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INDUCTION OF PARASITOID ATTRACTING SYNOMONE IN BRUSSELS SPROUTS PLANTS BY FEEDING OF *Pieris brassicae* LARVAE: ROLE OF MECHANICAL DAMAGE AND HERBIVORE ELICITOR

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Abstract—Induction of plant defense in response to herbivory includes the emission of synomones that attract the natural enemies of herbivores. We investigated whether mechanical damage to Brussels sprouts leaves (*Brassica oleracea* var. *gemmifera*) is sufficient to obtain attraction of the parasitoid *Cotesia glomerata* or whether feeding by *Pieris brassicae* caterpillars elicits the release of synomones not produced by mechanically damaged leaves. The response of the parasitoid *Cotesia glomerata* to different types of simulated herbivory was observed. Flight-chamber dual-choice tests showed that mechanically damaged cabbage leaves were less attractive than herbivore-damaged leaves and mechanically damaged leaves treated with larval regurgitant. Chemical analysis of the headspace of undamaged, artificially damaged, caterpillar-infested, and caterpillar regurgitant-treated leaves showed that the plant responds to damage with an increased release of volatiles. Green-leaf volatiles and several terpenoids are the major components of cabbage leaf headspace. Terpenoids are emitted in analogous amounts in all treatments, including undamaged leaves. On the other hand, if the plant is infested by caterpillars or if caterpillar regurgitant is applied to damaged leaves, the emission of green-leaf volatiles is highly enhanced. Our data are in contrast with the induction of more specific synomones in other plant species, such as Lima bean and corn.

Key Words—Lepidoptera, Pieridae, Hymenoptera, Braconidae, cabbage, Brussels sprouts, behavior, tritrophic interactions, green-leaf volatiles, her-

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bivore-induced synomones, elicitor, caterpillar regurgitant, *Brassica oleracea*, *Pieris brassicae*, *Cotesia glomerata*, parasitoid, wasp.

INTRODUCTION

Herbivory leads to mechanical damage of plants and thus to an augmented emission of plant volatiles. However, the chemical blend that is emitted from mechanically damaged plants and from herbivore damaged plants can be quite different. Natural enemies of herbivores use plant volatiles to locate the herbivores, and they can discriminate between the volatiles of a mechanically damaged plant and those of an herbivore damaged plant (for reviews see Dicke et al., 1990b; Vet and Dicke, 1992; Turlings et al., 1993b; Dicke, 1994). Herbivore damaged plants may emit volatile chemicals that are not emitted by undamaged or mechanically damaged plants. This has been shown for several plant species, such as Lima bean, cucumber, and corn (Dicke et al., 1990a,b; Turlings et al., 1990, 1993b; Takabayashi et al., 1994; Dicke, 1994). Such plant volatiles increase herbivore detectability to their natural enemies. Because the volatiles are produced by the plant in response to herbivore damage and attract the herbivore's natural enemies, whose activity is favorable to the plant, they are termed herbivore-induced synomones (Vet and Dicke, 1992).

Mechanical damage may differ from herbivore damage in physical attributes, such as the amount of cellular shearing or tearing, which might result in different blends of volatiles. Since the response of plants to damage may be mediated by endogenously derived damage cues such as cell-wall fragments or cell-wall-bound enzymes, one would expect different responses to different types of damage. Comparisons of plant responses to herbivory and mechanical simulations need to be accurate not only with respect to amount of damage, but also to the spatial and temporal pattern of the damage (Baldwin, 1990). Mechanical damage of plants results in an increased emission of C_6 alcohols, aldehydes, and their esters, so-called "green odors." Several parasitoid species are known to be attracted to these green odors (Nordlund et al., 1988; Takabayashi et al., 1991a; Whitman and Eller, 1990, 1992). However, the degree of attraction of natural enemies of herbivores to volatiles emitted from artificially damaged leaves is usually much lower than to volatiles of herbivore-damaged plants (Turlings et al., 1990; Takabayashi and Dicke, 1992; Steinberg et al., 1993).

In addition to differences in temporal and spatial aspects of mechanical damage and herbivore damage, herbivory also differs from mechanical damage in that herbivores may apply or inject oral secretions onto or into the plant. When regurgitant of *Spodoptera exigua* caterpillars is applied to a mechanical wound in a corn leaf, the emitted volatiles are similar to those emitted from a caterpillar-damaged plant, but different from those emitted from a mechanically damaged plant (Turlings et al., 1990).

That plants respond to herbivory by emitting specific herbivore-induced

synomones that are not emitted by mechanically damaged plants was first demonstrated for a tritrophic system of Lima bean plants, two-spotted spider mites, and predatory mites that consume the spider mites (Dicke and Sabelis, 1988; Dicke et al., 1990a,b). A very similar phenomenon has been described for the tritrophic system of corn plants, beet army worm larvae, and their parasitoids, for which it was also shown that caterpillar regurgitant can elicit the response of the plant (Turlings et al., 1990). A few other systems have been investigated but not in as much detail as the Lima bean and corn system (see Dicke, 1994, for review). It is of interest to investigate whether all plant species respond in a comparable way to herbivory. In this paper we present data for a tritrophic system consisting of Brussels sprouts plants, caterpillars of the large cabbage white butterfly, and one of its parasitoids. We show that these plants respond to herbivory in a way similar to corn plants but that the type of response by the plant is remarkably different.

