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New insights in analysing parasitoid attracting synomones: early volatile emission and use of stir bar sorptive extraction

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Summary. It is well known that feeding by *Pieris brassicae* caterpillars on cabbage leaves triggers the release of volatiles that attract natural antagonists such as the parasitoid *Cotesia glomerata*. The temporal dynamics in the emissions of parasitoid attracting volatiles has never been elucidated in this system. In a time course experiment, caterpillar infested leaves attracted the parasitoid within one hour after infestation. At such an early stage of infestation, as much as fifty percent of the parasitoids flew towards the infested plant in a wind tunnel bioassay, while only five percent flew towards the non-infested control plant. Three hours after infestation and later, the response to the volatiles from the infested plant reached its maximum and then continued at a constantly high level for the remaining 14 hours of the experiment. Chemical analyses of volatiles collected from infested leaves at short time intervals during the first 24 hours identified a total of ten compounds, comprising green leaf volatiles, terpenoids, and a nitrile. Significant increase of emission within the first 5 hours following initial herbivory was detected for (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, cineole and benzylcyanide. Subsequently, a coupled bioassay-chemical analysis procedure was developed allowing for testing and analyzing the same sample for future identification of the bioactive compounds. This was achieved by using stir bar sorptive extraction for the analysis of solvent extracts of caterpillar-damaged leaves.

Key words. Tritrophic interactions – time course experiment – *Brassica oleracea* – *Pieris brassicae* – *Cotesia glomerata* – stir bar sorptive extraction (SBSE) – synomones – parasitoid behaviour

Introduction

Volatile plant compounds released in response to caterpillar feeding can serve as chemical signals for predators and parasitoids (reviewed by Dicke 1999a). Temporal dynamics in volatile emissions are crucial for the attacked plant, as an early induction of emission is expected to rapidly attract natural antagonists, which subsequently interfere with the herbivore insect. In this study we report on the dynamics of induced volatiles released by a cabbage plant (Brussels sprouts plant, *Brassica oleracea* var. *gemmifera*; Brassicaceae) upon herbivory by *Pieris brassicae* (Lepidoptera: Pieridae) caterpillars, resulting in the attraction of the parasitoid *Cotesia glomerata* (Hymenoptera: Braconidae) (Steinberg *et al.* 1992, 1993). Changes in the volatile blend released by caterpillar infested cabbage plants appear to be quantitative in nature (Agelopoulos & Keller 1994; Mattiacci *et al.* 1994, Geervliet *et al.* 1997), in contrast to many other systems where both quantitative and qualitative changes have been reported (De Moraes *et al.* 1998; Dicke & Vet 1999b; Paré & Tumlinson 1999). Current knowledge on the chemical composition and behavioural effects of released volatiles is restricted to the period following at least 24 h of caterpillar feeding (Blaakmeer *et al.* 1994; Mattiacci *et al.* 1994), generally due to the fact that the detectability of chemicals is higher after a certain period of feeding (Turlings *et al.* 1990; McCall *et al.* 1994).

The first objective of this study was to establish the temporal dynamics of the release and subsequent behavioural effect of the volatile compounds from the very onset of herbivore damage. Analyses by gas-chromatography mass-spectrometry (GC-MS) were carried out with samples collected from infested cabbage at fixed time intervals during the first 24 hours. Behavioural bioassays with the parasitoid *C. glomerata* were performed throughout the first hours after infestation with *P. brassicae* caterpillars.

The identification of the chemicals responsible for guiding a host-seeking parasitoid to an infested plant is subjected to a number of methodological constraints. We developed a method to perform chemical analyses and behavioural tests on the same sample, as this allows a direct link between the compounds emitted and the behaviour observed.

Recently developed sorption-based techniques such as solid phase microextraction (SPME) (Arthur & Pawliszyn, 1990) offer the possibility for coupled chemical-behavioural

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analyses. SPME is based on sorptive enrichment of analytes on a polymer coated fiber. It received increasing interest in gas chromatography (reviewed by Baltussen *et al.* 2002) and has been applied, for example, to characterize the time course of volatile emission from apple fruits over a growing season (Hern & Dorn 2003).

Sorptive sampling techniques such as SPME, have major advantages over adsorptive sampling. Polydimethylsiloxane fibre (PDMS) and Tenax were compared to monitor volatiles emitted by living plants by means of dynamic headspace sampling (Vercammen *et al.* 2000). Tubes packed with PDMS particles (Baltussen *et al.* 1998) proved to be more inert than Tenax. Additionally, degradation products of Tenax such as benzaldehyde and acetophenone interfere with the analysis, and compounds with higher molecular weight were retained by the adsorbent. However, the principle shortcoming of SPME is its limited sensitivity due to the low amount of polymer present.

