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Analytical Methods

Headspace solid-phase microextraction and gas chromatography/ion trap-mass spectrometry applied to a living system: *Pieris brassicae* fed with kale

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ABSTRACT

The influence of *Pieris brassicae* feeding on kale was monitored, by evaluating its effect on the volatiles released by the plant through time. This is the first study applying headspace solid-phase microextraction (HS-SPME) and gas chromatography/ion trap-mass spectrometry to an isolated insect, as most studies analyse the insect–plant system as a whole, being unable to evaluate the contribution of the insect itself. Substantial differences were noticed between the volatiles composition of kale before and after the insect's attack. More than 60 compounds were found, including terpenes, lipoxygenase pathway by-products, ketones, norisoprenoids, etc. After insect attack, *l*-camphor, sabinene and α-thujene were found and limonene and eucalyptol suffered a noticeable increase. A considerable rise in (*Z*)-3-hexenyl acetate was also observed. *In vivo* accumulation of limonene and camphor by the insect was detected. The findings contribute to the knowledge of the ecological interactions between the two species.

1. Introduction

The role of volatile compounds in shaping insect–plant relations is a relatively new area of research which has attracted increased interest over the last few years.

In plants, there is a constitutive emission of volatile compounds that are released from the surface of the leaf and/or accumulated in storage sites. Terpenoids constitute the most important group of volatiles that are emitted by plants, consisting predominantly of monoterpenes, sesquiterpenes and their derivatives, homoterpenes. These volatiles play different roles in herbivore elimination, either by attraction of parasitoids that increase herbivore mortality (indirect defence) or by directly reducing herbivores. However, some environment stimuli, such as feeding (Howe & Jander, 2008) or oviposition (Meiners & Hilker, 2000), can change both qualitatively and/or quantitatively the blend of volatile constituents (Bukovinszky, Gols, Posthumus, Vet, & Van Lenteren, 2005).

This induced response is, in fact, a part of the plant's defence mechanisms against predation and has been revealed to be very complex, involving gene expression as a consequence of triggering signals that include jasmonic acid, abcisic acid, and systemin (a polypeptide first isolated from leaves of tomato plants), among others (Mello & Silva-Filho, 2002).

An additional set of compounds, which include C_6 alcohols, aldehydes, acetates, and methyl salicylate, are usually designated as "the green leaf volatiles" and their production is induced by herbivore attack (Mumm, Posthumus, & Dicke, 2008). These compounds are fatty acids derivatives that arise from the conversion of linolenic and linoleic acids *via* the lipoxygenase pathway (D'Auria, Pichersky, Schaub, Hansel, & Gershenzon, 2007).

The duration of volatiles emission is highly species-dependent, varying from a few hours in corn (Turlings & Tumlinson, 1992) to over 7 days in lima beans (Dicke, Sabeis, Takabayashi, Bruin, & Posthumus, 1990).

When working with living systems, some technical issues arise, as a consequence of the need to maintain the organisms' stress levels to a minimum, thus reducing interference in the results. A limited number of studies describe handmade systems designed to collect and analyse volatile compounds emitted by insects, which include glass-Teflon chambers with adsorption by means of Tenax traps (Mattiacci et al., 2001) or wind tunnels (Guerrieri, Poppy, Powell, Tremblay, & Pennacchio, 1999). However, in both cases the insect–plant complex is analysed as a whole and, therefore, the contribution of the insect itself cannot be assessed. A more complex and automated system, applied to a living insect (response of *Lycopersicon esculentum* Mill to *Spodoptera littoralis* attack) was described by Vercammen, Pham-Tuan, and Sandra

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(2001), involving an apparatus composed of two programmable temperature vaporisation injectors in series, a glass tube filled with PDMS, rotary valves, and vacuum pumps, among other devices. Regardless of the versatility of these systems, their complexity turns them into an expensive option, and they consist of specific material that may not be available in all laboratories. Again, the insect is analysed together with the plant.

In this work, the changes during 24 h of the volatiles profile of *Brassica oleracea* L. var. *acephala* (kale) induced by the predation by *Pieris brassicae* were monitored for the first time. Kale is a well established crop worldwide, having an important impact in local economic systems, which makes the study of the interactions with one if its most common pests a pertinent subject.

Additionally, volatiles emitted by the insect itself were analysed, before and after the feeding event, also for the first time, and comparisons with compounds released by *B. oleracea* var. *acephala* were made. To the best of our knowledge, this is the first time that *P. brassicae* has been screened *in vivo* for these kind of compounds or others.

To accomplish these objectives, a volatile collection system (HS-SPME) involving regular laboratory material was used. This system is highly sensitive and has proved to be useful in the analysis of a wide range of volatile compounds, as well as being a cheap and reliable method. The characterisation of the compounds was achieved using gas chromatograph/ion trap-mass spectrometry (GC/IT-MS).

In the system presented herein, no conduction tubes were used, with the plant or insect being placed near the volatile collector, which was a PDMS fibre. Although the use of liquid extraction of compounds from adsorbent material is well documented (Du et al., 1998; Guerrieri et al., 1999; Smid, van Loon, Posthumus, & Vet, 2002), relatively long sampling times, in addition to high sampling flow rates, are necessary to achieve sufficient sensitivity. For this reason, we used thermal-desorption of the PDMS fibre, which allowed a high throughput delivery of compounds, even at ambient extraction temperatures.

The results obtained will contribute to the knowledge of insect–plant interactions and plant responses to biotic stress, being particularly relevant for areas such as chemical ecology or pest management.

2. Materials and methods

2.1. Standards

Reference compounds were purchased from various suppliers: octanal, (*E*)-2-octenal, hexadecanoic acid methyl ester, geranylacetone, β-cyclocitral, α-pinene, β-pinene, linalool, limonene, eugenol, (*E*)-2-decen-1-ol; (*Z*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, 6-methyl-5-hepten-2-one and methyl dihydrojasmonate were from Sigma–Aldrich (St. Louis, MO); (*E*)-2-nonenal; hexanal, (*E*)-2-hexenal, phenylacetaldehyde, β-ionone, dimethyl disulphide; dimethyl trisulfide and (*Z*)-3-hexenyl acetate were obtained from SAFC (Steinheim, Germany); eucalyptol and *o*-cymene were from Extrasynthese (Genay, France); acetic acid, hexyl ester and menthol were obtained from Fluka (Buchs, Switzerland) and allyl isothiocyanate was from Riedel de Haën (Seelze, Germany).

2.2. Samples

Wild *P. brassicae* individuals were obtained from Cimo/Escola Superior Agrária do Instituto Politécnico de Bragança, north-east Portugal. The population was exclusively fed with *B. oleracea* var. *acephala* (kale). Twenty-five larvae at the fourth instar of development and with identical weight (~400 mg) and size (~2.5 cm) were chosen for analysis, transported and maintained at the Department of Pharmacognosy of the Faculty of Pharmacy of Porto University.

Samples of *B. oleracea* L. var. *acephala* were obtained from Bragança, north-east Portugal, in November 2008. In each experiment, a different vessel was used, totalizing 15 vessels.