Cotesia glomerata is a gregarious larval parasitoid of several pierid species such as the cabbage white caterpillars *Pieris brassicae* (L.) and *Pieris rapae* (L.). Female *C. glomerata* discriminate among undamaged, mechanically damaged, and caterpillar-infested cabbage plants (Steinberg et al., 1992, 1993). The plant–herbivore complex (PHC), where host larvae are actively feeding, is most attractive. Yet, after removing the host larvae, the herbivore-damaged plant (HD) remains very attractive to the parasitoids for at least several hours. In contrast, the attractiveness of mechanically damaged cabbage plants quickly wanes after the infliction of the damage is stopped (Steinberg et al., 1993). Apparently herbivore damage results in a different response from the plant than mechanical damage (Steinberg et al., 1993).

In the current study, we compared the effect of true and simulated herbivore damage by *P. brassicae* to Brussels sprouts plants, in order to know whether and how herbivory influences the production of synomones by the plant. We tested two hypotheses: (1) The release of synomones by cabbage plants is activated by the mechanical disruption of leaf tissue, which needs to be properly simulated for pattern and timing, in order to be comparable to the actual herbivore damage. (2) Synomones are produced as a direct physiological response of the cabbage plant, activated when the leaf cells get into contact with possible elicitors present in the regurgitant of *P. brassicae* larvae. The study comprises behavioral observations and chemical analyses of headspace collections.

METHODS AND MATERIALS

Rearing Procedures

Plants (Brussels sprouts, *Brassica oleracea* L. var. *gemmifera*, cv. Titurel), herbivores [*Pieris brassicae* (L.), Lepidoptera: Pieridae] and parasitoids [*Cotesia glomerata* (L.), Hymenoptera: Braconidae] were reared as described by Steinberg et al. (1992).

Bioassay

Steinberg et al. (1992) showed that the strongest and most consistent response of *C. glomerata* towards volatile infochemicals from the plant–herbivore complex was exhibited by 4- to 5-day-old female parasitoids that are experienced on leaves with host-feeding damage. The same conditions and the same flight chamber set-up were adopted here to examine the response of *C. glomerata* females to cabbage leaves in a series of dual-choice tests.

The day before the experiment, females were allowed to walk and antennate for at least 20 sec on a cabbage leaf where first- to second-instar *P. brassicae* caterpillars had been eating for 24 hr, in order to acquire host-damage experience. Larvae had been removed just prior to the introduction of wasps. Experienced wasps were then transferred individually into cotton wool-stoppered glass vials (6 ml) provided with a droplet of honey. The vials were held overnight in an incubator at 15 ± 1°C and transferred from the incubator to the flight chamber 30 min prior to the experiment.

The flight chamber set-up consisted of a "tent" made of white cotton sheets inside a greenhouse compartment, at 22 ± 2°C and 60 ± 10% relative humidity. Cabbage leaves, with the petiole in a glass vial with water, were placed on a table over which two electric fans generated an airstream of 30–40 cm/sec. The leaves formed an equilateral triangle with 40-cm sides with the release point, from where the wasps were individually released. In order to minimize visual stimuli, a white screened cloth (20 mesh) was placed between the release point and the test leaves (for details see Steinberg et al., 1992).

A choice was scored when the wasp completed a flight, landing on the screen area corresponding to one of the odor sources. A "no response" was scored when the wasp landed anywhere else in the bioassay chamber. Each wasp was allowed only one flight attempt. Wasps that did not fly within 20 min from being released in the bioassay arena were discarded.

Every bioassay was conducted at least on two days, in order to overrule a day-to-day variation in the response of *C. glomerata*, which was previously correlated to barometric pressure changes within the time period of the experiments (Steinberg et al., 1992). When the overall response level in a bioassay was lower than 40%, the same wasps were immediately retested in a standard experiment (three leaves infested with about 100 first- or second-instar *P. brassicae* (plant–herbivore complex, PHC) vs. three undamaged leaves (UND), in order to make sure that the low response level was not due to a low motivation of the wasps but due to the odor sources used.

Statistics

Bioassay data were subjected to a chi-square test for goodness-of-fit (Sokal and Rohlf, 1981) to determine if the response differed from a 50:50 distribution of wasps over the two odor sources.

Herbivory Simulation by Artificial Damage

Mechanical damage was inflicted on cabbage leaves with different patterns and timing. Prior to the experiments the leaves were excised and put in a glass vial with water, in order to be used for the bioassay.

"Old" Artificial Damage (ADold). About one third of the surface of one cabbage leaf was rubbed with 180-grit carborundum powder (BDH Chemicals) on a wet cotton wool pad. This kind of damage was inflicted one day before the bioassay. Old host damage (HDold) was represented by a leaf, of a different plant, where about 100 first- or second-instar larvae had been feeding for about 20 hr. The larvae were removed with a fine brush the day before the experiment.

The attractiveness of the ADold leaf was compared to an undamaged leaf (UND) excised from a different undamaged plant just before the bioassay and, in a separate bioassay, to one herbivore-damaged leaf (HDold).

"Continuous" Artificial Damage (AD7h). In order to compare the effect of prolonged feeding activity of the larvae with the administration of mechanical damage lasting for the same period of time, one cabbage leaf was punched with a cork borer (0.2 cm diam) every 30 min during 7 hr (AD7h) the day before the experiment. In a leaf of a different cabbage plant, one hole was punched and 100 first-instar *P. brassicae* larvae were added and allowed to feed (HD7h). In this way damage of the same size was inflicted starting from the same moment on the two leaf treatments (AD7h and HD7h). The larvae were removed after 7 hr. The attractiveness of the herbivore-damaged leaf (HD7h) was compared to the attractiveness of the artificially damaged leaf (AD7h) in the flight chamber bioassay.

