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Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant-volatile signals

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It is widely reported that plants emit volatile compounds when they are attacked by herbivorous insects, which may be used by parasitoids and predators to locate their host or prey. The study of herbivore-induced plant volatiles and their role in mediating interactions between plants, herbivores and their natural enemies have been primarily based on aboveground systems, generally ignoring the potential interactions between above and belowground infochemical- and food webs. This study examines whether herbivory by *Delia radicum* feeding on roots of *Brassica nigra* (black mustard) affects the behaviour of *Cotesia glomerata*, a parasitoid of the leaf herbivore *Pieris brassicae*, mediated by changes in plant volatiles. In a semi-field experiment with root-damaged and root-undamaged plants *C. glomerata* prefers to oviposit in hosts feeding on root-undamaged plants. In addition, in a flight-cage experiment the parasitoid also prefers to search for hosts on plants without root herbivores. Plants exposed to root herbivory were shown to emit a volatile blend characterized by high levels of specific sulphur volatile compounds, which are reported to be highly toxic for insects, combined with low levels of several compounds, i.e. beta-farnesene, reported to act as attractants for herbivorous and carnivorous insects. Our results provide evidence that the foraging behaviour of a parasitoid of an aboveground herbivore can be influenced by belowground herbivores through changes in the plant volatile blend. Such indirect interactions may have profound consequences for the evolution of host selection behaviour in parasitoids, and may play an important role in the structuring and functioning of communities.

The defence of plants against insect herbivores involves different strategies. Plants can defend themselves directly through the production of morphological structures on the leaf surface (i.e. trichomes) that deter herbivore colonization, and by producing or increasing levels of toxic compounds that deleteriously affect the development of phytophages (Dicke and Van Loon 2000). The plant defence system can also involve indirect mechanisms, including the production and release of plant volatile compounds as a response to herbivore feeding, which attract natural enemies (i.e. parasitoids and predators) of the attacking herbivores (Vet and Dicke 1992, Dicke 1999, Vet 1999). The role of herbivore-induced volatiles in mediating interactions between plants, herbivores and their natural enemies has received considerable attention over many years (Price et al. 1980, Turlings et al. 1990). Thus far, most studies have been primarily based on aboveground interactions, but similar plant-induced indirect defense responses have also been observed belowground (Van Tol et al. 2001, Rasmann et al. 2005).

It is known that root-associated organisms can affect the development of leaf associated herbivores sharing the host plant (Gange and Brown 1989, Masters 1995, Van der Putten et al. 2001, Bezemer et al. 2003), and higher trophic levels including parasitoids, and even hyperparasitoids of the fourth trophic level (Soler et al. 2005). Recently, there has been a growing interest in how soil organisms, such as root feeding insects, nematodes, or arbuscular mycorrhizal fungi, may also influence the levels of parasitism of aboveground herbivores (Masters et al. 2001, Gange et al. 2003, Bezemer and Van Dam 2005, Bezemer et al. 2005, Poveda et al. 2005). There is increasing evidence that the effect of root-associated organisms on the development of the aboveground trophic chains (herbivores–parasitoids–hyperparasitoids) is largely mediated by changes in quantity and/or quality of the shared host plant (reviewed by Bezemer and Van Dam 2005). However, little is known about the influence of root associated organisms on the behaviour of parasitoids of aboveground herbivores and the mechanisms mediating the interactions.

Recently, Guerrieri et al. (2004) showed that the behaviour of aboveground parasitoids can be affected by arbuscular mycorrhizal fungi. In absence of their hosts, aphid parasitoids were strongly attracted to mycorrhizal plants over conspecific plants without mycorrhizal association in the roots (Guerrieri et al. 2004). Although studies are scarce, there is also some evidence that soil organisms can influence indirect plant defense responses aboveground (Wäckers and Bezemer 2003, Bezemer and Van Dam 2005). For example, Rasmann et al. (2005) showed that root herbivory induces a volatile signal in the soil that attracts entomopathogenic nematodes, whilst simultaneously inducing the release of the same volatile compound aboveground from the leaves of the plant. However, the consequences of such belowground defence induction for the behaviour of aboveground natural enemies remain unexplored. Further studies integrating associations between plants, insect herbivores, and their natural enemies aboveground with the biological processes that occur in the soil, and the mechanisms mediating the interactions, are crucial for a better understanding of interactions between plants and other organisms in a multitrophic context.

Recently we showed that *Cotesia glomerata* (Hymenoptera: Braconidae), an aboveground parasitoid of caterpillars of the cabbage butterfly, *Pieris brassicae* (Lepidoptera: Pieridae) developed significantly slower and adults were smaller when roots of *Brassica nigra* (Brassicaceae) plants were damaged by larvae of the cabbage root fly, *Delia radicum* (Diptera: Anthomyiidae) (Soler et al. 2005). These negative effects appeared to be driven by increases in foliar levels of allelochemicals induced by the root feeding insects (induced direct defense). Parasitoid females are known to be well adapted to exploit chemical information coming from the host plants to select the most profitable host for their offspring (Godfray 1994, Dicke 1999). Considering that the performance of *C. glomerata* was significantly reduced when developing in hosts feeding on plants exposed to root herbivory, we tested whether *C. glomerata* uses cues induced by belowground herbivores to avoid root-damaged plants when suitable oviposition sites are not limiting.

