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COMPOSITION OF APHID ALARM PHEROMONES

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Abstract—Analysis of single cornicle droplets from six species of aphid showed the presence of volatile components in addition to (*E*)-β-farnesene. Compounds identified included (*Z,E*)-α- and (*E,E*)-α-farnesene for *Myzus persicae* and α- and β-pinene for *Megoura viciae*. With *Megoura viciae*, (−)-α-pinene was most important for alarm activity. The major component of the alarm pheromone of *Phorodon humuli* was (*E*)-β-farnesene even though farnesenes are present in the summer host *Humulus lupulus*.

Key Words—Aphid, pheromone, alarm pheromone, farnesenes, pinenes, behavior.

INTRODUCTION

When attacked or irritated, aphids can produce defensive secretions from their cornicles. In addition to their mechanical defensive action, these secretions generally release a volatile alarm pheromone, causing other aphids in the area to disperse. The alarm pheromone for several species of aphid is reported to be (*E*)-β-farnesene (Bowers et al., 1972; Edwards et al., 1973; Wientjens et al., 1973) and for *Therioaphis maculata* (−)-germacrene A (Nishino et al., 1977). However, we thought it likely that related terpenoids might occur and contribute to alarm activity in some aphid species. Therefore the main aim of the work described here was to identify, by chemical and biological studies, any additional components of aphid alarm pheromones.

METHODS AND MATERIALS

Aphids were obtained from laboratory cultures maintained, at ca. 20° C and day length of at least 16 hr, on the following food plants: Chinese cabbage

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(*Brassica pekinensis* Rupr.) for *Myzus persicae* (Sulz.); broad bean (*Vicia faba* L. dwarf varieties) for *Megoura viciae* Buckton (2 cultures), *Aphis fabae* Scop. (2 cultures) and *Acyrthosiphon pisum* (Harris); oats (*Avena sativa* L. cv. Manod) for *Sitobion avenae* (Fab.) (2 strains, pink and green).

Samples of *Phorodon humuli* (Schrank) on hops (*Humulus lupulus* L.) were obtained from laboratory cultures at East Malling Research Station.

Chemical Analysis

Gas chromatography (GC) employed a Pye 104 chromatograph fitted with a flame ionization detector. Gas chromatography coupled with mass spectrometry (GC-MS) used a Pye 204 chromatograph with the column effluent led directly to the source of the mass spectrometer (VG Micromass 70-70F) through a silanized glass-lined steel tube. The injection system in both cases consisted of a short length of silanized glass-lined steel tubing fitted into a modified Pye 104 or 204 injection port and connected directly to the chromatography column. Two 50 m × 0.25 mm ID glass capillary columns were employed in both systems, one wall-coated with heat-treated Carbowax 20M (PhaseSep) and the other wall-coated with Ucon 5100 (PhaseSep). In both systems helium at 10 psig was employed as carrier gas. Mass spectrometry was by electron impact at 70 eV with a source temperature of 200° C. In GC-MS, the effluent was either monitored by continuous integration of ion intensities between m/e 60 and m/e 120 or by single ion monitoring at m/e 93 and on occasions at m/e 204, 91, and 163.

Analysis of Alarm Pheromones

Analysis of Single Cornicle Droplets. Immature apterous aphids were immobilized on sticky tape and placed under a binocular microscope. A fine tube was drawn, immediately prior to use, from borosilicate glass tubing (ID ca. 5 mm) to give an ID of about twice the OD of the aphid cornicle. The tube was bent slightly at one end. The other end was held close to an aphid cornicle and the aphid gently prodded with a mounted needle until a cornicle droplet formed. This droplet was then drawn by capillarity directly into the tube which was placed immediately in the injection port of the chromatograph with the oven cool (ca. 35° C) and open. The bend in the tube prevented it from dropping below the injection block heater. After helium pressure had attained the operating level, the injection block heater was switched to a value that allowed a temperature of 150° C to be achieved in 4 min; this value was thereafter maintained. The chromatograph oven door was then closed and the oven temperature raised from 35° to 135° at 8° / min. This procedure ensured that volatile components were separated from the involatile cornicle lipids before chromatography.

