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The role of the plant in attracting parasitoids: response to progressive mechanical wounding

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Abstract

Based on the model system of Brussels sprouts [*Brassica oleracea* var. *gemmifera* (Brassicaceae)], the herbivore cabbage white caterpillar, *Pieris brassicae* (L.) (Lepidoptera: Pieridae), and the parasitoid wasp, *Cotesia glomerata* (L.) (Hymenoptera: Braconidae), the influence of plant damage type, and damage duration were assessed on plant volatile emission and subsequent recruitment of natural antagonists of the herbivore. Plants were damaged by three methods for a period of either 3 or 8 h: herbivore damage (HD), progressive mechanical damage, and final mechanical damage inflicted in a single event. Wind-tunnel bioassays evaluated whether the mode of damage affected female parasitoid oriented flight. After both periods of damage, all treatments were highly significantly preferred by naïve *C. glomerata* to undamaged control plants. After 3 h, herbivore-damaged plants were significantly preferred to plants with final damage (FD). Most remarkably, following 8-h damage, the parasitoid preferred both herbivore-damaged and progressively damaged plants to plants with FD and did not significantly discriminate between herbivore and progressively damaged plants, thus indicating a similarity in plant response to herbivore and progressive mechanical damage. In addition to wind-tunnel bioassays, emitted plant volatiles were collected and analysed by thermal desorption gas chromatography/mass spectrometry, following 3 and 8 h of damage in order to correlate volatiles released from different damage types with the attraction of the parasitoid. Differences in volatile profiles from all damage types were similar following both 3 and 8 h of damage, with only (*Z*)-3-hexenyl acetate found to be emitted in significantly higher quantities by final mechanical damage compared with HD after 3 h. In conclusion, the plant's response to progressive mechanical damage was more similar to HD than final mechanical damage deployed at a single point in time, irrespective of damage duration, and *C. glomerata* did not significantly discriminate between progressive damage and HD.

Introduction

Plants have evolved a range of defence mechanisms to protect themselves against a wide variety of pathogens and herbivores that pose a potential threat. While there have been intensive studies on how plants recognize infestation of a pathogen, with identification of many pathogen-derived exogenous elicitors of phytoalexins (Boller, 1995; Nürnberger, 1999; Nürnberger et al., 2004), little knowledge on the recognition of herbivore insects by plants is available. The chewing of insect larvae, such as caterpillars, results in wounding of plant tissue, or more specifically in crushing, rupturing, and damaging of plant cells, which in itself is sufficient to trigger a defence response against herbivory. Generally, plant responses can either directly affect the herbivore [e.g., enhanced production of toxins (Roda & Baldwin, 2003) or changes in plant volatile emission (Hern & Dorn, 2002)] or indirectly promote the effectiveness of natural enemies of herbivores, such as parasitoids (Vet & Dicke, 1992; Geervliet et al., 1997; Dicke et al., 1999; Pare & Tumlinson, 1999; Mattiacci et al., 2001a; Dorn et al., 2002). One strategy employed by plants in response to herbivory is the release of indirect defence compounds, which can comprise volatiles from the lipoxygenase pathway (green leaf volatiles), isoprenoid pathway (terpenoids), shikimic acid pathway (aromatics), and the myrosinase-glucosinolate system (glucosinolates) (Engelberth et al., 2004). Indirect defence compounds

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elicited upon herbivory differ from the blend of volatiles released by plants in response to artificial damage, which is typically represented by a single damage incident.

The induction of plant defence is believed to be triggered by elicitors present in herbivore oral secretions (Turlings et al., 1990; Mattiacci et al., 1994; Alborn et al., 1997, 2000; Bernasconi et al., 1998). So far, two types of elicitor have been isolated from lepidopteran herbivores, which result in an induced volatile release (Pare & Tumlinson, 1998; Halitschke et al., 2001; Kessler & Baldwin, 2002). These elicitors are β-glucosidase, isolated from *Pieris brassicae* larvae (Mattiacci et al., 1995) and *N*-(17-hydroxylinolenoyl)-L-glutanamine, or volicitin, from *Spodoptera exigua* larvae (Alborn et al., 1997; Roda et al., 2004).