We tested the hypothesis that there is a clear preference–performance correlation in the parasitoid, i.e. that females will preferentially search for and parasitize hosts on root-undamaged plants, where their progeny will develop most successfully. We examined the effects of root herbivory on the plant volatile blend, to correlate with our behavioural data. We specifically address the following questions: (1) is host acceptance of the parasitoid affected by belowground herbivory? (2) Is plant preference of the parasitoid affected by belowground herbivory? If so, does it differ when exposing the plants to different *Delia* larval stages? And (3) if variation in the parasitoid behaviour is observed, can it be attributed to changes in the plant volatiles triggered by root herbivory?

Material and methods

Our study is based on interactions involving a naturally occurring system in western Europe. Black mustard, *Brassica nigra* (Brassicaceae), is a widely distributed annual crucifer that is common along rivers in the Netherlands (Schaminee et al. 1998). *Brassica nigra*, like other members of the Brassicaceae, possesses potent inducible direct and indirect defenses via the production of glucosinolates and their breakdown products, providing excellent potential for studying multitrophic interactions (Feeny and Rosenberry 1982, Traw and Dawson 2002, Van Dam et al. 2003). It is attacked by several above and belowground specialist herbivores, including the large cabbage white butterfly, *Pieris brassicae* (Lepidoptera: Pieridae) whose larvae feed on the shoots and flowers of this and related species (Harvey et al. 2003). *Cotesia glomerata* is a gregarious endoparasitoid that attacks young larvae of several species of pierid butterflies; with *P. brassicae* as its preferred host in Europe (Feltwell 1982). To locate their hosts, *C. glomerata* females rely on infochemicals both from the plant and its host (Geervliet 1997). Mustard plants are also attacked by specialized root feeding herbivores, including the cabbage root fly, *Delia radicum* (Diptera: Anthomyiidae) (Coaker and Finch 1971). Females lay eggs on the soil surface around the plant stem, and the emerged larvae feed on the plant roots causing rapid infection by microorganisms.

D. radicum has associated gut microbial symbionts, which may be bacteria, yeast or protozoa that are assumed to enrich the nutritional value of the food by providing essential vitamins and amino acids or by digesting refractory plant materials. However, little is known about its digestive biochemistry, the source of these organisms and the maintenance between generations (Lukwinski et al. 2006). It is important to notice then, that for this system, the effects of root herbivory cannot be fully separated from the effects of the microorganisms associated to *D. radicum*.

Pieris brassicae (leaf herbivore) was obtained from an insect culture maintained at the Laboratory of Entomology of Wageningen University, the Netherlands and was cultured on *Brassica oleracea* plants. *Cotesia glomerata* (parasitoid) and *Delia radicum* (root herbivore) were obtained from cultures maintained at the Netherlands Institute of Ecology, Heteren, The Netherlands. The root herbivore was cultured on *Brassica napus* roots. *Brassica nigra* seeds were collected from a single population in the northwest of Wageningen, the Netherlands. Seeds were surface sterilized and germinated on a bed of glass-pearls (pearls of 1 mm ∅). One week after germination seedlings were transplanted into 1.2 l pots. The plants were grown in a greenhouse, at $22 \pm 1^{\circ}C$ (day) and $16 \pm 1^{\circ}C$ (night), 70% RH and 16:8 h day:night. Natural daylight was supplemented by metal-halide lamps (225 $\mu mol\ s^{-1}\ m^{-2}$ PAR). Plants were watered daily; only non-flowering plants were used in the experiments.

Host-acceptance in the semi-field experiment

In a two-choice experiment with 26 plants we examined if *C. glomerata* females prefer to parasitize their larval hosts (*P. brassicae*) feeding on root-undamaged plants over hosts feeding on plants of which roots were fed on by *D. radicum*. The experiment was performed outdoors (July 2005) in a transparent tent ($8 \times 4 \times 2.5$ m). On 13 plants, eight third instar (L3) *D. radicum* larvae per plant were introduced six days before the test (root herbivory treatment), while the 13 other plants were kept root-undamaged and served as control. One day prior to the test, all plants were infested with 10 L1 *P. brassicae* larvae. Each brood was placed on the youngest fully developed leaf of each plant. Plants of similar height and shape were used to avoid any potential effects of plant characteristics on parasitoid behaviour.

The following day, all plants were placed in the tent alternating root-damaged and root-undamaged plants. The distance between plants was approximately one m. Ten *C. glomerata* females were then released into the tent, allowed to forage freely during two h and then recaptured. Subsequently, the *P. brassicae* larvae were harvested and dissected to determine the number of parasitized and healthy larvae per plant. Parasitism was identified by the presence of parasitoid eggs, using a stereo-microscope. The number of plants with parasitized larvae was determined, and the percentage of root-damaged and root-undamaged plants selected by the parasitoid females calculated. In addition, the number of parasitized caterpillars per plant on which parasitized larvae were found was recorded.

