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Five Types of Olfactory Receptor Neurons in the Strawberry Blossom Weevil *Anthonomus rubi*: Selective Responses to Inducible Host-plant Volatiles

Helena Bichão[1,2], Anna-Karin Borg-Karlson[3], Jorge Araújo[1] and Hanna Mustaparta[2]

[1]Chemical Ecology Unit, Centre for applied Ecology, University of Évora, Apartado 94, PT-7001-Evora codex, Portugal, [2]Neuroscience Unit, Department of Biology, Norwegian University of Science and Technology, NO-7489 Trondheim, Norway and [3]Ecological Chemistry, Organic Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden

Correspondence to be sent to: Helena Bichão, Neuroscience Unit, Department of Biology, Norwegian University of Science and Technology, NO-7489 Trondheim, Norway. e-mail: Helena.Bichao@bio.ntnu.no

Abstract

Plants release hundreds of volatiles that are important in the interaction with herbivorous animals, but which odorants are detected by which species? In this study, single receptor neurons on the antenna of the oligophagous strawberry blossom weevil *Anthonomus rubi* were screened for sensitivity to naturally produced plant compounds by the use of gas chromatography linked to electrophysiological recordings from single cells. The narrow tuning of the neurons was demonstrated by responses solely to a few structurally related sesquiterpenes, aromatics or monoterpene hydrocarbons out of hundreds of plant constituents tested. We present five olfactory receptor neuron types, identified according to one primary odorant i.e. the compound to which the neurons are most sensitive. These odorants, (−)-germacrene D, (−)-β-caryophyllene, methyl salicylate, *E*-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, present in the intact strawberry plant, are induced in higher amounts by weevil feeding. This suggests that these compounds can provide information about the presence of conspecifics. We used protocols especially designed to allow comparison with previously investigated species. Striking similarities, but also differences, are demonstrated between receptor neuron specificity in the strawberry weevil and moths.

Key words: GC-MS, GC-SCR, insect olfaction, plant odours, terpenes, aromatics

Introduction

A large part of the olfactory system in herbivorous insects, including weevils, is evolved for the detection and processing of plant odour information, reflecting the importance of olfactory cues in host location for feeding and oviposition (Schoonhoven *et al.*, 1998; Mustaparta, 2002). Plants produce a large variety of secondary metabolites, many of which are in common, while some are restricted to certain plant groups. The blend of compounds released by a plant does not only vary with species and strain, but can also be influenced by abiotic or biotic factors like herbivore feeding (Paré and Tumlinson, 1999). While mechanical injuries increase the quantity of compounds released from the wounded site, feeding by insects often induces systemic production of new compounds and/or different relative amounts of the compounds. Species-specific compounds present in the oral secretions of herbivore larvae can induce these changes (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997). Among the compounds shown to be systemically induced by insect feeding activity are the sesquiterpenes germacrene D and β-caryophyllene, the monoterpene *E*-β-ocimene, the *homo*-monoterpene *E*-DMNT (*E*-4,8-dimethyl-1,3,7-nonatriene) and the aromatic compound methyl salicylate (Innocenzi *et al.*, 2001; Arimura *et al.*, 2004; Colazza *et al.*, 2004; reviewed by Paré and Tumlinson, 1999; Dicke and Van Loon, 2000). It has been shown that compounds released after herbivory appear to protect plants by repelling or deterring herbivores, or by attracting the enemies of herbivores (De Moraes *et al.*, 2001; Pichersky and Gershenzon, 2002; reviewed by Turlings and Wäckers, 2004). In addition to providing information about competitors or food quality, inducible plant compounds may be reliable cues that advertise the presence of conspecifics and thereby promote aggregation of individuals. Thus these compounds can be important synergists to aggregation pheromones (Rochat *et al.*, 2000; Yang *et al.*, 2004).

The strawberry blossom weevil, *Anthonomus rubi* (Herbst) 1795, is an oligophagous species that feeds and reproduces

on plants of the family Rosacea (Popov, 1996), mainly strawberry (*Fragaria* × *ananassa* Duchesne) and raspberry (*Rubus idaeus* L.). Adult weevils migrate early in the spring from overwintering shelters to the strawberry fields, where they feed on the foliage and start to mate at the onset of bud formation. After laying a single egg in an unopened bud, the female partially severs the bud pedicel. The larvae develop and pupate inside the withered bud, and emerge in late summer. This species constitutes a serious pest in strawberry throughout Europe, in some cases responsible for damages of up to 80% of the berry yield (Popov, 1996; Cross and Easterbrook, 1998). The role of olfaction for host location in *A. rubi* has not been completely clarified. The pheromone system has been investigated by Innocenzi *et al.* (2001), who showed that three components of a male-produced aggregation pheromone blend attract weevils to baited traps, but the role of host plant odours has not been examined.

An important question in insect–plant interactions is which of the numerous volatiles released by plants are detected by the insects and might constitute biologically relevant odorants for host location. In a preliminary study using gas chromatography linked to electroantennogram recordings (GC-EAG), the antennae of *A. rubi* were found to respond to several components present in the blend of the host plant (unpublished data). A more accurate method of identifying relevant odorants is recordings from single olfactory receptor neurons (RNs) linked to gas chromatography. With this method, each olfactory receptor neuron can be screened with numerous separated compounds of naturally produced blends, and repeatedly tested for confirming the responses. This allows determination of the molecular receptive range of the RNs. Gas chromatography linked to single cell recording (GC-SCR), in combination with GC linked to mass spectrometry (GC-MS), has been successfully used to identify host plant odours that are detected by several species of beetles and moths (Blight *et al.*, 1995; Wibe *et al.*, 1997, 1998; Røstelien *et al.*, 2000a,b; Stensmyr *et al.*, 2001; Barata *et al.*, 2002; Stranden *et al.*, 2002, 2003a,b; Bichão *et al.*, 2003). In the present study we identified the compounds that are detected by five types of RNs in the strawberry weevil and investigated structure–activity relationships. The protocols used also allowed the comparison with RN types described in other species. The primary odorants of the RNs were monoterpenes, sesquiterpenes and aromatic compounds that were emitted in greater amounts from flowers of strawberry subjected to weevil feeding.

Materials and methods

Insects

Adult *A. rubi* were collected from unsprayed strawberry fields (Dragvoll, Trondheim, Norway) during the summer seasons (mid-May to August) of 2002 and 2003. Prior to recordings, the insects were kept in the laboratory under natural scotophase at room temperature (22–25°C) for a maximum of 1 week. Fresh food material, consisting of young and mature leaves, flowers and flower buds of strawberry, was provided every third day. All insects used in this study were starved for at least 12 h before the experiments. The weevils were sexed after the experiments using the method described by Innocenzi *et al.* (2002).

Plants and collection of plant volatiles

In this study the host plant materials used were strawberry (*F.* × *ananassa*, Duschene, variety Korona) and raspberry (*R. idaeus* L.) from fields and green areas in Trondheim, Norway. Two different methods were used to collect volatiles from the plants, solid-phase micro-extraction (SPME) and dynamic headspace entrainment using the sorption material Porapak Q (80/100 mesh, Supelco).

Volatiles emitted by strawberry flowers, both intact and infested by overwintered weevils, were collected by SPME, using a 65 μm polydimethylsiloxane-divinylbenzene fibre (Supelco™) (Zhang and Pawliszyn, 1993; Borg-Karlson and Mozuraitis, 1996). The volatiles were collected from three individual plants before and after adding the beetles. The flowers were enclosed separately in small glass vials, of ~10 ml, the fibre was placed at a distance of 5 mm above the flower and the odours were sampled during 24 h. The samples obtained were analysed using the equipment and the GC-MS parameters specified below. These samples were used to characterize emissions from intact and infested flowers.

For the GC-SCR experiments, volatile compounds released by host plant materials were collected from freshly cut material of strawberry and raspberry (foliage and buds) using dynamic headspace techniques. The plant material was confined in an entrainment chamber (1500 ml) and a carrier gas (N_2 flow measured at the outlet: 50–60 ml/min) was blown through the chamber. The volatile compounds released by the plant sample were carried by the gas through two parallel glass tubes (6.6 × 0.5 cm i.d.) containing a solid trap, Porapak Q (80/100 mesh, Supelco), where the compounds were adsorbed and pre-concentrated. The adsorbed volatiles were eluted [with *n*-hexane (>99%), ethyl acetate (absolute) or a mixture of both (1:1)] and used as test samples in the experiments. Before use, the Porapak Q was rinsed with dichloromethane and *n*-hexane, and activated overnight in a heating chamber at 180°C while perfused with N_2. These samples were used to test the RNs for sensitivity to host odours.