Herbivory Simulation Using Caterpillar Regurgitant

Collection of Regurgitant. One day before the bioassay, regurgitant was collected from third- to fifth-instar *P. brassicae* larvae. Regurgitation was induced by gently squeezing the caterpillars and rapidly collecting with a 5-μl glass capillary tube (Einmal-Mikropipetten, Blau Brand, Germany) the regurgitant droplet produced by the larvae. Per caterpillar about 1–5 μl regurgitant was collected, depending on their size. After collection, the regurgitant was immediately applied to three cabbage leaves either to artificial damage or via incubation in an aqueous solution in the amounts explained below. Leaves treated with larval regurgitant were named "REG" leaves. Control leaves had always the same type of AD or of incubation as the test leaves, but no regurgitant smeared onto the wound surface or added to the solution.

Application onto Artificial Damage. The day before the bioassay, 100 μl of larval regurgitant was collected as described and applied on three leaves with old artificial damage. Two combinations were independently tested: REG on ADold vs. ADold, and REG on ADold + fresh vs. ADold + fresh. In this last

treatment the regurgitant was applied on the wound surface of old damage and the leaf was punched with an 0.8-cm-diam. cork borer every 15 min, starting 1 hr before and continuing for the duration of the bioassay.

Incubation in Regurgitant Solution. In order to test if the regurgitant, or some elicitor contained in it, was systemically transported throughout the leaf, three intact leaves were excised and incubated overnight with their petiole in 1 ml of a 100 μl/ml aqueous solution of larval regurgitant. As a control, three intact leaves were incubated in distilled water. The two groups were used in the bioassay on the following day. Two combinations were tested: incubated leaves with fresh artificial damage (REG on ADfresh vs. ADfresh), and undamaged leaves incubated in regurgitant solution or water (REG on UND vs. UND). To obtain fresh artificial damage (ADfresh), undamaged leaves were excised just prior to the bioassay and, in order to mimic the feeding bouts of the caterpillars (Steinberg et al., 1993), one 0.8-cm-diam. hole was punched with a cork borer every 15 min, starting 1 hr before the bioassay and continuing for its duration (2–3 hr).

Collection and Analysis of Headspace Volatiles from Cabbage Plants. Eight-week-old plants with 10–12 leaves of approximately the same leaf area were used. All samples were collected in the period April–July 1993. Ten cabbage leaves were cut from one plant and placed immediately in a 5-liter glass flask, with a 10-cm-ID opening. Care was taken not to cause any additional damage to the leaves, except for cutting the petiole. The leaves were placed with their cut petiole in a 200-ml glass beaker filled with water that was located inside the flask. The beaker was covered with a perforated glass lid, in order to reduce the evaporating surface. The petioles were inserted through the holes of the lid and put in contact with water. Four plant treatments were used: undamaged leaves (UND), leaves with old artificial damage (ADold), leaves on which about 100 first-instar caterpillars/leaf had been feeding for at least 24 hr (PHC), and leaves treated with larval regurgitant (REG, 10 μl regurgitant/leaf applied on old AD, 20 hr prior to headspace collection).

An airstream was generated in the flask at 500 ml/min by air pressure. The air was cleaned at the inlet of the flask through silica gel, molecular sieves, and activated charcoal as described by Takabayashi et al. (1991b). Filters and flask were connected through 0.8-cm-diam Teflon tubing. After the introduction of the leaves, the system was purged for 3 hr, in order to remove air contaminants from the flask, and then the flow was interrupted for 1 hr to increase the quantity of volatiles produced by accumulation. A Pyrex glass tube (160 × 6 mm OD) containing 90 mg Tenax-TA was connected to the outlet of the flask through a Teflon-coated plastic fitting and the airflow was restarted to last for 1 hr. After the collection period, the adsorption tube was disconnected from the flask and closed with 1/4-in. brass Swagelok caps, using Teflon ferrules. The analysis of

the collected headspace volatiles was performed by desorption in a Thermodesorption Cold Trap Unit (Chrompack) connected to a gas chromatograph–mass spectrometer system as described in Takabayashi et al. (1991b).

RESULTS

Herbivory Simulation by Artificial Damage. Steinberg et al. (1993) demonstrated that current AD inflicted to a cabbage plant attracted *C. glomerata* females. Here we show that even a day after the AD was done, an excised cabbage leaf attracted significantly more *C. glomerata* than a control undamaged

