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Methyl Salicylate, Identified as Primary Odorant of a Specific Receptor Neuron Type, Inhibits Oviposition by the Moth *Mamestra Brassicae* L. (Lepidoptera, Noctuidae)

S. Ulland[1], E. Ian[1], R. Mozuraitis[2,3], A.-K. Borg-Karlson[2], R. Meadow[4] and H. Mustaparta[1]

[1]Neuroscience Unit, Department of Biology, Norwegian University of Science and Technology, NO-7489 Trondheim, Norway, [2]Ecological Chemistry Group, Department of Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden, [3]Laboratory of Chemical and Behavioural Ecology, VU Institute of Ecology, Akademijos 2, LT-08412, Vilnius, Lithuania and [4]Bioforsk, Norwegian Institute for Agricultural and Environmental Research, NO-1432 Ås, Norway

Correspondence to be sent to: Hanna Mustaparta, Neuroscience unit, Department of Biology, Norwegian University of Science and Technology, NO-7489, Trondheim, Norway. e-mail: hanna.mustaparta@bio.ntnu.no

Abstract

The cabbage moth, *Mamestra brassicae* L. (Lepidoptera, Noctuidae), is a polyphagous species that is often choosing plants of *Brassica* as hosts for oviposition. In the search for biologically relevant odorants used by these moths, gas chromatography linked to electrophysiological recordings from single receptor neurons (RNs) has been employed, resulting in classification of distinct types of neurons. This study presents specific olfactory RNs responding to methyl salicylate (MeS) as primary odorant and showing a weak response to methyl benzoate, the 2 aromatic compounds occurring together in several plant species. In 2 cases, the neuron was colocated with another RN type responding to 6 green leaf volatiles: 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol, (3*Z*)-hexenyl acetate, (2*Z*)-hexen-1-ol, and an unidentified compound. Whereas the specific RNs detected the minor amounts of MeS in some plants, the compound was not found by gas chromatography linked to mass spectrometry in intact plants, but it was found after herbivore attack. The behavioral effect of MeS was studied in outdoor test arenas with *Brassica napus* and artificial plants. These experiments indicated that mated *M. brassicae* females avoid plants with dispensers emitting MeS. As it is induced by caterpillar feeding, this compound may mediate a message to mated *M. brassicae* females that the plant is already occupied.

Key words: behavioral responses, GC-SCR, *Mamestra brassicae*, methyl salicylate, olfactory receptor neurons

Introduction

Olfactory receptor neurons (RNs) in herbivore organisms may have evolved to detect volatiles that are specifically produced by certain species of host plants or that are widely present in many families of plants. In several species of moths, weevils, and beetles, distinct types of olfactory RNs have been found using gas chromatography linked to electrophysiological recordings from single RNs (Røstelien et al. 2000; Stranden, Liblikas, et al. 2003; Stranden, Røstelien, 2003; Bichão, Borg-Karlson, Araújo, et al. 2005; Bichão, Borg-Karlson, Wibe, et al. 2005; Røstelien et al. 2005; Ulland et al. 2006). The results have demonstrated RNs of polyphagous as well as oligophagous insects, which respond to compounds present in a wide range of plant species. However, each of the neuron types in these species has appeared with a relatively narrow molecular receptive range, by strongly responding to one primary odorant and weaker to a few structurally similar compounds, termed secondary odorants (Røstelien et al. 2000). In the related heliothine moths, the same functional types of RNs have been found in the polyphagous moths, using a broad range of plants in monocultures (cotton, corn, tomato, maize, etc.) and in oligophagous species, preferring a more narrow range of host plant within the family Solanaceae (Røstelien et al. 2005). In this context, one may ask whether other moth species, like *Mamestra brassicae*, preferring different plant species as hosts, may have evolved olfactory RNs detecting

different odorants used in their orientation toward suitable host plants.

Plant defence can be either "constitutive" or "induced"; constitutive defenses meaning that the compounds are continuously produced, stored in specialized structures, and released upon attack. Induced defenses on the other hand can be triggered by herbivore or pathogen attack (Paré and Tumlinson 1999; Gouinguené and Turlings 2002). Induction and release of volatile compounds can also be triggered by abiotic factors, such as UV radiation, ozone, and temperature (Johnson et al. 1999; Pichersky and Gershenzon 2002; De Moraes et al. 2004). An example of a well-known compound to be induced is the aromatic compound methyl salicylate (MeS), which is shown to be induced upon attack by herbivores in several plant species (van Poecke et al. 2001; Chen et al. 2003; Van den Boom et al. 2004; Bichão, Borg-Karlson, Araújo, et al. 2005). Further, plants can emit specific blends of volatiles that differ depending on the attacking species, even closely related species as shown for *Heliothis virescens* and *Helicoverpa armigera* (De Moraes et al. 1998). Altogether, insects in search for a suitable host plant needs to unravel the vast amount of nonrelevant and relevant components released by plants. The challenge is met by the use of a highly sensitive and specialized olfactory system.

The cabbage moth, *M. brassicae*, is widespread around the world and is common in the southern parts of Norway. The species overwinters as prepupae in the soil close to its host plant. From mid-June to August the adults emerge and after mating the females start searching for a host plant. *M. brassicae* is polyphagous and survives on many species of plants, and the caterpillars are often associated with host plants of the genus *Brassica* (CAB International 2005; Skou 1991). Feeding by the caterpillars causes severe damage on the plants, mostly due to chewing and fouling rather than the amount of plant tissue eaten (CAB International 2005). Plant production in agriculture has been dependent on insecticides for over half a century. The undesirable side effects of many of these insecticides have led to the current focus on research on other methods of protecting plants from insect pests. Thus, identification of biologically relevant odorants provides key compounds to be included in integrated control of herbivorous pest insects. As with other herbivorous moths, the important olfactory cues used by *M. brassicae* include pheromones and plant odors. Whereas the limited number of compounds produced by female moths makes the pheromone blend relatively simple to resolve, it is much more complicated to identify the broad range of numerous plant-produced volatiles that are detected by the olfactory RNs. In *M. brassicae*, specific tuning of single RNs to the female-produced sex pheromones is well described (Renou and Lucas 1994). Studies on the specificity of the plant odor RNs, including tuning to induced compounds, have just started by using gas chromatography linked to SCR (GC-SCR). In a previous study, we presented one RN type in *M. brassicae* tuned to linalool (Ulland et al. 2006). In the present study, we ask whether *M. brassicae* has other RN types specifically tuned to plant odorants, systemically produced or induced by herbivory. We here present 2 types of plant odor RNs, one tuned to MeS and the other to green leaf volatiles (GLVs). The behavioral effect of MeS was tested in a field bioassay. The results indicated that MeS has an inhibitory effect on the oviposition of mated *M. brassicae* females.