Other test samples

In addition to the host headspace mixtures, the neurons were tested with volatile constituents in extracts, headspace samples and essential oils of several plant materials (Table 1). For details of extraction of other sample mixtures used in this study, reference is made to Bichão *et al.* (2003), Stranden *et al.* (2003a) and Wibe and Mustaparta (1996). Standard samples

Table 1 Samples used to test single olfactory receptor neurons (RNs) in *A. rubi* via the gas chromatograph

Test samples	RN types				
	I	II	III	IV	V
Headspace					
Chrysanthemum sp.[a]	1	1	1	–	–
Maritime pine (*Pinus pinaster*), Aiton	–	3	3	2	–
Orange (*Citrus* sp.)[b]	1	–	–	1	1
Raspberry (*Rubus idaeus*)	2	2	2	2	2
Strawberry (*Fragaria* × *ananassa* var. Korona)	15	3	7	5	4
Spruce sawdust (*Picea abies* L.)[c]	1	–	–	–	–
Wild briar (*Rosa dumalis* Bechst.)[b]	1	–	–	–	–
Essential oils and fractions					
Cubebe pepper (*Piper cubeba* L.) containing (−)-β-caryophyllene and (−)-germacrene D[d]	11	2	1	–	–
Cinnamon oil (*Cinnamomum sp.*)[NMD]	–	1	1	–	–
Clove bud oil (*Syzygium aromaticum* L.)[NMD]	–	–	–	2	–
Lavender oil (*Lavandula angustifolia* Mill.)[e]	–	–	–	4	1
Lemon oil (*Citrus medica*, L)[NMD]	–	–	–	2	2
Orange oil (fractions 23–30) (*Citrus* sp.)[f]	4	–	–	–	–
Orange oil (fraction 60) (*Citrus* sp.)[f]	4	–	–	–	–
Orange oil (fractions 115–117) (*Citrus* sp.)[f]	4	–	–	–	–
Germacrene D, thermal degradation products (230°C)[f]	1	–	–	–	–
Germacrene D, UV degradation products (1 h)[f]	4	–	–	–	–
Germacrene D, UV degradation products (2 h)[f]	1	–	–	–	–
(+)-Germacrene D fraction [90% (+) and 10%(−)][g]	21	–	–	–	–
(−)-Germacrene D fraction [90%(−) and 10% (+)][g]	1	–	–	–	–
Ylang-ylang (*Cananga odorata*, Hook)[h]	12	5	4	3	–
Ylang-ylang (*Cananga odorata*, Hook)[i]	11	–	–	–	–

Numbers indicate the total of times each sample was tested on each olfactory receptor neuron (RN) type (I–IV). Sources: [a]Ulland, Norwegian University of Science and Technology, Trondheim, Norway; [b]Stranden *et al.* (2003); [c]Wibe and Mustaparta (1996); [d]Stranden *et al.* (2002); [e]Rolhoff, Norwegian University of Science and Technology, Trondheim, Norway; [f]Liblikas, Estonian Agriculture University, Tartu, Estonia; KIT, Stockholm, Sweeden; [g]König, University of Hamburg, Germany; [h]Dragoco; [i]Firmenich; [NMD]norsk medisinaldepot AS.

of chemical compounds (synthetic and authentic) were used to re-test the neurons and confirm identification of the active compounds. The source and purity of the chemical standards included in the experiments are presented in Table 2.

Reference samples of (−)- and (+)-germacrene D enantiomers, containing 90% of the main enantiomer and 10% of the opposite enantiomer [optical purity 79% *ee.* for the (+)-sample and 76% *ee.* for the (−)-sample; for details see Stranden *et al.*, 2002], were kindly provided by Prof. Dr W.A. König (University of Hamburg, Germany). The samples containing acid catalysed, photochemical and thermal induced rearrangement products of germacrene D were prepared by Dr Ilme Liblikas (Estonian Agriculture University, Tartu, Estonia and KTH, Stockholm, Sweden) according to the procedures described by Bülow and König (2000).

Gas chromatography linked to single cell recordings

Each weevil was fixed and immobilized in dental wax in a Plexiglas holder made to fit the insect. The antennae were exposed on a layer of wax and secured with tungsten hooks. Nerve impulses from single RNs on the antenna club were recorded using tungsten microelectrodes sharpened to a tip <0.3 μm (Mustaparta *et al.*, 1979). The tip of the recording electrode was inserted at the base of the sensilla, which are located in three bands on the antennal club. The reference electrode was inserted ventrally in the head. Most recordings were obtained from the two distal bands where the density of sensilla is higher.

Each RN was first screened for sensitivity to volatiles in the different test samples and in fresh plant materials. These tests were performed by puffing filtered air through glass cartridges containing either a filter paper with the test sample or fresh plant material. Neurons responding to any of these stimuli were classified as plant odour RNs and further examined by stimulation via the gas chromatograph (GC). A sample of 0.8–1 μl of the solution with the plant volatiles was injected into the GC-column through a cold on-column injector. Helium (99.9%) served as the carrier gas. A split at the end of the GC-column led half of the effluent to the GC-detector (Flame Ionization Detector, FID) and half to a clean airflow (300 ml/min) that blew over the insect antenna. This resulted in simultaneously recorded gas chromatograms and single cell responses to the separated compounds (Wadhams, 1982; Wibe and Mustaparta, 1996). The nerve impulse signal was amplified (×1800) and a spike integrator (Syntech, NL, Hilversum, The Netherlands) measured frequency. Spike activity was recorded in parallel with the computer programs Electro Antenno Detection (version 2.3, Syntech NL,) and Spike 2 (Cambridge Electronic Design Limited, Cambridge, UK).

The GC was equipped with two parallel columns allowing testing the neurons for the same mixture, with two different separation patterns. The types of columns used in this study were: one polar (J&W DBwax; 30 m; 0.25 mm i.d.; 0.25 μm film thickness) and one chiral [heptakis-(6-O-*t*-butyldimethylsilyl-2, 3-di-O-methyl)-β-cyclodextrin (50% in OV1701, 25m, i.d. 0.25mm)] (Schmidt *et al.*, 1998 König *et al.*, 1999;). The separation of the mixtures in the polar column used as a reference was obtained using the following temperature program: initial temperature of 80°C held isothermal for 2 min, then a

Table 2 Chemical standards and reference samples used to test single olfactory receptor neurons (RNs) in *A. rubi* via the gas chromatograph

Chemical standards (purity)	I	II	III	IV	V
Monoterpene hydrocarbons					
β-Myrcene[a]	–	–	–	11	–
Z- and *E*-β-Ocimene[b]	–	–	–	11	–
Limonene[c]	–	–	–	5	1
3,7-Dimethyl-1,6-octadiene[a]	–	–	–	5	1
Oxygenated monoterpenes					
Neral	–	–	–	3	3
Geranial	–	–	–	3	3
Myrtenal	–	–	–	1	1
Camphor[d] (98%)	1	1	3	–	–
Citronellol [32%(–) and 68% (+)][a]	–	–	–	5	1
3,7-Dimethyl-1-octanol	–	–	–	5	1
Geraniol	–	–	–	5	1
Racemic linalool[b] (97%)	1	1	3	5	1
Myrcenol[a]	–	–	–	5	1
(+)-*E*-Verbenol	1	1	3	–	–
iso-Borneol	2	1	3	1	1
Sesquiterpene hydrocarbons					
α-Bulnesene[e] (84%)	1	–	–	–	–
Cadinenes (from Scots pine)[a]	3	–	–	–	–
δ-Cadinene mix (from conifers)[a]	–	–	–	–	–
(–)-β-Caryophyllene[b] (99%)	1	12	11	–	–
α-Copaene (mainly (–)-enantiomer)[a]	3	–	–	–	–
(–)-α-Cubebene[a]	3	–	–	–	–
β-Cubebene[a]	3	–	–	–	–
(+)-Cyclosativene[b] (>99%)	1	–	–	–	–
β-Elemene[a]	3	–	–	–	–
(E,E)-α-Farnesene[a]	–	–	–	11	–
α-Guajene[e] (94.3%)	1	–	–	–	–
α-Humulene = α-Caryophyllene[e] (>95%)	1	–	–	–	–
(+)-Longicyclene[b] (97%)	1	–	–	–	–
(+)-Longifolene[b] (>99%)	1	–	–	–	–
(+)-Longipinene[b] (>99%)	1	–	–	–	–
α-Muurolene[e] (95,8%)	1	–	–	–	–
γ-Muurolene[e] (54,5%)	1	–	–	–	–
Muurolenes (distillation fraction from Scots pine)[a]	3	–	–	–	–
γ-Muurolene[a]	3	–	–	–	–
α-Muurolene[a]	3	–	–		–
Aromatic esters					
Methyl salicylate[f] (99%)	5	1	7	–	–
Ethyl benzoate[g] (99%)	5	1	7	–	–
Methyl benzoate[g] (99%)	5	1	7	–	–
Aliphatic compounds					
(2E)-Hexenal[f]	–	–	–	1	–
Butyl butyrate[h]	–	–	–	1	–
Butyl hexanoate[h]	–	–	–	1	–
Ethyl-2-methyl butyrate[h]	–	–	–	1	–
(2*E*)-Hexenyl acetate[h]	–	–	–	1	–
(3*Z*)-Hexenyl acetate[h]				1	
(3*Z*)-Hexenyl hexanoate[h]	–	–	–	1	–
2-Methylhexyl butanoate[h]	–	–	–	1	–
3-Methylbutyl hexanoate[h]	–	–	–	1	–
sec-Propyl butanoate[h]	–	–	–	1	–
Methyl jasmonate[g]	4	–	4	–	–

Numbers indicate the total of times each sample was tested on each olfactory receptor neuron (RN) type (I–V). Sources: [a]Borg-Karlson, Royal Institute of Technology RIT, Stockholm, Sweden; [b]Fluka; [c]Barata, University of Evora, Evora, Portugal; [d]Kebo; [e]Hartlieb and Remboldt (1996); [f]Merck; [g]Lancaster; [h]Liblikas, Estonian Agriculture University, Tartu, Estonia.

temperature increase rate of 6°C/min to 180°C, followed by a further increase rate of 15°C/min to 220°C. In several experiments the temperature program was altered in order to obtain optimal separation in certain areas. For better separation of the monoterpenes, the initial temperature was lowered to 50°C, and for complex mixtures like the orange headspace, the initial increase rate used was lowered to 2°C/min. Separations with the chiral column were performed by holding the temperature constant at 125°C.

For each cell type, protocols of stimulation sequences were established for the investigation of structure activity relationships. These were also designed to compare the functionally identified neuron types with those of other weevils and moths (Wibe *et al.*, 1997; Røstelien *et al.*, 2000b; Bichão *et al.*, 2003; Stranden *et al.*, 2003a,b).

Analysis of the recordings was carried out using the Spike 2 program. In this program construction of templates is based on the recorded data, and sorting of spikes is made by template matching. All counts were checked manually. In the experiments showing responses to several of the components by more than one RN, the selectivity of each neuron was resolved on the basis of different spike amplitudes and waveforms. Spontaneous activity rate of each neuron (expressed by minimum and maximum) was determined by random counts during 1 s intervals.

Chemical analysis

The chemical analyses were carried out by GC-MS, using the same type of GC-columns described previously for GC-SCR. Mass spectra were obtained with a Finningan SSQ 7000 spectrometer connected to a Varian 3400 gas chromatograph. The GC was equipped with a split/splitless injector (splitless mode 30 s; injector temperature 200°C; carrier gas helium). The MS source was kept at 150°C, and mass spectra were obtained at 70 eV, with a mass range 30–300 mass units. Temperature programs were adjusted to reproduce the separation obtained in the GC-SCR experiments.

