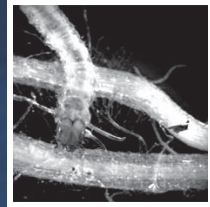
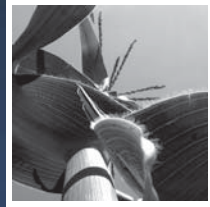
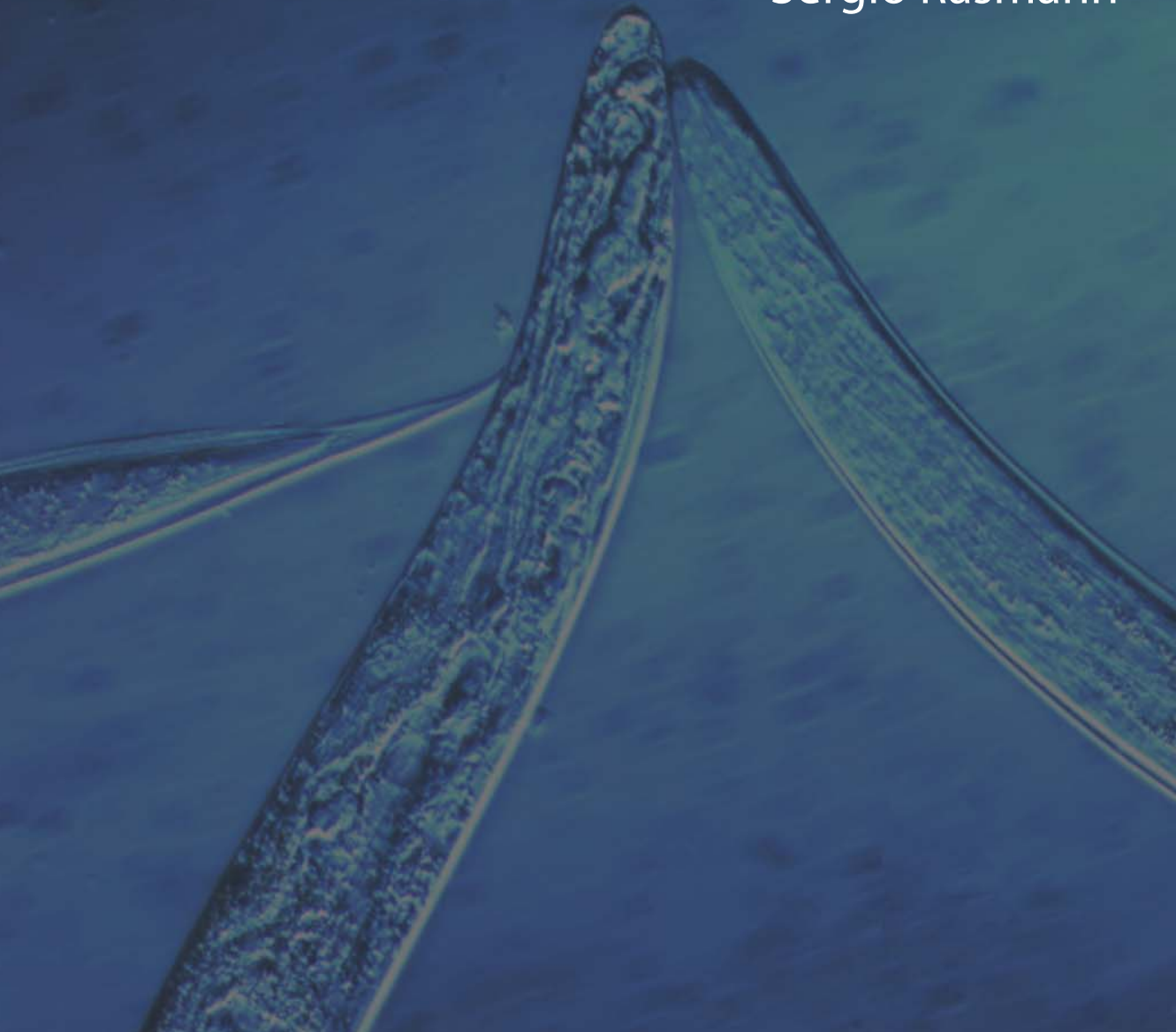


Belowground Tritrophic Interactions



Sergio Rasmann



IMPRIMATUR POUR LA THESE

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SUMMARY



Summary

In response to phytophagous insects attack, plants produce volatile compounds that can serve as cues for natural enemies of the herbivore to locate their host or prey. Very substantial progress has been made in understanding such tritrophic interactions aboveground. Recently, however, it is more and more recognized that aboveground communities are influenced through physiological and biochemical changes in plants driven by belowground communities and the current thesis aimed to provide new insight in these interactions. Corn (*Zea mais* L.) plants attacked by the leaf feeder noctuid butterfly (*Spodoptera littoralis* Boisduval) and the root feeder larvae of the western corn rootworm (*Diabrotica virgifera virgifera*) were used as a model system. We investigated belowground tritrophic interactions by developing a belowground six arm olfactometer. With the use of this device we discovered that *Diabrotica*-attacked plants emit an attractant for the entomopathogenic nematode *Heterorhabditis megidis* Poinar, Jackson & Klein. The attraction was mainly caused by the release of the sesquiterpene (*E*)- β -caryophyllene in the soil after root feeding. The importance of the compound in the attraction for the nematodes was further confirmed in field experiments using (*E*)- β -caryophyllene producing and non-producing corn varieties (Chapter I). To investigate cross effects between plant-mediated below- and aboveground interactions we connected an above- and a belowground olfactometer, and used this assembly to simultaneously study attraction of parasitic wasps and nematode to the odour emissions of maize after herbivory by either the above or the below ground herbivore, or by both. It was found that indeed root feeding influences aboveground tritrophic interactions, and vice-versa leaf feeding influences belowground tritrophic interactions (Chapter II). The specificity of the newly discovered belowground interaction was tested by using different plant, herbivore and nematode species (Chapter III). Besides a fundamental interest in the ecology and mechanisms involved multitrophic interactions, the work was also driven by an interest to find ways of enhancing a possible biological control of the two major maize pests that were under study. Overall the thesis contributed to our understanding of the role of induced plant volatiles in above- and belowground tritrophic interactions and of how these two interactions may influence one another. Moreover, the thesis is, to our knowledge, the first to demonstrate in the field that indirect plant defences can indeed be used to enhance the efficacy of a biological control agent.

Key words

Tritrophic interactions, induced indirect defenses, roots, belowground, maize, entomopathogenic nematodes, *Diabrotica*.

Résumé

En réponse à des dommages causés par les insectes phytophages, les plantes produisent des composés volatiles signalant aux ennemis naturels de l'herbivore la présence d'hôtes ou de proies. D'importants progrès ont été réalisés dans la compréhension de telles interactions trophiques se déroulant aux alentours de la partie aérienne de la plante. De nos jours, l'influence des communautés souterraines, par l'intermédiaire de changements physiologiques et biochimiques de la plante, sur les communautés aériennes est de plus en plus reconnue. Le contenu de cette thèse vise à apporter de nouveaux points de vue sur ces interactions. Le modèle étudié était composé de plantes de maïs (*Zea mays* L.) dont les feuilles étaient soumises aux attaques du ver du cotonnier (*Spodoptera littoralis* Boisduval) et les racines aux attaques de la chrysomèle du maïs (*Diabrotica virgifera virgifera* LeConte). Le développement d'un olfactomètre souterrain à 6 bras nous a permis de mener une étude sur les interactions tritrophiques se déroulant dans le sol. Grâce à l'utilisation de ce dispositif, nous avons découvert que les racines d'une plante attaquée par *Diabrotica* émettent une substance attirant les nématodes entomopathogènes *Heterorhabditis megidis* Poinar, Jakson & Klein. Cette attirance est principalement le résultat de l'émission dans le sol, par les racines dévorées, d'un sesquiterpène, le (*E*)- β -caryophyllène. L'importance de ce dernier lors du phénomène d'attraction des nématodes fût également confirmée au travers d'expériences en champ lors desquelles nous avons comparé des variétés de maïs productrices de (*E*)- β -caryophyllène avec des variétés ayant perdu cette faculté (Chapitre I). Afin d'analyser l'effet croisé entre les interactions aériennes et souterraines et le rôle de la plante en tant que vecteur dudit effet, nous avons connectés l'olfactomètre souterrain à un olfactomètre aérien. Grâce ce nouvel assemblage, nous avons étudié simultanément l'attraction de guêpes parasites et de nématodes en fonction des odeurs émises par les plantes de maïs attaquées par des herbivores soit dans leur partie aérienne, soit souterraine ou encore lors d'une attaque simultanée des deux parties. Il a été démontré que les ravages du système racinaire influencent les interactions tritrophiques aériennes de même que les ravages du système foliaire ont pour résultat la modification des interactions tritrophiques souterraines (Chapitre II). La spécificité de cette nouvelle interaction trophique souterraine fût testée en utilisant différentes espèces de plantes, d'insectes herbivores et de nématodes (Chapitre III). En parallèle à l'intérêt fondamental concernant l'écologie et les mécanismes régulant ces interactions multitrophiques, cette recherche fût également conduite dans l'optique d'améliorer les possibilités de lutte biologique contre les deux importants ravageurs étudiés. D'un point de vue plus général, cette thèse contribue à la compréhension du rôle joué par l'émission de volatiles induits lors d'interactions tritrophiques, qu'elle soit souterraine ou aérienne. Elle met également en lumière l'influence réciproque des interactions se déroulant dans ces deux milieux. De plus, cette thèse démontre pour la première fois, à notre connaissance, que les stratégies de défense indirecte des plantes peuvent améliorer, dans des conditions réelles, l'efficacité d'agents utilisés pour la lutte biologique.

Mots clés

Relations tritrophiques, défenses indirectes, racines, maïs, nématode, *Diabrotica*.

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INTRODUCTION



Plants and plant defences

For millions of years, plants have invaded and “greened” our living planet in almost every possible suitable location or habitat. After plant colonization, the first herbivore insects came about and exploited the plants sugar production and the balanced carbon/nitrogen content to optimize their own fitness (Schoonhoven, Jermy *et al.* 1998). Efficient counter-adaptations and specializations have allowed insects to deal with evolved plant defense traits and have allowed them to radiate into what we know today as one of the most species rich groups of organisms (May 1988). Among insects, the most strikingly abundant and diverse group is represented by herbivorous, representing $\frac{1}{4}$ of all living species (Strong, Lawton *et al.* 1984), continuously challenging the ruling of plants on earth (Schoonhoven, Jermy *et al.* 1998), and therefore creating a long and tremendous arms race between the two groups (Thompson and Cunningham 2002). Nowadays, this arms race is seen as coevolution between the two groups as cited in a seminal paper by (Ehrlich and Raven 1964), often reaching unexpected and marvelous patterns of biodiversity. Plants have then to defend themselves against the voracity of herbivores by more and more complexes and subtle armaments, provoking on the other side, various sophisticated resistant defense strategies in the insects (Schoonhoven, Jermy *et al.* 1998).

The plant defense arsenal is extraordinarily vast and complex. A first line of defense is constituted of physical features on the tissues (leaves, stem) that can drastically influence herbivore acceptance of host plants. The presence of trichomes and wax crystal structures on the plant surface, leaf thickness and toughness, sclerotization and high silica content may cause avoidance behavior and such plant trait are often assumed to fulfill a defensive function (Schoonhoven, Jermy *et al.* 1998). On the other hand, some plants continuously contain toxic or repellent

compounds against herbivores in their leaf tissues; this is considered a constitutive defense. Moreover, in order to save precious energy resources, plants produce toxic or repellent compounds only when they are attacked by phytophagous organisms, which is termed induced defense. Several books (Karban and Baldwin 1997; Agrawal, Tuzun *et al.* 1999) and reviews (Baldwin 1994; Karban, Agrawal *et al.* 1997; Agrawal and Rutter 1998; Agrawal and Karban 1999; Baldwin and Preston 1999; Dicke, van Poecke *et al.* 2003) have recently been devoted to the subject of induced plant defenses. Furthermore, induced and constitutive defenses can be direct, exerting a negative impact on herbivores, or indirect; including higher trophic levels of organisms involved in the own plant ecosystem (Price, Bouton *et al.* 1980). Direct defenses may prevent herbivores from feeding via physical barriers, such as spines, thorns, trichomes, and waxes or chemical ones; via secondary plant metabolites (metabolites that do not serve directly on plant physiology), such as phenylpropanoids, terpenoids, alkaloids and fatty acids; or via specialized defense proteins, such as proteinase inhibitors.

The term indirect defenses, refers to those adaptations that result in the recruitment and sustenance of organisms that protect the plants against herbivores attackers. These defenses range from the constitutive formation of domatia, which serve as homes for organisms such as ants, mites, and even bacteria to the production of foliar nectaries and nutritives structures which can also be used by natural enemies of the herbivores (for reviews see (Boethel and Eikenbary 1986; Whitman 1988)). In addition, plant indirect defenses can be induced. During the two last decades, it has been revealed that when herbivores feed on plant, the injured plants respond by producing and releasing odors (volatiles organic compounds or VOCs) that are exploited by natural enemies of the herbivores to locate their preys and hosts (for reviews see: (Turlings and Benrey 1998;

Dicke and Vet 1999; Dicke, van Poecke *et al.* 2003; Turlings and Wäckers 2004)).

A plant employs induced indirect defenses, when, right after herbivore attack, it starts to produce predator and parasitoid attracting volatiles.

Plant volatiles as induced indirect defenses

Herbivore induced plant volatiles, are known to play important roles in plant-arthropods interactions, other than natural enemies recruitment (Turlings and Wäckers 2004); as for example, by directly deterring oviposition by Lepidoptera (Landolt 1993). There is in addition growing evidence that herbivore-induced VOCs are involved in chemical transfer between plants (Engelberth, Alborn *et al.* 2004; Arimura, Ozawa *et al.* 2000; Kessler and Baldwin 2001; Baldwin, Kessler *et al.* 2002)

The chemical composition of herbivore-induced aboveground VOCs is known for many plant-herbivore systems (Paré and Tumlinson 1999). Some VOCs are taxon specific, such as the glucosinolate breakdown products in Brassica species (Mattiacci, Dicke *et al.* 1995), whereas other compounds appear to be common to many different plant families (Boom, Beek *et al.* 2004)

These compounds include six-carbon (C6)-volatiles or “green leaf volatiles” as they are also called, and generally released by plant leaves immediately after wounding. They include isomers of hexenol, hexenal, and hexenyl acetate (Hatanaka 1993). Generally, green leaf volatiles except (Z)-3-hexenyl acetate, are formed directly after wounding of the leaf (Matsui, Kurishita *et al.* 2000), and it seems they are also involved in the triggering of terpenoids production in plants (Farag and Paré 2002), the accumulation of endogenous jasmonic acid (JA) as well as the expression of defense genes (Bate and Rothstein 1998; Engelberth, Alborn *et al.* 2004). It has also

been suggested that C6-volatiles, besides being considered as antimicrobial, play a role as direct defense in plant. For example, C6-aldehydes and -alcohols reduce tobacco aphid fecundity (Hildebrand, Brown *et al.* 1993). Moreover, some C6-compounds may function as indirect defenses (Kessler and Baldwin 2001; D’Alessandro and Turlings 2005) or play a role in signaling within or between plants (Arimura, Ozawa *et al.* 2001). In contrast to C6-aldehydes and -alcohols, the emission of (Z)-3-hexenyl acetate can be observed a few hours after feeding or mechanical damage suggesting a similar signaling pathway as for some herbivore induced terpenoids (Turlings, Loughrin *et al.* 1995; Arimura, Ozawa *et al.* 2000). The green-leaf volatiles are derived from linolenic acid and various forms result from the jasmonic acid pathway (Paré and Tumlinson 1999; Arimura, Kost *et al.* 2005).

Herbivore-induced leaf volatiles also include terpenoids, encompassing monoterpenes (C10), sesquiterpenes (C15) and homoterpenes (C11 or C16). All terpenoids are synthesized through the condensation of isopentyl diphosphate and its allylic isomer dimethylallyl diphosphate in either the cytosol or the plastids (Paré and Tumlinson 1999; Arimura, Kost *et al.* 2005).

Indole is a nitrogenous compound has also been found as common and dominating compound in the blend of herbivore-induced VOCs (Frey, Stettner *et al.* 2000). It is derived from the shikimate acid pathway.

Recently it was found that continuous mechanical damage on Lima bean plants can result in the emission of volatile blends resembling those that occur after herbivore damage (Mithöfer, Wanner *et al.* 2005), but commonly the emission of these volatiles can be greatly enhanced and prolonged by eliciting factors coming directly from a feeding insect. These factor also elicit odor emission, when they are taken up via the stem of the plant, or even via the petiole of a leaf, and the response to these elicitors has been shown to be systemic (Dicke, Sabelis *et al.* 1990, Turlings, McCall

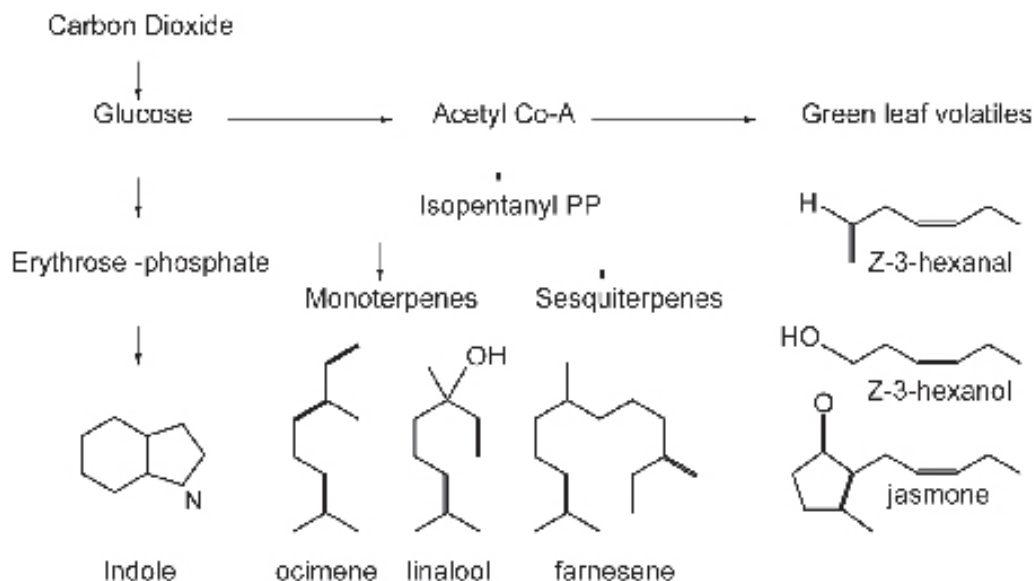


Figure 1 The biosynthetic routes leading to three classes of volatiles (indole, terpenoids, and green leaf volatiles). Modified after Pare *et al.* (1997)

et al. 1993). Plant defense responses have been ascribed to a wide variety of chemical elicitors that activate specific downstream signal transduction pathways (Pare, Farag *et al.* 2005). Two major classes of insect-derived elicitors are known so far. Beta-glucosidase, discovered in the regurgitant of *Pieris brassicae* larvae, which seems to facilitate the emission of glucosinolate breakdown products (Mattiacci, Dicke *et al.* 1995); and the fatty acid derivative volicitin and related compounds that, particularly in maize, induces the release of the full blend VOCs normally induced by caterpillar feeding (Alborn, Turlings *et al.* 1997). Volicitin (N-(17-hydroxylinolenol)-L-glutamine) was isolated from regurgitant of *Spodoptera exigua* larvae, and the biosynthesis of volicitin requires plant-derived linolenic acid, which is hydroxylated and conjugated with insect-derived glutamine (Paré, Alborn *et al.* 1998). The wide variety of elicitors often derive from slight changes in chemical structure of elicitors, this often

strongly affecting the profile of volatile blend of a plant (e.g. (De Moraes, Mescher *et al.* 2001; Kessler and Baldwin 2001; van Poecke and Dicke 2004)). Moreover, biosynthesis and release of herbivore-induced VOCs can be affected by biotic factors such as plant hormones (Farmer 2001; Thaler, Farag *et al.* 2002), microorganisms (e.g. : (Piel, Atzorn *et al.* 1997; Cardoza, Alborn *et al.* 2002)); and abiotic factors such as temperature and light (Takabayashi, Dicke *et al.* 1994; Gouinguéné and Turlings 2002), or O₃ and CO₂ (Vuorinen, Nerg *et al.* 2004; Vuorinen, Nerg *et al.* 2004). It should be noted that, although the series of specific defense responses that are activated depend on the precise plant-herbivore interaction, several common global responses have emerged. Herbivore feeding usually triggers defense responses mediated by ethylene and jasmonic acid that act synergistically (Kahl, Siemens *et al.* 2000; Schmelz, Alborn *et al.* 2003), whereas pathogen attack typically elevates

salicylic acid levels in a plant (Vranova, Inze *et al.* 2002). On the other hand, it seems that plant provided-signals can be highly variability depending on plant genotypes (Takabayashi, Dicke *et al.* 1991; Loughrin, Manukian *et al.* 1995; Gouinguéné, Degen *et al.* 2001), plant parts (Turlings, Wäckers *et al.* 1993), or between different growth stages of a plant (Gouinguene, Degen *et al.* 2001). What is amazing, however, is that plants seem to be able to respond differentially to different herbivores (De Moraes, Lewis *et al.* 1998; Turlings, Lengwiler *et al.* 1998), and to different life stages of an herbivore (Takabayashi, Takahashi *et al.* 1995). A wonderful example of selective specificity was shown in *Nicotiana tabacum* L. plants, where the odor emitted after caterpillar feeding is different during the night than during the day. The day-time volatiles are known to attract parasitoids (De Moraes, Lewis *et al.* 1998), whereas the night-time volatiles repelled female *Helicoverpa virescens* F. moth and kept them from laying eggs on the emitting plants (De Moraes, Mescher *et al.* 2001).

It has to be pointed out here that, so far, work on herbivore-induced VOCs and their involvement in the interaction between plant and arthropods, has focused on the aboveground parts of the plants and little has been done belowground. Van Tol *et al.* (2001), however, showed that plants may recruit entomopathogenic nematodes toward their herbivore-damaged roots. This was also tested in the current thesis on the maize system, and root-emitted, herbivore-induced volatiles were shown to be responsible for the recruitment of nematodes toward herbivore-damaged plants (Rasmann, Kollner *et al.* 2005) (See Chapter I). Other studies have shown that volatiles emitted by *Delia radicum* L. (Diptera: Anthomyiidae) infested turnips (*Brassica campestris* L.) were attractive for the specialist larval endoparasitoid *Trybliographa rapae* Westwood (Hymenoptera: Figitidae) (Neveu, Grandgirard *et al.* 2002). Similarly, rust mite (*Aceria tulipae* Keifer) infested

tulips bulb have been shown to be attractive to the predatory mite (*Neoseiulus cucumeris* Oudemans) (Aratchige, Lesna *et al.* 2004). In this context, more and more attention is being paid to the ecological role of belowground biota and how they influence the aboveground biota (Van der Putten, Vet *et al.* 2001; De Deyn, Raaijmakers *et al.* 2003; Wardle, Bardgett *et al.* 2004; Bezemer and van Dam 2005). Such interactions between above- and belowground tritrophic interactions were also tested during this thesis (See Chapter II).

The model system

Maize (*Zea mais* L.)

Maize (or corn) is the central primary trophic level organism of this thesis. It is said that corn is one of the most widely cultivated crop worldwide (Sattaur 1989), not only because the vegetative material is one of the major food component of cattle, but also because, its huge ears, each packed firmly attached kernels filled with starch, protein, and oil, make it an important food staple for humans (Fedoroff 2003). Hence, maize is a plant of special economic interest (Sattaur 1989). The origins of maize are still not completely understood (Jaenicke-Despres, Buckler *et al.* 2003). The general opinion, however, agrees nowadays on setting the wild grass, teosinte (*Zea mais* spp. *parviglumis*) as the origin of modern corn (*Zea mays* spp. *mays*). The earliest undisputed archaeological evidence of domesticated maize is 6250 years old (Piperno and Flannery 2001). Recent molecular data, suggest, however, that domestication could have begun as early as 9000 years ago in the Balsas River Valley in southern Mexico (Matsouka 2002). Also interesting in the context of this thesis is that different maize varieties reveal very high intraspecific variation in both quantity and quality of herbivore-induced VOCs released (Turlings, Lengwiler *et al.* 1998; Gouinguéné, Degen *et*

al. 2001), and a comparable polymorphisme



Figure 2 Maize plant.

among teosinte species (Gouinguéné, Degen *et al.* 2001). It seems that maize has retained a high degree of variability during the process of domestication (Wang, Stec *et al.* 1999).

Diabrotica virgifera virgifera (Col., Chrysomelidae) and related species complex

Out of the 354 described species of *Diabrotica* (Krysan 1999) there is a small complex that includes the most serious pest of corn (Levine and Oloumisadeghi 1991): the western corn rootworm, *Diabrotica virgifera virgifera* Le Conte (WCR); the mexican corn rootworm, *D. v. zea* Krysan and Smith (MCR); the northern corn rootworm, *D. barberi* Smith and Lawrence (NCR); the banded cucumber beetle, *D. balteata* Le Conte (BCB); and the southern corn rootworm, *D. undecimpunctata howardi* Barber (SCR) (Szalanski, Roehrdanz *et al.* 2000). The larvae of the corn rootworms cause great damages to maize (Fuller, Boetel *et al.* 1997). In addition, SCR is an economically important pest of cucurbits and peanuts, and BCB is a pest of sweet potatoes (Szalanski, Roehrdanz *et al.* 2000). *D. undecimpunctata howardi* is considered a sister to *D. balteata*, with *D. barberi*, *D. virgifera virgifera*, and *D. v. zea* representing another sister clade

(Szalanski, Roehrdanz *et al.* 2000).