Materials and methods

Electrophysiological experiments

Insect material

Mamestra brassicae pupae were from our cultures at The Norwegian Institute for Agricultural and Environmental Research, Ås, Norway. The sexed pupae were stored in separate containers placed in climate chambers (22 °C, 14:10 light:dark regime, onset of dark cycle at 10.00 AM). After eclosion, the adult insects were kept in cylindrical containers (approximately 1350 cm^3) with access to water containing sucrose (5%). The age of adult insects used in the experiments ranged from 3 to 15 days. Both sexes were used in the experiments.

Headspace samples and synthetic compounds

Volatiles were collected from several plant species using a headspace technique (Byrne et al. 1975; Pham-Delegue et al. 1989; Røstelien et al. 2000). The plants were placed in a closed oven bag (Look®) through which purified air (flow below 40 ml/min) was passed into glass tubes containing the adsorbents Tenax TA and Porapak Q (1:1). The air was purified by a filter of activated charcoal before the intake to the bag, and the collection was carried out for 24 or 48 h. The trapped volatiles were eluted by filling the glass tube with the solvent (hexane and ethyl acetate, ratio 1:1) and leading it drop by drop into different vials that were stored in a freezer. The plant materials used for collecting volatiles were *Brassica oleracea* (*L.*), *Brassica napus* (*L.*) as well as the related species *Arabidopsis thaliana*, a potential host of *M. brassicae*. With the known genome and pathways of biosynthesis, *A. thaliana* was considered as an interesting species as concerns relevant volatiles. Synthetic material of MeS (99%) and other compounds was also used to stimulate the RNs. Table I gives an overview of the plant material from which volatiles were collected as well as the tested synthetic compounds, standards, and essential oils. The concentrations of all synthetic compounds tested alone as well as those constituting the standards were 1 μg/μl.

Direct stimulation via cartridges

Direct stimulation via glass cartridges was used for screening the RNs for sensitivity to the various headspace samples of plants, essential oils, and synthetic mixtures. A 5-μl dilution

Table 1 Plant material and compounds used to stimulate the MeS and GLV RNs

Chemical standards					Essential oils	Headspace samples	Synthetic compounds
Standard 1	Standard 2	Standard 3	Standard 4	Standard 5			
(Z)- and (*E*)-β-Ocimene (70%)/	(2*E*)-Hexenal[a]	Ethyl 2-methylbutyrate[b]	Ethyl butyrate[b]	Ethyl 2-methylpropanoate[b]	Basil (*Ocimum basilicum* L.)[c]	*Arabidopsis thaliana sp*.[d]	Ethyl benzoate[e]
Limonene (25%)[f]	3-Octanone[g]	*i*-Propyl butanoate[b]	Butyl propionate[b]	Methyl methylbutyrate[b]	Lilac (*Syringa vulgaris* L.) [c]	*Brassica napus*[d]	Methyl salicylate[h]
Camphor[i]	(3*Z*)-Hexenyl acetate[a]	Butyl butyrate[b]	2-Methylbutyl propanoate[b]	Propyl butyrate[b]	Ylang ylang (Cananga odorate Hook)[a]	*Brassica oleracea*[d]	*o-*, *p-*, *m-* Methylanisol[g]
racemic Linalool[f]	1-Hexanol[j]	(2*E*)-Hexenal[b]	3-Methyl-1-butanol[b]	i-Butyl butyrate[b]			(-)- Verbenone
Methyl benzoate[e]	(3*Z*)-Hexen-l-ol[k]	(2*E*)-Hexenyl acetate[b]	2-Methyl-1-butyl butyrate[b]	(±)-2-Methyl-1-butanol[b]			(-)- *trans*-Verbenol
Isoborneol[g]	(2*E*)-Hexen-1-ol[j]	Butyl hexanoate[b]	Pentyl butyrate[b]	Hexyl formate[b]			
(+)-*trans*-Verbenol[g]	(2*Z*)-Hexen-1-ol[j]	2-Methylhexyl butanoate[b]	Hexyl propanoate[b]	Butyl pentanoate[b]			
Methyl salicylate[h]	1-Heptanol[l]	3-Methylbutyl hexanoate[b]	Hexyl-2-methyl butyrate[b]	i-Butyl hexanoate[b]			
	1-Octanol[k]	(3*Z*)-Hexenyl hexanoate[b]	2-Methyl-1-butyl hexanoate[b]	Hexyl butyrate[b]			
			Hexyl hexanoate[b]	Pentyl hexanoate[b]			
			Ethyl 2,4-decadienoates[b]	Butyl octanoate[b]			
				2,3-Butan-diol[b]			

[a]Dragoco, Totova, NJ.
[b]Synthesized by I. Liblikas, Royal Institute of Technology, Stockholm, Sweden.
[c]NMD ("Norsk Medisinal Depot"), Oslo, Norway.
[d]Headspace sample, Ulland, NTNU, Norway.
[e]Lancaster, Lancashire, UK.
[f]Fluka, Buchs, Switzerland.
[g]Borg-Karlson, Royal Institute of Technology, Stockholm, Sweden.
[h]Merck, Darmstadt, Germany.
[i]Kebo, Stockholm, Sweden.
[j]Aldrich, Steinheim, Germany.
[k]Sigma, Oslo, Norway.
[l]Janssen Chimica, Geel, Belgium.

of each sample (1 μg/μl) was applied to a filter paper placed inside the cartridge, letting the solvent evaporate before use. The RN was exposed for the test sample by puffing air (8 ml/s) through the cartridge and over the antenna. Direct stimulation via glass cartridges was also used for determining dose–response curves. In these tests, 100 μl of each MeS solution diluted in decadic steps was applied to a filter paper. The solvent was evaporated by N_2 flow before inserting the filter paper into the glass cartridge. In dose–response experiments, the tests were performed from low to high concentrations (range 0.12 ng to 1.2 μg on each filter paper). Between stimulations, the antenna was exposed to a continuous flow (500 ml/min) of purified air. The interstimulus interval varied from 1 min at low concentrations to 7 min at high concentrations.