2.3. Samples analysis

2.3.1. P. brassicae

Fifteen larvae were isolated and deprived of food for 6 h. During this period, non-attacked kale leaves were analysed. After this starving time, larvae were placed in three distinct kale flower pots, in groups of five elements, and fed *ad libitum* for 1 h, after which one larva from each kale pot and respective leaves were analysed separately. Insect specimens were also analysed after a period of starvation of 6 h.

Besides the insect and kale alone, we also proceeded to the analysis of the insect in conjugation with its host plant, that is, the insect–plant system as a whole. For this purpose, *P. brassicae* was in contact with kale for 1 h and, after this time, all the material was simultaneously analysed.

All analyses were performed in triplicate.

2.3.2. B. oleracea L. var. acephala

Kale leaves were analysed after 1, 4, 8, 12 and 24 h of insect predation. Non-attacked leaves were also analysed. All analyses were performed in triplicate.

2.4. Headspace solid-phase microextraction (HS-SPME)

2.4.1. SPME fibres

Several fibres with different characteristics and uses are commercially available. According to bibliography, recommendations of supplier (Supelco, Bellefonte, PA) and to our own knowledge (Guedes de Pinho et al., 2008, 2009) three of them are the most adaptable to the compounds and to the matrices under study. The fibre selected was coated with Divinylbenzene/PDMS (DVB/PDMS), 65 μm. Fibres were conditioned by inserting them into the GC injector at 250 °C for 30 min.

2.4.2. Volatiles extraction

For the *in vivo* analysis of *P. brassicae*, several temperatures and adsorption times were tested in order to determine which one interfered the least with the insects' behaviour, in an effort to minimise insect's stress, which could influence the results. The conditions assayed were 60 °C for 1 h, 40 °C for 1 h and 40 °C for 40 min.

A 15-ml vial, which was a suitable size for *P. brassicae*, was sealed with a polypropylene hole cap and PTFE/silicone septum (Supelco) and subjected to extraction, with magnetic stirring. During this period the operator had to be present, as a swift change in the insects' behaviour could result in its death by accident with the magnet, or in the attack of the exposed fibre, damaging it.

Afterwards, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 1 min the fibre was removed and conditioned in another GC injection port for 20 min at 250 °C. The same procedure was used to test the leaves of *B. oleracea* var. *acephala*, with the exception of the operator supervision during adsorption phase.

2.5. Gas chromatography/ion trap-mass spectrometry analysis

GC/IT-MS analysis was performed using a Varian CP-3800 gas chromatograph (Varian, Palo Alto, CA) equipped with a Varian Saturn 4000 mass selective detector and Saturn GC/MS workstation

Table 1
Average content (standard deviation), in percentage, of alcohols identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a]	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
1 3-Methylbutanol[e]	759	57/58/71/86	nd	nd	nd	nd	nd	nd	nd	nd	7.974 (0.711)	2.794 (0.243)
2 1-Penten-3-ol[e]	787	57/67	0.474 (0.016)	0.280 (0.011)	nd	0.300 (0.021)	0.210 (0.013)	0.565 (0.046)	0.065 (0.006)	nd	nd	nd
3 3-Pentanol[e]	807	41/59	0.188 (0.018)	0.041 (0.004)	0.247 (0.004)	0.057 (0.004)	0.040 (0.003)	0.183 (0.017)	nd	0.097 (0.007)	2.141 (0.187)	nd
4 (*Z*)-2-Penten-1-ol[e]	880	57/68	0.283 (0.026)	0.143 (0.003)	0.282 (0.013)	0.157 (0.018)	0.052 (0.002)	0.109 (0.008)	0.017 (0.001)	nd	nd	nd
5 2,4-Hexadien-1-ol[e]	907	55/79/83/98	nd	nd	nd	nd	nd	nd	0.304 (0.005)	nd	nd	nd
6 (*Z*)-3-Hexen-1-ol[d,e]	962	41/55/67/82	0.052 (0.001)	0.019 (0.002)	0.032 (0.003)	0.029 (0.001)	0.027 (0.002)	0.034 (0.002)	nd	0.068 (0.006)	3.834 (0.007)	nd
7 (*Z*)-2-Hexen-1-ol[d,e]	968	57/67/82	0.059 (0.005)	0.028 (0.002)	nd	nd	nd	nd	0.106 (0.009)	nd	nd	nd
8 2,6-Dimethyl-7-octen-2-ol[e]	1181	59	nd	nd	nd	nd	nd	nd	nd	nd	4.103 (0.355)	nd
9 (*E*)-2-Nonen-1-ol[e]	1213	41/43/55/57	0.078 (0.002)	0.192 (0.035)	0.282 (0.011)	0.207 (0.020)	0.088 (0.008)	0.277 (0.015)	0.097 (0.007)	0.186 (0.016)	4.745 (0.276)	4.047 (0.541)
10 Undecanol[e]	1298	55/69/83/97	nd	nd	0.401 (0.040)	0.083 (0.002)	0.095 (0.009)	0.191 (0.009)	nd	0.288 (0.021)	nd	nd
11 (*E*)-2-Decen-1-ol[d,e]	1309	41/43/55/57	0.017 (0.001)	nd	0.072 (0.006)	0.054 (0.004)	0.019 (0.002)	0.063 (0.004)	nd	0.105 (0.009)	2.002 (0.133)	1.316 (0.101)

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of *n*-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Identified by comparison with reference compound.
[e] Tentatively identified by NIST05; nd = not detected.

software, Version 6.8. The column used for samples analysis was VF-5ms (30 m × 0.25 mm × 0.25 μm) from Varian. A Stabilwax-DA fused silica column (60 m × 0.25 mm, 0.25 μm; Restek, USA) was used in order to confirm the identity of some compounds found using the first column. The injection port was heated to 220 °C. Injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 ml/min. The oven temperature was set at 40 °C for 1 min, then increasing at 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in electron impact (EI) mode. Ionisation commenced 2 min after injection. The ion trap detector was set as follows: the transfer line, manifold and trap temperatures were, respectively, 280, 50 and 180 °C. The mass range scanned was *m*/*z* 40–350, with a scan rate of 6 scans/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionisation time was 25,000 μs, with an ionisation storage level of 35 *m*/*z*. The analysis was performed in full scan mode.

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analysed under the same conditions, and by comparison of retention indices (as Kovats indices) with literature data. The comparison of MS fragmentation pattern with those of pure compounds and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral data base. Confirmation was also conducted using a laboratory-

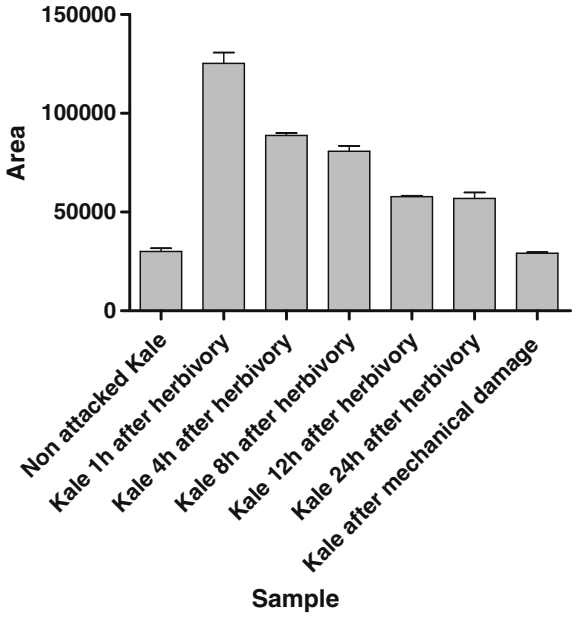

Fig. 2. Variation in total terpenes content in non-attacked kale, kale after insect's attack and after mechanical damage. Values show areas mean ± SE of three experiments.

