

First insights into specificity of belowground tritrophic interactions

Sergio Rasmann and Ted C. J. Turlings

S. Rasmann and T. C. J. Turlings (ted.turlings@unine.ch), *Evolutionary Entomology, Inst. of Biology, Univ. of Neuchâtel, Rue Emile-Argand 11 cp158, CH-2009 Neuchâtel, Switzerland. Present address for SR: Ecology and Evolutionary Biology, Cornell Univ., Corson Hall, Ithaca, NY 14853, USA.*

Tritrophic interactions involving plants, herbivores and parasites have been only recently documented for belowground systems, where entomopathogenic nematodes can exploit root herbivore induced volatile compounds to locate their hosts. Little is known, however, about whether the specificity of such interactions rivals that of the remarkable interactions found in aboveground studies. Using a belowground six-arm olfactometer that allows recording of nematode attraction, specificity of nine economically important species of different trophic levels, including plants, root feeders and entomopathogenic nematodes, was tested. We found that belowground tritrophic interactions are variable at the level of plant volatiles that are induced, elicitation by herbivores, as well as behavior of nematodes. We argue that studies on specificity and variability of belowground responses should be included in plant defense theories and in efforts to exploit tritrophic interactions to improve biological control practices.

The specificity of plant defensive traits can be of crucial importance in determining the ecological and evolutionary interactions among communities of insects interacting with plants (Karban and Baldwin 1997, Ohgushi et al. 2007). For example, specific defences may prevent the evolution of single plant strategies that are effective against all attackers (Maddox and Root 1990, Van Zandt and Agrawal 2004, Viswanathan et al. 2005). In addition, specificity may determine community structures by particular plant phenotypes attracting or repelling particular sets of organisms. In the context of tritrophic interactions, specificity may be generated from 1) constitutive plant traits that vary among species or genotypes, 2) induced responses generated by different herbivores species, or 3) different predators or parasitoids responding to different plant traits. Moreover, any one of these factors alone or in combination is likely to generate a temporally or spatially varying mosaic of specific interactions in the field.

When considering the specificity of herbivore-induced plant responses, it is important to distinguish between specificity in the elicitation of responses; i.e. the ability of the plant to generate distinct chemical responses to different damage types; and specificity of effect, i.e. the range of species affected by a given induced response (Karban and Baldwin 1997, Stout et al. 1998). Studies on the specificity of elicitation by arthropods and other stresses have revealed that plants possess the capacity to respond differentially to different biotic and abiotic challenges (Agrawal 2000, Viswanathan et al. 2005). Likewise, both herbivores and enemies of herbivores show a high level of specificity of

effect in their responses (De Moraes et al. 1998, Dicke 1999, Agrawal 2000, Van Zandt and Agrawal 2004, Viswanathan et al. 2005).

Although responses to herbivore induced volatile emissions are generally thought to be broad in their effects (Dicke and Vet 1999), this seems to be the exception rather than the rule (Takabayashi et al. 2006). For example, De Moraes et al. (1998) showed that the specialized parasitoid *Cardiochiles nigriceps* was able to discriminate between the odors emitted from plants attacked by its host, *Heliothis virescens*, compared to the odors coming from plants attacked by a closely related non-host herbivore, *H. zea*. Although this latter study is one of the most complete to date, in that it considers variation in plant and herbivore species, this topic is relatively unexplored and it remains largely unresolved how much specificity there is in tritrophic interactions.

The active role of plants in recruiting natural enemies of belowground herbivores has recently been demonstrated in a few plant species (van Tol et al. 2001, Neveu et al. 2002, Aratchige et al. 2004), but there is currently no information on the specificity of these interactions. Previously, we have shown that larvae of the leaf beetle *Diabrotica virgifera virgifera* feeding on maize roots (*Zea mays*) induce the production of an indirect defence signal (the sesquiterpene (E)- β -caryophyllene), which is attractive to the entomopathogenic nematode *Heterorhabditis megidis* in the laboratory and field (Rasmann et al. 2005). Here, we build on these findings and take a hierarchical approach to study the specificity of such belowground tritrophic interactions. We

examined variation at each of the three trophic levels (i.e. plants, root herbivores, and nematodes predators) separately. First, we compared the attractiveness of three plant species when damaged by the same herbivore species (specificity of plant species). Second, we studied whether three different herbivore species differentially induce a single plant species (specificity of elicitation). And finally, we tested if three different nematode species are differentially attracted to the same induced odour cue (specificity of effect). These experiments were conducted using a below-ground six arm olfactometer (Rasmann et al. 2005), which allowed simultaneous testing of the relative attractiveness of multiple odour sources to nematodes. In addition, at the end of each experiment, we collected volatiles from the roots of each plant for chemical analyses, allowing us to couple the underground plant volatile chemistry to the behaviour of entomopathogenic nematodes.

Material and methods

The three different trophic levels were investigated separately (details are provided below), using maize *Zea mays*, cotton *Gossypium herbaceum* and cowpea *Vigna unguiculata*; the beetles *Diabrotica virgifera virgifera*, *Diabrotica balteata*, *Agriotes usulatus* (Elateridae) and the phytopathogenic nematodes *Ditylenchus dipsaci* (Tylenchida) as herbivores. The entomopathogenic nematodes *Heterorhabditis megidis*, *Heterorhabditis bacteriophora* and *Steinernema feltiae* as parasites of herbivores.

