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LETTER

Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies

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Abstract

Herbivore-damaged plants emit volatile organic compounds that attract natural enemies of the herbivores. This form of indirect plant defence occurs aboveground as well as belowground, but it remains unclear how simultaneous feeding by different herbivores attacking leaves and roots may affect the production of the respective defence signals. We employed a setup that combines trapping of volatile organic signals and simultaneous measurements of the attractiveness of these signals to above and belowground natural enemies. Young maize plants were infested with either the foliar herbivore *Spodoptera littoralis*, the root herbivore *Diabrotica virgifera virgifera*, or with both these important pest insects. The parasitic wasp *Cotesia marginiventris* and the entomopathogenic nematode *Heterorhabditis megidis* were strongly attracted if their respective host was feeding on a plant, but this attraction was significantly reduced if both herbivores were on a plant. The emission of the principal root attractant was indeed reduced due to double infestation, but this was not evident for the leaf volatiles. The parasitoid showed an ability to learn the differences in odour emissions and increased its response to the odour of a doubly infested plant after experiencing this odour during an encounter with hosts. This first study to measure effects of belowground herbivory on aboveground tritrophic signalling and *vice-versa* reemphasizes the important role of plants in bridging interactions between spatially distinct components of the ecosystem.

Keywords

Above- and belowground, entomopathogenic nematodes, herbivory, indirect plant defence, parasitoids, plant–insect interactions, tritrophic interactions.

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INTRODUCTION

Plants are the key organisms that bridge above- and belowground subsystems into complex multitrophic environments (Wardle 2002; Blossey & Hunt-Joshi 2003; Strauss & Irwin 2004; Wardle *et al.* 2004), where herbivores, pathogens and mutualists have been identified as major drivers of plant diversity and ecosystem functioning (De Deyn *et al.* 2003). The interdependence of above- and belowground interactions, which are usually studied separately, has been acknowledged (van der Putten *et al.* 2001), but their joint effects have rarely been taken into account (Schroter *et al.* 2004; Poveda *et al.* 2007). Studies linking the two spatially separated systems, where above- and belowground herbivores share a common host plant, have recently emerged, and this work has focussed on how interactions at one level affect resistance at the other level (van Dam *et al.* 2004, 2005; Bezemer & van Dam 2005; Schwachtje *et al.* 2006). Indeed, it has been hypothesized that above- and belowground herbivores have strong potential to influence each other because of plant allocational changes in primary and secondary metabolites, the instruments of plant resistance and defence, including indirect defences (van der Putten *et al.* 2001; van Dam *et al.* 2003; Bezemer & van Dam 2005; Soler *et al.* 2007).

Ever since Price *et al.* (1980) popularized the tritrophic concept, which recognizes the important role of plants in mediating interactions between herbivores and their natural enemies, a vast number of studies have examined tritrophic interactions in aboveground systems (Dicke *et al.* 2003;

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Turlings & Wäckers 2004). Recently, it was found that such interactions also occur belowground among plants, root feeders and their parasites (van Tol *et al.* 2001; Boff *et al.* 2002; Neveu *et al.* 2002; Aratchige *et al.* 2004; Rasmann *et al.* 2005), adding another level to the recognized need for studies on plant-mediated cross effects between above- and belowground communities (van der Putten *et al.* 2001; Poveda *et al.* 2007). A few studies have considered effects of belowground herbivory on aboveground tritrophic interactions (Bezemer *et al.* 2005). Positive effects of root herbivory have been found in terms of increased aboveground production of extra-floral nectar in cotton plants (Wackers & Bezemer 2003) and enhanced recruitment of parasitoids (Masters *et al.* 2001; Poveda *et al.* 2005). In contrast, Soler *et al.* (2005) found that insect feeding on roots of black mustard plants negatively affected aboveground parasitoids and hyperparasitoids. In this latter system, *Cotesia glomerata*, a parasitoid of leaf-feeding *Pieris* larvae also shows reduced attraction to plants that have received root damage, which is correlated with an altered pattern of herbivore-induced leaf volatiles (Soler *et al.* 2007). How aboveground herbivory may affect belowground tritrophic interactions remains unstudied.

Information on such cross effects, especially when plants are simultaneously attacked by above- and belowground herbivores, is pertinent if we wish to understand the extent to which the belowground multitrophic community may affect the interactions among aboveground communities and *vice versa* (Schroter *et al.* 2004; Poveda *et al.* 2007). These plant-mediated cross-effects may depend on signal interactions or resource allocational changes within the plant. Signal interactions occur when induced responses by one herbivore modify responses induced by a later feeding herbivore (Bostock 1999; Kahl *et al.* 2000; Rodriguez-Saona *et al.* 2005; Stout *et al.* 2006) and are likely to be different from effects observed when multiple herbivores and/or pathogens attack the same plant tissues (e.g. Shiojiri *et al.* 2001; Cardoza *et al.* 2003; Rostás *et al.* 2006). Similarly, allocational changes following root herbivory frequently change water and nutrient intake, thus modifying the resource budget of the whole plant (Gange & Brown 1989; Ohgushi 2005). Differences in sink strengths between roots and shoots may also critically affect the intensity with which they respond to herbivory (Orians & Jones 2001; van Dam *et al.* 2004). Moreover, partitioning of resources among roots and shoots can shift dramatically upon herbivory (Schwachtje *et al.* 2006), which is likely to lead to considerable changes in the induced production of plant secondary compounds.