Stir bar sorptive extraction (SBSE) is a new analytical approach that consists of a PDMS coated stir bar used for the extraction of compounds from liquid matrices. Due to the higher amount of PDMS present on the stir bar, SBSE offers greater sensitivity as compared to SPME (Baltussen *et al.* 1999a). Moreover, this technique allows the performance of behavioural assays and chemical analysis on the same sample. The second objective of this study was to apply SBSE to extracts of herbivore damaged cabbage leaves and to examine its applicability for the investigation of compounds mediating host location by *C. glomerata*.

Materials and methods

Plants

Brussels sprouts seedlings (*Brassica oleracea* var. *gemmifera*; Brassicaceae) were reared in a walk-in climate room at 16L: 8D photoperiod, with 10,000 ± 1000 lux, 24 °C (day), 16 °C (night) and 50-70 % relative humidity (Mattiacci *et al.* 2001). Brussels sprouts plants with five to eight fully expanded leaves, which were similar with respect to appearance, size, and number of leaves, were used for experiments.

Insects

Herbivores (*Pieris brassicae*, Lepidoptera: Pieridae) and parasitoids (*Cotesia glomerata,* Hymenoptera: Braconidae) were reared as previously described (Gu & Dorn 2000). Briefly, *P. brassicae* larvae were maintained on Brussels sprouts plants, at 21 ± 1 °C, 50-70 % relative humidity, and a 16L:8D photoperiod. *C. glomerata* were reared in a climate room at 15 °C, 90 % relative humidity, and a 16L: 8D photoperiod. For parasitization, first- or second-instar of *P. brassicae* caterpillars were exposed to 4- to 10-day-old parasitoids. Care was taken to ensure that only one wasp laid eggs into one larva. Parasitized herbivores were subsequently provided with abundant Brussels sprouts plants and, after they reached the fourth instar, with Savoy cabbage leaves (*Brassica oleracea,* var. *sabauda*). Upon egression, parasitoid cocoons were separated from the remains of the herbivore and transferred into a Petri dish. Newly emerged adults were transferred into a nylon gauze cage where they had access to honey and water, and they were able to perform normal flight activities and to mate at random. Females were never exposed to host caterpillars or plants before bioassay and therefore were considered naïve (Gu & Dorn 2000; Mattiacci *et al.* 2001).

Behavioural assays

Behavioural experiments were conducted in a Plexiglas wind tunnel under the following conditions: 22 ± 2 °C, 50 ± 5 % relative humidity, 750-1000 lux and a wind speed of 26-30 cm/sec (Mattiacci *et al.* 2001).

For the bioassay, 4- to 5-day-old female parasitoids were used (Steinberg *et al*. 1992). Wasps were selected without bias in the cage and placed individually into cotton wool-stoppered glass vial (6 ml) 2 hours prior the experiment and transferred to the bioassay room. Each wasp was allowed two flight attempts. Based on preliminary observations, most females would not fly toward the odour sources at the first flight attempt. A choice was recorded when the wasp completed a flight, landing on the nylon gauze in front of one of the two odour sources. Landing elsewhere in the tunnel was recorded as no choice. Wasps that did not fly within 5 min from being released in the wind tunnel were discarded. For every experiment a total of 50 wasps were tested on at least two days, in order to account for day-to-day variation in their response (Mattiacci *et al.* 2001).

Odour sources were offered to the parasitoids in a dual-choice situation, where the two sources were placed 20 cm apart from each other and the parasitoid was released 30 cm downwind. The two odour sources were exchanged after testing three wasps to compensate for possible asymmetry in the setup. In order to minimize visual stimuli a white nylon gauze was suspended in front of the odour sources (Steinberg *et al*. 1992). The response of *C. glomerata* was tested in the following bioassays: *in a time-course experiment* using as odour sources caterpillar-infested Brussels sprouts vs. healthy (i.e., non infested) Brussels sprouts, *in a solvent extract experiment*, using hexane extracts of caterpillar-infested Brussels sprout leaves vs. solvent control, and *in a solvent elution experiment*, using the hexane elution of volatiles collected from caterpillar-infested Brussels sprout leaves *vs.* solvent control.