With research focussing increasingly on the interactions between plants and their herbivores (Landolt et al., 1999; Hern & Dorn, 2002), more attention needs to be given to the modes of artificial damage employed as control methods in the elucidation of the complex dynamics of plant response. Herbivory, especially by chewing insects, causes substantial injury of a mechanical nature to the plant, which may elicit a response in the plant, independent of chemical stimuli from the insect (Mithöfer et al., 2005). Numerous methods have been employed to inflict artificial mechanical damage, including the removal of plant tissue using a hole punch (Mattiacci et al., 2001a,b), leaves scratched with a razor blade (Schmelz et al., 2001), puncture wounds created with a fabric pattern wheel (Halitschke et al., 2001), or leaves rubbed with abrasive carborundum powder (Mattiacci & Dicke, 1995b). However, these mechanical damage treatments are generally deployed in a way that does not directly mimic true herbivory in space and time. This is due to two main factors: (i) treatments rarely damage a similar area of plant tissue to herbivore feeding, and (ii) mechanical damage is rarely deployed over the same time period as herbivore damage (HD), and therefore the plant may respond to progressively inflicted damage differently than to a single damage event (Mithöfer et al., 2005). A number of studies have found a plant response when herbivore oral secretions/regurgitant are added to mechanically damaged leaves (Mattiacci et al., 1994; Alborn et al., 1997; Pare & Tumlinson, 1997; Turlings et al., 1998; Schittko & Baldwin, 2003). However, we cannot exclude that this response may be a consequence, at least in part, of plant–plant interaction, as oral secretions ‘milked’ from lepidopteran larvae may include previously ingested plant components from the midgut. It is therefore necessary to examine the plant’s response to mechanical damage in the absence of exogenous plant or insect chemical stimuli.

Great progress was made by Mithöfer et al. (2005) in exploring differences in volatile emission from lima bean plants damaged mechanically compared with herbivore-damaged plants, which revealed a systemic response in the absence of caterpillar elicitors. Therefore, the question remains whether parasitic wasps, which are known to use plant-derived semiochemicals in host location (Turlings et al., 1990), will discriminate between HD and progressive mechanical damage treatments during host location.

Using the tritrophic system of Brussels sprouts [*Brassica oleracea* var. *gemmifera* (Brassicaceae)], cabbage white caterpillar, *P. brassicae* (L.) (Lepidoptera: Pieridae), and the gregarious endoparasitoid, *Cotesia glomerata* (L.) (Hymenoptera: Braconidae), we aimed to elucidate the plant’s response to progressive mechanical damage compared with HD and mechanical damage deployed at a single point in time, and the response of the parasitoid to any changes in plant volatile release. This system is well established in the study of parasitoid response to plants (Gu & Dorn, 2000; Wanner et al., 2006) and in particular, chemically mediated interactions (Mattiacci et al., 2001a,b; Wang et al., 2003). We hypothesized that *C. glomerata* would be attracted to herbivore-damaged plants over mechanically damaged and control plants regardless of the duration and mode of damage.

Materials and methods

Rearing procedures

Brussels sprouts (*B. oleracea* var. *gemmifera*) were grown in seedling trays in a glasshouse at 23/16 °C (day/night) with L12:D12 and an r.h. of approximately 60%. Single plants were transplanted to individual 400-ml pots 17 days after being sown in a commercially available mixture of peat and clay (H. Gilgen Optima-Werke, Arlesheim, Switzerland). The substrate mixture contained 400 mg per l total nitrogen, 200 mg per l phosphorous, 370 mg per l potassium, 2200 mg per l lime, and 0.32 mg per l magnesium. Seedlings were moved to a walk-in climate room 38 days after being sown with a 24/16 °C (day/night) on an L16:D8 regime, with an r.h. of 60 ± 10%. Plants used in experiments were 6–7 weeks old, with 5–8 fully developed leaves and were similar in size, appearance, and leaf number. In the stock rearing, *P. brassicae* larvae were maintained on Brussels sprouts plants until the third instar and on Savoy cabbage leaves (*B. oleracea* var. Sabaudo) thereafter at 21 ± 1 °C and 60 ± 10% r.h. *Cotesia glomerata* wasps were kept in a climate chamber at 15 °C, 90% r.h., and L16:D8 in insect cages (30 × 30 × 30 cm) allowing for natural post-emergence dispersal (Gu & Dorn, 2003; Scascighini et al., 2005). For parasitism, first or second instars of *P. brassicae* were exposed to 4- to 10-day-old parasitoids. Care was taken to avoid superparasitism (Gu et al., 2003). Parasitized *P. brassicae* larvae were subsequently