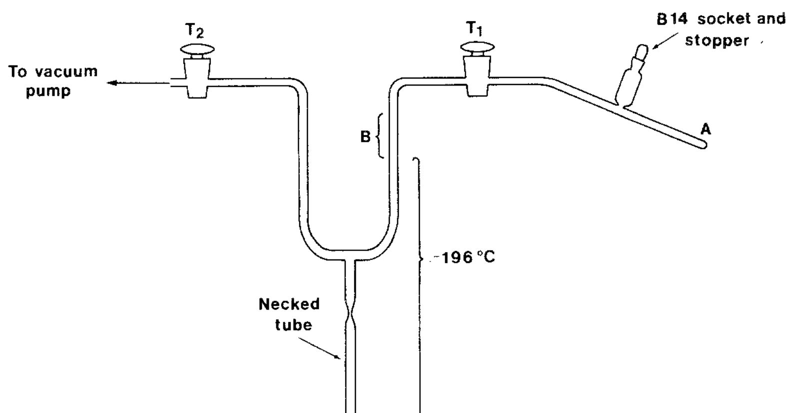

FIG. 1. Apparatus for vacuum distillation of aphid extract.

Preparation and Analysis of Vacuum Distillate from Whole Aphids. In a typical experiment ca. 5000 aphids (*Myzus persicae*) were extracted with cold, redistilled pentane (2× 10 ml). The extract was filtered and evaporated to 0.5 ml under a stream of nitrogen. The concentrated extract was then distilled in a specially designed apparatus (Figure 1) which allowed the volatile fraction to be conveniently isolated with minimum contamination. The ground glass joints of the apparatus were sealed with grease (Apiezon L) purified by molecular distillation. The pumps, rotary, and oil diffusion, were separated from the system by a trap cooled with liquid nitrogen which arrested any back-flow of oil vapor. The aphid extract, placed in A, was distilled under vacuum at 2×10^{-2} Torr for half a day and at 10^{-3} Torr for 2 days and the distillate collected in the upper limit of the U tube cooled to −196° with liquid nitrogen. After distillation was complete, taps T_1 and T_2 were closed and the distillate allowed to thaw and run into the necked tube. By differential heating (ca. 20°/−196° C), the pentane was allowed to reflux onto the region of the tube marked B, where most of the volatile components had condensed, to ensure that even the less soluble distillate was washed into the necked tube. The necked tube was then cooled, below the neck, to −196°, tap T_2 was opened, and the tube sealed off at the neck with an air/gas burner. The sample, thus sealed under vacuum in situ, was stored at −20° C until required for analysis by GC and GC-MS. Samples for analysis were injected through rubber septa and an oven temperature program of 40–110° C at 4°/min was employed.

Analysis of Headspace above Crushed Aphids. Aphids (ca. 50) were placed in a glass tube and crushed with a tightly fitting glass rod. A sample of air (5 μl) was removed using a 10-μl syringe and injected into the GC column. The chromatogram was developed at 80° or 40° C.

Identification of Compounds

Unless stated otherwise, identification was from mass spectral evidence and peak enhancement with authentic material on the two capillary columns. In each case, mass spectra were similar to those obtained from authentic materials and to published spectra: farnesenes, Murray (1969) and Anet (1970); monoterpenes, Ryhage and von Sydow (1963); 2-phenylethanol, Mass Spectrometry Data Centre (1975); 2-phenylethyl isothiocyanate, Kjaer et al. (1963).

Chemicals

Impure (*E*)-β-farnesene was prepared by dehydrating a mixture of (*E*)- and (*Z*)-nerolidol (Aldrich) with phosphoryl chloride in pyridine without heating (Anet, 1970). The product contained 16% of (*E*)-β-farnesene. Impurities in (*E*)-β-farnesene samples consisted almost entirely of isomeric farnesenes (Anet, 1970). A mixture of the four isomeric α-farnesenes was obtained from impure (*E*)-β-farnesene by liquid chromatography on silica gel impregnated with silver nitrate using a solvent gradient of ethyl acetate in hexane.

Purified (*E*)-β-farnesene was obtained by a method similar to that of Bowers et al. (1977) except that the farnesenes prepared from pure (*E*)-nerolidol were stored in air at 4° C after preparation. During several days storage, the proportion of (*E*)-β-farnesene was monitored by GC and rose from 32% to 75%. This product was then purified further by chromatography on silver nitrate-impregnated silica gel, as in the previously published method, to give a final product containing 85% (*E*)-β-farnesene, confirmed by ^{13}C nuclear magnetic resonance spectroscopy, spectrum published by Burger et al. (1978). Other chemicals were from commercial sources.