Olfactometer assays

The belowground olfactometer consisted of a central glass chamber (8 cm in diameter, 11 cm deep) with six equally distributed side arms with a female (24 mm Ø and 29 mm long) connector on the tip of the arm (Rasmann et al. 2005). These arms connected the central chamber with six glass pots (5 cm Ø, 11 cm deep) in which plants or other sources of attractants could be placed. Each pot also had a female connector (29/32) at 0.5 cm height. The connecting arms consisted of two detachable parts; one was a glass tube with ground-glass connectors (male, 24/29) on both sides, and the second part, a Teflon connector (24/29 to 29/32) was used to attach the glass tube to the odour source pot. The custom-made Teflon connectors contained an ultra-fine metal screen (2300 mesh) preventing the nematodes from reaching the odour source pots (Rasmann et al. 2005). For each experiment, the entire system was filled with sterilized, moist (10% water) white sand to about 5 cm from the rim of the pots. Nematodes were released in a drop of water in the centre of the central pot. One day after nematode release, the olfactometer was disassembled and the sand in each detachable glass tube was placed on a separate cotton filter disk 19 cm in Ø. The disk with the sand was placed in a baermann extractor (Hass et al. 1999), and the next day, nematodes in the collection tube were counted under a microscope on a counting plate.

Comparison of plant species

Three plant species were used. Maize *Z. mays*, var. delprim, cotton *G. herbaceum*, and cowpea *V. unguiculata* var. kpodii-guegue (Hoballah et al. 2002). Plants were sown in plastic pots (7 cm high, 9 cm Ø) with fertilized commercial soil and placed in a climate chamber (16L:8D, 25 000 lm m⁻²). To obtain comparable biomasses maize plants used in the experiment were 10–12 days, whereas cotton and cowpea plants were 20–22 days old when they were used in the experiments. Three days before the olfactometer experiments, plant roots were carefully washed with water to remove the soil around the roots and the plants were then transplanted in the glass pots of the olfactometer (see below) with moist (10% water) white sand.

To compare induction among different plant species we choose to work with the generalist *D. balteata*, which readily feeds on the roots of the three plant species tested; cotton, cowpea and maize. Four 2nd instar *D. balteata* larvae were added to each root system of the experimental plants. In total, one plant of each species per olfactometer and three control pots containing only sand were prepared for each replicate. Simultaneously, the connector glass tubes of the olfactometer, covered by the Teflon connectors, were also filled with sand and connected to the previously prepared pots. The end of the connector tube and the top of the treatment pot were covered with aluminium foil to avoid desiccation. One day prior to the experiment, all the treatment pots were connected to the central chamber of the olfactometer, via the connector tubes, which were also filled with sand. The next day, about 2000 infective juvenile entomopathogenic nematodes *H. megidis*, were placed about 2 cm below the sand surface in the middle of the central chamber. Nematodes had been propagated in *Galleria mellonella* larvae. All tested nematodes were between 10 to 15 days old. Nematodes were left to choose for 24 h, after which they were recollected and counted as described above. Thus, in this experiment we only examined specificity of plant species responses, as both the herbivores and responding entomopathogenic nematode species were held constant. After larvae were removed from the plants, roots of the three plant species were washed with deionized water and frozen in liquid nitrogen. The roots were pulverized in a mortar and 0.3 g of the resulting powder was placed in a glass vial (20 ml) with a septum in the lid. A 100 µm PDMS solid phase micro extraction fiber was inserted through the septum and exposed for 60 min at 40°C. The compounds adsorbed onto the fiber were analyzed by placing the fiber for 5 min into the injector port of a gas chromatograph heated at 250°C, and coupled to a quadrupole type mass spectrometer operated in electron impact mode (transfer line 230°C, source 230°C, ionisation potential 70 eV, scan range 33–280 amu). Immediately after inserting the fiber the sample was injected onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness). Helium at constant pressure (18.55 psi) was used as carrier gas flow. Following injection, the column temperature was maintained at 60°C for 1 min and then increased to 250°C at 20°C min⁻¹ followed by a final stage of 12 min at 250°C.

Using the same root material, a supplementary analysis was done using another type of column (HP-5, 30 m, 0.25 mm ID, 0.25 μ m film thickness), and with a slower heating procedure to obtain better separation. After injection, the column temperature was maintained at 40°C for 3 min and then increased to 250°C at 8°C per min. Volatiles were identified by comparison of their mass spectra with those of the NIST02 library, and by comparison of retention times with those in previous analyses (Rasmann et al. 2005). Since no authentic standards, except for (E)- β -caryophyllene, were tested in the chromatograph, the following identifications should be considered tentative. Approximate quantification of (E)- β -caryophyllene from attacked maize plants was obtained by spiking 0.3 g of powdered root tissue from non-attacked plants with known amounts (0; 4.5; 9.0; 45; 90 and 200 ng) of pure (E)- β -caryophyllene.

Comparison of herbivore species

Here, we tested whether the entomopathogenic nematode *H. megidis* responds differentially to maize roots damaged by different herbivore species: the specialist western corn rootworm *D. v. virgifera* (as opposed to *D. balteata* in the previous experiment); the wireworm *A. ustulatus*, and the generalist phytopathogenic nematode *D. dipsaci*, all commonly known pests on maize (Toth et al. 2003). Second instar *D. v. virgifera* larvae were obtained from a rearing culture. *A. ustulatus* larvae were collected in maize fields of northern Italy (Venezia region) and kept in sandy soil until the experiment. *D. dipsaci* nematodes were obtained from an onions rearing facility. Nematodes were extracted from onions by decantation in water prior to the experiment.