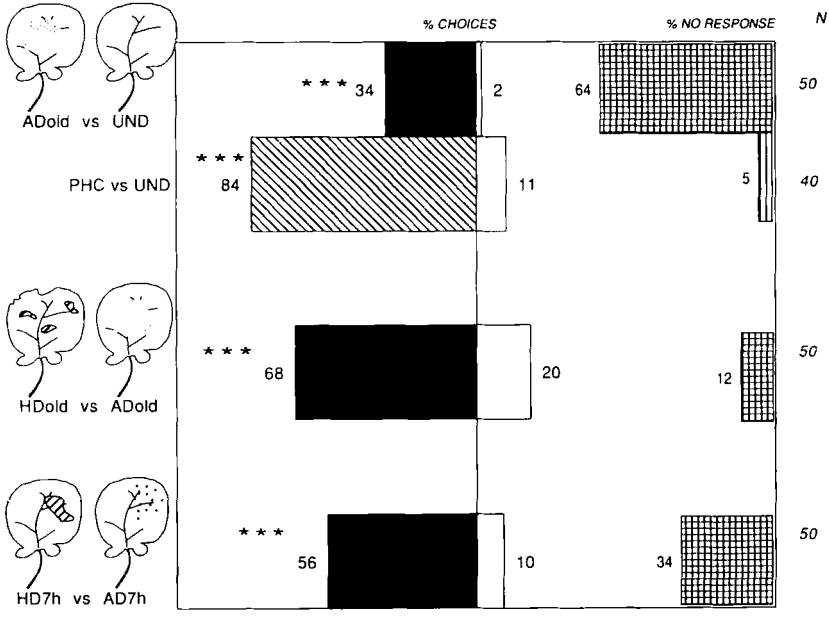

FIG. 1. Response of female *C. glomerata* to herbivory simulated by artificial damage. Drawings on the left side of the figure illustrate the types of larval or artificial damage or no damage; per pair, the left drawing refers to the left part on the choice bar, and the right drawing to the right part of the choice bar. Standard test is reported without drawings. Number of replicates (N) for every comparison are given on the right side of the figure. Numbers next to bar indicate percentage of parasitoids making a choice for one of the two odor sources, or not making a choice at all. The three percentages add up to 100%. Asterisks indicate significant differences within the choice test: *$0.01 < P \leq 0.01$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$, Chi-square test for goodness-of-fit (Sokal and Rohlf, 1981). For abbreviations of treatments see Methods and Materials.

leaf (ADold vs. UND, Figure 1). However, the overall response level was low (36% of all bioassayed females made a choice), indicating either a weak stimulation or absence of some important volatile in the blend. The same wasps, retested in the standard bioassay preferred PHC over undamaged leaves, with 95% of wasps making a choice (Figure 1), showing that atmospheric pressure fluctuation (cf. Steinberg et al., 1992) did not account for the low response level.

Host damage was significantly more attractive than artificial damage, since damage caused by *P. brassicae* larvae that had been feeding for 24 hr and then removed 20 hr before the test was more attractive than artificial damage that was inflicted 20 hr before the test (HDold vs. ADold, Figure 1). Damage from caterpillars feeding for 7 hr on the day prior to the bioassay was more attractive than AD done at regular intervals on a control leaf during the same 7 hr (HD7h vs. AD7h, Figure 1).

Herbivory Simulation Using Caterpillar Regurgitant. Female *C. glomerata* were given a choice between combinations of cabbage leaves that had been treated with caterpillar regurgitant and different types of artificial damage (Figure

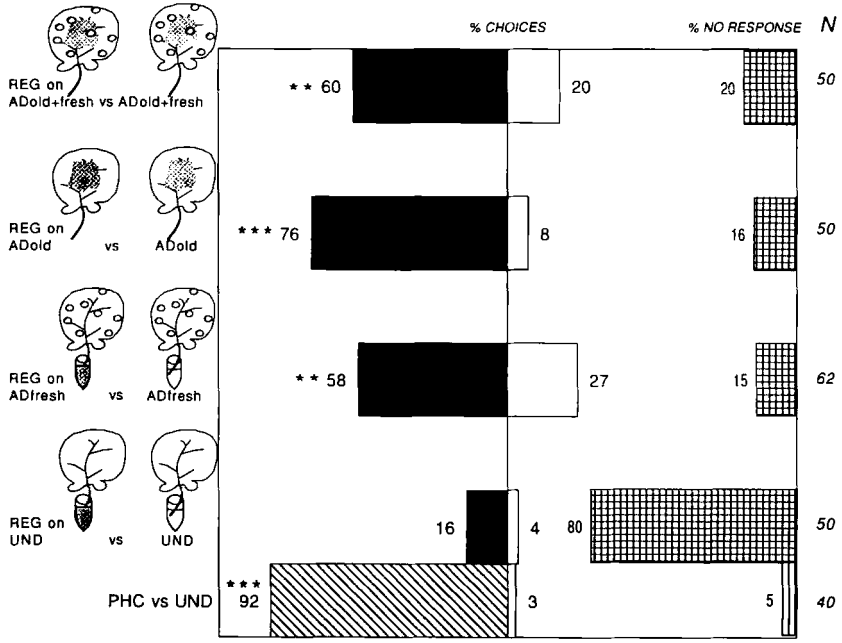

FIG. 2. Response of female *C. glomerata* to herbivory simulated by application of caterpillar regurgitant to artificial damage. For other explanations, see Figure 1 legend.

2). Wasps showed a clear preference for leaves treated with regurgitant over mechanically damaged leaves for all combinations. Only when undamaged leaves were incubated in a regurgitant solution, did the parasitoids not distinguish between the two odor sources (REG on UND vs. UND). Only 20% of the females made a choice in the bioassay, while, when they were retested in the standard bioassay, 95% of the wasps made a choice, almost exclusively in favor of the PHC.

Analysis of Headspace Volatiles of Cabbage Leaves. The composition of the volatile blends emitted by Brussels sprouts plants with different treatments (UND, ADold, PHC, REG on ADold) and the average total area of the identified peaks are given in Table 1. A very high number of chemicals was detected (73 among all treatments). The chemicals were identified as short-chain aldehydes, ketones, alcohols and esters, terpenes, fatty acids, and traces of sulfides and isothiocyanates. The variation between replicates of each treatment was considerable, with several compounds detected in only one replicate/treatment or consistently but in very small amounts.

The PHC produced the highest number of compounds (53) and amounts, since the average total peak area was more than 14-fold relative to the average total peak area of compounds produced by undamaged leaves (UND). Regurgitant-treated leaves produced a somewhat lower number of compounds (40) and in lower amounts (4.5-fold UND). Artificially damaged leaves produced fewer compounds (34) and in a lower amount (2.5-fold UND). Chromatographic profiles of headspace of plants with the four treatments are given in Figure 3.