In the current study, we used interconnected above- and belowground six arm olfactometers to investigate how simultaneous belowground and aboveground herbivory on maize may affect tritrophic signals from roots and leaves. Maize (*Zea mays* L.) has been studied extensively for caterpillar-induced volatile emissions from leaves that are attractive to parasitoids of the caterpillars (Turlings *et al.* 1990, 1998, 2002), and maize roots have recently been shown to respond to insect feeding damage with the production of the sesquiterpene (*E*)-β-caryophyllene, which attracts entomopathogenic nematodes (Rasmann *et al.* 2005). Our novel olfactometer setup allowed us to simultaneously compare the attraction of a parasitoid and an entomopathogenic nematode to plants infested with only a foliar herbivore, plants infested with only a root herbivore, and plants that were infested with both. Volatiles emitted by the leaves and roots were sampled and analysed for identification and quantification, thus revealing the impacts of double infestation on signal production and the consequences of such effects on attractiveness of the plant to the natural enemies of the herbivores.

MATERIALS AND METHODS

The study system

The system comprised maize plants (*Zea mays*) of the variety Delprim, the aboveground herbivore *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), the belowground herbivore *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomellidae) and as natural enemies of the herbivores the generalist endoparasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) and the entomopathogenic nematode *Heterorhabditis megidis* Poinar, Jackson and Klein (Heterorhabditidae). *S. littoralis* eggs were supplied weekly by Syngenta (Stein, Switzerland) and emerging larvae were reared on a maize-based artificial diet also furnished by Syngenta. Second instars *S. littoralis* larvae were used to rear the generalist endoparasitoid *C. marginiventris* as described in Turlings *et al.* (2004). Adult wasps were supplied with water and honey and were kept in incubators (25 °C; 16 L : 8 D) until the experimental day. *D. v. virgifera* larvae were obtained form CABI Bioscience (Delémont, Switzerland). The nematodes were provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland) and were kept in Ringer solution using culture flasks (Fisher Scientific AG, Wohlen, Switzerland) at 5 °C.

Maize seeds were sown in plastic pots (10 cm diam., 7 cm. deep) with fertilized commercial soil (Balkoneerde, Coop, Switzerland) and placed in a climate chamber (16 L : 8 D, 25 000 lm m^{-2}). Plants used for the experiments were 10–12 days old and had three fully developed leaves. Four days prior to the experiments, plant roots were gently washed and plants were transplanted in glass pots that could be connected to the olfactometer (see below for details). The pots contained moist white sand (10% water). Five such pots were prepared, three for the olfactometer and two that would serve as 'training' plants for the wasps.

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All pots were then transferred under light banks (16 L : 8 D, 8000 lm m^{-2}) and kept at 21 ± 2 °C.

Three days before the olfactometer experiments, four second-instar *D. v. virgifera* larvae were added to two of the olfactometer pots with a plant. The evening prior to the experiments, 20 s-instar *S. littoralis* larvae (3–5 days old) were placed in the whorl of the youngest maize leaf of all plants except for one of the experimental plants infested with *D. v. virgifera*. In this way, we obtained one plant infested with only *D. v. virgifera*, one plant only with *S. littoralis* and one plant infested by both herbivores. For wasp 'training' we had prepared similar pots with one plant with only *S. littoralis* and one plant with both herbivores. The glass pots were attached to glass vessels to keep larvae from escaping and to connect the odour sources to the aboveground olfactometer (Fig. 1). The system was assembled the day before responses of parasitoids and nematodes were tested. The experiments were replicated on 12 days, using new plants, insects and nematodes on each of the experimental days.