Gas chromatography linked to single-cell recordings

The insects were mounted in a Plexiglas holder, and the head and antennae were stabilized with tape and wax as described by Røstelien et al. (2000). Electrophysiological recordings from single RNs were made by the use of electrolytically sharpened tungsten microelectrodes; the recording electrode was placed into the base of an olfactory sensillum and the reference electrode into the base of the antenna. The neurons were initially screened for responses to the mixtures of plant odors and single compounds via cartridges. Each sample consisted of 0.5–1 μl of the solution, which was injected into the column of the GC. The column was equipped with a splitter at the end, leading half of the effluent to the flame ionization detector (FID) and the other half into a constant airflow (500 ml/min) blowing over the insect antenna (Røstelien et al. 2000). This made it possible, together with the simultaneous SCR, to determine which compounds in the mixture elicited the responses. The spike rate and the gas chromatogram were recorded using EAD software (Syntech, Netherlands), whereas the spikes were recorded and stored using Spike2 software (Cambridge Electronic Design Limited, Cambridge, Great Britain). The GC was installed with a polar column [DBwax, 25 m, inner diameter (i.d.) 0.25 mm, film thickness 0.25 μm; J&W Scientific, Folsom, CA]. Separation in the polar column was performed with 2 different programs, the first and most frequently used program started at an initial temperature of 80 °C with an increase in rate of 6 °C/min to 180 °C and a further increase in rate of 15 °C/min to 220 °C. The second program, used to achieve better separation of the compounds in some of the samples, was performed from the initial temperature of 50 °C isothermal for 2 min followed by a 3 °C/min increase to 180 °C and a final increase of 15 °C/min to 220 °C. The FID temperature was set to 230 °C for all programs. The GC was equipped with a cold on-column injector.

Spike analysis and cell classification

The spikes from RNs were analyzed using the software Spike2. Separation of the cell types in one recording was based on differences in spike amplitudes and waveforms. The RNs were classified according to which odorant elicited the strongest response (primary odorant) as well as those having weaker effects (secondary odorants).

Identification of MeS in the headspace of cabbage plants

The Solid Phase Micro Extraction (SPME) technique (Pawliszyn 1997) was used to collect volatiles released from individual potted cabbage plants (*B. oleracea* var. capitata L.), bearing 6–7 leaves. The collection lasted for 24 h, and it was carried out under laboratory conditions at 20:4 h light:dark regime. Two 400 W metal halide lamps (Philips HPI-T Plus) were used as the light source. The temperature was kept at 26 °C ± 2 °C during the photophase and 24 °C ± 2 °C during the scotophase. The aboveground part of the plant was placed into a glass jar. At the bottom, the jar was closed by folding plates covered with aluminium foil to prevent soil odors from entering the jar. Before the collection periods, the routine conditioning of the SPME fibre (100 mm polydimethylsiloxane) was done at 225 °C for about 10 min in a GC injector. The tip of the syringe with the cleaned fibre was then placed in the jar through the inlet hole, which was covered by aluminium foil. Volatiles were collected from 5 individual healthy plants and 5 caterpillar-wounded plants (*B. oleracea* var. *capitata* L.). Three *Pieris napi* (*L.*) caterpillars of about 8 mm body length were allowed to feed on each plant for 48 h, and afterward, they were removed from the plant just before sampling. Volatiles were analyzed by means of a Varian 3400 GC, connected to a Finnigan SSQ 7000 mass spectrometer (MS). A SPB-1 and DB-Wax silica capillary column (30 m, i.d. 0.25 mm, film thickness 0.25 μm; J&W Scientific) was used with a temperature program of 40 °C (1 min), increased by 4 °C/min to 200 °C, then by 10 °C/min up to 230 °C, and thereafter held isothermally at 230 °C for 6 min. The split/splitless injector temperature was 225 °C and the splitless period lasted for 60 s. Helium was used as the carrier gas, with an inlet pressure of 70 kPa. Electron ionization mass spectra were determined at 70 eV with an ion source at 150 °C. MeS was identified by comparison of mass spectral data and GC retention times of volatiles with the corresponding data of a synthetic standard.

Behavioral experiments

The behavioral experiments were carried out during 3 separate periods (June 2004, August 2004, and June 2005) at Ås, Akershus, in southern Norway. All experiments took place in outdoor test arenas, consisting of aluminium-framed cages (2 × 2 × 2 m) covered with plastic mesh (Figure 1). Experiments were performed in 3 arenas simultaneously. Real plants of *B. napus* var. *napobrassica* (*L.*) Wilhelmsburger and artificial plants were used separately. The artificial plants, made from paper (4 leaves) were placed over a black pot. The test odorants, diluted in hexane (0.15 mg/μl), were applied on a small piece of towel inserted into a 6-mm piece

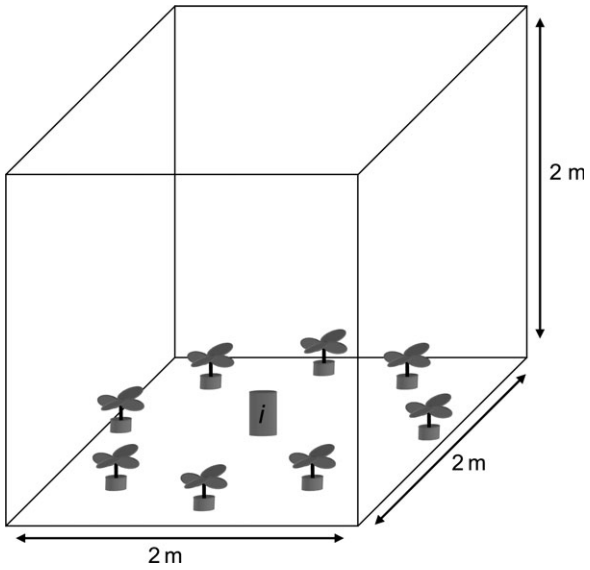

Figure 1 Scheme of the arena used in the behavioral experiments. The plants were placed on the ground, randomly distributed in a circle. The insects were released from the middle of the cage (*i*) prior to scotophase.