Three days before each experiment, three 10–12 days old maize seedlings were transferred into the olfactometer glass pots as described above, with three pots again left open as a control. There were three treatments: either 1) four second instar *D. v. virgifera* larvae, 2) three eight to eleventh instar (Furlan 1998) *A. ustulatus* larvae, or 3) approximately 1000 *D. dipsaci* nematodes, added to the roots of an individual maize plant. The difference in the numbers of individuals of each species placed on the plants is a consequence of exploratory experiments, in which we assessed how to obtain comparable amounts of root damage. For the phytopathogenic nematodes *D. dipsaci* we used a dose of nematodes that is commonly found on attacked maize seedlings in the field (J. Grunder pers. comm.). The pots of the olfactometer were attached to the connector tubes and the ends of these tubes covered with aluminium foil as above. The olfactometer was assembled the day before the release of approximately 2000 *H. megidis* nematodes in the center of the central chamber, which were then left free to choose for 24 h. The next day, nematodes were extracted and counted, and the roots of the plants were collected and washed for SPME analysis as described above. The experiment was replicated 12 times.

Comparison of nematode species

To test for specificity of attraction of entomopathogenic nematodes towards insect damaged maize roots, two other nematodes besides *H. megidis* were tested; *H. bacteriophora*

(Heterorhabditidae) and *S. feltiae* (Steinernematidae). Nematodes were propagated in *G. mellonella* larvae, and 10 to 15 days old infective juveniles were tested in the olfactometer. Three days prior to the experiment, two 10–12 days old maize seedlings were transplanted each into a separate olfactometer pot as described above, and here a single species of herbivore, four second instar *D. v. virgifera* larvae, were added to one of the plants. The other four pots were also filled with sand, and two days before the experiment, four second instar *D. v. virgifera* larvae were added to one of these pots. Thus, the treatment consisted of one pot containing a *D. v. virgifera* attacked maize plant, one containing a healthy maize plant, one containing only larvae, and three control pots containing only sand. This allowed us to infer the relative importance of plant vs larvae to nematode attraction. The procedure of assembling the olfactometer and releasing the nematodes was the same as described above. For all the experiments, about 2000 nematodes for each of the three species were released in the center of the olfactometer. The experiment was replicated 10 times for each nematode species.

Statistical analyses

The nematodes' behavioural responses to the different odour sources offered in the six-arm olfactometer were examined with a log-linear model. The entity computing a repetition in the statistical analysis corresponds to the response of a group of 2000 nematodes released, which was shown to follow a multinomial distribution (Ricard and Davison 2007). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasilielihood functions to compensate for the overdispersion of nematodes within the olfactometer (Turlings et al. 2004). 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).

Results

Comparison of plant species

In the experiment comparing the attractiveness of *D. balteata*-damaged roots of maize, cotton and cowpea to *H. megidis* nematodes, we found that the number of nematodes recovered from olfactometer arms connected to pots with maize and cotton plants was higher than those from the arms with cowpea plants (Fig. 1a) (maize vs cowpea $p < 0.0001$; cotton vs cowpea $p = 0.01$). No strong difference was found between the number of nematodes choosing maize and cotton ($p = 0.078$). As expected, the number of nematodes choosing the control arms (sand only) was lower compared to choices for any of the plant treatments (maize vs sand $p < 0.0001$; cotton vs sand $p < 0.0001$; cowpea vs sand $p = 0.015$).

Figure 1b shows typical chromatograms obtained for the roots of maize (healthy and *D. balteata* damaged), cotton (healthy and *D. balteata* damaged) and cowpea (*D. balteata* damaged), from the extended analyses on an HP-5 column.