Bioassays

Three different types of test were done, each with four replicates per treatment. Suitable controls, i.e., solvent alone, camphene (a monoterpene hydrocarbon not detected in any of the aphids), or air alone were included in each set of tests.

Arena Tests. The arena for *Myzus persicae* was a short glass tube 25 mm diameter and 15 mm deep affixed to an inverted piece of Chinese cabbage leaf by paraffin wax. Ten apterous adult aphids were placed in each ring and allowed to settle, their premature escape being prevented by a circle of polytetrafluoroethylene (PTFE, I.C.I.) at the top. The test substance was introduced on a small triangle of filter paper or as a 5-μl droplet in pentane solution applied directly to the surface of the leaf. Aphids that withdrew their

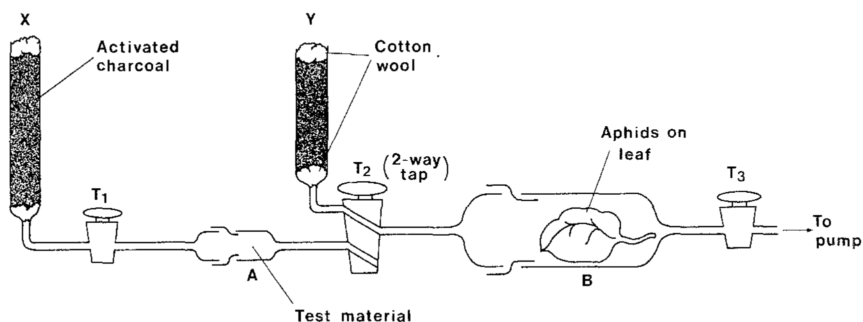

FIG. 2. Apparatus for glass tube test.

mouthparts and moved from their feeding sites were counted at 1 and 5 min, and expressed as a percentage of the total.

For *Megoura viciae* the arena was the inverted lid (50 mm diameter) of a glass Petri dish. A short length of bean stem was carefully cut from a plant infested with aphids and placed at an angle of ca. 20° in the Petri dish lid, supported at one end by a piece of cork. Each test substance (30 ng in 3 μl pentane) was introduced on a small triangle of filter paper that was slid beneath the stem. A second glass lid was then placed gently on top. Aphids that left the stem at 1 and at 5 min were counted and expressed as a percentage of the total.

Glass Tube Apparatus. The apparatus was constructed of glass with ground-glass joints sealed by means of PTFE seals (Fivac, Fisons) (see Figure 2). A colony of aphids on a freshly cut plant leaf was placed in tube B and allowed to settle in a clean air stream (5 ml/sec) entering via tube Y. Test material in pentane (1 μl) was placed in tube A. Taps T_1 and T_2 were then turned so that air containing the test material entered via tube X, and the behavior of the aphids was noted during 5 mins.

Syringe Tests. Test chemicals, i.e., (E)-β-farnesene (5 μl), (−)-β-pinene (5 μl), (−)-α-pinene (1 μl), (+)-α-pinene (1 μl), or a mixture of the first three in the ratio 5:5:1 were smeared on the inner surface of a 5-ml glass syringe. Air from the syringe (4 deliveries = 20 ml) was expelled slowly over separate colonies of aphids settled on intact plants (or in the case of *Phorodon humuli* on detached leaves). Photographs of the plants before and 1 min after treatment allowed counts to be made of the numbers of aphids that responded.

RESULTS AND DISCUSSION

Chemical Analysis. Analysis of single cornicle droplets from all six species of aphid showed the presence of (E)-β-farnesene. This had not