of rubber tube [outer diameter 0.75 mm, i.d. 0.30 mm]. To each of these "test dispensers," 3 µl of the odor solution was added, and the hexane was allowed to evaporate before starting the experiment. The release rate of the test odorants (50–100 ng/h) was measured by collecting a headspace sample of the "test dispenser" by the use of SPME. In all experiments, we used 8 plants in each arena. Four of the plants were supplied with a test dispenser, whereas the remaining 4 served as control plants with empty dispensers. The 8 plants were placed in a circle on the floor of the arena in a randomized order; for example, in one arena, every second plant was a control and in the second and third arena 2 test and 2 control plants were placed side by side. Prior to the main experiment, we carried out pilot experiments testing 4 different compounds, including MeS, in each arena. Individual plants were supplied with a test dispenser containing one of the compounds. In the main experiments, we only used test dispensers containing MeS. Before, as well as during the experiments, female and male *M. brassicae* were allowed to mate and drink sucrose water. We used up to 20 insects in each arena, more than half of them being females. Each experiment started shortly before dusk and ended the following morning.

Data analysis

The number of eggs was counted at the end of each experiment. Only eggs oviposited on the plants were included in the data analysis. The number of eggs on the test plants and the control plants was analyzed using the Wilcoxon matched pair test, by matching the total number of eggs on test plants contra control plants within each cage.

Results

Electrophysiology

RN responding to MeS

Out of the 43 olfactory RNs classified according to the compounds influencing their activity, 6 responded to the aromatic MeS. These neurons were tested in total 13 times via the GC (GC = 13) with various headspace samples of plants (Figure 2A) as well as synthetic MeS present in standard 1 (Figure 2B). No differences were noticed between RNs obtained in females and males. The recordings demonstrated high reproducibility of the responses, which appeared as increased firing rate. We did not observe any indications of inhibition, by reduced firing rate or stop of firing as responses to an eluted compound. The selectivity of the RNs appeared by a strong response to MeS (primary odorant) in headspace samples as well as in the standard and a weak response to the aromatic methyl benzoate (MB) (secondary odorant) in the standard. The amounts of MeS and MB in all headspace samples were below the detection limit by the FID. Two other, unidentified compounds (retention times 10.51 min and 15.01 min) present in *A. thaliana*, one of them also present in *B. oleracea* (retention time 15.01 min), activated the RN presented in Figure 2A. However, they had a lower effect than MeS that elicited a response of 25 spikes/s at the trace amount, below the FID detection limit. In comparison, the larger amounts of the unidentified compounds appearing in the gas chromatogram elicited a weaker response of ~20 spikes/s, suggesting a role as secondary odorants. Another MeS RN tested for the headspace sample of *A. thaliana* did not show responses to these compounds, probably because of a general lower sensitivity of this RN. Like the other MeS RNs, this RN also responded to the larger amount of MB present in the standard. Although the 2 other secondary odorants present in *A. thaliana* and *B. oleracea* could be detected by the FID, the amounts were too small to identify their mass spectra. The increased firing rate as a response to the GC eluted MeS followed the concentration profile of the GC peak, regaining the spontaneous activity within one or a few minutes after the peak. Spike analysis of the responses to MeS and MB showed similar amplitudes and waveforms (Figure 2B), indicating that they originated from the same RN. Dose dependency was shown by direct stimulation with decadic increase of MeS concentrations over 6 log units (Figure 2C). The threshold concentration for the most sensitive RN was in the range of 1.2–12 ng.

Responses originating from co-located RNs

In recordings from 2 of the 6 MeS RNs, an additional RN with large spike amplitudes responded to GLVs present in standard 2 (Figure 3A). Three of the activating compounds, 1-hexanol, (3*Z*)-hexen-1-ol, and (2*E*)-hexen-1-ol, elicited relative strong responses in this neuron, whereas 3 others,