Plant-preference in the flight-cage experiment

We used a flight-cage, comparable to a wind tunnel described in Geervliet et al. (1994), to examine if *C. glomerata* discriminates between plants fed on by *Delia* (root-damaged plant) and root-undamaged plants. A cage of $1.2 \times 1.0 \times 0.8$ m, with nets at the front and back and otherwise of plexiglas, was placed on a table inside a transparent cloth tent in a greenhouse compartment, at $22 \pm 1^{\circ}C$ and 70% RH. Plant preference was tested in a two-choice set-up. We performed the following choice-test comparisons: control test: undamaged plants vs *Pieris* (host caterpillar)-infested plants; test a: undamaged plants vs plants with roots damaged during the entire *Delia* larval development time (from L1 to L3); test b: undamaged plants vs plants with roots damaged by *Delia* L3 larvae; test c: undamaged plants vs plants with roots damaged by *Delia* L1-L2 larvae; test d: plants with *Pieris* feeding on it vs plants with *Pieris* feeding on it and roots damaged by *Delia* L3 larvae (below). For each choice test 10 pairs of plants were used, and the distance between the plants within a plant pair was 0.70 m. For each plant pair, 10 to 12 parasitoid females of five to eight days old were released from a distance of 1 m from the plants. A choice was recorded when the parasitoid alighted on the foliage of a plant. Each choice test was terminated after choices of 10 females were registered. Overall, 76% of the female wasps actively made a choice, independent of the choice test, adding to a total of approximately 100 *C. glomerata* females observed per comparison. In order to increase the parasitoid motivation to search, one first instar larva of *P. brassicae* was presented to each female wasp for oviposition immediately prior to each test (Vet et al. 1995). The offered first instar larvae of *P. brassicae* had been feeding on *Brassica oleracea*, a different host plant as presented in the test, to prevent a possible associate learning-induced bias in the plant choice by *C. glomerata* (Vet et al. 1995). Experimental plants exposed to root herbivory were checked after the test to ensure that *D. radicum* larvae were present and actively feeding. More than 80% of the larvae that were introduced were recovered in each test. To avoid the interference between plant-odours of different replicates

or treatments, the air inside the cage was ventilated between tests using a fan.

Control test

Undamaged plants vs *Pieris*-infested plants (control vs LH). Previous studies reported that *C. glomerata* is significantly more attracted to *Brassica* plants infested by its hosts (*P. brassicae*) than to uninfested plants (Steinberg et al. 1993). Therefore, to test our flight-cage set-up, we initially compared plant preference of *C. glomerata* between *B. nigra* plants with and without a brood of 10 first instar larvae of *P. brassicae*. Approximately 24 h before the test, the *P. brassicae* larvae were placed on the third new leaf of the plants, in a clip-cage of 5.5 cm diameter. Clip-cages were similarly placed on the control plants without *P. brassicae* larvae. The clip-cages were removed prior to the choice tests to fully expose the larvae to the parasitoids.

Test a, b, c

Undamaged plants vs plants with root herbivores (control vs different larval stages of RH). After checking the flight-cage set-up, parasitoid preference was compared between *B. nigra* plants without (control) and with *D. radicum* larvae (root herbivory treatment, performed by different larval stages). In all cases the *Delia* larvae were carefully placed with a brush next to the stem of the plant at the soil surface. Test a (L1-L3): eight *D. radicum* larvae were introduced as first instar (L1) and the test was performed when larvae had reached the end of the last (third) instar (L3), being approximately the entire larval developmental time (approximately 15 days). Test b (L3): damage caused by late instar larvae only: eight early-stage L3 *D. radicum* larvae fed during six days and the test was performed at the end of L3. Test c (L1-L2): damage caused by early instar larvae only: eight L1 *D. radicum* fed for nine days, and thus the test was performed when larvae were L2. Due to early flowering of some plants with *Delia*, five replicate plants instead of 10 were used for test c.

Test d

Plants with *Pieris* vs plants with *Pieris* and roots damaged by *Delia* L3 larvae (LH vs LH + RH_{L3}). *P. brassicae* larvae were placed on the plants 24 h before the test, following the same procedure described above in the control tests. Root herbivory was similar as in test b.

Plant volatiles

Volatiles were collected from plants exposed to root herbivory (RH), leaf herbivory (LH), both types of herbivory (LH + RH), or no herbivory (control). The entire plants (including the pots and soil) were placed in the chambers to collect the volatiles. There were six replicate plants per treatment. Plants of similar size were used for the volatile collection. *Delia* infested plants were inoculated with eight L3 *D. radicum* larvae six days prior to the collection of volatiles. Plants with leaf herbivory were inoculated with 10 L1 *P. brassicae* larvae 24 h prior to volatile collection. Plants were transferred to four separate 17 l glass bell-shaped collection chambers that had been placed in a controlled climate cabinet (21°C, 70% RH). The chambers were constantly supplied at the top with 300 ml of pressurized air (Hoekloos, the Netherlands) and were cleaned over a Zero Air generator to remove hydrocarbons (Parker Hannifin Corp, Tewksbury, MA, USA). Volatiles were collected in a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B using a vacuum pump. Collection flow rates were set to 100 ml min^{-1}. After one h the traps were removed from the pump and capped until analysis. In this way, we measured six full series of four plants and two background profiles from an empty glass chamber within one day.