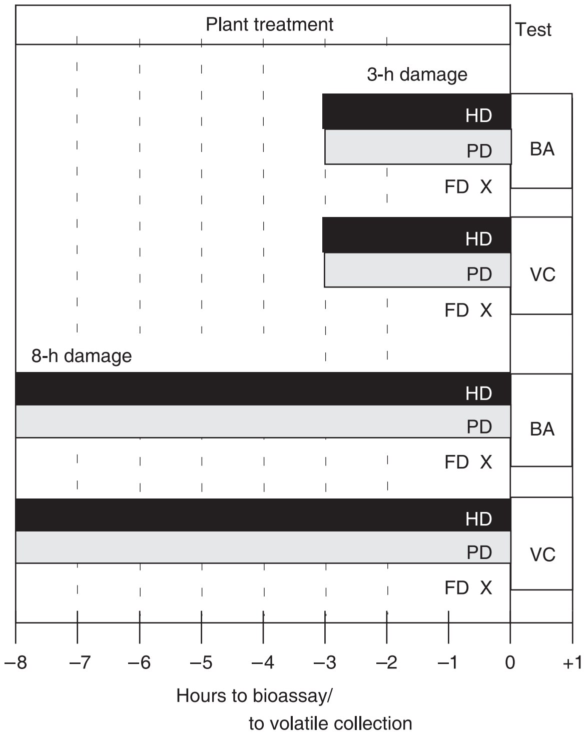

Figure 1 Temporal design of plant treatment prior to bioassay (BA) with *Cotesia glomerata* or to volatile collection (VC) both lasting for 1 h. Herbivore damage (HD, in black) with 50 *Pieris brassicae* larvae feeding on a single Brussels sprouts leaf; progressive damage (PD, in grey) inflicted by a herbivore simulator that mimics the continuous mechanical damage in space and time caused by the herbivore; final damage (FD, marked with X) inflicted by a hole punch to represent the final area of damage caused by the herbivore within the feeding period. Herbivore feeding lasted either 3 or 8 h, with according imitations by PD and FD. All treatments were carried out during photoperiod.

fed on Brussels sprouts plants (L1-3) and on Savoy cabbage leaves (L4-pupa). Upon egression, parasitoid cocoons were transferred to nylon gauze cages, where newly emerged wasps could perform normal flight activities and mate randomly. Wasps were provided with honey and water. Four 5-day-old female wasps were used in bioassays (Steinberg et al., 1992). Females were never exposed to *P. brassicae* or Brussels sprout plants prior to experiments and were therefore considered naïve (Mattiacci et al., 2001a).

Behavioural bioassay

Behavioural experiments with *C. glomerata* were conducted in a Plexiglas wind tunnel measuring 150 × 35 × 35 cm. Conditions inside the wind tunnel were 22 ± 2 °C, 50 ± 2% r.h., 26–30 cm s^{-1} wind speed, and 750–1000 lux light intensity (Scascighini et al., 2005). To minimize visual stimuli, white cotton gauze was hung in front of the test plants (Steinberg et al., 1992). Naïve female wasps were selected at random from rearing cages and allowed 2 h to acclimatize in the bioassay room. Wasps were released individually 30 cm downwind from the test plants and were allowed a second flight attempt if the first was unsuccessful (Scascighini et al., 2005). Wasps were only used in one bioassay. A flight ended when the wasp landed on the gauze in front of the plants, or on the side, top, or bottom of the wind tunnel. Wasps were allowed up to 5 min to begin flying, and were allowed unlimited time between take-off and landing. Test plants were placed 20 cm apart and their positions alternated half way through each 1 h bioassay. For each choice bioassay, at least 80 wasps were tested. One plant was used per treatment per bioassay in the hour immediately following the damage period. To avoid any day-to-day bias in results due to changing atmospheric and weather conditions, these tests were carried out over a minimum of 3 days per choice bioassay. To gain directly comparable results between the chemical study and behaviour bioassays, volatile collections and bioassays were both conducted during the first full hour following damage (Figure 1).

Plant treatments

All plant treatments were carried out during the photoperiod.

Progressive damage. The uppermost fully developed leaf was damaged using a hand-held miniature drill (Hobby drill 2000, Conrad Electronic GMBH, Hirschau, Germany) with a 3-mm diameter steel shaft. A conical steel cutter (2.3 mm in diameter) attached to the steel shaft served as a drill head. The drill was operated at a maximum current of 10 V and 10 000 rotations per min. This ‘herbivore simulator’ was used to cause damage to leaf material at a similar rate to HD. Progressively damaged plants were covered with a plastic sheet to stop leaf residue landing on undamaged leaves, as it is possible that leaf residue could induce a reaction in surrounding leaves (Engelberth et al., 2004). Damage was inflicted every 10 min for either 3 or 8 h and the area of damage was not significantly different from the area of damage caused by 50 second-instar *P. brassicae* (Mann–Whitney U-test at 3 h: U = 29, P = 0.753, n = 10; 8 h: U = 29, P = 0.326, n = 10).

Final damage. Preliminary feeding trials showed that 50 second-instar *P. brassicae* consumed an average of 1 cm^2 and 3 cm^2 leaf material after 3 and 8 h of feeding, respectively. Therefore, to simulate the final area of damage after 3 h of herbivory, a 1-cm^2 hole was punched into the uppermost fully developed leaf. To simulate 8-h herbivory, 3 × 1 cm^2 holes were punched into the uppermost fully developed leaf. Holes (1 cm in diameter) were made in a leaf using a hole punch, with care taken to avoid main veins and the midrib. Final damage (FD) was inflicted immediately prior to volatile collection or wind-tunnel bioassays, as preliminary tests showed that wounds inflicted using a hole punch dried during volatile collection within 3–8 h. This mode of damage was therefore deployed immediately prior to volatile collection so that comparison with other modes of damage would be possible. Furthermore, many previous studies have used such FD immediately before the experiment to reflect the mechanical damage by the herbivore (Steinberg et al., 1993; Vernède & Pak, 1994; Mattiacci & Dicke, 1995a; Wäckers & Wunderlin, 1999; van Poeke et al., 2001).