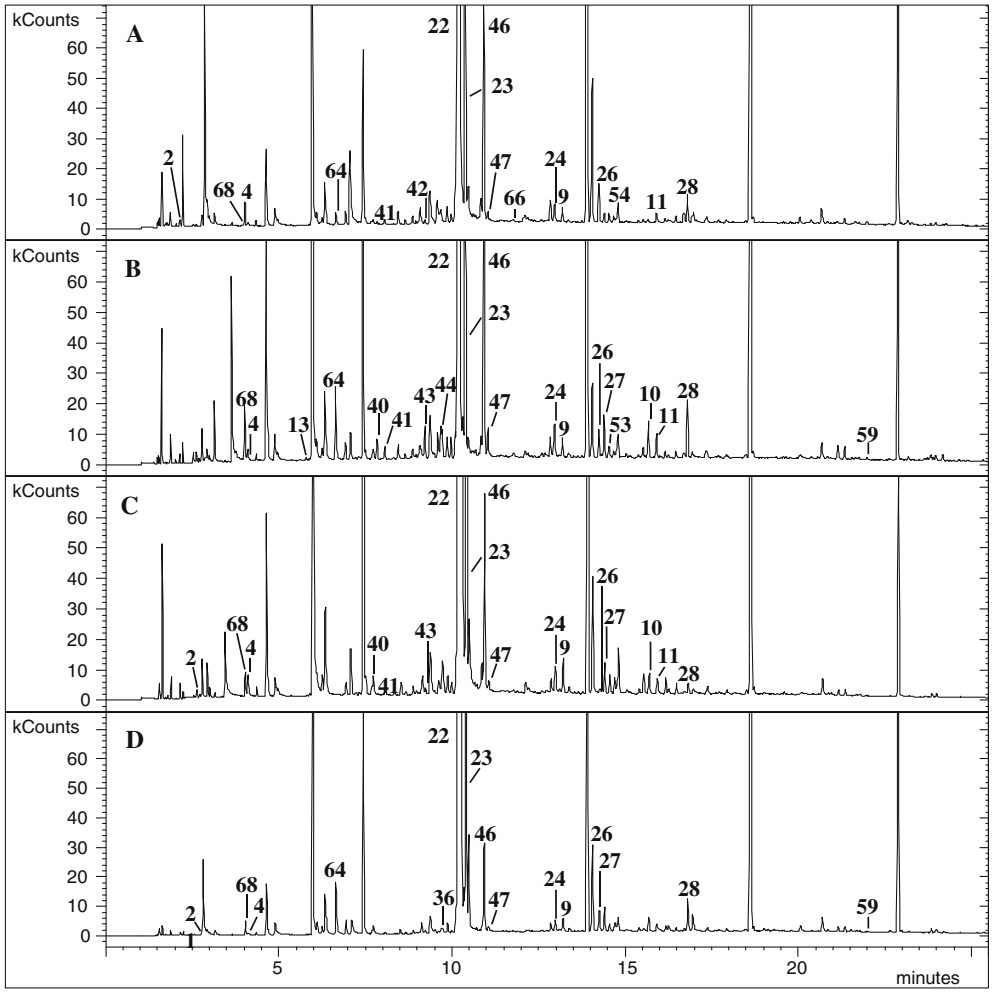

Fig. 1. Chromatographic profile of HS-SPME combined with GC/IT-MS using divinylbenzene/PDMS fibre. Non-attacked kale (A), kale after 4 h (B) and after 24 h of insect's attack (C) and kale after mechanical damage (D). Identity of compounds as in Table 1.

Table 2
Average content (standard deviation), in percentage, of aldehydes identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a] (min)	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
12	914	56/57/67/72	nd	0.110 (0.008)	0.348 (0.013)	0.061 (0.004)	0.083 (0.008)	0.168 (0.009)	nd	nd	1.083 (0.036)	0.927 (0.008)
Hexanal[d,e]												
13	958	41/55/69/83	nd	0.011 (0.000)	0.043 (0.002)	nd	nd	nd	nd	0.041 (0.002)	8.455 (0.525)	2.052 (0.044)
(*E*)-2-Hexenal[d,e]												
14	1014	55/57/70	nd	0.013 (0.001)	0.021 (0.000)	nd	nd	nd	nd	nd	nd	0.310 (0.021)
Heptanal[e]												
15	1116	43/56/69/84	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.854 (0.007)
Octanal[d,e]												
16	1159	91/120	nd	nd	nd	nd	nd	nd	nd	0.033 (0.003)	6.068 (0.608)	3.431 (0.142)
Phenylacetaldehyde[d,e]												
17	1172	41/55/70/83	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.266 (0.026)
(*E*)-2-Octenal[d,e]												
18	1272	43/55/70/83	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.233 (0.017)
(*E*)-2-Nonenald[e]												

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of *n*-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Identified by comparison with reference compound.
[e] Tentatively identified by NIST05; nd = not detected.

built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and under the same conditions. Peaks' areas were determined by re-constructed full scan chromatogram using some specific ions for each compound (quantification ions, see Table 1). By this way some peaks which were co-eluting in full scan mode (resolution value lower than 1) could be integrated with resolution value higher than 1.

The relative areas (RAs) of individual components are expressed as percentage of identified compounds.

3. Results and discussion

3.1. In vivo headspace solid-phase microextraction (HS-SPME)

For the optimisation of the sampling process, several conditions were tested. Initially, one larva was placed in a 15-ml vial at 60 °C for 1 h in contact with the fibre. However, these conditions resulted in the insect's death. The next experiment involved the same time of contact with the fibre, but was performed at 40 °C. Under these conditions the death of the insects also occurred.

The following analysis involved the contact of the insect with the fibre at 40 °C for 40 min, and the analysed specimen survived the complete procedure. All tests involved magnetic stirring at 40 rpm. This value was chosen as a way to minimise the insect's stress, which could interfere with subsequent results.

In all assays, permanent monitoring by the operator was required, as a way to control the integrity of the insect and of the fibre, as sometimes the insect would attack the fibre. When the physical integrity of the analysed specimen was at risk, stirring was turned off for a brief period.

3.2. Effect of P. brassicae predation on the volatile profile of kale

Several chemical classes of compounds could be found in kale, prior to and after the insect's attack. Herbivore attack changed the qualitative and quantitative profile of volatile compounds emitted (Tables 1–8, Fig. 1).