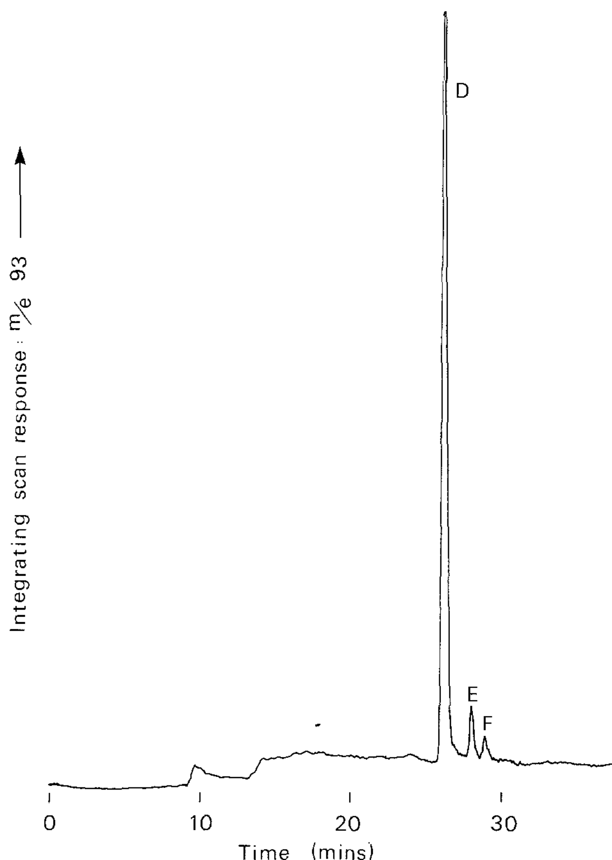

FIG. 3. GC-MS of single cornicle secretion from *Myzus persicae*.

previously been identified in *Aphis fabae*, *Phorodon humuli*, or *Megoura viciae*. However, in each case other volatile compounds were detected.

Most sensitive analysis of cornicle droplets was by GC-MS employing single ion monitoring at m/e 93, an abundant ion in the spectra of many terpene hydrocarbons, but this procedure discriminates against most other compounds. However, no other substantial peaks were observed by integrating intensities of ions from m/e 60 to 120 or by using a flame ionization detector. Typical chromatograms from *Myzus persicae* and *Megoura viciae* are shown in Figures 3 and 4. Peaks D are from (*E*)-β-farnesene. Relatively small peaks with retention times of E and F were also present in chromatograms from the other four aphid species but peaks A, B, and C were obtained only with *Megoura viciae*. Chromatograms from *Acyrthosiphon pisum* contained an additional peak with retention time near that of B, and *Sitobion*

avenae gave a peak immediately after F. Although amounts of volatile components varied considerably between different cultures of the same species of aphids, the same peaks were always present within the species and for the two strains of *Sitobion avenae* except for the component giving peak C which was frequently absent from *Megoura viciae.*

Peaks E and F, although relatively small, were investigated first because of their ubiquity. The aphid chosen for the investigation was *Myzus persicae.* Insufficient material was present in single secretions for mass spectra, although single ion monitoring at m/e 204 suggested that E and F were from sesquiterpene hydrocarbons (i.e., M = 204). Mass spectra were, however, obtained by GC-MS of the vacuum distillate obtained from a solvent extract of the whole aphids, Figure 5. Chromatograms from the two methods of analysis gave similar relative heights for peaks D, E and F. It is therefore unlikely that compounds giving peaks E and F could have been formed during the second, more lengthy, extraction procedure. Peaks E and F were

