MANIPULATION OF TRITROPHIC INTERACTIONS: A KEY FOR BELOWGROUND BIOLOGICAL CONTROL?

a dissertation submitted to the:

Faculty of Science

University of Neuchâtel

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thesis defended on: 23th of October 2008

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IMPRIMATUR POUR LA THESE

Manipulation of tritrophic interactions : a key for belowground biological control ?

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Summary

In response to attack by phytophagous insects, plants produce volatile organic compounds that serve as cues for natural enemies of the herbivore to locate their potential host or prey. Such tritrophic interactions are well understood aboveground. Recently, it has become evident that such interactions also occur belowground. Among the compounds that are involved in belowground tritrophic signalling is the sesquiterpene (*E*)-β–caryophyllene, a key compound emitted by insect-damaged maize roots (*Zea mais*) when attacked by larvae of *Diabrotica virgifera virgifera*. This sesquiterpene is attractive to the entomopathogenic nematode *Heterorhabditis megidis*, which infects and kills *D. v. virgifera* larvae. Aboveground, maize leaves subjected to insect herbivory emit a wide range of volatile compounds. To understand why the same plant when attacked belowground only emits a reduced pattern of compounds, largely dominated by (*E*)-β-caryophyllene, we studied the diffusion properties of this sesquiterpene. Of the potential compounds tested a few other sesquiterpenes diffused better than (*E*)-β-caryophyllene, but these are more costly to synthesize for the plant. The release of (*E*)-β-caryophyllene seems ideal balance between diffusion efficiency and production costs (Chapter I). Because of breeding, some maize varieties are not emitting this belowground signal anymore. The transformation with a terpene synthase gene from oregano into a maize line that normally does not produce this call-for-help signal successfully restored their ability to release (*E*)-β-caryophyllene. In presence of *H. megidis*, this release resulted in a better protection of the root system and fewer *D. v. virgifera* adults emerging near the transformed plants compared to their original lines under field conditions (Chapter II). In order to determine the most effective nematode species for biological control of *D. v. virgifera* in Europe, we compared the control efficiency of *H. bacteriophora*, *H. megidis* and *Steinernema feltiae*. The susceptibility of different *D. v. virgifera* development stages to the above mentioned nematodes species was assessed under semi-field conditions. All stages of the targeted pest were susceptible to entomopathogenic nematodes and *Heterorhabditis* species were slightly better in controlling the pest than *Steinernema* species (Chapter III). When tested in the field near (*E*)-β-caryophyllene producing and non-producing maize varieties, *H. megidis* and *S. feltiae* were considerably more effective in plots with the (*E*)-β-caryophyllene releasing line. *H. bacteriophora* appears to use other plant-produced signals. Promisingly *H. bacteriophora* level of control was

comparable to levels that can be achieved with pesticides (Chapter IV). In order to achieve an improved control of *D. v. virgifera*, was manipulated in the laboratory. We selected a strain of *H. bacteriophora* in belowground six-arm olfactometers over six generations for improved attraction to (*E*)-β-caryophyllene. This species does normally not respond to the signal (Chapter IV). In field trials it was confirmed that the selected strain, when applied near a (*E*)-β-caryophyllene producing maize variety, was more efficient in killing *D. v. virgifera* than the original strain. As expected, there was no such difference between the strains when released near a maize variety that did not emit (*E*)-β-caryophyllene (Chapter V).

In addition to the fundamental knowledge on belowground interactions obtained by the current study, it also provides key information on how to use entomopathogenic nematodes for biological control of *D. v. virgifera*. Moreover, this study is, to our knowledge, the first demonstration that biological control can be improved by manipulating the production of and responsiveness to a plant signal.

Key words: Tritrophic interaction, *Diabrotica virgifera virgifera*, western corn rootworm, maize pest, root pest, belowground chemical ecology, induced plant defence, *Zea mais*, entomopathogenic nematode, *Heterorhabditis megidis*, *Heterorhabditis bacteriophora*, *Steinernema feltiae*, (*E*)-β-caryophyllene.

Résumé

En réponse à des attaques d'insectes phytophages, les plantes produisent des composés organiques volatiles servant de signal aidant les ennemis naturels de l'insecte herbivore à localiser un hôte ou une proie potentielle. De telles interactions trophiques sont bien comprises pour la partie aérienne de la plante. Récemment, des systèmes tritrophiques souterrains ont été mis en évidence et des composés importants impliqués dans de telles interactions ont été identifiés. Parmi eux, un sesquiterpène, le (*E*)-β-caryophyllène, est considéré comme un composé clé dans le modèle tritrophique *Zea mais - Diabrotica virgifera virgifera - Heterorhabditis megidis*. Des racines de maïs attaquées par la larve du ravageur *D. v. virgifera* émettent dans le sol ce signal d'alerte et peuvent ainsi attirer le nématode entomopathogène *H. megidis* capable d'infecter et de tuer la larve de *D. v. virgifera*

Les feuilles de maïs attaquées par des insectes émettent un large spectre de composés volatiles. Afin de comprendre pourquoi les racines d'une même plante n'émettent qu'un éventail réduit de ces composés lors d'une attaque de *D. v. virgifera*, largement dominé par le (*E*)-β-caryophyllène, une étude concernant la diffusion de ces volatiles dans le sol fût menée. Parmi les composés testés, quelques autres sesquiterpènes diffusèrent mieux que le (*E*)-β-caryophyllène, mais la biosynthèse de ces derniers est plus coûteuse pour la plante. L'émission de (*E*)-β-caryophyllène semble donc être un compromis optimal entre les propriétés de diffusion de cette molécule et ses coûts de production pour la plante (Chapitre I). A cause de la sélection opérée sur le maïs depuis sa domestication, certains des cultivars ont perdu la capacité de produire et d'émettre ce signal souterrain. L'insertion d'un gène responsable de la synthèse du (*E*)-β- caryophyllène de l'origan dans une variété de maïs ne produisant plus ce signal d'appel à l'aide a rétabli la capacité de la plante à émettre du (*E*)-β-caryophyllène. Lors d'expériences en champs et en présence de *H. megidis*, cette émission résulte en une meilleure protection du système racinaire et moins d'adultes de *D. v. virgifera* émergent à proximité des plantes transformées que des plantes contrôles (Chapitre II). Dans un souci d'utilisation de la meilleure espèce de nématodes entomopathogènes en lutte biologique contre *D. v. virgifera* en Europe, l'efficacité de *H. bacteriophora, H. megidis et Steinernema feltiae* contre ce ravageur fût testée dans des conditions semi-naturelles. Une mortalité plus importante à tous les

stades pré-imagos du ravageur ciblé fût observée en présence des trois espèces de nématodes entomopathogènes susmentionnées. Cependant des résultats légèrement meilleurs furent obtenus avec les deux espèces du genre *Heterorhabditis* comparé à *Steinernema feltiae* (Chapitre III). Lors d'expériences en champs, *H. megidis* et *S. feltiae* se sont révélés considérablement plus efficaces dans les parcelles plantées de maïs émettant du (*E*)-β-caryophyllène que dans les parcelles n'en émettant pas. *H. bacteriophora* semble ne pas être influencé par la présence ou l'absence de ce volatile mais semble plutôt répondre à d'autres composés produits par une plante infestée. Néanmoins, *H. bacteriophora* a égalé le niveau de contrôle du ravageur atteint par les insecticides chimiques (Chapitre IV). Dans le but d'obtenir encore un meilleur contrôle de *D. v. virgifera*, *H. bacteriophora* a été manipulé en laboratoire. Une nouvelle souche de ce nématode a été sélectionnée pour une réponse améliorée au (*E*)-β- caryophyllène. Grâce aux olfactomètres souterrains à six bras, il a suffi de six générations pour accroître l'attraction de *H. bacteriophora* vers ce signal racinaire, alors que cette espèce ne répondait normalement pas à ce composé (voir Chapitre IV). Lors d'expériences en champs, la souche sélectionnée, lorsqu'elle était appliquée près d'une variété de maïs produisant du (*E*)-β-caryophyllène, s'est avérée plus efficace pour éliminer *D. v. virgifera* que la souche d'origine. De telles différences entre la souche sélectionnée et l'originale n'ont pas été observées auprès des plantes n'émettant pas de (*E*)-β-caryophyllène (Chapitre V).

En parallèle des connaissances fondamentales sur les interactions tritrophiques souterraines mises en lumière, la présente étude apporte des informations clés sur l'utilisation des nématodes entomopathogènes dans un contexte de lutte biologique contre *D. v. virgifera.* De plus, cette thèse démontre pour la première fois à notre connaissance que la lutte biologique peut être améliorée par la manipulation soit de la production d'un signal émis par une plante, soit de la réponse du troisième niveau trophique à ce même signal.

Mots-clés: Interaction tritrophique, *Diabrotica virgifera virgifera*, chrysomèle du maïs, ravageur du maïs, ravageur de racines, écologie chimique souterraine, défence induite des plantes, *Zea mais*, nématode entomopathogène, *Heterorhabditis megidis*, *Heterorhabditis bacteriophora*, *Steinernema feltiae*, (*E*)-β-caryophyllène.

Acknowlegements

mitakoye oyasin

More than three years… More than three years of collaboration and friendly relationships with many great people. How can I thank them without having to restrict myself? But shall I?

First of all, I would like to thank Prof. Ted Turlings for its supervision and its help during this period. You never had more than 5 min. at once, but ideas exchanged during these short periods of time were almost always successful (after some adaptation for a human feasibility, of course).

As already mentioned, this thesis is resulting of intense collaborations. I am grateful to Prof. Jonathan Gershenzon, Dr. Jörg Degenhardt and Dr. Tobias Köllner from the Max Planck Institute for Chemical Ecology (Jena, Germany) for their huge input in molecular biology. Doing experiment on maize pest includes the necessity to have it and thus I would like to express my gratitude to Dr. Tim Haye and co-workers form CABI Europe (Delémont, Switzerland) for rearing and supplying me with WCR larvae. From the same institution, Dr. Stefan Toepfer and Dr. Uli Kuhlmann made field work in Hungary realisable. In a field context, I am grateful to Prof. Bruce Hibbard from University of Columbia (Missouri, USA) for the great field experiment we conducted together. Both field and lab work were feasible thanks to Andermatt Biocontrol (Switzerland), e-nema (Germany) and Becker Underwood (United Kingdom) which provided me in entomopathogenic nematodes.

