

Discrimination of *Medicago truncatula* volatiles infested with prey or non-prey volatiles by the predatory mite *Phytoseiulus persimilis*



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Abstract

To find prey species carnivorous arthropods can use compounds produced by plants after infestation by herbivores. The composition of these induced volatile blends is influenced by the plant species and type of plant damage and other factors. However, not every plant and predator show these multitrophic interactions. We investigated the response of a predatory mite (*Phytoseiulus persimilis*) to damage induced volatiles produced by a legume species (*Medicago truncatula*). This research showed that *P. persimilis* is attracted to the prey (*Tetranychus urticae*) induced volatiles from *M. truncatula* as well as non prey (*Spodoptera exigua*) induced volatiles from *M. truncatula*. However they were not able to discriminate between the prey and non prey induced volatiles. There was no difference in response to the differently induced volatile blends between Lima bean reared and *M. truncatula* reared *P. persimilis*. Therefore the previous experiences of *P. persimilis* did not influence the response to the differently induced volatiles. The amount of herbivores inducing the volatiles does influence the response of *P. persimilis*. However even with damage of few *S. exigua* *P. persimilis* was not able to discriminate between the prey and non prey induced volatiles. Based on the results of this study, we conclude that the multitrophic relation between *M. truncatula* and *P. persimilis* doesn't seem to be ideal for indirect defence research.

Introduction

Plants are stationary, which means they cannot escape from animals attacking them. To be able to defend themselves against pathogens or herbivorous arthropods, plants have developed two types of defences: direct and indirect defence. Further, these mechanisms can be either constitutive, which means that they are always present, or induced. Induced plant defences mean that the defence is triggered by herbivore attacks (Arimura et al 2005). With direct defence plants defend themselves directly against herbivores, for example by having thorns, or producing chemicals that repel or deter herbivorous arthropods (Arimura et al 2005). Indirect defence means that plants defend themselves indirectly against attackers by attracting predators or parasitoids of the herbivore (e.g. Dicke and Hilker 2003, Arimura 2005). One mechanism of indirect defence is that plants produce certain volatile compounds when they are attacked by herbivores (e.g. Arimura et al 2005). These compounds can attract natural enemies of the herbivore (Dicke and Hilker 2003). Natural enemies can effectively reduce the number of herbivorous organisms and therefore can increase the fitness of the plant, since the

damage plants suffer from herbivory will be less (Dicke and van Loon 2000, Van Loon et al 2000).

Plants always produce volatiles, but when they are attacked by a herbivore they can change the amount and the composition of the emitted volatile compounds (Dicke and Van Loon 2000). The composition of induced volatiles can differ between plant species (Van den Boom et al 2004), plant age, biotic, and abiotic factors (De Boer and Dicke 2006, DeMoraes et al 1998). Plants also emit differently composed volatile blends when damaged by herbivores with different feeding types and therefore different type of plant damage (Leitner et al 2005). Furthermore, volatile blends emitted after mechanical damage can also differ from those induced by herbivory (Dicke and Van Loon 2000, Dicke and Hilker 2003, Mithöfer et al 2005, Van den Boom et al 2004). The increase of quantity and change of composition of the volatile blends can be a good indication for the presence of prey. Many predatory arthropods use the volatile blends produced by plants since the herbivore has a low biomass compared to the plants they feed on. The difference in biomass between herbivores and plants causes the delectability of chemical cues from herbivores to be lower than those of plants. Furthermore, prey organisms are under selective pressure to avoid producing cues indicating their presence (Dicke and Van Loon 2000).

Induced volatiles emitted by plants as a result of herbivore damage does not necessarily mean that a suitable prey of the carnivorous arthropod is present (De Boer and Dicke 2006). Therefore, the ability to distinguish between induced volatile blends as a result of prey or a non-prey species infestation is an important factor when using plant volatiles to locate prey organisms, which consequently should increase the fitness of the carnivorous arthropod. Since volatile blends can differ for certain plant species and type of damage and other factors, it is important for the carnivorous arthropod to be able to recognize and learn new volatile blend patterns. The ability of carnivores to learn new volatile blends has been shown in several studies (e.g. De Boer et al 2005B).

Many studies have shown that with certain plant species predatory mites can prefer spider mite induced volatiles to volatiles of uninfested plants (e.g. Krips et al 1999, Shimoda and Dicke 2000, Shimoda et al 2002, De Boer et al 2005B, De Boer and Dicke 2006). Most of these studies were done on Lima bean (Shimoda and Dicke 2000, Shimoda et al 2002, De

Boer and Dicke 2006). On Cucumber (De Boer and Dicke 2006) and *Gerbera jamesonii* the same response of *P. persimilis* was also found (Krips et al 1999).

As stated before plants can also produce volatiles when damaged by herbivores with different feeding types (De Boer et al 2004, Leitner et al 2005). So when a plant is damaged by caterpillars they also produce volatile blends, these induced volatiles can also attract *Phytoseiulus persimilis* prefers cucumber and Lima bean plants damaged by caterpillars over undamaged ones (Shimoda and Dicke 1999, Shimoda and Dicke 2000, De Boer and Dicke 2006).