The European Plant Protection Organization (EPPO) has been monitoring the presence of WCR from the neotropical region of Central America to the State of Ontario in Canada passing from Mexico and all of the corn belt of the United States. Since 1992, the WCR has been reported in Europe, spreading from Serbia to Hungary (1995), Croatia (1995) Romania (1996), Bosnia and Herzegovina (1997), Bulgaria (1998), Italy (1998) and southern Switzerland (2000) (www.eppo.org). Currently, WCR is found in most of the European countries except Scandinavia (Figure 4). *D. v. virgifera* was introduced into Europe by a series of multiple introduction events, which have led to the different outbreaks spots such as Central, and Southeastern Europe, Northern Italy and France (Miller, Estoup *et al.* 2005).

It is assumed that *D. v. virgifera* and maize evolved together in the tropics or subtropics of Mesoamerica (Branson and Krysan

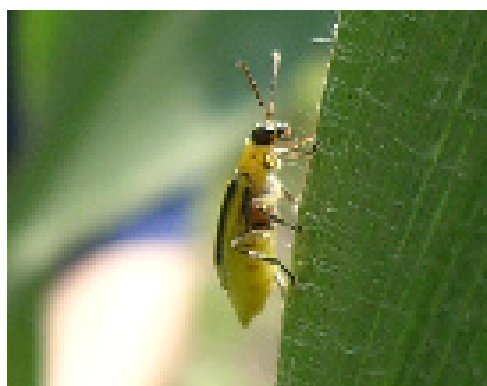


Figure 3 *D. v. virgifera* gravid female walking on a maize leaf.

1981). *Diabrotica* females lay their eggs in the proximity of the host-plant rhizosphere in moist soil at a maximum depth of 40 cm (Branson, Reyes *et al.* 1982). Egg survival is strongly effected by low temperatures and low moisture in the soil (Krysan 1978). WCR

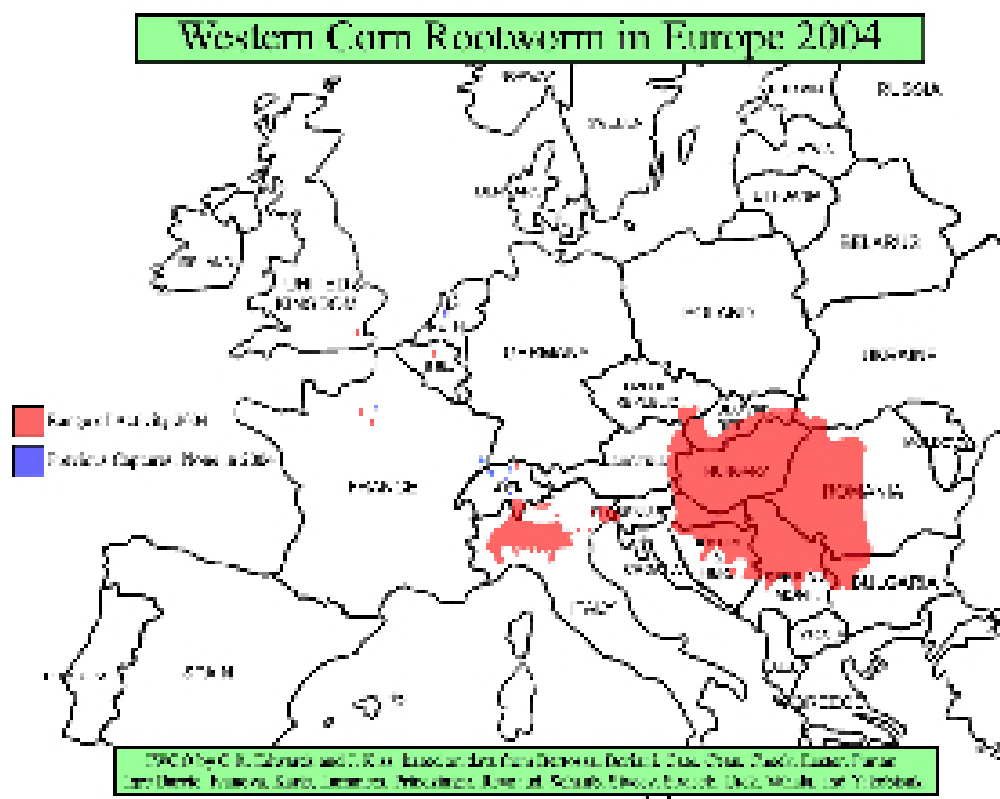


Figure 4 Map of Europe showing the spreading of *D. v. virgifera* until summer 2004.
Source: <http://www.entm.purdue.edu/wcr/>

has one generation per year and passes the cold (temperate) or dry (tropical) season as egg stage in the soil. There is evidence for a prolonged egg diapause on some *D. barberi* populations in a two crop rotation (maize and soybeans) system even though the percentage was very low (Levine and Oloumisadeghi 1991). Data collected in central regions of North America show that after the winter diapause larvae hatch from late May to mid-June (Branson and Krysan 1981). Larvae go through three instars and have an average developmental time to adult emergence of 26.3 days for males and 28.9 days for females (Jackson and Elliott 1988). Eggs are not laid directly on the roots or in the roots, therefore the larvae, after hatching, depend on their mobility to locate roots. Larvae of WCR are attracted to the root tip mainly by the CO₂ (Bernklau and Bjostad 1998). First instars larvae feed on seminal roots and roots of whorls 1 through 7, burrowing into root branches from 0.5 to 2 mm of diameter (Strnad and Bergman 1987), second instars feed on roots and whorls and third are only found on whorls (Krysan 1999). At maturity, the third instars transform into a pupa, which is inactive for a week or two. The pupae then turn into adult beetles, which emerge from the soil and start to feed on corn foliage, pollen, and silks around mid-July. The adults are active for about 10 to 12 weeks, during which they feed, mate, and deposit their eggs. Even though WCR adult proceed in the vegetation by using trivial flights (1min of flight or less), wind tunnel and field experiment had inferred a capacity of movement of about 62 km per generation (Chiang 1973).

In Europe, after the recent introduction of WCR in the Balkans (Figure 4) and the urgent need for a control of the pest, Toepfer and Kuhlmann (submitted) constructed life-tables of the pest using Hungary as model region. First instars larvae begin to emerge the end of May and the development until pupation ranged from mid July, when adults begin to emerge until the end of August. What

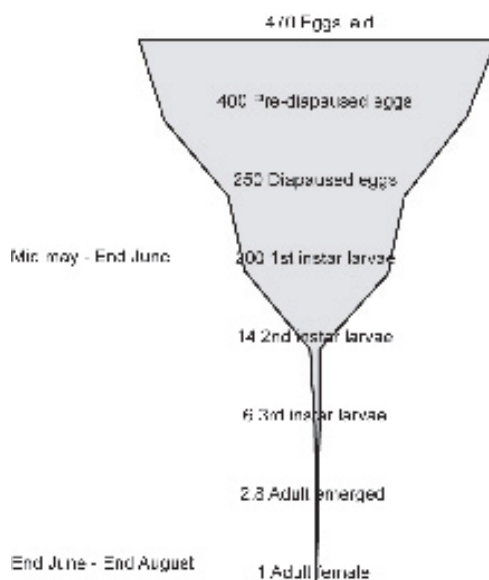


Figure 5 Age-specific survivorship of *D. v. virgifer* in southern Hungary and emergence dates for 1st instar larvae and adults - Modified after Toepfer and Kuhlmann.

is also interesting in the study by Toepfer, is the age-specific survivorship table: one adult female emerges from a maximum of 476 laid eggs, 251 diapaused eggs, 207 1st instars larvae and 14 2nd instars larvae. Thus, in Hungary WCR shows a huge bottleneck of mortality between first and second instars, due mainly to intrinsic fragility of the minute larvae emerging from the eggs, than to the pressure of natural enemies on the population (Kuhlmann and Burgt 1998; Toepfer and Kuhlmann submitted) (Figure 5).

WCR is considered the most important pest of maize in the USA and Canada, causing yield losses and chemical control costs of up to one billion US dollars annually (Krysan and Miller 1986). Alternative ways of controlling the pest, such as the use of pest monitoring systems, the release of natural enemies, cultural techniques to enhance the conservation of natural control, crop rotations and orientation disruption of adults (i.e. Integrated Pest Management or IPM strategies) are

therefore considered as ecologically sound control strategies (Kuhlmann and Burgt 1998; Moellenbeck, Peters *et al.* 2001). Corn rootworm management is currently limited to two basic strategies: annual rotation with non-host crop to break the insect life-cycle and soil insecticides application to limit corn root injury (Journey and Ostlie 2000). These existing corn rootworm management strategies may not be sustainable. As mentioned above, there is evidence for an adaptation to extended diapause for populations of *D. barberi* in Minnesota, Iowa, and South Dakota (Krysan, Foster *et al.* 1986). Moreover, corn following soybeans culture appears to favor females of another WCR strain that feeds also on soybean (Sammons, Edwards *et al.* 1997). The reliance on soil pesticides also presents increasing difficulties, including groundwater contamination, phytotoxic interactions with herbicides, and toxicity to applicators and nontarget organisms (Journey and Ostlie 2000). Nowadays, a newly commercialized genetically modified strain of corn is able to produce a complex of two Bt-proteins especially designed against WCR (Masson, Schwab *et al.* 2004).

We will focus here on the possibility to use biological control by introducing natural enemies of the pest in the field (Eilenberg, Hajek *et al.* 2001).

Krysan (1999), in his review, found a number of possible predators for WCR. Ants of the genus *Lasius* and mites of the genus *Androlaelaps* and *Stratiolaelaps* can attack eggs and larvae (Chiang 1970). Natural enemy surveys carried out in Mexico and Argentina revealed that parasitoids belonging to the genus *Celatoria* (Diptera: Tachinidae) and *Centistes* (Hymenoptera: Braconidae) are found to parasitize adults of *Diabrotica* species (Zhang, Toepfer *et al.* 2003; Zhang, Toepfer *et al.* 2004). WCR is also susceptible to some pathogens such as *Beauveria bassiana* fungus and *Wolbachia spp.*, which have been investigated also for possible biological control (Kuhlman 1970).

The most promising biological control agents against WCR are nematodes of the genera *Steinernema* and *Heterorhabditis* (Ehlers 1998; Toepfer, Gueldenzoph *et al.* 2005). In Southeastern Europe, after a two years survey study on adults, larvae and eggs, no natural enemies were found attacking WCR individuals except the fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Toepfer and Kuhlmann 2004).



Figure 6 *D. v. virgifera* first instars larvae feeding on maize roots and surrounded by *H. megidis* nematodes.

Entomopathogenic nematodes against *D. v. virgifera*

During the last twenty years, biological control methods have also focused on nematodes as possible control agents for insect pests (e.g. (Thurston and Yule 1990; Barbercheck 1993; Kaya and Gaugler 1993; Barbercheck, Wang *et al.* 1995; Choo, Koppenhofer *et al.* 1996; Ellsbury, Jackson *et al.* 1996; Strong, Kaya *et al.* 1996; Eben and Barbercheck 1997; Mortimer, Putten *et al.* 1999; Elliot, Sabelis *et al.* 2000; Journey and Ostlie 2000; van Tol, van der Sommen *et al.* 2001; Boff, van Tol *et al.* 2002)). Two families of soil nematodes have captured the attention of researchers: Steinernematidae and Heterorhabditidae. Both these families have evolved the ability to carry and introduce symbiotic bacteria into the body cavities of insects. They are also the only insect pathogens with a host range that includes the majority of insect orders and families, and they can be cultured on a large scale in artificial solid or liquid media (Poinar 1990). Moreover, steinernematid and heterorhabditid nematodes can kill insects within 48 hr, can form a durable, infective stage, which can be stored for long periods and applied by conventional methods, and persist in the natural environment (Poinar 1990). Fifteen years ago, nine recognized species of *Steinernema* and three of *Heterorhabditis* were listed (Poinar 1990). In 2002, more than 40 species of the two genera are described, showing the increasingly strong interest in the groups (Byron and Nguyen 2002).

The life cycle of entomopathogenic nematodes can be dissected in the following steps: 1) penetration into body cavity of the potential host, 2) release of bacteria, 3) development to mature adults, 4) mating and reproduction of infective juveniles, and 5) host searching and location by mobile infective juveniles (Poinar 1990) (see Figure 7 and 8).

Infection is initiated by a third-stage juvenile, which is morphologically and physiologically

adapted to remain in the environment for a prolonged period without ingesting food (Poinar 1990). When the nematode finds a host, it enters the insect's hemocoel through natural openings such as mouth, anus, spiracles or by breaking the soft outer cuticle with subventral teeth or hooks (Bedding and Molyneux 1982). Once in the insect's hemocoel, the nematodes start to release the symbiotic bacteria which then multiply. The bacteria are consumed and digested by the developing nematodes. *Xenorhabdus* and *Photorhabdus* are the two bacterial genera mutualistically associated with infective juveniles of *Steinernema* and *Heterorhabditis* respectively (Boemare 2002). In the *Steinernema* group, the infective juveniles develop into amphimictic females or males (sexual reproduction). In *Heterorhabditis*, each infective juvenile develops into a hermaphroditic female or male. The eggs can hatch when still in the mother (ovoviviparous) or the female can deposit the eggs (oviparous) (Poinar 1990). After the first developmental stages, the new infective juveniles move into the soil looking for a new host in which they can complete another life-cycle (Figure 9).

One of the major themes in applied entomopathogenic nematology is research into how nematodes forage for resources (hosts) (Lewis, Campbell *et al.* in press). In general, the behavior can be divided into a hierarchical process of host habitat location, host location, host acceptance, and host suitability (Doutt 1964). Moreover, foraging strategies can be divided into two broad categories; cruise (widely foraging) and ambush (sit-and-wait) (Pianka 1966; Schoener 1971). Cruise foragers have a higher probability of finding sedentary and cryptic resources (such as *D. v. virgifera* larvae) than ambushers, and ambush foragers are more effective at finding resources with high mobility (Lewis, Campbell *et al.* in press). The foraging strategies used by different infective juveniles species to find a host vary along a continuum between ambush and cruise foragers (Lewis, Gaugler *et al.*

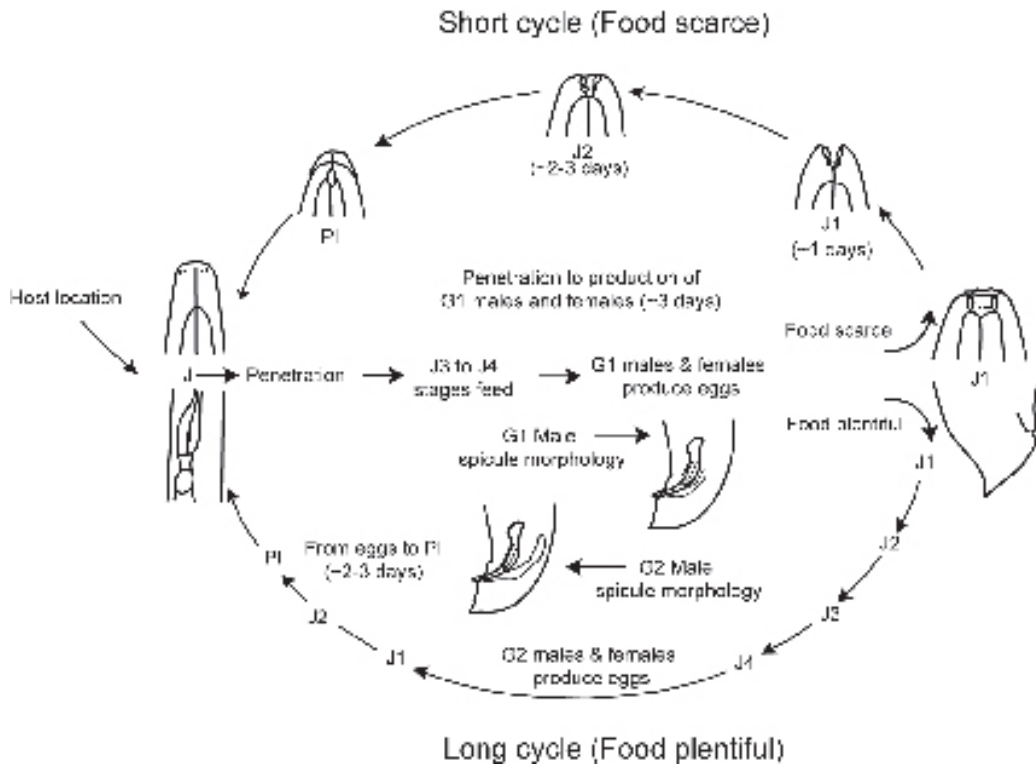


Figure 7 Diagram of the life cycle of *Steinernema*. G1 = first generation, G2 = second generation, J1 = first-stage juvenile, J2, second-stage juvenile, J3 = third-stage juvenile, J4 = fourth-stage juvenile, PI = pre-infective stage juvenile, J = infective juvenile. Modified after Adams and Nguyen (2002).

1992; Campbell and Gaugler 1993; Lewis, Gaugler *et al.* 1993; Campbell and Gaugler 1997). It is generally assumed that infective juveniles search behavior can be divided into two broad categories: crawling and standing on their tails (i.e. nictation) (Campbell and Gaugler 1993). Nictation takes many forms, and ranges from straight motionless behavior to partial lifting from the substrate and waving back and forth (Campbell and Gaugler 1993). Many *Steinernema* species exhibit jumping behavior, which is initiated from a standing posture, and is produced by forming a loop with the body, with the head held to the side of the body by the surface tension. The jump is performed by contracting and suddenly releasing the loop (Campbell and Kaya 1999). Figure 10 shows a schematic view of the cruiser and ambusher strategies.

The focus of this thesis is on how entomophagous nematodes use plant-provided signals to locate their hosts. It has been suggested that nematode attraction to a suitable host can integrate different possible cues such as temperature, electric potential, carbon dioxide, and various organic and inorganic substances (Jansson and Nordbringhertz 1979). The general focus has oriented, however, toward the chemically-mediated attraction (Riga 2004), either resulting from substances coming from the plant such as CO₂ (Gaugler, Lebeck *et al.* 1980), root and leaf homogenates (Bilgrami, Kondo *et al.* 2001), or from the host itself, such as feces (Lewis, Gaugler *et al.* 1992; Grewal, Gaugler *et al.* 1993), plasma (Khlibsuwan, Ishibashi *et al.* 1992), or specific host kairomones (Gaugler, Lebeck *et al.* 1980). Some past experiments with nematophagous fungus have shown that nematode attraction can be influenced by organic compounds such as sialic acid (Jansson and Nordbringhertz 1984). No specific compound has yet been found to be responsible for entomopathogenic nematodes attraction toward the insect host (Kaya 1990; Boff, Zoon *et al.* 2001) (But see chapter I); but it is mainly assumed that

nematode orientation and aggregation is due to unspecific signaling involving carbon dioxide. For example, Lewis (1993) found that *S. glaseri* (Steiner) responded positively to volatiles cues from an insect host and that this response was eliminated if CO₂ was removed. A similar response was found later by Grewal (1994) for other cruiser *Steinernema* and for two species of *Heterorhabditis*, and this strong response to volatile cues was extended to many *Steinernema spp* that are effective in finding sedentary hosts (Campbell, Lewis *et al.* 2003). On the other hand, CO₂ appears to be mainly a short-range attractant and may play a role in host penetration through the spiracles (Ishibashi and Kondo 1990). It seems also very unlikely that such a general signal can be unambiguously exploited by foraging nematodes looking for a specific host. Quite recently, (van Tol, van der Sommen *et al.* 2001) proposed that plants can produce inducible compounds which can be used by foraging nematodes, and later this notion was confirmed by testing *H. megidis* toward weevil infected roots (Boff, Zoon *et al.* 2001; Boff, van Tol *et al.* 2002).

The central theme of this thesis focuses on these insect-induced responses by plant roots and their role in recruiting entomopathogenic nematodes.

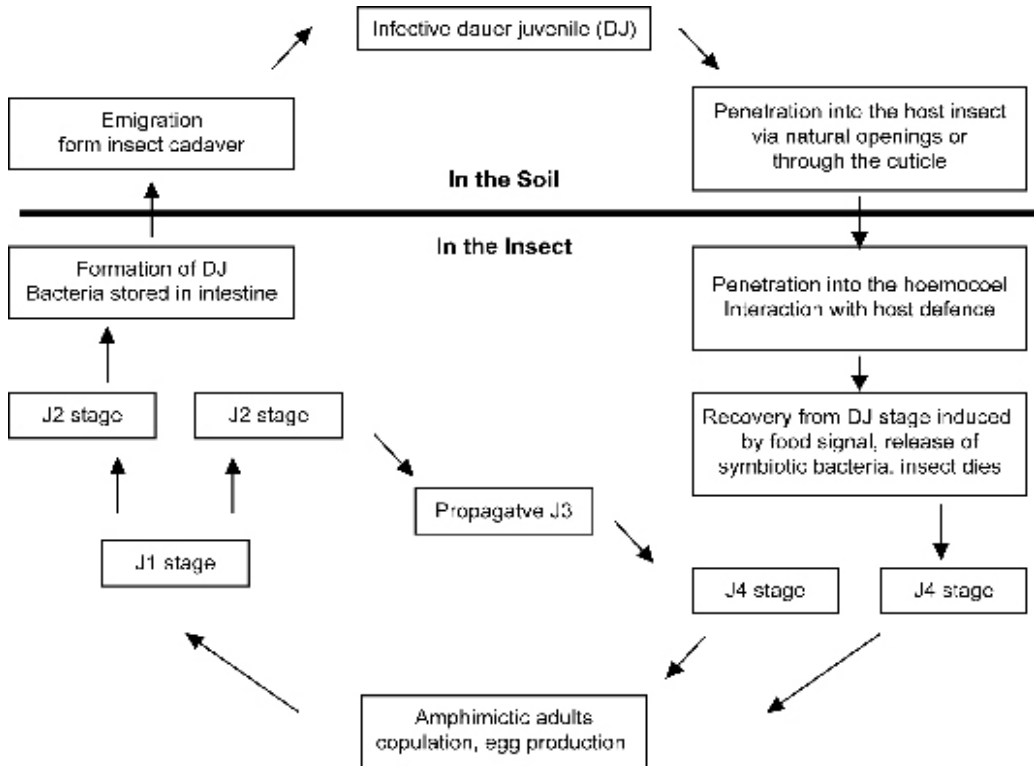


Figure 8 Diagram of entomopathogenic nematode different life stages from insect infection till propagation. J1-4 = 1-4 juvenile stages, DJ = Dauer juvenile (resistance stage).

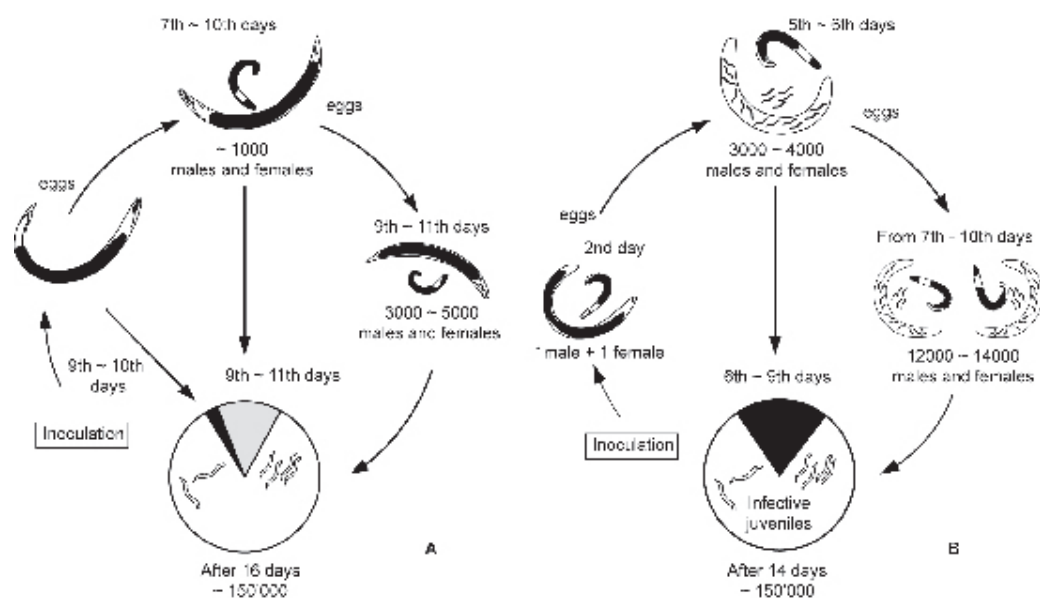


Figure 9 Population dynamics of A) *Heterorhabditis bacteriophora* and B) *Steinernema carpocapsae* in a larva of *Galleria mellonella* after injecting one or two DJ per insect respectively. The pie charts represent the number of DJ progeny recruited from each generation. (black): DJ progeny recruited from first generation females; (grey): DJ progeny recruited from second generation females; (white): DJ progeny recruited from third generation females. Modified after Wang and Bedding (1996).