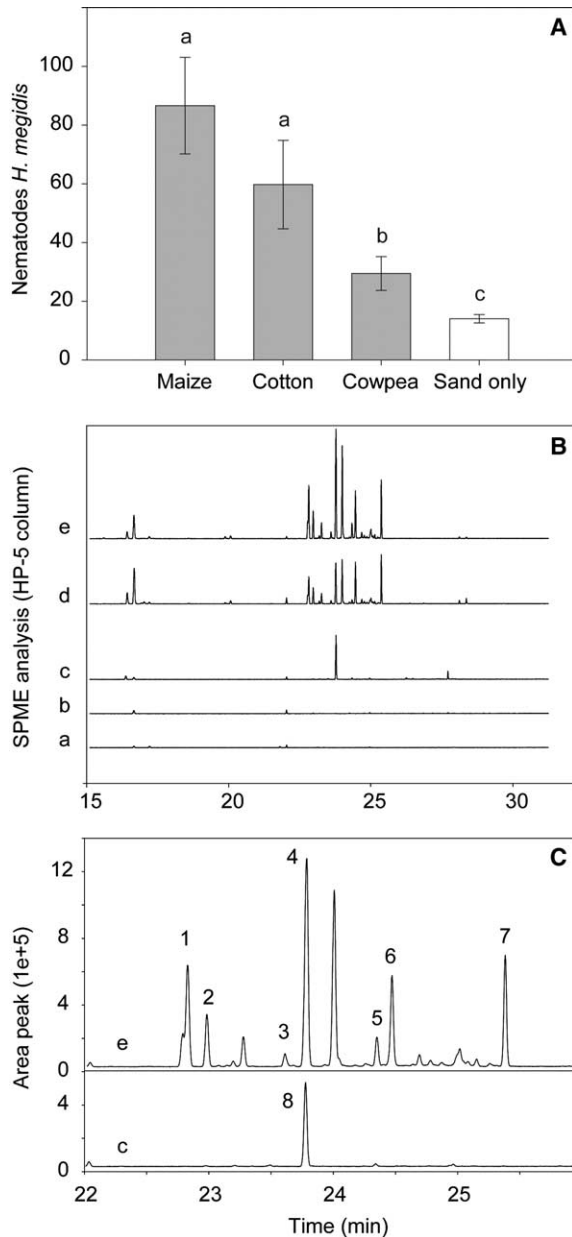


Fig. 1. Comparison of attractiveness of plant species. (a) Mean (\pm SE) number of nematodes *H. megidis* choosing maize, cotton or cowpea attacked by *D. balteata*, as compared to pots containing only sand, $n=11$. Letters above bars represent statistical differences ($p < 0.05$). (b) Chromatographic spectra obtained from SPME root analysis of maize, cotton and cowpea plants a = cowpea and four *D. balteata* larvae; b = healthy maize plants; c = maize and four *D. balteata* larvae; d = healthy cotton plants; e = cotton and four *D. balteata* plants. (c) "Close-up" of chromatograms showing volatiles collected from cotton and maize roots infested with *D. balteata* larvae. Labeled compounds are: 1) (+)-cycloisotavene; 2) (-)- α -copaene; 3) unknown sesquiterpene; 4) aristolene; 5) α -humulene; 6) unknown sesquiterpene 7) (-)- α -cubebene; 8) (*E*)- β -caryophyllene. Identification of peaks 1–7 should be considered tentative.

No induction of emissions was detected for the damaged cowpea roots, and the chromatogram is comparable to those obtained from the analysis of healthy maize roots. The induction of (*E*)- β -caryophyllene, however, was only

strongly visible in the *D. balteata*-damaged maize roots. Both attacked and healthy cotton roots produced a number of compounds and overall released considerably more volatiles than the other two plant species. In Fig. 1c, a close up shows the eight compounds that were tentatively identified using the NIST02 library.

Comparison of herbivore species

When offered a choice between attractants emitted by maize roots attacked by *D.v.virgifera*, *A. ustulatus* or *D. dipsaci*; *H. megidis* nematodes showed a clear preference for *D.v.virgifera*-attacked roots (Fig. 2a). *D. v. virgifera* damage was preferred over *A. ustulatus* damage ($p=0.013$), over *D. dipsaci* damage ($p=0.005$), and over the sand only controls ($p=0.001$). Nematodes did not discriminate between plants damaged *D. dipsaci* and *A. ustulatus* ($p=0.69$), but they were attracted more to plants damaged by these two herbivores than to sand only ($p=0.0005$ for *D. dipsaci* and 0.0001 for *A. ustulatus*). By using a standard curve obtained from the analysis of healthy maize roots spiked with different amounts of authentic (*E*)- β -caryophyllene, the quantification of the compound in the different treatments (Fig. 2b) showed that indeed *D.v.virgifera*-damaged roots

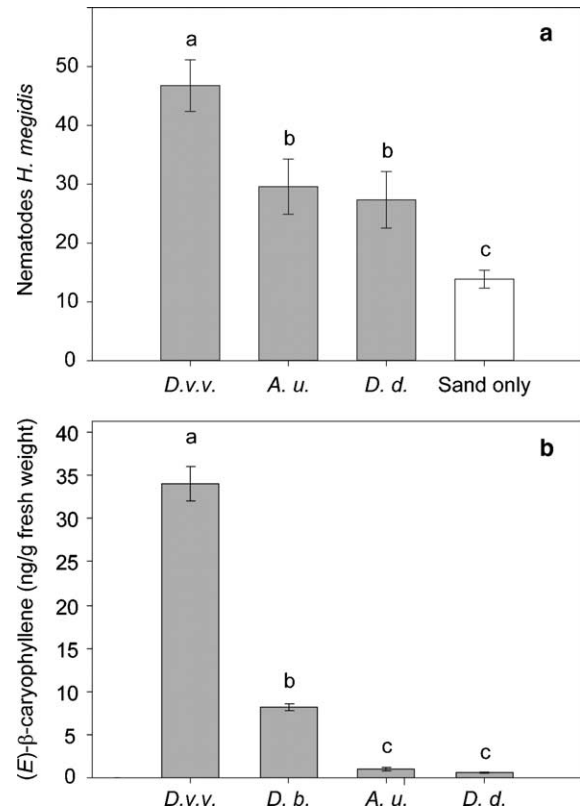


Fig. 2. Comparison of attractiveness of maize roots damaged by different herbivore species. (a) Mean (\pm SE) number of *H. megidis* nematodes choosing maize roots attacked by either *D.v.virgifera* (*D.v.v.*), *A. ustulatus* (*A. u.*), or *D.dipsaci* (*D.d.*), vs pots containing only sand, $n=12$. (b) Mean (\pm SE) amount of herbivore (*D.v.virgifera* (*D.v.v.*), *D. balteata* (*D.b.*), *A. ustulatus* (*A.u.*), and *D.dipsaci* (*D.d.*))-induced (*E*)- β -caryophyllene in maize roots, $n=12$. Letters above bars represent statistical differences ($p < 0.05$).

produced more than *D. balteata*, *A. ustulatus* and *D. dipsaci*-damaged roots.