The compounds detected included alcohols (**1**–**11**), aldehydes (**12**–**18**), esters (**19**–**32**), ketones (**33**–**35**), norisoprenoids (**36**–**39**),

Fig. 3. Variation of terpenes content in non-attacked kale, kale after insect's attack and after mechanical damage. Values show areas mean ± SE of three experiments.

Table 3
Average content (standard deviation), in percentage, of esters identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a] (min)	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
19 Acetic acid, butyl ester[e]	925	43/56/73	nd	0.054 (0.004)	0.080 (0.000)	nd	nd	nd	nd	0.084 (0.007)	nd	0.672 (0.056)
20 Acetic acid, pentyl ester[e]	1022	43/55/61/70	nd	nd	0.208 (0.020)	0.202 (0.013)	0.109 (0.004)	0.334 (0.008)	nd	nd	nd	nd
21 4-Penten-1-yl acetate[e]	1029	43/67/68	0.325 (0.020)	0.827 (0.056)	0.469 (0.025)	0.197 (0.021)	0.378 (0.031)	0.408 (0.033)	0.266 (0.003)	0.854 (0.012)	nd	nd
22 (*Z*)-3-Hexenyl acetate[d,e]	1119	43/55/67/82	92.708 (7.887)	90.156 (5.907)	70.943 (7.365)	82.890 (7.353)	87.612 (8.431)	78.259 (7.222)	88.359 (5.826)	82.483 (1.364)	nd	nd
23 Acetic acid, hexyl ester[d,e]	1123	43/56//61/84	2.421 (0.172)	3.162 (0.231)	17.249 (0.127)	10.036 (0.140)	8.338 (0.710)	14.190 (1.336)	5.800 (0.356)	8.556 (0.591)	nd	nd
24 Propanoic acid, 4-hexen-1-yl ester[e]	1206	57/67/82	0.266 (0.018)	0.251 (0.011)	0.882 (0.064)	0.244 (0.024)	0.130 (0.008)	0.572 (0.048)	0.194 (0.008)	nd	nd	nd
25 Acetic acid, heptyl ester[e]	1220	43/56/61/70	nd	0.055 (0.004)	0.102 (0.005)	0.051 (0.005)	0.030 (0.001)	0.120 (0.003)	0.024 (0.002)	0.057 (0.003)	nd	nd
26 Butanoic acid,4-hexen-1-yl ester[e]	1252	67/71/82	0.625 (0.046)	0.147 (0.005)	0.380 (0.032)	0.456 (0.038)	0.070 (0.005)	0.064 (0.005)	0.817 (0.079)	0.825 (0.069)	nd	nd
27 Acetic acid, 2-ethylhexyl ester[e]	1257	43/55/57/70	nd	0.210 (0.018)	0.785 (0.074)	0.202 (0.019)	0.106 (0.001)	0.292 (0.025)	0.323 (0.025)	0.431 (0.040)	nd	0.747 (0.049)
28 Pentanoic acid, 4-hexen-1-yl ester[e]	1322	57/67/82	0.328 (0.028)	0.224 (0.023)	0.571 (0.055)	0.478 (0.023)	0.112 (0.004)	0.206 (0.013)	1.346 (0.123)	2.064 (0.130)	nd	nd
29 (*Z*)-Valeric acid, 3-hexenyl ester[e]	1377	57/67/82/85	0.090 (0.009)	0.117 (0.012)	nd	0.075 (0.002)	0.060 (0.001)	0.085 (0.005)	0.360 (0.036)	0.367 (0.011)	nd	nd
30 (Z)-Hexanoic acid, 3-hexenyl ester[e]	1380	67/82/99	0.031 (0.002)	0.021 (0.002)	0.039 (0.001)	nd	0.035 (0.001)	0.018 (0.001)	0.019 (0.000)	0.032 (0.001)	nd	nd
31 Methyldihydrojasmonate[d,e]	1663	83/153	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.196 (0.016)
32 Hexadecanoic acid methylester[d,e]	1937	74/87/143/270	nd	nd	nd	nd	nd	nd	nd	nd	0.480 (0.031)	0.113 (0.006)

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of *n*-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Identified by comparison with reference compound.
[e] Tentatively identified by NIST05; nd = not detected.

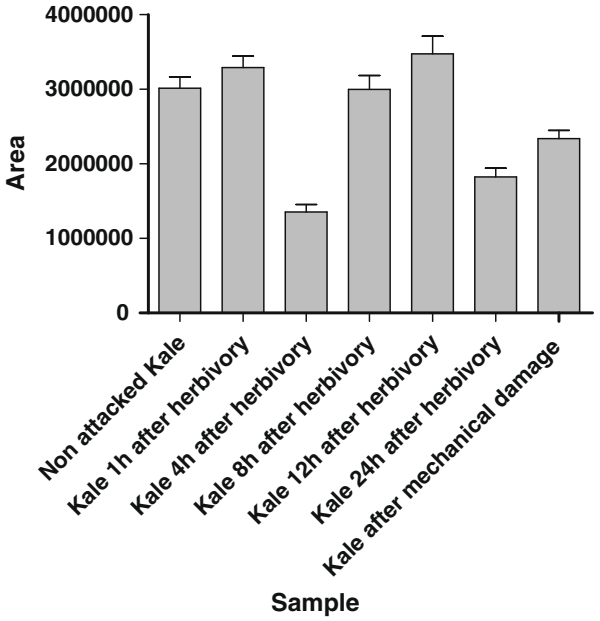

Fig. 4. Variation in (*Z*)-3-hexenyl acetate content in non-attacked kale, kale after insect's attack and after mechanical damage. Values show areas mean ± SE of three experiments.

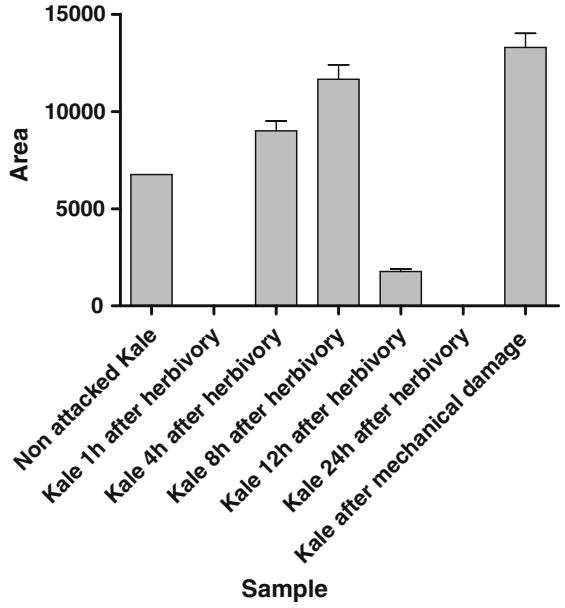

Fig. 5. Variation in allyl isothyocianate in non-attacked kale, kale after insect's attack and after mechanical damage. Values show mean areas ± SE of three experiments.

terpenes (**40**–**60**) (monoterpenes and sesquiterpenes), sulphur-containing compounds (**61**–**66**), among others (Tables 1–7).