Time course experiment

The volatile emission of a Brusselss sprout plant, infested with *P. brassicae* caterpillars, was monitored with behavioural assays and chemical analyses over time.

A whole cabbage plant, with two leaves infested with 50 second-instar *P. brassicae* caterpillars each, and an healthy plant of the same age and size were used for the behavioural assay, as explained above. In order to elucidate the temporal dynamics of volatile emission, six wasps were tested every hour throughout 17 hours starting immediately after infestation. The experiment was carried out during two days (0-8 h and 8-17 h) using different plants. To obtain additional information within the first phase of herbivory, a further experiment was carried out testing 10 wasps at each half hour interval for the first three hours after infestation.

For the chemical analyses, volatile sampling (see below) was performed on the two infested leaves of the test plant. Sampling was carried out during two time blocks. In the first time block, sampling was performed at one-hour intervals during the first five hours after infestation. In the second time block, sampling took place throughout a 19-hour period, from five to 24 hours following infestation. Both time blocks samplings were replicated five times.

Solvent extract

Solvent extracts of cabbage leaves were used to develop a consistent method allowing behavioural assay and chemical analyses to be performed on the same sample to improve synomones identification.

Infested leaves of Brussels sprout plants used for the extract consisted of leaves on which 50 second-instar *P. brassicae* were allowed to feed during 16 hours, to ensure the

emission of high amounts of volatile. Frass and caterpillars were carefully removed with a paintbrush, leaves were ground in a mortar and extracted in hexane (>99.5 %) for 30 minutes at RT. One hundred mg leaf material per ml solvent was extracted. After filtration through two filter papers (Schleicher & Schuell no. LS14), the extract was kept at –60 °C in glass vials with PTFE/silicon septa and used for bioassays and chemical analyses. The hexane extract was prepared using five leaves of five different plants. Five samples were collected.

The hexane extract was presented to the wasps in the bioassays (see above) using a system similar to the one described by Wang *et al.* (2003), i.e., 3 × filter papers (Schleicher Schuell 589, 1-2mm width and 90 mm length) were inserted for support into a capillary tube that was itself inserted in a 2 ml vial containing 500 µl solution, e.g., solvent extract or plain solvent for test and control.

Solvent elution

In order to attest the attractiveness of hexane extracts, a comparison to a commonly used method in synomone studies, such as solvent elution of volatiles collected from plant headspace, was performed.

Infested leaves of Brusselss sprout plants used for the volatile collection (see below) consisted of leaves where 50 second-instar *P. brassicae* were allowed to feed during 16 hours prior to collection. Volatile sampling was carried out during 24 hours thereafter from two infested leaves. Subsequently, volatiles were eluted from Tenax GR traps with 1 ml hexane (>99.5 %). Five sampling replicates were performed. The five elutes were pooled together and stored at –60 °C in a glass vial with PTFE/silicon septa and used for the bioassay. The solvent elution was presented to the wasps in the wind tunnel using the same system as for the solvent extract (Wang *et al.* 2003).

Volatile collection

Volatiles emitted by the cabbage leaves during the time course experiment and for the solvent elution were collected using the same equipment as described by Mattiacci *et al.* (2001). Activated charcoal-filtered air was drawn (at approx. 240 ml/min) through a glass-Teflon chamber and through a stainless steel thermal desorption tube packed with 300 mg Tenax GR (mesh sizes 60/80, Alltech Associates). Collections were carried out in a climate room at 20 °C, 60 % RH and a 16L:8D photoperiod. After collection the tubes were capped with swagelock brass end caps using PTFE ferrules. Thermal desorption tubes were conditioned for 15 hours at 325 °C with a 75 ml/min flow of purified helium through the trap prior to collection.

Stir bar sorptive extraction (SBSE)

The *solvent extracts* were analyzed using SBSE. Stir bars were prepared as described in Baltussen *et al.* (1999a). Steel rods (40 mm length) were enveloped in glass tubing and coated with Silastic® laboratory tubing (1.57 i.d. and 3.18 o.d., Dow Corning, Midland, MI, USA). For extraction, a stir bar was transferred in 10 ml sample extract and stirred for 2 h at RT. After extraction, the stir bar was introduced in a regular desorption tube (stainless steel tubes, Markes International) and capped with swagelock brass end caps using PTFE ferrules prior desorption. Stir bars were conditioned at 250 °C for 15 h with a 75 ml/min flow of purified helium, prior to use.