Herbivore damage. Fifty second-instar *P. brassicae* were placed on a leaf. Larvae were allowed to feed undisturbed for 3 or 8 h prior to volatile collection, or to a wind-tunnel bioassay. Herbivores were prevented from feeding on other parts of the plant by sticky strips wrapped around the petiole (Mattiacci et al., 2001a). All larvae and frass traces were removed before volatile collection or wind-tunnel bioassays were conducted. Plants damaged by one of the above methods were then used for 1 h in either wind-tunnel bioassays with *C. glomerat*a or for volatile collection (Figure 1).

Volatile collection and analysis

Collection chambers. Volatiles were collected using a Pyrex glass chamber, described by Agelopoulos et al. (1999), which consisted of two parts: one male and one female. The male part (7 cm long) has a ground glass connection (5 cm long) and an air inlet in its centre. The female part (10 cm long) has a slot (6 cm long, 0.5 cm wide) in its underside, an air inlet, and a ground glass connection. For all treatments, the leaf petiole was wrapped with cotton wool and Teflon band, passed through the slot and the two sections of the collection chamber connected around it. Any gap between the glass and petiole was closed with Teflon banding to stop any air passage in or out of the collection chamber. Each chamber was used to isolate one leaf from a healthy plant and allowed analysis of volatiles released in the hour following 3 or 8 h of a certain damage type. Plants were carefully selected and checked for damage prior to an experiment to avoid any induced response to damage. After each assay, the Pyrex glassware was washed with distilled water, and then rinsed with hexane and finally acetone. The glassware was then dried overnight in an oven at 250 °C.

Volatile collection. Headspace volatiles were collected from single Brussels sprout leaves isolated within a glass collection chamber, but still attached to the plant. Volatiles were collected in a steel trap containing 300 mg Tenax GR (mesh size 80/100; Alltech Associates, Deerfield, IL, USA) and sealed with Pyrex glass wool at both ends. Tubes were thermally conditioned prior to use for 15 h at 250 °C with a flow of 50–60 ml per min dried filtered helium (with an initial purity of 99.96%). Charcoal-filtered air entered the collection chamber through the inlet and was drawn out through the Tenax GR trap at a rate of 340–360 ml per min for the full duration of the collection. All collections were done in a climate room at 20 °C, 60% r.h., and an L16:D8 regime. A total of 10 plants were used for volatile collections, which were conducted between 14:00 and 16:00 hours following 3 h of damage and between 16:00 and 18:00 hours following 8 h of damage.

Volatile analysis. Headspace volatiles collected were analysed using thermal desorption followed by gas chromatography/mass spectrometery (GC/MS). Using the Unity system (Markes International Ltd^{TM}, Pontyclun, UK) (desorption flow: 30 ml per min; split flow: 10 ml per min), volatiles were eluted from the Tenax GR trap with helium (99.96%) for 5 min at 45 °C, increasing to 250 °C at a rate of 8 °C per min. Volatiles were then transferred to a cold trap (–10 °C) that was filled with Carbopack B (Supelco, Buchs, Switzerland) and Tenax TA 1:2. The cold trap was subsequently heated at a rate of 40 °C per min to a maximum of 300 °C for 3 min. Volatiles were transferred via a fused silica transfer line (heated to 280 °C) to the gas chromatograph/mass spectrometer (Hewlett Packard GC 6890 Mass selective detector 5973, Atlanta, GA, USA) and analysed following the method described by Scascighini et al. (2005). The GC column used was a phenyl methyl siloxane column (EC-5, Alltech; 30 M; inner diameter of 0.25 mm and film thickness of 0.25 μm). A post run was conducted for 5 min at 300 °C, with helium being used to flush out the system to remove any residual products from the previous sample. The desorption flow and column flow were kept at 30 ml per min and 1.5 ml per min for all analyses.

Volatile identification

All compounds were identified by comparison of their mass spectra with those in the NIST98 library and from our own library of phytochemical compounds. In addition, retention times were compared with standard commercial compounds (Fluka, Buchs, Switzerland). While a total of 60 compounds were detected in the headspace samples, detailed analysis focussed on those previously reported in literature to be of definite plant origin, in analogy to Vallat et al. (2005). Table 1 indicates the origin of compounds according to literature reports and gives the retention times measured.

Table 1 Mean peak areas of compounds detected in samples from plants with herbivore damage (HD), progressive damage (PD), and final damage (FD) Brussels sprout leaves following 3 and 8 h of damage