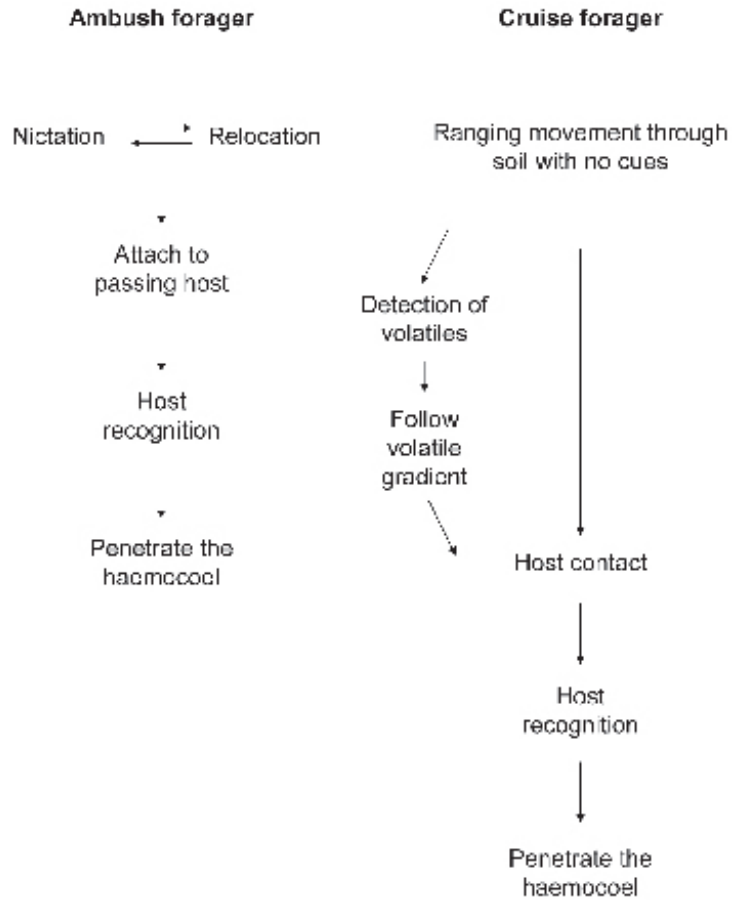


Figure 10 The order of events that occur during a bout of host finding for ambusher and cruiser nematodes. Modified after Lewis (2002).

Spodoptera littoralis and *Cotesia marginiventris*

Maize plants growing in the field are generally attacked by a wide variety of herbivore pests feeding on leaves (folivores) and stem. One of the chapters of the present thesis focusing on multi-enemy interactions between above- and belowground species feeding on maize plants, it will be here presented the major distinctive traits of the aboveground herbivore (*Spodoptera littoralis* Boisduval Lepidoptera: Noctuidae) and one of its parasitoids (*Cotesia marginiventris* Cresson Hymenoptera: Braconidae). Noctuid butterfly of the genus *Spodoptera* are likely to be found on maize plants. *S. littoralis*, the African cotton leafworm is a highly polyphagous species feeding primarily on cotton, but attacking other plant family including Solanaceae, Cruciferae, artichoke, strawberry, fodder crops, maize, cotton, tomato and capsicum (Brown and Dewhurst 1975).

The moth (adult) has a wingspan of 35 to 40 mm. Fore wings are brownish with bluish overtones and straw yellow along the median vein; the ocellus is marked by 2 or 3 oblique whitish stripes. The front of the wing tip has a blackish marking, more pronounced in the male. Hind wings are whitish, with a brown front edge. Egg: about 0.6 mm long, more or less spherical. Eggs are laid in clusters and covered with brownish-yellow hairs detached from the abdomen of the female. The neonate caterpillar is pale green with a brownish head; when fully developed (L 4) it is 35 to 45 mm long. Color varies from grey to reddish or yellowish, with a median dorsal line bordered on either side by two yellowish-red or greyish stripes, and small yellow dots on each segment. The edges, more or less dark grey, bear triangular markings which on the 1st and 8th segments become 2+2 large, triangular, velvety black markings. The underside of the caterpillar is greyish-red or yellowish. The pupa size range from 15 to 20 mm, is brick red color, and the cremaster are with a single pair of spines. In Egypt, its

place of origin, adults appear in early spring (as early as February in cold glasshouses). The moths emerge at twilight or at night. Eggs are laid in clusters covered in hairs from the abdomen of the female. The majority of clusters are sited on the lower parts of plants. At a temperature of 25 to 28°C, embryonic development takes 3 to 4 days. Larvae are firstly gregarious; the caterpillars become solitary from L4. Like the butterflies, they are mainly nocturnal, sheltering in the soil during the day. After about 2 weeks post emergence, pupation takes place in the soil at a depth of about 2 to 5 cm. The adult molt occurs in less than a week. Details on biology of the species were found at <http://www.inra.fr>.

In the previous section it was mentioned that plants subjected to feeding damage by insects respond with the release of characteristic blends of volatiles that attract parasitoids and predators, and these releases by attacked plants are triggered by elicitors in oral secretions of the herbivores (Dicke, Sabelis *et al.* 1990; Turlings, Tumlinson *et al.* 1990; Turlings, McCall *et al.* 1993; Mattiacci, Dicke *et al.* 1995). Alborn *et al.* (1997) identified a nonprotein elicitor from the regurgitant of *S. exigua* Hübner, N-(17-hydroxylinolenoyl)-L-glutamine, named volicitin. In maize plants, volicitin triggers a response similar to that triggered by *S. exigua* feeding. The induced odor is composed mainly of terpenoids and is highly attractive to the braconid parasitoids *C. marginiventris* and *Microplitis croceipes* Cresson (Alborn, Turlings *et al.* 1997; Turlings, Alborn *et al.* 2000).

C. marginiventris is a generalist parasitoid found on more than 30 different species of plants and reared on at least 3 butterfly families (Noctuidae, Plutellidae, and Pyralidae) (Turlings 1990). It has been reported on almost all the geographic zones of the Americas and the West Indies (Turlings 1990). The life-cycle of a typical endoparasitoid can be divided into eight steps; 1) adult parasitoids emerge from the cocoon, 2) adult female

search for the micro-habitats of potential host, 3) female examine faeces for host acceptance, 4) oviposition, 5) development from egg till last instars larvae inside the host, 6) final instars larvae chews its way out of the host, and 7) formation of the cocoon in which the parasitoid larva pupates (Boling and Pitre 1970). In laboratory conditions, *C. marginiventris* reared on *S. littoralis* larvae, develop from egg till final larval instars in

Parasitized young *S. littoralis* caterpillars will be strongly reduced in their developments, thus reducing feeding damages (Turlings 1990). After cocoon emergence, caterpillars will die, and the young, naïve parasitoid adult female will mate freely and many times (Boling and Pitre 1970) before searching for a new host. It has been mentioned that parasitic wasps may use plant odors induced by insect feeding to locate plants that carry their host



Figure 10 Three days old *S. littoralis* larvae being attacked by *C. marginiventris* adult females.

about 10 days, and from cocoon formation till adult emergence in 3-5 days. Adult maximum survival was recorded for a period of 20 days; but generally not oversteps 12 days of survival probability (Faria 2005). The third instars larvae emerge by biting their way out through the cuticle of the host larva with their well developed mouth parts, and immediately start spinning a crescent-shaped cocoon in the vicinity or on the host (Boling and Pitre 1970).

(Vet and Dicke 1992; Turlings, McCall *et al.* 1993). Associative learning of the most reliable cues (in this case the faeces of the host) may help the wasp to more efficiently find such plants (e.g. (Vet and Groenewold 1990; Lewis, Tumlinson *et al.* 1991; Zanen and Carde 1991; Eller, Tumlinson *et al.* 1992). This ability to learn is generally expected to be an adaptative strategy for parasitoids that have a broad host range or which can find

their hosts on multiple plant species (Vet and Groenewold 1990; Vet and Dicke 1992; Vet 1999). Indeed, generalist parasitoids such as *C. marginiventris* are often found to exhibit this learning ability (Steidle and van Loon 2003). The wasp *C. marginiventris*, like entomopathogenic nematodes, has been mentioned as possible biological control agent against lepidopteran pests (e.g. (Henneberry, Vail *et al.* 1991; Turlings and Tumlinson 1992; Tumlinson, Turlings *et al.* 1993; Tillman and Scott 1997; Bottrell, Barbosa *et al.* 1998; Hoballah, Degen *et al.* 2004). Strong learning abilities to associate host cues with induced plant volatile production, r-strategies life-cycle (i.e. reproductive investments seem to be mostly concentrated on quantity rather than on quality), and strong potential in reducing host populations (Lewis and Nordlund 1980), will thus surely select *C. marginiventris* in favour of a possible biological control agent against *S. littoralis* larvae.

Thesis outline

The present thesis addresses the following questions:

Do maize roots that are attacked by the larvae of the western corn rootworm produce induced volatiles organic compound that attract entomopathogenic nematodes? It has been clearly demonstrated that aboveground, plant-produced organic volatile compounds, induced by the feeding of folivores, are responsible for the attraction of natural enemies such as parasitoids (Turlings and Wäckers 2004). Only recently the focus has gone belowground (van Tol, van der Sommen *et al.* 2001). The aim of the study presented in chapter 1 was to asses if *Diabrotica virgifera virgifera* attacked maize plants do also produce signals that are responsible for the attraction of entomopathogenic nematodes *Heterorhabditis megidis*. The chapter also introduces a novel method to study nematode behaviur, and the characterization of a specific compound involved in the belowground tritrophic interaction. Moreover, it was tested if the compound is active in a field situation and might be used to enhance biological control of the pest.

Is there a plant-mediated interaction between above- and belowground tritrophic interactions? The aboveground tritrophic interactions have been subject to many studies over the last twenty years (Karban and Baldwin 1997). Belowground interactions, on the other hand, have only recently become subject to investigations (Bezemer and van Dam 2005). How one compartment of interactions can affect the other and vice versa is still an unanswered question. Two newly developed olfactometers (Turlings, Davison *et al.* 2004; Rasmann, Kollner *et al.* 2005) were connected together to study the aboveground system (maize leaves attacked by *Spodoptera littoralis* larvae and the parasitoid *Cotesia marginiventris*) and the belowground system (maize roots, *Diabrotica virgifera virgifera* and the entomopathogenic nematodes *Heterorhabditis megidis*) in interaction.

Is there specificity in the belowground signals involved in a tritrophic interaction? In this thesis it was shown that maize roots produce an organic volatile compound ((E)- β -caryophyllene) after the feeding by *Diabrotica virgifera virgifera* larvae (Rasmann, Kollner *et al.* 2005). This compound is attractive to the entomopathogenic nematode *Heterorhabditis megidis*. We tested if other plant species (cotton, *Gossypium hirsutum* and cowpea *Vigna unguiculata*) also produce belowground signals after root herbivory that are attracted to *H. megidis*. We also tested the responses of other species of nematodes (*H. bacteriophora* and *S. feltiae*) to such signals, and how the root-produced signals may differ after the attack of other herbivores and pathogens on maize roots, such as wireworms *Agriotes ustulatus* and phytopathogenic nematodes.

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CHAPTER I



RECRUITMENT OF ENTOMOPATHOGENIC NEMATODES BY INSECT-DAMAGED MAIZE ROOTS

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Recruitment of entomopathogenic nematodes by insect-damaged maize roots

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Plants under attack by arthropod herbivores often emit volatile compounds from their leaves that attract natural enemies of the herbivores. Here we report the first identification of an insect-induced belowground plant signal, (E)- β -caryophyllene, which strongly attracts an entomopathogenic nematode. Maize roots release this sesquiterpene in response to feeding by larvae of the beetle *Diatraea lineatella* (larvae), a maize pest that is currently invading Europe. Most North American maize lines do not release (E)- β -caryophyllene, whereas European lines and the wild maize ancestor, teosinte, readily do so in response to *D. lineatella* attack. This difference was consistent with striking differences in the attractiveness of representative lines in the laboratory. Field experiments showed a twofold higher nematode infection rate of *D. lineatella* larvae on a maize variety that produces the signal than on a variety that does not, whereas spiking the soil near the latter variety with authentic (E)- β -caryophyllene decreased the emergence of adult *D. lineatella* to less than half. North American maize lines must have lost the signal during the breeding process. Development of new varieties that release the attractant in adequate amounts should help enhance the efficacy of nematodes as biological control agents against root pests like *D. lineatella*.

Plants are not simply passive victims of attacking herbivores; they have evolved an arsenal of physical and chemical defences to protect themselves. Often these defences are mobilized only in response to herbivory^{1,2}. Among the proposed inducible defences is the production and release of volatile chemicals that could serve as signals to attract natural enemies of the herbivores³⁻⁵. Identifying these signals can help increase the effectiveness of these natural enemies as control agents⁶⁻⁸. The induced emission of chemical signals is not limited solely to aboveground plant parts. The entomopathogenic nematode *Heterorhynchus megidis* was found to be attracted to exudates emitted by plant roots after damage by weevil larvae^{9,10}, but the nature of the attractants involved is unknown. Here we show that maize roots damaged by larvae of the economically important coleopteran pest *Diatraea lineatella* (Lac.) are attractive to entomopathogenic nematodes, and we identify the chemical compound responsible for the attraction. *D. lineatella* or Western corn rootworm (WCR) is a voracious pest of maize that is responsible for the use of the bulk of pesticides applied in the cultivation of this crop in the USA¹¹. The recent introduction and rapid spread of WCR into Europe has caused major concern for maize production on this continent and has stimulated the search for new methods of maize protection^{12,13}. The use of nematodes to control WCR is an ecologically sound option^{14,15}, especially if researchers can optimize their efficacy at finding and killing WCR.

Attraction of nematodes by WCR-damaged roots

To determine whether or not WCR-infested maize plants would attract nematodes, three glass pots each containing one 10-day-old maize plant (var. Delfino) were attached to the arena of a custom-made six-arm olfactometer filled with moist (10% water) sand (Fig. 1a). The plants had been grown on clean sand in the pots, starting 5 days after seed germination. Three additional pots, containing only sand, were attached to the remaining three arms

of the olfactometer. Four such olfactometers, each containing three plants plus three sand controls, were prepared on a given day. One plant of each set of three received four second-instar or third-instar WCR larvae, the roots of a second plant were damaged daily by stabbing them five times with a metal corkborer 7 mm in diameter, and the third plant was left unharmed. On day 3 after initial damage, about 1,000 *Heterorhynchus megidis* nematodes were released in the centre of each olfactometer, where they were free to enter the arena until their passage was blocked by an ultra-fine metal screen (see Fig. 1a and Methods). One day after release, the number of nematodes in each arm was recorded. Significantly more nematodes were recovered from arms connected to the pots with the WCR-damaged plants than from the arms connected to the other treatments or controls (Fig. 1b), indicating that damage by WCR induces maize roots to release a nematode attractant.

Identification of the attractant

Maize leaves had previously been shown to emit a mixture of volatile compounds in response to damage by caterpillars¹⁶. To determine whether WCR damage induces similar changes in plant volatiles, the leaves and roots from WCR-damaged (3 days) and healthy maize plants were ground and volatiles collected by solid-phase microextraction (SPME) were analysed by gas chromatography-mass spectrometry (GC-MS). A marked difference between the treatments was that the sesquiterpene (E)- β -caryophyllene was present in roots damaged by WCR but was completely absent from undamaged roots (Fig. 1c). The damaged roots contained small amounts of α -humulene and caryophyllene oxide as well. To a smaller extent, the WCR-induced increase in (E)- β -caryophyllene content was also apparent in the leaves (Fig. 1d). To test whether (E)- β -caryophyllene was indeed attractive to *H. megidis*, an authentic standard (Sigma-Aldrich, more than 99% pure) was tested in the olfactometer. For this purpose the system was entirely filled with clean moist sand and a 0.1- μ l dose of (E)- β -caryophyllene was

injected in the centre of one of the pots, whereas the five remaining pots received no such treatment. Nematodes were released in the middle of the olfactometer and on the next day nematodes were recovered from the six arms. The arm attached to the pot that had received (*E*)- β -caryophyllene contained almost three times as many nematodes as the average control arm (Fig. 3a). Using a much lower dose of three injections with 300 ng of (*E*)- β -caryophyllene in pentane produced very similar results. In an additional experiment, a 10-day-old healthy maize plant (var. Delpino) was placed in each of two opposing pots of the olfactometer and the other four pots contained sand only. One of the pots with a plant was spiked with a 0.1- μ l dose of (*E*)- β -caryophyllene and nematodes were released in the olfactometer centre. On the next day, the arm with the caryophyllene-spiked plant contained on average almost fivefold as many nematodes as the control arms, whereas there was no statistical difference between the plant without (*E*)- β -caryophyllene and the control pots (Fig. 3b). We tested several other synthetic compounds that are commonly released from caterpillar-damaged maize leaves in three choice tests, always including (*E*)- β -caryophyllene as one of the choices (data not shown). These compounds either were not attractive (linalool) or were significantly less attractive than (*E*)- β -caryophyllene ((*Z*)-3-hexenyl acetate, methyl salicylate, (*E*)- β -farnesene, α -humulene and (*E*)-neolidol) at a 0.1- μ l dose per pot.

Loss of signal in North American maize genotypes

The very limited number of compounds in the volatile blend obtained from WCH-induced roots is in striking contrast to what is emitted from maize leaves in response to caterpillar feeding, a complex mixture of different terpenoids, aromatic compounds and green-leaf volatiles^{43,47}. Many of the maize lines that we have screened in the past for caterpillar-induced leaf volatiles do not emit

(*E*)- β -caryophyllene in detectable amounts. This is particularly characteristic for maize varieties that originate from North American breeding programmes⁴⁸. We tested whether this difference also holds true for the roots by measuring WCH-induced (*E*)- β -caryophyllene in six inbred lines selected from a study on caterpillar-induced leaf volatiles⁴⁹: those that emitted large amounts (*E*)- β -caryophyllene from their leaves (Dn1D1, F3 and E584) and three lines that released no or very little (*E*)- β -caryophyllene (E584, A654 and F7008). In this experiment we also included the closest wild ancestor of maize, teosinte (*Zea mays parviglumis*^{50,51}), which is known to release relatively large amounts of (*E*)- β -caryophyllene from its leaves in response to caterpillar feeding⁴⁷. Ten-day-old plants were subjected to 3 days of WCH feeding, after which (*E*)- β -caryophyllene levels were measured in the roots as above. Teosinte roots were found to release moderate amounts of (*E*)- β -caryophyllene in response to WCH damage (Fig. 3a). The experiment also confirmed a correlation between the levels of (*E*)- β -caryophyllene induced in the leaves and the roots (Fig. 3a): Dn1D1, F3 and E584 emitted considerable amounts of (*E*)- β -caryophyllene from the roots after WCH attack and E584, A654 and F7008 emitted hardly detectable amounts. These differences offered an excellent opportunity to test whether (*E*)- β -caryophyllene is a key compound for nematode attraction, because non-emitting varieties should be far less attractive than emitting varieties. This was tested with representative lines of commercial maize for which we had information on caterpillar-induced (*E*)- β -caryophyllene released⁴⁷.

Factor 1 is a commercial maize variety that releases no detectable amounts of (*E*)- β -caryophyllene from its leaves in response to caterpillar feeding, whereas Gof releases relatively large amounts, significantly more than the variety Delpino, which was used in the first experiments⁴⁷. Root extracts from WCH-damaged plants confirmed the presence of (*E*)- β -caryophyllene in Gof and Delpino

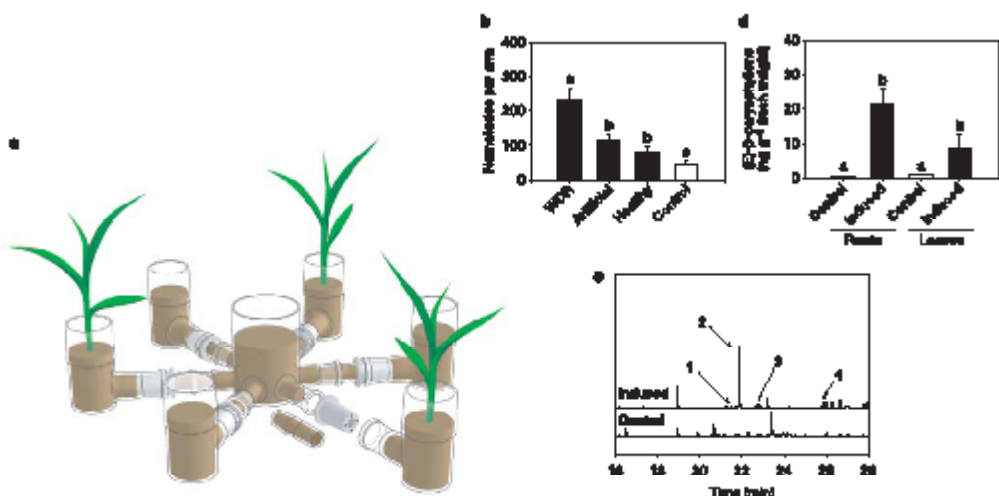


Figure 1 Attraction of entomopathogenic nematodes to a WCH-induced root signal. **a**, Drawing of a newly designed belowground six-arm olfactometer in which nematode attraction was tested. **b**, Choice between plants: the average number of nematodes recovered from olfactometer arms that were connected to pots holding either a maize plant with WCH-damaged roots, mechanically damaged roots or undamaged roots ($n = 12$). For each replicate, the total number of nematodes that went to the three control pots (only moist sand) were summed and divided by three. **c**, Typical

chromatographic traces obtained from the roots of a healthy plant and of a WCH-damaged plant. The labeled peaks are as follows: 1, unknown compounds; 2, (*E*)- β -caryophyllene; 3, α -humulene; 4, caryophyllene oxide. **d**, Quantification of (*E*)- β -caryophyllene in roots and leaves from healthy and WCH-damaged maize plants ($n = 8$). Lower letters bar indicate significant differences. Error bars indicate standard error.

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roots and its absence from Factorol roots (Fig. 3b). Next, individual plants of these three varieties were tested simultaneously in the olfactometer by letting four third-instar WCR larvae feed on their roots for 3 days and then releasing nematodes from the olfactometer centre as before. The numbers of nematodes recovered from the olfactometer arms revealed strong attraction to Graf and Delpino and no attraction to Factorol (Fig. 3c). The importance of (E)- β -caryophyllene for this difference in attractiveness was confirmed in a nearly identical experiment with the three varieties, except that on the third day of WCR feeding 0.2 μ l of (E)- β -caryophyllene was added to the sand in the pot with the Factorol plant. After this treatment the Factorol plant was as attractive to the nematode as the two other plants (Fig. 3d).

Attractiveness in the field

To verify the importance of (E)- β -caryophyllene as an attractant for *H. megidis* under realistic conditions, we conducted two types of field experiment in Hungary, where WCR is already an established pest. For each experiment, six maize plants were planted at an equal distance from each other in circles 1 m in diameter. For the first experiment, those of the plants in each of 33 circles were of the variety Graf, alternated with three plants of the variety Factorol (Fig. 4a). Eight weeks after planting each plant was infested with six second-instar WCR larvae. Seven days after this infestation we released about 10,000 *H. megidis*, three times at 2-day intervals, in the centre of each circle. Larval infection rate by nematodes was determined by collecting the roots with larvae for 15 circles at 3 days after the last nematode release. For the remaining 18 circles, larvae were left to pupate and sleeve cages were placed around the plants at least 1 week before expected adult emergence. In circles with

nematode release, the infection rate for larvae on Graf (43.6% of the recovered larvae) was more than fivefold that for larvae on Factorol (8.3% of the recovered larvae; Fig. 4b). This nematode effect was also evident from a significantly lower emergence of adults from Graf roots (Fig. 4c).

More direct evidence for the importance of (E)- β -caryophyllene was obtained with a second experiment with only the Factorol variety planted in the six-plant circles. Again, all plants were infested with six WCR larvae. The soil directly next to those of the plants per circle was spiked on a daily basis with 2 μ l of (E)- β -caryophyllene for 5 days (Fig. 5a). One day after the first spiking (7 days after WCR infestation), about 10,000 nematodes were released in the centre of each circle; this was repeated twice at 2-day intervals. We recovered relatively few larvae (18% as opposed to 40% for the Factorol-Graf experiment) from the 12 circles that had been reserved to estimate infection rates. This was probably due to poor lodgement of these circles, which could also explain why we did not observe a difference in infection rate between treatments. However, the results from the 24 circles that were left to estimate adult emergence showed a significant effect of (E)- β -caryophyllene, with a more than twofold decrease in adult emergence for the plants that had been spiked with the signal (Fig. 5b).

The possibility that there could have been a direct effect of (E)- β -caryophyllene on the WCR larvae or on the quality of the plant was tested in subsequent laboratory experiments. Equal amounts of (E)- β -caryophyllene to those in the field experiments were injected in 15 0.5-litre pots each containing a maize plant and five WCR larvae, whereas 15 other pots each containing a plant and five larvae

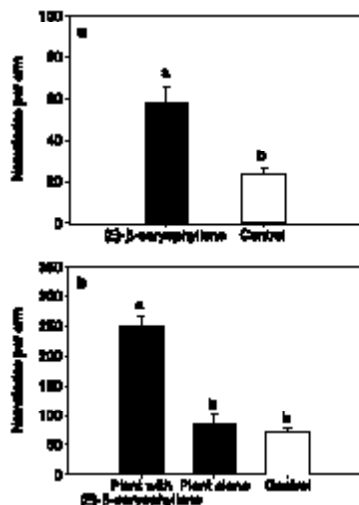


Figure 2 Attraction of *H. megidis* to synthetic (E)- β -caryophyllene. a, Average number of nematodes recovered from olfactometer arms connected to a pot spiked with 0.2 μ l of (E)- β -caryophyllene compared with those recovered from arms connected to unspiked pots ($n = 12$). b, Average number of nematodes recovered from olfactometer arms connected to a pot with a healthy maize plant and spiked with 0.2 μ l of (E)- β -caryophyllene, an arm connected to a pot with a healthy plant only, and four control pots with moist sand only ($n = 12$). For each replicate, the results for control pots were summed and divided by the number of control pots. Different letters above bars indicate significant differences. Error bars indicate standard errors.

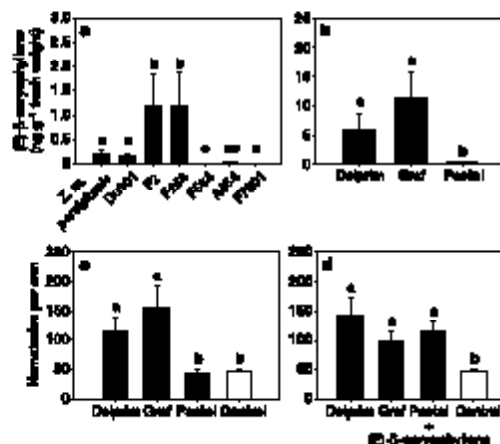


Figure 3 The absence of the (E)- β -caryophyllene signal in certain maize genotypes renders these plants unattractive to the nematode. a, Average amount of (E)- β -caryophyllene detected from the WCR-damaged roots of *Zea mays pungens* (Deltol), of three lines (Deltol, F2 and F200) that we know to release (E)- β -caryophyllene from their leaves in response to caterpillar damage and of three lines (Graf, Factorol and F700) that release no detectable amount of (E)- β -caryophyllene from their leaves. b, Average amount of (E)- β -caryophyllene detected from WCR-damaged roots of the commercial maize varieties Deltol, Graf and Factorol ($n = 6$). c, Average number of nematodes recovered from olfactometer arms connected to pots holding WCR-damaged maize plant of the varieties Deltol, Graf and Factorol ($n = 12$). d, Average number of nematodes recovered from olfactometer arms connected to pots holding WCR-damaged maize plant of the varieties Deltol, Graf and Factorol after the pots with Factorol were spiked with 0.2 μ l of (E)- β -caryophyllene ($n = 12$). Statistical differences are indicated with different letters above the bars. Error bars indicate standard errors.