With the exception of aldehydes, all the referred classes of compounds were found before and after the insect's attack; however, some compounds of each class could be detected only after insect feeding, mainly terpenes. Compounds such as α-thujene (**40**), sabinene (**43**), β-pinene (**44**), *psi*-cumene (**45**), *m*-cymene (**48**), *o*-cymene (**49**), *p*-cymene (**50**), *l*-camphor (**53**), longifolene (**58**) and geranylacetone (**60**) are examples of such compounds (Fig. 1 and Table 6).

Terpenes were the class most affected by predation (Tables 6 and 8). After 1 h of insect attack, the amount of kale terpenes' amount had increased by over 315%. Although there was a tendency for this quantity to decrease through time (Fig. 2), after 24 h their amounts were still ca. 90% higher than those prior to the attack. After 1 h, alcohols had decreased by about 30% and aldehydes, that were absent in non-attacked leaves, appeared, with hexanal (**12**), (*E*)-2-hexenal (**13**) and heptanal (**14**) being detected. Hexanal (**12**) was the only aldehyde that could be detected in kale after 24-h predation (Table 2).

A pattern in the time of appearance of the compounds could be noticed. Among the 10 terpenoids that could be found exclusively after insect predation, five were only detected 4 h after the attack, being absent 1 h after herbivory (Fig. 3 and Table 6). On the other hand, three ester compounds acetic acid, butyl ester (**19**); acetic acid, heptyl ester (**25**); acetic acid 2-ethylhexyl ester (**27**) were absent before the attack, but were found immediately 1 h after predation (Table 3). These results strongly suggest that the synthesis of terpenes occurs *de novo*, while the referred esters are probably accumulated in the leaves and released after predation. Further data support this hypothesis; if a compound is accumulated in leaves, it would be expected that its release would occur both by insect predation or mechanical damage. In fact, we conducted an experiment in which leaves of kale were mechanically damaged and the above referred esters were identified. However, terpenes, such as α- and β-thujene (**40** and **42**, respectively), sabinene (**43**) or β-pinene (**44**) were absent, which is in line with the hypothesis of their *de novo* synthesis.

In all experiments, esters were the main class of compounds, always accounting for more than 90% of the volatiles. In fact, this value results from the contribution of one single compound, (*Z*)-3-hexenyl acetate (**22**), which, alone, accounted for 70–92% of the identified compounds in the different experiments. Although high amounts of this compound have been reported (Geervliet, Posthumus, Vet, & Dicke, 1997), to the best of our knowledge this is the first time that such a high proportion of (*Z*)-3-hexenyl acetate (**22**) has been found. This compound has been extensively described in literature as being crucial in shaping insect–plant interactions (Mattiacci et al., 2001; Paré & Tumlinson, 1999). In this study, the amounts of (*Z*)-3-hexenyl acetate (**22**) suffered an increase of ca. 10% 1 h after insect's attack. Analysis 12 h after the attack revealed an increase by some 15% (Fig. 4). Interestingly, if the damage to the leaf was caused mechanically, instead of an increase in (*Z*)-3-hexenyl acetate its quantities compared with basal emissions would diminish by ca. 25% (Fig. 4). This result, as well as the absence of the alcohol (*Z*)-3-hexen-1-ol (**6**), had already been described in a similar study involving one plant from the same species, although from a different cultivar, *B. oleracea* var. *gemnifera* (Mattiacci et al., 2001). These authors describe the absence of β-caryophyllene (**59**) in mechanically damaged leaves, but in our study this compound was found in leaves that were mechanically damaged, albeit in much lower quantities than those registered after insect feeding (Table 6). Moreover, β-caryophyllene was not detected in the headspace of other *B. oleracea* varieties, such as white cabbage (*B. oleracea capitata* L. var. *alba* cv. Langedijker de Waar) and red cabbage (*B. oleracea capitata* L. var. *rubra* (DC) (Geervliet et al., 1997)).

Regardless of the differences between volatiles emitted after predation and those that result from mechanical damage, it can be said that in both situations the chemicals released are similar, albeit different from a quantitative point of view. Regarding the volatile blends emitted by plants when challenged by insect predation or mechanical damage, two kinds of plant groups exist: one, in which the compounds released in the two situations are completely different and a second, in which the volatiles released share chemical similarities. Examples of the first are corn (Turlings, Tumlinson, & Lewis, 1990) and lima beans (Dicke et al., 1990), in which terpenoids are found as a response to herbivory but not to mechanical damage. In the second case, cabbage is an example

(Geervliet et al., 1997) as only subtle qualitative differences were found. The results described herein are in line with those of other cabbages, from which kale is taxonomically very close.

The vegetable species used in these experiments, kale, contains glucosinolates (a group of amino acid-derived thioglucosides), which can be found through most cruciferous vegetables. These secondary metabolites are the precursors of volatile isothiocyanates and are involved in defence against predation, as isothiocyanates are toxic upon ingestion, contact, or when present in the gas phase (Agrawal & Kurashige, 2003).

This defence system consists of glucosinolates and myrosinases, which are thioglucoside glucohydrolases that hydrolyse the thioglucosidic bond of the glucosinolates, yielding glucose and an unstable aglycone. Spontaneous rearrangement of the aglycone then leads to the formation of an isothiocyanate (Mumm et al., 2008). In this study, allyl isothiocyanate (**64**) was detected in small amounts in plants that had not been attacked by insects or mechanically damaged (Table 7 and Fig. 5). Given the fact that, in intact plant tissue, glucosinolate hydrolysis is prevented by spatial separation of myrosinases and glucosinolates by storage in different cells (Andréasson & Jørgensen, 2003), the presence of allyl isothiocyanate (**64**) in non-attacked leaves must mean that some kind of damage has been delivered to the leaf. In fact, the volatile analysis was not performed in the plant as a whole, as leaves were gently removed from the plant and analysed. After 1-h predation, this compound was not noticed in kale, a fact that we cannot yet fully understand, with further studies being needed. As expected, the amounts of this compound rose in the sequence of insect predation, having increased by over 70%. As expected, the amounts of allyl isothiocyanate in the leaves of the mechanically damaged plant were far higher than the basal values, over 96% (Table 7). Overall, increase in allyl isothiocyanate was much higher in mechanically damaged leaves than those that suffered insect attack (Fig. 5). As the mechanism involved in this compound's release is strictly a consequence of tissue damage, it is understandable that our provoked damage occurs to a higher extent than that from insect's chewing.

Methylthiocyanate (**62**) was detected in the samples of herbivore-infested kale, while it was absent in infested red and white cabbages (Geervliet et al., 1997). However, this compound has already been found in infested leaves of nasturtium (*Tropaeolum majus* cv. Mahogany), another species containing glucosinolates (Geervliet et al., 1997).

One of the most interesting findings about volatiles emission after herbivory in kale is its distinct differences in the release mechanism when compared with a plant from the same species but different cultivar, *B. oleracea* var. *gemnifera* (Mattiacci et al., 2001). Although the compounds emitted are related, in *B. oleracea* var. *gemnifera* the compounds induced by insect feeding could be detected only after mechanical damage, as otherwise they would remain trapped inside leaves. In our study, however, such compounds could be detected simply by insect feeding, mechanical damage of leaves was not necessary. In fact, *B. oleracea* var. *gemnifera* seems to be an exception, as volatile emissions in other species such as lima bean (Dicke et al., 1990), cotton (Röse, Manukian, Heath, & Tumlinson, 1996) or corn (Turlings &Tumlinson, 1992) follow the same process as in *B. oleracea* var. *acephala*.