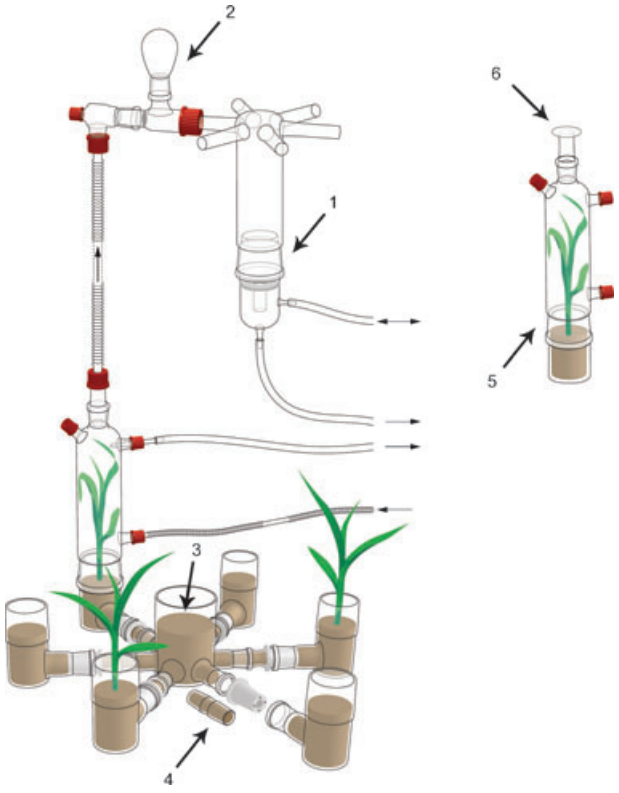

Figure 1 Schematic representation of the above- and belowground olfactometers connected together (only one arm out of six is shown). (1) Wasp release point; (2) wasp trapping bulb; (3) nematode release point; (4) nematode collection tube; (5) odour source vessel; (6) wasps training tube. The small arrows represent air flows (see text for further explanation). Drawing by Thomas Degen. For further details see Turlings *et al.* (2004), and Rasmann *et al.* (2005).

The tested wasps were divided into three groups: NAIVES, no oviposition experience; EXPS, wasps that experienced three to five ovipositions in 2–4 days old *S. littoralis* larvae, while they perceived the odour from a plant attacked by *S. littoralis* only; and EXPSD, wasps that experienced three to five ovipositions in the presence of the odour from plants that were simultaneously attacked by *S. littoralis* and *D. v. virgifera*. To give them experience, the wasps were introduced into a tube (3 cm height, 2.5 cm diameter) with 10 host larvae. The tube was attached to the top opening of one of the vessels containing an infested plant, and wasps were prevented from entering the odour vessel by a nylon screen (Fig. 1). After three to five ovipositions, wasps were considered experienced.

During each of the 12 experimental days, two groups of three wasps of each treatment (naïve, ovipositions in the presence of singly infested plants, ovipositions in the presence of doubly infested plants) were released alternately into the olfactometer. After each release, the six wasps were allowed to choose between the odours for 30 min, after which wasps were recovered from the trapping bulbs of the aboveground olfactometer and their choices were recorded. In a previous study, it was shown that releasing the wasps in groups has no effect on each other's choices (Turlings *et al.* 2004). Wasps were only used once.

The release of about 2000 2-week old infective juveniles of the entomopathogenic nematode *H. megidis* in the centre pot of the belowground olfactometer occurred around 9:00 h on the same day of the wasp releases. Twenty-four hours after release of the nematodes, the olfactometer system (see below) was disassembled and the sand in each detachable glass connector tube was placed on a separate cotton filter disk (19 cm diam.; Hoeschele Gmbh, Remshalden, Germany). The disks were then placed in a Bearmann extractor (Hass *et al.* 1999), and nematodes were counted the next day. Roots of the plants were then collected, water washed and frozen in liquid nitrogen for further analyses (see below).

Olfactometer set-up

For all experiments, an above- and a belowground olfactometer were connected together and run simultaneously (Fig. 1). For each experiment, a plant attacked by the aboveground herbivore, a plant attacked by the belowground herbivore and a plant attacked by both herbivores were placed each in an odour source vessel and connected to the system. These three treatment vessels were alternated with three control vessels, which only contained sand in the bottom pot. For each of the 12

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replicates, the treatments were positioned randomly around the centre of the olfactometers.

The belowground olfactometer, which was used to test the attractiveness of entomopathogenic nematodes toward infested maize roots, connected the six vessels via their bottom glass pots (5 cm diam., 11 cm deep) to a central glass pot (8 cm diam., 11 cm deep) by glass tubes (8 cm long; 24/29 male connectors on both sides). The glass tubes and the treatment pots were each connected by an additional Teflon connector tube that contained a ultra-fine meshed metal screen (2300 mesh; Small Parts Inc., Miami Lakes, FL, USA), which prevented the nematodes from entering the odour source pots (for more details see Rasmann *et al.* 2005). All pots and tubes contained moist (10% water) white sand (Migros, Switzerland), allowing passive diffusion of chemical substances from the treatment pots to the centre pot. The top of each glass vessel was connected via a 50/55 male ground connector to a female ground glass connector of the aboveground olfactometer. The aboveground six arm olfactometer (Turlings *et al.* 2004) was used to test the attractiveness of the wasps towards the treatment plants, and, simultaneously, collecting the volatiles emitted by the plants. The six odour vessels were connected to an air supply just above the sand and 1.2 L min^{-1} of purified air was pushed into each vessel. Half of this airflow (0.6 L min^{-1}) was pulled out of the vessel through a trap containing Super-Q adsorbent (25 mg, 80/100 mesh; Alltech, Deerfield, IL, USA), which was attached to the vessel at plant height. The other half of each airflow was pushed and pulled via Teflon tubes into the upper part of the olfactometer, where the 6 air streams entered a central chamber in which the wasps were released. This release chamber was connected via a Tygon tube to a water-filled glass U-tube that served as a pressure gauge to balance incoming and outgoing air, minimizing pressure differences with the outside (Turlings *et al.* 2004).