Chemical analyses

Samples were analyzed using a thermal desorption system (Unity Markes International) connected to a Hewlett Packard GC-MS (GC 6890 mass detector 5973).

Volatiles were desorbed from the Tenax GR trap for 5 minutes at 300 °C and transferred to the cold trap (packed with a 4 cm bed of Tenax TA and a 2 cm bed of Carbopack B) which was held at –10 °C throughout the tube desorption process then heated at a rate of 60 °C/min to 300 °C, the total desorption time was 3 minutes. The unity was used with a double split flow, i.e., during both tubes desorption and cold trap desorption (split flow ratio 7.2:1). The transfer line to the GC-MS was held at 200 °C. The GC column used was a polydimethylsiloxane column (HP-1; 30M; ID of 0.25 mm and film thickness of 0.25 m) fitted with a fused silica retention gap (HP1, 5M, 0.25 mm ID). The initial temperature was 40 °C for 5 minutes, then a 8 °C per minute ramp to 300 °C.

The stir bars were desorbed at 200 °C for a period of 5 minutes after 10 minutes purge flow. The cold trap was packed with a 6 cm bed of 10 % OV-101 (PDMS) on Chromosorb W-HP 80/100 from Alltech and was held at –10 °C throughout the SBSE desorption process. The cold trap was subsequently heated at a 60 °C per minute rate to 300 °C, with a total cold trap desorption time of 3 minutes. The unity was used with a single split flow, i.e. only during stir bar desorption (split flow ratio 7.7:1). The transfer line to the GC-MS was held at 150 °C. The GC column used was a phenyl methyl siloxane column (EC-5; 30M; ID of 0.25 mm and film thickness of 0.25 µm) fitted with a fused silica retention gap (HP1, 5M 0.25 mm ID). The initial temperature was 45 °C for 5 minutes, then an 8 °C per minute ramp to 250 °C.

For both analysis types the GC-MS transfer line was held at 280 °C and the MS quadropole and MS source temperatures were 150 and 230 °C respectively. The sample spectra were identified by comparison to spectra from the Nist98 and from our own library. Subsequently, the spectra and the retention time of the compounds were matched to those of authentic standards in all cases. Quantification of the compounds was based on the peak area of an internal standard (hexylbenzene) that was injected directly into the Tenax GR trap or calculated on the basis of SBSE analyses performed with authentic standards. Standards for this process were purchased from chemical suppliers (Fluka, Sigma, Aldrich, Avocado and Acros).

Statistical analysis

Data from the bioassays were analyzed with a χ^2-test for goodness of fit (Sokal & Rohlf 1981). Wasps that did not make a choice within 5 min were excluded from the statistical analysis.

A Kruskall-Wallis non-parametric test was performed on chemical data of the time course experiment to evaluate changes for each compound over the first 5 hours after infestation.

Results

Time course experiment

In dual choice bioassays in the wind tunnel, parasitoids were attracted to infested plants soon after the beginning of *P. brassicae* caterpillar feeding (Fig. 1A). Females showed a significant preference for the infested plant within each sampling interval ($P < 0.001$; χ^2-test for goodness-of-fit) except for the first 30 minutes after infestation ($P > 0.05$). The response to the infested plant reached a maximum after 3 hours and then continued at a constant level for the subsequent 14 hours (Fig. 1B). In both choice experiments, the healthy control plants evoked a small response from the parasitoids.

The time course of the emission of major compounds after the infestation of cabbage leaves with *P. brassicae* caterpillars was monitored by sampling hourly during the first 5 hours following infestation, and throughout 19 hours (5 to 24 h) after infestation (Fig. 2). The release