I am grateful to Prof. Ralph-Udo Ehlers, Prof. Bruce Hibbard and Dr. Stefan Toepfer for the expertise and advices during my thesis.

I would like to thank the jury of my thesis: Prof. Ted Turlings, Prof. Ralph-Udo Ehlers, Prof. Bruce Hibbard, Prof. Jean-Michel Gobat and Dr. Stefan Toepfer.

A special thought to the FARCE and e-vol lab members. They supported me over these years and became more than colleagues. A particular thank to Matthias (le Held, of course) and Georg my patient officemates; Vio et Yann pour m'avoir hébergé dans leur fauteuil; Mättu et Anahi pour votre soutient et amitié sans limites; Russ, Sahra and Claudia for their stimulating discussions and their corrections of manuscripts; Greg pour nos sorties alpines pas toujours réparatrices mais au combien

bienfaisantes; Nadir mon coach professionnel, mentor et ami; Sergio tu n'es plus vraiment dans le labo, mais merci pour tout ces grands moments. Because of your presence every day, I was waking up with a smile and was glad to come at work.

Yann, notre relation a fait jaser (…), mais je t'en remercie du fond du cœur! Ces années passée à partager nos vies furent pour moi de toute beauté. Si je devais résumer ce que j'ai en tête en ce moment, je dirais: « Comme un lundi… ». Merci pour tout et encore d'avantage!

L'amitié est peut-être un des meilleurs moteurs que j'ai pu expérimenter ces dernières années. Je voudrais donc remercier toutes les personnes qui m'ont accompagnée sur mon chemin parfois tortueux parcouru durant cette période. Sans votre présence rassurante et aimante, cela ferait peut-être longtemps que je me serai assis sur le bas côté et que j'aurai attendu, qui sait ?

Merci à papa, maman, Fanny et Annelaure. C'est bon de vous savoir là, près de moi. Votre soutient et amour m'ont toujours permis d'aller de l'avant sans jamais trop douter. Vous avez été les confidents de mes peines, mais surtout les artistes de mes joies et réussites. Merci d'avoir cru en mes choix et de m'avoir parfois remis sur les rails de la raison. Je vous aime!

… et pour terminer, merci Lanilà, tu es venue illuminer ma vie. Notre aventure ne fait que commencer…

Introduction

&

Thesis outline

Ivan Hiltpold

2008

Introduction

Since humans became sedentary and started to domesticate plants for agronomy (~10,000 years ago), crop production has steadily increased (Hillel and Rosenzweig 2005). Besides nutritional concerns (Larsen 2003), primitive farmers also had to compete with insect pests (Hillel and Rosenzweig 2005). In early times, small scale fields and crop diversification minimized the impact of herbivory (Tonhasca and Byrne 1994). However, the increasing need for food resources led to a reduction of crop biodiversity and, according to the "resource concentration hypothesis", the impact of pests also increased (Root 1973). Through the ages, human beings never stopped the creative development of strategies to protect their crops (Hillel and Rosenzweig 2005) and during the last century the profuse use of chemical pesticides has been the answer to the pest problem (Pimentel 1997). The plants themselves have been engaged in a continuous arms race with herbivores during millions of years of evolution and have evolved abilities to protect themselves against herbivore pressures (Thompson and Cunningham 2002). Currently considered as a coevolution process, this arms race has led to a spectacular pattern of biodiversity. While plants have to defend themselves against voracious organisms by more and more complex and fine armaments, insects in turn had and continue to evolve strategies in order to resist plant defences and further exploit plants as sources of nutrients (Schoonhoven, Jermy *et al.* 1998).

The defence artillery of plants is incredibly diverse and complex. The first fence built against insect feeding is of a physical nature and consists of the plant tissues themselves. The presence of trichomes or wax on the leaf surface, leaf thickness or toughness, high silica content or sclerotization may reduce food acceptance behaviour or decrease the food accessibility for insects (Schoonhoven, Jermy *et al.* 1998). The second defence line is formed by "chemical soldiers". Plants may store toxics and/or repellents against phytophagous organisms in their tissue. While this constitutive defence is efficient, it is costly for the plant to continuously produce such compounds. Therefore, to limit costs and stay as vigorous as possible, plants have developed the ability to produce certain toxic or repellent compounds only when they are fed on by herbivores. Defence mechanisms triggered by insect damage triggered defence mechanisms

are termed induced defences. Many recent studies have focused on this strategy (books: Karban and Baldwin 1997; Agrawal and Rutter 1998; Agrawal, Tuzun *et al.* 1999; reviews: Baldwin 1994; Karban, Agrawal *et al.* 1997; Baldwin and Preston 1999; Dicke and van Loon 2000; Dicke, van Poecke *et al.* 2003; Bruce and Pickett 2007; Heil 2008).

Plant constitutive/induced defence does not only target their enemies directly by influencing the herbivore feeding behaviour or fitness, but also indirectly by enhancing the involvement of higher trophic levels (Price, Bouton *et al.* 1980). Such indirect defence aims to provide sustenance, housing or attraction of organisms able to protect the plants against phytophagous attacks. These adaptations range from formation of special structures serving as home for ants, mites or bacteria to the production of nectar foliar glands used by natural enemies of herbivores as food sources (constitutive indirect defence) (Janzen 1966; Stapley 1998). The induced indirect plant defence, following an herbivore attack, results in the recruitment of natural enemies of the herbivore by producing and emitting cues or volatile organic compounds (VOCs). Such interactions with a third trophic level were revealed in many studies during the two last decades (for review see: (Turlings and Benerey 1998; Dicke and Vet 1999; Dicke, van Poecke *et al.* 2003; Turlings and Wäckers 2004). Exploiting such plant ability in crop protection strategies would lead to an ecological sound way of controlling pests.

Having a deep understanding of such tritrophic interaction in order to enhance belowground crop protection is the global aim of the following thesis. To achieve this objective, the interaction between the crop plant maize (*Zea mays* L.), the root feeder larvae of the western corn rootworm (*Diabrotica vigifera virgifera* LeConte) and the entomopathogenic nematodes (*Heterorhabditis* ssp. and *Steinernema feltiae* Filipjev) was used as a model system.

The model system

Maize (Zea mays mays)

Maize is the first trophic level of the model previously mentioned. Maize is one of the most widely cultivated crops worldwide, not only as fodder for cattle but also for human consumption, thanks to the starch, proteins and oils contained in the kernels (Fedoroff 2003). As such, maize is one of the most important crops worldwide (Sattaur 1989). Even so, its domestication

history remains poorly understood. Despite decades of research by botanists, molecular biologists and archaeologists, the theories of maize domestication are still controversial.

Depending on the authors, the presumed start of its domestication is fluctuating between 10'000 ^{14}C B. P. and 5'000 ^{14}C B. P. (Piperno and Flannery 2001; Sluyter and Dominguez 2006). However, scientists do agree on the ancestor, the wild grass teosinte (*Zea mays* ssp. *parviglumis* Iltis and Doebley) (Fukunaga, Hill *et al.* 2005), of our modern maize (*Zea mays* ssp. *mays*). Evidences are designating two main locations as basins of maize domestication, the Southern Mexican Highlands around Tehuacán and Oaxaca (at an elevation of 1200–2000m) and the Balsas River Valley on the Pacific slope (at an elevation of 400–1200m) both areas located in Central Mexico (Piperno and Flannery 2001; Sluyter and Dominguez 2006).

Because of breeding selection, the different maize varieties accessible to farmers have high intraspecific variation in both qualitative and quantitative release of VOCs (Gouinguené, Degen *et al.* 2001; Degen, Dillmann *et al.* 2004). Such chemical polymorphisim is also present among teosinte subspecies (Gouinguené, Degen *et al.* 2001).

Western Corn Rootworm (Diabrotica v. virgifera LeConte, Col. : Chrysomelidae)

The subtribe of Diabroticina contains many polyphagous and oligophagous herbivorous species feeding on crops, ornamental and wild plants. Among these, the genus *Diabrotica* (Chevrolat) contains the greatest number of pests, including some of the most important crop pests around the world. The *virgifera* group includes the northern (*Diabrotica barberi* (Smith

Figure 1 WCR adult feeding on corn silks. Even if adults' damages are fewer than larvae's, they may lead to yield reduction as well.

and Lawrence)), the Mexican (*D. virgifera zea* (Krysan and Smith)) and the western (*D. v. virgifera*, WCR (fig. 1)) corn rootworms. These corn rootworms are among the most economically important maize pests in terms of annual losses in crop yields and control costs (Walsh 2003). WCR is considered as 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).

WCR life cycle and ecology

In order to survive the cold (in temperate areas) or the dry (in tropical areas) season eggs are oviposited belowground. According to Gray et al. (1992), 66% of the eggs are laid in the top 10 cm of the soil. It has been shown that the eggs are highly susceptible to extremes of temperature and moisture (Krysan 1999). Such a depth seems to provide environmental conditions which are favorable enough for egg survival. At the end of this quiescence period, from late May to mid-June (Branson and Krysan 1981), WCR larvae hatch and go through three larval instars before pupation. Small variations between male and female development time to the adult stage are observed, it takes on average 26.3 days for males, whereas females need 28.9 days to complete their larval and pupal development (Jackson and Elliot 1988). Eggs are not systematically hatching close to or into the root system, therefore finding host-roots is critical for the survival of the first larval instar. Newly hatched larvae follow CO_2-gradients to locate roots (Strnad and Bergman 1987; Bernklau, Fromm et al. 2004). Other behavioural experiments indicate that 6-methoxy-2-benzoxazolinone (Bjostad and Hibbard 1992) and long-chain free fatty acids (Hibbard, Bernklau et al. 1994) are involved in orientation of second instar WCR larvae. Once in the root system, these oligophagous larvae start feeding. It has recently been shown that a combination of simples sugars and free fatty acids act as a feeding stimulant for WCR neonates (Bernklau and Bjostad 2008). At maturity, the third-instar larva pupates and stays in soil as pupa for approximately two weeks. Then the pupa gives rise to an adult and the beetle emerges from late June to late September depending on location (Governatori, Frausin et al. 2002). Between eggs laying and the emergence of adults, as many as 98.9% of the individuals die. The 1.1% surviving adults feed on the aboveground part of maize in order to get enough energy for mating at the end of the season (Kuhlmann and van der Brugt 1998;