The induced volatiles produced by plants as result of different type of damage show resemblances, many of the same compounds are produced (De Boer et al 2004, Leitner et al 2005). However the composition of compounds in these induced volatiles can differ among plants and different feeding types (De Boer et al 2004, Van den Boom et al 2004, Leitner et al 2005). Several studies already showed that on Lima bean *P. persimilis* could distinguish between prey induced and non prey induced volatile blends (Shimoda and Dicke 2000, De Boer et al 2005B, De Boer and Dicke 2006).

During this research the predatory mite, *P. persimilis*, was used. *P. persimilis* is a specialised predator on the two spotted spider mite (*Tetranychus urticae*). The caterpillar of *Spodoptera exigua* represented the non-prey species for *P. persimilis*. *T. urticae* is a herbivore with piercing-sucking mouthparts, whereas *S. exigua* is a herbivore with chewing mouthparts.

In this study, *Medicago truncatula* (Fabales, Fabaceae) was used. *M. truncatula* is an interesting plant for research on indirect defence since it is shown that it reacts differently to different type of herbivore damage (Leitner et al 2005). Since it has a small genome, and has the ability to be genetically transformed this species can also be useful for research on the molecular level. As a legume, *M. truncatula* can form a symbioses with mycorrhizal fungi (Young et al 2003, Frugoli and Harris 2001). Several studies suggest that the interaction of the belowground and aboveground compartments of a plant can influence also the aboveground multitrophic interactions (Van der Putten et al 2001, Van Dam et al 2003, Bezemer and Van Dam 2005). Furthermore, studies on the influence of the mycorrhizal fungi on indirect defence of plants are rare (Van Putten et al 2001).

In a preliminary study *P. persimilis* showed a preference to volatiles of either spider mite or caterpillar infested *M. truncatula* compared to volatiles of uninfested *M. truncatula*. However, *P. persimilis* did not discriminate between *M. truncatula* volatiles infested by *T. urticae* or caterpillars. This could be caused by the rearing history of the predatory mites. *P. persimilis* used in that study were reared on Lima bean, but in the bioassays they were exposed to volatiles of *M. truncatula*. Previous experiences of *P. persimilis* could influence their preference for certain induced volatile blends (Dicke et al 1998, Kripps et al 1999, De Boer et al 2004, De Boer et al 2005B). Shimoda and Dicke (2000) showed that *P. persimilis* could distinguish between volatiles induced by a prey and by non-prey herbivore species on a plant it did not experienced before. However, other studies showed that *P. persimilis* was not able distinguish between these different induced volatiles of a plant they did not experience before (De Boer et al 2005B, De Boer and Dicke 2006). The latter studies shows that rearing history could influence this ability since *P. persimilis* could distinguish between prey and non prey induced volatiles of plants they experienced before (De Boer et al 2005B, De Boer and Dicke 2006).

The preliminary study showed that *P. persimilis* might not be able to discriminate between the volatiles emitted by *M. truncatula* infested by a prey from the volatiles emitted by *M. truncatula* infested by caterpillars. To see if the amount of herbivores influences this discrimination the amount of *S. exigua* during the tests was decreased. Some literature suggested that the amount of herbivores could influence the choice of *P. persimilis* (Horiuchi et al 2003, De Boer et al 2005B). We expected that less herbivores induce less quantity of the volatile blend. By decreasing the amount of *S. exigua* it might be easier for *P. persimilis* to discriminate between *S. exigua* and *T. urticae* induced volatile blends.

The general research question in this research is: Is the predatory mite, *P. persimilis*, able to discriminate between volatile of *M. truncatula* infested by either a prey species (*T. urticae*) or by a non-prey (*S. exigua*) species? The following hypotheses will be tested:

Hypotheses

- 1) Volatiles of *M. truncatula* infested by *T. urticae* will be more attractive to *P. persimilis* than uninfested ones.

2) Volatiles of *M. truncatula* infested by *S. exigua* will be more attractive to *P. persimilis* than uninfested ones.

3) *P. persimilis* will show no preference for volatiles of *M. truncatula* either infested by *T. urticae* or infested by *S. exigua*.

4) The density of *S. exigua* will influence the ability of *P. persimilis* to discriminate between spider mite infested and caterpillar infested *M. truncatula* volatiles.

5) The host plant on which the predatory mites were reared will influence the ability of *P. persimilis* to discriminate between spider mite infested and caterpillar infested *M. truncatula* volatiles

Material and Methods

Plant species:

Seeds of *M. truncatula* were placed in a Petri dish with a moisturised paper and were allowed to germinate for 4 days in the dark. Seedlings were then transferred to pots filled with soil and were kept in a climate chamber at 23°C, 60% r.h., and L:D 16h:8h. *M. truncatula* used in tests were between 6 to 8 weeks old.