Comparison of nematode species

Of the three nematode species tested in the olfactometer, only *H. megidis* and *H. bacteriophora* showed movement and attraction toward damaged maize roots (Fig. 3). *H. megidis* nematodes preferred *D. v. virgifera* damaged plants over the other three treatments (*D. v. virgifera* attacked plant vs healthy plant $p = 0.0063$; *D. v. virgifera* attacked plant vs *D. v. virgifera* larvae only $p < 0.0001$; *D. v. virgifera* attacked plant vs sand $p < 0.0001$). Healthy plants were also somewhat attractive to *H. megidis*, and were preferred over larvae alone or sand (healthy plant vs larvae only $p = 0.0148$; healthy plants vs sand only $p < 0.0001$). But even the larvae alone were slightly more attractive than the control pots (larvae only vs sand only $p = 0.019$).

H. bacteriophora nematodes preferred *D. v. virgifera* damaged plants over the healthy plants, the larvae alone, and sand only (*D. v. virgifera* attacked plant vs healthy plant $p = 0.0012$; *D. v. virgifera* attacked plant vs *D. v. virgifera* larvae only $p = 0.018$; *D. v. virgifera* attacked plant vs sand $p = 0.00091$). However, there was no difference in the attractiveness of nematodes between the healthy plant, larvae only

and sand only (healthy plant vs larvae only $p = 0.179$; healthy plant vs sand only $p = 0.15$; larvae only vs sand only $p = 0.88$).

In none of the 6 replicates conducted with *S. feltiae* did we recover any nematodes from the arms of the olfactometer, and four additional replicates of the experiment showed that, after 24 h, most of the released nematodes were present in the same spot where they were released, i.e. the center of the olfactometer (data not shown).

Discussion

Induced indirect defenses are generally accepted as a part of a plant's arsenal to counter-attack and diminish herbivore damage (Karban and Baldwin 1997, Walling 2000, Arimura et al. 2005). However, specificity in tritrophic interactions has been addressed only in few studies (Agrawal 2000, Cipollini et al. 2003, Van Zandt and Agrawal 2004, Arimura et al. 2005). More such studies are needed as specificity is expected to affect community structure and the evolution of defense strategies (Karban and Baldwin 1997, Underwood and Rausher 2002). To our knowledge, the present study is the first to assess specificity and variability in belowground induced responses. By taking a hierarchical approach to study specificity at three different trophic levels we show that herbivore-induced changes in root volatiles is a common phenomenon, and nematodes are differentially attracted to different plant species, as well as to plants of the same species attacked by different herbivores. The type of compounds induced in roots by herbivory, and the responses of the nematodes to the root signals vary considerably. Previous work (Köllner et al. 2004) shows that changes in terpenoid production in maize tissues is a precise reflection of what is emitted from these tissues and that no storage occurs and recent head-space measurements in the rhizosphere have confirmed this (unpubl.). For cotton and cowpea this has not been studied and some of the measured volatiles may not be emitted.

Specificity of induction can be caused by the type of feeding damage (Takabayashi and Dicke 1996, Stout et al. 1998, Walling 2000), salivary constituents (Turlings et al. 1990, Mattiacci and Dicke 1995, Alborn et al. 1997), and the extent of damage imposed by the herbivore (Karban 1987, Lin et al. 1990).

The different plant species tested produced different volatile blends in the roots upon herbivory, and this differential production was largely correlated with the attraction of nematodes (Fig. 1). For cowpea roots we detect no volatile organic compound, and there was correspondingly low attraction of predatory nematodes. Low induced emissions from cowpea roots corroborate what has been found in an aboveground study on the same variety, where cowpea leaves damaged by *Spodoptera littoralis* larvae were found to produce almost exclusively green leaf volatiles and hardly any of the terpenoids that were found in other plant species (Hoballah et al. 2002). In the same study, however, the responses of the generalist endoparasitoid *Cotesia marginiventris* was stronger to cowpea odor than to maize odor, indicating that quantity of induced odor emission is not always a good predictor of parasitoid or nematode attraction.

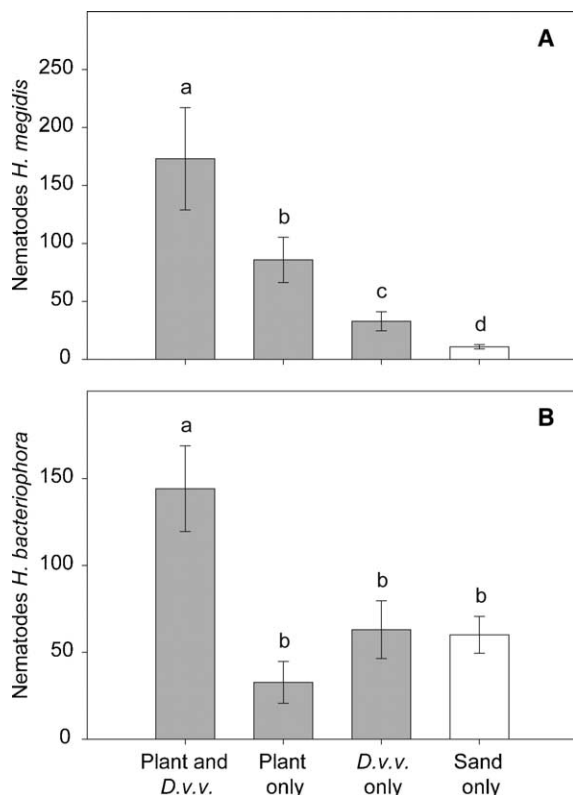


Fig. 3. Comparison of responses of nematode species. (a) Mean (\pm SE) number of *H. megidis* nematodes choosing the olfactometer arm containing maize plants attacked by *D. v. virgifera* larvae, healthy plants only, larvae only or sand only. (b) Mean (\pm SE) number of *H. bacteriophora* nematodes choosing one of the olfactometer arm containing maize plants attacked by *D. v. virgifera* larvae, healthy plant only, larvae only or sand only, $n = 10$. Letters above bars represent statistical differences ($p < 0.05$).

