heating the cold trap to 220°C . After an initial column temperature of 40°C for 4 min, the temperature was raised to 280°C at $4^\circ\text{C}/\text{min}$. The column was directly coupled to the ion source of a Finnigan MAT 95 mass spectrometer, which was operating in the 70-eV electron impact (EI) ionization mode with scanning from mass 24 to 300 at 0.5 scan/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 library and in the Wageningen Mass Spectral Database of Natural Products, and by checking the retention indices.

Cucumber Volatiles were released from Tenax traps with a thermodesorption cold trap setup (Markes, UK) by heating at 200°C for 10 min, with a He-flow of 30 ml/min. The desorbed volatiles were collected in the cold trap at -10°C . Volatiles were injected in splitless mode into the RTX-5Silms column (Restec, 30 m \times 0.32 mm ID, 0.33 μm film thickness) by heating of the coldtrap to 270°C . After an initial column temperature of 40°C for 2 min, the temperature was raised to 95°C at $3^\circ\text{C}/\text{min}$, then to 165°C at $2^\circ\text{C}/\text{min}$, and subsequently to 250°C at $15^\circ\text{C}/\text{min}$. The column was directly coupled to the ion source of a Finnigan quadrupole mass spectrometer, which was operated in the 70-eV EI ionization mode and scanned from mass 33 to 300 at three scans/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 and

Wiley 7th edition spectral libraries, and by checking the retention indices.

Analyses Not all compounds were detected in every replicate of every treatment. We therefore added one peak area unit to all measurements for lima bean and 10 units to all measurements for cucumber. These were the lowest recorded non-zero values for bean and cucumber, respectively. This enabled us to plot volatile emission on a log

scale (Figs. 1a and 2a), and to determine ratios among treatments (see below).

For cucumber, we first determined which volatile compounds were induced by herbivory by using Kruskal–Wallis tests per compound. Variation in volatile emission levels of uninfested plants was low, indicating that other types of inducing factors (e.g., general stress) were absent or had only minor effects. Only herbivore-induced volatiles were used in subsequent analyses. We assumed that all