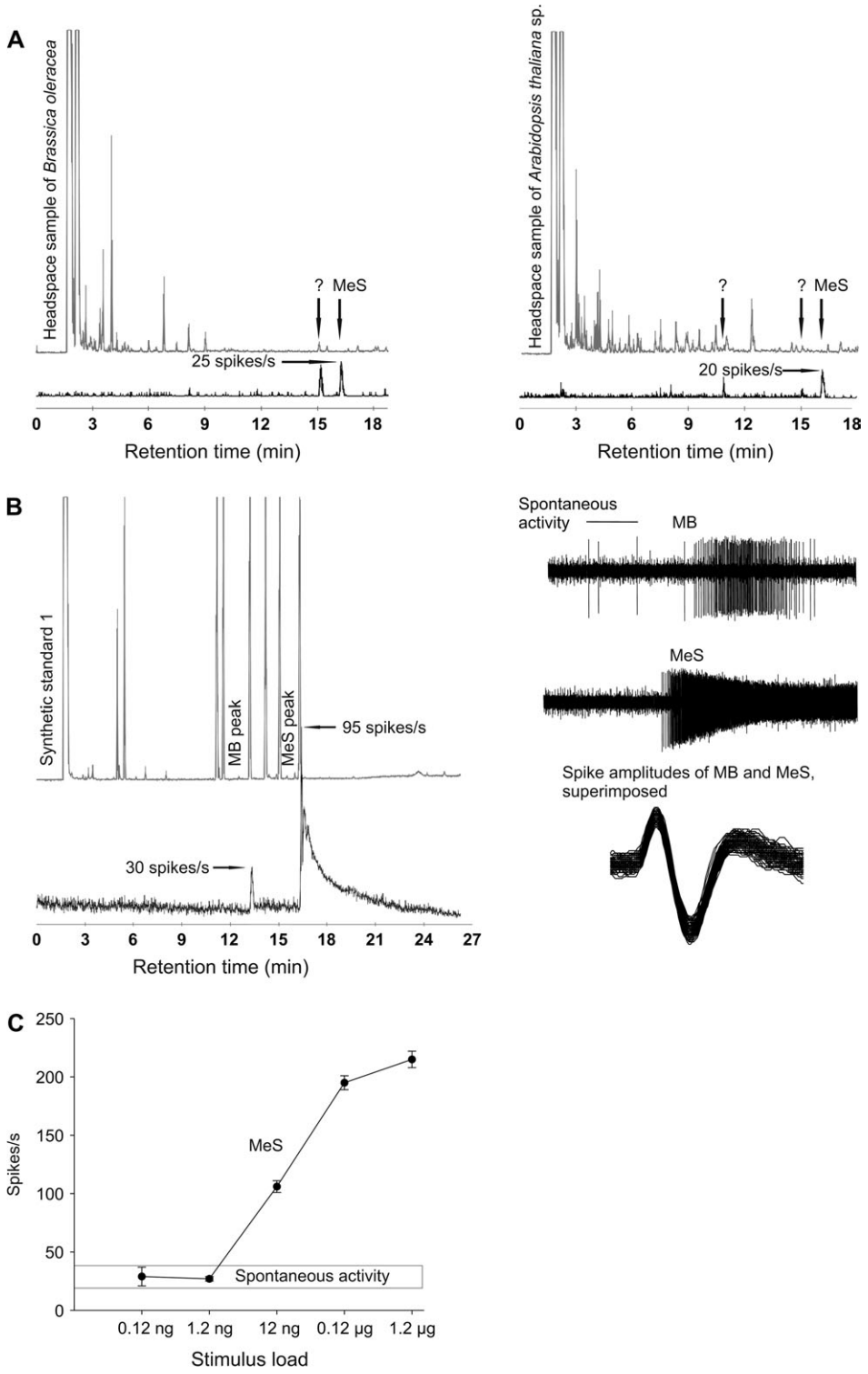

Figure 2 **(A, B)** Gas chromatogram (upper trace) of samples injected in the polar GC column and simultaneously recorded activity (lower trace) of a RN. (A) The RN responded to 2 compounds in the sample of *B. oleracea*, one unidentified (15.01 min) and MeS (16.12 min). The trace amount of MeS in both headspace samples was below the detection limit of the FID but still detected by the RNs (left). The RN responded to 3 compounds in the headspace sample of *Arabidopsis thaliana* sp., 2 unidentified (10.51 and 15.01 min) and MeS (16.12 min) (right). (B) The RN responded to MB (13.13 min) and MeS (16.12 min) present in the synthetic standard 1 (left). Spike traces of spontaneous activity and responses to MB and MeS (right). Spike analysis of the 2 responses showed overlap of spike shape and amplitude. **(C)** Dose–response curve based on stimulation via cartridges containing MeS in decadic concentration steps from 0.12 ng to 1.2 µg. Threshold concentration of the RN was in the range of 1.2–12 ng.

(3*Z*)-hexenyl acetate, (2*Z*)-hexen-1-ol, and an unidentified compound were less potent. Coinjection of standard 1 and standard 2 containing nearly equal amounts of MeS, MB, and the GLV (Figure 3B) showed a much stronger response by the MeS RN (100 spikes/s) than the GLV RN (30 spikes/s). A clear difference between the spike amplitudes and waveforms of the 2 RNs appeared as shown by the spike analysis in Figure 3B. Thus, the response to the GLVs originated from a different RN, colocated with the MeS RN.

Induction of MeS in cabbage plants

The SPME analyses of headspace samples from intact *B. oleracea* showed no detectable amount of MeS in the GC-MS. Because caterpillars of *M. brassicae* were not available at the time of these experiments, *P. napi* was used. After the feeding by *P. napi* caterpillars on the plants for 48 h, MeS could be identified in samples of all 5 test plants. The amount produced was found to be 0.9 ± 0.4 ng/day (Figure 4).

Behavioral experiments

Out of 44 behavioral experiments, 14 resulted in oviposition and are included in the results. In the initial 6 "pilot experiments" using real plants, we never observed oviposition on the plants having dispensers with MeS, only on the control plants and the plants having dispensers with other odorants. In the experiments designed for the present study, testing plants with MeS dispensers' contra control plants, eggs were laid in 9 of 38 experiments. One of the 9 experiments was terminated because of heavy rain and low temperature. Thus, the presented results are based on 8 experiments, 5 with real plants and 3 with artificial plants (Figure 5A). We never observed differences in oviposition preference between experiments with real and artificial plants. In 6 experiments, 4 with real plants and 2 with artificial plants, oviposition was exclusively on the control plants. The total number of eggs deposited in each of these experiments was in the range of 52–300. In only 2 of the experiments, a few eggs were deposited on the plants with MeS dispensers; in one of the