For cotton, as is known for their leaves (Loughrin et al. 1994), the undamaged roots of contained relatively large amounts of various terpenoids, which is in clear contrast to the other two plant species studied, and feeding by *D. balteata* larvae caused only a small increase in the amounts of total compounds detected in the cotton roots (Fig. 1b). It is perhaps somewhat surprising that the damaged cotton roots were not more attractive than the damaged maize roots, given that cotton produced at least seven more possible attractants. However, we only measured what was present in the roots and this may, in the case of cotton, not have been indicative of what was actually emitted by the roots. This result may also imply that some substances are more attractive than others, and that in this case, (E)- β -caryophyllene, which is the main compound emitted by maize roots, is the most potent attractant in these species.

Studies on aboveground plant–insect interactions have found similar differences between maize and cotton. Cotton plants store terpenoids in special pigmented glands on the surface of their leaves. These compounds, which offer a direct defense mechanism against lepidopteran larvae (Hedin et al. 1992), are liberated when the leaf tissues are damaged (Turlings and Wäckers 2004). Roots do not display such glands, but our findings imply that some quantities of terpenoids are also stored in cotton root tissue. Studies on root feeding by *A. lineatus* larvae support this notion, as feeding resulted in increased levels of already presents terpenoid aldehydes in cotton roots plants (Bezemer et al. 2004). Interestingly, peak number 4 in Fig. 1c, which was tentatively identified as aristolene, is very similar to (E)- β -caryophyllene and was difficult to separate from the latter by chromatography. Further studies will have to confirm its identity and attraction potential. *H. megidis* was similarly attracted to cotton and maize, suggesting that terpenoids in general are attractive to nematodes. A previous comparison of attractiveness to *H. megidis* of several terpenoids such as linalool, nerolidol and (E)- β -farnesene revealed that these compounds were less attractive than (E)- β -caryophyllene (Rasmann et al. 2005).

Clear differences were also found in the (E)- β -caryophyllene production induced by the different herbivores that were tested. Maddox and Root (1990) proposed that selection for a plant resistance trait may be driven by suites of herbivores displaying a similar feeding habit. In our example, the similarly feeding coleopteran species induced the emission of at least some volatiles, compared to the root knot nematode, which induced hardly any detectable amounts (Fig. 2). This is possibly due to the piercing-sucking feeding behavior of the nematodes compared to the chewing type of feeding by the coleopterans.

The observed poor response of the roots to nematode feeding is in contrast to the reported induction of direct defenses by phytopathogenic nematodes (Ogallo and McClure 1995, Tsao and Yu 2000). For example, the root-knot nematode *Meloidogyne incognita* increases the amount of terpenoid aldehydes in roots of cotton seedlings, thus increasing the resistance of the plant (Khoshkhoo et al. 1994). In terms of indirect defense, it can be argued that there is no need for plants under *D. dipsaci* attack to attract entomopathogenic nematodes because the latter infect only

insects. The same appears to be true for *A. ustulatus* larvae; induced production of (E)- β -caryophyllene was also surprisingly low (1.1 ng g⁻¹) compared to the amount of the same volatile induced by *D.v.virgifera* feeding (34 ng g⁻¹), but still sufficient to attract nematodes. There are no reports of entomopathogenic nematodes using wireworm larvae as hosts (Eidt and Thurston 1995), and in this sense selective forces should have acted to reduce caryophyllene production after *Agriotes* feeding. Although the numbers of herbivores placed on the plants were chosen to obtain comparable amounts of damage, it cannot be fully excluded that the observed differences in emissions reflect differences in amount and type of feeding damage. The *A. ustulatus* larvae used in the experiments were between 8th and 11th instar and may feed considerably less than younger larvae (Furlan 1998), perhaps explaining a lower induction. Additionally, the relatively poor induction by *D. balteata* of (E)- β -caryophyllene may be explained by lower feeding rates by this less specialized root herbivore (Mithofer et al. 2005) compared to *D. v. virgifera*; and lower specialization in this sense can be seen as a lower tolerance to a non-host plant (Karban and Baldwin 1997).

The responses of the infective juveniles of the three tested nematode species to *D.v.virgifera*-infected maize plants differed considerably, and this result conformed to our expectations given their different lifestyles. Entomopathogenic nematodes display a wide variety of foraging behaviors, which are situated in a continuum between the “cruiser type” and the “ambusher type” (Grewal et al. 1994). Cruisers crawl towards their hosts, whereas ambushers use a sit-and-wait strategy, standing on their tail (nictation) waiting for motile prey to pass nearby (Campbell and Gaugler 1997). In general it is assumed that Heterorhabditidae nematodes are of the cruiser type, actively foraging for new hosts, whereas Steinernematidae display all types of foraging strategies (Campbell and Gaugler 1997). *S. feltiae* nematodes are considered to display an intermediate foraging behavior, where standing on the tail is rare in occurrence and short in duration (Grewal et al. 1994). Long-range chemical cues are predicted to be used by cruisers for resource location, but such cues are apparently unimportant for ambush foragers (Bell 1991). For example, *H. bacteriophora* has been shown to respond to volatiles and water soluble chemical cues in a wide variety of experiments (Grewal et al. 1994, O’Halloran and Burnell 2003). Here too, we found an attraction of *H. bacteriophora* to the plant–insect complex, and probably to volatile organic compounds emitted by the damaged roots. This was already known for *H. megidis* (van Tol et al. 2001, Rasmann et al. 2005).