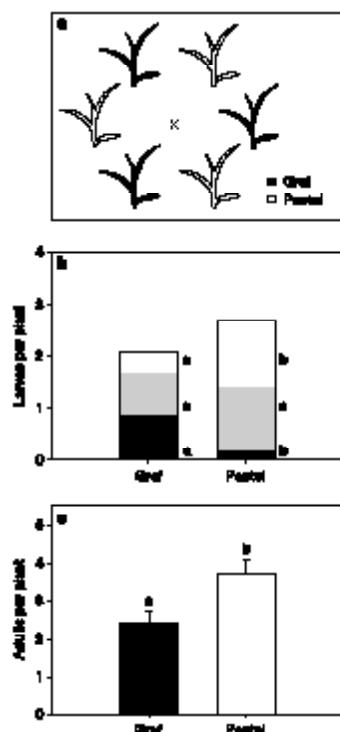


Figure 4 MicroHCH larvae were collected with nematodes and lower adults emerged near Gai plants than near Pectol plants. **a**, Design of field circle experiment for which maize plants of the varieties Pectol and Gai were alternated. The rows mark the spot at which nematodes were released. **b**, Mean number of larvae per plant that were healthy (white bars), infected by fungi (gray bars) or infected by nematodes (black bars). Statistical differences between the proportions of the three larval types are indicated with different letters. **c**, The mean number of adults that emerged for each plant was significantly different for the two varieties ($P < 0.01$). Error bars indicate standard error.

served as controls. No difference was found in the total number of adults that emerged from these pots (data not shown), supporting the hypothesis that nematode attraction to (*E*)- β -caryophyllene was responsible for the difference observed in the field.

Suitability of (*E*)- β -caryophyllene as a belowground signal

(*E*)- β -Caryophyllene is a common secondary plant compound that is also emitted from the silk of mature maize plants and has been shown to be weakly attractive to adult WCH females²¹. This sesquiterpene is probably not the only attractant for *H. megidis*, because some degree of nematode attraction was also found to healthy and mechanically damaged plants (Fig. 1b), even though emission of (*E*)- β -caryophyllene from maize leaves and roots has been detected only after herbivory. Indeed, several plant metabolites, including CO₂, are known to be attractants for entomopathogenic nematodes²². Cues that come directly from host larvae might also guide nematodes^{23–25}, but these have been shown to be attractive only over short distances. The overriding importance of (*E*)- β -caryophyllene as a long-range attractant is best indicated by its abundance in the root exudates of the most attractive varieties and

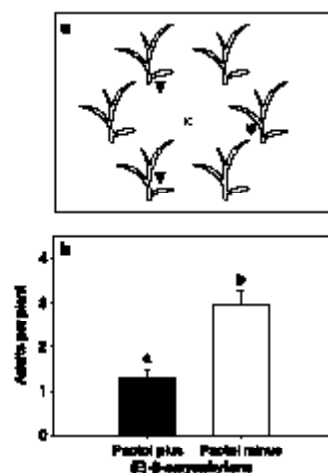


Figure 5 Fewer WCH adults emerged near Pectol plants that were spiked with (*E*)- β -caryophyllene than near Pectol plants that received no (*E*)- β -caryophyllene. **a**, Design of field circle experiment with only plants of the variety Pectol. They signs mark the sites at which five times 2 μ l of (*E*)- β -caryophyllene was injected into the soil; the rows mark the spots at which nematodes were released. **b**, The mean number of adults that emerged near the spiked plants was significantly lower than for the unspiked plants ($P < 0.0001$). Error bars indicate standard error.

the fact that supplementing sand with (*E*)- β -caryophyllene renders an otherwise unattractive variety highly attractive (Fig. 3d).

To test the ability of (*E*)- β -caryophyllene to diffuse in moist sand, 1 μ g of this sesquiterpene were pipetted into one spot in a sand-filled glass dish. At a distance of 10 cm from this spot a SFMB fibre was inserted into a hole in the sand (see Methods). Every half hour the compounds adsorbed on the fibre were desorbed and analysed by GC-MS, starting with the fibre hour before the addition of (*E*)- β -caryophyllene. (*E*)- β -Caryophyllene travelled rapidly through the sand and was already trapped on the fibre during the first half hour after it had been introduced to the sand. The amount trapped increased steadily for 1 h, after which it decreased sharply (Fig. 6a). A similar experiment in a sand-filled olfactometer, with an arm modified to permit the introduction of a SFMB fibre, revealed the presence of (*E*)- β -caryophyllene in the centre part of an arm 1 h after injecting 0.2 μ l into a pot connected to that arm (not shown). To determine whether the rapid decrease in (*E*)- β -caryophyllene detection was due to evaporation from the sand, an additional experiment was performed by which a drop containing 1 μ g of (*E*)- β -caryophyllene was placed on the bottom of a beaker, which was immediately covered by 5 cm of moist sand. The beaker was placed in a closed-loop volatile-collection system where the headspace above the sand was continuously sampled at intervals of 30 min. A very similar time course of (*E*)- β -caryophyllene diffusion was obtained, with the first detection after 30 min and a peak after 1 h (Fig. 6b), indicating rapid evaporation. Recovery was more than 50%, which implies that the degradation of (*E*)- β -caryophyllene or its immobilization to sand particles is not significant under these conditions. The rapid diffusion of (*E*)- β -caryophyllene in moist sand and its chemical stability seem to make it exceptionally suitable as a belowground signal. In the olfactometer assays described above, the nematodes were released 25 cm from the treatment pots. Therefore, after detecting the signal they move a distance of more than 250 times their body length within a day.

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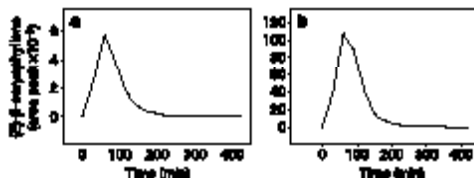


Figure 3 (E)- β -Caryophyllene diffuses readily through sand and then evaporates rapidly without breakdown or irreversible adsorption. a, Detection of volatile (E)- β -caryophyllene with a SPME fibre in headspace of 10 min from a reference vial, away from the root system. b, Detection of (E)- β -caryophyllene in the headspace above a beaker containing 5 cm of moist sand after (E)- β -caryophyllene had been placed at the bottom of the beaker.

Discussion

The failure of most North American maize lines to release (E)- β -caryophyllene suggests that the ability to produce this compound has been lost during breeding. Indeed, the closest wild ancestor of maize, *Zea mays* ssp. *parviglumis*^{23,24}, was also found to release (E)- β -caryophyllene from its roots in response to WCH damage (Fig. 3a). The loss of direct defences to herbivores during plant domestication has been amply documented²⁵. However, to our knowledge this is the first example of the loss of a signal involved in indirect defence.

WCH has already caused large economic losses to maize in Central Europe. Since 2003 it has been detected in almost all European countries south of Scandinavia, and will inevitably become a major threat to maize cultivation throughout Europe²⁶. Effective, ecologically sound control methods are needed. Botanical-pathogenic nematodes could be an option^{24,27}, but they have not yet been employed with sufficient efficacy. The results of this study led us to speculate that the absence of an attractive signal in many American maize lines could explain why attempts to control WCH with nematodes have yielded only mixed results on the North American continent²⁸. Reintroduction of this signal in newly developed maize varieties might aid in effective control of this voracious pest.

This first identification of an inducible belowground plant signal that attracts enemies of root-feeding herbivores underlines the breadth and sophistication of indirect plant defences. With a growing interest in belowground plant-mediated interactions and their effects on various trophic levels^{24,29} our results should prompt new studies into the evolutionary history and ecological consequences of multitrophic-level interactions and should lead to the exploitation of the signal for crop protection. □

Methods

Nematode assays

The survival of nematodes to plant-produced substances was tested in a belowground bioassay consisting of a central glass chamber (5 cm in diameter, 11 cm deep) with six equally distributed side arms at 0.5 cm height with a female (34 mm diameter \times 12 mm long) connector (Fig. 3a). Three arms connected the central chamber with six glass pots (5 cm in diameter, 11 cm deep) in which plants or other source of stimulus could be placed. Each pot also had a female connector (RSC1) at 0.5 cm height. The connecting arms contained either two detachable parts: one was a glass tube with ground-glass connectors (male, 24/28) on both sides, and the second part, a Teflon connector (RSC2 to RSC3) was used to attach the glass tube to the other source pot. The connectors made Teflon connectors (Analytical Research Systems) consisted of thin-line metal screens (3.00 mm mesh; Farn Film Inc.) surrounding the nematode lines reaching the other source pots (Fig. 3a). For each experiment, the entire system was filled with sterilized white sand (94 litre) to about 5 cm from the rim of the pots. Nematode were released in a drop of water in the centre of the central pot. One day after nematode release, the effectiveness was determined and the sand in each detachable glass tube was put on a separate clean filter disk (12 cm in diameter) (Göteborg GmbH). The disk with the sand was placed in a Beaman carrier³⁰, and nematodes in the collection tube were counted on the next day.

Statistical differences in choices made by the nematodes were determined with log-linear models on the basis of the assumption that the nematodes would disperse equally among the arms in the absence of any stimulus. The model was adapted to account for possible overdispersion due to directional bias³¹.

Root samples

For the analysis of root systems, root of WCH-damaged and undamaged maize plants were washed with water and frozen in liquid nitrogen; they were then powdered in a mortar and 0.4 g of root powder was placed in a glass vial with a septum in the lid. A 200- μ m polydimethylsiloxane (PDMS) SPME (Supelco) fibre was inserted through the septum and exposed for 60 min at 40 °C. The compounds adsorbed on the fibre were analysed by GC-MS with an Agilent 6890 Series GC system QES50A, coupled to a quadrupole-mass selective detector (Agilent 5973 transfer line 250 °C, source 250 °C, ionization potential 70 eV). The fibre was heated manually to the injector port (350 °C) and desorbed and chromatographed on an apolar column (DBS-MS, 50 m, 0.25 mm internal diameter, 0.25 μ m film thickness; I & W-GmbH). Helium at a constant pressure of 35.5 kPa (\times 0.25 MPa) was used for carrier gas flow. After fibre insertion, the column temperature was maintained at 50 °C for 3 min and then increased to 300 °C at 15 °C min⁻¹ followed by a final stage of 3 min at 300 °C. Approximately 0.5 μ l of the eluate was collected with an internal standard by performing analysis on 0.4 g of powdered root tissue from maize line 102 (which produces only trace of (E)- β -caryophyllene)²⁸ spiked with known amounts (0.5, 1.0, 2.5 and 10 ng) of this compound.

The (E)- β -caryophyllene in the roots was provisionally identified as the (-)-enantiomer by chromatography on a chiral column using published procedures for the separation of the two enantiomers³². However, the lack of a standard for the (+)-enantiomer prevented final confirmation.

Field experiments

Field experiments were conducted at the Plant Health Station in Hódmezővásárhely, in southern Hungary (46°13'30" N, 16°08'30" E), from April to October 2004. Six plants were grown from seed in 1-m-diameter circles and with a 1-m distance between circles.

Two types of circle were formed: one contained the two variable line maize var. *Facet* (*Bravura*) and *Z. mays* var. *Onix* (Land) and the other contained only the *Facet* variety. Eight weeks after planting, each plant was infested with six WCH larvae by digging out 5 cm of soil near the base of the plant and dropping the larvae with some potting soil into the hole. The larvae came from a laboratory colony that had been established with 1000 collected adults the year before. At 7, 9 and 31 days after infestation, about 2000 \pm 500 maize nematodes were released in the centre of the treatment circles at a depth of about 10 cm. Additionally, half of the plants in the circle with only the *Facet* variety were spilt daily with 1 μ l of (E)- β -caryophyllene (more than 99% pure; Sigma-Aldrich) for 5 days, starting on the sixth day after infestation with WCH larvae (3 day before nematode release).

Two measurements were taken to determine the effects of the treatments on nematode effectiveness. Part 1 of the *Facet*-*Onix* circles and 11 of the *Facet*-*Facet* circles, the central part of the plants was removed and with a 1-litre cone sample the roots and soil around it were collected. Larvae were extracted by crushing the soil over a black plastic sheet and observing the roots. Each recovered larva was placed on a moist filter paper in a plastic Petri dish (5 cm in diameter, 1 cm deep) and stored at 37 °C for 1 month. They were checked weekly under a microscope for nematode infection, characterized by red pigmentation resulting from gut contents, and for nematode emergence. Infections by other pathogens were also noted.

In addition, adult emergence was measured in another 16 *Facet*-*Onix* circles and 24 *Facet*-*Facet* circles. For this, cylindrical down cages (30 cm \times 70 cm; M4400; Ichnos Education Services Co. Ltd) were fixed on plastic cylinders 10 cm in diameter and 15 cm deep that were placed about 30 cm in the soil around each plant. The upper part of each down was slightly stretched around the stem of the plant to prevent adults from escaping. Once a week, from the beginning of July until the end of August, adults in the emergence cages were counted and collected until no more adults were found. The same log-linear models as employed for the effectiveness data were used to determine differences between treatments.

Nematode measurements

A glass dish (15 cm in diameter, 8 cm deep) was filled with a 5-cm layer of moist (10% water) sand. With a microscope, 1 μ l of synthetic (E)- β -caryophyllene (99% pure; Aldrich) in 30- μ l oil-solvent was placed 5 cm deep in the sand at 1 cm from the dish edge. Immediately after which the hole was covered. At a distance of 30 cm from this spot, a hole 1 mm wide and 5 cm deep was made with a metal rod and a 200- μ m PDMS SPME fibre was placed in the hole. Every 25 min the compounds adsorbed on the fibre were analysed by GC-MS essentially as described above, except that the mass selective detector was operated in the selective ion mode, scanning only for the characteristic ions at molecular masses 204.135 and 75. After the 5-min desorption period, the fibre was placed back in the hole in the sand for a further 25-min collection. The first collection started 70 min before the (E)- β -caryophyllene sample was added to the sand, and the last collection was 7 h later. To measure the time course of evaporation from sand, a 10- μ l drop of dichloromethane containing 1 ng of (E)- β -caryophyllene was placed on the bottom of a 25-mm diameter glass beaker and was immediately covered by a 5-cm layer of 50 μ m mesh sand. The beaker was placed in a closed-loop volatile collection system consisting of a 1-litre desiccator in which the headspace above the sand was continuously collected by pulling air through a 75- μ m activated charcoal filter at a rate of 31 ml min⁻¹. The filter was connected with dichloromethane at intervals of 50 min and the eluate was analysed by GC-MS as described above.

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Note

During the present thesis, and particularly throughout Chapter I, field soil type that was encountered in Hungary was not discussed.

The experimental field consisted of a dense clay-loam soil (sand 14%, loam 44%, clay 42%, pH 8.3) with a soil bulk density of 1.1 g/cm³ (SD 0.13), and a soil moisture of 18.5% SD 2.1) between May and June (analysed by I. Hiltbold, University of Neuchâtel, Switzerland). In its report Hiltbold (2005) characterized the soil as being a Vermic Csernozom soil (Gobat, Aragno *et al.* 1998). For details of the analysis refer to (Hiltbold 2005).

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CHAPTER II



PLANT-MEDIATED CROSS EFFECTS BETWEEN ABOVEGROUND AND
BELOWGROUND TRITROPHIC INTERACTIONS

Sergio Rasmann
Ted Turlings

Abstract

Indirect plant defences, whereby plants attract natural enemies of herbivores, have been studied extensively for aboveground interactions and such tritrophic interactions now have also been found to take place belowground. We studied plant-mediated cross effects between above- and belowground tritrophic interaction in maize using a novel setup that combines collections of above and belowground signal emissions and simultaneous measurements of attractiveness of these signals to the natural enemies of herbivores. 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 specific 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, which was less evident for the leaf volatiles, confirming an important role for minor compounds in parasitoid attraction. The parasitoid could learn the differences in odour and increased its response to the odour associated with double infestation after experiencing this odour during an encounter with hosts. This is the first study to measure an effect of belowground herbivory on aboveground tritrophic signalling and *vice-versa*.

Introduction

Plants are the key organisms that bridge above- and belowground subsystems in complex multitrophic environments (Wardle 2002; Blossey and Hunt-Joshi 2003; Strauss and Irwin 2004; Wardle, Bardgett *et al.* 2004), where herbivores, pathogens and mutualists have been identified as major drivers of plant diversity and ecosystem functioning (De Deyn, Raaijmakers *et al.* 2003). The interdependence of above- and belowground interactions, which are usually studied separately, has been acknowledged (van der Putten, Vet *et al.* 2001), but their joint effect has rarely been taken in account (Bardgett and Wardle 2003). Studies linking the two spatially separated system, where above- and belowground herbivores share a common host plant, have mainly focused on the direct defences of plants affecting the herbivores (e.g. (Masters, Jones *et al.* 2001; Bezemer, Wagenaar *et al.* 2004; Schroter, Brussaard *et al.* 2004; van Dam, Witjes *et al.* 2004; Bezemer and van Dam 2005; van Dam, Raaijmakers *et al.* 2005; Wolfe, Husband *et al.* 2005).

Ever since Price *et al.* (1980) introduced the concept that recognizes the important role of plant in mediating interactions between herbivores and their natural enemies, a vast number of studies have looked at tritrophic interactions in aboveground systems (for reviews see: (Dicke, van Poecke *et al.* 2003; Turlings and Wäckers 2004). Only few recent studies have considered possible effects of belowground herbivory on aboveground tritrophic interactions (Bezemer and van Dam 2005). For example, Poveda *et al.* (2005) and Masters *et al.* (2001) found a positive effect of root herbivory on the recruitment of parasitoids aboveground, whereas Soler *et al.* (Soler, Bezemer *et al.* 2005) found a negative effect of root feeding on aboveground parasitoids and hyperparasitoids. Tritrophic interactions have been also found to occur belowground among plants, root feeders and their parasites

(van Tol, van der Sommen *et al.* 2001; Boff, van Tol *et al.* 2002; Neveu, Grandgirard *et al.* 2002; Aratchige, Lesna *et al.* 2004; Rasmann, Kollner *et al.* 2005), adding another level to the recognized need (van der Putten, Vet *et al.* 2001) for studies on plant-mediated cross effects between above- and belowground communities.

In the current study, we used interconnected above- and belowground six arm olfactometers to investigate in maize plants a possible effect of belowground herbivory on aboveground tritrophic signalling and *vice versa*. Maize (*Zea mais* L.) has been studied extensively for caterpillar-induced volatile emissions that are attractive to parasitoids of the caterpillars (Turlings, Tumlinson *et al.* 1990; Turlings, Lengwiler *et al.* 1998; Turlings, Gouinguéné *et al.* 2002). Maize roots have also been shown to respond to insect feeding damage with the production of the sesquiterpene (*E*)- β -caryophyllene, which attracts entomopathogenic nematodes (Rasmann, Kollner *et al.* 2005). The novel experimental 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. During and after the assays volatiles emitted by the leaves and roots were sampled and could subsequently be analyzed for identification and quantification, thus revealing, for the first time, the effects of double infestation on signal production and the consequences of such effects on attractiveness of the plant to the natural enemies of the herbivores.

Material & Methods

The study system

The system comprised maize *Z. mays* plants 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 as described in (Turlings, Davison *et al.* 2004). Second instars *S. littoralis* larvae were used to rear the generalist endoparasitoid *C. marginiventris* as described in (Turlings, Davison *et al.* 2004). Adults wasps were supplied with water and honey and were kept in incubators (25°C; 16L:8D) until the experimental day. *D. v. virgifera* larvae were obtained from CABI Bioscience (Delémont, Switzerland). The nematodes were supplied by Andermatt Biocontrol AG (Grossdietwil, Switzerland) and were kept in culture flasks (Fisher Scientific AG, 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 (16L:8D, 25000 lm/m²). Plants used for the experiments were 10 to 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. Three additional pots were filled with moist sand only, to serve as controls in the olfactometer. Two additional plants were transplanted as above in glass pots and would serve as “training” plants for the wasps (see below). All pots were then transferred under light banks (16L:8D, 8000 lm/m²) and kept at 21±2 C°.

Three days before the olfactometer experiments, four second instars larvae were added to two of the olfactometer pots with a plant and to one of the learning pots. The evening prior to the experiments, 20 second instars *S. littoralis* larvae (3-5 days old) were placed in the whorl of the youngest maize leaf of plants except for one of the experimental plants infested with *D. v. virgifera*. This way we obtained for the olfactometer 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 above ground olfactometer (Figure 1). The system was assembled the day before responses of parasitoids and nematodes were tested.

The tested wasps were divided into three groups: NAIVES, no oviposition experience; EXPS, wasps that experienced 3-5 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 3-5 ovipositions in the presence of the odour from plants that were simultaneously attacked by *S. littoralis* and *D. v. virgifera*. For these experiences 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 odour vessel by a nylon screen (Figure 1). After three to five ovipositions, wasps were considered experienced.

During each experimental day, 2 groups of 6 wasps of each experience 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 6 wasps were allowed to choose between the odours for 30 minutes, after which, wasps were recovered from the trapping bulbs of the aboveground olfactometer and their choices were recorded.

The release of about 2000 two-week old infective juveniles of the entomopathogenic nematode *H. megidis* in the centre pot of the belowground olfactometer occurred around 9h00 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, Griffin *et al.* 1999), and nematodes were counted the next day. Roots of the plants were then collected, water washed and frozen in liquid nitrogen. 0.3 grams of frozen roots were then placed in sampling vials (22.5 mm diam and 75.5 mm deep) for further analysis (see below).

Olfactometers set-up

For all experiments, an above- and a belowground olfactometer were connected together and run simultaneously (Figure 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 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. It 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 fine meshed metal screen (2,300 mesh; Small Parts Inc.), which prevented the nematodes from entering the odour source pots (for more details see (Rasmann, Kollner *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, Davison *et al.* 2004) was used to test the attractiveness of the wasps towards the treatment plants, and, simultaneously, collecting the plants odours. The 6 odour vessels were connected to an air supply just above the sand and 1.2 L/min of purified air was pushed into each vessel. Half of this airflow (0.6 L/min) was pulled out of the vessel through a trap containing Super-Q adsorbent (25 mg, 80/100 mesh, Alltech, Deerfield State), 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 (see above).

Volatile analysis

During each bio-assay, aboveground volatiles were collected for 4 hrs. The super-Q traps were then extracted with 150 µl of dichloromethane (Merk, 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 next

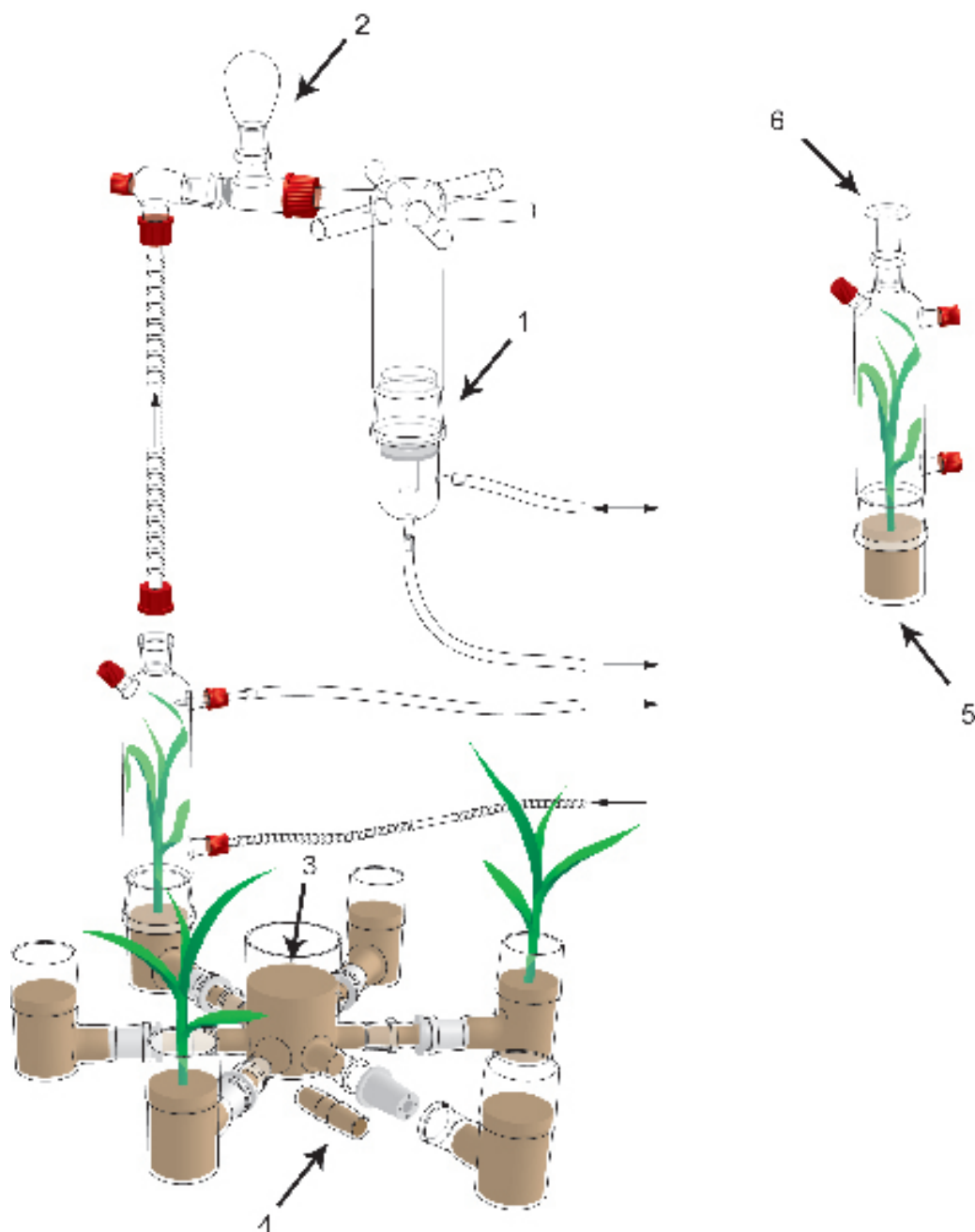


Figure 1 Schematic representation of the above- and belowground olfactometers connected together (only one arm out of six is shown). 1) Wasp release point; 2) wasp trapping bulb; 3) nematode release point; 4) nematode collection tube; 5) odour source vessel; 6) wasps training tube. The small arrows represent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

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; transfer line 230°C, source 230°C, ionisation 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, 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 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, Tamo *et al.* 2002; D'Alessandro and 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 in Figure 3 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 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) fiber was inserted through the septum and exposed for 60 min at 40°C. The compounds adsorbed onto the fiber were analyzed by placing it for 5 minutes

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 fiber 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 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

The responsiveness of the wasps and the nematodes observed in the six-arm olfactometers was analysed using a log linear model (GLM). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of the organisms in the olfactometers (Turlings, Davison *et al.* 2004). The model was tested in the software package R, version 1.9.1. To test for effects of experience, the choices of wasps from the different experience groups were analysed with a Two-Way ANOVA using SigmaStat, version 2.0. The amounts of volatiles collected from the different treatment plants, were compared with a paired t-Test analysis (SigmaStat, version 2.0), whereby the two treatment plants (i.e.: plants induced with *S.littoralis* only *versus* plants induced with both herbivores) of one replicate were the functional variables.