Compound	PD				HD				FD			
	3 h	8 h	Increase/ decrease[a]	n[b]	3 h	8 h	Increase/ decrease	n	3 h	8 h	Increase/ decrease	n
Alcohol/phenol												
(*Z*)-3-hexen-1-ol*[1,2,3,4,5]	145 ± 24	190 ± 95	↑	2/3	93 ± 26	178 ± 31	↑	2/4	157 ± 12	1152 ± 499	↑	3/5
Aldehyde												
(*E*,*E*)-2,4-hexadienal*[4]	n.d.	n.d	–	–	3007 ± 1609	1378	↓	2/1	n.d.	n.d	–	–
Esters												
(*Z*)-3-hexenyl acetate*[1,2,3,4,5]	1939 ± 602	5056 ± 1433	↑	7/9	830 ± 204	1995 ± 550	↑	9/8	2057 ± 408	9246 ± 2898	↑	10/10
(*Z*)-3-hexenyl butanoate[2,3,5]	147	225 ± 68	↑	1/7	n.d.	101	↑	0/1	n.d.	215 ± 42	↑	0/3
Terpenes												
α-pinene*[3,5]	182 ± 72	76 ± 10	↓	5/4	146 ± 15	72 ± 35	↓	4/3	109 ± 17	91 ± 17	↓	4/4
β-pinene*[5]	n.d.	n.d.	–	–	95 ± 4	41	↓	2/1	211	161 ± 69	↓	1/2
β-myrcene*[5]	177 ± 38	127 ± 12	↓	5/9	205 ± 15	197 ± 60	↓	5/7	163 ± 32	149 ± 20	↓	5/5
Limonene*[1,2,3,4,5]	741 ± 220	376 ± 52	↓	9/10	657 ± 128	372 ± 115	↓	9/9	565 ± 105	355 ± 39	↓	10/8
Eucalyptol*[3,5]	295 ± 30	346 ± 52	↑	2/5	611 ± 134	466 ± 1	↓	6/2	466 ± 66	426 ± 67	↓	2/3
Ketones												
6-Methyl-5-hepten-2-one*[4]	334 ± 01	617 ± 120	↑	7/9	286 ± 34	597 ± 229	↑	5/5	502 ± 134	402 ± 80	↓	6/9
Aromatics												
1,3-Dimethylbenzene*[1]	505 ± 120	383 ± 47	↓	8/10	446 ± 122	382 ± 87	↓	7/7	367 ± 66	417 ± 76	↑	9/8
Benzonitrile*	311 ± 65	382 ± 71	↑	8/9	362 ± 56	292 ± 70	↓	8/8	527 ± 129	514 ± 98	↓	9/8
Isothiocyanates												
Cyclohexane isothiocyanate*[1]	n.d.	196 ± 40	↑	0/2	460 ± 178	n.d.	↓	3/0	n.d.	139	↑	0/1

*Definitively identified.

[a] Relative increase or decrease from 3 to 8 h (↑↓).

[b] Number of samples of the 10 original volatile collections containing a compound following 3 and 8 h damage.

n.d., not detected.

Compounds of plant origin previously detected by: [1]Scascighini et al. (2005); [2]Smid et al. (2002); [3]Mattiacci et al. (2001b); [4]Geervliet et al. (1997); [5]Blaakmeer et al. (1994).

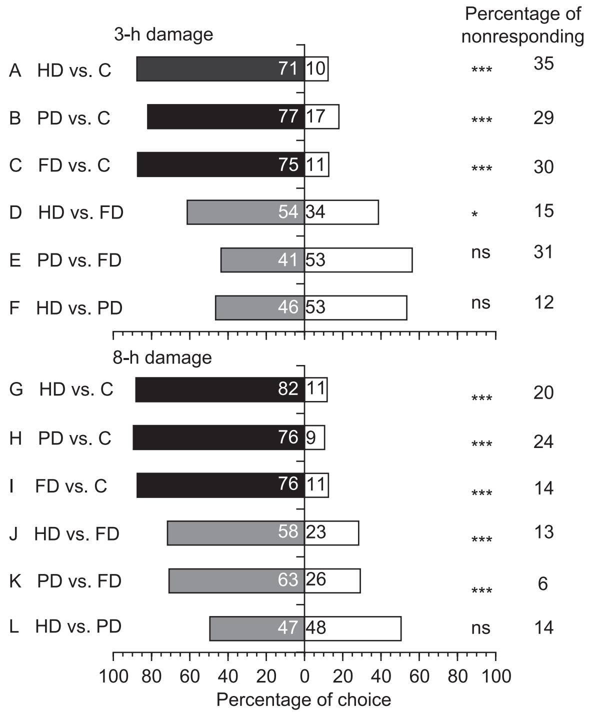

Figure 2 Response of female *Cotesia glomerata* to different damage types in wind-tunnel bioassays following 3 h (A–F) and 8 h (G–L) of damage to a single leaf of Brussels sprouts plants. Bars indicate the choice of female *C. glomerata* to leaves damaged by herbivore damage (HD) (50 *Pieris brassicae* larvae feeding on a single leaf), progressive damage (PD) (damage made using a herbivore simulator that is spatially and temporally similar to HD), final damage (FD) (damaged using a hole punch to represent final area of damage), and undamaged control leaves (C). Numbers within bars represent the total number of wasps responding to a treatment. * indicates a significant difference within the choice test at $P<0.05$ (χ^2 test) and *** indicates a significant difference at $P<0.001$; ns, not significant.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 for Mac OS X statistical package (SSPS, Chicago, IL, USA). χ^2 tests were used to test differences in the response of female *C. glomerata* to different damage types. A general linear model (GLM) was fitted to the bioassay data to include the variation between the dates tested. A binomial distribution was assumed with the logit link to model the influence of day and damage treatment on the net choice of the parasitoids. Models were corrected for overdispersion (scale parameter = 2.4). Differences in the quantity of chemical compounds were tested using the non-parametric Mann–Whitney U-tests. Canonical discriminant analysis (by forward stepwise method) was used to classify the emitted volatiles according to the three different damage treatments. Wilks' lambda was used to select discriminant variables ($P<0.05$).