Results

In this study we present the results from five olfactory receptor neuron (RN) types (Figure 1) that respond specifically to induced compounds identified in the volatiles of infested flowers. The volatiles emitted by the healthy uninfested flowers were minute amounts of benzaldehyde, germacrene D and methyl salicylate (Figure 2). After 24 h of infestation the emission of flower volatiles had increased and was dominated by *E*-β-ocimene, germacrene D, (*E,E*)-α-farnesene, and methyl salicylate. Other sesquiterpenes such as α-muurolene, δ-cadinene, β-caryophyllene and β-bourbonene were also present together with linalool and benzylalcohol. After only 2 h of infestation large amounts of terpenes were produced. The volatile profile was consistent among plants.

The results are based on recordings from single RNs located in sensilla within the two distal bands of the antennal club of male and female *A. rubi*, representing the data of 175 GC-SCR experiments. Recordings from the single neurons, lasting for periods of 40 min to several hours, allowed up to 23 GC-SCR experiments to be performed from each neuron. The responses of the neurons appeared consistently at the same retention time when stimulated with the same or different mixtures containing the active components. In all experiments reported here responses were recorded as excitation, i.e. increased spike firing activity, which in general followed the concentration profile expressed by the GC-peak of the active components. In some cases the neurons showed a slow decay of the response,

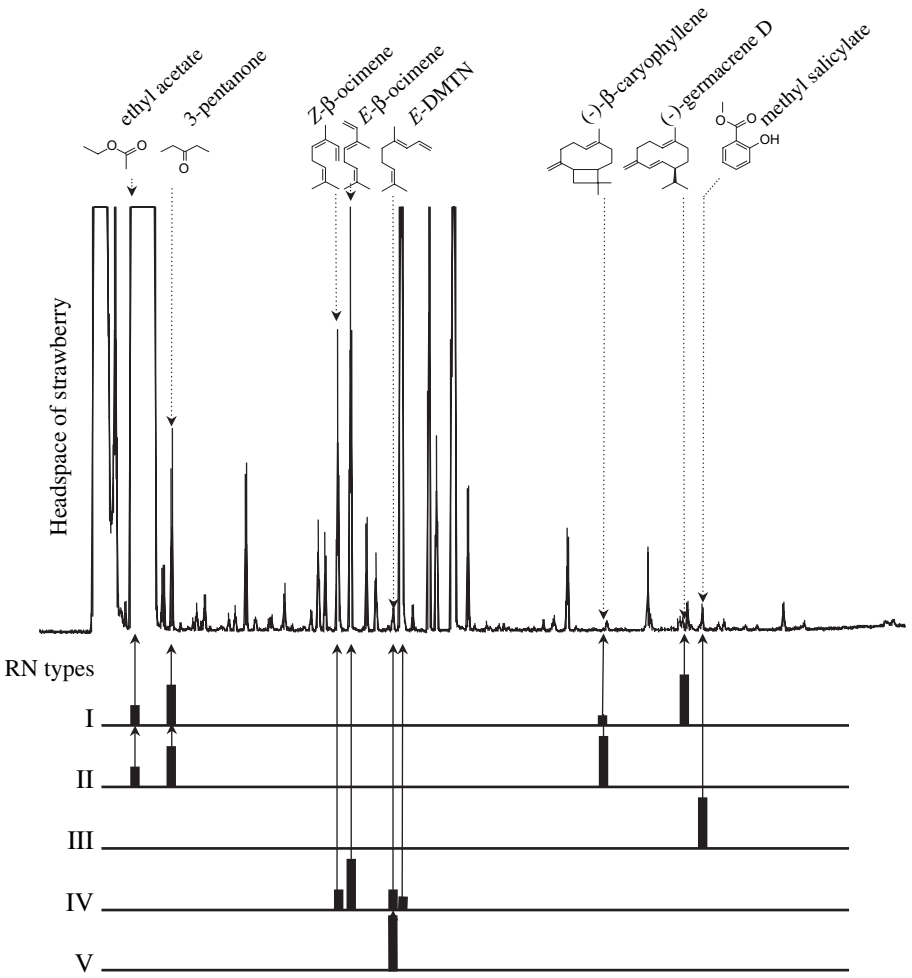
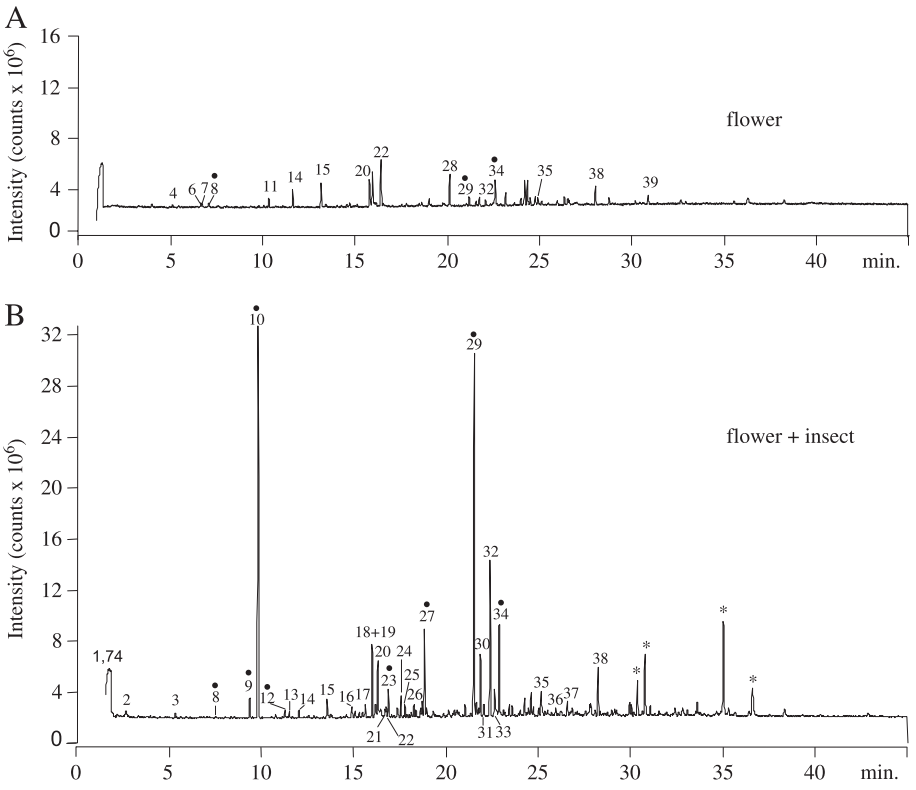


Figure 1 Overview of the responses of the five olfactory receptor neuron (RN) types identified in *A. rubi*. Top: gas chromatogram of the headspace volatiles from cut materials of strawberry (*F.* × *ananassa* var. Korona). Structures of the active components are shown. Bottom: vertical bars indicate the selective responses of the RN types (I–V). Within each RN type bar height indicates the relative response strength.



• Compounds with electrophysiological activity

Peak #	Compound	Flower	Flower+ insect
1	Acetone		x
2	2-Butenal**		x
3	1-Butene-2-one**		x
4	*α*-Pinene	x	
5	2-Methyl furane**		x
6	Hexanal	x	
7	3-Carene	x	
8	Myrcene	x	x
9	*Z*-*β*-Ocimene		x
10	*E*-*β*-Ocimene		x
11	Octanal	x	
12	*E*-DMNT		x
13	(3*Z*)-Hexenyl acetate		x
14	Sulcatone	x	x
15	Nonanal	x	x
16	Alloocimene**		x
17	Linalool oxide (furane type)		x
18	2-Pinen-10-ol**		x
19	Myrtenal**		x

Peak #	Compound	Flower	Flower + insect
20	Decanal	x	x
21	Alcanfor**		x
22	Benzaldehyde	x	x
23	Bourbonene		x
24	Linalool		x
25	Cinerone**		x
26	*β*-Ylangene		x
27	*β*-Caryophyllene		x
28	*β*-Farnesene	x	
29	Germacrene D	x	x
30	*α*-Muurolene		x
31	Elemene		x
32	(*E*,*E*)-*α*-Farnesene	x	x
33	*δ*-Cadinene		x
34	Methyl salicylate	x	x
35	Benzylalcohol	x	x
36	2-Phenylethanol		x
37	*β*-Ionone		x
38	Anisaldehyde	x	x
39	Benzoic acid	x	

* oxygenated sesquiterpenes,

** identified by MS-library reference spectra

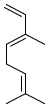
Figure 2 Gas chromatograms of the volatiles released from flowers on potted strawberry plants (*F.* × *ananassa* var. Korona) collected by solid-phase micro-extraction (SPME). Clear differences are shown both in the composition and in the relative amounts of the various compounds emitted from intact flowers **(A)** and from the flowers with weevils feeding **(B)**. Numbers indicate the identified structures listed below.

which outlasted the GC-peak. All neurons were tested with the sample of the host strawberry (*F.* × *ananassa*), which contained >90 compounds detected by the GC flame ionization detector. In addition, mixtures of volatiles from many other plants were tested. In all cases the responses to the most active components were confirmed by re-testing with standard mixtures of synthetic or authentic materials. In this investigation, a total of 29 RNs were classified into five types, according to the

compound that elicited the best response (Table 3). The RNs responded selectively to a few compounds (in the range of 3–12), one or two of which had a markedly better effect (primary odorant). All neuron types were found in both males and females. No particular distribution of the different RN types was found on the two antennal bands.