Results

Aboveground

The general wasp responsiveness (number of wasps choosing any arm) was 84.72% for naive wasp; 84.72% for EXPSD wasps and 84.03% for EXPS wasps. The number of wasps choosing odours coming from a plant damaged by *Diabrotica* only was always similar to the number of wasps choosing an arm connected to an empty bottle (For NAIVES, $p = 0.53$; EXPS, $p = 0.1$; EXPSD, $p = 0.54$). Wasps significantly more often

choose the odours coming from plants induced by *S. littoralis* (Figure 2). For NAIVES: *S. littoralis* attacked plant versus empty, $p < 0.0001$; doubly infested plant versus empty, $p = 0.0002$; *D. v. virgifera* infested plants versus *S. littoralis* infested plants, $p < 0.0001$; *D. v. virgifera* infested plants versus doubly infested plants, $p = 0.0024$. For EXPS: *S. littoralis* attacked plant versus empty, $p < 0.0001$; doubly infested plant versus empty, $p < 0.0001$; *D. v. virgifera* infested plants versus *S. littoralis* infested plants, $p < 0.0001$; *D. v. virgifera* infested plants versus doubly infested plants, $p = 0.0001$. For EXPSD: *S. littoralis* attacked plant versus empty, $p <$

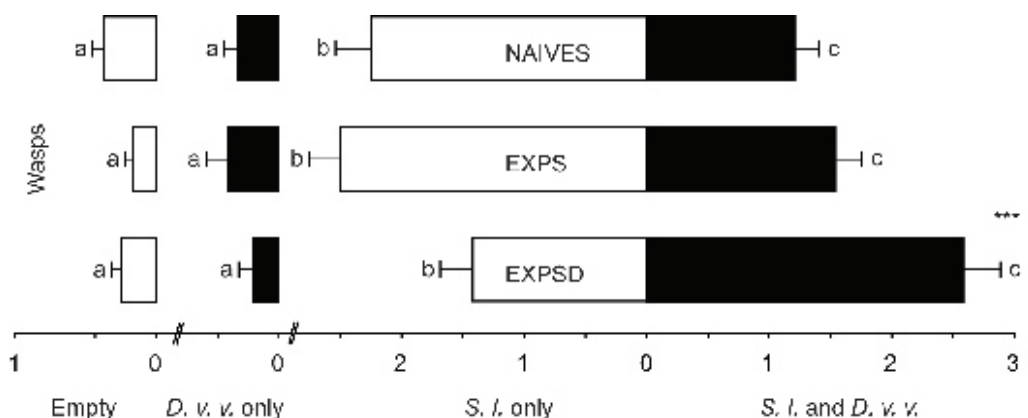


Figure 2 Mean (\pm SE) number of wasps choosing one of the treatment arms; i.e. vessels containing only humidified sand (Sand only), vessels containing plants attacked by 4 *D. v. virgifera* larvae only (*D. v. v. only*), vessel containing plants attacked by 15 *S. littoralis* caterpillars (*S. l. only*) and vessels containing plants attacked by both herbivores (15 *S. littoralis* and 4 *D. v. virgifera*). Tested wasps were either NAIVES, no previous oviposition experience; EXPS, 3-5 ovipositions in presence of odours coming from plants attacked by *S. littoralis* only; and EXPSD, 3-5 ovipositions in presence of odours coming from plants attacked by both herbivores. Different letters next to the bars indicate significant differences in the number of wasps choosing the odour of a treatment for a given experience ($p < 0.05$). Asterisks indicate significant effect of interaction ($p < 0.0001$). $N = 12$ replicates of 2 consecutive releases of six wasps.

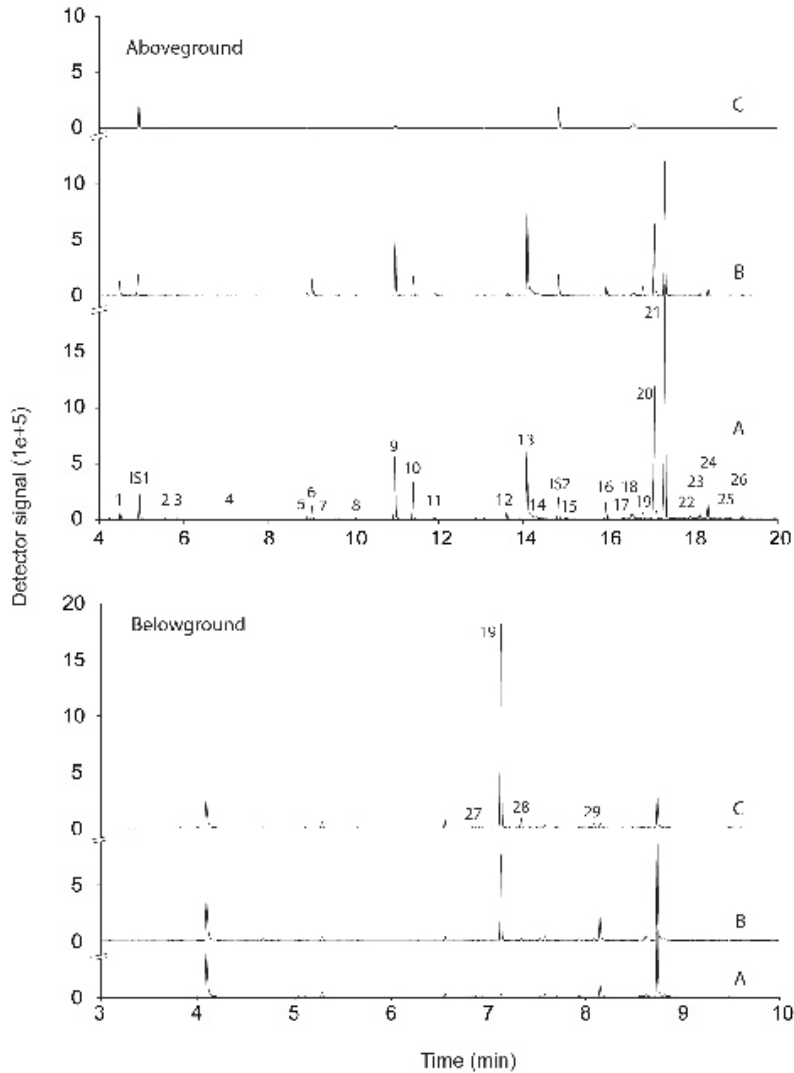


Figure 3 Representative examples of GC-MS chromatograms obtained with the collected volatiles of 10-days old maize seedlings. **Belowground** SPME analysis of pulverized roots. **Aboveground** Analysis of Super-Q filter extract from 4 hrs headspace collection of maize leaves. See Methods for details. 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-hexenal, 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) phenethyl 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). N = 12.

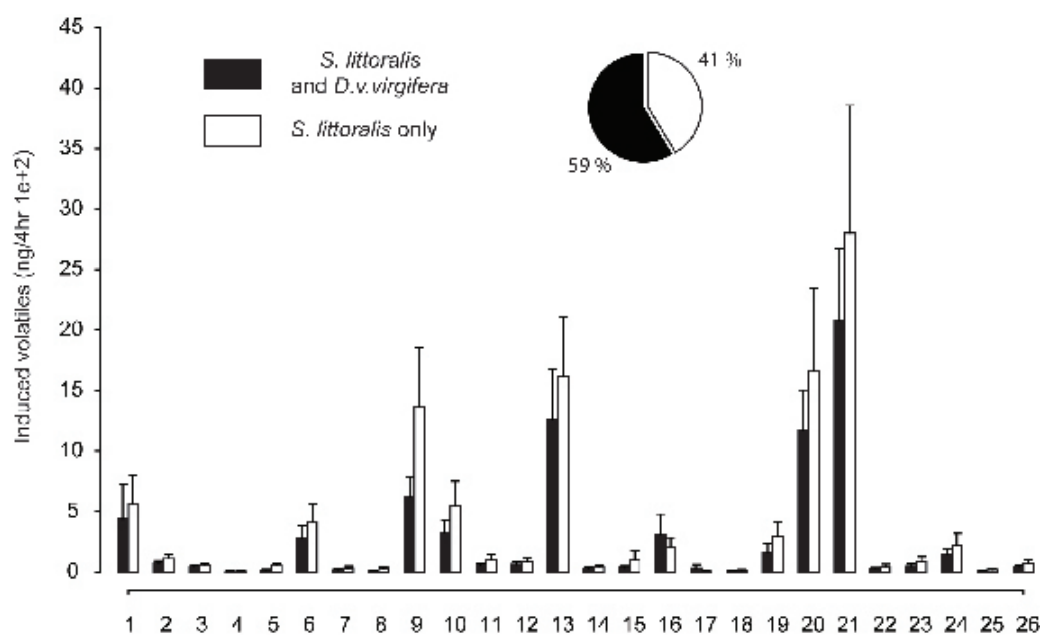


Figure 4 Mean (\pm SE) amount of volatiles collected from the leaves of plants attacked by *S. littoralis* only and plants attacked by both herbivores (*S. littoralis* and *D. v. virgifera*). For the complete list of identified compounds 1 – 26 refer to Figure 3. $N = 12$.

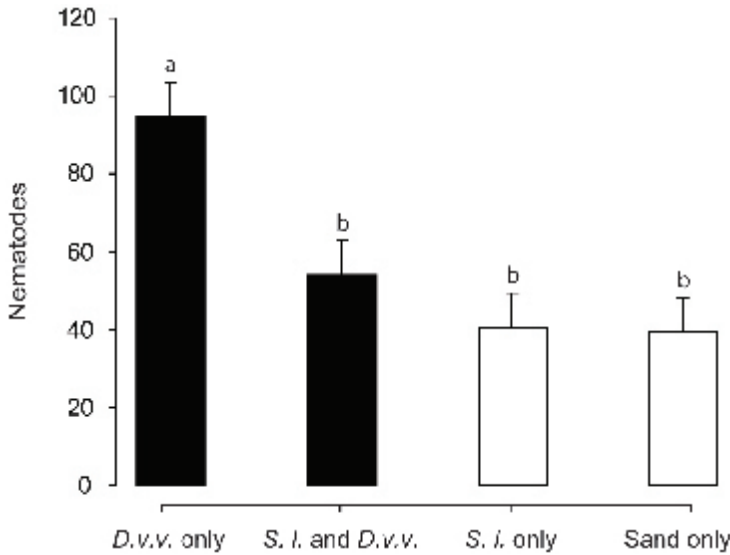


Figure 5 Mean (\pm SE) number of nematodes recovered from the six treatment arms of the belowground olfactometer; containing a plant attacked by 4 *D. v. virgifera* larvae (*D. v. v. only*), a plant attacked by 20 *S. littoralis* and 4 *D. v. virgifera* (*S. l. and D.v.v.*), a plant attacked by 20 *S. 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 ($p < 0.05$). $N = 12$.

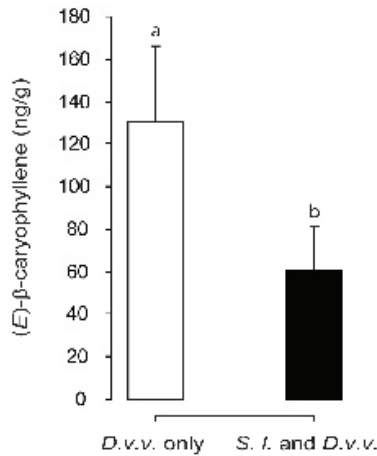


Figure 6 Mean (\pm SE) amount of (*E*)-β-caryophyllene collected from freshly frozen maize roots from plants attacked by 4 *D. v. virgifera* larvae, and from plants attacked by both herbivores (i.e. 20 *S. littoralis* and 4 *D. v. virgifera*). Different letters above bars indicate differences in the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

0.0001; doubly infested plant versus empty, $p < 0.0001$; *D. v. virgifera* infested plants versus *S. littoralis* infested plants, $p = 0.0005$; *D. v. virgifera* infested plants versus doubly infested plants, $p < 0.0001$.

When given a choice between the odours of a plant attacked by *S. littoralis* only and a plant attacked by both herbivores naive wasps significantly preferred the former ($p = 0.01$). This was also the case for wasps that were experienced in the presence of the odour infested only by *S. littoralis* (EXPS; $p = 0.039$). 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 ($p = 0.013$). A two-way ANOVA with the two experience groups (EXPS and EXPSD) and the plant treatments (doubly or singly infested plants) as factors confirmed a highly significant effect of learning and plant treatment (treatments $p < 0.0001$; experience, $p = 0.997$; treatment*experience $p < 0.0001$).

The analyses of the volatiles collected from the aboveground parts of the treatment plants showed that plants attacked by *D. v. virgifera* only, did not emit any detectable amounts of induced volatile organic compound (Figure 3). In contrast, the plants infested by *S. littoralis* emitted considerable amounts of typically induced compounds. Plants infested by *S. littoralis* only and plants infested by both herbivores produced a very similar pattern of volatiles, and quantitative analysis of 26 major compounds showed no significant difference between the two treatments (Figure 4). A more detailed analysis of the volatile blend revealed another 19 minor peaks presents in all of the 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.

Belowground

The choices of the *H. megidis* nematodes that had been released in the centre of the belowground olfactometer showed a clear effect of the aboveground herbivore (*S. littoralis*) on nematodes attraction (Figure 5). The number of nematodes choosing an arm with a doubly infested plants was significantly lower compared to the number of nematodes choosing an arm containing a plant with *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 and to the control pots containing only sand; no significance was found ($p = 0.38$ and $p = 0.21$ respectively). Neither was there a difference between the number of nematodes choosing the arm connected to a pot with a *Spodoptera*-infested plant and arms connected to the control pots ($p = 0.87$).

From the 8 measures taken for each treatment, i.e. plants with *S. littoralis* only, healthy plants, plants with *D. v. virgifera* only and plants with both herbivores, only the last two showed the presence of (*E*)- β -caryophyllene after the SPME analysis (Figure 2). Quantification was done using the standard curve obtained from the analysis of healthy Delprim root material that was spiked with 0, 4.5, 9, 45, 90, 200 and 450 ng of pure (*E*)- β -caryophyllene (Sigma-Aldrich), and adapting a linear regression curve. A pair-wise comparison between plants attacked by *D. v. virgifera* only and plants attacked by both herbivores (Figure 6) confirmed a negative effect of *S. littoralis* on the production of (*E*)- β -caryophyllene ($p = 0.036$).

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*. The results of this first study to measure cross effects between above- and belowground tritrophic interactions thus 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 and Dicke 1992; Dicke 1994; Turlings and Wäckers 2004). Parasitoids are thought to predominantly use plant-provided signals to find herbivorous host because the hosts themselves have been selected to emit as little and as cryptic as possible, whereas plants may benefit from emitting a clear signals to lure in the enemies of their enemies (Vet & Dicke 1992). Similar arguments can be used for the attraction of nematodes by the root-produced signals. The existence of belowground tritrophic interactions have only been recently demonstrated (van Tol, van der Sommen *et al.* 2001; Boff, van Tol *et al.* 2002; Neveu, Grandgirard *et al.* 2002; Aratchige, Lesna *et al.* 2004; Rasmann, Kollner *et al.* 2005), but in maize, unlike for the leaf signals that attract parasitoids, a specific compound that is emitted by its roots when they are damaged by *D. v. virgifera* larvae has already been identified and shown to attract *H. megidis* also under natural conditions (Rasmann, Kollner *et al.* 2005). In the current study, the emission of this key compound, the sesquiterpene (*E*)- β -caryophyllene, was negatively affected when plants were attacked by *Spodoptera* larvae in addition to *D. v. virgifera* larvae, which explains the reduced nematode attraction.

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 CO₂. Although such a physiological constraint

would be a likely explanation for the observed reduction in (*E*)- β -caryophyllene emission, 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, Harvey *et al.* 2003), although induced resistance against one phytophage does not automatically result in enhanced resistance against others (Agrawal, Tuzun *et al.* 1999; Walling 2000; Rostas, Simon *et al.* 2003).

Also, if the emissions are costly for the plants, it should be considered that, when confronted with two herbivores, they may “chose” to invest more in one defence strategy than in the other. In general, the fitness costs of inducible defences are relatively low (Gerhenzon 1994; Hoballah, Kollner *et al.* 2004), but this may change 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, Witjes *et al.* 2004). If in the current study there was an allocation “decision” made by the plant as part of its optimal defence strategy, it appears that it was in favour of the leaf signal.

Aboveground, the recruitment of *C. marginiventris* by *S. littoralis* damaged maize leaves was also affected when the other herbivores was feeding on the roots, but this change in attractiveness was not reflected in the measure odour emissions (Figure 3). There was a minor non-significant trend indicating that doubly infested plants release less of some compounds. This is in contrast to the assumption that root feeding has an effect on plant physiology that is comparable to drought stress (Masters, Brown *et al.* 1993), leading to an increase of both direct and indirect defences (Karban and Baldwin 1997; Turlings and Wäckers 2004; Bezemer and van Dam 2005). Indeed, *Agriotes lineatus* larvae,

when feeding on cotton roots, enhance the production of extrafloral nectar aboveground (Wackers and 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) have shown an increased parasitoids number of seed predators of the marsh thistle (*Cirsium palustre*) when root herbivores were not removed, but this seemed to be correlated to the higher number of herbivores on plant and does not necessarily imply a change in signal emission. It has also been shown that root herbivory increases flower visitation by pollinators (Poveda, Steffan-Dewenter *et al.* 2003; Poveda, Steffan-Dewenter *et al.* 2005), which is possibly explained by an increased induction of floral nectar following root feeding.

The flexible responsiveness of *C. marginiventris* to the induced leaf volatiles remains intriguingly complicated. It has become evident that the bulk of the truly induced maize volatiles only become important in the attraction of the wasp after it has experienced them in association with a contact with hosts (Steidle and van Loon 2003; Hoballah and Turlings 2005; D'Alessandro and Turlings 2006). Inexperienced (naïve) wasps are mainly attracted to volatiles that result from fresh damage (Hoballah and Turlings 2005) and which are the same for many different plant species. It appears that the wasp relies on such generic signals until it encounters hosts and obtains more specific information on which signals can be most reliably associated with host presence. This is when the induced terpenoids and other compounds become important. These compounds are much more specific and can provide information on plant genotype (Gouinguene, Degen *et al.* 2001; Krips, Willems *et al.* 2001; Degen, Dillmann *et al.* 2004) and possibly herbivore species (De Moraes, Lewis *et al.* 1998; Dicke and Vet 1999). 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 an particular time (Turlings, Wackers *et al.* 1993; Vet, Lewis *et al.* 1995). Induced volatile emissions do not only differ between plant genotypes and as a result feeding by different herbivores, but variability in the emissions is also influenced by abiotic factors such as temperature, light, UV-radiation (D'Alessandro and 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* increases the attractiveness of the infested plants to the parasitoid *Cotesia glomerata*, but decreased the attractiveness to *Cotesia plutellae* (Shiojiri, Takabayashi *et al.* 2001), demonstrating that the consequences for the various interactions are species-dependent (Bezemer, De Deyn *et al.* 2005). Here naïve *C. marginiventris* females showed an innate preference to 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. The chemical analyses detected no clear differences in the odour profiles of the two treatments, but the wasp behaviour implies that there are differences. This supports the notion that minor, as yet unidentified, compounds play a key role in the attraction of this wasp (D'Alessandro and Turlings 2005) and suggests that feeding by *D. v. virgifera* larvae has an effect on the emission of these compounds. It should be noted that *Spodoptera* species, *Diabrotica* species and *C. marginiventris* commonly occur together, in particular in Mexico. The reduced attraction of the wasp to *D. v. virgifera*-infested plant response might therefore be an adaptation. In our experiments, we found no evidence that root feeding by *D. v. virgifera* larvae induces the production of volatiles aboveground, nor does feeding by *S. littoralis*

caterpillars induce the production of volatiles belowground (Figure 2). Still the wasp may be avoiding plants that it perceives as being infested by the “wrong” herbivore or as plants that have been rendered less suitable as a food source for its hosts due to the belowground infestation. An encounter with suitable hosts in the presence of the normally less attractive odour increases the odours attractiveness to the wasp.

Induced plants defences play an important role in interactions between belowground and aboveground organism (Bezemer and van Dam 2005), and this notion has found further support in the present work. In nature, plants are almost constantly exposed to aboveground and belowground herbivores. It has become evident that herbivores can influence each other through changes in the shared host plant, and that the host plants’ responses can influence their respective natural enemies. A good understanding of these cross effects will help to further elucidate the relationships between plants and their communities. The present work was also motivated by the aim to improve biological control methods against the two major maize pests that were studied. Recent studies have shown that indirect defences can be manipulated to enhance the attractiveness of plants to beneficial arthropods (Kappers, Aharoni *et al.* 2005; Schnee, Köllner *et al.* In Press). Especially in the case of parasitoids, however, it remains to be determined which are the compounds that are most important for the attraction (D’Alessandro and Turlings 2006) before the full potential of this strategy of exploiting the plants’ indirect defences for biological control can be assessed. The same is true for a fundamental understanding of how above and belowground tritrophic interactions affect each other. To further disentangle the complex mechanisms of plant defences and the effects of the plants responses on their “friends and foes” it is pertinent to identify the key compounds that are involved.

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CHAPTER III



FIRST INSIGHTS INTO SPECIFICITY OF BELOWGROUND TRITROPHIC
INTERACTIONS

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Abstract

Tritrophic interactions involving plants, herbivores and natural enemies of the herbivores have only recently been discovered in belowground systems, whereby entomopathogenic nematodes exploit root herbivore induced volatile compounds to locate their hosts. Obtaining further knowledge on such belowground interactions will be one of the key steps for a better understanding of how natural communities in terrestrial ecosystems are fashioned. Little is known, for instance, about the specificity of the plant-provide signals in terms of signal production by different plant species and the plants' responses to different herbivores. Using a belowground six-arm olfactometer that allows recording of nematode behavior and attraction, we obtained first insight into this specificity by measuring signal production and attraction using three plant species (maize, cotton and cowpea), four root herbivores and three entomopathogenic nematodes. Results indicate considerable differences in quantities and composition of signal blends among plant species, as well as in the intensity of how roots may respond to damage inflicted by different herbivore species and in how different nematode species respond to the induced signals attract.

Introduction

The question of what drives the structuring of natural plant communities has been a recurrent theme in ecology (Wardle, Bardgett *et al.* 2004). It is recognized that soil organisms have important effects on plant communities in space and time (Wardle 2002; De Deyn, Raaijmakers *et al.* 2003; De Deyn and Van der Putten 2005), and it seems that plant defense responses belowground are strongly involved in this process (Van Der Putten 2003). Plant defensive strategies may include tolerance, resistance, direct and indirect defense, or movement away from their above- and belowground herbivores or pathogens (Karban and Baldwin 1997). Plant defense theory that includes the recruitment of natural enemies of the herbivores (indirect defense) (Price, Bouton *et al.* 1980), in particular through plant-derived herbivore-induced volatile organic compounds, has been almost solely developed and tested for aboveground interactions (Karban and Baldwin 1997; Van der Putten, Vet *et al.* 2001; Dicke, van Poecke *et al.* 2003; Turlings and Wäckers 2004). Recently, the active role of the plants in recruiting natural enemies of the belowground herbivore has been acknowledged in a number of studies, whereby it was found that: fly larvae (*Delia radicum* L.) that feed on turnip roots induce the plant to emit volatiles that attract the parasitoid *Trybliographa rapae* L. (Neveu, Grandgirard *et al.* 2002); the rust mite *Aceria tulipae* Keifer induces tulip bulbs to attract the predatory mite *Neoseiulus cucumeris* Oudemans (Aratchige, Lesna *et al.* 2004); larvae of the weevil *Otiorhynchus sulcatus* F. causes *Thuya sp.* and strawberry roots (*Fragaria vesca* L.) to emit attractants for the entomopathogenic nematode *Heterorhabditis megidis* Poinar (Boff, Zoon *et al.* 2001; van Tol, van der Sommen *et al.* 2001); and larvae of the leaf beetle *Diabrotica virgifera virgifera* LeConte induces maize roots (*Zea mais* L.) to emit a sesquiterpene that is also attractive to

the nematode *H. megidis* (Rasmann, Kollner *et al.* 2005).