Volatile analysis

During each bio-assay, aboveground volatiles were collected for 4 h. The super-Q traps were then extracted with 150 μL of dichloromethane (Merck, Dietikon, Switzerland), and two internal standards (*n*-octane and nonyl-acetate, each 200 ng in 10 μL dichloromethane) were added. The traps were washed with 3 mL of dichloromethane before reusing them for a next collection. The samples were either analysed immediately or stored at −70 °C before analysis. Samples were analysed with an Agilent 6890 Series gas chromatograph equipped with an automated column injection system (G1530A), coupled to a mass spectrometer operated in electron impact mode (Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA, USA); transfer line 230 °C, source 230 °C, ionization potential 70 eV, scan range 33–280 amu). A 3 μL aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 μm film thickness, Alltech Associates, Inc., Deerfield, IL, USA). Helium at constant pressure (18.55 psi) was used as a carrier gas flow. Following injection, the column temperature was maintained at 40 °C for 30 min and then increased at a rate of 8 °C min^{-1} to 250 °C. Mass spectra were compared with those of the NIST02 library, and by comparison of retention times with those from previous analysis (Hoballah *et al.* 2002; D'Alessandro & Turlings 2005), and where necessary, spectra and retention times were compared with those of authentic standards. Compounds that were not identified by comparing retention times and spectra with those of authentic standards are labelled with a superscript ^{N} in the text, and their identification should be considered tentatively. The detected volatiles were quantified based on a comparison of their peak areas with those of the internal standards (*n*-octane for compounds 1–14, *n*-nonyl acetate for compounds 15–26).

To measure the production of the belowground volatile (*E*)-β-caryophyllene analysis, roots of maize plants were, after each experiment, washed with water and then frozen in liquid nitrogen. The frozen roots were pulverized in a mortar and 0.3 g powder was placed in a glass vial (20 mL) with a septum in the lid. A 100 μm PDMS solid phase micro extraction (SPME; Supelco c/o Sigma-Aldrich Chemie GmbH Buchs, Switzerland) fibre was inserted through the septum and exposed for 60 min at 40 °C. The compounds adsorbed onto the fibre were analysed by placing it for 5 min into the injector port of a gas chromatograph heated at 230 °C, and coupled to the quadrupole type mass selective detector described above. Immediately after inserting the fibre the sample was pulse injected onto an apolar HP-1 column. Helium at constant pressure (18.55 psi) was used as carrier gas flow. Following injection, the column temperature was maintained at 50 °C for 3 min and then increased to 180 °C at 5 °C min^{-1} followed by a final stage of 3 min at 250 °C. For approximate quantification we obtained calibration curves by spiking 0.3 g of powdered root tissue from healthy maize (Delprim variety) with known amounts (0, 4.5, 9.0, 45, 90 and 200 ng) of (*E*)-β-caryophyllene and used the same SPME method to measure emissions.

Statistical analysis

Wasps and nematodes behavioural responses to the different odour sources offered in the above- and belowground six-arm olfactometers were examined using a log-linear model. The responses consist of counts of the

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number of wasps/nematodes choosing any of the six possible arms of the olfactometers for every release of six wasps or 2000 nematodes, which have been shown to follow a multinomial distribution (Ricard & Davison 2007). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasilikelihood functions to compensate for the overdispersion of wasps/nematodes within the olfactometer [see Turlings *et al.* (2004) for a detailed explanation]. The model was fitted by maximum quasi-likelihood estimation in the software package R (http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings *et al.* 2004). With the same model, we also tested for interaction between experience (i.e. wasps that had previous experience on plants attacked by *S. littoralis* only vs. plants attacked by both herbivores), and treatments (i.e. odour sources coming from plants attacked by *S. littoralis* only, and plants attacked by both herbivores). To test the effect of pseudo-replication due to releasing two groups of wasps of the same treatment on the same day, the parameter 'release' was added to the model. The amounts of volatiles collected from the different treatment plants (i.e. plants infested with *S. littoralis*, and plants infested with *D. v. virgifera* and *S. littoralis*), were compared with a paired *t*-test analysis (SigmaStat, version 2.0, Systat Software, Inc., San Jose, CA, USA) for every single volatile compound separately.

