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Leaf volatiles and polyphenols in pear trees infested by *Psylla pyricola*. Evidence of simultaneously induced responses

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Summary. Feeding by the homopteran *Psylla pyricola* on leaves of pear trees induces the production of volatile compounds, such as (*E,E*)- α -farnesene and methyl-salicylate, as well as the production of polyphenols. The inference on induction is based on GC-MS and HPLC chromatograms from the same samples of *Psylla* infested leaves, leaves from the same pear tree before *Psylla* infestation and uninfested leaves from other pear trees. *Psylla* infestation greatly enhanced the production of volatiles ((*E,E*)- α -farnesene, methyl-salicylate and others) and triggered the production of new polyphenols, characterized by much longer retention times.

However, the responses to infestation depend critically on leaf age (defined by leaf distance to apex). With respect to the leaf volatiles it appears that infested, old leaves produce fewer compounds and lower amounts of the volatiles than infested, young leaves. Moreover, there seem to be differences in pattern. Relative to (*E,E*)- α -farnesene, methyl-salicylate was found in much lower amounts in heavily infested, old leaves. With respect to polyphenols it was found that infested old leaves collected in August have polyphenols with the same retention times, but more or less equal amounts as uninfested young leaves collected in May. This shows that *Psylla* infestation causes the induced response mostly in young leaves.

The induced leaf volatiles may act as synomones to heteropteran bugs. As shown elsewhere, *Anthocoris nemoralis* responds significantly to (*E,E*)- α -farnesene and methyl-salicylate when offered in pure form against clean air in a Y-tube olfactometer. The effect of polyphenols on the performance of *P. pyricola* is not yet known. Hence, a role in direct defence is still to be investigated.

Key words. volatiles – induced responses – α -farnesene – methyl-salicylate – polyphenols – pear tree – Homoptera – *Psylla pyricola* – Hemiptera – *Anthocoris nemoralis*

Introduction

In a series of laboratory and field studies it was demonstrated that pear trees (*Pyrus communis* L.), when attacked by psyllids (*Psylla pyricola* Forster and *P. pyri* L.), start producing volatiles that are attractive to the anthocorid predators *Anthocoris nemorum* (L.), *A. nemoralis* (Fabricius), and *Orius* spp. (Drukker & Sabelis 1990; Drukker *et al.* 1995). This plant response may be interpreted as a form of induced indirect defense (*sensu* Price 1986) through which a plant promotes the effectiveness of a herbivore's natural enemies. Also the presence of direct defenses in pear trees against psyllids has been suggested. For example, Matias *et al.* (1990) have found that some pear cultivars (Leclerc, Beurré Hardy, Passe Crassane and Doyenne du Comice), showed a higher content of polyphenols and a lower level of infestation than other pear cultivars (Williams and Rocha). Here, we report on induced responses of both volatiles and polyphenols in the same pear trees under attack by the psyllid *P. pyricola*.

Materials and methods

The pear trees used in the experiments were young potted trees of variety 'Conference' grafted on pear rootstocks, and purchased from commercial nurseries. These trees were infested with *Psylla pyricola* (Homoptera, Psyllidae) collected in a pear orchard in Watergraafsmeer (Amsterdam, The Netherlands).

Experiment 1

In early April 1993 one 4-year-old tree was transferred from outdoors, where it had been for the previous years, into a cage in a climate room ($\pm 20^\circ\text{C}$; 60–70% RH; L:D = 17:7h). Field-collected twigs with *P. pyricola* adults and eggs were added to infest this tree. In July when the *Psylla* population had reached a high density, two other uninfested trees of similar age were transferred from the field to the same cage, now in another climate room ($\pm 23^\circ\text{C}$; 65–70% RH; L:D = 16:8h). In May (shortly after the trees had started to flush) and in August (shortly after the trees had stopped to flush) samples of ten leaves each were randomly collected from these trees. The leaf samples were used for (1) head space analysis of volatile compounds, (2) assessment of infestation levels, and (3) analysis of polyphenols. The results were compared to those from a tree of exactly the same age, placed outdoors. Regular leaf inspections showed that this tree was free of psyllids and any other herbivore throughout the season.

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Experiment 2

In January 1994, two 1-year old trees were placed in separate cages in a climate room ($\pm 23^{\circ}\text{C}$; 65–70% RH; L:D = 16:8h). One tree gradually became infested after the release of *P. pyricola* adults, the other tree was kept uninfested to serve as control. At different moments (eight times) samples of 5–10 young leaves were taken from each of both trees, and used for analysis (as in experiment 1).