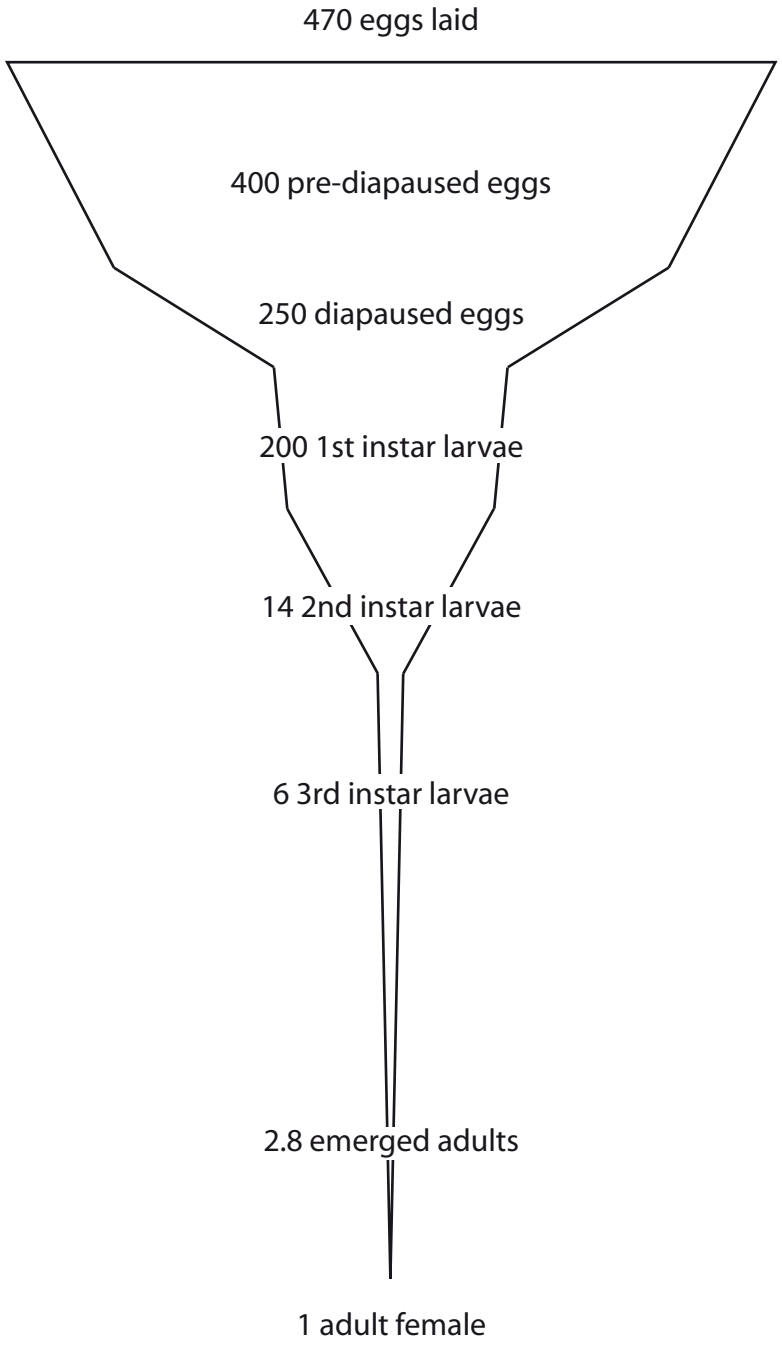

Figure 2 Age-specific survivingship of WCR in Southern hungary. Modified after Toepfer and Kuhlmann (2005).

Toepfer, Zhang et al. 2001) (fig. 2).

Geographic distribution

WCR is native to Central America from where it spread to northern parts of the new continent (Branson and Krysan 1981). WCR was introduced into Europe in the 1990s (Baca 1994; Sivcev, Manojlovic *et al.* 1994). Observed first in Serbia (1992), the beetle quickly spread to Hungary (1995), Croatia (1995), Romania

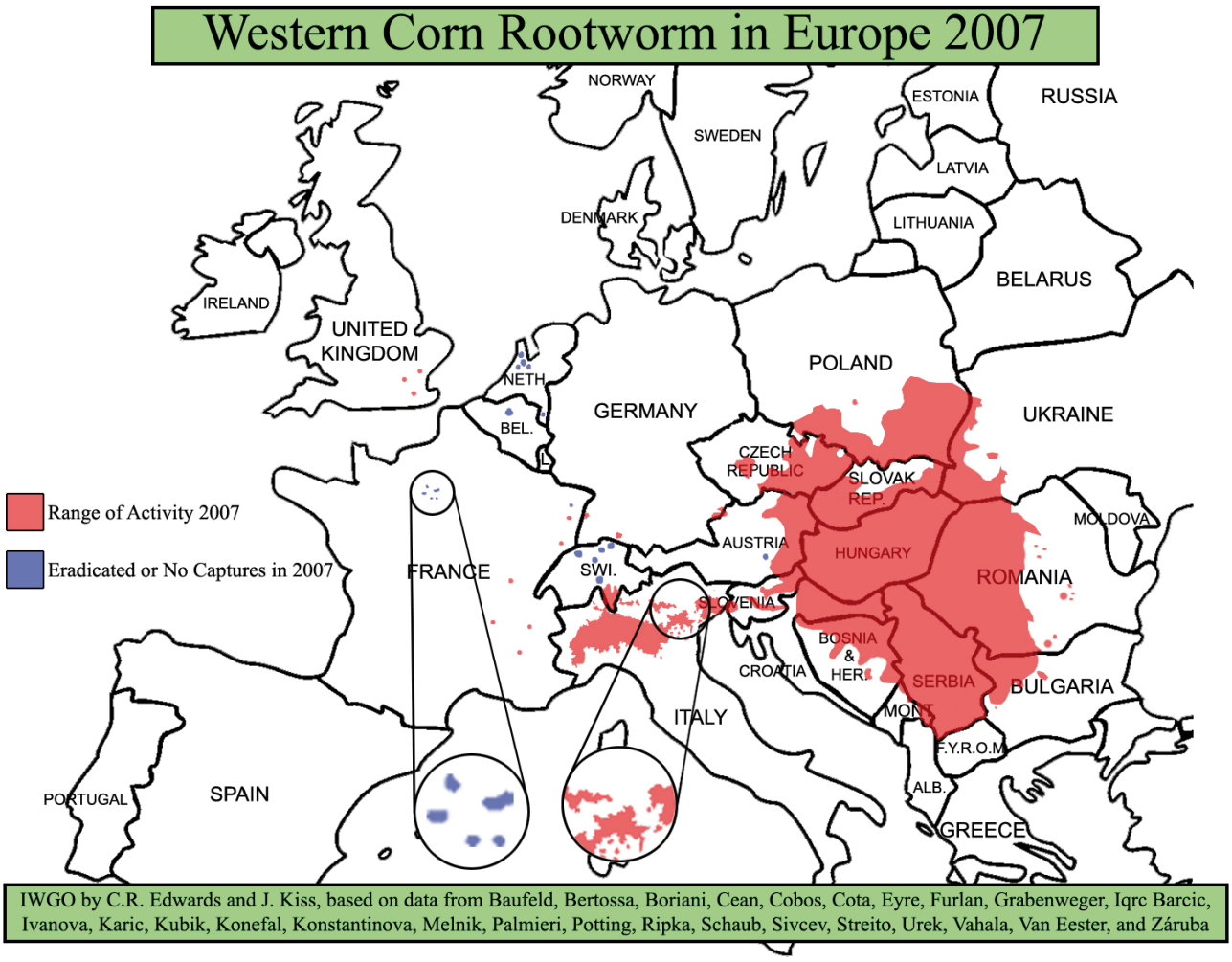

Figure 3 spreading of WCR in Europe until summer 2007. Source: www.eppo.org/QUARANTINE/ Diabrotica_virgifera/diabrotica_virgifera.htm

(1996), Bosnia and Herzegovina (1997), Bulgaria (1998), Italy (1998), southern (2000) and northern (2003) Switzerland and to many other European countries (EPPO 2004) (fig. 3).

New evidence resulting from molecular studies imply multiple introductions in Europe (Miller, Estoup *et al.* 2005) (fig. 4). This indicates that the European populations do not all originate from the first introduction in Serbia and that new colonizations are possible and have occurred.

Current control strategies against WCR

Several approaches have been tried to control WCR populations, but three strategies are mainly used: crop rotation in order to break the annual beetle life cycle, soil insecticide applications and transgenic BT maize with resistance to WCR larval feeding.

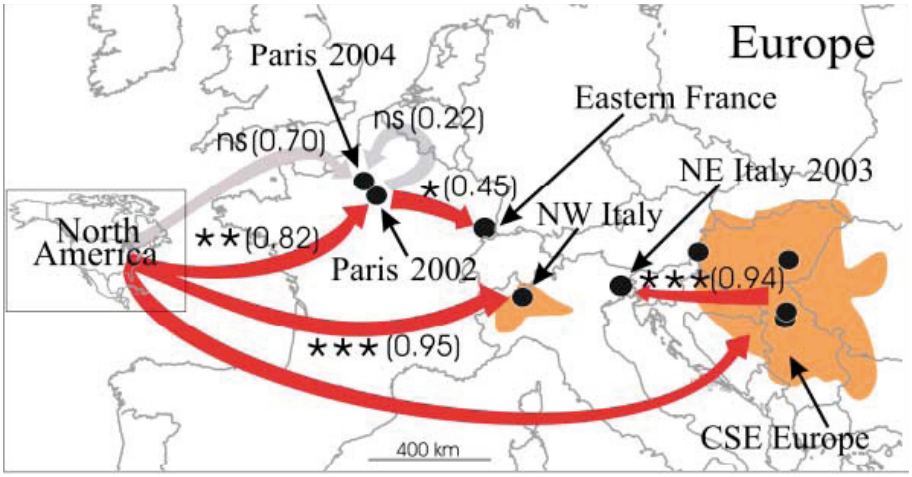

Figure 4 The most likely scenario of invasion into Europe of WCR. Gray arrows represnents unresolved scenarios. Large areas where WCR is present are shown in orange. After Miller et. al *(2005)*

The success of crop rotations is limited in certain region because of adaptation of WCR. Since the beginning of the 90s, problems with this technique have been reported in part of the US Corn Belt. Females able to lay eggs in soybean and other crop fields developed after the extensive use of crop rotation (Levine and Oloumi 1996; O'Neil, DiFonzo et al. 2002). A two-year rotation rhythm can still break the cycle and seems to be efficient for the control of WCR (Levine and Oloumi-Sadeghi 1991), but due to the apparition of extended diapause this approach is losing efficiency (Tollefson 1988; Levine, Oloumi et al. 1992). In Europe, where WCR reached the economic threshold six to eight years after initial infestation, crop rotation is still efficient and it is not expected that the selection pressure is high enough to soon result in a rotation-resistant WCR population (Kiss, Komáromi et al. 2005).