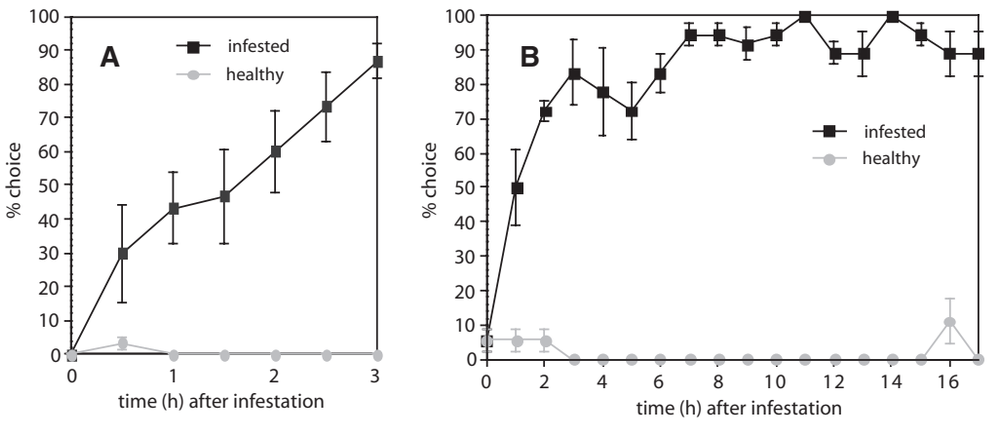

Fig. 1 Choice response of female *Cotesia glomerata* to Pieris brassicae infested plant vs. healthy plant in a time course experiment. The percentage of individuals choosing infested or healthy plant is presented, and the difference to 100% accounts for the individuals making no choice.
A. 0 to 3 hours after infestation, 10 wasps were tested each at half an hour intervals.
B. 0 to 17 hours after infestation, 6 wasps were tested each at one hour intervals.
N = 3, bars indicate SE. $P < 0.001$ except for time 0 h after infestation ($P > 0.05$, χ^2-test)

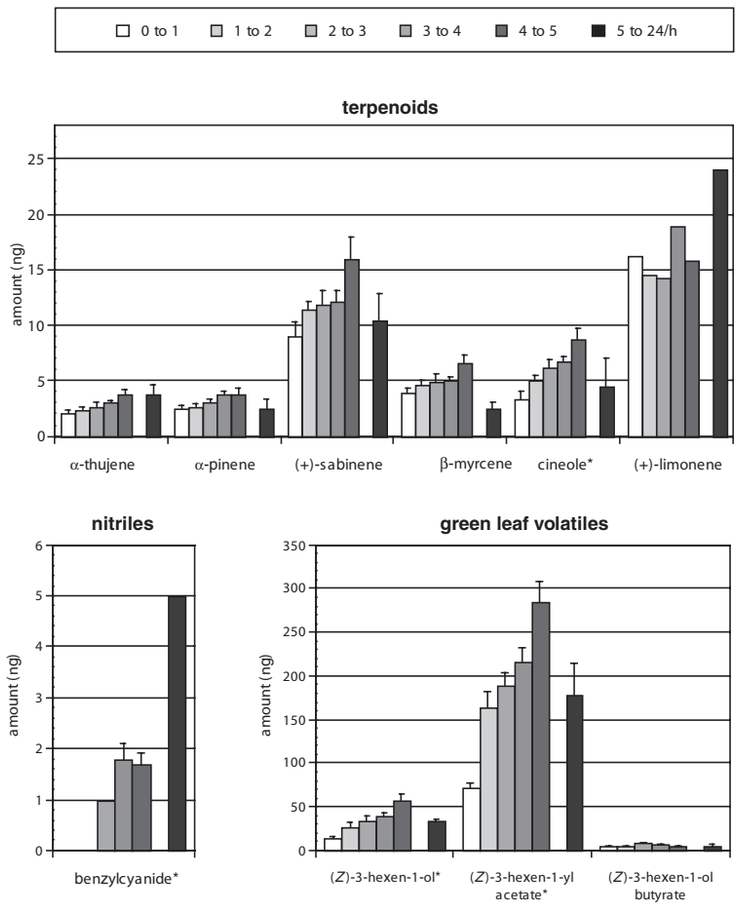

Fig. 2 Time course experiment: average amounts (ng ± SE) released per hour from 0 to 5 hours and from 5 to 24 hours after the infestation of two Brussels sprouts leaves with 50 *Pieris brassicae* caterpillars each.* Indicates significant increase in the emission during the first 5 hours (Kruskall Wallis non parametric test: * = $P < 0.01$). N = 5, the SE is given as a bar for compounds detected more than once. Note the different scales for the different groups of compounds

of cineole, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate and benzylcyanide significantly increase over the first 5 hours (Kruskall Wallis non-parametric test: $P < 0.01$).

Bioassays: extract vs. elution

This experiment was designed to assess whether the solvent extraction of compounds from infested cabbage leaves and the solvent elution of headspace samples of infested cabbage leaves contained the compounds responsible for the olfactory response of *C. glomerata* (Fig. 3). Both, the extract and the eluate were significantly attractive (χ^2-test: $P < 0.005$; $P < 0.001$, respectively), yet the extract elicited a significantly higher response when compared to the eluate (χ^2-test: $P < 0.005$).