Differences were consistently observed in the total amounts but not in the ratios of the compounds. An exception was (*Z*)-3-hexen-1-ol, which comprised 31% in PHC and 4–10% in the other three treatments. In order to evaluate the quantitative variation of single compounds across treatments (ADold, PHC, REG on ADold), we assumed that the chemicals that would show an evident increase from the amounts emitted by undamaged leaves (UND) would cause attraction of the parasitoid, although a synergistic effect with constitutive cabbage compounds could not be excluded. Therefore, the amount of a certain chemical relative to that emitted by UND leaves was calculated by dividing its average peak area in a treatment (ADold, PHC, REG on ADold) by the average peak area of the same compound in the UND treatment (lowest degree of attraction for the wasps). When a chemical was not present in undamaged leaves, a peak area value of 1 was given, in order to avoid a division by 0. This transformation was performed only for 24 compounds that were common to the treatments that proved to be most attractive during the bioassays (PHC and REG on ADold), assuming that a complete synomone blend would certainly be emitted by the leaves of these treatments. The 24 compounds comprised 85–90% of the average total quantity of identified chemicals.

The amounts relative to the amount emitted by UND leaves for compounds

TABLE 1 PERCENTAGES[a] OF TOTAL AREA OF GC PEAKS FOR COMPOUNDS DETECTED IN HEADSPACE OF CABBAGE LEAVES WITH DIFFERENT TREATMENTS[b]

	treatments			
Compounds	UND	ADold	PHC	REG on ADold
Aldehydes				
2-Butenal			0.1	
(*E*)-2-Pentenal			0.2 (0–800)	
(*Z*)-2-Pentenal			0.1 (0–250)	
Hexanal	0.3	0.6 (0–236)	0.9 (1262–1395)	0.7 (0–544)
(*E*)-2-Hexenal		0.4	5.5 (2958–18006)	1.7 (0–1324)
(*Z*)-2-Hexenal			2.1 (0–8240)	
2,4-Hexadienal			0.1	
Octanal		0.5 (0–300)	tr	0.2
Nonanal	1.1	1.5 (0–871)	tr	0.9
Decanal	2.5	4.3 (0–2135)	tr	1
Dodecanal	4.3			
Tetradecanal	2.1			
Ketones				
1-Cyclopropyl-2-propen-1-one			0.6	
3-Pentanone	5.5 (0–1370)	4.3 (0–2117)	2.6 (3556–4959)	5.9 (2195–4391)
3-Methyl-2-pentanone			tr	
1-Penten-3-one			1.1 (949–2867)	
3-Penten-2-one			0.1(0.12)	
3-Octanone				0.5 (66–555)
2,3-Pentanedione			tr	
Alcohols				
Ethanol			0.1	
2-Methyl-1-propanol	0.6	0.1	0.4	
2-Propanol				tr
1-Butanol		0.2		
3-Methyl-1-butanol			0.1	
Pentanol			0.1	
1-Pentanol			tr	

3-Pentanol	0.9	0.8 (0–636)	0.3	1.7 (0–1919)
2-Penten-1-ol				0.2
1-Penten-3-ol	0.4	2.5 (0–1612)	1.6 (2548–2696)	0.9 (0–1000)
1-Hexanol		1.1 (158–444)	1.3 (1200–2668)	0.9 (0–1038)
(*E*)-2-Hexen-1-ol			2.1 (1189–6092)	0.3
(*Z*)-2-Hexen-1-ol			tr	
(*Z*)-3-Hexen-1-ol	5.6 (213–612)	9.6 (1306–4376)	31.2 (33297–53600)	4.3 (674–4336)
1-Octen-3-ol				0.3 (0–262)
1-Nonanol				0.1
Esters				
Isobutyl acetate				0.1
1-Butyl acetate		0.5		0.9 (0–1158)
3-Methyl-3-buten-1-yl acetate		0.1	0.6 (0–2596)	
Isopentyl acetate		0.9	0.1	0.5
1-Pentyl acetate		0.2		3.7 (0–3289)
1-Hexyl acetate	2.2 (0–537)	6.5 (0–4388)	2.2 (1260–7010)	7.7 (445–5837)
(*E*)-2-Hexen-1-yl acetate		0.6 (0–382)	1.4 (669–5272)	1.4 (0–1386)
(*Z*)-3-Hexen-1-yl acetate	39.5 (1474–9266)	46.2 (201–23468)	32.8 (28972–79270)	39.6 (3196–32951)
3-Hexen-1-yl propanoate		0.5 (0–307)	0.2	1.2 (0–1097)
3-Hexen-1-yl		0.5 (0–436)		0.9 (0–1011)
3-Methylbutanoate				
(*Z*)-3-Hexen-1-yl butyrate		0.2	2.3 (1145–6484)	0.4
(*Z*)-3-Hexen-1-yl isobutyrate			0.9 (0–2960)	
(*Z*)-3-Hexen-1-yl isovalerate			2.2 (1227–6026)	0.1
3-Hexen-1-yl caproate			0.1	
(*Z*)-3-Hexen-1-yl hexanoate			0.6 (0–1710)	
Heptyl acetate				0.2
Fatty acids				
Isobutyric acid			0.5	
Caproic acid	1		0.1	
Isovaleric acid			0.8	
Terpenoids				
α-Pinene	0.4	0.3 (0–245)		0.7 (243–499)
β-Pinene	0.5	0.3 (0–153)	tr	0.6 (179–325)
α-Thujene	1.6	1.5 (302–580)	0.2 (0–847)	1.3 (544–703)