Although soil insecticides still work well in North America (Boetel, Fuller *et al.* 1998; Sundermeier and Eisley 2001; Viana and Marochi 2002; Boetel, Fuller *et al.* 2003), ecological problems are linked to this practice. Effects on non-target organisms, like WCR predators and humans or on plants themselves (chemical reactions can occur between insecticides and herbicides and produce phytotoxic compounds) (Journey and Ostlie 2000) and therefore restrictions on application have been imposed.

Disruption of early WCR larval instars' host location appears work in reducing *Diabrotica v. virgifera* population. Bernklau, Fromm *et al.* (2004) increased the level of carbon dioxide in soil by adding CO_2 generating granules, resulting in significantly lower root damage.

Transgenic maize lines producing *Bacillus thurigiensis* crystal proteins specific to

Chrysomelidae have been the focus of recent research in the USA. Once ingested, these crystals turn toxic and seem to be effectove against WCR larvae (Moellenbeck, Peters *et al.* 2001; Ellis, Stockhoff *et al.* 2002; Baum, Chu *et al.* 2004; Hibbard, Vaughn *et al.* 2005; Vaughn, Cavato *et al.* 2005). However, the Cry3Bb1 Bt toxin, targeted at WCR, has no evident effect on WCR adults (Nowatzki, Zhou *et al.* 2006). This transgenic maize seems to have a high specificity against WCR larvae and its effect against non-target soil organisms is low (Al-Deeb and Wilde 2003; Al-Deeb, Wilde *et al.* 2003; Ahmad, Wilde *et al.* 2005; Ahmad, Wilde *et al.* 2006).

Biological control may be an alternative to combat WCR, as a large number of pathogens, predators and parasitoids are available to kill WCR. Fungal and bacterial pathogens such as *Beauveria bassiana* (Deuteromycetes) or *B. thuringiensis* (specific strains) can be used to reduce WCR populations (Krueger and Roberts 1997; Kuhlmann and van der Brugt 1998). Some predatory mites feed on corn rootworm eggs, but Stoewen and Ellis (1991) concluded that egg predation is not a factor worth pursuing for WCR control. Predation by insects does not seem to be effective enough to be used in WCR management, even if the ant species *Lasius neoniger* (Emery) (Hymenoptera: Formicidae) significantly reduces WCR larval population (Chiang 1970; Kirk 1981; Governatori, Frausin *et al.* 2002). Studies on parasitoids belonging to the genus *Celatoria* (Diptera: Tachinidae) and *Centistes* (Hymenoptera: Braconidae) show that these organisms are potential control agents against corn rootworm (Kuhlmann and van der Brugt 1998; Zhang, Toepfer *et al.* 2004).

After a two-year study on adults, larvae and eggs, no natural enemies of WCR described above were found in south-eastern Europe except the fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Toepfer, Zhang *et al.* 2001; Pilz, Wegensteiner *et al.* 2008).

A promising group of WCR enemies is represented by the two nematode families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida). These entomopathogenic nematodes (EPN) significantly reduced WCR populations in laboratory bioassays (fig. 5). Although field results have been variable (Jackson and Brooks 1995; Choo, Lee *et al.* 1996; Jackson 1996; Ben-Yakir, Efron *et al.* 1998; Journey and Ostlie 2000; McCoy, Stuart *et al.* 2002), EPN

can play a major role in the biological control of corn rootworms (Ehler 1990) and efficiently reduce WCR population (Rasmann, Köllner *et al.* 2005; Hiltpold, Baroni *et al.* in prep.; Hiltpold, Toepfer *et al.* in prep.; Degenhardt, Hiltpold *et al.* in prep.). Moreover, EPN do not adversely affect non-target arthropod populations in the medium term (Georgis and Gaugler 1991; Bathon 1996). Used worldwide as biological agents (Grewal, De Nardo *et al.* 2001), it also appears that if they are applied in an integrated approach together with certain insecticides, their efficacy in controlling insect pests increases (Nishimatsu and Jackson 1998; Lacey, Frutos *et al.* 2001). Similar synergies can be expected when EPN are used in combination with transgenic plants that contain insecticidal proteins.

Entomopathogenic nematodes (Heterorhabditis ssp and Steinernema feltiae)

Since late 20th century, potential and possibilities of the use of EPN as biological control agents against insect pests have been studied (e. g. Thurston and Yule 1990; Barbercheck 1993; Kaya and Gaugler 1993; Barbercheck and Wang 1995; Choo, Koppenhofer *et al.* 1996; Ellsbury, Jackson *et al.* 1996; Eben and Barbercheck 1997; Mortimer, van der 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). Out of the two taxonomic families of EPN (Steinernematidae and Heterorhabditidae), researchers are focussing their work on two genera, *Steinernema* ssp. and *Heterorhabditis* ssp. As all other known EPN, both families carry symbiotic bacteria in their guts. Because of this symbiosis, they can, by releasing the bacteria in the body cavity of insects, kill their host within 24-48h (Dowds and Peters 2002; Lewis 2002) (fig. 5). Even though EPN infect and kill a broad spectrum of arthropod species in laboratory experiments, it seems that their non-target effect in the field are rather low (Bathon 1996). In the field, the host range of EPN is dependent on the temporal

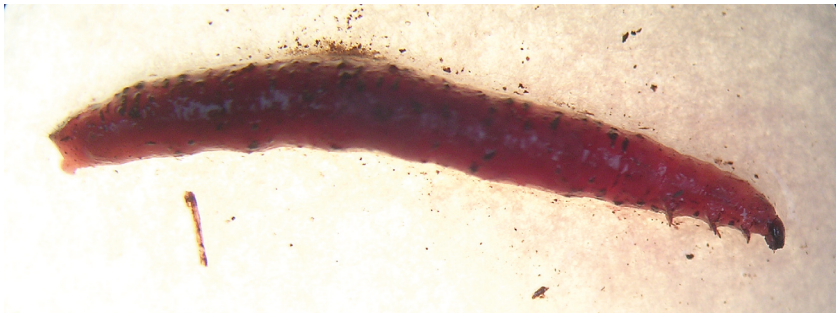

Figure 5 WCR 2nd-instar larva infect by H. megidis. *The typical red colour is due to the presence of the symbiotic bacteria* Photorhabdus *sp.*

and spatial occurrence of potential hosts, as well as their distribution, which leads to a more restricted host range. EPN are used in many countries in almost all continents, but there are no reports that their application (indigenous or exotic) significantly affects local non-target populations. Moreover, due to their low potential to spread, the impact of nematodes is restricted to the application area (Bathon 1996; Grewal, Grewal *et al.* 2002; Puza and Mracek 2005). In addition, EPN exhibit traits that facilitate their effectiveness, including their host finding ability (Boff, van Tol *et al.* 2002), compatibility with conventional agricultural spraying equipment (Fife, Ozkan *et al.* 2005; Laczynski, De Moor *et al.* 2006), compatibility with pesticides (Head, Walters *et al.* 2000), amenability for commercial production in large scale liquid culture (Ehlers 2001) and persistence in natural environment (Poinar 1990).

In the early 1990th, only nine *Steinernema* ssp. and three *Heterorhabditis* ssp. were described. Today, more than 50 species of the two genera are known. The enormous increase of description shows the interest in this group of organisms during the last two decades (Adams, Fodor *et al.* 2006).

The life cycle of EPN (fig. 6) can be subdivided into five events: (1) penetration into the haemocoel of the potential host, (2) recovery into the haemocoel, (3) development to mature stage and reproduction, (4) development of new infective juveniles (IJs) and (5) host searching and host acceptance by IJs (Dowds and Peters 2002; Forst and Clarke 2002).

Infection starts when EPN enter the host through natural openings such as mouth, anus or spiracles (Dowds and Peters 2002). Heterorhabditid EPN can actively cross the cuticle of the host thanks to teeth-like appendices (Bedding and Molyneux 1982). Once EPN have passed through the mechanical barriers (e. g. sieve plates protecting the spiracles (Forschler and Gardner 1991) or avoided crushing by insects' mandibles (Gaugler and Molloy 1981) and defence chemicals (e. g. up to 40% of invading EPNs can be killed by gut fluids (Wang, Campbell *et al.* 1995)), they still have to cross the epithelial membrane of the intestine to reach the heamocoel. Heterorhabditid nematodes again use their head appendices for this final step. There is evidence that Steinernematids use enzymes secretions that provoke histolysis, allowing penetration into the haemocoel (Peters and Ehlers 1994; Abu Hatab, Selvan *et al.* 1995). Once in the