From studies on aboveground indirect defense signals it is known that they vary enormously between plant species (Hoballah, Tamo *et al.* 2002; Turlings and Wäckers 2004). Considerable variation also exists between different genotypes of the same plant species (Gouinguene, Degen *et al.* 2001; Krips, Willems *et al.* 2001; Degen, Dillmann *et al.* 2004) and even between different plant parts (Turlings, Wäckers *et al.* 1993). This variation can also be observed between individuals of the same plant genotype if they are attacked by different herbivore species (Rodriguez-Saona, Crafts-Brandner *et al.* 2003; De Boer, Posthumus *et al.* 2004) or stages (Takabayashi, Takahashi *et al.* 1995). For instance, the specialized parasitoid *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) can discriminate between the odor emitted by tobacco or cotton plants attacked by its host, *Heliothis virescens* F. (Lepidoptera: Noctuidae) and odors coming from plants attacked by a non-host herbivore (De Moraes, Lewis *et al.* 1998). Abiotic factors such as temperature, light, soil and air humidity can also significantly affect the induced volatile emissions (Takabayashi, Dicke *et al.* 1994; Gouinguene and Turlings 2002).

The few studies on belowground tritrophic interactions have not yet identified specific compounds that are emitted in response to root herbivory, except in the case of maize plants, where corn root larvae have been shown to induce the release of a sesquiterpenoid ((*E*)- β -caryophyllene) (Rasmann, Kollner *et al.* 2005), which was found to be involved in the attraction of the entomopathogenic nematode *H. megidis*.

A good understanding of the mechanisms and strategies of host finding by entomopathogenic nematodes (Rhabditidae: Heterorhabditidae and Steinernematidae) can help to exploit their full potential in the control of soil insect pests (Gaugler and Kaya 1990). The infective stage of these nematodes

is the third-instar juvenile, which is adapted to find, penetrate and infect new hosts in the soil. The nematodes penetrate the haemocoel of the host via body openings such as mouth, anus and spiracles. Steinernematidae and Heterorhabditidae live in symbiosis with bacterial cells of the genus *Xenorhabdus* and *Photorhabdus* respectively, which, when released in the haemocoel of the hosts, multiply and kill the host. The resulting bacterial soup offers suitable conditions for the nematodes to develop to adulthood and reproduce. The dioecious nematodes may undergo a few generations within the cadaver before shortage on nutrients triggers infective juvenile formation and the subsequent exodus from the host (Poinar 1990). Appropriate foraging cues are essential for the nematodes to find a new suitable host (Grewal, Lewis *et al.* 1994), and it has been acknowledged that different nematode species have adopted a wide variety of foraging behaviors, ranging from sessile forms (sit-and-wait) to very active foragers (Gaugler 2002; Gaugler and Bilgrami 2004).

Here we present a first study on the specificity of induced root signals that are implicated in the host location behavior of actively foraging entomopathogenic nematodes. Several members of each of the three different trophic levels were included in the study: maize (*Zea mays* L.), cotton (*Gossypium herbaceum* L.) and cowpea (*Vigna unguiculata* L.) as plants; the beetles *D.v.virgifera*, *D. balteata*, *Agriotes ustulatus* Schaller (Elateridae) and the phytopathogenic nematodes *Ditylenchus dipsaci* Filipjev (Tylenchida) as herbivores and the entomopathogenic nematodes *H. megidis*, *H. bacteriophora* Poinar and *Steinernema feltiae* Filipjev as parasites.

Materials & Methods

Three principle experiments were performed that each aimed to give prominence to one of the three trophic levels involved in the interaction; i.e. the plant, the herbivore and the parasite. All the experiments were conducted using a belowground six arm olfactometer (Rasmann, Kollner *et al.* 2005), which allows to simultaneously test the relative attractiveness of multiple odor source to entomopathogenic nematodes.

Olfactometer assays

The belowground olfactometer consisted of a central glass chamber (8 cm in diameter, 11 cm deep) with six equally distributed side arms at 0.5 cm height with a female (24mm diameter and 29mm long) connector (Rasmann, Kollner *et al.* 2005). These arms connected the central chamber with six glass pots (5 cm in diameter, 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 (Analytical Research Systems, Florida, USA) contained an ultra-fine metal screen (2,300 mesh; Small Parts Inc., Florida, USA) preventing the nematodes from reaching the odour source pots (Figure 1). For each experiment, the entire system was filled with sterilized white sand (Migros, Switzerland) 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 diameter (Hoeschele GmbH, Germany). The disk with the sand was placed in a baermann extractor (Hass, Griffin *et al.* 1999), and nematodes in the collection

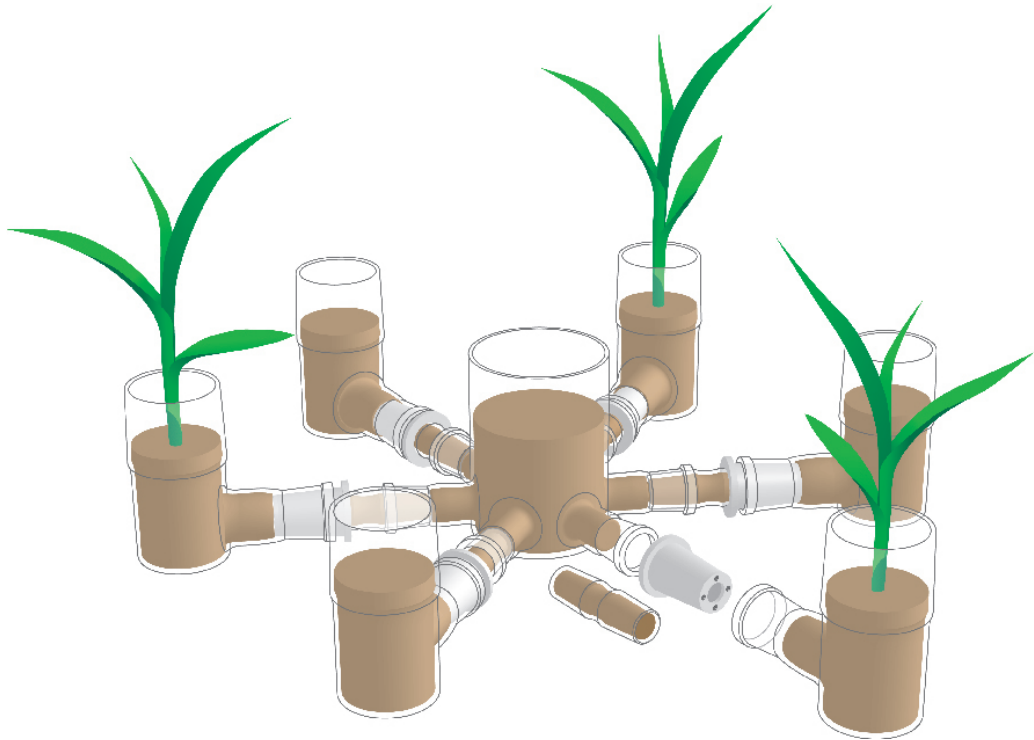


Figure 1 Drawing of the belowground six arm olfactometer. The detachable glass connector arm is shown separated from Teflon connector. Nematodes are released in the middle of the central chamber and recollected in the fraction of the soil contained in the connector arm. Drawing by Thomas Degen.

tube were counted on the next day. The responsiveness of the nematodes observed in the six-arm olfactometer was analysed using a log linear model (GLM) (Turlings, Davison *et al.* 2004). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of the organisms in the olfactometers in the software package R, version 1.9.1.

Comparison of plant species

Three plant species were used. Maize (*Zea mays* L., var. Delprim from Fenaco, Switzerland), Cotton (*Gossypium herbaceum* L. from Samen Mauser, Switzerland), and Cowpea (*Vigna unguiculata* L. var. kpodii-guegue, obtained from IITA, Benin, Africa (Hoballah, Tamo *et al.* 2002)). Plants were sown in plastic pots (7 cm high, 9 cm diameter) with fertilized commercial soil (Balkoneerde, Coop, Switzerland) and placed in a climate chamber (16L:8D, 25000 lm/m²). Maize plants used in the experiment were 10-12 days old and had three fully developed leaves. Cotton and Cowpea plants were 20-22 days old when used in the experiments. Three days before the olfactometer experiments, plant roots were carefully water washed to remove the soil around the roots and the plants were then transplanted in the glass pots of the olfactometer (see below) with humidified (10 % water) white sand (Migros, Switzerland). Four 2nd instars *D. balteata* larvae, provided by Syngenta, Switzerland, were then added to each root system of the experimental plants. In total, one plant of each species (cotton, cowpea, maize) 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 up 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 chamber. Nematodes were provided by Andermatt Biocontrol AG, Switzerland and had been propagated in *Galleria mellonella* L. larvae. All tested nematodes were between 10 to 15 days old. Nematodes were left to choose for 24 hours, after which time they were recollected and counted as described above. In addition, larvae were removed from the plants and 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 (SPME, Supelco) 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 minutes 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 (Agilent 5973 Network Mass Selective Detector; 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, Alltech Associates, Inc, USA). 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 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 µm film thickness, Alltech Associates, Inc, USA), and with a slower

heating procedure to obtain better separation. After injection, the column temperature was maintained at 40°C for 3 minutes and then increased to 250°C at 8°C per minute. 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 analysis (Rasmann, Kollner *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 Delprim attacked plants was obtained 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 pure (*E*)- β -caryophyllene (Sigma-Aldrich). The amount of volatiles collected, were analyzed on SigmaStat, version 2.0.

Comparison of herbivore species

To assess specificity of the root response in maize, three different herbivores were tested: the western corn rootworm *Diabrotica virgifera virgifera*; the wireworm *Agriotes ustulatus* and the generalist phytopathogenic stem nematode *Ditylenchus dipsaci*, all commonly known pests on maize (Toth, Furlan *et al.* 2003).

Second instars *D.v.virgifera* larvae were obtained from a rearing culture at CABI Bioscience, Delémont, Switzerland. *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 onion rearing at Agroscope, Wädenswil, Switzerland. Nematodes were extracted from onion by decantation in water, prior to the experiment.

Three days before each experiment, 10-12 days old Delprim seedlings were transferred into the olfactometer glass pots as described above. Four second instars *D.v.virgifera* larvae, three eight to eleventh instars (Furlan 1998) *A. ustulatus* larvae, and approximately

1000 *D. dipsaci* were added to the roots of the plants. The pots of the olfactometer were attached to the connector tubes and the ends of these tubes covered with aluminum foil as mentioned 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 left for 24 hrs. The next day, nematodes were extracted and counted, and 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 specificity in terms of attraction of entomopathogenic nematodes toward insect damaged maize roots, two other active foragers nematodes (Lewis 2002), besides *H. megidis* were tested; *H. bacteriophora*, Poinar (Heterorhabditidae), and *Steinernema feltiae*, Filipjev (Steinernematidae), both provided by E-Nema GmbH, Germany. 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 – 15 days old maize (var. Delprim) seedlings were transplanted each into a separate olfactometer pot as described above, and four second instars *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 2nd instars *D.v.virgifera* larvae were added to one of these pots. Thus, the treatment consisted of one pot containing a *Diabrotica* attacked maize plant, one containing a healthy maize plant, one containing only larvae, and three control pots containing only sand. 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.

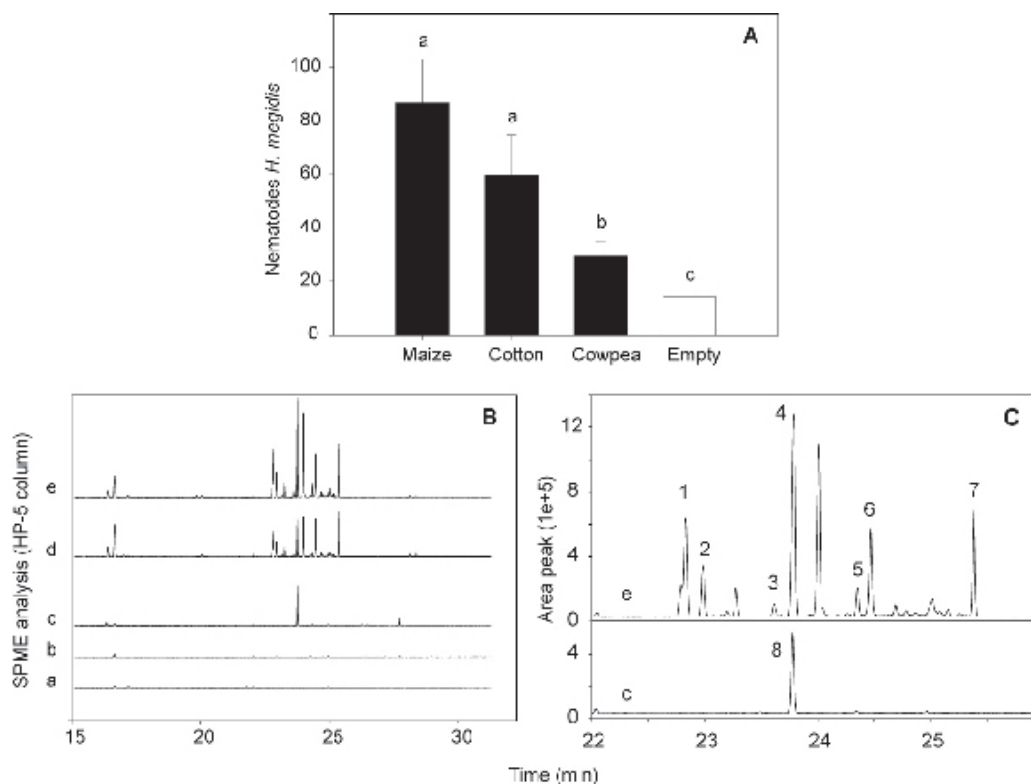


Figure 2 Comparison of attractiveness of plant species. **A)** Mean (\pm S. E.) number of nematodes *H. megidis* choosing maize, cotton or cowpea attacked by *D. balteata*, as compared to pots containing only sand. $N=11$. Letters above bars represent statistical differences ($p < 0.05$).

B) Chromatographic spectra obtained from SPME root analysis of maize, cotton and cowpea plants *a* = cowpea and 4 *D. balteata* larvae; *b* = healthy maize plants; *c* = maize and 4 *D. balteata* larvae; *d* = healthy cotton plants; *e* = cotton and 4 *D. balteata* plants. **C)** “Close-up” of chromatograms showing volatiles collected from cotton and maize roots treated infected with *D. balteata* larvae. Labelled compounds are: 1) (+)-cycloisotativene; 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.

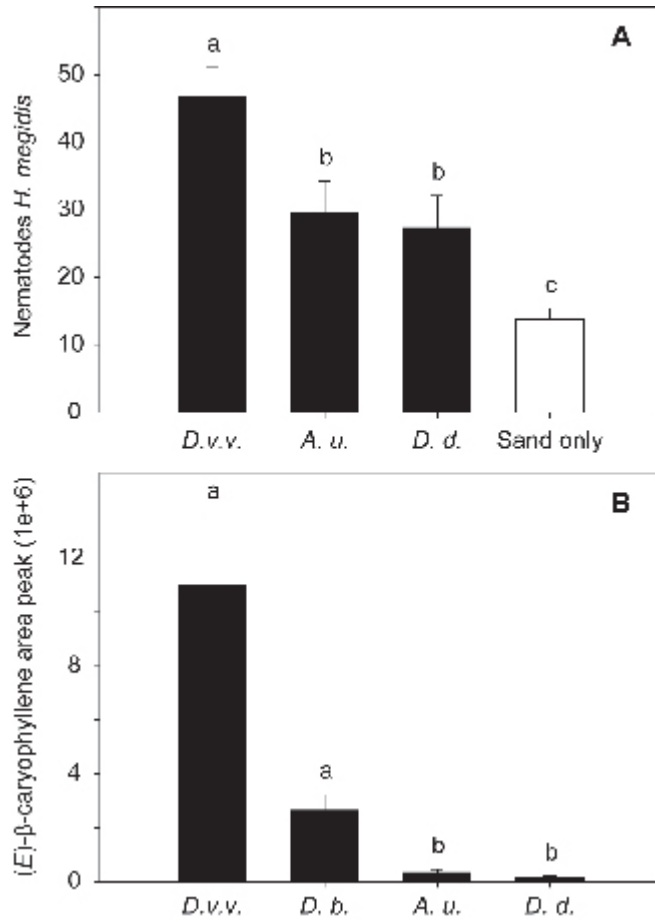


Figure 3 Comparison of attractiveness of maize roots damaged by different herbivore species. **A)** Mean (\pm S.E.) number of *H. megidis* nematodes choosing maize roots attacked by either *D.v.virgifera* (*D.v.v.*), *A. ustulatus* (*A. u.*), or *D.dipsaci* (*D.d.*), versus pots containing only sand. $N=12$. **B)** Mean (\pm S.E.) amount of herbivore (*D.v.virgifera*, *D. balteata*, *A. ustulatus*, and *D.dipsaci*)-induced (E)-β-caryophyllene in maize roots. $N=12$. Letters above bars represent statistical differences ($p < 0.05$).

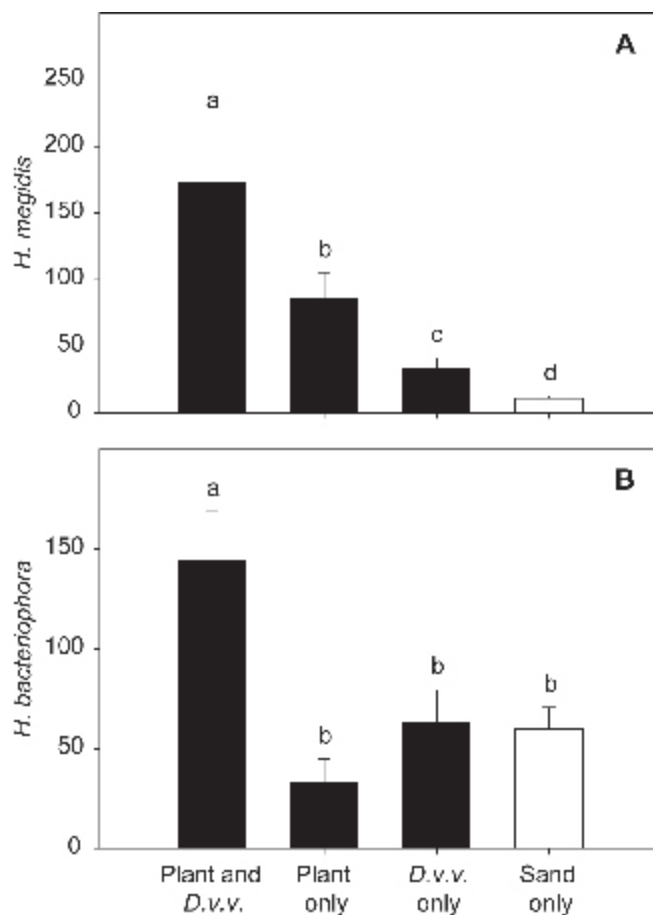


Figure 4 Comparison of responses of nematode species. **A)** Mean (\pm S.E.) number of *H. megidis* nematodes choosing the olfactometer arm containing plants (maize, Delprim var.) attacked by *D. v. virgifera* larvae, healthy plants only, larvae only or sand only. **B)** Mean (\pm S.E.) number of *H. bacteriophora* nematodes choosing one of the olfactometer arm containing plants (maize, Delprim var.) attacked by *D. v. virgifera* larvae, healthy plant only, larvae only or sand only. $N=10$. Letters above bars represent statistical differences ($p < 0.05$).

Results

Comparison of plant species

The experiment aimed to compare the attractiveness of *D. balteata*-damaged roots of maize, cotton and cowpea to *H. megidis* nematodes. The number of nematodes recovered from olfactometer arms connected to pots with maize and cotton plants was significantly higher compared to the number of nematodes choosing the arms with cowpea plants (Figure 2a) (Maize vs. Cowpea $p < 0.0001$; Cotton vs. Cowpea $p = 0.01$). No difference was found between the number of nematodes choosing maize and cotton ($p = 0.078$). The number of nematodes choosing the control arms (sand only) was lower compared to choices for the plant treatments (Maize vs. Sand $p < 0.0001$; Cotton vs. Sand $p < 0.0001$; Cowpea vs. Sand $p = 0.015$). Figure 2b 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. Now 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, is very visible in the *D. balteata*-damaged maize roots. The analysis of volatiles from pulverized cotton roots where very similar, independent of whether or not they had been damaged by *D. balteata*. Attacked and healthy cotton roots, both produced a number of compounds and overall released considerably more than the other two plant species. In Figure 2c, a close up shows the compound shows 10 possible 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 (Figure 3a) *D.v.virgifera* vs. *A. ustulatus* $p = 0.013$; *D.v.virgifera* vs. *D. dipsaci* $p = 0.005$; *D.v.virgifera* vs. sand $p < 0.001$). The roots attacked by *Agriotes* and those attacked by the phytopathogenic nematode were equally attractive (*A. ustulatus* vs. *D. dipsaci* $p = 0.69$), and both were more attractive than the control pots (*A. ustulatus* vs. sand $p = 0.0001$; *D. dipsaci* vs. sand $p = 0.0005$). Quantification of (*E*)- β -caryophyllene in the roots of the different treatments (Figure 3b) showed that indeed *D.v.virgifera*-damaged roots produced more than *A. ustulatus*- and *D. dipsaci*-damaged roots. Using the standard curve obtained from the analysis healthy maize roots spiked with pure (*E*)- β -caryophyllene resulted in an estimation that *D. v. virgifera* damage resulted in a production of 113.6 ng of (*E*)- β -caryophyllene per grams of root fresh weight, compared to 27.44 ng/g produced by *D. balteata* attacked plants, and 3.54 ng/g produced by plants attacked by *A. ustulatus*.

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 (Figure 4). As we knew from a previous study (Rasmann, Kollner *et al.* 2005) healthy plants were also somewhat attractive to *H. megidis*, and were preferred over larvae alone and sand (*D.v.virgifera* healthy plant vs. larvae only $p = 0.0148$; healthy plants vs. sand only $p < 0.0001$). Even the larvae alone were slightly more attractive when compared to the control pots (larvae only vs. sand only $p = 0.019$). *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

$p < 0.0001$)

H. bacteriophora nematodes preferred *D.v.virgifera* damaged plants over the healthy plant and the larvae alone (*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 $p = 0.00091$). On the other hand, 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. Four additional replicates of the experiment were performed, whereby we also collected the sand from the central glass chamber (were the nematodes had been released). A portion of the released nematodes were recovered from this center pot, but again nothing from the arms (results not shown).

Discussion

Overall, the results from the current study imply that herbivore-induced emissions of attractants for entomopathogenic nematodes is a common phenomenon, but that the types of emissions and the responses of the nematodes to the signals can vary considerably. The different plant species tested produced different volatile blends in the roots upon herbivory (Figure 2b), and this differential production was largely correlated with the attraction of the nematode (Figure 2a). For cowpea roots we could hardly detect any compounds. In maize roots considerable amounts of (*E*)- β -caryophyllene were detected, but only after feeding by *D. balteata* larvae. As is known for their leaves (Loughrin, Manukian *et al.* 1994), the healthy roots of cotton plants already contained relatively large amounts of various terpenoids, which is in clear contrast to the other two plants.

Feeding by *D. balteata* larvae caused only a small increase in the amounts detected in the cotton roots (Figure 2c). Considering that cowpea produces very little, it is logical that nematode attraction was poor. Less logical perhaps is that the damaged cotton roots were not more attractive than the damaged maize roots, which one could expect from the differences in the amounts emitted. This might imply that some substances are more attractive than others and in this case it confirms the high attractiveness of (*E*)- β -caryophyllene, as we observed in an earlier study (Rasmann, Kollner *et al.* 2005). It should be noted; however, that the methodology used here does not allow us to infer what the plants emitted, but only what was produced inside the roots. It is therefore possible that the maize plants, although containing less, may have emitted more. 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 mechanisms against lepidopteran larvae (Hedin, Parrott *et al.* 1992), are liberated when the leaves 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; feeding results in increased levels of already presents terpenoid aldehydes in cotton roots plants (Bezemer, Wagenaar *et al.* 2004). Interestingly is the peak number 4 in Figure 2c, which was tentatively identified as aristolene. It is very similar to (*E*)- β -caryophyllene and could barely be separated from the latter by chromatography. Further studies will have to confirm its identity and attraction potential. The nematodes were 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, but each of these compounds was found to be less attractive than (*E*)- β -caryophyllene (Rasmann, Kollner *et al.* 2005).

The 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, Tamo *et al.* 2002). In the same study, however, the responses of the generalist endoparasitoid *Cotesia marginiventris* (Turlings, Loughrin *et al.* 1995; D'Alessandro and Turlings 2005) was stronger to cowpea odor than to maize odor, again indicating that quantity of induced odor emission is not always a good predictor of parasitoid or nematode attraction.