Results

Wind-tunnel bioassays

Female *C. glomerata*, given a choice between damaged plants in wind-tunnel bioassays, showed a very high responsiveness following 3 h of damage with at least 65% of wasps tested completing flights. The highest responsiveness was found in choice tests between progressive damage (PD) vs. FD and PD vs. HD with at least 85% of wasps completing flights (Figure 2D,F). No influence of the day-to-day variation on the net choice of the parasitoids ($\chi^2 = 0.267$, $P = 0.606$) and the interaction between day and treatment ($\chi^2 = 4.623$, $P = 0.201$) on the net choice of the parasitoids was found in the GLM. A clear preference for all three damage types was found when female wasps were offered a choice between damaged and undamaged control plants, with a minimum of 82% of responding wasps preferring the damage treatment (GLM: $\chi^2 = 77.39$, $P<0.001$; Figure 2A, B, and C). However, parasitoids showed no significant preference for progressively damaged leaves over FD leaves with 44% choosing PD and 56% choosing FD (Figure 2D). Similarly, wasps did not significantly discriminate between PD and HD, with 46% choosing PD and 54% choosing HD (Figure 2F). In the choice tests between the three damage types after 3 h, only one comparison yielded a significant preference: 61% of female wasps preferred plants with HD over 39% choosing plants with FD ($\chi^2 = 4.5$, $P<0.05$; Figure 2E).

Following 8 h of damage, responsiveness in wind tunnel choice tests was very high, with at least 76% responding females in all comparisons. The highest responsiveness was found in choice tests between HD and FD with 94% of wasps tested completing flights. The GLM showed no influence of the day-to-day variation ($\chi^2 = 0.169$, $P = 0.681$) and the interaction between day and treatment ($\chi^2 = 4.623$, $P = 0.201$) on the net choice of the parasitoids. Highly significant preferences of female *C. glomerata* to the three different damage types following 8 h of damage were found with over 87% of females choosing damaged over undamaged control leaves (GLM: $\chi^2 = 175.62$, $P<0.001$, Figure 2G, H, and I). Female *C. glomerata* showed a clear preference for both progressively damaged and herbivore-damaged plants when either of these treatments was offered against leaves with FD, with 71 and 72% of females choosing progressively damaged and herbivore-damaged leaves, respectively. These results were both highly significant ($\chi^2 = 15.4$ and 15.1; $P<0.001$, Figure 1J,K). When given a choice between progressively damaged and herbivore-damaged leaves, female wasps did not significantly discriminate, with 51% choosing PD and 49% choosing HD (Figure 2L).

Volatile analysis

Following 3 h of damage, the volatile profiles from the three different damage types showed that herbivore-damaged and progressively damaged plants released similar quantities of volatile compounds, which were generally lower than amounts released from the FD treatment (Table 1). This trend was observed in seven of the 13 compounds identified, with only one compound, the green leaf volatile (*Z*)-3-hexenyl acetate, being released in significantly lower quantities by herbivore-damaged plants than in plants with FD (Mann–Whitney U-test: U = 19.0, P = 0.033; Table 1). A much greater amount of (*Z*)-3-hexenyl acetate was released from progressively damaged plants than from herbivore-damaged plants, although this difference was non-significant (Mann–Whitney U-test: U = 15.0, P = 0.091; Table 1). In fact, the canonical discriminant analysis shows that this ester significantly contributes to the classification of volatiles according to damage type (Wilks' lambda = 5.63, d.f. = 3,34, P<0.003), followed by the terpenes, β-myrcene, and eucalyptol, which altogether explain 98.4% of the variance (Figure 3A). Herbivore-damaged plants also released lower quantities of (*Z*)-3-hexen-1-ol, another green leaf volatile, and the ketone 6-methyl-5-hepten-2-one than both types of mechanically damaged plants; however, these differences were non-significant (both P>0.05). Two compounds were found only in samples from herbivore-damaged leaves following 3-h damage. These were an aldehyde, (*E*,*E*)-2,4-hexadienal, and cyclohexane isothiocyanate.