Analysis of volatiles

Volatiles from freshly cut leaves (blades and petioles) were collected during 120 min on Tenax adsorbance tubes (airflow: ca 100 ml/min.). Then, fresh weight of the blades was measured. The tubes were stored in the dark at room temperature until they were analysed by means of combined gas chromatography and mass spectrometry (GC-MS). Trapped compounds were released from the adsorbance in a thermodesorption cold trap unit at 250°C for 10 min, with a He-flow of 10 ml/min. Desorbed compounds were cryofocused in a cold trap at -90°C and subsequently analysed on a Supelcowax-10 capillary column. The temperature program used was: 4 min at 40°C , from 40 to 140°C at $3^{\circ}/\text{min.}$, from 140 to 270°C at $6^{\circ}/\text{min.}$ The column was connected to a mass spectrometer. Compounds were identified by comparison of mass spectra with those in the Wiley-Library and our own specialised library of natural products, and by comparison of retention times (for further details see Scutareanu *et al.* submitted). To normalize gaschromatograms the peak areas were divided by the total fresh weight of the leaves used for volatile collection.

Analysis of polyphenols

After collection of volatiles all psyllid stages, excretions and excrements were removed from the leaves by gently whipping with paper tissue. Fresh leaves (1 g fresh weight) were macerated in 15 μl acetone. After filtration, the dried extract was dissolved in 250–500 ml ethanol 96% and stored at -20°C until it was used for HPLC determination. Before running the HPLC, each extract was centrifuged and then diluted $10\times$ in ethanol 96%. In the first experiment, HPLC-chromatograms were obtained using a LKB 2152 controller, under the conditions described by Nieman *et al.* (1992). In the second experiment, HPLC conditions were as follows: gradient elution on a C-18 column; solvent A, 95% water + 5% acetonitrile; solvent B, 25% water + 75% acetonitrile + phosphate (pH = 2); detection at $\lambda = 280\text{ nm}$; flow rate, 0.8 ml/minute. To normalize HPLC chromatograms, peaks were expressed as a percentage of the total peak area (first experiment) or of the peak height sum (second experiment).

Results and discussion

Psylla infestation on experimental trees

By the time of leaf picking for volatile collection and subsequent polyphenol analysis, the mean numbers of *P. pyricola* nymphs per leaf are as shown in Figure 1. Not shown are eggs, adults and honeydew which were abundantly present on the infested leaves in both experiments.

Volatile compounds from *Psylla*-infested and uninfested leaves

Both qualitative and quantitative differences were found in the composition of the volatile blends from *Psylla*-infested leaves versus uninfested control leaves. In both experiments the number of compounds was higher in the samples from the infested leaves, than in samples from the controls. An extensive treatise of the

Fig. 1 The abundance (mean number per leaf, and st. dev.) of *Psylla* nymphs on leaves. **A** first experiment: **1** uninfested tree; **2, 3, 4** infested trees; **B** second experiment: **T0** before infestation; **T1** appearance of nymphs; **T5** after 5 d; **T6** after 14 d; **T7** after 30 d; * performed GC-MS and HPLC

head space analyses will be published elsewhere (Scutareanu *et al.* submitted).

Here, we focus specifically on two compounds: (*E,E*)- α -farnesene and methyl-salicylate. (*E,E*)- α -farnesene was identified by Miller *et al.* (1989) in both *P. communis*, which is susceptible to *Psylla*-infestation, and in *P. callieriana*, which is resistant to *Psylla*-infestation. Methyl-salicylate is reported to play a role in various plant-herbivore-natural enemy interactions (e.g. Dicke *et al.* 1990; Hardie *et al.* 1994).

Normalized chromatograms, obtained from Experiment 1, show that the sample of young infested leaves collected in May (ca 40 *Psylla* nymphs/leaf; Fig. 1A) contained about ten times more (*E,E*)- α -farnesene and methyl-salicylate than the sample from uninfested control leaves (Table 1). Samples from old infested leaves, collected in August, also showed higher levels of (*E,E*)- α -farnesene and methyl-salicylate than uninfested control leaves. At a nymph density of ca 95 individuals per leaf (Fig. 1A), the head space sample contained about twice as much (*E,E*)- α -farnesene than at a density of 5 nymphs per leaf (Table 1). Levels of methyl-salicylate were lower in samples from older leaves than in the

Table 1 The amounts of (*E,E*)- α -farnesene and methyl-salicylate in the head space of uninfested and infested young and old pear tree leaves (first experiment) and related to increasing infestation levels (second experiment)

			Normalized GC-areas*	
Leaf age	Treatment	Time of sampling	(<i>E,E</i>)- α -farnesene	Methyl-salicylate
First experiment				
Young	Uninfested	May	95	376
	Heavily infested	May	1129	3145
Old	Uninfested	August	51	—
	Mildly infested	August	1028	1438
	Heavily infested	August	1917	125
Second experiment				
Young	Uninfested	T0**	—	35
		T1	—	3
		T5	—	Trace
		T7	—	—
Young	Before infestation	T0	—	14
	Infested	T1	—	109
		T5	8	181
		T7	20	259

* Normalized GC-areas = absolute GC-areas (10E6 = 3ng)/total fresh leaf weight

** T0 before infestation; T1 appearance of nymphs; T5 after 5 d; T7 after 30 d

sample from infested younger leaves, and the sample from the heavily infested old leaves contained about ten times less methyl-salicylate than the sample from the mildly infested old leaves (Table 1). This suggests that the emission of (*E,E*)- α -farnesene and methyl-salicylate is differently related to the level of infestation of pear tree leaves by *Psylla* nymphs.