Clear differences were also found in the (*E*)- β -caryophyllene production induced by the different herbivores that were tested. For the roots infected by the phytophagous nematode *D. dipsaci* no (*E*)- β -caryophyllene was detected. This is in contrast to the role that nematodes may play in the induction of direct defenses (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, Hedin *et al.* 1994). Our SPME analyses may have missed such compounds in maize roots. 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; there are no reports of nematodes using wireworm larvae as hosts (Eidt and Thurston 1995; Peters 1996). It will be interesting to see if, for instance, predatory nematodes are attracted to *D. dipsaci*-infected plants. The differences in the amount of (*E*)- β -caryophyllene produced, also readily explain the differences in attractiveness. For

nematode-infected plants, virtually nothing was detected, and production induced by *A. ustulatus* damage was also surprisingly low (3.54 ng/g) compared to the amount of the same volatile compound induced by *D.v.virgifera* feeding (113.6 ng/g). We cannot exclude, however, that this difference was merely a reflection of the difference in the amount of feeding damage that the larvae made. The *A. ustulatus* larvae used in the experiments were between 8th and 11th instars and may feed considerably less than younger larvae (Furlan 1998), perhaps explaining a lower induction. In the same line, relatively poor induction by *D. balteata* of (*E*)- β -caryophyllene can be explained by lower feeding rates by this less specialized root herbivore (Mithöfer, Wanner *et al.* 2005). In fact, complementary studies with 6 additional analyses of *Agriotes*-induced roots under the same conditions as explained above, but without studying nematode behavior, resulted in relatively high amounts of (*E*)- β -caryophyllene, i.e. 20 ng/g of fresh weight material, which is still considerably less than what was produced by the *D.v.virgifera*-damaged roots. Overall, the results confirm our notion from a previous study (Rasmann, Kollner *et al.* 2005) that continuous mechanical damage is sufficient to induce some (*E*)- β -caryophyllene production in maize roots, but the production is much higher when *D.v.virgifera* feeds on the roots. In that study Delprim maize roots were mechanically damaged once a day for three days with a cork borer and produced on average 20.65 ng/g of (*E*)- β -caryophyllene.

The response of the infective juveniles of the three tested nematode species to *D.v.virgifera*-infected maize plants differed considerably. This was expected as entomopathogenic nematodes display a wide variety of foraging behaviors, which are situated in a continuum between the cruiser type and the ambusher type (Lewis, Gaugler *et al.* 1992; Grewal, Lewis *et al.* 1994). Cruisers crawl towards their hosts, whereas ambushers use a sit-and-wait strategy, standing on their tail (nictation)

waiting for a motile prey to pass nearby (Campbell and Gaugler 1997). In general it is assumed that Heterorhabditidae nematodes are of the cruiser type (Boff, Zoon *et al.* 2001; van Tol, van der Sommen *et al.* 2001), actively foraging for new hosts, whereas all Steinernematidae display all type of foraging strategies. *S. feltiae* nematodes are considered to display an intermediate foraging behavior (Grewal, Lewis *et al.* 1994), where standing on the tail is rare in occurrence and short in duration (Lewis 2002). Long-range chemical cues are heavily used by cruisers for locating resources, but such cues are relatively unimportant for the ambush foragers (Bell 1991). *H. bacteriophora* has been shown to respond to volatiles and water soluble chemical cues in a wide variety of experiments (e.g. (Grewal, Lewis *et al.* 1994; O'Halloran and Burnell 2003)). Here too we found strong attraction of *H. bacteriophora* to the plant-insect complex, and probably to volatile organic compounds emitted by the damaged roots. The same is true for *H. megidis*, the nematode for which this was already known (Boff, Zoon *et al.* 2001; van Tol, van der Sommen *et al.* 2001; Rasmann, Kollner *et al.* 2005). The current study, in addition showed that the rootworm larvae alone were hardly attractive to the nematodes, implying that the plant, as expected, is the key source of attractants for both *H. bacteriophora* and *H. megidis*. Interesting was the fact that very few *H. bacteriophora* were recovered from the arm with the healthy plants, there might even be a tendency for the healthy plants to be repellent for this species. An ability to distinguish damaged from healthy plants would certainly be an advantage to find hosts. This seems not to be the case for *H. megidis*, which also significantly attracted to healthy plants, but notably less than to damaged plants.

Not one individual of *S. feltiae* was recollected 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, Lewis *et al.* 1994). *S. feltiae* is, in terms of behavior, considered to be an intermediate between a cruiser and an ambusher, possibly it responds only to short range cues (Grewal, Lewis *et al.* 1994; Lewis, Grewal *et al.* 1995; Hui and Webster 2000). These cited studies have found evidence for a direct effect of larvae on the attraction of cruiser nematodes. This was not found during the present study, also indicating that cues coming from the host are more important in short range recognition.

This study aimed to provide insight in the specificity in terms of signals and responses of belowground tritrophic interactions. Species at all trophic levels were found to contribute to variation in the interactions. It has to be emphasized again that we had no control over the feeding rate of the herbivores and therefore differences in the responses to different herbivores may in part be explained by differences in the amounts of damage. We also tested for attraction at a set distance from the sources (about 20 cm). Responses of the nematodes may be different at shorter distances and it cannot be excluded that *S. feltiae* also used plant-provided cues.

Recently, Van der Putten *et al.* (2001) 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, plant affect soil organisms, and soil organisms reciprocally affect plants, leading to a feedback that drives changes in plant communities over space and time (Bever, Westover *et al.* 1997). The current study illustrates that these interactions can vary in intensity and that there are differences in signals and the responses they evoke. Here we focused on agricultural plant species and associated pest with one of the aims to explore how root signals may be better exploited to enhance the efficacy of entomopathogenic nematodes in biocontrol management strategies (Hazir,

Kaya *et al.* 2003; Toepfer, Gueldenzoph *et al.* 2005). However, for a better understanding of the still controversial role of inducible plant volatiles (Holopainen 2004) in shaping plant communities more natural ecosystems will have to be studied and eventually this will have in the field. Before this will be possible a better comprehension of the mechanisms that are involved will be needed and novel methodologies will have to be developed.

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CONCLUSIONS & OUTLOOK



Conclusions and outlook

The following questions were asked in the introduction and are briefly answered here

Do maize roots that are attacked by the larvae of the western corn rootworm produce induced volatiles organic compound that attract entomopathogenic nematodes ?

Yes, leaf beetle larvae of the western corn rootworm (*D. v. virgifera*) feeding on the roots of maize seedlings, induce the production of predominately a sesquiterpenoid ((*E*)- β -caryophyllene), which it is shown; using a newly developed six arm belowground olfactometer, to be attractive to entomopathogenic nematodes. Different maize varieties produce different amounts of (*E*)- β -caryophyllene, and some varieties have lost the ability to produce it. The importance of (*E*)- β -caryophyllene was tested in a field experiment, where maize varieties producing the volatiles produced more entomopathogenic nematode infected larvae and less adults of the western corn root worm compared to non-producing maize varieties.

Is there a plant-mediated interaction between above- and belowground tritrophic interactions?

Yes, aboveground herbivory by *S. littoralis* caterpillars reduces belowground recruitment of entomopathogenic nematodes; and *vice-versa*, belowground herbivory by *D. v. virgifera* leaf beetle larvae reduces the attractiveness for parasitic wasps *C. marginiventris*. This seems mainly due to changes in the induced odours profiles above- and belowground emitted by maize plants attacked by both herbivores simultaneously.

Is there specificity in the belowground signals involved in a tritrophic interaction?

The results from the study imply that herbivore-induced emissions of attractants for entomopathogenic nematodes is a common phenomenon, but that the types of emissions and the responses of the nematodes to the signals can vary considerably. This seems to be true when either looking at plants, the first trophic level, at herbivores, the second trophic level, or at the parasites or predators, the third trophic level.

Based on the presented results, new questions arose

- Belowground herbivory does not induce the production of volatile organic compounds on maize from the leaf, but slight changes in the aboveground induced odors profiles can be detected by parasitic wasps. This needs to be more deeply investigated, by analyzing the importance of minor compound in the attraction by parasitoids (D'Alessandro and Turlings 2005).
- Belowground herbivory has shown to influence aboveground direct defenses (Bezemer and van Dam 2005). This need to be proven in the present maize system. Interestingly would be to investigate the metabolite changes aboveground in the plant after root feeding, followed by the direct effects on leaf herbivores, and finally measuring plants fitness.
- After root feeding, some maize varieties are induced to produce volatile organic compounds, while some others not (Rasmann, Kollner *et al.* 2005). The future step would be to integrate biochemical and molecular knowledge with the aim to investigate genetic basis of this adaptation. If belowground induction of the sesquiterpenoid (*E*)- β -caryophyllene is controlled by simple genomic; the manipulation will open new possibilities to asses the importance of

the compound in the attraction of entomopathogenic nematodes (Degenhardt, Gershenzon *et al.* 2003; van Poecke and Dicke 2004). Restoring the ability to attract nematodes in maize varieties, will hopefully help enhance biological control methods against root feeders such as *D. v. virgifera*.

- Fitness benefits by the action of parasitoids have been shown aboveground (Hoballah, Kollner *et al.* 2004). We hypothesize that added root herbivory will influence plant fitness, and future field studies including more than one herbivore will be needed to assess the importance of both above- and belowground tritrophic interactions for the costs and benefits of the plant.
- The newly developed six arm belowground olfactometer (Rasmann, Kollner *et al.* 2005) will open new possibilities to study entomopathogenic nematodes behavior, and selection experiments will possibly help future biological control methods against root feedings pests.
- The six arm belowground olfactometer will also be helpful in all kind of belowground interaction studies, involving plants, herbivores, pathogens and motile predators or parasites. An adapted version will also be helpful in studying plant-plant communication belowground (Dicke, Agrawal *et al.* 2003), or interactions between the rhizosphere and soil fauna such as earthworms, thus enhancing the understanding and the dynamic of soil communities (Wardle 2002; De Deyn and Van der Putten 2005).
- The present thesis helped a deeper investigation in belowground tritrophic interactions, but has focused mainly on a maize system (Rasmann, Kollner *et al.* 2005). The present methods can be adapted to investigate other natural and semi-natural systems (van Tol, van der Sommen *et al.* 2001).

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ANNEX



PLANT-MEDIATED CROSS EFFECTS BETWEEN ABOVEGROUND AND
BELOWGROUND TRITROPHIC INTERACTIONS

Sergio Rasmann
Ted Turlings

Abstract

The release of volatiles by plants in response to insect attack can function as an indirect plant defence by attracting natural enemies of the herbivores. Such tritrophic interactions have been studied for simplified systems with the plant usually being attacked by just one herbivore. Here we studied the consequence of a simultaneous attack of maize plants by an above and a below ground herbivore for the production of induced leaf volatiles and their attractiveness for the parasitoid *Cotesia marginiventris*. The common maize pests *Spodoptera littoralis* and *Diabrotica virgifera virgifera* served as the respective above and below ground herbivores. *D.v.virgifera* does not induce any aboveground volatiles, but long induction results in a decrease of constitutive volatile production. *C. marginiventris* readily attacks *S. littoralis* and is highly attracted to volatiles emitted by maize plants under *Spodoptera* attack. Olfactometer assays in which wasps were given a choice between the odours of singly (*S. littoralis* only) and doubly (both herbivores) infested plants, revealed that infestation by both herbivores significantly influence the attractiveness of the plants to the parasitoid. Interestingly, associative learning of the respective odours significantly affected the wasps' preferences. This first demonstration of an effect of below ground herbivory on above ground tritrophic interactions corroborates the complexity of plant-insect interactions and illustrates the adaptability of natural enemies to deal with this complexity.

Introduction

Under the attack of herbivores, plant can produce a suite of defence mechanisms that are classified constitutive or induced defences. While constitutive defences act on the herbivore directly such as waxes, trichomes, spines or chemical deterrents; the induced defences has been defined as changes in plants following damage or stress. These changes can, in the broadest sense, increase the plant protection to further herbivore attack by reducing the preference for, or reducing the effect of, herbivores on the damaged plant (Karban and Baldwin 1997; Agrawal 1998). Moreover, the induced defences can also be classified as direct or indirect, where, in the first case, plants protect themselves to an herbivores attack by producing deterrents or digestibility reducers, the indirect defence promote the effectiveness of carnivores such as predators or parasitoids by induced chemical volatiles emitted by the attacked plant (for reviews see: (Agrawal, Tuzun *et al.* 1999; Dicke and Hilker 2003; Turlings and Wäckers 2004)). Recently, an increasing amount of work is focusing on how different organisms, acting on different locations of the same plant, can influence one another; and understanding the effects of below- and aboveground communities acting on a same plant has been highlighted as one of the most important issues in understanding ecosystem functioning (Van der Putten, Vet *et al.* 2001; De Deyn, Raaijmakers *et al.* 2003; Wardle, Bardgett *et al.* 2004), and for recent reviews see: (Bezemer and Nicole M. 2005; De Deyn and Van der Putten 2005).

The role of how direct defences can fashion interspecific above- and belowground interactions can often lead to argued observations (Gange and Brown 1989; Brown and Gange 1990; Moran and Whitham 1990; Masters and Brown 1992; Baldwin, Schmelz *et al.* 1994). The same is valid when looking at the effects of root feeding on the aboveground indirect defences (i.e. recruitment of predators

or parasites (Dicke, van Poecke *et al.* 2003; Turlings and Wäckers 2004)) have also led to different conclusions. It has been shown that plants may benefit from a belowground herbivore by being more attractive to pollinators and parasitoids (Masters, Jones *et al.* 2001; Poveda, Steffan-Dewenter *et al.* 2003; Poveda, Steffan-Dewenter *et al.* 2005). On the other hand, it has also being concluded that root herbivory can lower induced leaf volatile production (Rodriguez-Saona, Crafts-Brandner *et al.* 2003), thus reducing the parasitism rate aboveground (Rodriguez-Saona, Chalmers *et al.* 2005).

Larvae of *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), one of the most destructive pest on corn in the United States (Krysan and Miller 1986) that has been recently introduced into Europe (Miller, Estoup *et al.* 2005); are specialized on maize (*Zea mays* L.) roots (Moeser and Vidal 2005), and share the food source with a guild of other herbivores, above- and belowground (Harwood, Wallin *et al.* 2005). The generalist noctuid butterfly pest *Spodoptera littoralis* Boisduval is also voracious of maize leaves (Hoballah, Degen *et al.* 2004), and it has been shown that upon caterpillar feeding, maize plant produce a blend of volatile organic compounds which are attractive to *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae), a generalist endoparasitoid wasp (e.g. (Turlings, Loughrin *et al.* 1995; Turlings and Wäckers 2004; D'Alessandro and Turlings 2005)), this having strong fitness impact on the plant (Hoballah, Kollner *et al.* 2004). Using a six arm olfactometer which allows simultaneous observation of wasp's behaviour and volatile collection (Turlings, Davison *et al.* 2004), we investigated the effect of *D.v.virgifera* larvae feeding on maize roots, on the aboveground *S. littoralis* induced volatiles emission, and on the recruitment of *C. marginiventris* parasitoids. We expected that root herbivory alter aboveground induced volatile emission and thus modify parasitoid behaviour.

Materials and Methods

Organisms

Delprim variety of maize (*Zea mays* L.) plants used for the experiments were grown in plastic pots (6 cm high, 8 cm diam) using fertilized commercial soil (Balkonerde, Coop, Switzerland) in a climate chamber (23°C, 60% relative humidity, and 16L:8D, 50'000 lumens/m²). All maize plants were 10 to 12 days old (three fully developed leaves) at the beginning of each experiment. Three days previous the experiments, plants were carefully transplanted in glass pots (5 cm diam, 11 cm high) belonging to the six arm olfactometer (see below).

D. v. virgifera larvae were obtained from the rearing culture at CABI Bioscience Centre in Delémont, Switzerland and kept in plastic boxes (15x9x5 cm) containing maize seedlings planted in commercial potting soil (Coop, Switzerland) until the experimental day.

S. littoralis caterpillars and eggs were supplied weekly by Syngenta (Stein, Switzerland). Eggs were incubated in Petri dishes (9 cm diam, 1.5 cm high) on moist filter paper, and newly emerged caterpillars were fed with artificial wheat-germ-based diet (provided by Syngenta) in plastic-boxes (15x9x5 cm) under ambient laboratory conditions (Hoballah, Tamo *et al.* 2002).

For all bioassays, 2-3 day old solitary endoparasitoid *C. marginiventris* parasitoids were used. The rearing was done using 3-4 days old *S. littoralis* larvae, and, after parasitisation, caterpillars were kept on a same diet as above in an incubator (25°C and LD 16:8 h) till cocoon formation. Cocoons were kept in Petri dishes until adult emergence, for later being transferred in cages (30x30x30 cm). Adults were fed with drops of honey and distilled water on cotton wool (for details see Turlings *et al.* (2004)).

Parasitoids females were considered naives, if no oviposition occurred before the

experiments, but considered experienced if they were allowed to oviposit in presence of plant produced volatiles (see below).

The olfactometer set-up

As shown in Figure 1, maize seedling of each experimental replicated were transplanted in a glass pots (11cm high and 5 cm diam), which was furnished with a male ground connector (50/55 mm). This pot fitted the female ground glass joint of a 28 cm long vessel covering the whole plant. Just above the female fitting, a horizontally connected glass port allowed fixing a Teflon air supply tube, into which charcoal purified and humidified air (1.2 l/min) was pushed through. On the top of the vessel, two air ports were arranged. An identical horizontal port allowed to fix an odour trapping filter (25 mg of 80-100 mesh Super Q adsorbent (Altech, Deerfield, Illinois), so that half of the air pumped into the vessel (0.6 l/min) was pulled through the filter. Trapped odours were extracted using 150 µL of methylene chloride, and 10 µL of two internal standards were added (200 ng each of *n*-Octane and Nonyl acetate, Sigma, Switzerland diluted in methylene chloride). The samples were either analysed immediately or stored at -70°C before analysis. (see below for chemical analysis). The rest of the air was sucked through the second air port narrowed into a 22/25 mm female ground connector. A glass male joint formed the connection to a 3/4" corrugated Teflon tube that transfers the air from the odour sources into the arms of the top parts of the olfactometer, where wasps' behaviour was recorded. The air was pulled through a series of three glass connecting parts. The first was a glass elbow so, that a second piece was arranged horizontally. This one was provided with a male (22/25 mm) connector for the insect trapping bulb (50 ml). A 325 mesh stainless metal screen (Small Parts Inc., Miami Lakes, Fl.) was placed just after horizontal male connector and served to block the passage of the choosing insects. The second connecting part was jointed to

one of the arm of the central glass chamber. This chamber consisted of six arms (15 mm diam) attached to a 6 cm internal diam, 22 cm long vertically placed cylinder, where the wasps were released through the bottom opening. The top part of the olfactometer was surrounded with cardboard, and illuminated from the top with a bulb light 25 cm above it. The insects that choose one of the six arms, were easily counted and recollected after when, attracted by the upper light; entered the glass bulb (see Turlings *et al.* (2004) for more details). After an experimental day, all parts of the olfactometer were water washed and rinsed with acetone and hexane. The glass parts were then placed in a drying oven (250°C) overnight.

Chemical Analysis of volatiles

Extracted volatiles organic compounds were analysed by injecting 3µl aliquot of each sample in pulsed splitless mode into a gas chromatograph (Agilent 6890 Series GC system G1530A) equipped with an apolar column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness, Alltech Associated, Inc, USA). Helium at a constant pressure of 18.55 psi was used a carrier gas flow. Following injection, temperature was maintained at 40°C for 3 min and then increased to 230°C at 3°C/min followed by a postrun of 290°C for 9.5 min. To identify specific compounds, the system was coupled to a mass spectrometer operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70eV, scan range 33-280 amu). 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 analysis (Turlings, Lengwiler *et al.* 1998; Gouinguene, Degen *et al.* 2001; Hoballah, Tamo *et al.* 2002; D'Alessandro and Turlings 2005). Volatiles that were not identified by comparing retention times and spectra with those of pure standards are labelled in Figure 3b with a superscript N in

the text. The detected volatiles were quantified based on comparison peak area with those of the internal standards (n-octane for IS1, and n-nonyl acetate for IS2, see Figures 3b, 4b).

Timing of belowground herbivory on the aboveground volatile emission and effect on parasitoid recruitment

The first experiment consisted of adding four 2nd instars *D.v.virgifera* larvae to three experimental plants, which were alternated with three healthy plants in the olfactometer set up. The above parts of the vessels, containing the plants and connecting the corrugated Teflon tubing, was sealed with a Teflon cap; and all the air that was pulled in from the bottom of the vessel, was directly pulled out through the filter (0.6 l/min). After infestation, plants were kept in laboratory conditions, and 16L:8D light conditions. Larvae were let feed for two hours before placing the filters to each bottle for a first collection period of two hours; after which air flow was stopped and filters were removed. The same was repeated 5 (first day), 25, 30 (second day) and 50 (third day) hours later.

A second experiment was done to correlate effect of belowground herbivory to wasps' behaviour. Six maize seedlings were transplanted in the glass pots belonging to the olfactometer as described above. Four *D.v.virgifera* larvae were added to half of the plants, and let feed for three days. The third day, the six arm olfactometer was assembled for wasps' behavioural observations. To test oviposition experienced wasps, an additional *Diabrotica*-infested plant was transplanted, but the vessel containing the plant (i.e. learning vessels) was connected with a glass tube (6 cm high, 2 cm diam) furnished with a male ground opening. Between the glass tube and the vessel; a fine meshed nylon gauze was interposed, so that 10-15 *S. littoralis* could have been placed just above plant leaves. *C. marginiventris* wasps were then individually inserted into the glass, and after 3-5 ovipositions, wasps were considered as experienced.

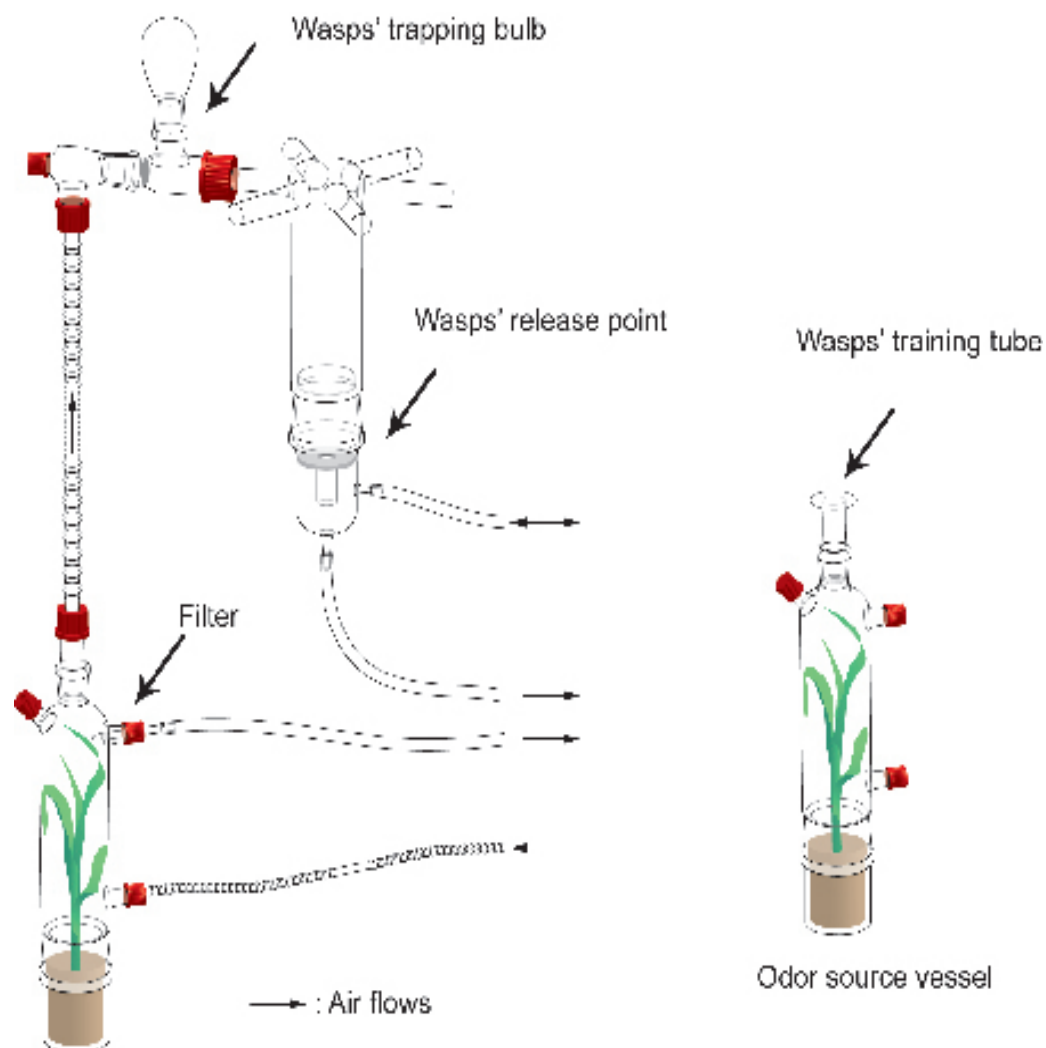


Figure 1 Schematic representation of the six-arm olfactometer used to record parasitic wasps' behaviour and plant volatile collection. Only one arm out of six is presented. Representation of a learning vessel used for wasp learning process. Drawing: Dr. Thomas Degen.

Naives (no experience) and experienced wasps were then released twice in groups of six into the central glass chamber of the olfactometer. Wasps that did not enter any of the six glass bulbs or the glass pieces supporting the bulbs after 30 min were considered having made “no choice”. Odours were collected for three hours and the experiment was replicated three times.

Effect of below- and aboveground herbivores on aboveground indirect defences

This experiment was done to assess the effect of *D. virgifera virgifera* herbivory on aboveground *S. littoralis* induced volatiles and subsequent *C. marginiventris* attraction. Three days previous to the experiment, two plants were transplanted into the glass pots of the olfactometer and 4 *D.v.virgifera* larvae were applied to one of them. On the evening of the second day, 20 *S. littoralis* larvae were applied to both of the plants, so that plants were either *S. littoralis*-only attacked (singly infested plants) or, *S. littoralis* and *D. v. virgifera* attacked (doubly infested plants). The learning vessels contained plant attacked by *S. littoralis* only, so that experimental wasps had two types of experience, i.e. no experience, and oviposition experience in presence of odours coming from a *S. littoralis* only attacked plant. On the third day the olfactometer was assembled with the six odour sources coming from a *S. littoralis*-infested plant, a doubly infested plant and four empty vessels (no plant treatment). Wasps were released in groups of six for a total of 6 times. Odours were collected for three hours.