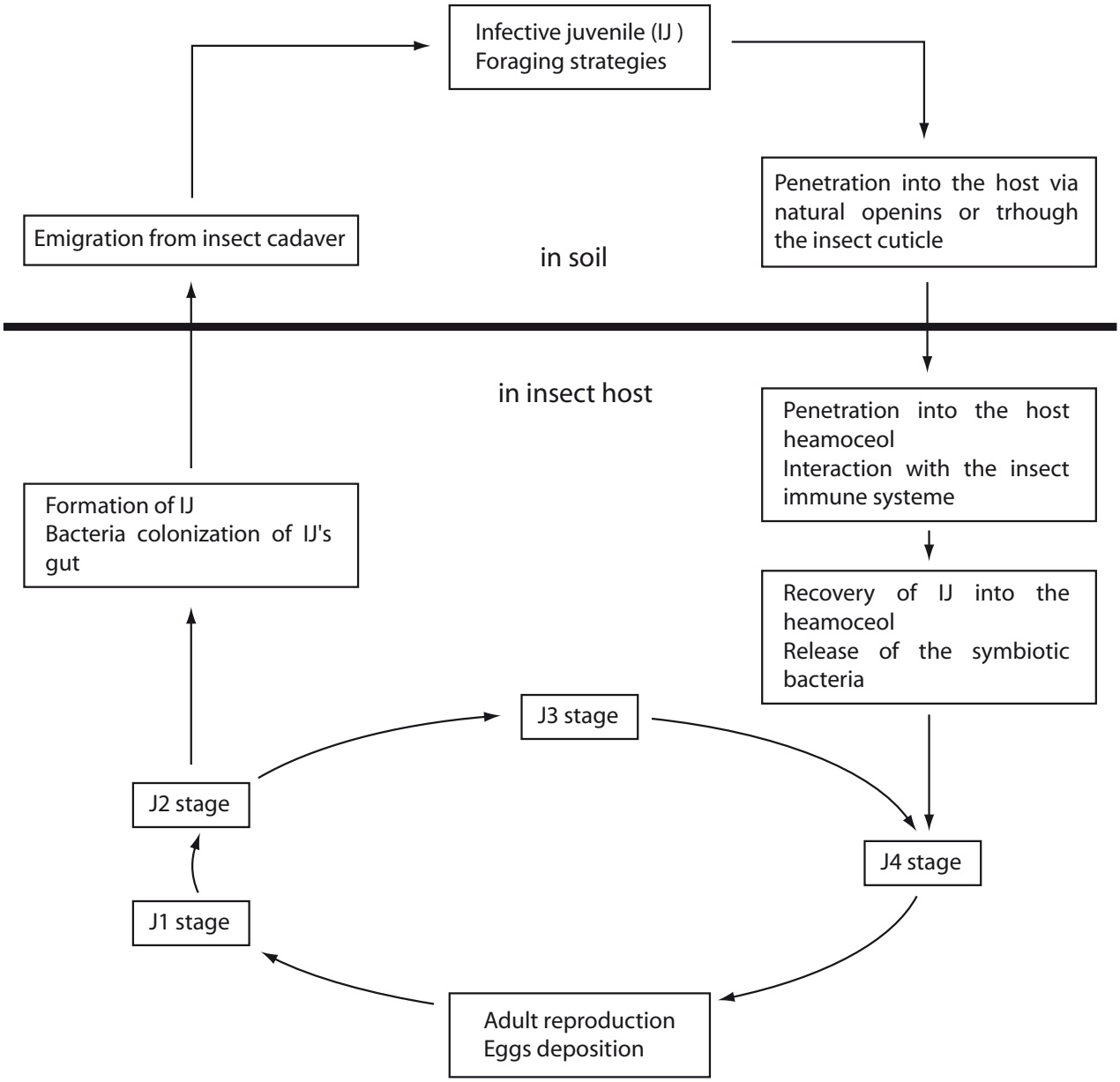

Figure 6 Life cycle scheme of EPN with both free living IJ stage and stages needing a host to develop. J1-4 = 1-4 juvenile stage. IJ = infective juvenile.

haemocoel EPN have to face the immune system of the host. The immune reaction is mainly characterized by encapsulation and phagocytosis (Trenczek 1998). Nematodes may resist encapsulation in insects by either avoidance of being recognized (evasion, 1), by tolerating the encapsulation response (tolerance, 2) or by actively suppressing the immune response (suppression, 3). (1) Evasion has been reported by Dunphy and Webster (1987), who discovered that a lack of non-self recognition prevents *Steinernema carpocapsae* from being encapsulated by the wax moth *Galleria mellonela*. The authors concluded that

lipid components on the nematode surface are responsible for protection against recognition. IJs may innately secrete these surface components or acquire them from the host during penetration. In *Heterorhadbitis* ssp., the second juvenile stage cuticle plays an important role in evasion. Peters and Ehlers (1994) suggested that *Heterorhadbitis* ssp. avoid non-self recognition by slipping off the second juvenile stage cuticle just before entering the insect haemocoel. (2) Tolerance depends on the number of EPN invading a host. The number of nematodes present in the haemocoel may overwhelm the immune response (Dowds and Peters 2002). (3) Immune suppression is resulting from coating proteins. Some of these surface proteins have been shown to reduce the number of haemocyte and their ability to phagocytose or do decrease the melanization. , As bacteria can overcome the insect immune system, this EPN resistance strategy mainly depends on the duration of the establishment of these symbiotic cells (Dowds and Peters 2002).

Once EPNs are established in their host they release their symbiotic bacteria. The immune system of the insect is then reacting mainly by phagocytizing these cells. But as bacteria have a high reproductive rate, they can generally quickly kill the insect (Forst and Clarke 2002). *Xenorhabdus* and *Photorhabdus* are the two bacterial genera mutualistically associated with infective juveniles of *Steinernema* and *Heterorhabditis* respectively (Boemare 2002). While bacteria are killing the insect by releasing toxins, they are exuding many other compounds as enzymes and antibiotics (Webster, Chen *et al.* 2002). Enzymes can help to break down the host tissues and thus make them available for nematode feeding and reproduction (Boemare, Ehlers *et al.* 1996). The production of antibiotics contributes to prevent the colonisation of the insect cadaver by bacterial and fungal micro-organisms, thus keeping the suitability of the nematode habitat allowing them to go through their life cycle (Ehlers 1996). Some of the symbiotic bacteria are bioluminescent (*P. luminsecens*) (Poinar, Thomas *et al.* 1980), which may help protect the nematode habitat as many soil dwelling organisms show negative phototrophic behaviour (Ehlers 1996).

In addition to feeding on degraded host tissues the nematodes also eat bacterium cells in order to get enough energy to mature and mate. Two reproductive strategies are observed (fig. 7). Heterorhabditis juveniles

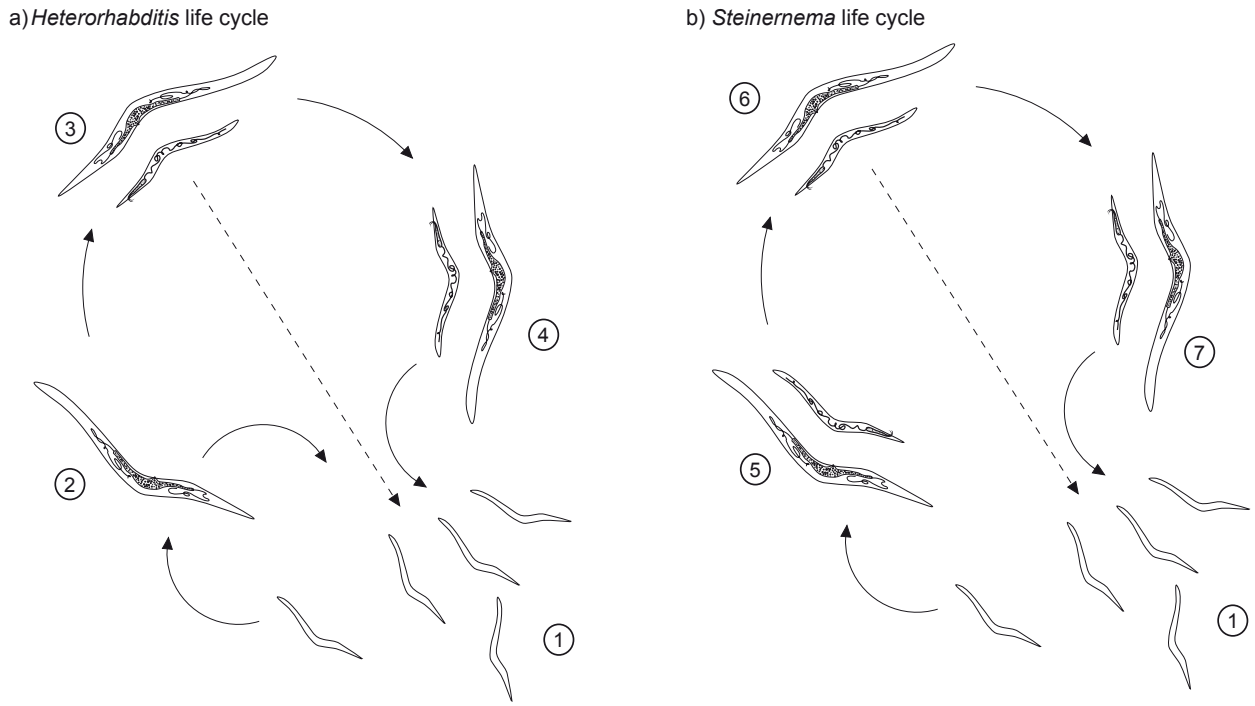

Figure 7 The reproductive cycles of a) Heterorhabditis and b) Steinernema entomopathogenic nematodes. 1. Infective juveniles; 2. first generation of hermaphrodite females; 3. second generation females (can either be amphimictic or hermaphrodite) and rare males; 4. third generation hermaphrodite females and rare males; 5. first generation amphimictic females and males; 6. second generation amphimictic females and males; 7. third generation amphimictic females and males. Dotted arrows imply some recruitment from an adult stage to the infective juvenile stage. Modified after Burnel (2002)

mature to hermaphrodite females (Burnell 2002). This first generation of Heterorhabditis females gives rise to a second generation of amphimictic males and females and also to some hermaphrodite females and IJs (Dix, Burnell et al. 1992; Strauch, Stoessel et al. 1994; Wang and Bedding 1996). By contrast, a Steinernematid juvenile usually matures to either a male or an amphimictic female (Burnell 2002). However, Griffin, Callaghan et al. (2001) have recently identified a hermaphrodite strain of Steinernema. At the end of the reproduction cycles, colonization of the new IJs by bacteria occurs. Colonization requires specific interactions and chemical signalling between nematodes and bacteria. The exact nature of the relationship between these partners remains poorly understood (Ehlers 1996; Forst and Clarke 2002). Once the host has been fully exploited, IJs have to leave the insect cadaver and search for new hosts. Recently, it has been demonstrated that the emergence of Steinernema feltiae is triggered by the presence of ammonia, a side product

of nematode defection (San-Blas, Gowen et al. 2008).