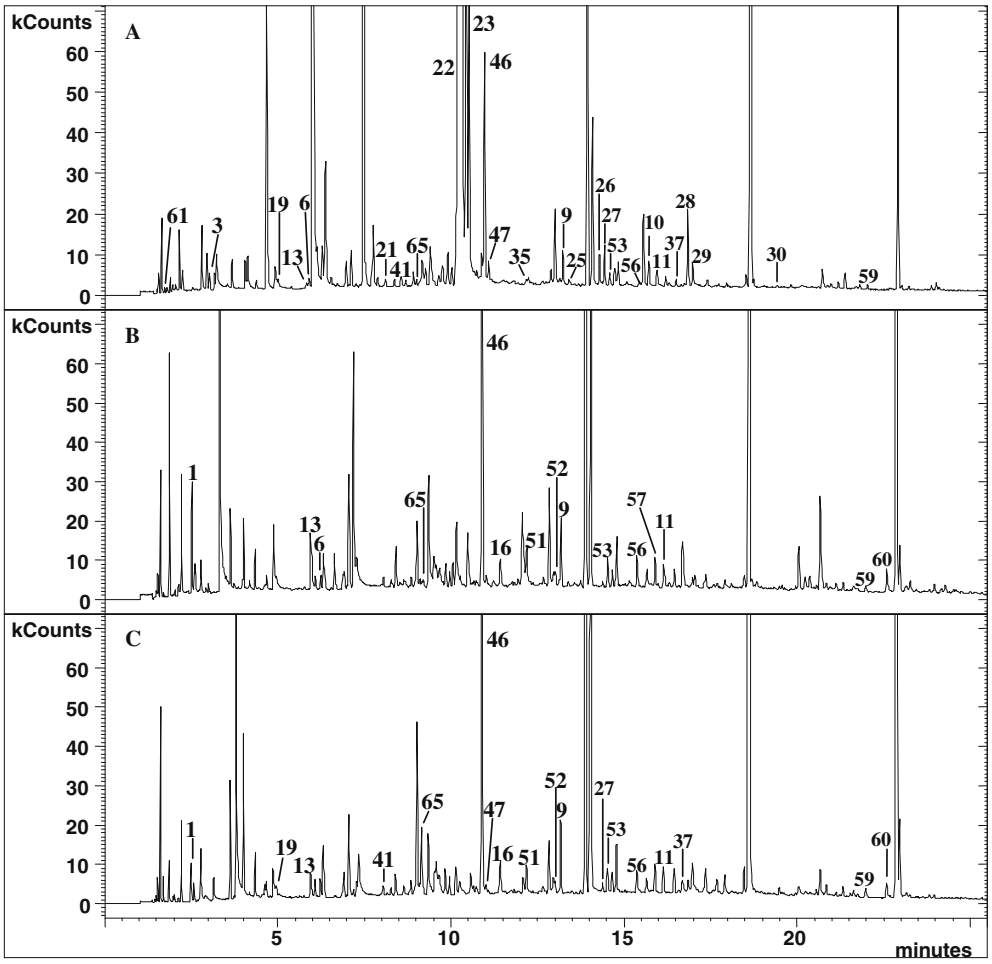

Fig. 6. Chromatographic profile of HS-SPME combined with GC/IT-MS using divinylbenzene/PDMS fibre. Insect–plant complex (A), *P. brassicae* 1 h after feeding (B) and *P. brassicae* after 6 h starvation (C). Identity of compounds as in Table 1.

Table 4
Average content (standard deviation), in percentage, of ketones identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a] (min)	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
33 3-Pentanone[d]	806	57/86	0.169 (0.015)	nd	0.280 (0.027)	0.264 (0.023)	0.175 (0.007)	0.387 (0.030)	nd	nd	nd	nd
34 3,5-Dimethyl-2-octanone[d]	861	43/57/72	0.020 (0.000)	nd	nd	nd	nd	nd	nd	nd	nd	nd
35 α,α-Dihydroxyacetophenone[d]	1179	77/105	nd	0.132 (0.006)	0.206 (0.014)	0.099 (0.009)	0.034 (0.002)	0.137 (0.010)	nd	0.184 (0.013)	nd	nd

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of *n*-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Tentatively identified by NIST05; nd = not detected.

Table 5
Average content (standard deviation), in percentage, of norisoprenoids identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a] (min)	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
36 6-Methyl-5-heptene-2-one[d,e]	1096	43/55/69/108	nd	0.136 (0.013)	0.101 (0.008)	0.033 (0.003)	0.023 (0.001)	0.029 (0.001)	0.017 (0.001)	0.029 (0.003)	0.657 (0.051)	0.452 (0.042)
37 β-Cyclocitral[d,e]	1320	109137/152	0.004 (0.000)	nd	nd	nd	0.013 (0.000)	0.027 (0.002)	0.016 (0.001)	0.018 (0.000)	nd	0.206 (0.003)
38 β-Ionone[d,e]	1494	177	0.003 (0.000)	nd	nd	nd	nd	nd	0.003 (0.001)	0.010 (0.012)	nd	nd
39 β-Methylionone[e]	1676	57/149/191	0.008 (0.001)	0.018 (0.001)	0.024 (0.001)	0.010 (0.001)	nd	nd	0.025 (0.001)	0.004 (0.000)	0.198 (0.007)	nd

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of n-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Identified by comparison with reference compound.
[e] Tentatively identified by NIST05; nd = not detected.