Arthropods:

T. urticae was continuously reared on Lima bean plants (*Phaseolus lunatus cv Sieva*) at 25 ± 5 °C, 50 – 70% humidity and a 16L:8D photoperiod. *S. exigua* was reared on an artificial diet (Smits 1987) at 23 °C. The *S. exigua* used in the test were 3 to 4 days old and of the L1 instar. *P. persimilis* was continuously reared on Lima bean leaves infested with *T. urticae* at 23 ± 1 °C, 50 – 70% humidity and permanent light. In order to test whether the rearing history has an influence on the subsequent behavioural choice of *P. persimilis* a rearing on *M. truncatula* that was infested with *T. urticae* was established. The conditions for this rearing were the same as used for the rearing on Lima beans (23 ± 1 °C, 50 – 70% humidity and permanent light). All experiments were done with adult females of *P. persimilis*. The *P. persimilis* were starved for 2 hours prior to the bioassays.

Y-tube olfactometer

During all experiments *P. persimilis* were given a choice in a Y-tube olfactometer (Takabayashi and Dicke 1992) between odours of 2 groups of plants which were differently treated. 4 plants of the same treatment were used as an odour source. A constant purified airflow was provided at 4 L/min. Individual *P. persimilis* were introduced at the base of the olfactometer on a metal wire running through the centre of the tube. The individual *P. persimilis* was observed in the olfactometer for 10 minutes. Their choice for a certain odour source was recorded. When they did not make a choice within the 10 minutes a “no choice” was recorded. The odour sources were interchanged after 10 individual predatory mites. The experiments were repeated on different days, with new odour sources and new predatory mites.

Treatment of *M. truncatula*

M. truncatula was infested with 50 *T. urticae* per plant. *T. urticae* was allowed to feed on the *M. truncatula* for 48 hours.

4 or 2 *S. exigua* per plant (depending on the test) were also allowed to feed on *M. truncatula* for 48 hours. During this period the plants were kept at the same conditions as described above.

Olfactory bioassays

Experiment 1: To answer hypothesis 1, 2 and 3 different plant treatments were tested against each other with *P. persimilis* reared on Lima bean (Table 1).

Experiment 2: To test hypothesis 4 different densities of *S. exigua* were used. 4 *S. exigua* per plant were used in experiment 1. The same test was also done with 2 *S. exigua* per plant (Table 1).

Experiment 3: To be able to compare the different rearing histories (hypothesis 5), both new *P. persimilis* reared on Lima bean and *P. persimilis* reared on *M. truncatula* were tested on the same plant batches (Table 1).

Table 1: The different plant treatments tested against each other in the Y-tube olfactometer for the different experiments.

Experiment	Predatory mites reared on	Treatment A	Treatment B
1	Lima Bean	Plants infested with 50 <i>T. urticae</i>	Uninfested plants
1	Lima Bean	Plants infested with 4 <i>S. exigua</i>	Uninfested plants
1	Lima Bean	Plants infested with 50 <i>T. urticae</i>	Plants infested with 4 <i>S. exigua</i>
2	Lima Bean	Plants infested with 2 <i>S. exigua</i>	Uninfested plants
2	Lima Bean	Plants infested with 50 <i>T. urticae</i>	Plants infested with 2 <i>S. exigua</i>
3	Lima Bean	Plants infested with 50 <i>T. urticae</i>	Uninfested plants
3	<i>Medicago truncatula</i>	Plants infested with 50 <i>T. urticae</i>	Uninfested plants
3	Lima Bean	Plants infested with 4 <i>S. exigua</i>	Uninfested plants
3	<i>Medicago truncatula</i>	Plants infested with 4 <i>S. exigua</i>	Uninfested plants
3	Lima Bean	Plants infested with 50 <i>T. urticae</i>	Plants infested with 4 <i>S. exigua</i>
3	<i>Medicago truncatula</i>	Plants infested with 50 <i>T. urticae</i>	Plants infested with 4 <i>S. exigua</i>

Statistical analysis

Predatory mites that did not make a choice (2.4% of 1020 *P. persimilis*) were excluded from the statistical analyses. For all hypotheses the difference in predatory mites attracted by the different treatments will be determined by a two-sided Binomial test. Also a GLIM was used to determine differences between two different olfactory bioassays. When necessary, the model was corrected for over dispersal.

Results

Experiment 1:

Figure 1 shows that *P. persimilis* significantly preferred volatiles emitted by spider mite infested *M. truncatula* to volatiles of uninfested *M. truncatula* (N= 79, binomial test: P <0.01) (Fig. 1). *P. persimilis* also preferred volatiles produced by *M. truncatula* infested by 4 *S. exigua* over volatiles produced by an uninfested *M. truncatula* (N=79, binomial test: P<0.001). However, *P. persimilis* did not discriminate between volatiles of *M. truncatula* infested by 50 *T. urticae* from those infested by 4 *S. exigua* (N=78, binomial test: P=0.308).