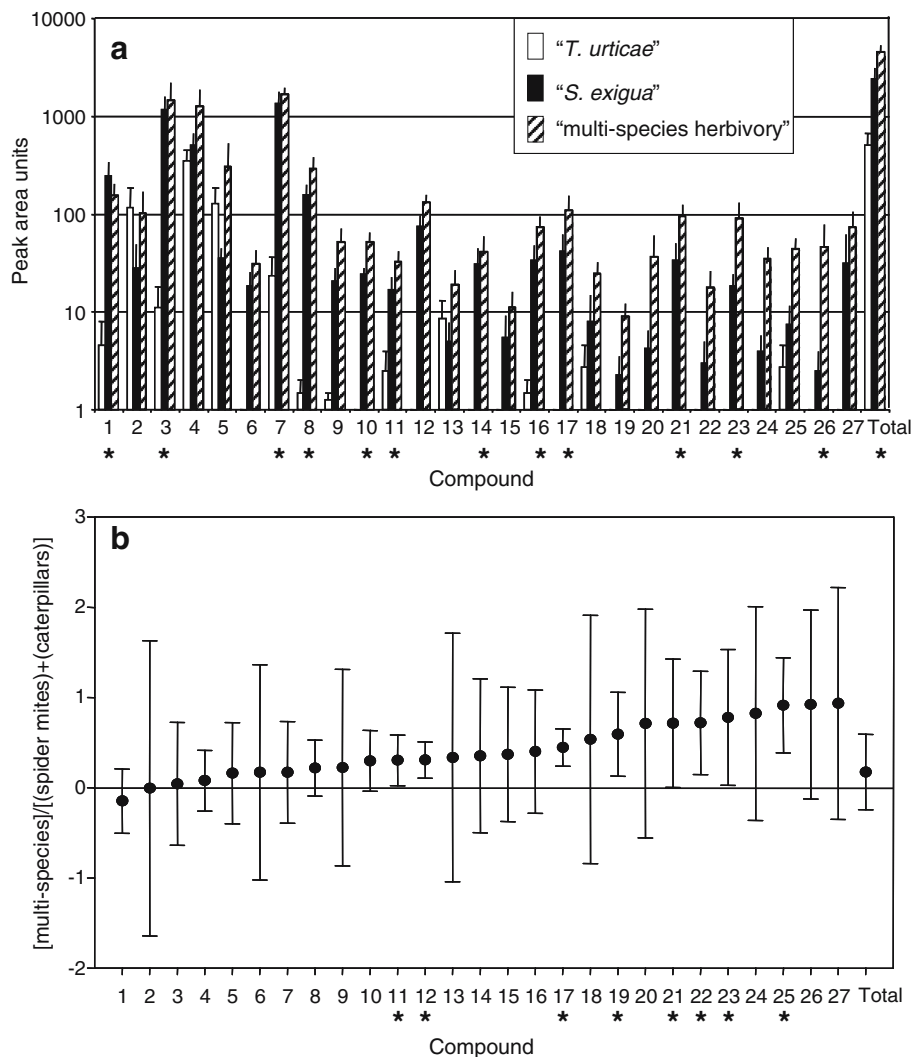


Fig. 1 Effect of multi-species herbivory on volatile emission by lima bean plants. **(a)** Volatile emission (mean + SE peak area units) upon single herbivory by *Tetranychus urticae* (20 mites per leaf) (open bars), single herbivory by *Spodoptera exigua* (two larvae per leaf) (filled bars), or multi-species herbivory (hatched bars). Asterisks indicate compounds that were significantly affected by herbivore treatment ($P < 0.05$, Kruskal–Wallis tests). **(b)** Ratio of emission rates upon multi-species herbivory to the sum per volatile emitted by the two single herbivore treatments (see “Methods and Materials” for detailed explanation). Symbols indicate mean ratio, and error bars indicate upper and lower 95% confidence limits. Asterisks indicate compounds that were significantly more strongly induced by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* (lower

95% confidence limit larger than 0). $N=4$ for all treatments. Compound numbers: (1) (*Z*)-3-hexen-1-ol, (2) methyl salicylate, (3) (*E*)- β -ocimene, (4) (*E*)-4,8-dimethyl-1,3,7-nonatriene, (5) (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (6) *p*-mentha-1,3,8-triene, (7) (*Z*)-3-hexen-1-ol acetate, (8) 2-methylbutanal-*O*-methyl oxime, (9) 3-methylbutanal-*O*-methyl oxime, (10) linalool, (11) 1-octen-3-ol, (12) hexyl acetate, (13) limonene, (14) β -caryophyllene, (15) nonanal, (16) indole, (17) (*E*)-2-hexen-1-ol acetate, (18) 3-pentanone, (19) 3-octanone, (20) 2-methylbutanal nitrile, (21) (*Z*)- β -ocimene, (22) rose furan, (23) unknown 95B, 150, (24) 3-methylbutanal nitrile, (25) unknown 91B, 148, (26) unknown 41, 69B, 164, (27) 2-methylpropanal-*O*-methyl oxime