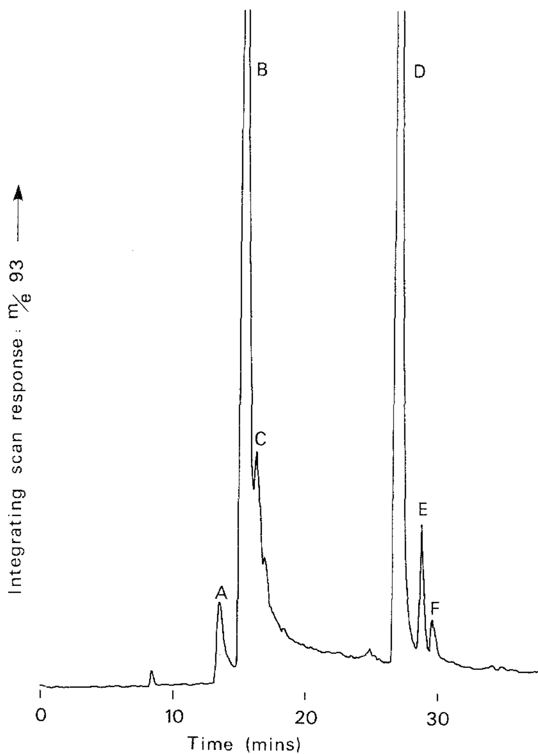

FIG. 4. GC-MS of single cornicle secretion from *Megoura viciae.*

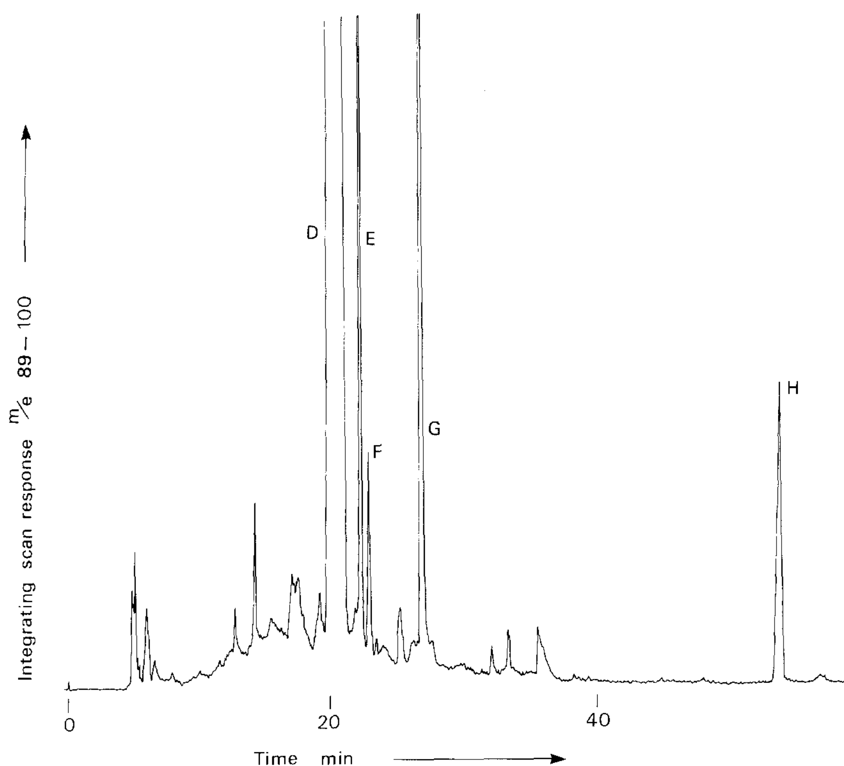

FIG. 5. GC-MS of vacuum distilled extract from *Myzus persicae.*

subsequently identified as arising from (*Z,E*)-α-farnesene and (*E,E*)-α-farnesene, respectively, by MS and peak enhancement in GC on the two columns. For peak enhancement a mixture of α-farnesenes was used, the published order of elution (Anet, 1970) having been confirmed for columns employed here by MS and by comparison between chromatograms obtained with farnesene samples from (*E*)-nerolidol and from the *E* and *Z* mixture. Compounds giving peaks G and H were identified from mass spectral evidence alone as being, respectively, 2-phenylethanol and 2-phenylethyl isothiocyanate. These latter compounds were probably derived from glucosinolates in the food plant, Chinese cabbage (*Brassica pekinensis*). Their absence from the alarm pheromone was confirmed by single ion monitoring studies at *m/e* 91 (i.e., $C_6H_5CH_2^+$) and *m/e* 163 (i.e., $C_6H_5CH_2CH_2NCS^+$) on single cornicle droplets.

Peaks A, B, and C in the chromatogram for *Megoura viciae* were found to be from α-pinene, β-pinene, and limonene, respectively, by GC-MS of single cornicle droplets. It was necessary to make peak enhancement studies

for α-pinene on headspace samples from crushed aphids because of the relatively small proportion of this compound present. Identification of limonene was from mass spectra only. α-Pinene can be formed by thermal rearrangement of β-pinene. However, peak A was undiminished when volatiles from the cornicle droplet were chromatographed at a lower temperature (i.e., 40° C). The monoterpene hydrocarbons were also present as major volatile components together with compounds giving peaks D, E, and F in the vacuum distillate from this aphid. Insufficient material was obtained for determination of chirality by optical measurements.

Approximate amounts of (E)-β-farnesene in total cornicle secretion obtained from single insects were estimated, by comparison with chromatograms from known amounts of the compound, to range from 0.1 to 3 ng with an average of 1 ng for *Myzus persicae* and from 0.05 to 5 ng with an average of 2 ng for *Megoura viciae* from both cultures. Levels of β-pinene varied between 8 and 50 ng with averages of 27 and 8 ng for *Megoura viciae* from the two cultures. The ratio of α-pinene to β-pinene was almost constant at 1:8.5 and suggests biosynthesis by a common route.