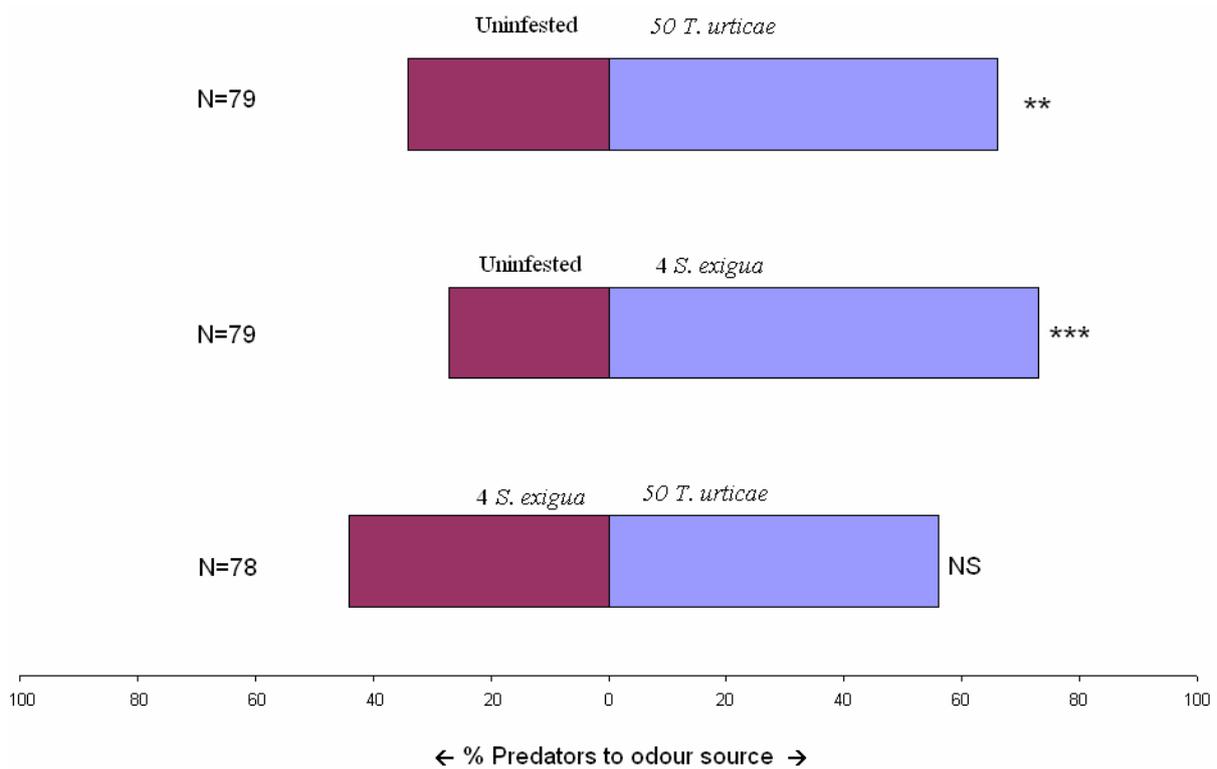


Figure 1: Response of *P. persimilis* to volatiles of *M. truncatula* damaged by either prey (50 *T. urticae* per plant), non prey (4 *S. exigua* per plant) or were left undamaged. The bars represent the percentage of *P. persimilis* choosing each odour source. NS= $P > 0.05$; *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$.

Experiment 2:

The plant damage as a result of amount of herbivores influences the response of *P. persimilis* (figure 2). *P. persimilis* does not show a preference between the 2 *S. exigua* per plant induced volatiles compared to uninfested plants (N=137, binomial test: $P=0.608$). However even though the amount of *S. exigua* per plant is halved, *P. persimilis* still does not discriminate between the prey induced volatile blends from the non prey induced volatile blends (N=135, binomial test: $P=0.085$), although this is almost a significant difference.