The current study also showed that rootworm larvae alone were not attractive to the nematodes, implying that the plant, as expected, is the key source of attractants for both *H. bacteriophora* and *H. megidis*. This is a parallel result to many studies of parasitoids, which are thought to predominantly use plant-provided signals to find herbivorous hosts. Hosts themselves have presumably been selected to emit as little and to be as cryptic as possible, whereas plants may benefit from emitting a clear signal to lure in the enemies of their enemies (Vet and Dicke 1992).

Surprisingly, not one individual of *S. feltiae* was recaptured from any of the olfactometer arms. Thus, contrary to expectation, *S. feltiae* infective juveniles do

not appear to use any long-range chemical signals to find their hosts (Grewal et al. 1994). *S. feltiae* is, in terms of behavior, considered to be an intermediate between a cruiser and an ambusher and previous studies have found evidence for a direct effect of larvae on the attraction of cruiser nematodes (Grewal et al. 1994, Lewis et al. 1995, Hui and Webster 2000). This was not found during the present study, also indicating that cues coming from the host are more important in short range recognition.

Van der Putten et al. (2001) have argued that persistence of plants in a community may depend on their defense belowground and that it is necessary to study such effects for a complete understanding of ecosystem functioning. Clearly, plants affect soil organisms, and soil organisms reciprocally affect plants, leading to a feedback that drives changes in plant communities over space and time (Poveda et al. 2007). Here we focused on agricultural plant species and associated pests, with one of the aims to explore how root signals may be better exploited to enhance the efficacy of entomopathogenic nematodes in biocontrol strategies (Toepfer et al. 2005). However, for a better understanding of the still controversial role of inducible plant volatiles in shaping plant communities (Holopainen 2004), natural ecosystems will have to be studied and eventually this will have to occur in the field. Here we take the first step in showing that belowground induction of volatiles can mediate tritrophic interactions, and that sufficient specificity exists to be a structuring force in field communities.

Acknowledgements – We would like to thank Ivan Hiltbold, Marco D’Alessandro, Cristina Faria and Matthias Held for stimulating discussions and/or technical assistance. Anurag Agrawal and John Parker for helpful comments on the manuscript. We are grateful to Lorenzo Furlan, Univ. of Padova, Italy for providing us with wireworms; Jürg Grunder, FAL Agroscope, Wädenswil, Switzerland for providing us with phytopathogenic nematodes; Ulli Khulmann at CABI Bioscience, Switzerland for providing us with *Diabrotica virgifera virgifera* larvae; and Evelyn Scherrer for providing us *D. balteata* larvae. This project was funded by the Swiss National Centre in Competence in Research “Plant Survival”.

References

- Agrawal, A. A. 2000. Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. – *Oikos* 89: 493–500.
- Alborn, H. T. et al. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. – *Science* 276: 945–949.
- Aratchige, N. S. et al. 2004. Below-ground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. – *Exp. Appl. Acarol.* 33: 21–30.
- Arimura, G. et al. 2005. Herbivore-induced, indirect plant defences. – *Biochim. Biophys. Acta-Mol. Cell Biol. Lipids* 1734: 91–111.
- Bell, W. J. 1991. Searching behaviour: the behavioural ecology of finding resources. – Chapman and Hall.
- Bezemer, T. M. et al. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. – *J. Chem. Ecol.* 30: 53–67.
- Campbell, J. F. and Gaugler, R. R. 1997. Inter-specific variation in entomopathogenic nematode foraging strategy: dichotomy or variation along a continuum? – *Fundam. Appl. Nematol.* 20: 393–398.
- Cipollini, D. et al. 2003. Costs of induced responses in plants. – *Basic Appl. Ecol.* 4: 79–89.
- De Moraes, C. M. et al. 1998. Herbivore-infested plants selectively attract parasitoids. – *Nature* 393: 570–573.
- Dicke, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? – *Entomol. Exp. Appl.* 91: 131–142.
- Dicke, M. and Vet, L. E. M. 1999. Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. – In: Olf, H. et al. (eds), *Herbivores: between plants and predators*. Blackwell Science, pp. 483–520.
- Eidt, D. C. and Thurston, G. S. 1995. Physical deterrents to infection by entomopathogenic nematodes in wireworms (Coleoptera, Elateridae) and other soil insects. – *Can. Entomol.* 127: 423–429.
- Furlan, L. 1998. The biology of *Agriotes ustulatus* Schaller (Col., Elateridae). II. Larval development, pupation, whole cycle description and practical implications. – *J. Appl. Entomol.* – *Z. Angew. Entomol.* 122: 71–78.
- Grewal, P. S. et al. 1994. Host finding behavior as a predictor of foraging strategy in entomopathogenic nematodes. – *Parasitology* 108: 207–215.
- Hass, B. et al. 1999. Persistence of *Heterorhabditis* infective juveniles in soil: comparison of extraction and infectivity measurements. – *J. Nematol.* 31: 508–516.
- Hedin, P. A. et al. 1992. Relationships of glands, cotton square terpenoid aldehydes, and other allelochemicals to larval growth of *Heliothis virescens* (Lepidoptera, Noctuidae). – *J. Econ. Entomol.* 85: 359–364.
- Hoballah, M. E. F. et al. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality or quantity important? – *J. Chem. Ecol.* 28: 951–968.
- Holopainen, J. K. 2004. Multiple functions of inducible plant volatiles. – *Trends Plant Sci.* 9: 529–533.
- Hui, E. and Webster, J. M. 2000. Influence of insect larvae and seedling roots on the host-finding ability of *Steinernema feltiae* (Nematoda: Steinernematidae). – *J. Invertebrate Pathol.* 75: 152–162.
- Karban, R. 1987. Environmental conditions affecting the strength of induced resistance against mites in cotton. – *Oecologia* 73: 414–419.
- Karban, R. and Baldwin, I. T. 1997. Induced responses to herbivory. – Univ. of Chicago Press.
- Khoshkhou, N. et al. 1994. Terpenoid aldehydes in root-knot nematode susceptible and resistant cotton plants. – *J. Agric. Food Chem.* 42: 204–208.
- Köllner, T. G. et al. 2004. The sesquiterpene hydrocarbons of maize (*Zea mays*) form five groups with distinct developmental and organ-specific distribution. – *Phytochemistry* 65: 1895–1902.
- Lewis, E. E. et al. 1995. Hierarchical order of host cues in parasite foraging strategies. – *Parasitology* 110: 207–213.
- Lin, H. C. et al. 1990. Induced resistance in soybean to the Mexican bean beetle (Coleoptera, Coccinellidae) – comparisons of inducing factors. – *Environ. Entomol.* 19: 1852–1857.
- Loughrin, J. H. et al. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. – *Proc. Natl Acad. Sci. USA* 91: 11836–11840.
- Maddox, G. D. and Root, R. B. 1990. Structure of the encounter between goldenrod (*Solidago altissima*) and its diverse insect fauna. – *Ecology* 71: 2115–2124.
- Mattiacci, L. and Dicke, M. 1995. Host-age discrimination during host location by *Cotesia glomerata*, a larval parasitoid of *Pieris brassicae*. – *Entomol. Exp. Appl.* 76: 37–48.