After 8 h of damage, herbivore-damaged and progressively damaged leaves produced lower mean quantities of seven of the 13 compounds detected than leaves with FD, although all these differences were non-significant (Table 1). This shows a continuation of the trend in amounts of chemicals released from damaged leaves following 3 h of damage. The canonical discriminant analysis used to classify the volatiles according to the damage type showed that 93.8% variance could be explained by the first two functions (Figure 3B). The volatiles that most effectively separate the damage types by stepwise forward entry are the aromatic 1,3-dimethylbenzene (Wilks' lambda = 6.11, d.f. = 3,34, P<0.002) and the esters (*Z*)-3-hexenyl acetate (Wilks' lambda = 4.74, d.f. = 6,66, P<0.001), and (*Z*)-3-hexenyl butanoate (Wilks' lambda = 4.67, d.f. = 9,78, P<0.003). Herbivore-damaged leaves alone were found to emit (*E*,*E*)-2,4-hexadienal. Lower mean amounts of (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate

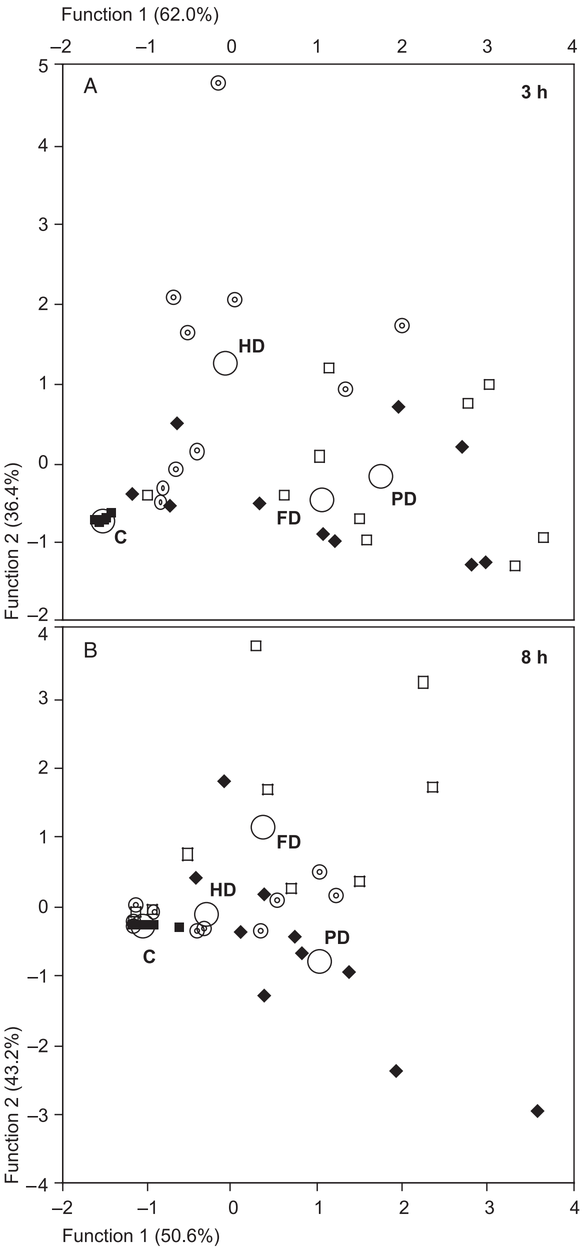

Figure 3 Canonical discriminant analysis used to classify the emitted volatiles according to the different damage treatments following (A) 3 h (Function 1: 62.0% variance explained; canonical correlation = 0.73; Function 2: 36.4% variance explained; canonical correlation = 0.63) and (B) 8 h of damage (Function 1: 50.6% variance explained; canonical correlation = 0.63; Function 2: 43.2% variance explained; canonical correlation = 0.60). Data points of different treatments are represented as follows: HD = ◎, PD = ◆, FD = □, and C = ■. Group centroids are represented by ❍.

were detected in volatile samples from herbivore-damaged leaves when compared with progressively damaged or leaves with FD. A comparison of the amount of each volatile released following 3 and 8 h showed that there were no significant differences within damage treatments (Mann–Whitney U-test, HD: U = 23, P = 0.232; PD: U = 15, P = 0.091; and FD: U = 27, P = 0.089). Changes in the quantity of compounds released were generally very small. The main exceptions to this trend were (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, which were released in much greater quantities following 8 h of damage in all three treatments. In PD and HD, 6-methyl-hepten-2-one was released in much greater amounts following 8 h, but in FD, the difference was very small.

Discussion

This study clearly highlights that the plant's response to progressive mechanical damage was more similar to HD than final mechanical damage deployed at a single point in time, irrespective of damage duration. While volatile emissions were quantitatively higher in PD and FD compared to HD, the wasp *C. glomerata* did not discriminate between PD and HD. This result is particularly interesting, as many previous studies have shown that mechanical damage alone does not elicit a strong response in parasitoids (Geervliet et al., 1994; Vernède & Pak, 1994; van Poeke et al., 2001). However, recent studies have shown that progressive mechanical damage over a period of time can elicit a physiological response in the plant resulting in a change in volatile production (Mithöfer et al., 2005; Röse & Tumlinson, 2005).