Volatiles were desorbed from the traps using an automated thermodesorption unit (model Unity, Markes, Pontyclun, United Kingdom) at 200°C for 10 min (He flow 30 ml min^{-1}) and focused on an cold trap (-10°C). After 1 min of dry purging, trapped volatiles were introduced into the GC-MS (model Trace, ThermoFinnigan, Austin, Texas, USA) by heating the cold trap for 3 min to 270°C. The split rate was set to 1:4 and the column used was a 30 m × 0.32 mm ID RTX-5 Silms, film thickness 0.33 µm. Temperature program: from 40°C to 95°C at 3°C min^{-1}, then to 165°C at 2°C min^{-1}, and finally to 250°C at 15°C min^{-1}. The volatiles were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, 3 scans s^{-1}). Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with Nist 98 and Wiley 7th edition spectral libraries and by comparing their linear retention indices. Additionally, mass spectra and/or linear retention indices were compared with values reported in the literature, obtained by interpolating homologous series, or by analyzing reference substances (farnesene, benzonitrile, dimethyldisulfide, dimethyltrisulfide and limonene; Sigma-Aldrich, Zwijndrecht, NL). Identified peaks in the chromatogram were integrated by Xcalibur software (version 1.3, Finnigan). To exclude potential interference by co-eluting compounds, specific quantifier ions were carefully selected for each individual compound of interest. In general, these quantifier ions were similar to the most intense model ions extracted from the raw mass spectrum by AMDIS. The integrated absolute signal of the quantifier ion(s) were used for

comparison between the treatments. Peak areas in each sample were divided by the total volume in ml that was sampled in the trap, to correct for small differences in sampling time and flow rates over individual traps.

Statistical analyses

For the host-acceptance semi-field experiment, proportion of plants with parasitization between control plants and plants exposed to root herbivory were compared using a two-sample binomial test. Percentages of parasitism per plant with parasitism were arc-sine transformed and tested using one-way ANOVA. Normality and homogeneity of variance were checked by inspection of the residuals after model fitting. For the plant preference flight-cage experiments, a binomial test was used to determine whether plant preferences of the wasps differed significantly from a non-preference situation ($p = q = 0.5$, two-tailed, $\alpha = 0.05$).

A total of 249 compounds were registered in the volatile blends of plants exposed to the different treatments. A subset of 26 compounds belonging to the isothiocyanates, monoterpenes, sesquiterpenes, terpenes, nitric compounds, sulfur and other/aromatic compounds were selected for detailed analysis, as they are known to play a role in plant–insect interactions in cruciferous plants (Reddy et al. 2002, Ibrahim et al. 2005, Kappers et al. 2005). Differences and similarities in the volatile blends between treatments were analyzed by canonical discriminant analysis (CDA) (McGarigal et al. 2000). CDA is a dimension-reduction technique similar to principal component analysis which calculates linear combinations of the original variables (i.e. volatile compounds). The linear combinations, usually referred to as canonical variates, summarize the information contained in the original variables and maximize between-treatment variation. This allows for the identification of those compounds that discriminate most clearly between the treatments. To visualize multivariate similarities in volatile blend among the treatments, we plotted the location of each sample with respect to their position on the first and second canonical variates (CDA plot). To quantify the relative (dis)similarity between treatments in volatile blends we calculated Mahalonobis distances which measure the distance between treatment-centroids in multivariate space (McGarigal et al. 2000). We used standardized canonical coefficients to assess the relative contribution of each compound to the canonical variates. For each compound separately we tested the effect of leaf herbivory (LH), root herbivory (RH) and the interaction ($LH \times RH$) using analysis of variance on log-transformed peak areas. Normality, independence and homogeneity of variance were checked for each variable by inspection of the residuals after model fitting. For some variables (beta-farnesene, dimethyl disulfide and dimethylnonatriene) a non-parametric test was used (Kruskal–Wallis) since their distribution could not hold the assumptions for a standard parametric analysis of variance.

Results

Host-acceptance in the semi-field experiment

Root herbivory significantly affected the oviposition behaviour of *C. glomerata*. Parasitized hosts were found on 85% of the control plants, compared to 46% on plants with root herbivory ($Z = 2.06$; $p = 0.03$). When parasitization had occurred on a plant, the percentage of parasitism between plants with and without root herbivory did not differ (control: 57 ± 10; root herbivory: 83 ± 13; $F_{1,15} = 2.55$; $p = 0.13$).

Plant-preference in the flight-cage experiment

C. glomerata showed a clear preference for *B. nigra* plants damaged by the caterpillar host *P. brassicae*, as compared to plants without the leaf herbivore ($Z = 5.58$; $p < 0.0001$; data not shown). Eighty percent of the released wasps alighted on plants that were infested with their host larvae, confirming that the flight cage set-up was appropriate to measure the plant preference of the parasitoid.