Foraging strategies are divided into two broad categories: cruise (widely foraging) and ambush (sit-and-wait) (Pianka 1966; Schoener 1971; Eckhardt 1979; Huey and Pianka 1981; McLaughlin 1989; Gaugler, Lewis *et al.* 1997) (fig. 8). Cruise foragers allocate more of their foraging time to scanning for resources-associated cues when moving through their environment (Lewis, Campbell *et al.* 2006). Ambush foragers scan during long pauses in a nictation posture (Gaugler, Lewis *et al.* 1997; Lewis, Campbell *et al.* 2006). These differences are significant because the duration of scanning pauses influences the types of resources that

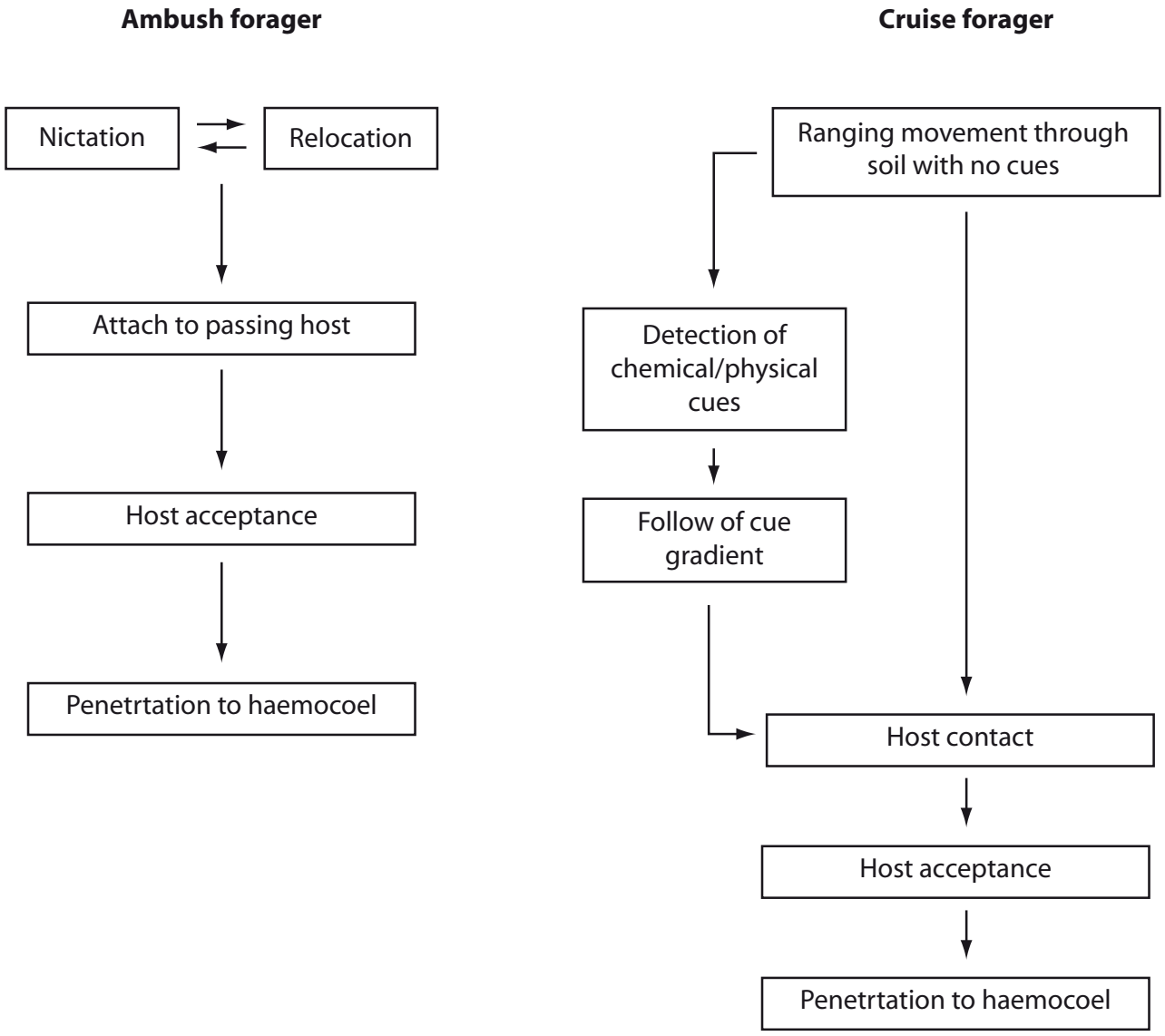

Figure 8 The order of events that occur during a bout of host finding for ambushing and cruising nematodes. Modifided after Lewis (2001).

the EPN is likely to encounter (Lewis, Campbell *et al.* 2006), and thus the type of pest the IJs can target. Cruise foragers have a higher probability of finding sedentary and cryptic hosts than ambushers, whereas ambush foragers are expected to be more effective against highly mobile insects (Campbell and Gaugler 1993). Extreme ambush and cruise foraging represent endpoints of a continuum of foraging strategies, rather than a clear dichotomy (Lewis, Campbell *et al.* 2006). Two of the three EPN species used in the current study (*H. megidis* and *H. bacteriophora*) are considered to be cruisers, whereas the third one (*S. feltiae*) exhibits a less clear foraging behaviour. Cruise foragers range through the environment and are attracted to, or arrested by, cues that may indicate the location of a potential host. During foraging search, cruisers typically respond to volatile cues, or cues dissolved in the water film, emanating from the host or its immediate environment (Lewis, Campbell *et al.* 2006). For instance, cues emanating from plant roots, a potential habitat for hosts, can influence EPN behaviour (Bird and Bird 1986; Choo, Kaya *et al.* 1989; Lei, Rutherford *et al.* 1992; Boff, Zoon *et al.* 2001; van Tol, van der Sommen *et al.* 2001; Neveu, Grandgirard *et al.* 2002). Chemical signalling from root damaged by insect feeding provide more specific information about the presence of potential hosts and these chemical cues have been shown to increase attraction and infection of pests by *H. megidis* (Rasmann, Köllner *et al.* 2005; Rasmann and Turlings 2008; Hiltpold, Baroni *et al.* in prep.; Hiltpold, Toepfer *et al.* submitted; Degenhardt, Hiltpold *et al.* in prep., Chap. 1-2 & 4). Cruiser EPN also respond to specific cues emitted by the hosts themselves (Gaugler and Campbell 1991; Lewis, Gaugler *et al.* 1993; Grewal, Lewis *et al.* 1994). Recently, response to chemical signalling was extended to many *Steinernema* ssp. that are effective at finding sedentary hosts (Campbell, Lewis *et al.* 2003). Once a nematode has reached the insect, it must change its behaviour to evaluate the suitability of this potential host and to accept or not the insect as host. Host recognition and acceptance is also mainly mediated by chemical stimuli or by the physical structure of the insect tegument sensed by the IJ (for review Lewis, Campbell *et al.* 2006). Apart from various organic compounds, nematodes use other suitable signals such as temperature, electric potential and carbon dioxide or various inorganic compounds (Jansson and Nordbringhertz 1979) and vibrations (Torr, Heritage *et al.* 2004).

As mentioned, chemical-mediated attraction of EPN involves chemicals from the host, such as faeces volatiles (Grewal, Gaugler *et al.* 1993; Lewis, Gaugler *et al.* 1993) or other specific host kairomones (Gaugler, Lebeck *et al.* 1980), and chemicals coming from plants, such as CO_2 (Gaugler, Lebeck *et al.* 1980) or root and leaf substances (Bilgrami, Kondo *et al.* 2001). Until recently, no specific plant attractant for EPN attraction towards insect hosts were known (Kaya 1990; Boff, Zoon *et al.* 2001). It was common knowledge that nematode orient to the unspecific signal carbon dioxide (Gaugler, Lebeck *et al.* 1980). However, it is unlikely that such a common compound can be unequivocally exploited by nematodes foraging for specific hosts. It was hypothesised by van Tol, van der Sommen *et al.* (2001) that plants can produce inducible compounds attractive for cruiser nematodes. This hypothesis was confirmed when *H. megidis* was found to be attracted towards weevil-infected strawberry roots (Boff, Zoon *et al.* 2001). Based of these results, Rasmann, Köllner *et al.* (2005) conducted a study in which they identified a belowground signalling compound emitted by maize roots damaged by WCR, the sesquiterpene (*E*)-β-caryophyllene.

The central theme of this thesis focuses on this specific belowground signal and the understanding of the tritrophic interaction between maize, the WCR and EPN. Such knowledge seems essential to fully exploit the biological control potential of EPN against belowground pests.

Thesis outline

The present study addresses the following questions:

Why (E)-β-caryophyllene is such a good belowground signal? (Chapter I)

The release by the roots of only one dominant compound in response to herbivory (Rasmann, Köllner *et al.* 2005; Rasmann and Turlings 2008) contrasts strongly with insect-damaged maize leaves, which release a complex blend of green leaf volatiles, aromatic compounds, and various terpenoids (Degen, Dillmann *et al.* 2004). This difference between roots and leaves VOC emission can be the consequence of the chemical properties of EβC, which might make it particularly suitable for belowground diffusion. In Chapter I, the diffusion of EβC was tested and compared with those of other typical maize volatiles that a root could potentially emit when attacked. In addition, the soil moisture impact on EβC diffusion was assed.

Is manipulation of trophic levels feasible in the context of belowground tritrophic systems exploitation in a biological control strategy? (Chapters II & V)

Plants emit blends of VOC in response to being damaged by herbivorous insects (Paré and Tumlinson 1999). These emissions have been proposed to serve a variety of physiological and ecological functions (Dudareva, Negre *et al.* 2006), including an indirect defence function through the attraction of natural enemies of the herbivores (Turlings, Tumlinson *et al.* 1990; De Moraes, Lewis *et al.* 1998; Dicke and Sabelis 1998; Hilker, Kobs *et al.* 2002). It has been confirmed that inducible volatiles attract natural enemies under field conditions (Bernasconi, Turlings *et al.* 1998; De Moraes, Lewis *et al.* 1998; Thaler 1999) and thus can help plants to reduce the damage inflicted by the herbivores (Kessler and Baldwin 2001). A specific enhancement of volatile signals in crop plants has been suggested to increase the effectiveness of predators and parasitoids as biological control agents (Bottrell and Barbosa 1998; Degenhardt, Gershenzon *et al.* 2003; Aharoni, Jongsma *et al.* 2005; Pickett, Bruce *et al.* 2006; Turlings and Ton 2006). Laboratory studies with genetically modified volatile emissions in model plants have provided the first evidence that this approach has potential (Kappers, Aharoni *et al.* 2005; Beale, Birkett *et al.* 2006; Schnee, Köllner *et al.* 2006; Cheng, Xiang *et al.* 2007). In Chapter II, the manipulation of the first trophic level, i.e. the maize plant, was

tested in the laboratory and in the field. The aim of this study was to evaluate the restorability of the EβC emission of a non-producing maize variety. Chapter V aimed to test the manipulation of the third trophic level, i.e. the nematodes as natural enemies, by selecting EPN for an improved responsiveness to EβC. Tests were conducted in the laboratory as in the field.