RESULTS

Aboveground

The wasps' responsiveness was high, with > 80% of the wasps of each experience type entering an arm of the olfactometer. The wasps were not attracted by the odour from plants damaged by *Diabrotica* only, but were in all cases significantly attracted by the odour from plants damaged by *S. littoralis*, and this was consistent for all experience types (Fig. 2). Naive wasps were more attracted to the odour of a plant attacked only by *S. littoralis* than the odour of a plant attacked by both herbivores ($P = 0.01$). This was also the case for wasps that were experienced in the presence of the odour infested only by *S. littoralis* ($P = 0.039$). However, when wasps were experienced in the presence of the odour produced by a doubly infested plant (EXPSD), they shifted their preference in favour of this odour ($P = 0.013$). The analysis of the interaction between type of experience and odour source confirmed the shift in the wasps' choice ($P = 0.0001$). Analyses of the volatiles collected from the aboveground parts revealed that plants attacked by *D. v. virgifera* only did not emit any detectable amounts of induced volatile organic compound (Fig. 3). In contrast, the plants infested by *S. littoralis* emitted considerable amounts of the typically induced compounds. Plants infested by *S. littoralis* only and plants infested by both herbivores produced a very

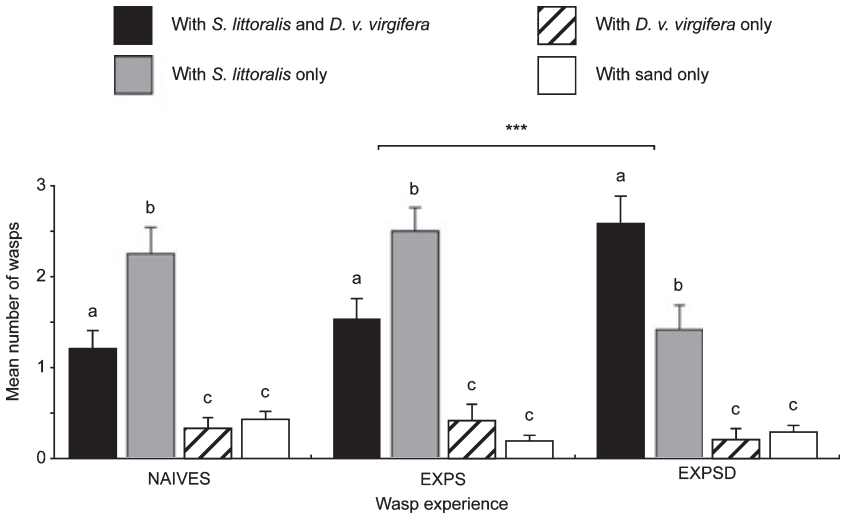

Figure 2 Mean (± SE) number of wasps choosing one of the treatment arms; i.e., a vessel containing only humidified sand (sand only), a vessel containing plants attacked by four *D. v. virgifera* larvae only (*D. v. virgifera.* only), a vessel containing plants attacked by 15 *S. littoralis* caterpillars (*S. littoralis* only) and vessels containing plants attacked by both herbivores (*S. littoralis* and *D. v. virgifera*). Tested wasps were either NAIVES, no previous oviposition experience; EXPS, three to five ovipositions in presence of odours coming from plants attacked by *S. littoralis* only; and EXPSD, three to five ovipositions in presence of odours coming from plants attacked by both herbivores. Different letters above bars indicate significant differences in the number of wasps choosing the odour of a treatment for a given experience ($P < 0.05$, $n = 12$). Asterisks indicate significant effect of interaction ($P < 0.0001$).

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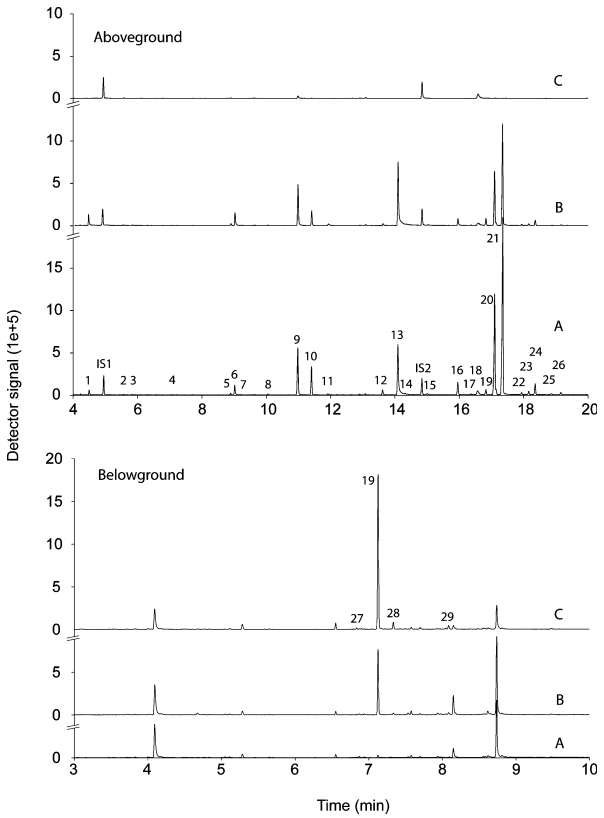