In absence of the caterpillars, root herbivory significantly affected plant preference of the parasitoid, and the effect differed with root herbivory intensity (Fig. 1a–c). Parasitoids showed a clear preference for root-undamaged *B. nigra* plants and generally avoided plants exposed to root herbivores feeding on the roots during almost the entire larval development time (L1-L3: Fig. 1a) ($Z = 2.40$; $p = 0.01$). Similarly, parasitoids clearly preferred root-undamaged plants to plants that had been exposed to root herbivores feeding on the roots during the final larval instar (L3: Fig. 1b) ($Z = 2.81$; $p = 0.004$). However, the parasitoid did not avoid *B. nigra* plants that had solely been exposed to early instar larvae of the root herbivores (L1-L2: Fig. 1c) ($Z = 0.1$; $p = 0.92$). Plant preference of the parasitoid was also not significantly affected by root herbivory when its host *P. brassicae* was present on the leaves of both root-damaged and root-undamaged plants (Fig. 1d), with approximately fifty percent of the released female parasitoids alighting on each plant choice ($Z = 0.49$; $p = 0.62$). The response time, defined as the time between release of the parasitoid and the parasitoid landing on the plant, was similar in all the experiments and did not depend on the choice offered ($F_{4,40} = 1.79$; $p = 0.14$).

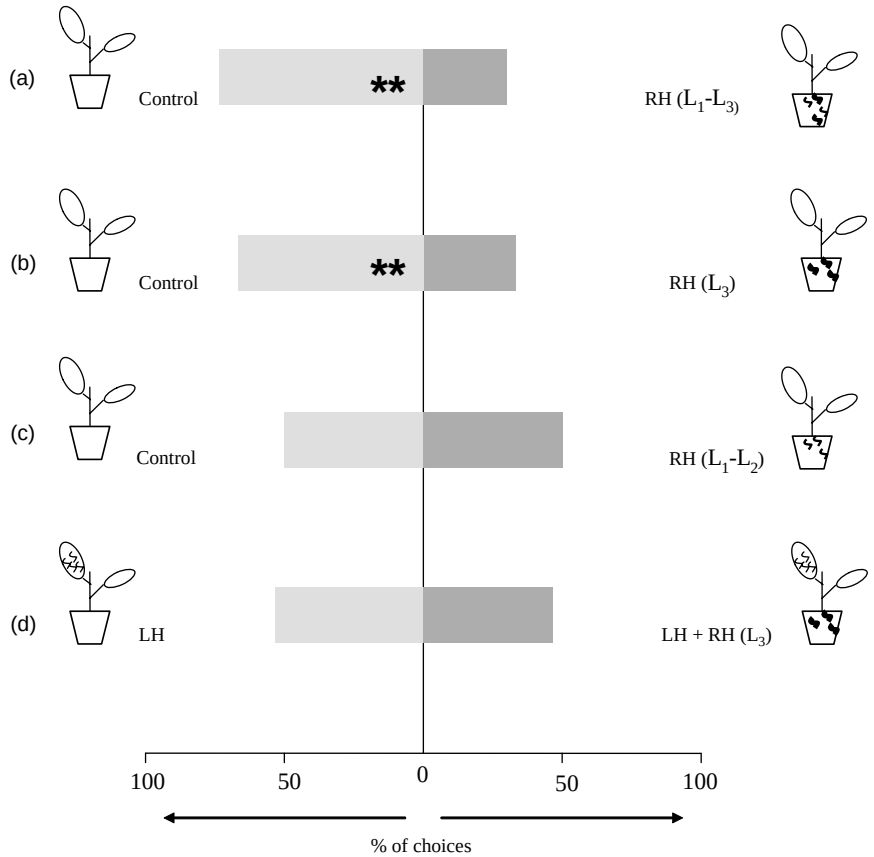
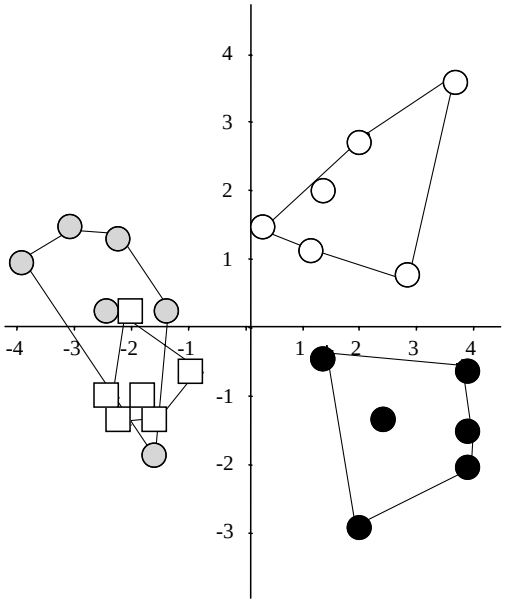


Fig. 1. Percentage of choices of *C. glomerata* females, in the two-choice experiment, between plants exposed to root herbivory (RH) [(by the entire larval root fly development time: L1-L3); (by late instar root fly larvae: L3); (by young instar root fly larvae: L1)], leaf herbivory (LH), both types of herbivory (LH+RH), or no herbivory (control). Root herbivory was caused by *D. radicum* larvae and leaf herbivory was caused by the parasitoid's host larva *P. brassicae*. Asterisks indicate significant preferences within tests (**: $p \leq 0.01$).