TABLE 1 CONTINUED

Compounds	treatments			
	UND	ADold	PHC	REG on ADold
Terpenoids				
Sabinene	11 (242–2100)	5.3 (1217–1757)	0.8 (600–1700)	7.2 (2349–4431)
Myrcene	4.4 (0–843)	2.6 (346–1023)	0.1	3.2 (1299–2266)
Limonene	9.1 (199–1564)	6 (1047–2587)	0.7 (711–1748)	6.7 (1720–4732)
1,8-Cineole	2.8 (114–450)	2 (331–899)	0.2 (236–422)	2.3 (645–1584)
β-Elemene	4.2 (0–910)	2.2 (0–917)	0.4 (119–1300)	1.5 (0–1132)
4,8-Dimethyl-1,3(*E*),7-nonatriene				0.2
4,8,12-Trimethyl-1,3(*E*),7(*E*)11-tridecatetraene				0.2
γ-Terpinene				tr
trans-Sabinenehydrate		0.1		0.1
α-Farnesene			0.1	
Furans				
2-Ethyl-furan			0.3	
Sulfides				
Dimethyldisulfide			0.6 (154–1741)	
Dimethyltrisulfide			0.1 (0–1600)	
Isothiocyanates				
Methyl-ITC			1.3 (1213–3451)	
Average total area	11,175	27,887	162,878	50,475
SD	(5,725)	(14,980)	(50,099)	(17,886)

[a] Percentages are calculated by dividing the average peak area of a compound in every treatment by the total average area of compounds detected in that treatment. The range of peak area found in all replicates is indicated, in parentheses, only for compounds that were found in more than one analysis. $N = 3$ for all treatments, except artificially damaged leaves (ADold) where $N = 4$

[b] Abbreviations: UND = undamaged, ADold = artificial damage, PHC = plant-herbivore complex, REG on ADold = ADold + larval regugitant, tr = chemicals found only in one analysis and in amounts smaller than 0.1%.

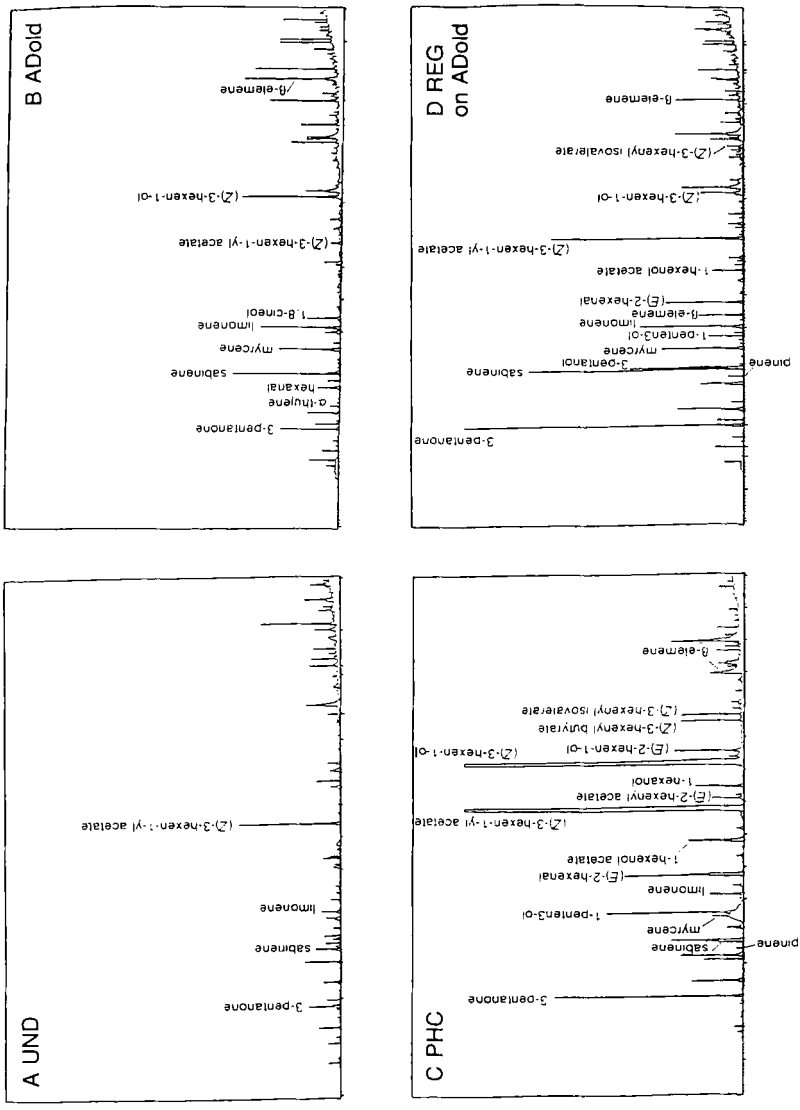

FIG. 3. Chromatographic profiles of headspace collections of Brussels sprouts plants that underwent different treatments. A = undamaged (UND). B = "old" artificial damage (ADold). C = plant–herbivore complex (PHC). D = "old" artificial damage + caterpillar regurgitant (REG on ADold).

common to the behaviorally active treatments are given in Figure 4. Clearly, all treatments (ADold, PHC, REG on ADold) show a similar trend in the increase of emission of chemicals compared to undamaged leaves. For the treatments resulting in highest behavioral attraction, PHC and REG on ADold, eight of the 24 compounds screened [(*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-2-hexen-1-yl acetate, 3-hexen-1-yl propanoate, (*Z*)-3-hexen-1-yl butyrate, (*Z*)-3-hexen-1-yl isovalerate, and octanal] showed an increase more than 20-fold UND. The PHC shows the most amplified emission, with an increase of 200–8000 times of (*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-2-hexenyl acetate, 3-hexen-1-yl propanoate, (*Z*)-3-hexenyl butyrate, and (*Z*)-3-hexenyl isovalerate. The emission of volatiles produced by REG on ADold-leaves is 50–800 times UND, and it is definitely higher than ADold. It is important to note that the ADold and REG on ADold treatments both had the same type of artificial damage, but they differ in the application of regurgitant. Artificially damaged