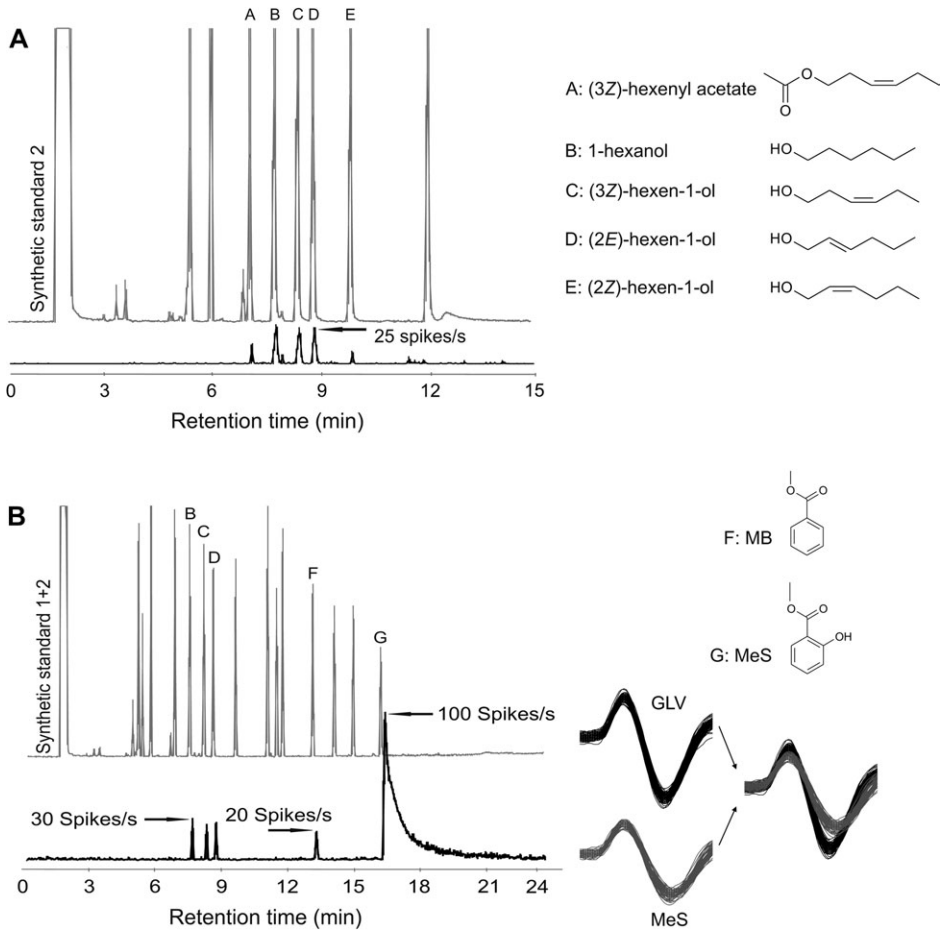

Figure 3 Gas chromatogram (upper trace) of sample injected in the polar GC column and simultaneously recorded activity (lower trace) of the RNs. **(A)** The RN responded to the eluents of (3*Z*)-hexenyl acetate, 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol, and (2*Z*)-hexen-1-ol. **(B)** Coinjection of synthetic standard 1 and 2 and the responses of the RNs to the eluents of 1-hexanol, (3*Z*)-hexen-1-ol, (2*E*)-hexen-1-ol, MB, and MeS (right). Spike analysis showed that the first 3 responses originated from a different RN than the 2 last responses (left).

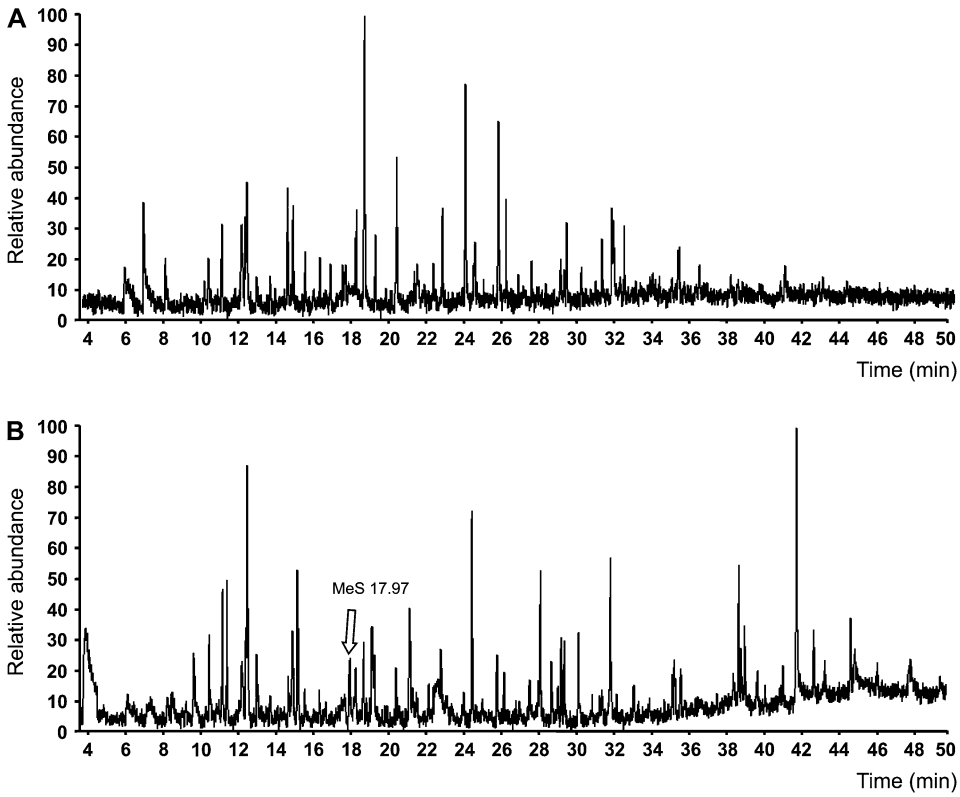

Figure 4 Total ion chromatogram records of volatiles obtained from *Brassica oleracea* plants. **(A)** Intact plant before introduction of caterpillars. **(B)** Plant wounded by 3 *Pieris napi* caterpillars. Total ion chromatogram in the range *m/z* 30–400; SPB-1 fused silica capillary column; peak size represents about 1 ng of MeS.

2 experiments, 4 eggs were placed on these plants, as compared with 150 eggs on the control plants. Statistical analysis of the number of eggs deposited on the control plants versus the test plants showed a preference for the control plants (Wilcoxon matched pair test, $P < 0.02$). The overall percentage of eggs deposited on the test plants contra the control plants are shown in Figure 5B.