The results of the second experiment showed that the levels of both (*E,E*)- α -farnesene and methyl-salicylate in the head space of young pear leaves increased with increasing *Psylla* infestation (Table 1). While nymph density gradually increased from zero to 30–35 individuals per leaf (Fig. 1B), the level of methyl-salicylate gradually increased to almost twentyfold the level in the leaf sample from the same tree prior to infestation. Also, the level of (*E,E*)- α -farnesene seemed to increase at increasing damage level, although the volatilization of this compound seems to take place at a different time scale than that of methyl-salicylate. This again suggests that these two volatiles are differently related to levels of infestation by pear *Psylla* nymphs.

Overall, the levels of (*E,E*)- α -farnesene and methyl-salicylate were lower in the second experiment than in the first experiment (Table 1). This is also true for the samples with comparable levels of *Psylla*-infestation: the heavily infested young leaves sampled in May in the first experiment versus the infested young leaves sampled at T7 in the second experiment (Table 1, Fig. 1). These different outcomes indicate that the amounts of the two volatile compounds are not merely dependent on the levels of infestation, other factors may play a role, too. Differences between the two experiments – and hence possible causes for the different results –

were, *e.g.*, the age of the trees used (in the first experiment the trees were four years old, in the second only one year), the history of the trees used (although we can be sure that the four-year-old trees were uninfested in the growing season when our experiment started, we cannot be certain that the trees had been undamaged throughout the preceding years as well); it is well known that various kinds of delayed or long-term induced responses to damage may occur in trees (examples in Tallamy & Raupp, 1991)), or the time of year in which the leaves were sampled (May *vs* March, in first and second experiment, respectively). Differences like these, and one can easily think of several others, make especially quantitative comparison of results between experiments very difficult.

Additional Y-tube olfactometer tests showed that adults of *A. nemoralis* respond significantly positive to (*E,E*)- α -farnesene and to methyl-salicylate in pure form solved in hexane, when tested against clean air with solvent (Scutareanu *et al.* in prep.). This suggests that the volatile compounds associated with leaf infestation by psyllids, may be used in distant prey location by anthocorid predators (Drukker *et al.* 1995).

Polyphenols in Psylla-infested and uninfested leaves

In the first experiment, a group of (as yet unidentified, work in progress) polyphenols that is dominant in chromatograms of infested leaves collected in May, is completely absent in samples from uninfested leaves (Fig. 2). Moreover, it is also absent in the sample from the infested old leaves collected in August. Its peak pattern closely resembles that of the uninfested (young and old) leaves (Fig. 2). This group of peaks (numbers 41–43) eluted from the HPLC column after 33–36 min.

In the second experiment, the most striking result is the increase of one peak (peak number 30) with progressing level of infestation (Fig. 3). In the original chromatogram, this specific group of polyphenols became manifest at a retention time of ± 46 min from infested young leaves sampled at day 5 after the appearance of nymphs, and in much higher quantities at day 14 and 30 (Fig. 3F–H). This peak is absent in samples of leaves collected before infestation from the same tree (Fig. 3E) and in leaves of the uninfested control tree. (Fig. 3A–C), except for the last sample, where it is present at a very low level (Fig. 3D). Repeated HPLC and first mass-spectra of the fraction collected from 'peak 30' indicated that this peak consisted of a group of polyphenols, probably flavonoids (work in progress).

Conclusions

Based on these experiments, we conclude that in pear trees infested by *P. pyricola* two simultaneous responses are triggered: changes in the composition of the volatile blend in the head space of the leaves, as well as changes in the production of polyphenolics in the same leaves. These induced responses are most manifest in infested

Fig. 2 Normalized HPLC peak areas of polyphenol compounds in **A** uninfested and **B** *Psylla*-infested, young and old leaves (first experiment)

Fig. 3 Normalized HPLC peak heights of polyphenol compounds in uninfested (**A–E**) and *Psylla*-infested (**E–H**) young leaves related to development of infestation: T0, T5, T6, T7 as in Figure 1B (second experiment)

young leaves. Note that in the first experiment the control tree was outdoors and the treated trees were in a climate room, which makes interpretation of the results less straightforward. However, confirmation of these results in the second experiment suggests that the environmental differences between field and climate room were less relevant.

Two volatile compounds that were shown to emanate in higher amounts from infested leaves are (*E,E*)- α -farnesene and methyl-salicylate. These compounds can mediate attraction of anthocorid predatory bugs towards *Psylla*-infested pear trees: *Anthocoris nemoralis* responds significantly to (*E,E*)- α -farnesene and to methyl-salicylate in pure form when tested against clean air. Thus, the damage-related changes in the blend of volatiles can be interpreted as induced indirect defensive responses of pear trees against psyllids. Although polyphenols are reported to have detrimental effects on various species of herbivores (e.g. Fisk 1980), it remains yet to be shown that the induced changes in polyphenolics in *Psylla*-infested pear trees, as found in this study, negatively influence the psyllids. Until then, one should be careful in interpreting this induced response as part of induced (direct) plant defense.

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