Is the virulence of EPN affected by the different WCR development stages? (Chapter III)

From egg hatching to the adult stage, WCR go through three different larval stages and one pupae instar. This implicates many differences in morphological structures and behaviour. Several studies have shown an effect of the host developmental stage on the efficacy of EPN (Peters and Ehlers 1994; Chyzik, Glazer *et al.* 1996; Ebssa, Borgemeister *et al.* 2001), however such information was not completely available for WCR. This study aimed to compare the susceptibility of each larval instar and the pupae of WCR to three promising and commercially available EPN strains (Toepfer, Gueldenzoph *et al.* 2005). Standard bioassays involving EPN applications in sand (Peters 2005) or soil-filled trays containing WCR larvae and pupae were used to assess EPN-induced mortality of the different pest life stages. The use of semi-natural conditions i.e. soil-filled trays, allowed determination of optimal EPN application timing relative to WCR phenology. This information will be critical for the development of an effective nematode-based biological control product.

How different species of nematodes react to different belowground signal blends in the field? (Chapter IV)

Whilst EPN respond to chemical signals, not all maize plants emit the same volatile blends. Commercial maize varieties show large intraspecific variation in both the qualitative and quantitative release of VOCs (Gouinguené, Degen *et al.* 2001; Degen, Dillmann *et al.* 2004) and these differences have been shown to be important for the recruitment of EPNs in the laboratory and the field (Rasmann, Köllner *et al.* 2005 and Chapter 1). It is imperative to assess the influence of these differences on the biological control abilities of EPN in the field. Therefore, the aim of the Chapter 4 was to investigate the influence of the VOC blends of two commercial maize varieties (one with EβC emission, the second without) on the efficacy of three promising EPN species (*H. bacteriophora*, *H. megidis* and *S. feltiae*) against WCR larvae under field conditions (Toepfer, Gueldenzoph *et al.* 2005). Moreover, the timing of EPN application was examined in order to link it to the control efficacy of WCR.

References

Abu Hatab, M. A., S. Selvan*, et al.* (1995). “Role of proteases in penetration of insect gut by the entomopathogenic nematode *Steinernema glaseri* (Nematoda, Steinernematidae).” Journal of Invertebrate Pathology **66**(2): 125-130.

Adams, B. J., A. Fodor*, et al.* (2006). “Biodiversity and systematics of nematode-bacterium entomopathogens.” Biological Control **37**(1): 32-49.

Agrawal, A. A. and M. T. Rutter (1998). “Dynamic anti-herbivore defense in ant-plants: the role of induced responses.” Oikos **83**(2): 227-236.

Agrawal, A. A., S. Tuzun*, et al.* (1999). Induced plant defenses against pathogens and herbivores biochemistry, ecology, and agriculture. Saint Paul, MN, APS.

Aharoni, A., M. A. Jongsma*, et al.* (2005). «Volatile science? Metabolic engineering of terpenoids in plants.» Trends in Plant Science **10**(12): 594-602.

Ahmad, A., G. E. Wilde*, et al.* (2005). “Delectability of coleopteran-specific Cry3Bb1 protein in soil and its effect on nontarget surface and below-ground arthropods.” Environmental Entomology **34**(2): 385-394.

Ahmad, A., G. E. Wilde*, et al.* (2006). “Evaluation of effects of coleopteran-specific Cry3Bb1 protein on earthworms exposed to soil containing corn roots or biomass.” Environmental Entomology **35**(4): 976-985.

Al-Deeb, M. A. and G. E. Wilde (2003). “Effect of Bt corn expressing the Cry3Bb1 toxin for corn rootworm control on aboveground nontarget arthropods.” Environmental Entomology **32**(5): 1164-1170.

Al-Deeb, M. A., G. E. Wilde*, et al.* (2003). “Effect of Bt corn for corn rootworm control on nontarget soil microarthropods and nematodes.” Environmental Entomology **32**(4): 859-865.

Baca, F. (1994). “New member of the harmful entomofauna of Yugoslavia *Diabrotica virgifera virgifera* LeConte (Coleoptera, Chrysomelidae).” Zastita Bilja **45**: 125-131.

Baldwin, I. T. (1994). Chemical changes rapidly induced by folivory. Insect-Plant Interactions. A. Bernays, Boca Raton. **5:** 1-23.

Baldwin, I. T. and C. A. Preston (1999). “The eco-physiological complexity of plant responses to insect herbivores.” Planta **208**: 137-145.

Barbercheck, M. E. (1993). "Tritrophic level effects on entomopathogenic nematodes." Environmental Entomology **22**(5): 1166-1171.

Barbercheck, M. E. and J. Wang (1995). "Adaptation of entomopathogenic nematodes to the southern corn rootworm and its hosts plants." Ann Mtg Soc Invertebr Pathol **28**: 4-5.

Bathon, H. (1996). "Impact of entomopathogenic nematodes on non-target hosts." Biocontrol Science and Technology **6**(3): 421-434.

Baum, J. A., C. R. Chu*, et al.* (2004). "Binary toxins from *Bacillus thuringiensis* active against the western corn rootworm, *Diabrotica virgifera virgifera* LeConte." Applied and Environmental Microbiology **70**(8): 4889-4898.

Beale, M. H., M. A. Birkett*, et al.* (2006). "Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior." Proceedings of the National Academy of Sciences of the United States of America **103**(27): 10509-10513.

Bedding, R. A. and A. S. Molyneux (1982). "Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp (Heterorhabditidae, Nematoda)." Nematologica **28**(3): 354-359.

Ben-Yakir, D., D. Efron*, et al.* (1998). «Evaluation of entomopathogenic nematodes for biocontrol of the European corn borer, *Ostrinia nubilalis*, on sweet corn in Israel." Phytoparasitica **26**(2): 101-108.

Bernasconi, M. L., T. C. J. Turlings*, et al.* (1998). "Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis*." Entomologia Experimentalis et Applicata **87**(2): 133-142.

Bernklau, E. J. and L. B. Bjostad (2008). «Identification of feeding stimulants in corn roots for western corn rootworm (Coleoptera : Chrysomelidae) larvae.» Journal of Economic Entomology **101**(2): 341-351.

Bernklau, E. J., E. A. Fromm*, et al.* (2004). "Disruption of host location of western corn rootworm larvae (Coleoptera : Chrysomelidae) with carbon dioxide." Journal of Economic Entomology **97**(2): 330-339.

Bilgrami, A. L., E. Kondo*, et al.* (2001). "Attraction response of *Steinernema glaseri* in presence of plant roots and leaves." Annals of Plant Protection Science **9**: 258-253.

Bird, A. F. and J. Bird (1986). "Observations on the use of insect parasitic nematodes as a means of biological control of root-knot nematodes." International Journal for Parasitology **16**(5): 511-

516.

Bjostad, L. B. and B. E. Hibbard (1992). "6-Methoxy-2-benzoxazolinone - a semiochemical for host location by western corn rootworm larvae." Journal of Chemical Ecology **18**(7): 931-944.

Boemare, N. (2002). "Interactions between the partners of the entomopathogenic bacterium nematode complexes, *Steinernema-Xenorhabdus* and *Heterorhabditis-Photorhabdus*." Nematology **4**: 601-603.

Boemare, N., R.-U. Ehlers*, et al.* (1996). Symbiosys and pathogenicity of the nematode-bacterium complexes. Luxembourg, Brussels, European Commission, Directorate-General XII, Science, Research and Development, Environment Research Programme.

Boetel, M. A., B. W. Fuller*, et al.* (1998). "Fecundity and egg viability of northern and western corn rootworm (Coleoptera: Chrysomelidae) beetles surviving labelled and reduced soil insecticide applications." Journal of Economic Entomology **91**(1): 274-279.

Boetel, M. A., B. W. Fuller*, et al.* (2003). "Emergence of adult northern and western corn rootworms (Coleoptera: Chrysomelidae) following reduced soil insecticide applications." Journal of Economic Entomology **96**(3): 714-729.

Boff, M. I. C., R. van Tol*, et al.* (2002). "Behavioural response of *Heterorhabditis megidis* towards plant roots and insect larvae." Biocontrol **47**(1): 67-83.

Boff, M. I. C., F. C. Zoon*, et al.* (2001). "Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer." Entomologia Experimentalis et Applicata **98**(3): 329-337.

Bottrell, D. G. and P. Barbosa (1998). "Manipulating natural enemies by plant variety selection and modification: a realistic strategy?" Annual Review of Entomology **43**: 347-367.

Branson, T. F. and J. L. Krysan (1981). "Feeding and oviposition behavior and life-cycle strategies of *Diabrotica*: an evolutionary view with implications for pest-management." Environmental Entomology **10**(6): 826-831.

Bruce, T. J. and J. A. Pickett (2007). "Plant defence signalling induced by biotic attacks." Current Opinion in Plant Biology **10**(4): 387-392.

Burnell, A. (2002). Genetics and genetic improvement. Entomopathogenic Nematology. R. Gaugler, CABI International**:** 241-263.

Campbell, J. F. and R. Gaugler (1993). Nictation behavior and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae): 155-169.

Campbell, J. F., E. E. Lewis, *et al.* (2003). «Evolution of host search strategies in entomopathogenic nematodes.» Journal of Nematology **35**(2): 142-145.

Cheng, A. X., C. Y. Xiang, *et al.* (2007). "The rice (E)-beta-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes." Phytochemistry **68**(12): 1632-1641.