Plant volatiles

Using GC-MS technique we examined the volatile blend of plants exposed to root (RH) or leaf herbivory (LH), both types of herbivory (LH+RH), or no herbivory (control). The results of the canonical discriminant analysis show a clear separation of the four treatments based on the volatile blends (Fig. 2). The first and second axis explained 67% and 22% of the total variation in volatile compounds, respectively. The first axis clearly discriminated between LH and LH+RH plants on the one hand, and RH and control plants on the other hand (Fig. 2). The second axis mainly separated RH from control plants (Fig. 2). The LH+RH and the LH group of plants partially overlapped, and the similarity in volatile blends between these two groups is also evidenced by the small Mahalonobis distance between them, compared to the higher distance recorded between RH and control plants (Fig. 2). The terpenes beta-farnesene and dimethylnonatriene showed the closest correlation with the first canonical variate (highest values for score 1 in Table 1) and were both negative. LH and LH+RH plants had negative canonical coefficients for the first axis, implying that the volatile blends of the LH and the LH+RH plants were characterized by a higher amount of these two compounds than the RH and control plants. The second axis was strongly determined by the sulfur compounds dimethyl disulfide and dimethyl trisulfide (Table 1). In the second axis, RH plants had negative coefficient values and control plants had positive coefficient values. High negative values were then associated with higher levels of these compounds, which was the case for the RH plants. The pattern showed by the CDA is consistent with the analysis of variance, which showed that beta-farnesene and dimethylnonatriene were significantly higher in plants exposed to leaf herbivory, and dimethyl disulfide



	Mahalonobis distances			
□ LH+RH	0			
○ Control	21	0		
● RH	22	12	0	
○ LH	2	21	32	0
	LH+RH	Control	RH	LH

Fig. 2. Two dimensional canonical discriminate analysis (CDA) plot of the volatile blends of plants exposed to root herbivory (black circles, RH) or leaf herbivory (grey circles, LH), no herbivory (white circles, control) or both types of herbivory (white squares, LH + RH). Each point represents the volatile blend of a sampled plant. Below, Mahalonobis distances between the volatile blends are presented.

and dimethyl trisulfide were the two volatile compounds significantly higher and associated with plants exposed to root herbivory (Table 1).

Discussion

The results of this study show that root feeding insects can influence plant preference and oviposition behaviour of a parasitoid of an aboveground herbivore sharing the host plant. The underlying mechanism points to changes in the volatile blend of the plant. Overall, the parasitoid *C glomerata* exhibited a clear preference for plants that had not been attacked by larvae of the root herbivore, *D. radicum*. An earlier study (Soler et al. 2005) reported that the performance, i.e. the development time and pupal weight of *C. glomerata* is negatively affected by foliar increases in phytotoxins (direct defense) induced by the root herbivore *D. radicum*, sharing the host plant. In the present study we find that the parasitoid is able to recognize plants on the basis of the presence or absence of root herbivores, and prefers to search and lay its offspring in root-undamaged plants. Hence we find a clear preference–performance pattern for the parasitoid. Theoretical models predict that oviposition decisions by parasitoid females lead to the selection of the most profitable host for their off spring (Van Alphen and Visser 1990, Godfray 1994). These models have been exclusively based on aboveground model systems, whereas our results suggest that *C. glomerata* females are also able to exploit root-induced signals to evaluate and select the most suitable host for their off spring.

The volatile blend of plants differed depending on whether the plant was exposed to root herbivory, leaf herbivory (by the parasitoid's host) or was undamaged. Overall, plants exposed to leaf herbivory were characterized by a high amount of beta-farnesene and dimethylnonatriene and plants exposed to root herbivory were characterized by high amounts of dimethyl disulfide and dimethyl trisulfide. Beta-farnesene and dimethylnonatriene are volatile compounds reported to act as attractants for herbivorous and carnivorous insects (Fukushima et al. 2002, Ansebo et al. 2005). By contrast, dimethyl disulfide and dimethyl trisulfide are reported to exert insecticidal neurotoxicity through mitochondrial dysfunction (Dugravot et al. 2003). Plants exposed to both root and leaf herbivory had volatile blends with higher levels of sulfides and lower levels of attractants compared with plants exposed to only leaf herbivory by the host. Consequently, the avoidance by the parasitoid for the plants exposed to root herbivory may be partly attributed to the higher amount of toxic volatiles combined with the lower production of attractants present in the blend of the root damaged plants.