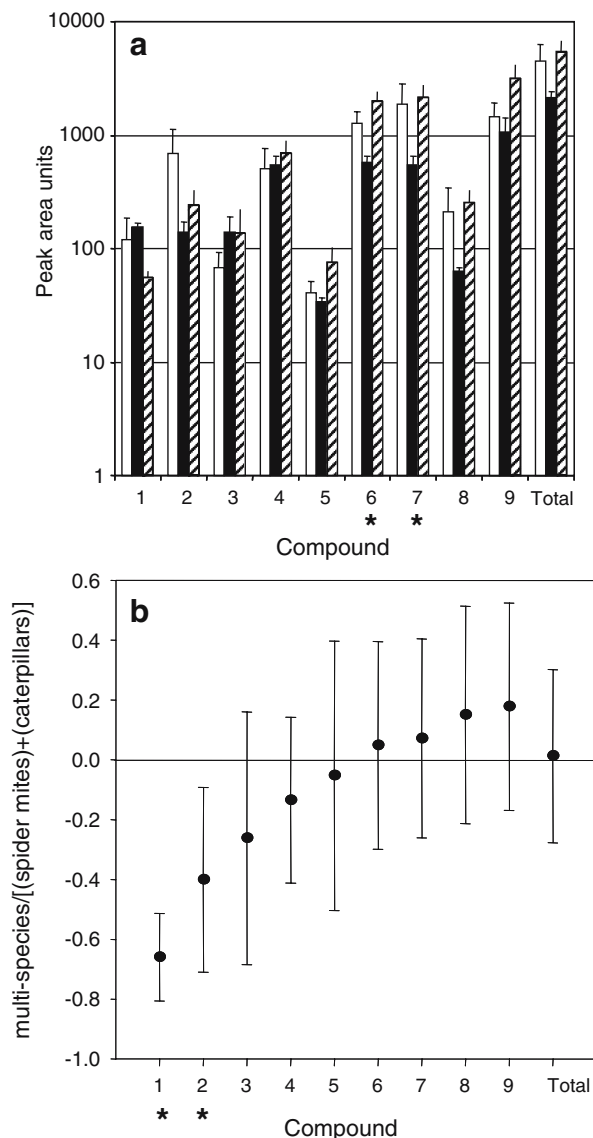


Fig. 2 Effect of multi-species herbivory on volatile emission by cucumber plants. **(a)** Volatile emission (mean \pm SE peak area units) upon single herbivory by *Tetranychus urticae* (100 mites per leaf, $N=5$, open bars), single herbivory by *Spodoptera exigua* (2 larvae per leaf, $N=4$, filled bars), or multi-species herbivory ($N=4$, hatched bars). Asterisks indicate compounds that are significantly affected by herbivore treatment ($P<0.05$, Kruskal–Wallis tests). **(b)** Ratio of emission rates upon multi-species herbivory to the sum per volatile emitted by the two single herbivore treatments (see “Methods and Materials” and for detailed explanation). Symbols indicate mean ratio, and error bars indicate upper and lower 95% confidence limits. Asterisks indicate compounds that were significantly less induced by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* (upper 95% confidence limit smaller than 0) ($N=4$) Compound numbers: (1) (*E*)-2-hexenal + (*Z*)-3-hexenal, (2) (*Z*)-3-hexen-1-ol acetate, (3) indole, (4) (*E,E*)- α -farnesene, (5) butyl aldoxime, (6) 3-methylbutanal-*O*-methyl oxime, (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene, (8) (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (9) (*E*)- β -ocimene. Note that peak area units in Fig. 2a cannot be compared with Fig. 1a because measurements were done on a different GC-MS system

compounds emitted by infested lima bean plants were induced by herbivores based on previous measurements of volatile emissions by uninfested lima bean plants (e.g., de Boer et al. 2004). Per plant species, we then used Kruskal–Wallis tests to analyze which compounds were differentially induced by the three herbivore treatments (*T. urticae*, *S. exigua*, or multi-species herbivory) ($\alpha=0.05$). Note that we did not statistically compare absolute levels of volatile emission across plant species because volatiles were collected for different periods of time, and analyses were run on two different GC-MS systems.

We also tested whether the effect on HIPV of multi-species herbivory was the same as the combined effect of the two herbivores feeding alone, i.e., *T. urticae* + *S. exigua*. Per replicate, the amount of each volatile compound of the multi-species herbivory treatment was divided by the sum of the amounts emitted by *T. urticae* and *S. exigua*. The ratios were log-transformed before taking their averages. An average ratio of 0 means the effect of multi-species herbivory and the sum of *T. urticae* and *S. exigua* is equal. We concluded that a compound was significantly more strongly induced by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* if the lower 95% confidence limit was larger than 0. Similarly, we concluded a significantly weaker effect of multi-species herbivory if the upper 95% confidence limit was smaller than 0 (see Figs. 1 and 2). We also used a χ^2 test to test whether the number of compounds with a ratio >0 and a ratio <0 differed from a 50:50 distribution. A 50:50 distribution would be expected if the effect of multi-species herbivory and the sum of *T. urticae* and *S. exigua* is equal. One replicate of the *T. urticae* treatment in cucumber was excluded from this analysis because we had no corresponding replicates of the *S. exigua* and multi-species herbivory treatments. Note that these tests reflect volatile responses to multi-species herbivory relative to responses to single-species herbivory. This allows us to compare these data between lima bean and cucumber because the data no longer depend on the absolute levels of volatiles detected by the different GC-MS systems.