In some recordings two types of RNs appeared together, indicating co-location in the same sensillum. For example, in some recordings of type III RNs, tuned to the aromatic ester methyl salicylate, additional activity of one neuron of type I, or type II, appeared [tuned to the sesquiterpenes (−)-germacrene D and (−)-β-caryophyllene, respectively]

Table 3. Summary of the olfactory receptor neurone (RN) types in *Anthonomus rubi*, classified according to specificity of response to odours. The primary odorant (eliciting best response) and secondary odorants are indicated. Number of neurones identified (n) in a number of GC-SCR experiments (e) is indicated. Total number of times the response to each active compound was observed (r), is given with indication of the stimulation method (GC, gas chromatograph equipped with polar column; Ch, gas chromatograph equipped with chiral column; D, direct stimulation). Numbers in superscript indicate methods used for identification of the compounds (No number means use of comparison of mass spectra and retention times, in polar or chiral column, and re-testing on the neurones with reference samples; [1] Comparison of mass spectra and retention times on a polar column; [2] Comparison of mass spectra)

RN types	Primary odorant		Secondary odorants	
I	**Germacrene D**	r=52 (GC)	(+)-Germacrene D	r=16(Ch)+35(D)
n=10	**(-)-Germacrene D**	r=33(Ch)+40(D)	(-)-β-Caryophyllene	r=33(GC)
e=100			α-Humulene [1]	r=27(GC)
			(-)-β-Bourbonene [2]	r= 6 (GC)
			3-Pentanone [1]	r=16(GC)
			3-Hexanone [1]	r= 5 (GC)
			Ethyl acetate	r=18(GC)
II	**(-)-β-Caryophyllene**	r=28 (GC)	α-Humulene [1]	r=5 (GC)
n=3			3-Pentanone [1]	r=8 (GC)
e=31			3-Hexanone [1]	r=2 (GC)
			Ethyl acetate	r=6 (GC)
III	**Methyl salicylate**	r=13 (GC)	Methyl benzoate	r=8 (GC)
n=5			Ethyl benzoate	r=8 (GC)
e=24				
IV	***E*-β-Ocimene**	r=39 (GC)	*Z*-β-Ocimene	r=22(GC)
n=8			β-Myrcene	r=23(GC)
e=42			Dihydromyrcene	r=4 (GC)
			E-DMNT [1]	r=3 (GC)
			Limonene	r=17(GC)
			Terpinene [1]	r=4 (GC)
			β-Phellandrene	r=5 (GC)
			Geraniol	r=5 (GC)
			Citronellol	r=2 (GC)
			Neral	r=1 (GC)
			Geranial	r=4 (GC)
V	***E*-DMNT [1]**	r=6 (GC)	Neral	r=5 (GC)
n=3			Geranial	r=4 (GC)
e=25			Geraniol	r=6 (GC)
			Citronellol	r=2 (GC)

(Figure 3A). The type IV neurons tuned to the monoterpene *E*-β-ocimene were sometimes recorded simultaneously with type V tuned to the monoterpene *E*-DMNT (Figure 3A). In these recordings of co-localized RNs, the relative size of the spike amplitudes of each type were consistent, i.e. the type II RNs showed larger spikes than type III RN and the type IV RN larger spikes than type V (Figure 3B).

Type I

One neuron type responded best to (−)-germacrene D (n = 10) and to another early eluting compound in the host plant mixture. Examples of GC-SCR recordings from this type of RNs are given in Figure 4. In a tentative identification, the second active component was indicated to be 3-pentanone. Weaker responses to (−)-β-caryophyllene and α-humulene were also recorded when mixtures containing high amounts of these compounds were injected into the GC (Figures 4C and 5A), as well as weak responses to 3-hexanone. When tested with an UV degraded germacrene D sample, a secondary response to the rearrangement product β-bourbonene appeared (structure shown in Figure 5D). An additional response appeared when the neurons were stimulated with ylang-ylang essential oil (Firmenich) and the *Chrysanthemum* headspace (Figure 4C,D). This response was elicited by a sesquiterpene that could not be identified. A sustained response during the elution of the solvent ethyl acetate was also observed in all recordings (Figure 4A).

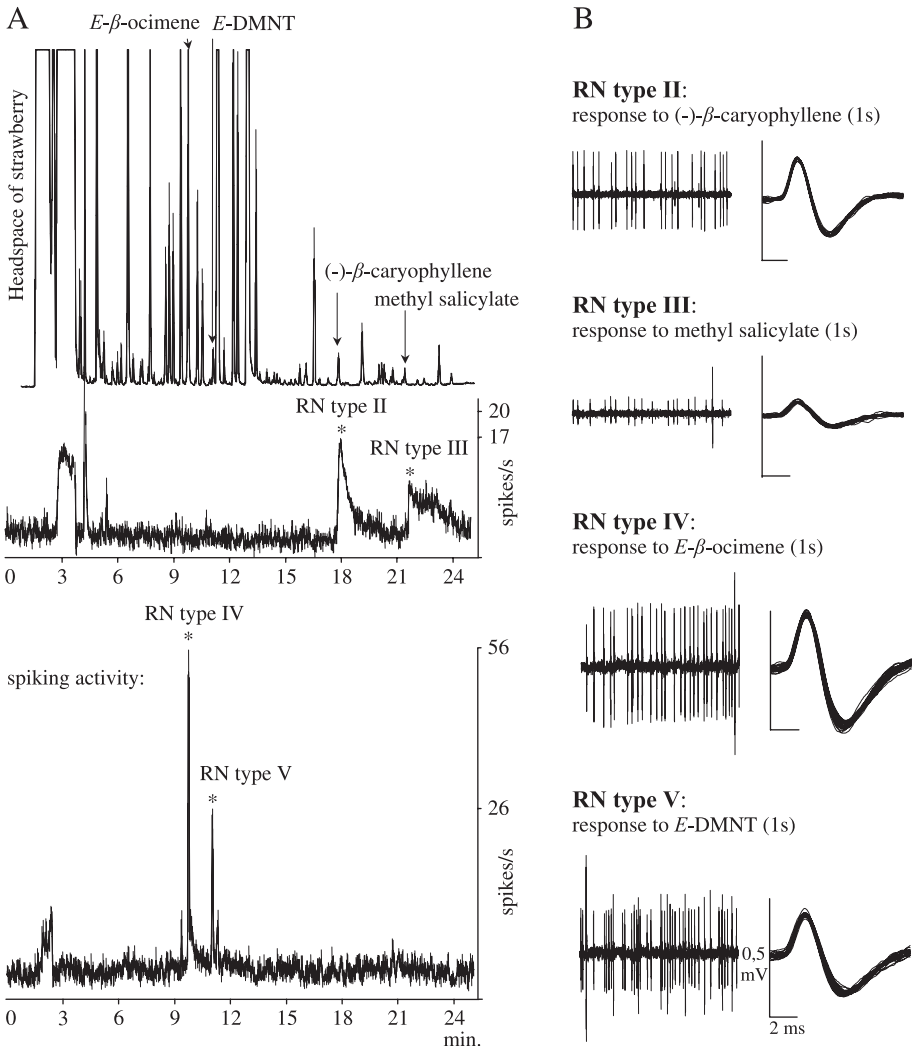


Figure 3 Responses of olfactory receptor neurons (RNs) located in the same sensillum of the weevil *A. rubi.* **(A)** Top: gas chromatogram of the headspace volatiles of strawberry (*F.* × *ananassa* var. Korona) and recording from a RN of type II responding primarily to (−)-β-caryophyllene and a type III RN responding to methyl salicylate (middle). The responses to the early-eluted compounds, ethyl acetate, 3-pentanone and 3-hexanone, originated from the RN type II. The recordings from the two other co-located RNs in the lower trace show responses to *E*-β-ocimene and to *E*-DMNT by a type IV and a type V RN, respectively. **(B)** Spike activity during 1 s after the start of each of the responses (marked with * in A). The differences in spike amplitude and waveform allowed each response to be ascribed to the RN types II–V. The spikes of each response are presented as overlays.