Figure 3 Representative examples of GC-MS chromatograms of volatiles collected from 10-days old maize seedlings. (Belowground) SPME analysis of pulverized roots. (Aboveground) Analysis of Super-Q filter extract from 4 h headspace collection of maize leaves (see Materials and methods for details). (A) Seedlings infested with 15 *S littoralis* larvae. (B) Seedlings infested with 15 *S littoralis* and 4 *D. v. virgifera* larvae. (C) Seedlings infested with 4 *D. v. virgifera* larvae. Labeled compounds are: (**1**) (*Z*)-3-hexenal, (**2**) (*E*)-2-hexanal, **(3)** (*Z*)-3-hexanol, (**4**) (*Z*)-2-penten-1-ol acetate^{N}, (**5**) β-myrcene, (**6**) (*Z*)-3-hexenyl-acetate, (**7**) (*E*)-2-hexenyl acetate, (**8**) (*Z*)-β-ocimene, (**9**) linalool, (**10**) (*3E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (**11**) benzyl acetate, (**12**) phenetyl acetate, (**13**) indole, (**14**) unknown, (**15**) methyl anthranilate, (**16**) geranyl acetate, (**17**) unknown, (**18**), unknown, (**19**) (*E*)-β-caryophyllene, (**20**) (*E*)- α-bergamotene, (**21**) (*E*)-β-farnesene, (**22**) unknown sesquiterpenoid, (**23**) unknown sesquiterpenoid, (**24**) β-sesquiphellandrene^{N}, (**25**) (*E*)-nerolidol, (**26**) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), (**27**) (-)-α-copaene^{N}, (**28**) α-humulene^{N}, (**29**) caryophyllene-oxide^{N}. **IS1** and **IS2**, internal standards (*n*-octane and nonyl-acetate). Compounds with superscript N were only tentatively identified (see text), $n = 12$.

similar pattern of volatiles, and quantitative analysis of 26 major compounds showed no difference between the two treatments (Fig. 4). A more detailed analysis of the volatile blend revealed another 19 minor peaks present in all replicates of both treatments. Quantitative analysis of these peaks also showed no difference between the plants attacked by *S. littoralis* only and plants attacked by both herbivores (data not shown).

Belowground

The choices of *H. megidis* nematodes were clearly affected by the aboveground herbivore (*S. littoralis*; Fig. 5). The number of nematodes choosing an arm with a doubly infested plant was 43% lower than those choosing an arm containing a plant damaged by *D. v. virgifera* only ($P = 0.0044$). When a doubly infested arm was compared to an arm containing a plant attacked by the aboveground herbivore only or to the controls containing only sand, no difference was found ($P = 0.38$ and 0.21, respectively). Finally, there was no difference between the number of nematodes choosing the arm connected to a pot with a *Spodoptera*-infested plant and arms connected to the controls ($P = 0.87$).

(*E*)-β-Caryophyllene was only detected in the SPME analysis of plants infested with *D. v. virgifera* alone and plants infested with both herbivores, but not in the analyses of healthy plants or those with *S. littoralis* only (Fig. 3). Using quantification curves obtained from healthy maize root material spiked with pure (*E*)-β-caryophyllene (Sigma-Aldrich Chemie GmbH Buchs, Switzerland), a pair-wise comparison between plants attacked by *D. v. virgifera* only and plants attacked by both herbivores confirmed a negative effect of *S. littoralis* on the production of (*E*)-β-caryophyllene (Fig. 6, $P = 0.036$).

DISCUSSION

The double infestation of the maize plants negatively affected the attractiveness of the maize plants to the parasitoid *C. marginiventris* and the nematode *H. megidis*. Such cross effects between above- and belowground tritrophic interactions add a new level of complexity to the highly variable signals that these natural enemies are confronted with.

Parasitoid attraction to herbivore-induced plant volatiles have been studied extensively (Vet & Dicke 1992; Dicke 1994; Turlings & Wäckers 2004), whereas the role of plant signals in belowground tritrophic interactions has only been recently demonstrated (van Tol *et al.* 2001; Boff *et al.* 2002; Neveu *et al.* 2002; Aratchige *et al.* 2004; Rasmann *et al.* 2005). In the current study, the emission of the key nematode attractant (*E*)-β-caryophyllene was reduced when *Spodoptera* larvae in addition to *D. v. virgifera* larvae attacked the maize plants. The drop in emission of the sesquiterpene is reflected in a highly reduced nematode attraction (Fig. 5). Although it is clear from these results that simultaneous root and leaf feeding can have important consequences for plant-mediated tritrophic interactions, it should be noted that the