SBSE analysis of solvent extract

Twenty-one compounds were identified in the hexane extracts of *P. brassicae* infested-cabbage leaves (Table 1):

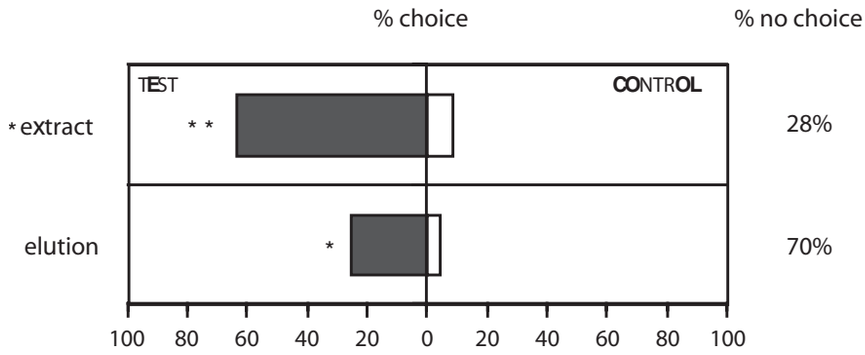

Fig. 3 Flight response of *Cotesia glomerata* females towards either solvent extract or solvent elution of Brussels sprouts leaves infested by second-instar *Pieris brassicae* vs. solvent control. N = 50. Asterisks indicate significant differences within and between the behavioural assays ($^{*}P < 0.005$; $^{**}P < 0.001$, χ^2-test)

Table 1 Compounds identified using the SBSE method in hexane extracts of herbivore damaged leaves of Brussels sprouts

RT (min)	Compound	ng/500 µl[a]	Class of compound
7.54	(*E*)-2-Hexenal [13b]	1038.1 ± 60.7	aldehyde
7.64	(*Z*)-3-Hexen-1-ol[123]	699.6 ± 24.7	alcohol
7.88	1,3-Dimethylbenzene	37.9 ± 1.6	benzene derivatives
8.35	1,2-Dimethylbenzene	271.3 ± 15.3	benzene derivatives
8.69	(*E*,*E*)-2,4-Hexadienal[1]	19.1 ± 3.9	aldehyde
10.19	2-Ethyltoluene	10.3 ± 0.8	benzene derivatives
10.47	1,2,3-Trimethylbenzene	7.9 ± 0.6	benzene derivatives
10.52	(*E*,*E*)-2,4-Heptadienal	4.5 ± 0.1	aldehyde
10.76	(*Z*)-3-Hexen-1-yl acetate[123]	158.9 ± 42.4	ester
10.90	Hexyl acetate[123]	59.3 ± 13.7	ester
11.23	(+)-Limonene[123]	28.4 ± 4.5	terpenoid
11.50	Phenylacetaldehyde[3]	3.5 ± 0.9	aldehyde
12.78	Nonanal[1]	7.7 ± 1.0	aldehyde
13.45	Benzylcyanide[3]	5.6 ± 0.9	nitrile
14.19	Octanoic acid	32.3 ± 2.0	acid
14.80	Decanal[13]	42.7 ± 8.5	aldehyde
15.07	2-Phenoxyethanol	5.3 ± 0.4	alcohol
15.33	Cyclohexanisothiocyanate	610.3 ± 280.4	isothiocyanate
15.44	Benzenepropanenitrile	1.4 ± 0.2	nitrile
15.99	Nonanoic acid	69.5 ± 40.7	acid
17.79	n-Decanoic acid	257.5 ± 204.6	acid

[a]500 µl corresponds to the amounts offered to the wasps in the bioassay. Average amounts (ng ± SE) were calculated on the basis of analyses performed with authentic standards. N = 5
[b]Compounds detected by: [1]Mattiacci *et al*. 1994; [2]Blaakmeer *et al*. 1994; [3]Geervliet *et al*. 1997

they comprise aldehydes, alcohols, acids, esters, terpenoids, nitriles, isothiocyanates, and benzene derivatives. In addition, n-alkanes ranging from C8 to C21 were detected (not shown in the table). Quantification was performed using stir bar sorptive extraction (SBSE) with authentic standards.