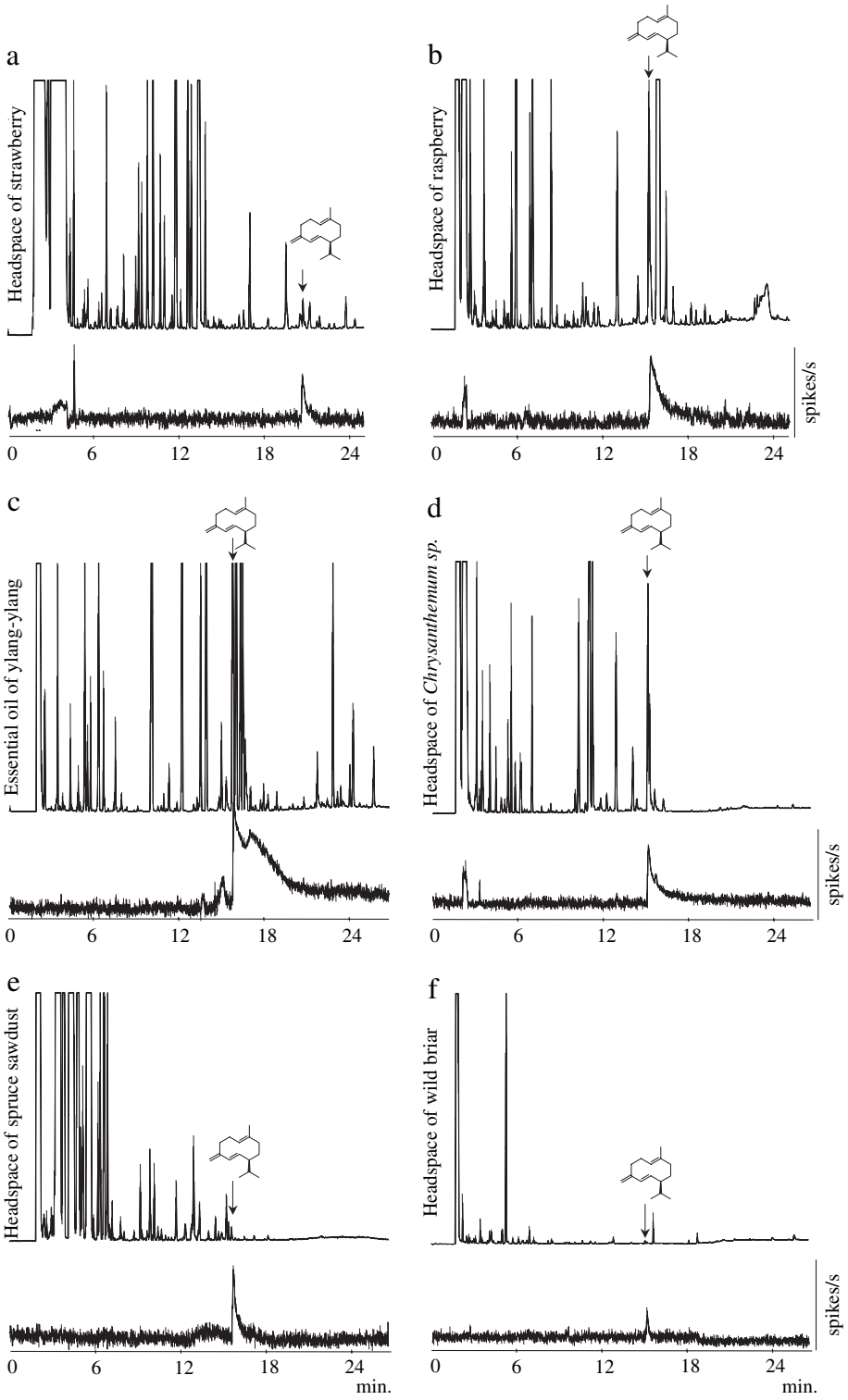


Figure 4 Selective responses of an olfactory receptor neuron (RN) type I in *A. rubi* to germacrene D and related compounds in the headspace of the host plant strawberry (*F.* × *ananassa* var. Korona) **(a)**, raspberry (*R. idaeus*) **(b)** and of four non-host plant materials **(c–d)**, separated by a polar GC column. Simultaneously recorded activity of a type I RN is shown below each chromatogram. Selective responses are shown to the primary odorant germacrene D, both at a high (c) and at a low concentration (f). Weaker responses are visible to secondary odorants in four of the recordings (a–d).

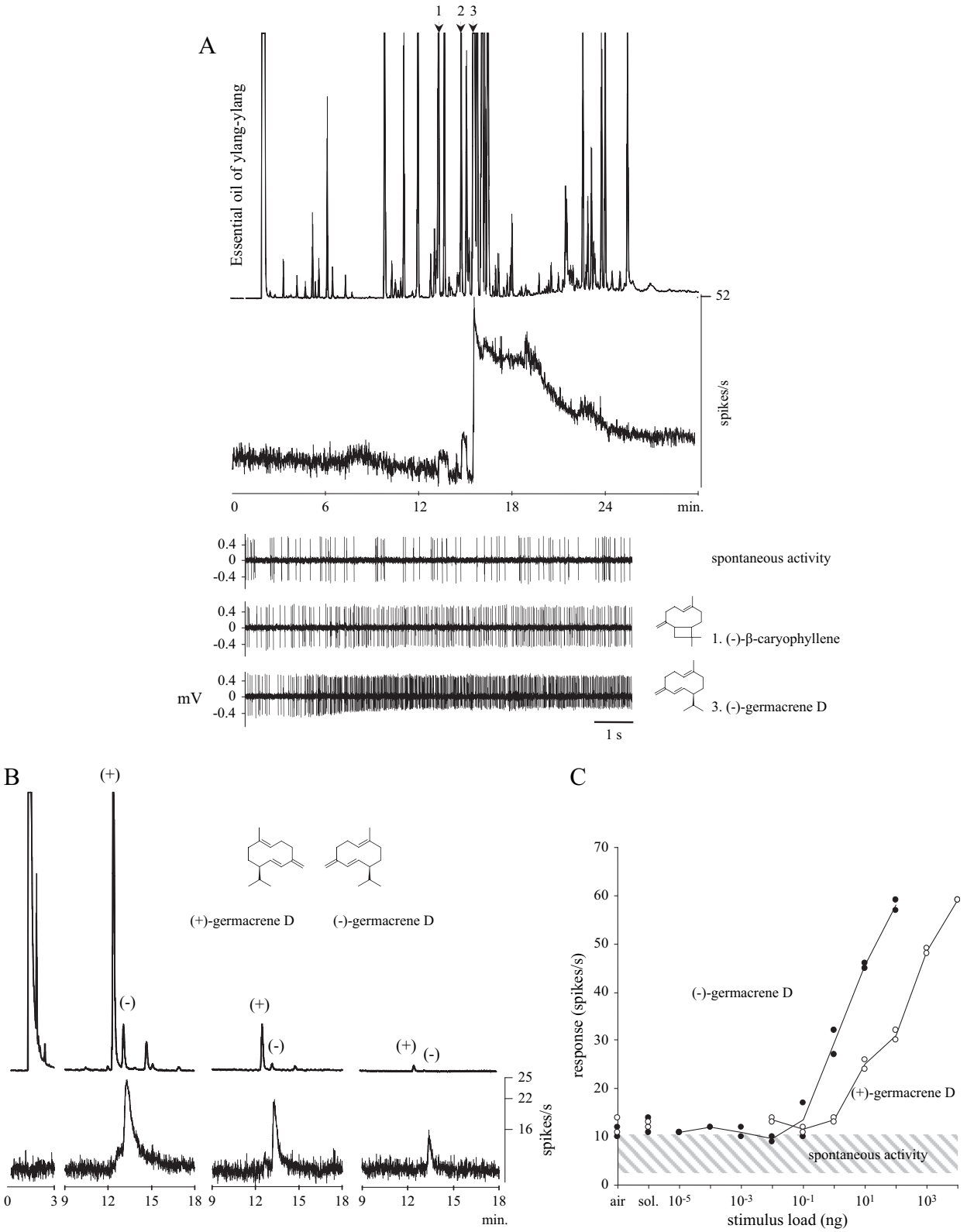


Figure 5 Sensitivity and molecular receptive range of the olfactory receptor neuron (RN) type I in *A. rubi*. **(A)** Gas chromatogram of an essential oil of ylang-ylang separated in the polar column, and simultaneously recorded responses of a type I RN. The primary response are shown to the sesquiterpene (−)-germacrene D (3) and the weak secondary responses to the related compounds (−)-β-caryophyllene (1) and α-humulene (2). **(B)** Decadic dilutions of a reference sample of (+)-germacrene D [containing 90% (+)- and 10% (−)-germacrene D] separated in the chiral column and simultaneously recorded activity of a type I RN, showing stronger response to the (−)-germacrene D than to the (+)-enantiomer (detection limit of the chiral column = 0.5 ng). **(C)** Dose–response curves

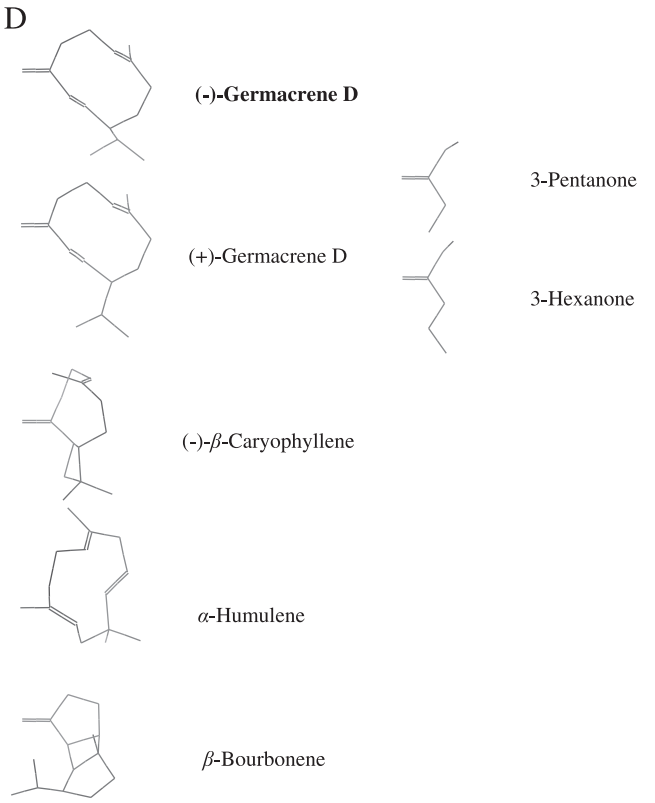


Figure 5 Continued.

We tested the neurons for sensitivity to the (−)- and (+)-germacrene D enantiomers by stimulating with reference samples separated in the chiral GC-column. Dose-dependent responses to both enantiomers were demonstrated for seven of these neurons. This was accomplished by separation in the chiral column of decadic dilutions of the (+)-germacrene D standard sample (Figure 5B). The neurons showed a higher sensitivity to (−)-germacrene D than to the (+)-germacrene D. This was confirmed by the dose–response curves obtained by direct stimulation of the neurons with decade step concentrations of the (+)- and (−)-germacrene D standard samples. Figure 5C presents an example of duplicated dose–response curves obtained for one neuron. The (+)-germacrene D curve is shifted ~1 log unit to the right of the curve for the (−)-enantiomer, indicating at least a 10-fold higher sensitivity for (−)-germacrene D. In one of the recordings activity of one type III RN was recorded simultaneously (details are given below). No overlap of the molecular receptive ranges of the two RN types was found.

Type II

Three neurons were specifically tuned to (−)-β-caryophyllene and responded also to the component identified as 3-pentanone. Secondary responses of these neurons to α-humulene and 3-hexanone were also recorded (Figure 6), as well as a sustained high firing rate during the elution of the solvent ethyl acetate. Dose–response studies performed by injecting decadic dilutions of a reference sample of (−)-β-caryophyllene into the GC-column revealed a high sensitivity of this neuron type, which responded to amounts below the minimum GC-detector threshold [in the range of picograms (data not shown)]. Injection of several mixtures of plant volatiles and standards into the chiral column of the GC showed that none of the samples used contained detectable amounts of (+)-β-caryophyllene [the detection limit of the chiral column is 0.5 ng as calculated by Stranden *et al.* (2002)]. Whether the neurons also respond to the (+)-enantiomer remains to be tested.