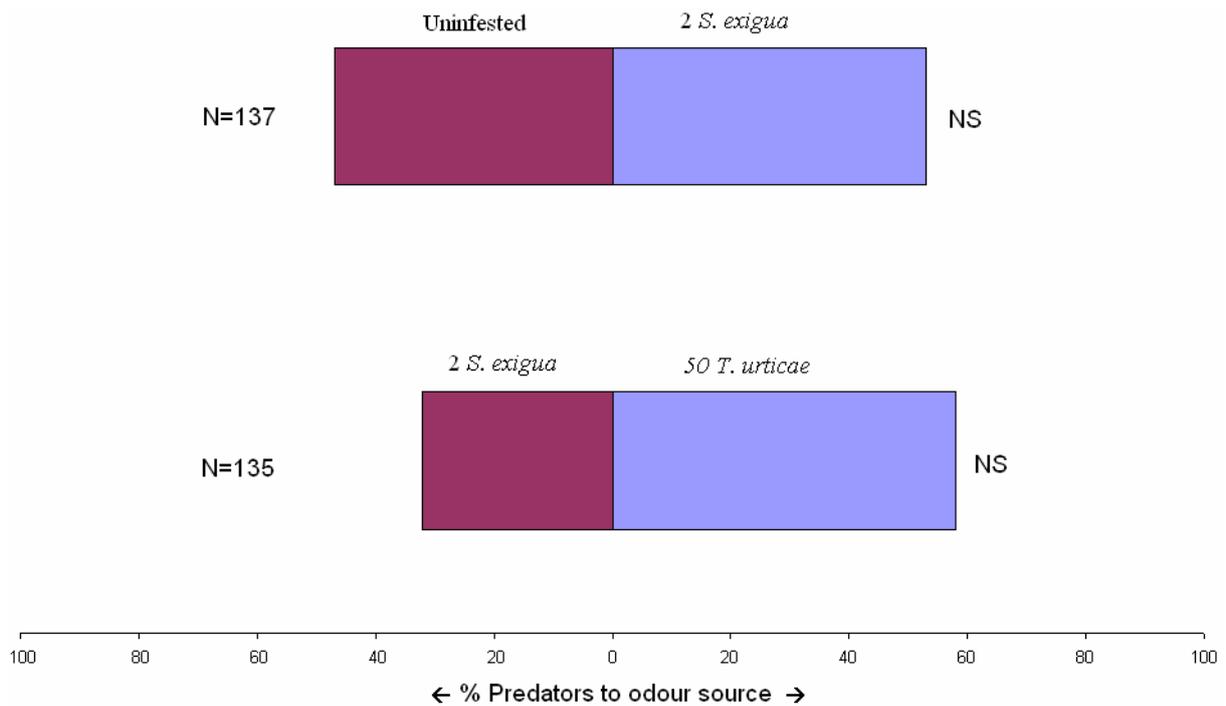


Figure 2: Response of *P. persimilis* to the volatiles of *M. truncatula* damaged by either prey (50 *T. urticae* per plant), fewer non prey (2 *S. exigua* per plant) or were left undamaged.. The bars represent the percentage of *P. persimilis* choosing each odour source. NS= $P > 0.05$; *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$.

Experiment 3:

Just like in the test of hypothesis 1 there is a significant preference by *P. persimilis* reared on Lima bean for the volatile produced by *M. truncatula* when damaged by *T. urticae* over undamaged plants (N=98, binomial test: $P < 0.003$). Also when the *P. persimilis* was reared on *M. truncatula* they preferred volatiles emitted by *M. truncatula* when damaged by *T. urticae* over undamaged (N=120, binomial test: $P < 0.003$) (Figure 3). There was also no difference in the response between *P. persimilis* reared on *M. truncatula* and *P. persimilis* on Lima bean (GLIM: DF=1, Chi-square = 5.99, $P = 0.844$).