Table 6
Average content (standard deviation), in percentage, of terpenes identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a] (min)	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
40 α-Thujene[e]	1036	77/91/93	nd	nd	0.053 (0.001)	0.013 (0.001)	0.051 (0.004)	0.022 (0.002)	nd	nd	nd	nd
41 α-Pinene[d,e]	1047	77/93	0.015 (0.001)	0.043 (0.001)	0.069 (0.005)	0.035 (0.003)	0.060 (0.008)	0.066 (0.005)	0.013 (0.001)	0.056 (0.003)	nd	0.716 (0.042)
42 β-Thujene[e]	1076	77/91/93	0.020 (0.001)	nd	nd	nd	nd	nd	nd	0.165 (0.002)	nd	0.682 (0.068)
43 Sabinene[e]	1082	77/91/94	nd	nd	0.147 (0.008)		0.043 (0.003)	0.070 (0.005)	nd	nd	nd	nd
44 β-Pinene[d,e]	1099	69/93	nd	nd	0.148 (0.003)	0.068 (0.006)	nd	nd	nd	nd	nd	nd
45 *psi*-Cumene[e]	1102	105/120	nd	nd	nd	nd	0.086 (0.009)	nd	nd	nd	nd	nd
46 Limonene[d,e]	1137	68/93	0.769 (0.067)	2.904 (0.204)	3.764 (0.088)	1.851 (0.109)	1.125 (0.112)	1.878 (0.166)	0.877 (0.039)	2.321 (0.128)	3.638 (0.236)	38.904 (2.927)
47 Eucalyptol[d,e]	1145	81/93/108	0.045 (0.003)	0.212 (0.019)	0.109 (0.012)	0.079 (0.001)	0.073 (0.006)	0.077 (0.007)	0.025 (0.002)	0.185 (0.002)	0.858 (0.031)	0.524 (0.050)
48 *m*-Cymene[e]	1185	91/119/134	nd	nd	nd	nd	0.005 (0.000)	nd	0.005 (0.000)	0.011 (0.001)	nd	nd
49 *o*-Cymene[d,e]	1190	91/119/134	nd	nd	0.024 (0.002)	0.012 (0.001)	0.008 (0.000)	nd	0.009 (0.000)	0.019 (0.001)	nd	nd
50 *p*-Cymene[e]	1193	91/119/134	nd	nd	nd	nd	0.011 (0.001)	nd	0.012 (0.001)	0.028 (0.001)	nd	nd
51 *cis*-Linalool oxide[e]	1196	59/68/94/111	nd	nd	nd	nd	nd	nd	nd	nd	0.643 (0.029)	0.176 (0.002)
52 Linalool[d,e]	1208	55/71/93/121	nd	nd	nd	nd	nd	nd	nd	nd	1.002 (0.096)	0.704 (0.025)
53 *l*-Camphor[e]	1261	69/81/95/108	nd	0.173 (0.013)	0.216 (0.005)	0.116 (0.009)	0.096 (0.007)	0.252 (0.025)	0.097 (0.003)	0.204 (0.020)	5.996 (0.461)	3.355 (0.154)
54 *p*-Menthone[e]	1268	55/69/112	0.015 (0.001)	0.021 (0.001)	0.057 (0.003)	nd	nd	nd	nd	nd	nd	nd
55 Isomenthone[e]	1277	55/69/112	0.008 (0.000)	nd	nd	0.015 (0.001)	0.007 (0.000)	0.019 (0.001)	0.006 (0.001)	0.016 (0.001)	nd	nd
56 L-(−)-Menthol[d,e]	1289	55/71/85/138	0.030 (0.002)	0.034 (0.003)	0.030 (0.002)	0.025 (0.001)	0.007 (0.000)	0.040 (0.002)	0.043 (0.004)	0.054 (0.003)	4.991 (0.383)	1.188 (0.100)
57 Terpineol[d,e]	1303	59/93/121/136	nd	nd	nd	nd	nd	nd	nd	nd	2.730	nd
58 Longifolene[e]	1425	94/107/161/204	nd		0.018 (0.001)	0.008 (0.001)	0.005 (0.000)	0.012 (0.001)	0.009 (0.001)	0.021 (0.001)	0.094 (0.006)	0.128 (0.008)
59 β-Caryophyllene[e]	1434	79/91/93/133	0.025 (0.002)	0.014 (0.001)	0.014 (0.000)	0.009 (0.009)	0.003 (0.000)	nd	0.007 (0.000)	0.023 (0.001)	0.885 (0.062)	0.357 (0.015)
60 Geranylacetone[d,e]	1459	43/69	nd	0.024 (0.001)	nd	0.005 (0.000)	0.004 (0.000)	nd	nd	nd	3.085 (0.147)	0.843 (0.017)

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of *n*-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Identified by comparison with reference compound.
[e] Tentatively identified by NIST05; nd = not detected.

Table 7
Average content (standard deviation), in percentage, of sulphur, nitrogen and miscellaneous compounds identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compound	RI[a]	QI[b] (*m*/*z*)	RA[c] (% ±SD)								
			Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
				1 h	4 h	8 h	12 h	24 h				
Sulphur compounds												
61 Dimethyl sulfide[e]	337	47/62	0.007 (0.000)	nd	nd	nd	nd	nd	nd	0.052 (0.005)	0.568 (0.036)	1.257 (0.062)
62 Methylthiocyanate[e]	818	45/72/73	0.200 (0.018)	nd	nd	0.169 (0.015)	0.130 (0.013)	0.083 (0.006)	nd	nd	nd	nd
63 Dimethyl disulfide[d,e]	854	45/79/94	0.028 (0.000)	nd	nd	nd	nd	nd	nd	nd	12.622 (1.229)	9.935 (0.452)
64 Allyl isothiocyanate[d,e]	997	41/72/99	0.208) (0.000	nd	0.472 (0.037)	0.322 (0.029)	0.045 (0.004)	nd	0.503 (0.039)	nd	nd	0.177 (0.002)
65 Dimethyl trisulfide[d,e]	1093	45/79/126	nd	nd	nd	nd	nd	nd	nd	0.029 (0.003)	0.521 (0.048)	10.183 (0.716)
66 2-Methylbutyl isothiocyanate[e]	1169	57/72/129	0.005 (0.000)	nd	nd	nd	nd	nd	nd	nd	nd	nd
Nitrogen compounds												
67 2-Butenenitrile[e]	762	41/67	0.215 (0.020)	nd	nd	nd	nd	nd	nd	nd	nd	nd
Miscellaneous compounds												
68 Toluene[e]	881	91/92	0.149 (0.013)	nd	0.825 (0.067)	0.298 (0.029)	0.183 (0.014)	0.453 (0.029)	0.236 (0.015)	nd	20.655 (1.744)	11.483 (1.043)
69 Phenol[e]	1099	66/94	nd	0.231 (0.001)	nd	0.412 (0.038)	nd	0.061 (0.005)	nd	nd	nd	nd
70 Eugenol[d,e]	1386	77/131/164	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.773 (0.040)

[a] RI = retention indices as determined on HP-5 capillary column using the homologous series of *n*-alkanes.
[b] QI = quantification ions.
[c] RA = relative area in percentage ± standard deviation.
[d] Identified by comparison with reference compound.
[e] Tentatively identified by NIST05; nd = not detected.

Table 8
Total content (standard deviation), in percentage, of the several volatile compounds' classes identified in kale before and after herbivory attack, kale mechanically damaged and *Pieris brassicae* 1 h after feeding and after 6 h starvation and insect–plant complex.

Compounds	RA[a] (% ±SD)									
	Non-attacked Kale	Kale after herbivory attack					Kale mechanically damage	Insect–plant complex	Insect 1 h after feeding	Insect after a 6 h starvation
		1 h	4 h	8 h	12 h	24 h				
Total compounds identified	35	32	38	38	41	35	32	36	26	33
Alcohols (%)	7	6	6	7	7	7	5	5	6	3
	(1.151)	(0.703)	(1.316)	(0.887)	(0.531)	(1.422)	(0.589)	(0.744)	(24.800)	(8.157)
Aldehydes (%)	0	3	3	1	1	1	0	2	3	7
		(0.134)	(0.412)	(0.061)	(0.083)	(0.168)		(0.074)	(15.607)	(8.073)
Esters (%)	8	11	11	10	11	11	10	10	1	4
	(96.794)	(95.224)	(91.708)	(94.831)	(96.980)	(94.548)	(97.508)	(95.753)	(0.480)	(1.728)
Ketones (%)	2	1	2	2	2	2	0	1	0	0
	(0.189)									
		(0.132)	(0.486)	(0.363)	(0.209)	(0.524)		(0.184)		
Norisoprenoids derivatives (%)	3	2	2	2	2	2	4	4	2	2
	(0.015)	(0.154)	(0.125)	(0.043)	(0.036)	(0.056)	(0.061)	(0.061)	(0.855)	(0.658)
Terpenes (%)	8	8	12	12	15	9	11	12	10	11
	(0.927)	(3.425)	(4.649)	(2.236)	(1.584)	(2.436)	(1.103)	(3.103)	(23.891)	(47.307)
Sulphur compounds (%)	5	0	1	2	2	1	1	2	3	4
	(0.448)		(0.472)	(0.491)	(0.175)	(0.083)	(0.503)	(0.081)	(13.712)	(21.552)
Nitrogen compounds (%)	1	0	0	0	0	0	0	0	0	0
	(0.215)									
Miscellaneous compounds (%)	1	1	1	2	1	2	1	0	1	2
	(0.149)	(0.231)	(0.825)	(0.710)	(0.183)	(0.514)	(0.236)		(20.655)	(12.256)