Interestingly, the RNs of type I and II, tuned to different sesquiterpenes [(−)-germacrene D and (−)-β-caryophyllene, respectively], showed overlapping molecular receptive ranges for several secondary compounds, as well as response of type I to (−)-β-caryophyllene. The common secondary odorants were α-humulene, 3-pentanone and 3-hexanone, as well as the solvent ethyl acetate (Figure 6 and Table 3). Another similarity was the pronounced phasic pattern of the responses to 3-pentanone and 3-hexanone, as compared with the phasic-tonic, long-lasting responses to the primary odorants in both neuron types.

Type III

The RN type III (n = 5) responded best to methyl salicylate, as shown by separation of the volatiles of the host plant, strawberry (Figure 3A). Three of these neurons appeared in the same recordings as RN type II, whereas the fourth type III neuron appeared together with a type I neuron and the fifth was recorded alone. Secondary responses (for three of the neurons) to methyl benzoate and ethyl benzoate were demostrated by injection of a standard mixture, as shown in Figure 7A (the remaining two neurons were not tested for these compounds). Figure 7B shows dose–response curves obtained by injection of three dilutions of another mixture containing the active compounds. It is worth noting that the spontaneous activity of these neurons was low (0–2 spikes/s) and that the response to the primary odorant showed a tonic, long-lasting firing rate even at relatively low concentrations. The high sensitivity and tonic response

obtained by direct stimulation with samples of (−)- and (+)-germacrene D (both containing 10% of the opposite enantiomer), showing 10–100 times stronger effect of the (−)-germacrene D. The curves show the mean of two responses obtained by repeated stimulation with the same cartridge. **(D)** Structures of the compounds that constitute the molecular receptive range of the RN type I.

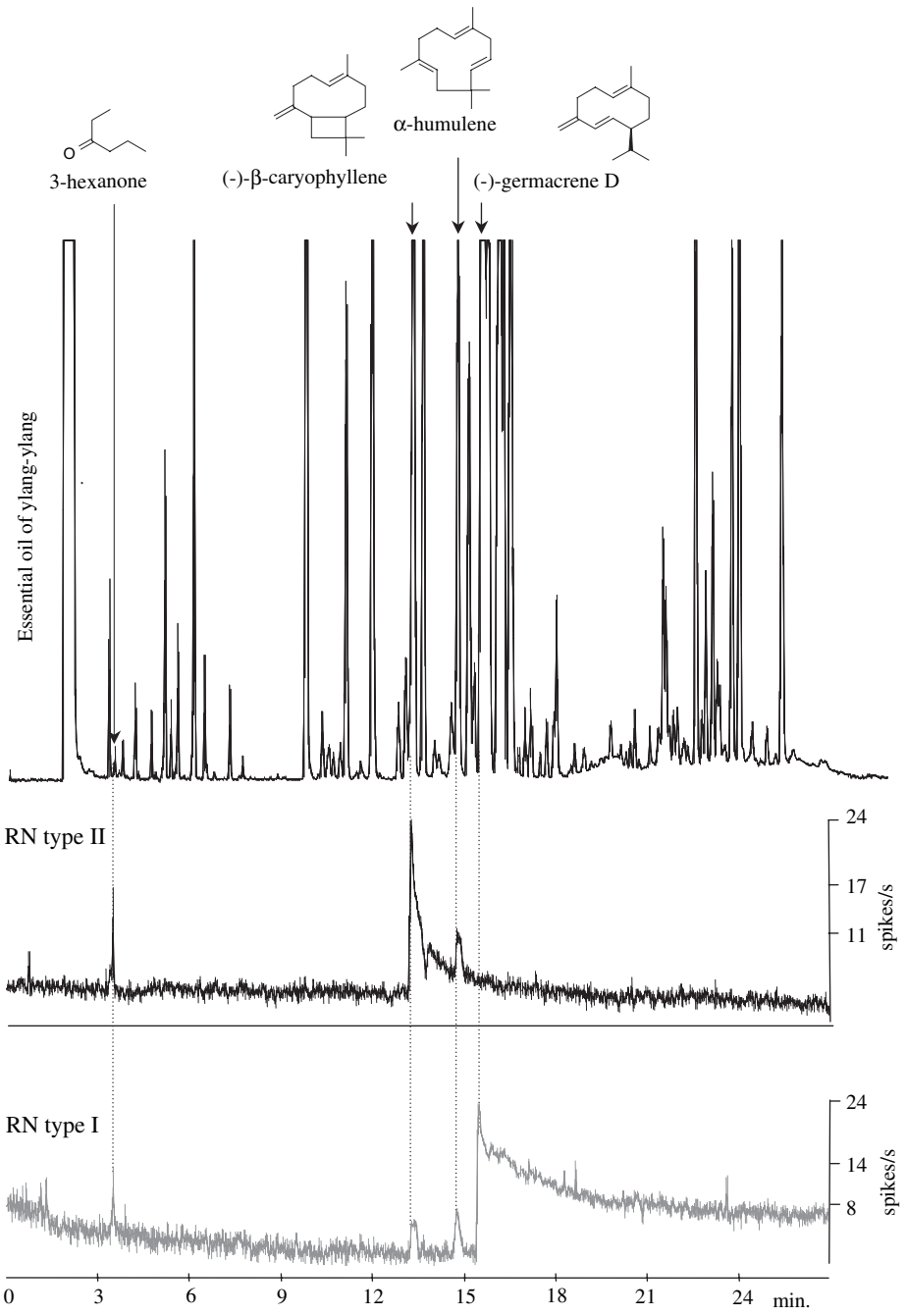


Figure 6 Gas chromatogram of an essential oil of ylang-ylang separated in the polar column and simultaneously recorded activity of a type II olfactory receptor neuron (RN) showing the strongest response to (−)-β-caryophyllene and weaker responses to α-humulene and 3-hexanone. The overlap between the molecular receptive ranges of the RN types I and II is shown by presenting (in grey) a recording from a type I RN (obtained in the same individual).

property of this RN type to the primary odorant was further shown by injecting a small amount of methyl salicylate. A 0.5 ng amount elicited a response that outlasted the GC-peak (65 s), although the highest firing frequency was only 13 spikes/s. In contrast, the responses to methyl benzoate and ethyl benzoate were relatively brief, lasting from 10 to 20 s. In the recording showing activity of only one type III neuron, the firing rate was much higher, i.e. maximum firing rate of the response was 31 spikes/s, and the spontaneous activity varied from 0 to 6 spikes/s.

Type IV

The neurons showing highest sensitivity to the acyclic monoterpene *E*-β-ocimene (n = 8) were classified as type IV. Altogether, 11 compounds elicited secondary responses in these RNs. Examples of GC-SCR recordings from this type

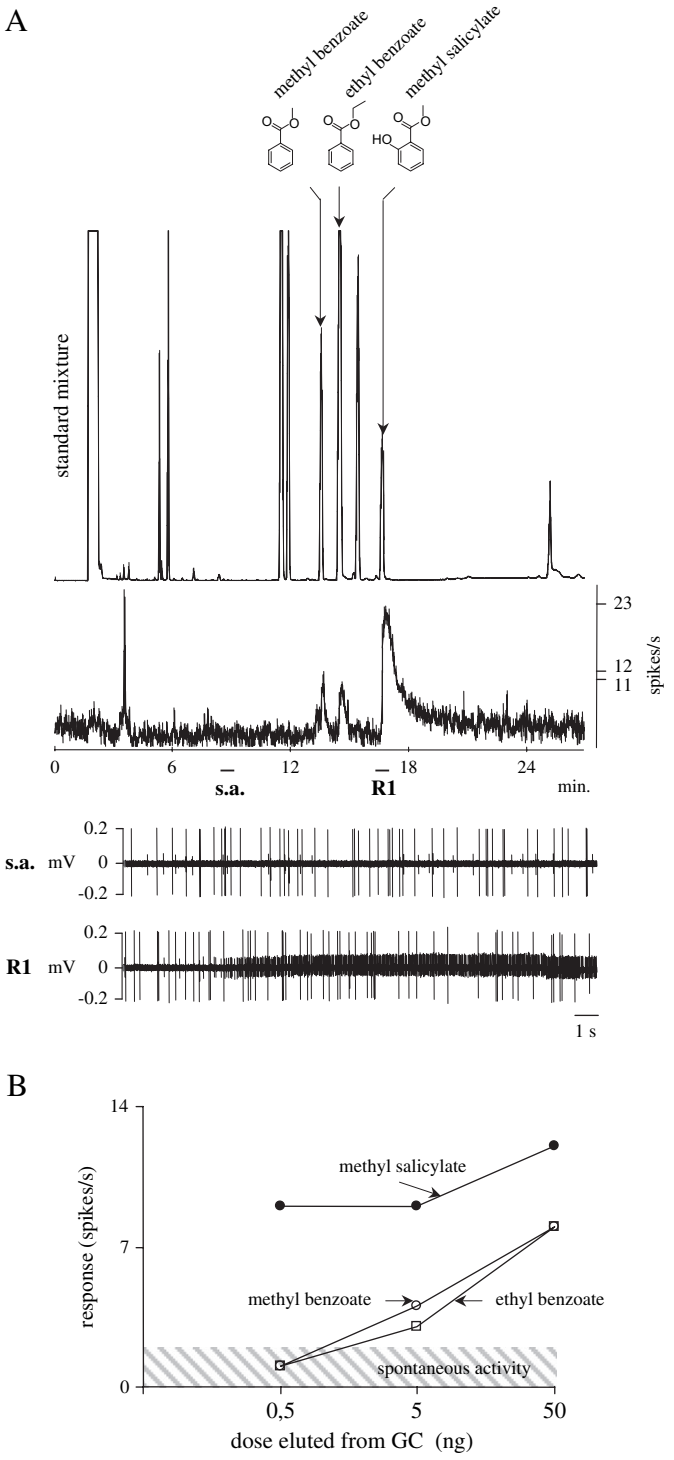


Figure 7 **(A)** Gas chromatogram of a standard mixture separated in the polar column and simultaneous recorded activity of a type III receptor neuron (RN) showing response to three components. Top: the molecular structures are shown of the three active compounds. Bottom: spike activity of the neuron during 20 s (black bars) of spontaneous activity (s.a.) and during the response to methyl salicylate (R1). **(B)** Dose–response curves for the three odorants activating the type III RN, obtained by stimulation via the polar column showing a stronger effect of the methyl salicylate (primary odorant) than of methyl benzoate and ethyl benzoate (secondary odorants).

of neurons are shown in Figure 8, with secondary responses to *Z*-β-ocimene and *E*-DMNT. In addition, dihydromyrcene, citronellol, geraniol, geranial and neral present in some mixtures (lavender and lemon essential oils) elicited weak responses. Responses to the cyclic monoterpenes limonene, γ-terpinene and β-phellandrene appeared when the neurons were stimulated with mixtures containing very large amounts (μg) of these compounds. Verification of the identity of all active compounds on this RN type was performed by stimulating the neurons with standard samples (except γ-terpinene). Dose–response experiments, performed by injecting standard decadic dilutions into the GC-column, indicated the specificity of these neurons by *E*-β-ocimene having a stronger effect than the geometrical isomer *Z*-β-ocimene and β-myrcene (Figure 8B). Three of these neurons appeared together with a RN of type V. Interestingly, the two neuron types had overlapping molecular receptive ranges, i.e. both responded to the odorants *E*-DMNT, citronellol, geraniol, geranial and neral (Figure 9B). Neuron type IV responded weakly to *E*-DMNT, the primary odorant for neuron type V. The synchronous responses of the two neurons to the stereoisomers neral and geranial are shown in Figure 9B.