Discussion

Chemical and behavioural time course experiments proved that the volatiles induced in cabbage plants upon herbivory are emitted, and attract *C. glomerata* females within one hour. The percentage of parasitoids responding to these volatiles increased steadily during the first three hours, and remained at a constantly high level for the remaining fourteen hour period of the experiment. Chemical analyses indicate the presence of green leaf volatiles and terpenoids from the onset of damage. Moreover, within the first five hours, we observed a significant increase in the emission of a terpenoid, cineole, two green leaf volatiles, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate, and one nitrile, benzylcyanide. The strong behavioural response of the parasitoid observed after the first three hours of herbivore damage most likely account for a quantitative increase in the emitted volatile signals.

In cotton, labeling experiments with $^{13}CO_2$ were carried out to analyze the sequence of biochemical reactions occurring in the plant upon herbivory. Paré and Tumlinson (1997a, b) reported that feeding by beet armyworm induces *de novo* synthesis of certain compounds (mostly acyclic terpenes and indole) whereas other compounds (cyclic

terpenes, butyrates and green leaf volatiles) are released from storage or synthesized from stored intermediates. Nearly all compounds considered in our study are released from the onset of damage. It is interesting to note that benzylcyanide was emitted from caterpillar infested plants only two hours after infestation. Recently, benzylcyanide was identified in frass of *P. rapae* caterpillars (Wittstock *et al.* 2004). The constant high response of *C. glomerata* to herbivore infested cabbage from three hours onward might be the result of the emission of the rapidly induced compounds. In this experiment, only compounds that were found in quantifiable amounts during the one hour collection periods were monitored. It cannot be excluded that other compounds, below the detection limit of the analytical method, may have contributed to the observed behavioural responses of the parasitoid.

In previous studies, volatiles have been collected after herbivores had been feeding for at least 24 h on cabbage leaves (Blaakmeer *et al.* 1994; Geervliet *et al.* 1997; Mattiacci *et al.* 1994) and not from the very onset of damage. Time course experiments of volatile emission were carried out in cotton plants (Loughrin *et al.* 1994), maize seedlings (Turlings *et al.* 1998), ashleaf maple leaves (Ping *et al.* 2001), and apple fruits (Hern & Dorn 2001). For example, early emissions from herbivore-induced cotton consisted of high levels of lipoxygenase derived volatile compounds, e.g. (*Z*)-3-hexenal, (*Z*)-3-hexen-1-yl acetate, and several terpene hydrocarbons, e.g. α-pinene, myrcene, caryophyllene (Loughrin *et al.*, 1994). As damage proceeded, high levels of other terpenes, all of them acyclic, e.g. (*E*)-β-ocimene, (*E*)-β-farnesene, were released. However, these time course analyses were not coupled to behavioural assays. This prevents a more comprehensive interpretation of the behavioural effect of the identified chemicals. GC-EAG-analysis is frequently applied in synomone studies with the assumption that those compounds eliciting higher EAG responses are behaviourally active (Marion-Poll & Thiéry 1996). However, the effect of these potentially bioactive constituents on the involved organism often remains unknown.

Dynamic headspace sampling is the most common method used for volatile sampling. The compounds are thereby enriched on an adsorbent such as porous polymer (Cole 1980a) followed by liquid or thermal desorption (Griffiths *et al.* 1999). However, this method, along with other volatile collection methods (i.e. zNose, Kunert *et al*. 2002), has major shortcomings when trying to link a specific insect behaviour to certain chemical information. In particular, volatiles are selectively trapped for the analysis by the porous polymer and in order to perform chemical analyses and behavioural tests with them, concentrating of the eluted volatiles-samples is needed (see for example Smid *et al.* 2002). The concentration process raises additional problems including possible loss of volatile compounds or changes in the ratios between compounds. In addition, low sensitivity may result from the injection of small volumes of eluted volatile-samples into GC-MS.

Our approach of using the same samples for both, the behavioural and chemical analyses, was made possible by using solvent extraction of cabbage leaves. This allows a much closer coupling of the insects' behavioural response to the chemical composition of the extract. The cabbage leaf extract proved to be significantly attractive to *C. glomerata* wasps and therefore contained bioactive compounds. Moreover, when compared to the more traditional solvent elution of volatile-samples, the percentage of responding parasitoid to the leaf extract was significantly higher. Furthermore, the same extracts have been analyzed using GC-MS by means of stir bar sorptive extraction (SBSE) (Baltussen *et al.* 1999a). As explain above, sorption has clear advantages over adsorption, e.g., analytes can be desorbed at lower temperatures, and as a result, losses of thermolabile solutes and the production of artifacts or permanent adsorption of solutes due to high desorption temperatures are minimized (Griffiths *et al.* 1999; Baltussen *et al.* 1999b). Additionally, degradation products of PDMS can be easily identified as siloxane mass fragments (for more technical details see Baltussen *et al.* 1998, 1999a, 2002; Vercammen *et al.* 2000). SBSE analysis offers several advantages to headspace analysis, as it combines the straightforward handling of SPME with the higher resolution of PDMS. As a consequence contamination risks are reduced, and the use of storable extracts allows standardizing the bioassay replicates and facilitates the process of identifying compounds with GC-MS.