Discussion

In principle, there are 2 approaches to study and identify important plant volatiles used by herbivorous insects: a top down and a bottom up approach. The former starts with the behavioral responses to the full bouquet of volatiles released by a plant and then narrow down the blend by separating fractions containing the effective compounds (D'Alessandro and Turlings 2005). The present study can be considered a bottom-up approach, starting with identifying the plant-released volatiles that are detected by the RNs and leaving behavioral studies to the final stage. The 2 approaches have particular advantages and challenges, the former giving direct behavioral information but having difficulties in identifying the minor components in the effective fractions. The advantage of the latter approach, using GC-SCR, is that relevant odorants present in minor amounts, even below the detection limit of the GC, can be identified because of the very sensitive RNs. This is indeed shown in the present study for the MeS RN that responded to the odorant in the headspace samples of the host plants at amounts below the detection limit of the FID (Figure 2A). By testing the synthetic standard, the RN showed a strong response to the MeS peak having the same retention time as the effective component of the headspace sample (Figure 2B). Similarly, the structurally related MB, present as a minor component or absent in some headspace samples, was identified as a second compound eliciting a weak response in this neuron type. The 2 other compounds present in *A. thaliana* and *B. oleracea*, eliciting marked weaker responses than MeS, could not be identified. They were present in too small amounts for GC-MS identification, and they were not among available compounds present in the standards. A way to identify these compounds would be to test the RNs for headspace samples containing a larger quantity of them, for example, samples of other plant species (Røstelien et al. 2000).

The results of the present study add to the growing data on narrowly tuned RNs responding to plant odorants in herbivorous insects. Each RN, tested for hundreds of volatiles naturally produced by a plant, responds to only one or to a few compounds of related chemical structures. In the latter case,

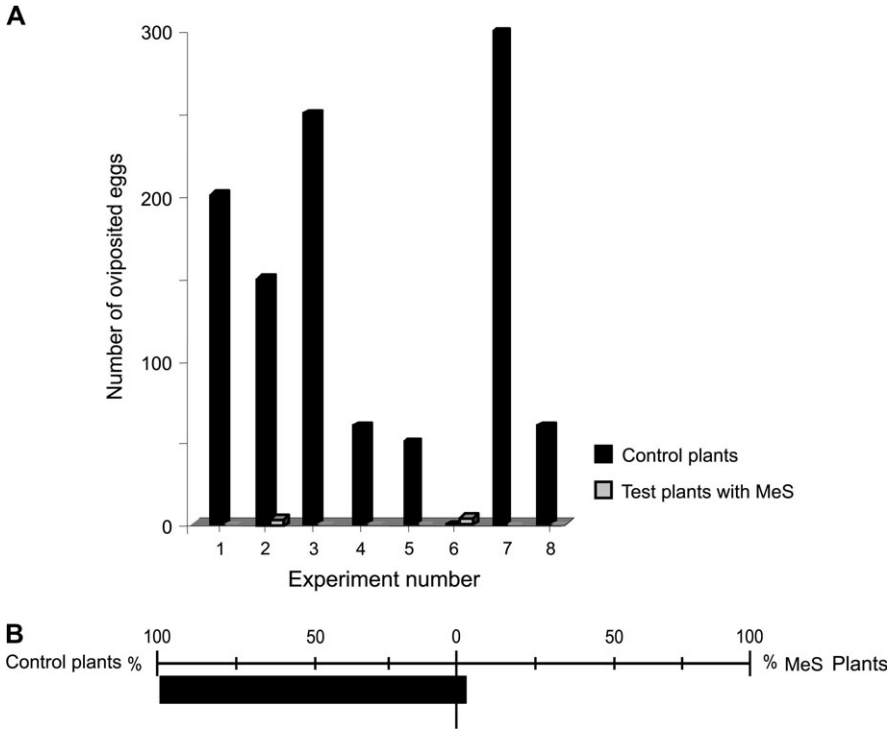

Figure 5 **(A)** Histogram of eggs oviposited on plants with MeS and control plants, which resulted from 8 experiments carried out at night. Experiments 1–5, natural plants. Experiments 6–8, artificial plants. **(B)** Percentage of eggs oviposited on the control plants as compared with plants with MeS dispensers, showing a significantly higher number of eggs deposited on the control plants (99.6%, Wilcoxon matched pair test, $P < 0.02$).

one compound has a marked strongest effect and is termed primary odorant, whereas those of weaker effects are termed secondary odorants. The MeS RN type typically exemplifies this. The marked higher effect of MeS qualified for the term primary odorant, whereas MB and the 2 unknown compounds were termed secondary odorants. The other RN type, appearing colocated with the MeS RN in 2 recordings, responded to 6 structurally related compounds; 3 of them, 1-hexanol, (3*Z*)-hexen-1-ol, and (3*Z*)-hexenyl acetate, having a marked best effect. These rarely appearing GLV RNs need to be further tested for dose–response properties, in order to precisely determine the specificity. A relevant question is whether one of the compounds has a marked best effect as primary odorant or if all the 3 flexible molecules fit into the same receptor pocket with similar receptor affinity. Although responding to 6 odorants, these RNs are also quite narrowly tuned, considering the numerous compounds in the various mixtures that had no effect. On the basis of the primary odorant and the molecular receptive ranges, the plant odor RNs in *M. brassicae* as well as in all species studied by GC-SCR fall into distinct functional types (Blight et al. 1995; Wibe et al. 1997; Røstelien et al. 2000; Stensmyr et al. 2001; Barata et al. 2002; Stranden, Liblikas, et al. 2003, 2003b; Røstelien et al. 2005; Bichão, Borg-Karlson, Araújo, et al. 2005; Bichão, Borg-Karlson, Wibe, et al. 2005). This correlates well with the general principle that subsets of olfactory RNs express only one type of receptor proteins in insects (Clyne et al. 1999; Störtkuhl and Kettler 2001; Wetzel et al. 2001; Keller and Vosshall 2003; Hallem and Carlson 2004). Thus, the recorded responses from each RN reflect the specificity of one expressed receptor protein type, for example, specified for MeS.