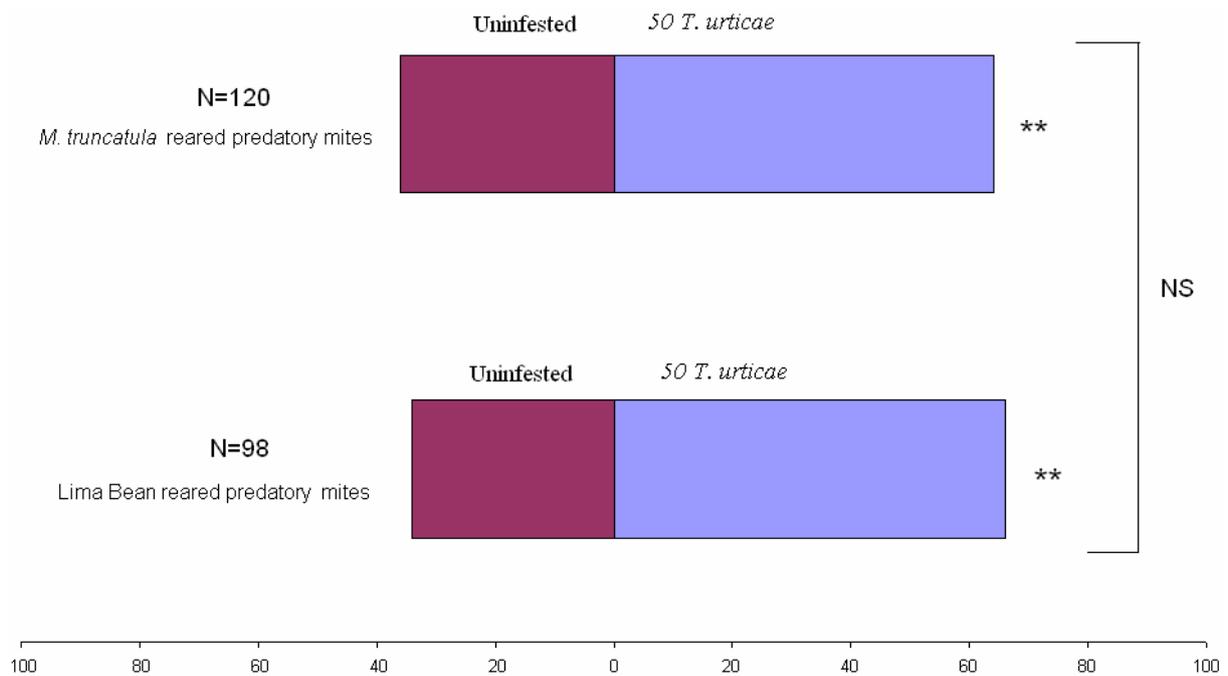


Figure 3: Response of *P. persimilis* with different rearing history to the volatiles of *M. truncatula* damaged by either prey (50 *T. urticae* per plant) or were left undamaged. The bars represent the percentage of *P. persimilis* choosing each odour source. NS= $P > 0.05$; *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$.

P. persimilis also preferred volatiles emitted by *M. truncatula* when damaged by *S. exigua* to undamaged plants. The preference was significant both when *P. persimilis* was reared on *M. truncatula* (N=77, binomial test: $P < 0.002$) and when they were reared on Lima bean (N=56, binomial test: $P < 0.001$) (Figure 4). No difference occurred in the response between *P. persimilis* reared on *M. truncatula* and *P. persimilis* on Lima bean (GLIM: DF=1, Chi-square = 9.58, $P = 0.336$).

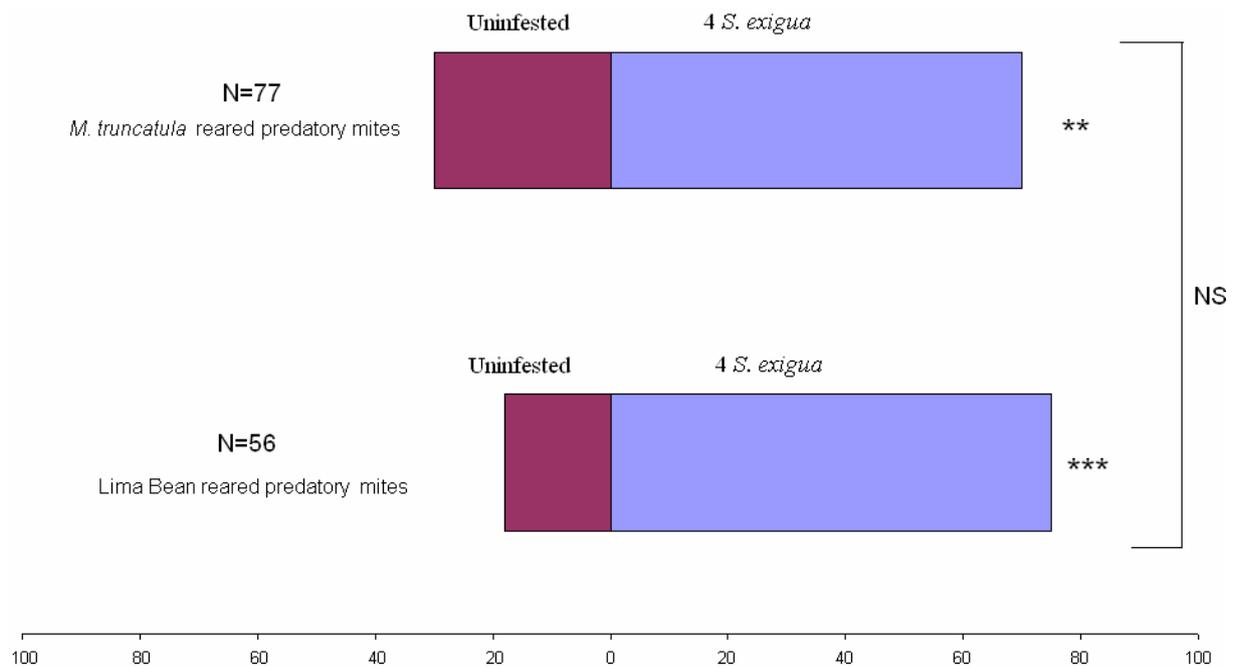


Figure 4: Response of *P. persimilis* with different rearing history to the volatiles of *M. truncatula* damaged by either non-prey (4 *S. exigua* per plant) or were left undamaged. The bars represent the percentage of *P. persimilis* choosing each odour source. NS= $P > 0.05$; *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$.

Figure 5 shows that there was no difference between the attractiveness of *T. urticae* induced volatiles emitted by *M. truncatula* compared to the *S. exigua* induced volatiles emitted when *P. persimilis* was reared on Lima bean (N=60, binomial test: $P=1.00$). When *P. persimilis* was reared on *M. truncatula* there was also no preference (N=80, binomial test: $P=0.428$). Again there was also no difference in response between *P. persimilis* with different rearing histories (GLIM: DF=1, Chi-square = 9.33, $P=0.616$).