Type V

The RNs of type V responded strongest to the monoterpene *E*-DMNT ($n = 3$) when tested with the host volatiles (Figure 8A). The compound *E*-DMNT eluted simultaneously with (3*E*)-hexenyl acetate in the strawberry headspace mixture, but re-testing with a (3*E*)-hexenyl acetate standard showed no response. Furthermore, testing the neurons for the alternative host raspberry that contained a clean *E*-DMNT peak confirmed the response. Strong responses to neral and weaker responses to geranial were detected when the neurons were stimulated with lemon essential oil and confirmed by injection of decadic dilutions of reference samples (results for one dilution shown in Figure 9A). Additional weaker responses to geraniol and citronellol were recorded when the neurons were stimulated with lavender essential oil (Figure 9B).

Discussion

The present results on functional types of olfactory receptor neurons (RNs) in the strawberry weevil *A. rubi* elucidate peripheral mechanisms involved in the detection of plant odour information. The results add to the growing knowledge about functional types of RNs in general, and in a comparative perspective, i.e. in relation to RNs of closely (weevils) and distantly (moths) related insect species that have adapted to different host plants. The use of GC-SCR has allowed each single neuron to be tested for hundreds of naturally produced volatiles in various host and non-host plants. The consistent and selective responses to one or a few primary and secondary odorants indicate that these compounds are biologically significant to the strawberry weevil. The narrowly

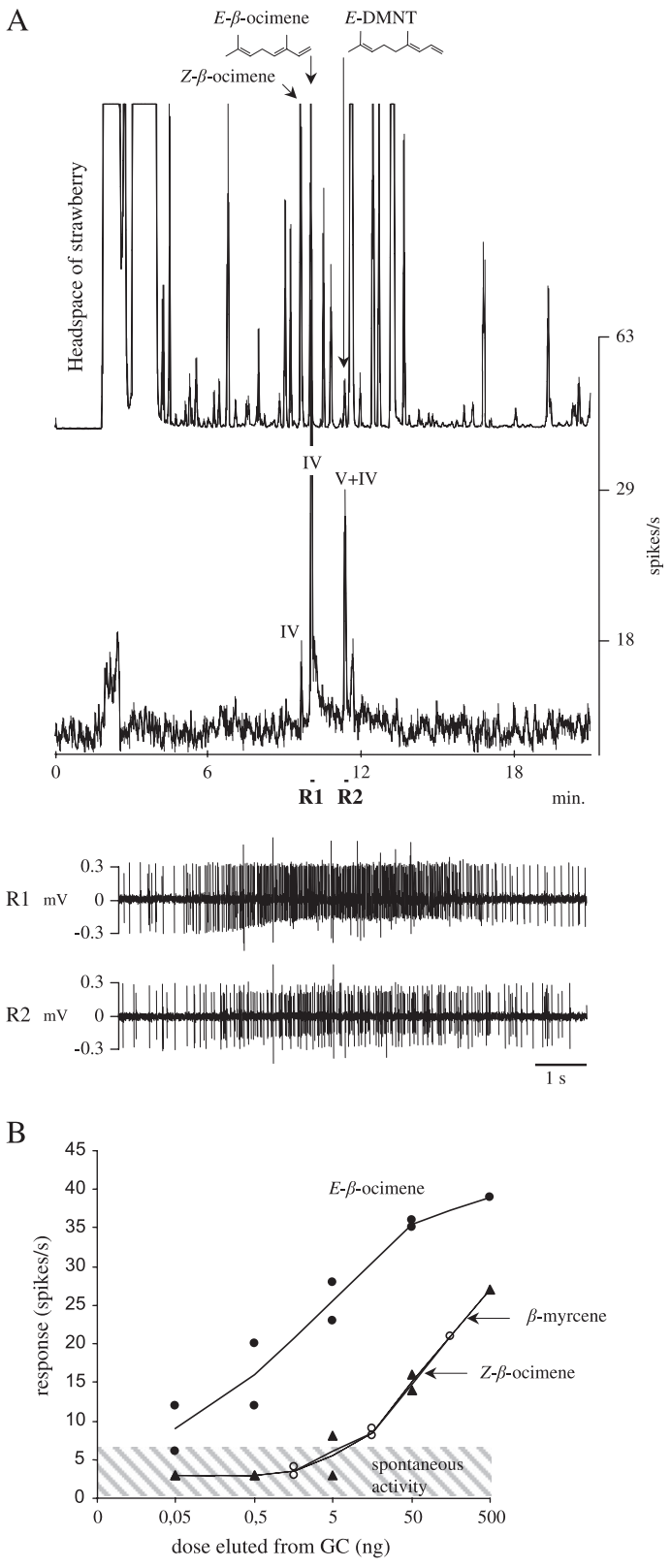


Figure 8 **(A)** Gas chromatogram of the headspace volatiles of strawberry (*F.* × *ananassa* var. Korona) and simultaneously recorded activity of a type IV and a type V olfactory receptor neurons (RN). Molecular structures of the active compounds are presented above. Spike activity elicited by the two most potent odorants, *E*-β-ocimene and *E*-DMNT, is shown during 10 s of the responses (indicated by black bars) R1 and R2. **(B)** Dose–response curves for the RN type IV stimulated via the GC with three odorants, *Z*-β-ocimene, *E*-β-ocimene and β-myrcene, showing 10–100 times stronger effect of the primary odorant, *E*-β-ocimene.

tuned RNs presented here could be classified into five distinct types, the neurons of each type having similar molecular receptive range and similar ranking of the primary and secondary odorants. The uniform response pattern within one neuron type correlates well with the current information from molecular biology studies showing that subsets of olfactory RNs express one type of receptor protein in insects and vertebrates (Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001; Keller and Vosshall, 2003; Hallem *et al.*, 2004). The primary odorants (eliciting the strongest response) and the majority of the secondary odorants (eliciting weaker responses) were shown to be inducible compounds, i.e. produced in higher amounts by the strawberry inflorescences on which weevils were feeding (Figure 2).

Structurally similar molecules have a higher probability to bind to the same odorant-binding and receptor protein. This explains the molecular similarity among the primary and secondary odorants activating one RN type, and also the pattern of overlap of molecular receptive ranges of different types. The principle that RNs which respond to the same chemical group show some overlap, whereas no overlap is found between RN types that respond to different chemical groups, e.g. monoterpenes versus sesquiterpenes, is shown in the present as well as in previous studies of weevils, other beetles and moth species (Wibe and Mustaparta, 1996; Wibe *et al.*, 1997; Stensmyr *et al.*, 2001; Barata *et al.*, 2002; Bichão *et al.*, 2003). In this study, the monoterpene molecules activating RN type IV and V are similar. For RN type IV, the primary and secondary odorants *E*-β-ocimene and *Z*-β-ocimene are geometrical isomers, and one other secondary odorant, β-myrcene, differs only in the position of one of the double bonds (Figure 9). The weaker effect of two other secondary compounds, dihydromyrcene and *E*-DMNT, is probably due to higher flexibility and the effect of one additional carbon atom, thus allowing less interactions with the receptor compared to the primary odorant. The RN of type V responded strongest to *E*-DMNT, which has a chain one carbon longer than ocimene and myrcene. The aldehyde group in neral, the second most active compound for type V RNs, might have an electrophilic similarity with the methylene group of *E*-DMNT, which may explain the relatively high secondary effect. In addition, the acyclic monoterpenes geranial and geraniol elicited weak responses in both type IV and V, whereas the cyclic monoterpenes activated only the type IV RNs.