Among the compounds identified and quantified in cabbage leaf extracts using SBSE are green leaf volatiles (aldehydes, alcohols, and esters), products of the shikimic acid pathway (benzene derivatives, an isothiocyanate and nitriles), and a terpene. A comparison of the compounds detected with SBSE in cabbage extracts to previous studies on the headspace of cabbage plants (Blaakmeer *et al.* 1994; Geervliet *et al.* 1997; Mattiacci *et al.* 1994) shows that some of the compounds detected in the extract were not found in previous studies. In particular, four benzene derivatives (1,3-dimethylbenzene, 1,2-dimethylbenzene, 2-ethyltoluene and 1,2,3-trimethylbenzene, all of them not found in the blank) resultant from shikimic acid (Torssell, 1997) have never been identified in cabbage or cruciferous plants before (Wallbank & Wheatley 1976; Cole 1980b; McEwan & Smith 1998). Several combinations of these aromatics have been detected in solvent extracts of Japanese mugwort leaf (Umano *et al.* 2000), crushed guava fruit (Vernin *et al.* 1998), and peach fruit (Sumitani *et al.* 1994), altough it can not be excluded that they are air pollutants. The extract further contained three glucosinolate breakdown products benzylcyanide, cyclohexanisothiocyanate and benzenepropanenitrile (Wittstock & Halkier 2002). Benzylcyanide is known as a component of the headspace of cabbage leaves infested with *P. brassicae* (Geervliet *et al.* 1997), and it evoked GC-EAG reactions in the antennae of *C. glomerata* (Smid *et al.* 2002). So far, cyclohexanisothiocyanate and benzenepropanenitrile have not been identified in the headspace of cabbage leaves. Their detection in the present study might have been facilitated by the use of a leaf extract. Benzenepropanenitrile was recently detected in headspace analyses from leaves that were mechanically damaged (A.S. Rott pers. com.). Interestingly, (+)-limonene was the only terpene detected by using SBSE. Terpenoids are a major class among herbivore-induced synomones that attract arthropod carnivores (Dicke 1994; Dutton *et al.* 2002). However, Mattiacci *et al.* (1994) found that the qualitative and quantitative variations of the terpenes identified

from cabbage plants of different treatments (caterpillar damage, artificial damage, artificial damage with regurgitant, and undamaged plant) are very low and thus their relative contribution to the headspace information might not be of relevance in this system. This is supported by our time course experiment results which show little increase in the emissions of terpenoids over time, and by molecular work on gene activation indicating little induction of the isoprenoid pathways upon herbivory (N. Scascighini, A. Schaller, L. Mattiacci and S. Dorn, submitted).

Current applications of SBSE comprise the analyses of aqueous matrices, for example water, and other beverages (Benanou *et al.* 2003; Ochiai *et al.* 2003; Tredoux *et al.* 2000), as well as the analyses for residues of pesticides in wine and tea (Sandra *et al.* 2001; Bicchi *et al.* 2003). In addition, SBSE has found application for the dynamic headspace analyses of aromatic and medicinal plants (Bicchi *et al.* 2000) and PDMS, used as adsorbent in a trap for headspace sampling, has been employed for monitoring volatiles from *Arabidopsis* plants (Vercammen *et al.* 2001).

In a recent study a six-arm olfactometer was used for the simultaneous observation of insect attraction and odour trapping (Turlings *et al*. 2004). Alternatively, the present study shows that SBSE is a very promising technique for studies aimed at establishing causal links between compound emitted and behavioural responses, as chemical analyses and behavioural tests can be easily carried out using the same sample. The use of solvent extraction might present limitations since directly emitted volatiles are not necessarily identified. However, our most recent data provide conclusive evidence that SBSE is a valid tool for the identification of the synomones from cabbage attracting female *C. glomerata* (N. Scascighini, A. Hern, L. Mattiacci, A.S. Rott, S. Dorn, unpublished).

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