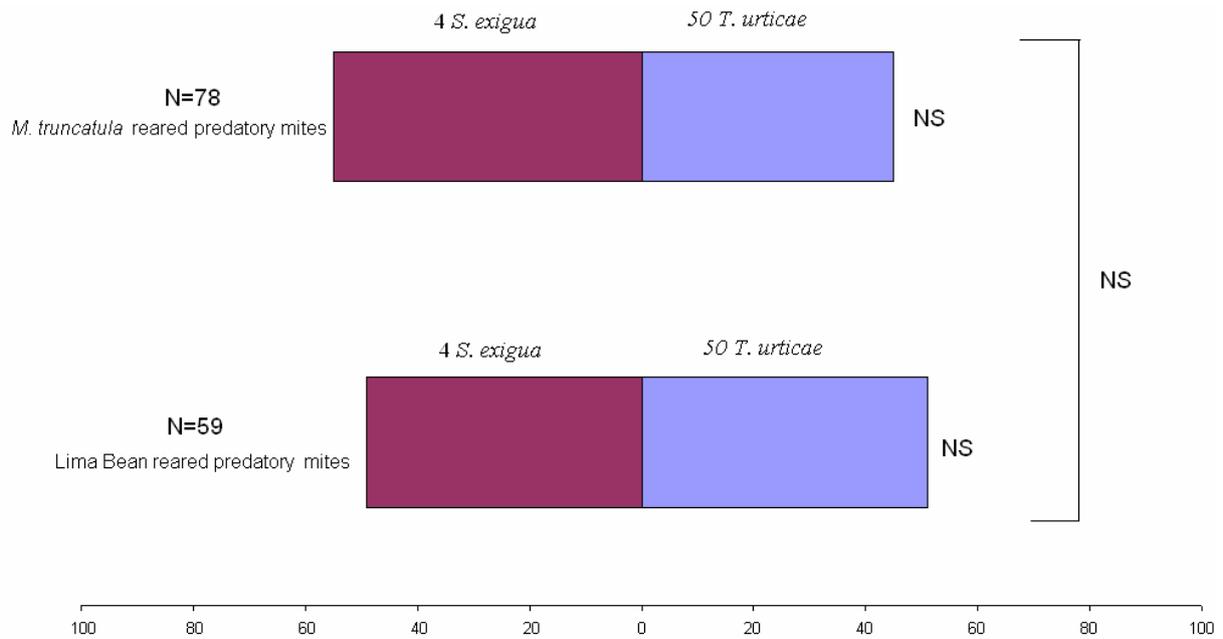


Figure 5: Response of *P. persimilis* with different rearing history to the volatiles of *M. truncatula* damaged by either Prey (50 *T. urticae* per plant) or non-prey (4 *S. exigua* per plant). The bars represent the percentage of *P. persimilis* choosing each odour source. NS= $P > 0.05$; *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$.

Discussion

Experiment 1 shows that hypothesis 1,2 and 3 are all accepted. When *P. persimilis* is reared on Lima bean it prefers the volatiles from a damaged *M. truncatula* (either by prey or non prey) over the volatiles from a undamaged *M. truncatula*. This shows that *P. persimilis* has a preference for damaged plants, which could inhabit a prey. However when *P. persimilis* is reared on Lima bean it does not discriminate between prey induced and non prey induced volatile blends.

Experiment 2 also shows that the herbivore density influences the attractiveness of *M. truncatula* volatiles to *P. persimilis*. This is in agreement with other studies, which also found that the density of herbivores influenced the attractiveness of the volatile blends by *P. persimilis* (Horiuchi et al 2003, De Boer et al 2005B). The 2 *S. exigua* induced volatile are not attractive enough to *P. persimilis* to distinguish between uninfested and damaged plants. But it is attractive enough for *P. persimilis* not to discriminate between prey and non-prey. Therefore hypothesis 4 is not accepted since the amount of herbivores did not influence the ability of *P. persimilis* to discriminate between prey and non prey induced volatiles. Apparently the 2 *S. exigua* induced volatile blend does not contain enough specific compounds for *P. persimilis* to discriminate between damaged and undamaged. However,

since there is no difference in preference between prey and 2 *S. exigua* induced volatiles the volatile blend seems to contain enough specific chemicals so that *P. persimilis* is not able to discriminate between non-prey and prey.

Experiment 3 was done to answer hypothesis 5, to see the influence of rearing history on the response of *P. persimilis*. If the rearing history has an influence on the response of *P. persimilis* then it shows that *P. persimilis* can learn and discriminate between the different induced volatile blend patterns. The rearing on *M. truncatula* does not seem to influence the ability of *P. persimilis* to discriminate between the volatiles produced after infestation with a prey or a non-prey species. This is surprising, since with other plant species with different volatile blend compositions as a result of different type of damage the rearing history does seem to influence this ability (Kripps et al 1999, De Boer et al 2004, De Boer et al 2005B). With the study that did not show an influence of rearing history *P. persimilis* was already able to discriminate between prey and non prey induce volatiles from a plant it had not experienced before (Shimoda and Dicke 2000). These results call for the following question: is *P. persimilis* able to learn the prey induced volatiles by *M. truncatula*, and discriminate them from non prey induced volatiles? Different studies showed that *P. persimilis* can be influenced by the host plants they previously experienced, and they have the ability to learn (Kripps et al 1999, De Boer et al 2004, De Boer et al 2005B). It is also known that *M. truncatula* produces different volatile blends for different type of damage (Leitner et al 2005). So why do the *P. persimilis* that were reared on *M. truncatula* not show that they are influenced by their rearing history compared to the *P. persimilis* that was reared on Lima bean? They do not show that they are attracted by the volatile blend they have experienced during their rearing, and therefore know it means prey species.