In the plant preference flight-cage experiment *C. glomerata* strongly distinguished between plants with and without root herbivory (RH vs control), preferring to search for hosts on root-undamaged plants. The ability of *C. glomerata* to discriminate between plants with or without root herbivores was found to depend on the stage of *D. radicum* feeding on the root system. Parasitoids only exhibited a clear discrimination when final (L3) instars of *D. radicum* were present, as opposed to solely younger (L1, L2) instars. In most holometabolous insects (insects with defined feeding and reproductive stages), the vast majority of damage is incurred by the feeding larva during the final stadium, in some instances exceeding 90% (Slansky 1986). This may suggest that a critical level of root damage may be an important pre-requisite for the parasitoid to detect the presence of root herbivores, and to actively make foraging decisions on this basis. Alternatively, different root fly larval stages may have induced different

Table 1. Log transformed peak area (mean ± SE) of volatile compounds, standardized canonical coefficients for the first two axes of the CDA plot (score 1 and 2) and two-way analysis of variance (Kruskal–Wallis -non parametric- analysis of variance for linalool, beta-farnesene and dimethyl disulfide) for individual volatile compounds of *Brassica nigra* plants exposed to root (RH) or leaf herbivory (LH), both types of herbivory (LH + RH), or no herbivory (Control). Root herbivory was caused by *D. radicum* larvae and leaf herbivory was caused by *P. brassicae* larvae. Non significant p values ($p > 0.05$) are indicated by ns.

Volatiles	Control	LH	RH	LH + RH	Score 1	Score 2	$p_{(LH)}$	$p_{(RH)}$	$p_{(LH \times RH)}$
alpha-farnesene	2.79 ± 0.63	2.84 ± 0.57	2.87 ± 0.64	3.81 ± 0.26	−0.073	−0.122	ns	ns	ns
alpha-humulene	1.90 ± 0.61	1.68 ± 0.75	1.98 ± 0.88	2.13 ± 0.68	0.0004	0.077	ns	ns	ns
alpha-muurolene	2.56 ± 0.53	2.79 ± 0.57	2.57 ± 0.52	2.20 ± 0.70	−0.023	0.058	ns	ns	ns
alpha-pinene	3.30 ± 0.67	2.87 ± 0.68	3.31 ± 0.66	3.38 ± 0.69	0.025	0.008	ns	ns	ns
alpha-ylangene	3.40 ± 0.15	3.61 ± 0.07	3.58 ± 0.18	3.02 ± 0.61	0.055	0.018	ns	ns	ns
aromadendrene	1.13 ± 0.51	2.06 ± 0.44	1.25 ± 0.57	1.33 ± 0.60	−0.076	0.001	ns	ns	ns
benzene	1.73 ± 0.78	1.94 ± 0.62	1.59 ± 0.71	0.53 ± 0.53	−0.040	0.087	ns	ns	ns
benzonitrile	0.98 ± 0.62	1.93 ± 0.61	1.09 ± 0.70	0.94 ± 0.59	0.020	0.021	ns	ns	ns
beta-cubebene	0.52 ± 0.52	1.06 ± 0.67	1.70 ± 0.76	0.94 ± 0.60	−0.057	−0.148	ns	ns	ns
beta-farnesene	0.00 ± 0.00	2.59 ± 0.53	0.54 ± 0.54	1.82 ± 0.82	−0.280	−0.133	0.004	ns	ns
2-beta pinene	2.27 ± 0.72	2.62 ± 0.54	3.38 ± 0.16	3.24 ± 0.07	0.027	−0.294	ns	ns	ns
3-butenenitrile	1.33 ± 0.84	2.08 ± 0.93	0.66 ± 0.66	1.42 ± 0.89	−0.069	0.066	ns	ns	ns
cadinene	1.38 ± 0.62	1.36 ± 0.61	1.46 ± 0.65	0.46 ± 0.46	0.042	0.082	ns	ns	ns
camphene	3.07 ± 0.13	2.97 ± 0.27	3.19 ± 0.16	3.33 ± 0.13	−0.089	−0.157	ns	ns	ns
delta-3-carene	2.71 ± 0.56	3.33 ± 0.20	2.87 ± 0.59	3.46 ± 0.20	−0.086	−0.090	ns	ns	ns
dimethyl disulfide	0.00 ± 0.00	0.00 ± 0.00	1.72 ± 1.09	2.42 ± 1.08	0.012	−0.353	ns	0.01	ns
dimethyl trisulfide	1.54 ± 0.69	2.73 ± 0.57	3.43 ± 0.18	3.34 ± 0.11	−0.095	−0.474	ns	0.01	ns
dimethylnonatriene	0.79 ± 0.79	5.17 ± 0.15	1.48 ± 0.93	5.12 ± 0.21	−0.499	−0.293	<0.0001	ns	ns
junipene	3.29 ± 0.13	3.27 ± 0.11	3.33 ± 0.17	3.29 ± 0.10	0.088	−0.033	ns	ns	ns
limonene	3.24 ± 0.66	3.91 ± 0.20	4.09 ± 0.17	3.99 ± 0.21	0.003	−0.269	ns	ns	ns
linalool	0.00 ± 0.00	1.58 ± 0.72	0.80 ± 0.50	0.84 ± 0.53	−0.115	−0.177	ns	ns	ns
m-cymene	1.11 ± 0.70	1.34 ± 0.85	0.59 ± 0.59	1.89 ± 0.84	−0.02	0.027	ns	ns	ns
methyl thiocyanate	1.08 ± 0.69	1.54 ± 0.69	0.56 ± 0.56	1.07 ± 0.67	−0.087	0.087	ns	ns	ns
thujone	1.74 ± 0.59	1.63 ± 0.73	2.25 ± 0.72	1.56 ± 0.70	0.082	−0.049	ns	ns	ns
trans-caryophyllene	4.09 ± 0.27	3.6 ± 0.55	2.7 ± 1.23	4.03 ± 0.46	0.046	0.046	ns	ns	ns
trimethyl tridecatetraene	2.53 ± 0.80	4.09 ± 0.11	2.97 ± 0.60	3.42 ± 0.69	−0.126	−0.051	ns	ns	ns