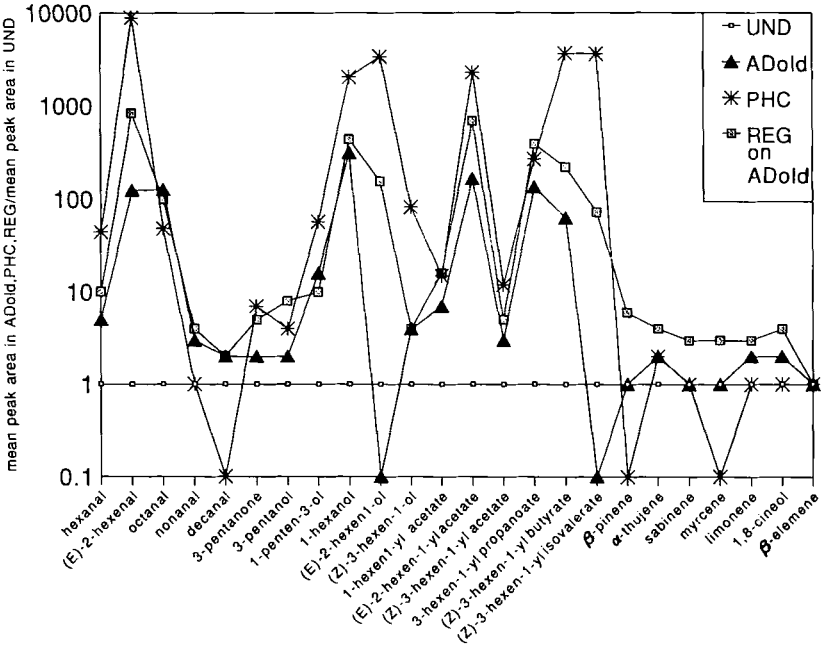

FIG. 4. Relative increase of selected compounds (*x* axis) common to behaviorally active treatments (ADold, PHC, REG on ADold), calculated in proportion of the amounts emitted by undamaged leaves (UND). Values of *y* axis (log scale) indicate the ratio between the average peak area of every compound in the ADold, PHC, and REG on ADold treatments and the average peak area of the same peak in the UND treatment.

leaves still show differences in comparison to undamaged leaves, but the emission scale is less than 10-fold UND for most compounds, exceeding a 100-fold UND increase only for (*E*)-2-hexenal, octanal, 1-hexanol, (*E*)-2-hexenyl acetate, and 3-hexenyl propanoate.

DISCUSSION

It has been shown for several tritrophic systems that an interaction is needed between the herbivore and the plant for volatile synomones to be emitted that attract predators or parasitoids of the herbivores (Sabelis et al., 1984; Dicke et al., 1990a,b; Turlings et al., 1990). Turlings et al. (1990) were the first to demonstrate that herbivore oral secretions applied into a mechanical wound can effectively mimic herbivory in this respect. Steinberg et al. (1993) showed that *C. glomerata* is strongly attracted to cabbage plants on which *P. brassicae* larvae are feeding, while infochemicals emitted by the herbivore alone or its products, such as frass, were of much less importance in parasitoid attraction. The PHC was more attractive to the parasitoids than herbivore-damaged plants from which the herbivores had been removed and mechanically damaged plants (Steinberg et al., 1993). Here we show that cabbage leaves, mechanically damaged, can produce synomones even without being in contact with *P. brassicae* larvae. Our study provides chemical data that demonstrate quantitative differences between the volatiles emitted from the PHC, from mechanically damaged plants treated with caterpillar regurgitant, and from mechanically damaged plants (treatments in decreasing order of quantity of emitted volatiles). No major qualitative differences between the blends from these treatments were recorded. The qualitative differences in minor components may be explained by these components being above the detection threshold at higher emission rates. The main components of the volatile blend released from cabbage are terpenoids and green-leaf volatiles. Terpenoids are a major class among herbivore-induced synomones that attract arthropod carnivores (reviewed by Dicke, 1994). Interestingly, the qualitative and quantitative variations of the terpenoids identified from cabbage plants of different treatments are very low. Due to an increase in the emission of other chemicals from PHC and regurgitant-treated plants, their relative contribution to the headspace of these plants is lower than in undamaged or artificially damaged plants. Their absolute quantities are hardly affected by damage. Terpenoids comprise, on average, 25–30% of the volatiles obtained from undamaged and artificially damaged leaves, while this percentage is only ca. 2.5% for volatiles from PHC. On the other hand, some green-leaf volatiles [(*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-2-hexen-1-yl acetate, 3-hexen-1-yl propanoate, (*Z*)-3-hexen-1-yl butyrate, and (*Z*)-3-hexen-1-yl isovalerate] that have not been recorded in the headspace of undamaged leaves constitute

only 2% of the blend in artificially damaged leaves but more than 13% in PHC. Green-leaf volatiles are saturated and unsaturated six-carbon alcohols, aldehydes, and derived esters formed by oxidative degradation of plant lipids, through the so-called "lipoxygenase pathway" (Hatanaka, 1993). They have been reported as volatile components of numerous plant species belonging to a variety of plant families (Visser et al., 1979). Given the dramatic increase of green-leaf volatiles in the headspace of damaged cabbage compared to undamaged plants, it is likely that these chemicals are involved in the attraction of *C. glomerata* to infested or regurgitant-treated cabbage plants.