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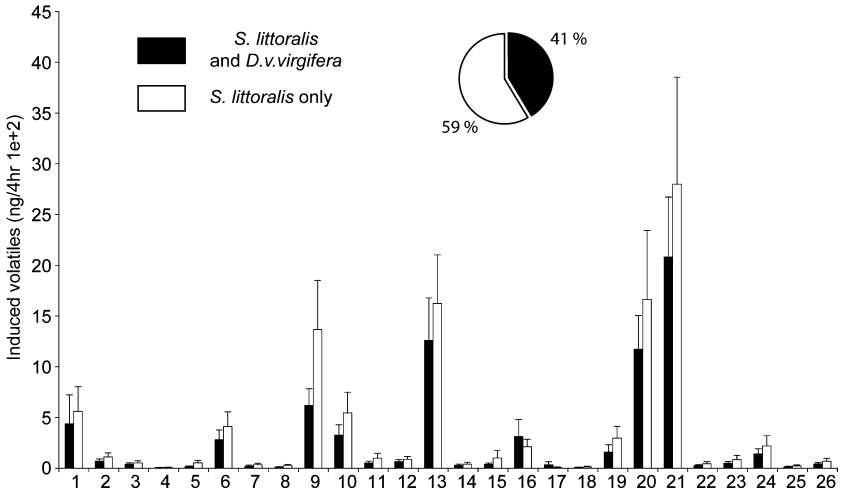

Figure 4 Mean (± SE) amount of volatiles collected from the leaves of plants attacked by *S. littoralis* only and plants attacked by both herbivores (*S. littoralis* and *D. v. virgifera*,). For the complete list of identified compounds **1**–**26** refer to Fig. 3. (n = 12).

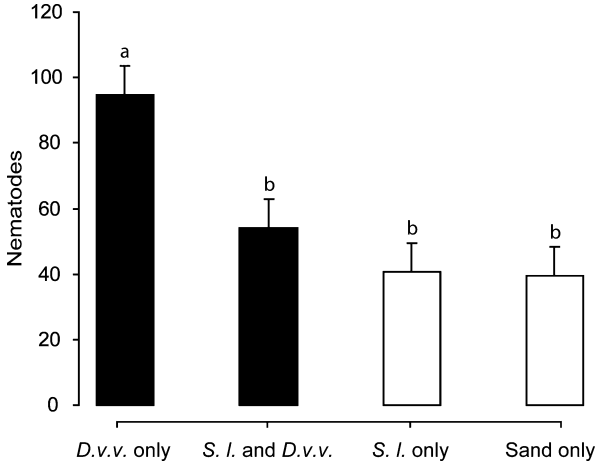

Figure 5 Mean (± SE) number of nematodes recovered from the six treatment arms of the belowground olfactometer; containing a plant attacked by 4 *D. v. virgifera* larvae (*D. v. v.* only), a plant attacked by 20 *S. littoralis* and 4 *D. v. virgifera* (*S. l.* and *D.v.v.*), a plant attacked by 20 *S. littoralis* (*S. l.* only), and humidified sand only (Sand only). The 'Sand only' bar is obtained by averaging the mean number of nematodes choosing the three arms containing only sand. Different letters above bars indicate differences in the choices made by the nematodes for the different treatments (n = 12, $P < 0.05$).

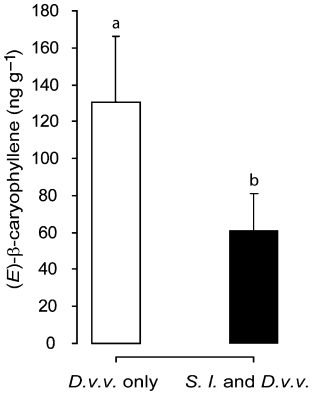

Figure 6 Mean (± SE) amount of (*E*)-β-caryophyllene collected from freshly frozen maize roots from plants attacked by 4 *D .v. virgifera* larvae, and form plants attacked by both herbivores (i.e. 20 *S. littoralis* and 4 *D. v. virgifera*). Different letters above bars indicate differences in the quantities between treatments ($P < 0.05$, n = 8).

effects might be different if the order in which the insects feed on the plant is reversed or at different time points after feeding commences.

The mechanistic reason for the altered emissions remains to be determined. Masters *et al.* (1993) propose that foliar herbivory reduces plant growth belowground, thereby limiting quality and quantity of belowground tissues and decreasing the production of root exudates and CO_2. A recent study with tobacco plants showed that leaf feeding causes translocation of sugars from leaves to roots, possibly to allow the plant to tolerate and recover from the damage (Schwachtje *et al.* 2006). Such shifts in resource allocation may result in physiological constraints, which could explain the observed reduction in (*E*)-β-caryophyllene emission, but other explanations should be considered. For instance, a systemic defence reaction in the plant in response to *S. littoralis* feeding might deter *D. v. virgifera* larvae and thus reduce their feeding rate and signal induction. Indeed, induction of one plant part may promote overall resistance (van Dam *et al.* 2003), although induced resistance against one herbivore does not automatically result in enhanced resistance against others (Agrawal *et al.* 1999; Walling 2000; Rostás *et al.* 2003).