chemical profiles in *B. nigra* plants. More studies are required to understand the mechanisms mediating this differentiation by the female parasitoid.

C. glomerata preferentially attacked caterpillars on plants where *D. radicum* larvae were absent. In the time allowed for foraging, hosts on 65% of the plants had parasitization, and more that two thirds of those plants were root-undamaged. It is known that parasitoids only spend certain amounts of time in host patches before 'moving on', depending on the profitability of the patch (Waage 1979, Vet et al. 1995, Vos et al. 1998). In a confined setting, it is likely that if the experimental time had been extended most or even all of the plants could have been visited and every host parasitized. Our aim was to allow the parasitoids to forage just long enough to determine whether plants with or without root herbivores were preferred.

Because many parasitoids, including *C. glomerata*, are quite specialized and have limited host ranges, they have unquestionably evolved highly efficient mechanisms to perceive chemical cues that are associated with high quality hosts (Geervliet et al. 1998). Our study provides clear evidence of this adaptive trait in *C. glomerata*. However, when testing plant preference in the flight-cage, the parasitoid did not distinguish between root-damaged and root-undamaged plants when its host was present in both plants (LH vs LH + RH). The CDA plot (and the low Mahalonobis distance) clearly illustrate that the volatile blend of the root-damaged plants with hosts (LH + RH plants) is fairly similar and partially overlaps with the volatile blend of the root-undamaged plants with hosts (LH plants). It is possible that the enclosed environment of the flight-cage leads to less contrasting odour-sources which can affect plant preference by parasitoids (Mumm and Hilker 2005) or may have caused volatile interference. The latter may be expected when the volatile compounds of plants with hosts that are responsible for attraction of parasitoids show overlap with volatile compounds released by other plant/plant-herbivore association (Vos et al. 2001), and this may be enhanced in an enclosed environment. Alternatively, it is possible that *C. glomerata* females decided not to oviposit in hosts on plants with root herbivory, although they landed on them but we did not study this foraging decision. More studies are clearly required to further understand the indirect interactions between belowground organisms and parasitoids of aboveground herbivores, and the mechanisms mediating the interactions.

Previous studies showed that the influence of mutualistic (arbuscular mycorrhizal fungi) organisms as well as of root herbivores enhanced the intensity of aboveground tritrophic interactions, by increasing the

percentage of parasitism of leaf herbivores feeding on plants exposed to soil organisms (Masters et al. 2001, Gange et al. 2003, Poveda et al. 2005). In our study we clearly show that influences of interactions in the soil domain may also result into completely different complex aboveground processes. Here, the decision of *C. glomerata* to avoid root infested plants points to a preference-performance correlation, since parasitoid off spring performs better in root uninfested than in root infested plants (Soler et al. 2005).

It is important to note however, that for *D. radicum* and related species the effects of root herbivory can not be fully separated from the effects of microorganisms directly and indirectly associated with them. It is reported that *D. radicum* possesses microorganisms in the alimentary tract that contribute to host nutrition directly as a food source as well as by providing increased digestive potential (Lukwinski et al. 2006). Moreover, the damage caused in the roots as a consequence of feeding provides a point of entry for subsequent infection by endemic root rot pathogens (Soroka et al. 2004). The gram-bacilli symbiotic bacteria found in the root herbivore gut as well as the rot root associated microorganisms may also influence the plant defense system being responsible for inducing the release of certain compounds in the volatile blend of the plant, or might even directly emit specific volatile compounds. Little is know however, about interactions between root herbivores and their associated microorganisms, and the role that this may play in aboveground interactions. Further studies are required to better understand the mechanisms mediating these complex interactions.

Our study shows that root herbivores can influence the behaviour of a third trophic level aboveground, as mediated by changes in plant volatiles of the shared host plant. Changes in the plant-volatile blend induced by root feeding insects may alert the aboveground parasitoids about the presence of the root herbivores on the host plant, which has potentially negative consequences for offspring fitness of the parasitoid. We conclude that understanding the ecology and evolution of above-belowground interactions requires an in-depth understanding of behavioural choices and preferences of the key players in the community. Further studies conducted in the field under more heterogeneous conditions and over longer time frames are needed in order to identify the above and belowground processes that maintain community structure and stability.

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