This could be caused by the composition of the volatile blend produced by *M. truncatula* when damaged by *T. urticae*. In the study of Leitner and co-workers (2005) the composition of the volatile blend produced by *M. truncatula* as a result of damage by *T. urticae* and as a result of damage by a caterpillar was shown. Leitner and co-workers (2005) shows that *M. truncatula* produces much more different compounds and higher quantity of volatiles when damaged by a caterpillar. Furthermore, *M. truncatula* does not produce certain compounds when damaged by *T. urticae* that it does produce when it is damaged by a caterpillar. One of these compounds is methyl salicylate (MeSA) of which it is shown that it plays an important role in forage decision making of *P. persimilis* (De Boer et al 2004, De Boer et al 2005B, De

Boer and Dicke 2005A). These studies showed that the presence of MeSA could attract *P. persimilis*.

Furthermore nearly all compounds produced by *M. truncatula* found in the study of Leitner and co-workers (2005) that were produced as a result of damage by *T. urticae* were also produced as a result of damage by caterpillars. So even if *P. persimilis* was reared on *M. truncatula* and had experience with the volatile blends produced as a result of the damage done by their prey, it might be difficult for *P. persimilis* to use specific compounds, that are only produced by *M. truncatula* when damaged by *T. urticae* and not when damaged by non-prey, to discriminate between the volatile blends produced by *M. truncatula* as a result of damage by a prey and a non-prey. The other compounds that are not present in the *T. urticae* induced volatile blends but are present in the *S. exigua* induced volatile blend could be regarded as background odours, since they are present but they are extra compounds to the in rearing experienced compounds. These background odours do not necessarily influence the choice of *P. persimilis* (De Boer and Dicke 2006). So that could also be a factor in the inability of *P. persimilis* to discriminate between the volatile blends produced by *M. truncatula* as a result of damage by a prey from a non-prey.

This brings another question mark, if *P. persimilis* is attracted to volatile blends which contains MeSA (Shimoda et al 2002, De Boer et al 2004, De Boer and Dicke 2005A), and when most compounds produced as a result of damage by *T. urticae* are also produced as result of damage by *S. exigua*, then why is *P. persimilis* reared on Lima bean not attracted to the *S. exigua* induced volatile blends, since those volatile blends consist of among others MeSA? *M. truncatula* probably produces more compounds then tests in recent studies are able to pick up. Maybe there are other compounds that were produced by *M. truncatula* as a result of *T. urticae* infestation that were not detectible in the tests of Leitner and co-workers (2005) which also attracted *P. persimilis*. To explain the results of this study, these compounds should not attract *P. persimilis* that much that they show a preference for volatile of *T. urticae* infested *M. truncatula* over the volatiles of *S. exigua* infested *M. truncatula*.

So to answer the overall research question: Is the predatory mite, *P. persimilis*, able to discriminate between volatile of *M. truncatula* infested by either a prey species (*T. urticae*) or by a non-prey (*S. exigua*) species? This research shows that *P. persimilis* was not able to discriminate between differently induced volatile patterns of *M. truncatula*. Other research

showed that *P. persimilis* was able to discriminate between prey and non prey induced volatile of Lima bean (Shimoda and Dicke 2000, De Boer et al 2005B, De Boer and Dicke 2006). But this is not the case with the induced volatile blends of *M. truncatula*. Therefore the multitrophic relation between *M. truncatula* and *P. persimilis* doesn't seem to be an ideal model relation for indirect defence research.

Outlook for future research

In this study the amount of non-prey varied. In future research it might be useful to vary the amount of prey per plant. This might influence the attractiveness of the volatile blends for *P. persimilis* more then the amount of non-prey (De Boer et al 2005B).

The amount of herbivores feeding on *M. truncatula* was controlled, however amount of herbivores does not necessarily have a linear correlation with feeding damage, since herbivores can die, or eat less. Especially with only 2 *S. exigua* feeding per plant the loss of one herbivore is huge. So in further research controlling the damage done by the herbivores instead of the amount of herbivores could lead to even more reliable data.

Also in this research *P. persimilis* was reared on *M. truncatula* leaves in Petri dishes for the tests on the influence of rearing history. Because the leaves were cut from a plant and put in Petri dishes the volatile blend could have been changed. For further research on rearing history it might be good to rear *P. persimilis* on complete *M. truncatula* plants.

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