Effect of root mechanical damage on Aboveground indirect defences

To assess the effect of belowground mechanical damage on aboveground indirect defences, two plants per experiment were transplanted into glass pots three days previous to the experiment. Each day, once a day, one plant was root mechanically damaged by stubbing five times randomly near the root area with a dissection cutter. On the evening

of the second day, 20 *S. littoralis* were applied to both plants. Additionally, a plant was prepared for learning, and was induced with 20 *S. littoralis* larvae as explained above. On the third day two groups of six wasps were released (non-experienced and *S. littoralis* experienced), and odours were collected for three hours. The experiment was replicated six times.

Statistical analysis

Wasps' behavioural responses in the six-arm olfactometer were analysed using software package R (version 1.9.1) by fitting the data to a log linear model (GLM) as described in (Turlings, Davison *et al.* 2004), but in comparison with the method described, the statistical analysis was improved by using a stochastic model developed specifically to allow for the significant overdispersion of the olfactometer data relative to that seen in a standard log-linear model (Tamò, Ricard *et al.* Submitted). The amounts of volatiles were analysed using paired t-Test analysis on SigmaStat (version 2.0).

Results

Effect on belowground herbivory on aboveground volatile production and parasitoids recruitment

D. v. virgifera has no effect on the induction of new volatiles aboveground. However, the constitutive production of volatiles is affected. As shown in Figure 2a, Delprim varieties of maize constitutively produce linalool from the leaves, and after three days of feeding, the production of linalool has significantly dropped compared to non-Diabrotica-treated plants (regression analysis, $p = 0.007367$). This has no effect on the recruitment of *C. marginiventris* wasps (Figure 1b), where no difference between the attractiveness of Diabrotica-treated plants and non treated plants has been detected for the non-experienced wasps ($p = 0.71$); and

	With Diabrotica experiment		Damaged experiment	
	p-value	significance	p-value	significance
Treatment	0	***	6.26E-09	***
Experience	0.603576	NO	7.75E-01	NO
Treatment*Exp	0.016235	*	3.15E-02	*
S - SD	8.72E-02	NO	0.462385365	NO
S - E	No exp 4.25E-09	***	0.002019182	***
SD - E	2.10E-05	***	0.003187901	**
S - SD	5.04E-04	***	2.16E-02	*
S - E	Exp S.l. 2.12E-11	***	1.18E-07	***
SD - E	2.11E-06	***	2.92E-03	**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table1 Interaction analysis of two experiments. 1) Wasps that choose plants attacked by *S. littoralis* only (S), plants attacked by *S. littoralis* and *D. v. virgifera* (SD), or empty bottles (E) - With Diabrotica experiment. 2) Wasps that choose plants attacked by *S. littoralis* only (S), mechanical damaged roots of plants attacked by *S. littoralis* (SD), or empty bottles (E) – Damaged experiment. The attraction of naive (No exp) and experienced (Exp S. l.) wasps to the three treatment (S, SD, E), was analysed with Two-Way ANOVA and Tukey's post-hoc test.

the same for experienced wasps ($p = 0.23$). The overall responsiveness (i.e. the number of choosing wasp compared to wasps that did not choose any of the six olfactometer arms) for non-experienced was of 19.44% and for experienced wasps was of 13.89%.

Effect of belowground herbivory to aboveground tritrophic interactions

The overall responsiveness for the non-experienced wasps, and experienced on a *Spodoptera*-infested plant was 74% and 78 % respectively; *D. v. virgifera* significantly affecting the attraction of *S. littoralis*-induced plants for *C. marginiventris* wasps (Figure 3a, Table 1). Non-experienced wasps could not differentiate between a singly (*S. littoralis*-only) plant and a doubly infested plant ($p = 0.87$), but both plants were more attractive than the empty bottles (*S. littoralis* attacked plant versus empty; and doubly infested plant versus empty, both $p < 0.0001$). When wasps

experienced oviposition in presence of odours coming from a plant attacked by *S. littoralis* only, the preference was shifted toward the equally treated plant (doubly versus *S. littoralis* only attacked plants, $p < 0.0001$), and both were more attractive to empty bottles (*S. littoralis* attacked plant versus empty, and doubly infested plant versus empty, both $p < 0.0001$). In Table 1 it is also highlighted the effect of experience (Exp S. l. and No exp) on the treatment (S, SD, E) chosen by the wasps (treatment*exp, $p = 0.016$).

On Figure 3b are listed 22 major compounds found on both induced plant with *S. littoralis* only and with *S. littoralis* and *D. v. virgifera*. Paired *t*-Test analysis for each compound could not detect any effect of belowground feeding on the aboveground *S. littoralis* induced volatiles, except for the compound (E)- β -caryophyllene ($p = 0.049$).

The overall responsiveness for the non-experienced wasps, and experienced on a

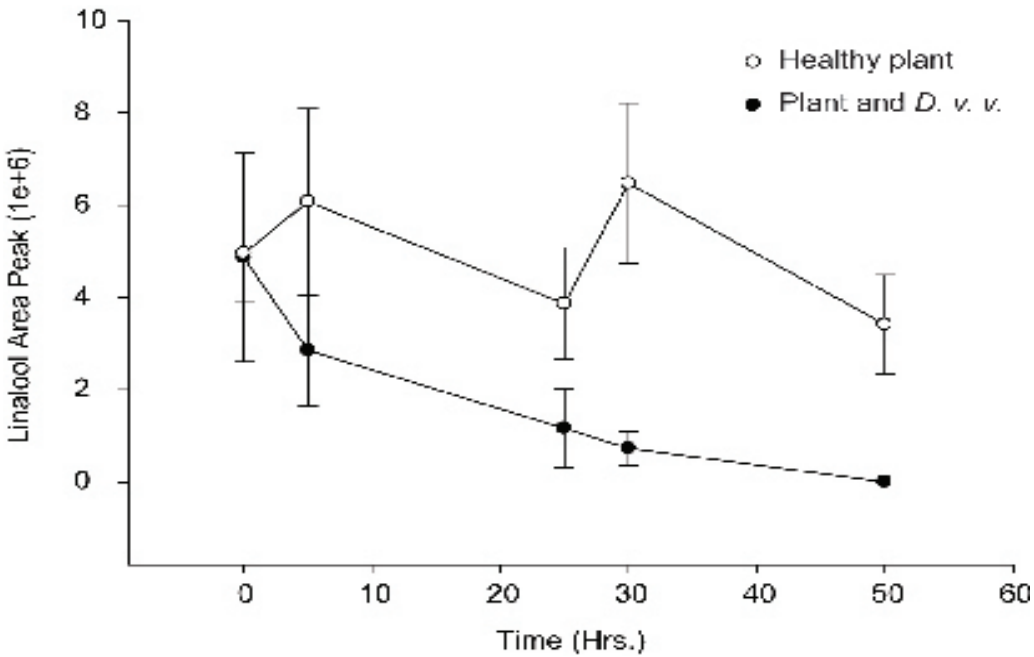


Figure 2a Mean (\pm SE) amount of linalool collected for three hours on healthy plants (white dots) and plants attacked by 4 *D. v. virgifera* larvae (black dots), during three consecutive days. $N = 6$.

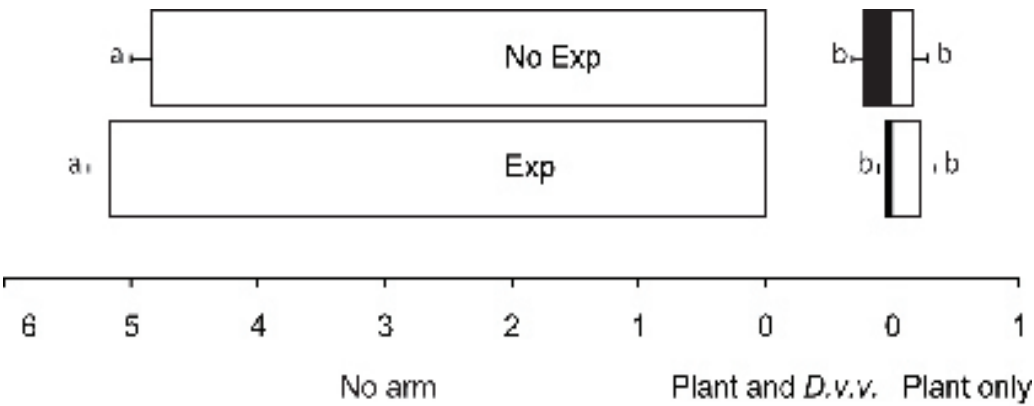


Figure 2b Mean (\pm SE) number of experienced and non-experienced wasps (*C. marginiventris*) that choose any of the three bottles containing healthy plants (Plant only), any of the three bottles containing plants attacked by four *D. v. virgifera* larvae (Plant and *D. v. v.*), or that did not choose any of the olfactometer arms (No arm); $N = 6$. Different letters above bars indicate differences in the number of wasps choosing any of the olfactometer arms for each experience treatment separately.

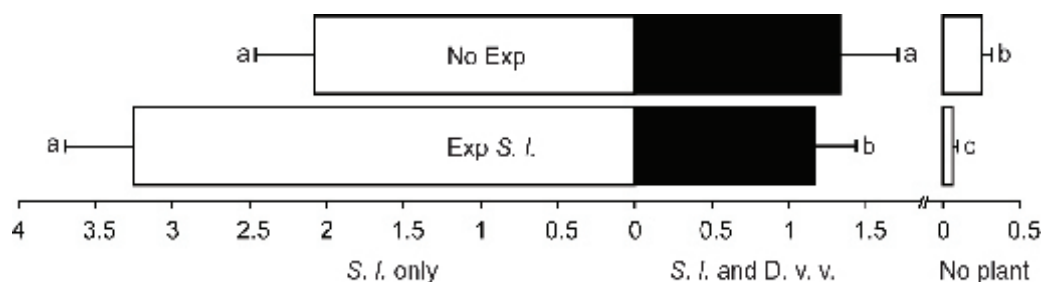


Figure 3a Mean (\pm SE) number of wasps non-experienced (No Exp), and experienced on plants attacked by *S. littoralis* only (Exp S.l.), choosing singly, doubly infested plants, or empty bottles (No Plant); $N = 6$. Different letters above bars indicate differences in the number of wasps choosing any of the olfactometer arms for each experience treatment separately.

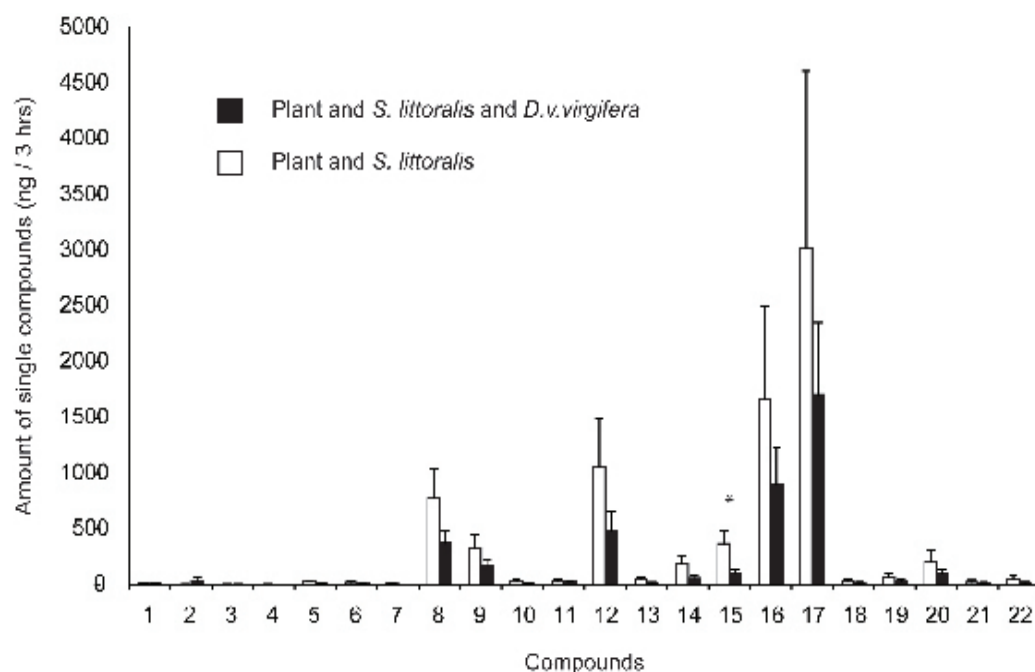


Figure 3b Mean (\pm SE) amount of major single volatile compounds of a blend collected for 3 hrs from: maize plants induced with *S. littoralis* only (white bars), and maize plants induced with *S. littoralis* and *D. v. virgifer* (black bars); $N = 6$. 1, (Z)-3-hexanal; 2, (E)-2-hexanal; 3, (Z)-3-hexanol; 4, (Z)-2-penten-1-ol^N; 5, *b*-myrcene; 6, (Z)-3-hexenyl acetate; 7, (Z)- β -ocimene; 8, linalool; 9, (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT); 10, benzyl acetate; 11 phenethyl acetate; 12, indole; 13, methyl anthranilate; 14, geranyl acetate; 15, (E)- β -caryophyllene; 15, (E)- α -Bergamotene; 16, (E)- β -Farnesene; 17, Unknown; 18, Unknown; 20, β -Sesquiphellandrene^N; 21, (E)-nerolidol; 22, 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMNT). Quantification of peaks 1-14 was done by comparison with IS1, whereas peaks 15-22 were calculated by comparison with IS2. Asterisks represent significant differences between pairs of bars.

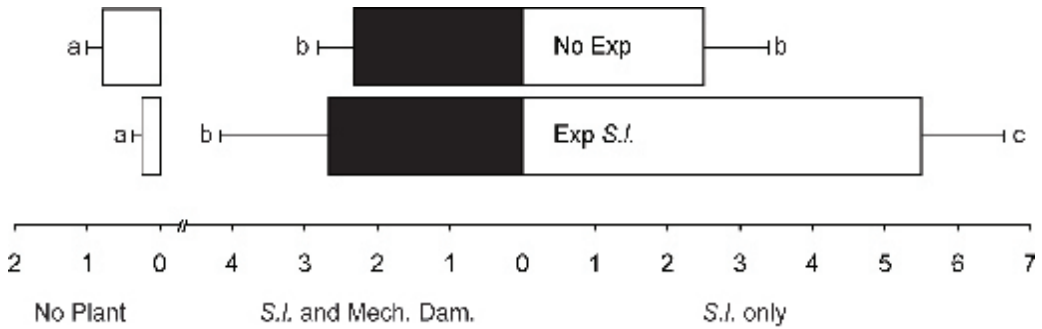


Figure 4a Mean (\pm SE) number of wasps non-experienced (No Exp), and experienced on plants attacked by *S. littoralis* only (Exp S.I. only), choosing singly attacked plants (S.I. only), and mechanical damaged roots (S.I. and mech. Dam.), or empty bottles (No Plant); $N = 12$. Different letters above bars indicate differences in the number of wasps choosing any of the olfactometer arms for each experience treatment separately.

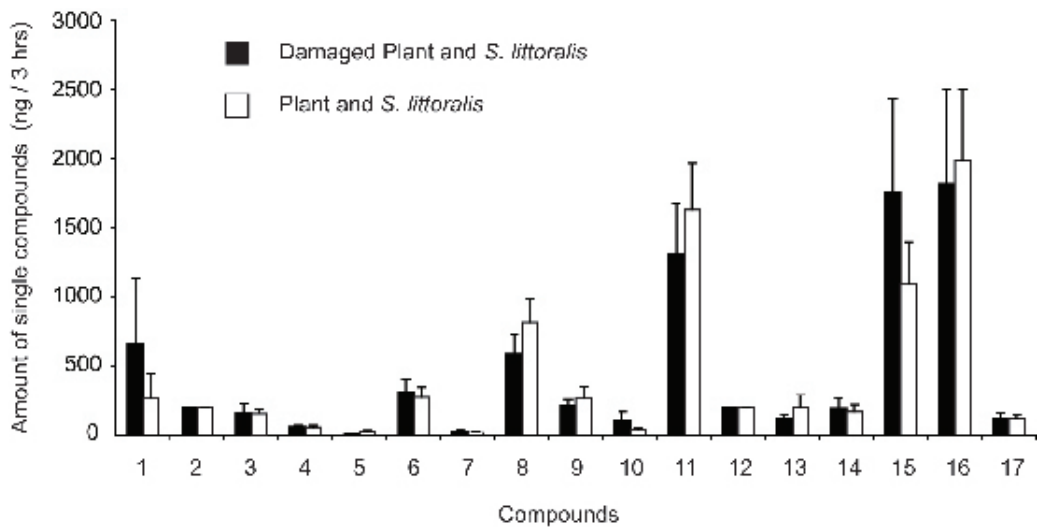


Figure 4b Mean (\pm SE) amount of major single volatile compounds of a blend collected for 3 hrs from: maize plants induced with *S. littoralis* only (white bars), and maize plants induced with *S. littoralis* and *D. v. virgifera* (black bars); $N = 6$. 1, (Z)-3-hexanal; 2, (E)-2-hexanal; 3, (Z)-3-hexanol; 4, (Z)-2-penten-1-ol^N; 5, *b*-myrcene; 6, (Z)-3-hexenyl acetate; 7, (Z)- β -ocimene; 8, linalool; 9, DMNT; 10, benzyl acetate; 11 phenethyl acetate; 12, indole; 13, methyl anthranilate; 14, geranyl acetate; 15, (E)- β -caryophyllene; 15, (E)- α -bergamotene; 16, (E)- β -farnesene; 17, unknown; 18, unknown; 20, β -sesquiphellandrene^N; 21, (E)-nerolidol; 22, TMNT. Quantification of peaks 1-14 was done by comparison with IS1, whereas peaks 15-22 were calculated by comparison with IS2. Asterisks represent significant differences between pairs of bars.

Spodoptera-infested plant was 67%, 76% respectively, and mechanical damaged roots also influenced wasps recruitment aboveground (Figure 4a, Table1). Non-experienced wasps could not distinguish between *S. littoralis* only induced plants and *S. littoralis* plus root mechanical damaged ($p = 0.46$) but both plants were more attractive than the empty bottles (*S. littoralis* attacked plant versus empty, $p = 0.002$; and *S. littoralis* infested plant plus mechanical damage versus empty, $p = 0.003$). An effect of experience was however detected when wasps had an oviposition experience in presence of odours coming from *S. littoralis* infested plants (*S. littoralis* only attacked plant versus *S. littoralis* attacked plant plus mechanical damage, $p = 0.021$), and again both plants were more attractive than the empty bottles (*S. littoralis* attacked plant versus empty, $p < 0.0001$, and *S. littoralis* infested plant plus mechanical damage versus empty, $p = 0.0029$). Effect of experience on wasps' treatment choice; treatment*exp, $p = 0.03$.

After volatile quantification, no differences on the amounts were detected for pairs of the 15 major compounds analysed (Figure 4b).

Discussion

Root feeding of *D. v. virgifera* larvae did not result in the induction of volatile production in the leaves, but, on the other hand, continuous feeding over a period of three days, resulted in the dramatic reduction of constitutive volatile production (Figure 2a). The plant induced with *D. v. virgifera* larvae only were then strongly unattractive, and were similarly attractive compared to healthy plants, for the generalist parasitoid wasps *C. marginiventris* (Figure 2b), this in accordance with previous studies (Turlings, Loughrin *et al.* 1995; Hoballah, Tamo *et al.* 2002). The present study, however demonstrates that root feeding has an effect on aboveground defences, this in accordance with other works.

Baldwin (1994) observed that the *de novo* induced nicotine production by damaging the leaves of *Nicotiana sylvestris* (Solanaceae) produces a signal that dramatically increases the *de novo* synthesis of nicotine in the roots, which in turn, by the mediation of the whole plant nicotine pool, make the plant resistant to further herbivore attack. On the other hand, Masters (1992) showed that a root chewer can increase the pupal weight of the leaf miner feeding on the same plant thus showing a positive direct effect between the two herbivores. Moreover, Moran and Whitham (1990) found no effect of root herbivory on foliar feeding insects, while others have found strong effects on the physiological changes in foliage, such as decrease in nitrogen and a reduction in water content (Gange and Brown 1989; Brown and Gange 1990). Masters (1993) proposed a general model explaining the positive effect of the root feeders to the above ground herbivores by suggesting that the plant root under stress diminishes leaf water content and thus improves leaf quality. With this in mind, we could have assumed that root herbivory will also lead to an increase of induced volatile production, and the subsequent parasitoid recruitment. No clear rule has been highlighted yet for the effect of belowground herbivory on aboveground parasitoid recruitment (Masters, Jones *et al.* 2001; Shiojiri, Takabayashi *et al.* 2001; Shiojiri, Takabayashi *et al.* 2002; Poveda, Steffan-Dewenter *et al.* 2003; Poveda, Steffan-Dewenter *et al.* 2005). Here we demonstrated that *D. v. virgifera* root feeding alters aboveground *S. littoralis* induced volatiles, and this can be learned by *C. marginiventris* wasps. This seems not to be very specific, since also mechanically damaged plants are clearly discriminated by experienced wasps. Put in a broader ecological sense this makes sense. It has been demonstrated that induced volatile profiles of a same plant can be altered by a suite of abiotic and biotic factors such as light, temperature, plant hormones, herbivore-derived elicitors, and microorganisms (see

Review in (D'Alessandro and Turlings 2005)). For this, generalists parasitoids such as *C. marginiventris* benefit strongly of associative learning behaviour (Turlings, Wackers *et al.* 1993; Vet, Lewis *et al.* 1995). In natural environment, plants have to cope with enemies above- and belowground (Bezemer and van Dam 2005), and plants become media through which aboveground communities can shape belowground ones, and vice-versa (De Deyn and Van der Putten 2005). Here is an example corroborating the present hypothesis.

Another similar study showed the effect of root feeding on aboveground tritrophic interactions (Soler, Bezemer *et al.* 2005), demonstrating that belowground herbivory can reduce aboveground herbivore and parasitoid fitness. The next logical step will be to integrate these complementary works, and asses if changes in volatile emissions due to root feeding, will also influence parasitism rate, herbivore and parasitoid performance. Furthermore, it will be capital to know if induced plant volatiles mediated interactions between above- and belowground organisms could also influence natural communities (Wardle, Bardgett *et al.* 2004). For this field studies will also be needed to improve knowledge on semi-natural agricultural systems and thus improve possible biological control management strategies (Hoballah, Degen *et al.* 2004). Finally, continuous integration of broader range above- and belowground systems will facilitate having a better understanding of complex multitrophic interactions.

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CURRICULUM VITAE



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Personal

Birth date	28.07.1975
Place of birth	Lugano- Switzerland
Status	Single
Citizenship	Swiss

Education

PhD Thesis in Biology, Université de Neuchâtel, Switzerland.	2002 - 2006
Biology at Université de Neuchâtel, Switzerland	1997 - 2002
Electronic engineering, 2 semesters at ETH Zürich.	1995 - 1997
Liceo cantonale di Bellinzona, Switzerland. Scientific Type.	1990 - 1995
Exchange student program, Greenwood High school, Texas, USA.	1992 - 1993

Languages

Italian	Mother tongue
French	Very good skills
English	Very good skills
German	Basic knowledge
Spanish	Basic oral skills

Teaching experiences

Supervisor of master student in biology
Course on biological control
Practical courses on entomology and insects systematic
Practical courses on soil biology

Working experiences

Private teaching in biology for college (maturity) degree	2005- 2006
Guide for natural history museum (Neuchâtel)	2000 - 2001
Coordinator for Enviro01 (Annual meeting of the Swiss students in environment)	2001
Practice on mechanical machines (Mikron, Switzerland)	1996

Attendance of congresses

NCCR Plant Survival International Conference 2005. <i>Leysin, Switzerland</i>	2005
Annual Symposium of the Swiss Biological Societies. <i>Basel, Switzerland</i>	2005
10th EPPO ad hoc Panel and FAO Network Group Meeting. <i>Bratislava, Slovak Republic</i>	2005
Host recognition by parasites and parasitoids. <i>Neuchâtel, Switzerland</i>	2004
Workshop “Entomopathogenic Nematode Application”. COST Action 850. <i>Helsinki Finland</i>	2004
Annual Graduate School Meeting. <i>Neuchâtel, Switzerland</i>	2004
Annual Symposium of the Swiss Biological Society. <i>Fribourg, Switzerland</i>	2004
10th IWGO Diabrotica Subgroup meeting. <i>Engelberg, Switzerland</i>	2004
International Symposium on the Ecology and Management of Western Corn Rootworm. <i>Göttingen, Germany</i>	2003
Annual Symposium of the Swiss Biological Societies. <i>Zurich, Switzerland</i>	2003
Annual Graduate School meeting. <i>Neuchâtel, Switzerland</i>	2003
Annual Symposium of the Swiss Biological Society. <i>Bern, Switzerland</i>	2002

Attended courses

Planning a Career Strategy – Job Finding Methodology and Networking,	2005
An Introduction to the Practice of Statistics using R	2005
How to Make Scientific Presentations and Posters Interesting	2005
Effective Public Speaking	2004
Peer Review and Writing Manuscripts	2004
Genetics of Biodiversity and Applications	2004
Identify Natural Compounds by Gas and Liquid Chromatography coupled to Mass Spectrometry	2003
Biodiversity and Species Interaction	2003
Risk Assessment of Genetically Modified Crops	2003
Microbiology, General ecology, Fauna and Flora systematics, Soil biology	1999-2002
Basics in biology, physics and chemistry	1997-1999

Publications

- Rasmann, S., T. G. Köllner, J. Degenhardt, I. Hiltbold, S. Töpfer, U. Kuhlmann, J. Gershenzon, and T. C. J. Turlings. 2005. *Recruitment of entomopathogenic nematodes by insect-damaged maize roots*. *Nature* 434: 732-737.
- Gotthard, K., N. Margraf, S. Rasmann and M. Rahier. 2005. *The Evolution of Larval Foraging Behaviour in Response to Host Plant Variation in a Leaf Beetle*. *OIKOS* 109(3): 503-512.

Affiliations

Swiss Zoological society, WWF, Greenpeace, Mountain Wilderness

Personal interests

Fauna and Flora observation

Alpinism

Sailing

Movies and Reading