Y-tube Olfactometer Experiments A closed system Y-tube olfactometer setup was used to test the responses of predatory mites to volatiles induced by multi-species herbivory (Takabayashi and Dicke 1992; de Boer et al. 2004). Odor sources consisted of four leaves of the same treatment that were cut from the plant just before an experiment; the petioles were wrapped in wet cotton wool and aluminum foil. Individual female predators were observed for a maximum of 5 min. A choice was recorded when the finish line, halfway one of the olfactometer arms, was reached within this period. Otherwise, it was recorded as “no-choice”. Sixteen to 23 predators were tested per

experimental day, and each experiment was repeated on 4 different days. Per replicate, new groups of predatory mites and new sets of leaves were used. We tested the responses of predatory mites to volatiles from bean or cucumber plants of the multi-species herbivory treatment vs. volatiles from plants of the *T. urticae* or *S. exigua* treatments.

The choices of predatory mites between two odor sources in the Y-tube olfactometer were analyzed with two-sided binomial tests to investigate whether the distribution of the predators differed from 50:50. Predators that did not make a choice were excluded from statistical analyses.

Results

Volatile Emission by Lima Bean Plants The main compounds emitted by *T. urticae*-infested bean plants were methyl salicylate (MeSA; compound number 2 in Fig. 1a), (3*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] (4), and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E*,*E*)-TMTT] (5). Herbivory by *S. exigua* alone resulted in the emission of similar amounts of (*E*)-DMNT (4), but amounts of MeSA (2) and (*E*,*E*)-TMTT (5) were small. Instead, *S. exigua*-infested bean leaves emitted large amounts of (*E*)- β -ocimene (3) and (*Z*)-3-hexen-1-ol acetate (7). Multi-species herbivory on bean plants induced the same volatile compounds as *S. exigua* feeding alone. Multi-species-infested plants emitted larger amounts of all volatile compounds than plants infested with a single herbivore species, except for (*Z*)-3-hexen-1-ol (1) that was emitted in larger amounts by *S. exigua*-infested plants, and MeSA (2) that was emitted in somewhat larger absolute amounts by *T. urticae*-infested plants.

In lima bean, herbivore treatment had a significant effect on the emission of (*Z*)-3-hexen-1-ol (1), (*E*)- β -ocimene (3), (*Z*)-3-hexen-1-ol acetate (7), 2-methylbutanal-*O*-methyl oxime (8), linalool (10), 1-octen-3-ol (11), hexyl acetate (12), β -caryophyllene (14), indole (16), (*E*)-2-hexen-1-ol acetate (17), (*Z*)- β -ocimene (21), and two unknown compounds (23 and 26) (all $P < 0.05$, Kruskal–Wallis tests, Fig. 1a). These compounds were all induced in larger amounts by *S. exigua* and/or multi-species herbivory than by *T. urticae*. Total volatile emission was also significantly affected by treatment ($P < 0.006$, Kruskal–Wallis test, Fig. 1a).

We compared the ratio of volatiles emitted in the multi-species herbivory treatment to the sum of volatiles emitted by the *T. urticae* and *S. exigua* treatments to determine whether infestation by two herbivore species simultaneously had additive, synergistic or antagonistic effects. Figure 1b shows the average ratio of multi-species herbivory to the sum of *T. urticae* and *S. exigua* for lima bean. Eight of

27 compounds were significantly more strongly induced by multi-species herbivory than by the sum of the two herbivore species feeding alone, suggesting synergistic effects (Fig. 1b). These compounds included members of various groups of chemicals, for example, (*E*)-2-hexen-1-ol acetate (17) and (*Z*)- β -ocimene (21). In addition, more compounds had a ratio > 0 than expected ($\chi^2 = 9.80$, $P = 0.002$). None of the compounds was significantly less induced by multi-species herbivory, suggesting the absence of antagonistic effects of *T. urticae* and *S. exigua* feeding simultaneously on lima bean.

Volatile Emission by Cucumber Plants Nine compounds were induced by herbivory on cucumber (all $P < 0.10$, Kruskal–Wallis tests; data not shown). The main compounds emitted by *T. urticae*-infested cucumber plants were (*Z*)-3-hexen-1-ol acetate (compound number 2 in Fig. 2a), (*E*,*E*)- α -farnesene (4), 3-methylbutanal-*O*-methyl oxime (6), (*E*)-DMNT (7), and (*E*)- β -ocimene (9) (Fig. 2a). Similar compounds were emitted in the largest amounts by *S. exigua*-infested cucumber plants. Multi-species herbivory resulted in a volatile blend that consisted of the same compounds as the blends induced upon herbivory by each of the herbivore species alone. The multi-species herbivory treatment resulted in the strongest induction of 3-methylbutanal-*O*-methyloxime (6) and (*E*)- β -ocimene (9), while *T. urticae*-infested plants emitted the largest amounts of (*Z*)-3-hexen-1-ol acetate (2). Herbivory by *T. urticae* alone and multi-species herbivory induced larger amounts of (*E*,*E*)-TMTT (8) than single herbivory by *S. exigua* alone. No MeSA was detected in any of the cucumber samples.