The results on the narrow tuning of the plant odor RN types of *M. brassicae* is in accordance with results obtained in other species using the method of GC-SCR. In the studies of heliothine moths and herbivorous weevils, carried out in the same laboratory using similar test protocols, has made it possible to compare the RN specificity across the species (Røstelien et al. 2000; Stranden, Røstelien, et al. 2003; Bichão, Borg-Karlson, Araújo, et al. 2005; Bichão, Borg-Karlson, Wibe, et al. 2005; Røstelien et al. 2005). For instance, in the strawberry blossom weevil *Anthonomus rubi*, one type of RN responding to MeS has been functionally characterized (Bichao et al. 2005a). Like in *M. brassicae*, they showed a weak response to MB. However, the RNs in *A. rubi* in addition responded weakly to ethyl benzoate, in contrast to the MeS RNs of *M. brassicae*. Thus, in the adaptation to different host plants, these 2 distantly related insect species have evolved RNs for MeS with slightly different molecular receptive ranges. Olfactory RNs responding to MeS have also been shown in 2 other species, the cabbage seed weevil, *Ceutorhynchus assimilis* (Blight et al. 1995) and the fruit chafer, *Pachnoda marginata* (Stensmyr et al. 2001), by the use of GC-SCR. However, because of different test protocols,

comparison of the RN specificity cannot be done between these species and *M. brassicae* and *A. rubi*. Sensitivity to MeS and some of the GLVs presented in this study have previously been shown in *M. brassicae* by the use of GC-electroantennograms (EAGs) (Rojas 1999a). Whereas the tool of EAG recordings is suitable for screening the antennae for general sensitivity to various volatiles, the techniques of GC-SCR give precise information about the specificity of single RNs. Large groupings of RNs responding weakly to secondary odorants may result in relatively strong EAG responses, whereas small groups of RNs specifically responding to a primary odorant may elicit small EAGs (Van der Pers and Löfstedt 1983). Thus, the contribution of the present results is that *M. brassicae* has evolved specific RNs for detecting MeS as well as GLVs.

Another interesting aspect of olfactory RNs is how the molecular receptive ranges correlate with the products of the biosynthetic pathways in host plants. In recent studies, the biosynthesis of MeS and MB has been resolved in several plant species (Dudareva et al. 1998, 2004; Chen et al. 2003). Both the MeS and the MB synthesis are catalyzed by methyltransferases, whereby a methyl group is transferred from the donor molecule *S*-adenosine-*L*-methionine to the carboxyl group of salicylic acid or benzoic acid, respectively (Chen et al. 2003). Like in many species, including the snapdragon, *Antirrhinum majus*, it has been shown that a methyltranferase catalyses the production of MeS along with a small amount of MB (Negre et al. 2002). The large number of plant species emitting MeS with a smaller amount of MB suggests that MeS is the important compound, whereas MB apparently does not play a significant role in insects using these plants as hosts. In fact, no MB was detected by the RNs in our headspace samples. The 2 compounds MeS and MB, activating the same RN, may be perceived by *M. brassicae* as the same odor quality but with different intensity. In contrast, humans clearly discriminate the 2 floral compounds that are perceived as 2 distinctly different odor qualities; MeS containing the hydroxyl group is experienced as a sweet odor, whereas MB is not as pleasant.

The second part of this study concerns the behavioral effect of MeS. The present finding that *M. brassicae* in general avoids ovipositing on plants with MeS dispensers is in accordance with results obtained in a previous behavioral study of *M. brassicae*. By the use of wind tunnel experiments, Rojas (1999a) found that *M. brassicae* did not show upwind flight to a cotton wool wick loaded with MeS. In our study, we only found a few eggs on plants with MeS dispensers in 2 of the experiments. In comparison, most experiments showed 50–300 eggs oviposited on the control plants, indicating that the females reject plants with MeS that they are able to detect by the very sensitive and narrowly tuned olfactory RNs on the antennae. MeS is shown to be induced in several plant species during herbivore attack (Chen et al. 2003; Van den Boom et al. 2004; Bichão, Borg-Karlson, Araújo, et al. 2005). For instance, herbivory by *Pieris rapae* larvae has previously been shown to induce emission of MeS in *A. thaliana* (van Poecke et al. 2001), a potential host plant of *M. brassicae*. In this study, MeS was shown to be induced in the host plant *B. oleracea*. We used caterpillars of *P. napi* but assume a similar induction of MeS caused by *M. brassicae* caterpillars. In any case, it might be important for mated *M. brassicae* females to detect which host individuals that are already occupied. These findings seem contradictory to those of wind tunnels experiments presented by Rojas (1999b). He found that *M. brassicae* females are attracted to and oviposit more eggs on plants (cabbage and tomato) damaged by female locusts (*Schistocerca gregaria*) and orient more often to cabbage plants damaged by conspecific caterpillars than to undamaged plants. Several reasons may underline the different results. Obviously, numerous plant-released volatiles are involved in host plant attraction and avoidance, and damaged plant releases larger quantities of volatiles than undamaged plants. Whereas our results were based on comparison of the behavioral effect of only MeS added to artificial or to intact plants, the results of Rojas involves the whole bouquet of systemically or induced plant-released volatiles. Thus, other induced compounds with strong attractive effect may have been involved in the experiments by Rojas.

In the only experiment terminated because of heavy rain, eggs were found on test plants with MeS dispensers. The reason for terminating this experiment was that the release of volatiles would be low in the cold weather and the motivation of the moths might be to search for shelter under such conditions rather than a suitable oviposition site. In a few other experiments carried out during cold weather and heavy rain resulting in no oviposition, the moths flew to the closest plant apparently for finding shelter. However, the possibility exists that one female actually chose the plant emitting MeS. The existence of individuals, with a different preference (e.g. for plants with MeS), may reflect a built-in flexibility that enables species to cope with changing conditions in the environment as discussed by Schoonhoven et al. (2005). In the case of a very polyphagous species like *M. brassicae*, this possibility should not be ignored.

The present study has elucidated the sensory significance and indicated a behavioral effect of MeS showing its specific effect as primary odorant on one RN type, which mediates avoidance or inhibition of oviposition on artificial and intact plants to which MeS is added. Future experiments should follow up concentration dependency with additional behavioral studies. This should include tests with MeS added to undamaged as well as damaged plants. Also, the behavioral significance of the GLVs, activating the other RN type in this study, should be tested. One might expect that these compounds have the opposite effect of MeS, because one of them, (3*Z*)-hexenyl acetate, has previously been shown to induce upwind flight of mated *M. brassicae* females in wind tunnel experiments (Rojas 1999a). Obviously, many more odorants are involved in host plant localization and

selection, and the challenge in future experiments are to find out the behavioral relevance of all plant odorants and their mixtures. For practical application of the results, supplemental studies should be done to determine whether mechanical damage in the absence of insect feeding would induce MeS production. If this is the case, well-timed mechanical damage could help prevent attack by *M. brassicae*.

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