In the case of the RN type I, the most effective molecules are (−)-germacrene D and (+)-germacrene D. The direction of the isopropyl group on the 10-carbon ring might explain the different effect of the two enantiomers. The much lower

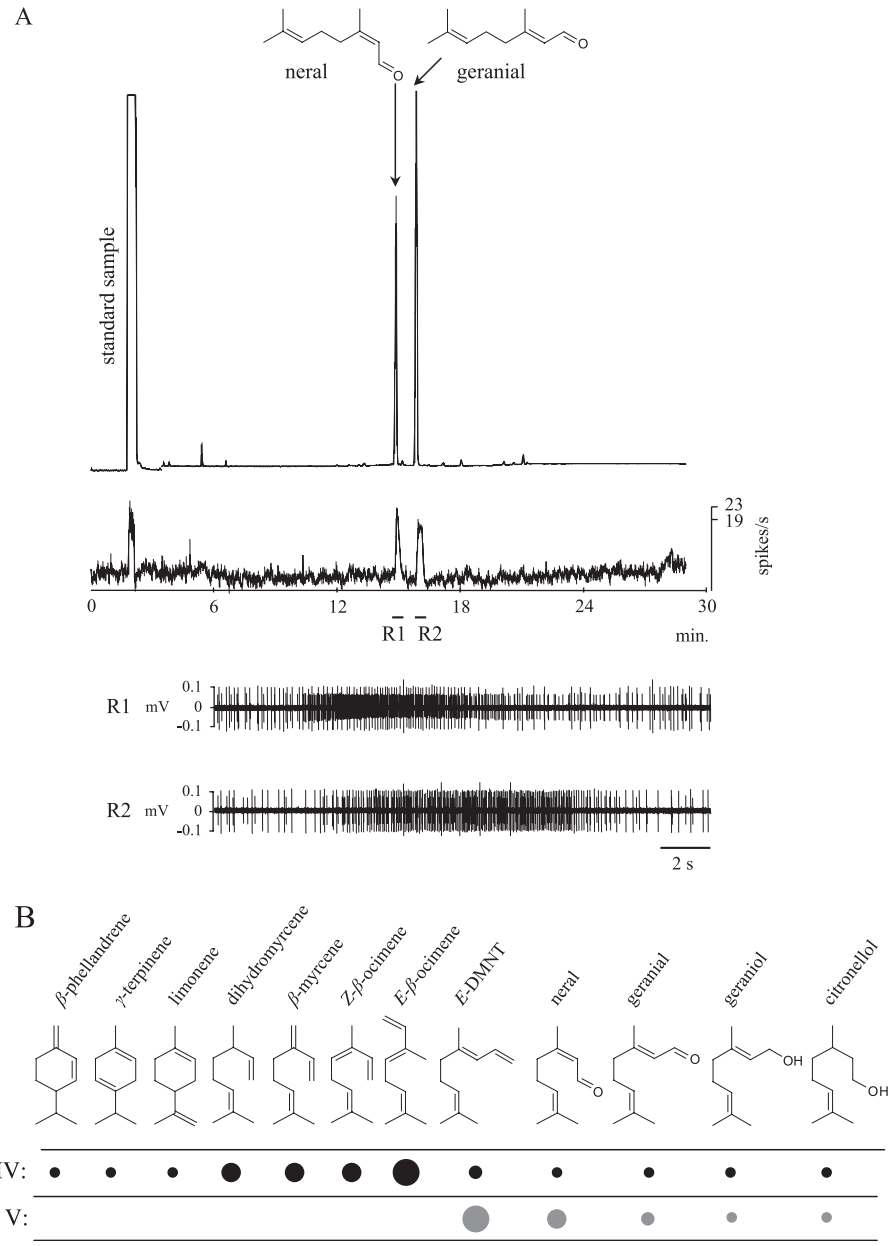


Figure 9 **(A)** Gas chromatogram of a standard mixture of neral and geranial, and simultaneously recorded responses of the two olfactory receptor neuron (RN) types (IV and V) located in the same sensillum. Top: molecular structures of the active compounds are shown. Bottom: spike activity during the 20 s periods R1 and R2 (indicated by black bars), of the type IV (large amplitude spikes) and the type V (small amplitude spikes) RNs, shows responses of both neurons to the two odorants. **(B)** The molecular receptive ranges of the two RN types are shown with indication of the relative response strength within each RN type (size of the circles).

effect of (−)-β-caryophyllene and α-humulene, which are molecules with similar hydrophobicity as germacrene D, can be explained by the different ring systems, and the absence of the isopropyl group (Figure 5D). In the case of the type II RNs tuned to (−)-β-caryophyllene, the receptor seems to be slightly different, so that the germacrene D does not fit well into the receptor. More surprising was the response of these two sesquiterpene RN types to the structurally unrelated molecules 3-pentanone, 3-hexanone and ethyl acetate. However, these are small molecules that might easily fit into the receptor pocket. The similarities in electrophilic properties between the carbonyl group in the ketones and the methylene group in the sesquiterpene molecule may be important features. Possibly the interaction is only with a part of the receptor site, which correlates with the responses to the small molecules showing a short and phasic pattern compared with the long-lasting responses elicited by the sesquiterpenes (cf. Figure 3A). Alternatively, the responses to the small molecules could have originated from a different neuron recorded simultaneously. However, since the spike

amplitude and waveform of the responses were similar, and the responses always occurred in the numerous recordings of these neurons, they seem to originate from the same neuron.

Consistent co-location of functional RN types in one sensillum has been found in this study as well as in previous studies of weevils and moths, and other insects (Blight *et al.*, 1995; Røstelien *et al.*, 2000b; De Bruyne *et al.*, 2001; Stranden *et al.*, 2003b), but the functional importance of co-location is not known. It is interesting to note, however, that in *A. rubi*, the five functional types occurred in three different pairs, two of them showing no overlap of the molecular receptive range between the two elements of the pair: I and III [(−)-germacrene D and methyl salicylate], and II and III [(−)-β-caryophyllene and methyl salicylate], i.e. type III occurred together with both types I and II. In the other neuron pair, IV and V, both RNs were tuned to related terpenes [*E*-β-ocimene and *E*-DMNT] and showed an extensive overlap of the molecular receptive range (cf. Figure 9).

From an evolutionary point of view it is interesting to compare the specificity of RNs responding to plant volatiles in closely and distantly related insect species. In this study we used experimental protocols designed to allow comparison with studies of weevils and heliothine moths carried out in our laboratory. Particularly interesting is the RN type tuned to (−)-germacrene D, which constitutes the largest number of the five RN types in *A. rubi* and is also the most abundant RN in the heliothine moths (Røstelien *et al.*, 2000a; Stranden *et al.*, 2002, 2003a). In both insect groups these RNs show similar enantioselectivity to the dominant enantiomer in higher plants (−)-germacrene D, which has a 10- to 100-fold stronger effect than (+)-germacrene D (Figure 5C). However, the secondary odorants were different. In the weevil *A. rubi*, the neurons responded secondarily to (−)-β-caryophyllene, α-humulene and β-bourbonene, whereas in the heliothine moths the germacrene D RN type responds to ylangenes and copaenes (Stranden *et al.*, 2003a). Thus, the RN type evolved for the detection of (−)-germacrene D in *A. rubi* is different in specificity from the type evolved in the three heliothine moths, indicating that they have evolved independently in the adaptation to their different host plants. An RN type corresponding to type IV tuned to *E*-β-ocimene in *A. rubi* has also been identified in heliothine moths (Røstelien *et al.*, 2000b; Stranden *et al.*, 2003b). In this case, a striking similarity occurred in the secondary responses to *Z*-β-ocimene and β-myrcene in both insect groups. However, in *A. rubi* these RNs responded weakly to several cyclic monoterpenes and oxygenated monoterpenes, which was not the case in the heliothine moths. In two other weevil species, *Pissodes notatus* and *Hylobius abietis*, which live on conifers, no RNs have been found that were tuned primarily to germacrene D or to *E*-β-ocimene (Wibe *et al.*, 1997; Bichão *et al.*, 2003), although the headspaces of the host plants contain large quantities of these compounds. In the cabbage moth *Mamestra brassicae*, one RN type is tuned to methyl salicylate (S. Ulland, personal communication) and responds secondarily to methyl benzoate, similar to the RN type III in *A. rubi*. But again differences emerge when considering the secondary responses, *A. rubi* showing an additional response to ethyl benzoate.

Receptor neurons specified for the same odorants as RN types II and III, are also found in other species of beetles. A RN type with primary odorant β-caryophyllene is found in *P. notatus* (Bichão *et al.*, 2003) and RN types with primary odorant methyl salicylate are found in the weevil *Ceuthoynchus assimilis* (Blight *et al.*, 1995) using brassica plants as hosts, and in the fruit chafer *Pachnoda marginata* (Stensmyr *et al.*, 2001). However, in these cases different protocols were used and therefore the comparison concerning secondary responses is limited. Whereas the mentioned studies are based on GC-SCR, screening with synthetic odorants has also revealed RN types for the same primary odorants in the cotton weevil *Anthonomus grandis*, e.g. RN types tuned to *E*-β-ocimene and to β-caryophyllene (Dickens, 1990). Using yet another method, GC-EAG, Kalinová *et al.* (2000) recorded responses to caryophyllene in the apple blossom weevil *Anthonomus pomorum.* Altogether these comparisons show that species of weevils and moths have evolved RNs tuned to the same primary odorants, but these RNs show differences in the secondary odorants. We do not know the significance of secondary odorants, but one can speculate that these differences reflect independent (convergent) evolution in the adaptation to different host plants. Alternatively, these RNs may have evolved from a common ancestral RN type that has later undergone chance mutations.

In recent years the advances in plant chemistry have shown that the blends of volatiles emitted from plants contain a large number of compounds most of which are common to many plants. The insects are equipped with RNs that detect these odorants. Thus, they probably use for host location a large number of compounds that are common to many plant species. This is in contrast to the idea that the plant odour RNs are specified for a few compounds characteristic of the particular host plants. Another principle, emerging from the results of this and other studies, is that plant odour RN types in insects are specified for compounds emitted in particular conditions by the host plant (Stranden *et al.*, 2003b). In this study, all the primary odorants identified for the five RN types were collected in higher amounts in the headspace of strawberry flowers on which *A. rubi* had been feeding than on intact flowers (cf. Figure 2). Whether these compounds contribute to enhanced or reduced attraction to the host plant is not known. In the case of attraction, the ability to recognize a plant on which conspecifics are feeding could have an adaptive role by favouring aggregation and therefore encounters of potential mates. Since these weevils lay only one egg per flower bud, it is also possible that induced volatiles might be a cue for the females to avoid attacked flower buds.

The five functional types of RNs in the antennal sensilla of *A. rubi* presented here are narrowly tuned and all respond to compounds which are common secondary metabolites present in many host and non-host plants. However, these compounds are inducible, i.e. emitted systemically in altered ratios when the weevils are feeding on the host plants, suggesting that *A. rubi* uses plant-produced compounds as information about the presence of conspecifics on the host plant. In addition, the results show that RNs tuned to the same compounds exist in unrelated species that utilize host plants belonging to different plant groups, suggesting a general importance of these compounds for plant herbivore interactions.

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