- Mithofer, A. et al. 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. – *Plant Physiol.* 137: 1160–1168.
- Neveu, N. et al. 2002. Systemic release of herbivore-induced plant volatiles by turnips infested by concealed root-feeding larvae *Delia radicum* L. – *J. Chem. Écol.* 28: 1717–1732.
- O'Halloran, D. M. and Burnell, A. M. 2003. An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*. – *Parasitology* 127: 375–385.
- Ogalle, J. L. and McClure, M. A. 1995. Induced resistance to *Meloidogyne hapla* by other *Meloidogyne* species on tomato and *Pyrethrum* plants. – *J. Nematol.* 27: 441–447.
- Ohgushi, T. et al. (eds) 2007. Ecological communities: plant mediation in indirect interaction webs. – Cambridge Univ. Press.
- Poveda, K. et al. 2007. Plant-mediated interactions between below- and aboveground processes: decomposition, herbivory, parasitism, and pollination. – In: Ohgushi, T. et al. (eds), Ecological communities: plant mediation in indirect interaction webs. Cambridge Univ. Press, pp. 147–163.
- Rasmann, S. et al. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. – *Nature* 434: 732–737.
- Ricard, I. and Davison, A. C. 2007. Statistical inference for olfactometer data. – *J. R. Stat. Soc.: Ser. C* 56: 479–492.
- Stout, M. J. et al. 1998. Specificity of induced resistance in the tomato, *Lycopersicon esculentum*. – *Oecologia* 113: 74–81.
- Takabayashi, J. and Dicke, M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. – *Trends Plant Sci.* 1: 109–113.
- Takabayashi, J. et al. 2006. Can plants betray the presence of multiple herbivore species to predators and parasitoids? The role of learning in phytochemical information networks. – *Ecol. Res.* 21: 3–8.
- Toepfer, S. et al. 2005. Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. – *Bull. Entomol. Res.* 95: 473–482.
- Toth, M. et al. 2003. Identification of pheromones and optimization of bait composition for click beetle pests (Coleoptera: Elateridae) in central and western Europe. – *Pest Manage. Sci.* 59: 417–425.
- Tsao, R. and Yu, Q. 2000. Nematicidal activity of monoterpenoid compounds against economically important nematodes in agriculture. – *J. Essential Oil Res.* 12: 350–354.
- Turlings, T. C. J. and Wäckers, F. 2004. Recruitment of predators and parasitoids by herbivore-injured plants. – In: Cardé, R. T. and Millar, J. G. (eds), Advances in insect chemical ecology. Cambridge Univ. Press.
- Turlings, T. C. J. et al. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. – *Science* 250: 1251–1253.
- Turlings, T. C. J. et al. 2004. A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. – *Physiol. Entomol.* 29: 45–55.
- Underwood, N. and Rausher, M. 2002. Comparing the consequences of induced and constitutive plant resistance for herbivore population dynamics. – *Am. Nat.* 160: 20–30.
- Van der Putten, W. H. et al. 2001. Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. – *Trends Ecol. Evol.* 16: 547–554.
- van Tol, R. et al. 2001. Plants protect their roots by alerting the enemies of grubs. – *Ecol. Lett.* 4: 292–294.
- Van Zandt, P. A. and Agrawal, A. A. 2004. Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. – *Oikos* 104: 401–409.
- Vet, L. E. M. and Dicke, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. – *Annu. Rev. Entomol.* 37: 141–172.
- Viswanathan, D. V. et al. 2005. Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. – *Ecology* 86: 886–896.
- Walling, L. L. 2000. The myriad plant responses to herbivores. – *J. Plant Growth Regulation* 19: 195–216.