Wind-tunnel bioassays following 3 and 8 h of damage showed that all damage treatments were significantly more attractive to female *C. glomerata* than undamaged control plants. After only 3 h of damage, the wasps already discriminated between damaged and undamaged plants. This is concurrent with other studies, which showed that *C. glomerata* females prefer mechanically damaged plants to undamaged control plants (Steinberg et al., 1993). This is likely to be the result of constitutive and induced compounds in Brussels sprouts being qualitatively similar (Mattiacci et al., 2001a; Scascighini et al., 2005).

However, wasps apparently could not discriminate between PD and FD after 3 h, which suggests that quantitative rather than qualitative differences in the volatile blend composition may be responsible for wasp preference in these bioassays (Bukovinszky et al., 2005). Following 8 h of damage, the plants evoked a strong preference of the wasp for PD and HD. The volatile profiles showed a trend for lower quantities of volatiles in herbivore and progressively damaged than in leaves with FD. For example (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate were both released in lower quantities from herbivore-damaged samples than in both mechanical damage treatments. It is well known that Brussels sprout plants release green-leaf volatiles such as (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate following damage (Mattiacci et al., 1994; Scascighini et al., 2005). Another compound produced by the lipoxygenase pathway, (*E*,*E*)-2,4-hexadienal, was only found in herbivore-damaged samples, as was one isothiocyanate.

Geervliet et al. (1994) showed that mechanical damage inflicted 24 h prior to analysis caused higher release of volatiles than HD. Interestingly, *C. glomerata* was able to distinguish between damage types after only 3 h in our study. Numerous studies have compared mechanical damage with HD, and have observed the response of the plant, in terms of volatile release and attraction of parasitoids to the two types of damage (Steinberg et al., 1993; Blaakmeer et al., 1994; Mattiacci & Dicke, 1995a; Smid et al., 2002), although most studies inflicted damage 24 h prior to volatile collection (Geervliet et al., 1994; Halitschke et al., 2001). To truly compare mechanical damage with herbivory, however, it is imperative for the damage to be spatially and temporally similar (Mithöfer et al., 2005). The number of plant cells damaged in a single wounding event is different to that of continuous (i.e., progressive) mechanical damage or HD, and plants appear to respond to the progressive nature of damage with a different volatile blend (Mithöfer et al., 2005).

Green-leaf volatiles produced from linolenic and linoleic acids through the lipoxygenase pathway (Pare & Tumlinson, 1996) are thought to be major constituents of attractants to parasitoids such as *C. glomerata* (Scascighini et al., 2005). It is possible that female *C. glomerata* would have reacted differently to our damage treatments had they been given experience of the volatile cues from the plant-host complex prior to wind-tunnel bioassays (Vet & Dicke, 1992). Experience of host-derived cues has been shown to be important in adult parasitoid learning (Tumlinson et al., 1993). In our study, female parasitoids were not given prior experience of host cues (except those encountered prior to pupation and emergence) and it would be interesting to see if experienced wasps would find the progressive mechanical damage treatment as attractive as plants damaged by *P. brassicae*, as we found with naïve females.

We provided the plant with mechanical damage in the absence of herbivore oral secretions. It is possible that oral secretions include previously ingested plant parts, which could induce a plant response due to plant–plant interaction. Our PD treatment provided a spatial and temporal mimic of herbivore feeding in the absence of salivary enzymes. The attraction of the wasp to this treatment is concurrent with other recent studies (Mithöfer et al., 2005; Röse & Tumlinson, 2005), which have shown that plants respond to solely progressive mechanical damage in a similar fashion to HD. Indeed at least one study has found that products within herbivore saliva can suppress, rather than promote, plant response to herbivory (Musser et al., 2002) and it would be interesting to see more studies investigating whether the role of herbivore salivary elicitors in the elicitation of a plant response has been overemphasized when compared with the role of progressive mechanical damage.

It is possible that our herbivore and progressive damaged plants responded to self-produced green-leaf volatiles (Arimura et al., 2001; Engelberth et al., 2004), methyl salicylate (Durner et al., 1997), or methanol (Von Dahl et al., 2006) released from wounded leaves during the 3 or 8 h of damage. In contrast, leaves with FD were treated immediately prior to bioassays, which may not have given the plant enough time to express a response to volatile organic compounds (VOC). Plants have been shown to ready themselves for herbivore attack after receiving herbivore-induced VOC from neighbouring plants (Karban & Baldwin, 1997; Baldwin et al., 2006; Kost & Heil, 2006). However, these studies often use quantities of volatiles that are several orders of magnitude higher than those found in natural systems, or experiments are performed in a closed system (Preston et al., 2004). The response to autogenous products is another factor possibly eliciting a systemic plant response that requires further investigation.

Other studies (Mithöfer et al., 2005; Röse & Tumlinson, 2005) have explored the plant's volatile response to progressive mechanical damage, but these were not coupled with behavioural assays. Indeed, our bioassay data with *C. glomerata* provide conclusive evidence that the plant plays a more important role than previously assumed (Mattiacci et al., 1995; Spiteller et al., 2001). This underlines the importance of performing both chemical analyses and behavioural bioassays in order to fully understand ecological processes and to relate minor differences in plant physiological responses to plant parasitoid interactions.

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