For another tritrophic system, it has been demonstrated that herbivore-damaged leaves produce C_6 volatiles that serve as synomones for the braconid parasitoid, *Microplitis croceipes*. In a wind-tunnel bioassay female parasitoids oriented to both cowpea plants damaged by the host herbivore, *Heliotis zea*, and to individual synthetic green-leaf volatiles (Whitman and Eller, 1990). In particular green-leaf esters elicited the greatest percentage of successful orientation flights when offered to the wasps at different concentrations (Whitman and Eller, 1992). This parasitoid is also strongly attracted to plants that release only minor amounts of C_6 volatiles, but large amounts of terpenoids (McCall et al., 1993; Turlings et al., 1993a).

Further studies are needed to test the attractiveness for *C. glomerata* of an artificial mixture of the volatiles identified in cabbage and to verify qualitative and quantitative characteristics of the blend.

Chemical analyses of the volatiles emitted by plants of the same cabbage cultivar used in our study, when infested by either *P. brassicae* or *P. rapae* caterpillars showed that the blends of those two PHCs did not differ qualitatively but that only the amount of emitted volatiles per individual caterpillar differed (Blaakmeer et al., 1994). Thus, it seems that damage inflicted to this cabbage cultivar results in a blend of volatiles that is qualitatively similar but that the emitted quantities may differ with treatment. A comparison of the data of Blaakmeer et al. (1994) on headspace composition of *P. brassicae*-infested and uninfested Brussels sprouts plants with our data shows many similarities. However, apart from differences in minor components, which must be the result of sampling from quite differently sized collection vessels, some larger differences occurred. In *P. brassicae*-infested plants we find a markedly higher percentage of (*Z*)-3-hexen-1-ol and a lower percentage of terpenoids than did Blaakmeer et al. (1994). The different percentages of (*Z*)-3-hexen-1-ol may be related to differences in the amount of mechanical damage done by the different amount of caterpillars (1000 caterpillars/10 leaves vs. 50 caterpillars/10–12 leaves plant) or by the use of excised leaves vs. intact plants. In this context it is also remarkable that other compounds produced through the lipoxygenase pathway [(*E*)-2-hexenal and (*E*)-2-hexen-1-ol] are recorded in our PHC headspace and not by Blaakmeer et al. (1994).

In all our bioassays, undamaged cabbage leaves were the least attractive infochemical source for foraging *C. glomerata*. The experiments with artificial damage showed that timing and pattern of infliction are important for the induction of attractive volatiles. Steinberg et al. (1993) found that the response to artificial damage inflicted immediately prior to the bioassay wanes quickly unless the damage is repeated at regular intervals. In addition, we observed (Figure 1) that artificial damage inflicted the day before the experiment (ADold) is significantly attractive to the parasitoids when compared to undamaged leaves, although the responsiveness is low. However, the wasps always preferred herbivore damage over artificially damaged leaves, regardless of pattern and timing of infliction of both damage types. Furthermore when *P. brassicae* regurgitant was applied on ADold, the responsiveness of the wasps is high: 84% make a choice and the wasps clearly discriminate between REG on ADold and ADold (Figure 2). These observations lead to the conclusion that attractive volatiles are produced upon mechanical damage but that herbivore damage results in a different plant response in terms of quantity of emitted volatiles. In previous studies, the term herbivore-induced synomone (HIS) was used for chemicals whose production was induced by herbivory. In addition to the data of Steinberg et al. (1993), our data show that caterpillar infestation or application of regurgitant of the caterpillar *P. brassicae* on cabbage plants of the cultivar Titurel leads to effects that are different from mechanical damage. However, the induction process does not lead to the emission of different volatiles than those emitted by mechanically damaged plants but rather to higher emission rates that remain detectable during a longer period after treatment.

In other tritrophic systems the application of an exogenous (Turlings et al., 1993a) or endogenous (Dicke et al., 1993) elicitor through the petiole induces emission of HIS from undamaged leaves. Synomones in corn are released by plants incubated in an aqueous solution of regurgitant without any further damage on the leaf surface (Turlings et al., 1993a). Undamaged uninfested Lima bean leaves, incubated in water in which spider mite-infested leaves had been present for the previous seven days, became attractive to predatory mites, demonstrating that an elicitor transported out of infested leaves was taken up by uninfested undamaged leaves that subsequently initiated the release of synomones (Dicke et al., 1993). When we incubated cabbage leaves in the *P. brassicae* regurgitant solution, we could only observe an attraction by the parasitoids if the leaf surface was wounded (Figure 2). If the leaves were incubated in regurgitant but not wounded, the wasps were far less responsive and not able to discriminate between these and the control leaves. In the *P. brassicae*–cabbage interaction, the regurgitant itself or an endogenous elicitor activated by the regurgitant in the place of entrance, is transported throughout the leaf that starts producing attractive volatiles. The final reaction that leads to the production or emission of synomones requires leaf-surface wounding. It can be hypothesized

that synomones are produced inside the leaf, without being able to permeate the cell walls or the wax layer protecting the leaf. Another possibility is that the elicitor contained in the regurgitant is transported through intracellular spaces in the leaf. The cell wall breakdown, caused by mechanical damage, allows the contact between the elicitor and its receptor and, therefore, the activation of the biochemical pathway that leads to synomone emission.

Our chemical data contrast with those from studies on other PHCs (Dicke and Sabelis, 1988; Dicke et al., 1990b; Turlings et al., 1990, 1993a,b; Dicke, 1994). For several plant species, such as Lima bean, cucumber, and corn, these studies have recorded that plants that are infested with herbivores or plants that are treated with herbivore products emit volatile chemicals that are not emitted by mechanically damaged plants or by undamaged plants.

The production of induced synomones by plants in response to herbivory was first documented in 1988 (Dicke and Sabelis, 1988). Since then, it has been studied for many plant species (see Dicke, 1994, for review). By incorporating more plant species, we are discovering that the response of different plant species varies. It will be a challenge to unravel the causes for this variation.

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