In cucumber, herbivore treatment had an effect on the emission of 3-methylbutanal-*O*-methyloxime (6) and (*E*)-DMNT (9) ($P < 0.05$, Kruskal–Wallis tests, Fig. 2a). Both compounds were induced in larger amounts by *T. urticae* or multi-species herbivory than by *S. exigua*. Total volatile emission was not affected by herbivore treatment in cucumber ($P > 0.10$, Kruskal–Wallis test, Fig. 2a).

Two volatile compounds [(*E*)-2-hexenal/(*Z*)-3-hexenal (1) and (*Z*)-3-hexen-1-ol acetate (2)] were induced significantly less by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* (Fig. 2b), suggesting the presence of antagonistic effects of the two herbivore species feeding on cucumber. The seven other compounds were equally induced by multispecies herbivory and the sum of the two herbivores feeding alone, indicating an additive effect of *T. urticae* and *S. exigua*. The distribution of compounds with a ratio > 0 or < 0 did not differ from 50:50 ($\chi^2 = 0.06$, $P = 0.81$).

Responses of *P. persimilis* We tested the preferences of the predatory mite *P. persimilis* for the volatile blends analyzed above in a Y-tube olfactometer. On bean and cucumber plants, predatory mites preferred HIPV induced by multi-



Fig. 3 Responses of *P. persimilis* to volatile blends in the Y-tube olfactometer. Odor sources consisted of four leaves from lima bean (**a** and **b**) or cucumber plant (**c** and **d**) simultaneously infested by *Tetranychus urticae* and *Spodoptera exigua* or infested by one of the herbivore species alone (*S. exigua*: **a** and **c**, *T. urticae*: **b** and **d**). Bars

present the overall percentages of predators choosing for each odor source. Numbers in bars are the total numbers of predators responding to each odor source. Choices between odor sources were analyzed with a two-sided binomial test (* $P < 0.05$; *** $P < 0.001$)

species herbivory to blends induced by either *T. urticae* or *S. exigua* alone (Fig. 3, $P \leq 0.02$, binomial tests).

Discussion

We investigated volatile emissions by lima bean and cucumber plants upon single and multi-species herbivory by *T. urticae* and *S. exigua*. In lima bean, we found that most compounds were more strongly induced by multi-species herbivory than by the sum of each of the herbivores feeding separately (Fig. 1b). This suggests that the two herbivores feeding together on the same plant have more than an additive effect on volatile emission, potentially indicating that biosynthetic pathways may act synergistically. It is also possible that one or both herbivore species inflicted more damage on multi-species-infested plants than on plants with conspecifics only. However, it is unlikely that spider mite feeding was affected by the presence of *S. exigua* because in a preliminary experiment oviposition rates of mites, which are correlated with food intake rates, were similar on leaf discs with or without *S. exigua* damage (de Boer et al., unpublished). Some studies have observed

that feeding by *S. exigua* is enhanced by the presence of aphids or a fungal pathogen (Rodríguez-Saona et al. 2005; Cardoza and Tumlinson 2006), but other studies have found no effect of the presence of whiteflies or a fungus (Rodríguez-Saona et al. 2003; Rostás et al. 2006).

In contrast to bean, we found that in cucumber plants, two volatile compounds were induced less by multi-species herbivory than by the sum of the two herbivores feeding separately, suggesting that their induction was suppressed by simultaneous feeding of *T. urticae* and *S. exigua*. Whether this reduction is caused by a negative interaction between the signal transduction pathways induced by spider mites and beet armyworms, or to reduced feeding of one or both herbivore species as a result of feeding on the same plant, remains to be investigated. From our data, it appears that plant species is an important determinant in the way in which herbivore-induced defense pathways interact upon multispecies herbivory.