Alternatively, if the emissions are costly, plants confronted with two herbivores may 'choose' to invest more in one

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defence strategy than in the other. In general, the costs of inducible defences are expected to be relatively low (Gerhenzon 1994; Hoballah *et al.* 2004), but physiological costs may increase in a multi enemy context, where there might be direct competition for the products of induction when differences in sink strength between organs affect the distribution of induced compounds above- and belowground (van Dam *et al.* 2004). A recent molecular study shows a strict shoot-root segregation of the inducible maize genes that are responsible for terpene synthesis (unpublished data). Only double infestation induces expression of the caryophyllene-synthase gene in both tissues. If resources needed for synthesis are limited, allocation shifts and an overall drop in production can be expected. If in the studied system there is an allocation 'decision' made by the plant as part of its optimal defence strategy, it appears that it favours the leaf signal.

Aboveground, the recruitment of *C. marginiventris* parasitoids by *S. littoralis*-damaged maize leaves was negatively affected by *D. v. virgifera* feeding on the roots, but this change in attractiveness was not reflected in the measured odour emissions (Fig. 3). There was a non-significant trend indicating that doubly infested plants released less of some compounds, but we may not have detected all relevant changes. Indeed, our recent work suggests that minor, non-detectable compounds may be essential for attraction of naïve *C. marginiventris* (D'Alessandro & Turlings 2005). A reduction in emissions contradicts the general assumption that root feeding leads to an increase of both direct and indirect defences aboveground (Birch *et al.* 1992; Karban & Baldwin 1997; Pieterse *et al.* 2002; Bezemer *et al.* 2004, 2005). For example, *Agriotes lineatus* larvae, when feeding on cotton roots, enhance the production of extrafloral nectar aboveground (Wackers & Bezemer 2003) and Baldwin *et al.* (1994) found that nicotine levels in either roots or shoots increase following damage to the other organs of tobacco plants. When looking at higher trophic levels, Masters *et al.* (2001) showed an increase in parasitoid numbers of seed predators of the marsh thistle (*Cirsium palustre*) when root herbivores were not removed, but this may have been correlated with the higher number of herbivores on plants and does not necessarily imply a change in signal emission. It has also been shown that root herbivory increases flower visitation by pollinators (Poveda *et al.* 2003, 2005), which may be explained by an increased induction of floral nectar following root feeding.

Associative learning is common among parasitoids and is assumed to allow them to focus their foraging efforts on plants that carry hosts in a particular area during a particular time (Turlings *et al.* 1993; Vet *et al.* 1995). Induced volatile emissions do not only differ between plant genotypes (Gouinguené *et al.* 2001; Krips *et al.* 2001; Degen *et al.* 2004) and as a result of feeding by different herbivores (De Moraes *et al.* 1998; Dicke & Vet 1999), but variability in the emissions is also influenced by abiotic factors such as temperature, light, UV-radiation (Gouinguené & Turlings 2002; D'Alessandro & Turlings 2005). Evidently, simultaneous infestation by multiple herbivores and/or pathogens may add to this variability and to the need for the parasitoids to learn. For instance, simultaneous infestation of cabbage plants by *Plutella xylostella* and *Pieris rapae* increased the attractiveness of infested plants to the parasitoid *Cotesia glomerata*, but decreased the attractiveness to *Cotesia plutellae* (Shiojiri *et al.* 2001), demonstrating that the consequences for the various interactions are species-dependent (Bezemer *et al.* 2005). Here naïve *C. marginiventris* females showed an innate preference for odours emitted by plants attacked by *Spodoptera* only, but when the wasps were given oviposition experiences while perceiving the odour from a doubly infested plant, their preferences shifted in favour of the latter. Although chemical analyses detected no clear differences in the odour profiles of these two treatments, the wasps' behaviour implies that there was a difference. It should be noted that *Spodoptera* species, *Diabrotica* species and *C. marginiventris* commonly occur together, in particular on maize and its wild ancestors in Mexico. Therefore, in the field, the wasp will be regularly confronted with the odours that were tested here and can be expected to have adapted to optimize the use of these odours. Root feeding by *D. v. virgifera* larvae did not result in detectable emissions of volatiles aboveground, nor does feeding by *S. littoralis* caterpillars induce the emission of volatiles belowground (Fig. 2). Yet, in a previous study, we did find minor amounts of (*E*)-β-caryophyllene in the leaf tissue of *Diabrotica*-damaged plants (Rasmann *et al.* 2005). Possibly wasps detect such minor changes and may avoid plants that they perceive as being infested by the 'wrong' herbivore or plants that have been rendered less suitable as a food source for their hosts due to the belowground infestation. However, once the wasps have encountered suitable hosts in the presence of the normally less attractive odour, they increase their responsiveness to that odour.

In the field, plants are constantly being exposed to aboveground and belowground herbivores and it has become evident that herbivores can influence each other through changes in the shared host plant (Bezemer & van Dam 2005). Here we show that such effects can be extended to herbivores influencing their respective natural enemies. An understanding of these cross effects will help to further elucidate the relationships between plants and their communities.

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