[a] RA = relative area in percentage ± standard deviation.

3.3. Volatiles profile of the insect-plant complex

Kale–insect complex was directly analysed by HS-SPME and GC–MS (Fig. 6). Generally, the compounds from all classes suffered a dramatic increase during insect predation when compared with the results obtained for kale 1, 4, 8, 12 and 24 h after attack (Tables 1–8). Few studies address the question of the analysis of the combined insect–plant complex; however, such increase in all compounds has been previously reported in a plant taxonomically close to kale, *B. oleracea* var. *gemnifera* (Mattiacci et al., 2001). The higher contents may be a consequence of the immediate response of kale to insect's attack.

Even so, the information provided by this kind of analysis is somewhat limited, as it is not possible to differentiate which compounds are released by kale's leaves or the insect. No compounds were detected that had not been also identified in attacked leaves.

3.4. Volatiles emitted by P. brassicae

To the best of our knowledge, this is the first study aiming to determine the profile of volatile compounds of a living isolated insect, *P. brassicae*.

Two kinds of assays were conducted in order to study the fate of volatiles in the insect through time, one in the insect 1 h after feeding and another after a 6-h starvation period subsequent to feeding. As expected, many of the volatiles found after 1 h had already been found in kale and probably resulted from the ongoing digestion process occurring in *P. brassicae* (Fig. 6).

Predictably, many of the compounds found in the insect after 1 h were absent in the analysis conducted after 6 h (Tables 1–8). However, some exceptions were noticed. Limonene (**46**) was a compound whose amount was extremely high after a 6 h starvation, over five times its amounts in leaves (Table 6). Given the fact that by this time digestion was already over, such contents can only be due to accumulation by the insect. The accumulation described herein must constitute a mechanism by which the insect takes benefit from bioactive constituents from the diet. In fact, the insecticidal activity of limonene has been described (Hebeish, Moustafa, Hamdy, EL-Sawy, & Abdel-Mohdy, 2008), and it is possible that *P. brassicae* accumulates this compound for its own defence, as the sequestration of terpenoids for defence purposes is known (Nishida, 2002). Moreover, this terpenoid has already been implicated as a sex pheromone in the cerambycid beetle *Megacyllene caryae*, and the same function in *P. brassicae* should be considered (Lacey, Moreira, Millar, & Hanks, 2008).

Even more surprising is the presence of eugenol (**70**) (Table 7), a phenylpropanoid that was not detected in kale. This result can have two explanations. This compound may exist in the plant in very low amounts, below the limit of detection of the instrumental techniques used. However, this is unlikely to happen, as GC/IT-MS is a very sensitive technique, and a bioconcentration process of several thousand folds would be necessary. The second hypothesis is that eugenol is formed from other compounds present in kale, by a process of metabolism by the insect. However, in kale, the only slightly related compounds would be phenol and toluene, which are also aromatic compounds. Even so, eugenol (**70**) synthesis from these compounds would involve many reaction steps, and therefore seems unlikely to happen *in vivo*.

Allyl isothiocyanate (**64**) was a compound also found in the insect after a 6-h starvation period, albeit in low amounts (Table 7). This was an expected result as these compounds are toxic to insects and, therefore, a detoxification process probably took place. This detoxification process is not common to all insects, as only some species have co-evolved together with its host plant, allowing them to feed on the very compounds that the plant synthesises to serve as herbivore deterrents (Mello & Silva-Filho, 2002), which

is the case of glucosinolates and *P. brassicae*, a specialist in crucifers. In some cases, high levels of adaptation by the insect can result in sequestering deterrent compounds from the plant for its own use against predators, turning the insect less attractive (Mello & Silva-Filho, 2002).

We also noticed that, from the wide range of esters detected in kale before and after insect predation, very few were found in *P. brassicae*. Concerning ketones, none were found in *P. brassicae*, although they existed in kale leaves. An opposite phenomenon was registered with aldehydes, as non-attacked kale displayed none of these compound and several were found in insect after 6 h starvation, but not 1 h after feeding. Therefore, it is possible that aldehydes constitute markers for starvation stress in *P. brassicae*. The same possibility could be stated for eugenol.

In conclusion, compounds emitted by insect-damaged leaves share remarkable chemical similarities, as was carefully described by Mattiacci et al. (2001).

Such structural resemblance displayed by a wide range of species could indicate that a common group of biosynthetic pathways are triggered (Paré & Tumlinson, 1999). According to Paré and Tumlinson (1996) volatiles released as a result of herbivory can be divided between lipoxygenase by-products, isoprenoid-derived terpenoids, and shikimic acid-derived aromatics.

The production of induced terpenoids is regulated by two pathways, one of which is mevalonate-dependent and the other is mevalonate-independent, also named as the deoxyxylulose pathway. The deoxyxylulose pathway appears to be important in the release of inducible monoterpenes after elicitation with jasmonic acid, which has a similar effect to herbivore infestation (Dicke, Gols, Ludeking, & Posthumus, 1998). Contrarily, constitutive compounds are synthesised through the mevalonate-dependent pathway.

Taking into account this division, in the current work no shikimic acid pathway derivatives were found. Even with *B. oleracea* var. *gemnifera*, only trace amounts of this class of compounds could be found (Mattiacci, Dicke, & Posthumus, 1994; Mattiacci et al., 2001).

This leads to the hypothesis that plants might use a defensive strategy that is more complex than a shared set of biosynthetic pathways within a plant family. In fact, specific pathways and/or limited biosynthetic capabilities within different species or different cultivars may explain the amazing variability of different plants when facing herbivory challenge.

Also, some biological processes in *P. brassicae* were described, such as limonene (**46**) accumulation, which was verified for other compounds, like *l*-camphor (**53**) or L-(−)-menthol (**56**). With this, terpenoids sequestering and accumulation was proved.

Some questions remain open, such as the origin of eugenol in the insect, as this compound was absent in kale and related compounds that could originate eugenol by a biotransformation process were not found.

With this work, further knowledge concerning the specialist *P. brassicae* and its interactions with one of its host plants was provided, which can be important in pest management, chemical ecology and entomology.

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