The plant hormones salicylic acid (SA) and jasmonic acid (JA) play important roles in plant defense pathways and the production of HIPV (e.g., Kessler and Baldwin 2002; van Poecke and Dicke 2004). Interaction between these pathways may occur, for example, when SA inhibits JA-dependent defense pathways (e.g., Doares et al. 1995; Niki et al. 1998; Engelberth et al. 2001). To determine whether

synergistic (lima bean) and antagonistic (cucumber) interactions between biosynthetic defense pathways caused the changes in HIPV blends that we observed, endogenous levels of JA and SA should be determined in future studies. Arimura et al. (2002) showed that both JA and SA levels are significantly elevated in lima bean after 3 d of spider mite feeding, whereas several *Spodoptera* species are known to increase JA levels in corn and broad bean (Blechert et al. 1995; Schmelz et al. 2003). We are not aware of analyses of SA levels in response to *Spodoptera* feeding. No studies of JA and SA levels in plants upon multi-species herbivory are known to us either.

Another important question that needs to be addressed is the effect of herbivore sequence on volatile emission patterns, since in nature two herbivore species may infest an individual plant sequentially. In fact, some herbivore species are known to be attracted to HIPV themselves (e.g., Bolter et al. 1997; Kalberer et al. 2001), but spider mites are not attracted to *S. exigua*-infested lima bean plants (Horiuchi et al. 2003).

To determine whether the differences in volatile profiles upon multi-species herbivory and single-species herbivory are relevant in an ecological context, we investigated the behavior of the predatory mite *P. persimilis*, a specialist natural enemy of spider mites. Our results show that *P. persimilis* prefers volatiles induced by multi-species herbivory by spider mites and the non-prey caterpillar *S. exigua* to volatiles induced by one of the herbivore species feeding alone (Fig. 3). The strong preference of *P. persimilis* for the volatiles induced by multi-species herbivory may be explained by the relatively large amount of some specific compounds. Of the three major compounds emitted by spider mite-infested lima bean, two, namely (*E,E*)-TMTT and especially (*E*)-DMNT, were induced more strongly by multi-species herbivory, whereas induction of the third, MeSA, was somewhat reduced (Fig. 1a). Of the five major compounds emitted by spider mite-infested cucumber, four were induced more strongly by multi-species herbivory: (*E*)-DMNT, (*E*)- β -ocimene, (*E,E*)- α -farnesene, and 3-methylbutanal-*O*-methyl oxime, while (*Z*)-3-hexen-1-ol acetate emission was reduced (Fig. 2a). (*E*)-DMNT, (*E,E*)-TMTT, and (*E*)- β -ocimene are known attractants for *P. persimilis* (Dicke et al. 1990a, de Boer et al. 2004), and (*E,E*)-TMTT can be used by *P. persimilis* to discriminate between complex blends of HIPV (de Boer et al. 2004). Similarly, when *S. exigua*-induced volatiles are compared to the blend induced by multi-species herbivory, all of these compounds are produced in larger amounts in the latter. This suggests that a greater emission of (the sum of) attractive compounds may be the main determinant in attracting the predatory mite *P. persimilis*.

Previous studies on the responses of parasitoid wasps to HIPV also demonstrated a preference for HIPV induced by multi-species herbivory compared to single-species her-

bivory (Shiojiri et al. 2000; Vos et al. 2001; Cardoza et al. 2003; Moayeri et al. 2007). Rodriguez-Saona et al. (2005) even showed that naïve *Cotesia marginiventris* females were only attracted to tomato plants infested with their host *S. exigua* when the same plant was also infested with aphids. The presence of aphids thus made the host-infested plants more detectable. The opposite, i.e., multi-species herbivory-induced volatiles being less attractive than volatiles induced by only the host has been found in two cases: *C. plutellae* wasps preferred volatiles induced by their host, *P. xylostella*, to volatiles induced by their host and the non-host caterpillar *P. rapae* (Shiojiri et al. 2000), and *C. marginiventris* as well as the entomopathogenic nematode *H. megidis* were less attracted to volatiles from maize plants that were infested by a non-host in addition to their host (Rasmann and Turlings 2007). In such cases, the presence of non-host or non-prey herbivores may decrease the detectability or reliability of HIPV cues for natural enemies. For example, feeding by a non-prey or non-host herbivore may suppress the induction of volatile compounds used by natural enemies. Multi-species herbivory thus adds a new dimension to the trade-off between reliability and detectability of searching cues used by natural enemies of herbivorous arthropods (Vet and Dicke 1992).

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