Estimation of Activity. Table 1 gives results of bioassays with *Myzus persicae*. In glass tube tests the aphids were sensitive to small quantities of

TABLE 1. RESULTS FROM BIOASSAY WITH *Myzus persicae* (% RESPONSE AT 5 MIN)

	Type of test			
		Glass tube (standard dose of (E)-β-farnesene: 0.16 ng) Test number		
Material	Arena (standard dose of (E)-β-farnesene: 900 ng)	1	2	3
Control				
Solvent	8			
Comphene		5		
Standard dose				
× 1/100 Purified	4			28
Impure	30			31
× 1 Purified	100		57	
Impure	90		81	
× 1000 Impure		71		
1 Crushed *Myzus persicae*		56		
L.S.D. $P = 0.05$	22	41	NS	NS

pheromone, and whereas only 5% responded to the control, camphene, over 80% responded to 0.16 ng of (*E*)-*β*-farnesene (glass tube test 2). As this is less than one fifth the amount obtained on average from individual aphids, it may be assumed that the synthetic material has at least the activity of the natural alarm pheromone from this species. However, this response could not be increased even by employing a dose 1000 times greater (glass tube test 1).

Impure (*E*)-*β*-farnesene gave at least as large a response as the purified product in three out of four comparisons, and this could prove a considerable advantage if these responses were used in practical control measures, as impure material is easily prepared in bulk.

Isomeric farnesenes in the synthetic (*E*)-*β*-farnesene samples included (*Z,E*)-*α*- and (*E,E*)-*α*-farnesene identified as components of the cornicle droplets from *Myzus persicae* and giving peaks E and F. (*E,E*)-*α*-Farnesene and a mixture of this compound with (*Z,E*)-*α*-farnesene have been found inactive with *Myzus persicae* during studies by Bowers et al. (1977) on molecular structure–alarm activity relationships. It is possible that these compounds synergize the activity of (*E*)-*β*-farnesene but mixtures with the exact quantitative composition of the natural material were not tested because it was considered unlikely that the activity of the samples of farnesenes already tested could be surpassed by this approach.

Bioassay results with *Megoura viciae* are given in Table 2. In the arena tests, crushed aphids elicited large responses but these were variable, probably because of differences in amounts of alarm pheromone released: (−)-*α*-pinene

TABLE 2. RESULTS FROM BIOASSAY WITH *Megoura viciae* (% RESPONSE AT 5 MIN, ARENA TEST; 1 MIN, SYRINGE TEST)

	Type of test			
	Arena Test number			
Material	1	2	3	Syringe
5 crushed *M. viciae*	83	38		
Pinenes				
(−)-*α*-			31	35
(±)-*α*-		8	3	
(+)-*α*-				9
(−)-*β*-	19			12
(*E*)-*β*-Farnesene				4
Mixture				56
Control[a]	3	4	0	6
L.S.D. ($P = 0.05$)	18	10	17	28

[a]Control = camphene in arena test 1; solvent in arena tests 2 and 3; air alone in syringe tests.

was also active. However, presence of (+)-α-pinene in the racemic mixture appeared to inhibit activity of the (−) isomer. The glass tube test was insensitive with this species of aphid and was replaced by the syringe test. In this test a positive response was again obtained with (−)-α-pinene, but the best response was with the mixture of (−)-α-pinene, (−)-β-pinene, and impure (*E*)-β-farnesene. When tested alone (*E*)-β-farnesene, (−)-β-pinene, and (+)-α-pinene were relatively inactive.

As reported above, (*E*)-β-farnesene was found to be a major component of the cornicle secretions of *Phorodon humuli*. When tested by the syringe method on several colonies of *P. humuli*, impure (*E*)-β-farnesene elicited alarm response (55 ± 2% of aphids responded). However, the summer host of *P. humuli* is the perennial hop *Humulus lupulus* which is also known to contain an unspecified stereoisomer of β-farnesene (Sorm et al., 1949) and (*E,E*)-α-farnesene (Tressl and Friese, 1978), and it must be assumed that, in natural conditions, the presence of farnesenes in the plant does not deter settling and feeding.

In conclusion, the present study shows that aphids can contain, in addition to (*E*)-β-farnesene, alarm pheromone components not previously identified. The pheromone of *Megoura viciae* contains large proportions of α- and β-pinene in addition to (*E*)-β-farnesene, and the (−)-α isomer is important in producing the alarm response. Alarm activity of the natural pheromone from *Myzus persicae* can be imitated by a mixture of farnesenes easily available in large amounts. This could facilitate exploitation of alarm behavior in attempts to reduce damage by this important pest.

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