Chiang, H. C. (1970). "Effects of manure application and mite predation on corn rootworm applications in Minnesota." Journal of Economic Entomology **63**: 934 - 936.

Choo, H., D. Lee, *et al.* (1996). "Noneffective results of Steinernematid and Heterorhabditid nematodes against pill bug, *Armadillidium vulgare* (Isopoda : Armadillidae)." Korean Journal of Applied Entomology **35**(1): 91-93.

Choo, H. Y., H. K. Kaya, *et al.* (1989). "Entomopathogenic nematodes - Host-finding ability in the presence of plant-roots." Environmental Entomology **18**(6): 1136-1140.

Choo, H. Y., A. M. Koppenhofer, *et al.* (1996). «Combination of two entomopathogenic nematode species for suppression of an insect pest.» Journal of economic entomology **89**(1): 97-103.

Chyzik, R., O. Glazer, *et al.* (1996). "Virulence and efficacy of different entomopathogenic nematode species against western flower thrips (*Frankliniella occidentalis*)." Phytoparasitica **24**(2): 103-110.

De Moraes, C. M., W. J. Lewis, *et al.* (1998). «Herbivore-infested plants selectively attract parasitoids.» Nature **393**(6685): 570-573.

Degen, T., C. Dillmann, *et al.* (2004). "High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines." Plant Physiology **135**(4): 1928-1938.

Degenhardt, J., J. Gershenzon, *et al.* (2003). "Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies." Current Opinion In Biotechnology **14**(2): 169-176.

Degenhardt, J., I. Hiltpold, *et al.* (in prep.). "Restoring the communication between maize roots and entomopathogenic nematodes helps to control a major pest insect."

Dicke, M. and M. W. Sabelis (1998). "How plants obtain predatory mites as bodyguards." Netherlands

Journal of Zoology **38**(2-4): 148-165.

Dicke, M. and J. J. A. van Loon (2000). "Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context." Entomologia Experimentalis et Applicata **97**: 237-249.

Dicke, M., R. M. P. van Poecke*, et al.* (2003). "Inducible indirect defence of plants: from mechanisms to ecological functions." Basic And Applied Ecology **4**(1): 27-42.

Dicke, M. and L. E. M. Vet (1999). Plant-carnivore interactions: evolutionary and ecological consequences for plat, herbivore and carnivore. Herbivores: between plants and predators. H. Olff, V. K. Brown and R. H. Drent. Oxford, Blackwell Science**:** 483-520.

Dix, I., A. M. Burnell*, et al.* (1992). "The identification of biological species in the genus *Heterorhabditis* (Nematoda, Heterorhabditidae) by cross-breeding 2nd-generation amphimicticadults." Parasitology **104**: 509-518.

Dowds, B. C. A. and A. Peters (2002). Virulence Mechanisms. Entomopathogenic Nematology. R. Gaugler, CABI International**:** 79-98.

Dudareva, N., F. Negre*, et al.* (2006). "Plant volatiles: Recent advances and future perspectives." Critical Reviews in Plant Sciences **25**(5): 417-440.

Dunphy, G. B. and J. M. Webster (1987). "Partially characterized components of the epicuticle of dauer juvenile *Steinernema feltiae* and their influence on hemocyte activity in *Galleria mellonella*." Journal of Parasitology **73**(3): 584-588.

Eben, A. and M. E. Barbercheck (1997). "Host plant and substrate effects on mortality of southern corn rootworm from entomopathogenic nematodes." Biological Control **8**(2): 89-96.

Ebssa, L., C. Borgemeister*, et al.* (2001). "Impact of entomopathogenic nematodes on different soil-dwelling stages of western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), in the laboratory and under semi-field conditions." Biocontrol Science and Technology **11**(4): 515-525.

Eckhardt, R. C. (1979). "The adaptive syndromes of two guilds of insectivorous birds in the Colorado Rocky Mountains." Ecological Monographs **49**(2): 129-149.

Ehler, L. E. (1990). Some contemporary issues in biological control of insects and their relevance to the use of entomopathogenic nematodes. Entomopathogenic nematodes in biological control. R. Gaugler and H. K. Kaya. Boca Raton, CRC.

Ehlers, R. U. (1996). Symbiotic relation within the nematode-bacterium complex. Symbiosis and pathogenicity of the nematode-bacterium complexes. N. Boemare, R. U. Ehlers, A. Fodor and A. Szentirmai, European Commision**:** 45-51.

Ehlers, R. U. (2001). "Mass production of entomopathogenic nematodes for plant protection." Appl Microbiol Biotechnol **56**: 623–633.

Elliot, S. L., M. W. Sabelis*, et al.* (2000). "Can plants use entomopathogens as bodyguards?" Ecology Letters **3**(3): 228-235.

Ellis, R. T., B. A. Stockhoff*, et al.* (2002). "Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte." Applied and Environmental Microbiology **68**(3): 1137-1145.

Ellsbury, M. M., J. J. Jackson*, et al.* (1996). "Efficacy, application distribution, and concentration by stemflow of *Steinernema carpocapsae* (Rhabditia: Steinernematide) suspensions applied with a lateral-move irrigation system for corn rootworm (Coleoptera: Chrysomelidae) control in maize." Journal of Economic Entomology **85**(6): 2425 - 2432.

EPPO (2004). Report of the 8th Meeting of the EPPO ad hoc Panel on *Diabrotica virgifera virgifera* held jointly with the 10th International IWGO Workshop on *Diabrotica virgifera virgifera*. Engelberg (CH), European and Mediterrian Plant Protection Organization**:** 1-8.

Fedoroff, N. V. (2003). "Prehistoric GM corn." Science **302**(5648): 1158-1159.

Fife, J. P., H. E. Ozkan*, et al.* (2005). "Viability of abiological pest control agent through hydraulic nozzles." Transactions of the Asae **48**(1): 45-54.

Forschler, B. T. and W. A. Gardner (1991). "Parasitism of *Phyllophaga hirticula* (Coleoptera, Scarabaeidae) by *Heterorhabditis heliothidis* and *Steinernema carpocapsae*." Journal of Invertebrate Pathology **58**(3): 396-407.

Forst, S. and D. Clarke (2002). Bacteria-nematode symbiosis. Entomopathogenic Nematology. R. Gaugler, CABI International**:** 57-77.

Fukunaga, K., J. Hill*, et al.* (2005). "Genetic diversity and population structure of teosinte." Genetics **169**(4): 2241-2254.

Gaugler, R. and J. F. Campbell (1991). "Selection for enhanced host-finding of scarab larvae (Coleoptera, Scarabaeidae) in an entomopathogenic nematode." Environmental Entomology **20**(2): 700-706.

Gaugler, R., L. Lebeck*, et al.* (1980). "Orientation of the entomogenous nematode *Neoaplectana carpocapsae* to carbon-dioxide." Environmental Entomology **9**(5): 649-652.

Gaugler, R., E. Lewis*, et al.* (1997). "Ecology in the service of biological control: The case of entomopathogenic nematodes." Oecologia **109**: 483-489.

Gaugler, R. and D. Molloy (1981). "Instar susceptibility of *Simulium vittatum* (Diptera, Simuliidae) to the entomogenous nematode *Neoaplectana carpocapsae*." Journal of Nematology **13**(1): 1-5.

Georgis, R. and R. Gaugler (1991). "Predictability in biological-control using entomopathogenic nematodes." Journal of Economic Entomology **84**(3): 713-720.

Gouinguené, S., T. Degen*, et al.* (2001). "Variability in herbivore-induced odour emission among maize cultivars and their wild ancestors (teosinte)." Chemoecology **11**: 9-16.

Governatori, G., C. Frausin*, et al.* (2002). "*Diabrotica virgifera virgifera* LeConte in Friuli: biology, distribution and control measures to contain its spread in the region." Notiziario ERSA **15**(6): 30-35.

Gray, M. E., G. L. Hein*, et al.* (1992). "Western and northern corn rootworm (Coleoptera, Chrysomelidae) egg densities at 3 soil depths - Implications for future ecological studies." Journal of the Kansas Entomological Society **65**(3): 354-356.Grewal, P. S., E. A. B. De Nardo*, et al.* (2001). "Entomopathogenic nematodes: potential for exploration and use in South America." Neotropical Entomology **30**(2): 191-205.

Grewal, P. S., R. Gaugler*, et al.* (1993). "Host recognition by entomopathogenic nematodes - Behavioral response to contact with host feces." Journal of Chemical Ecology **19**(6): 1219-1231.

Grewal, P. S., S. K. Grewal*, et al.* (2002). "Differences in susceptibility of introduced and native white grub species to entomopathogenic nematodes from various geographic localities." Biological Control **24**(3): 230-237.

Grewal, P. S., E. E. Lewis*, et al.* (1994). "Host finding behavior as a predictor of foraging strategy in entomopathogenic nematodes." Parasitology **108**: 207-215.

Griffin, C. T., K. M. Callaghan*, et al.* (2001). "A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution amongst entomopathogenic nematodes?" Parasitology **122**: 181-186.

Head, J., K. F. A. Walters*, et al.* (2000). "The compatibility of the entomopathogenic nematode, *Steinernema feltiae*, and chemical insecticides for the control of the South American leafminer, *Liriomyza huidobrensis*." Biocontrol **45**(3): 345-353.

Heil, M. (2008). "Indirect defence via tritrophic interactions." New Phytologist **178**(1): 41-61.

Hibbard, B. E., E. J. Bernklau*, et al.* (1994). "Long-chain free fatty-acids - Semiochemicals for host location by western corn rooworm Irvae." Journal of Chemical Ecology **20**(12): 3335-3344.

Hibbard, B. E., T. T. Vaughn*, et al.* (2005). "Effect of Cry3Bbl-expressing transgenic corn on plant-to-plant movement by western corn rootworm larvae (Coleoptera : Chrysomelidae)." Journal of Economic Entomology **98**(4): 1126-1138.

Hilker, M., C. Kobs*, et al.* (2002). "Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids." Journal Of Experimental Biology **205**(4): 455-461.

Hillel, D. and C. Rosenzweig (2005). The role of biodiversity in agronomy. Advances in Agronomy, Vol 88. San Diego, Elsevier Academic Press Inc. **88:** 1-34.

Hiltpold, I., M. Baroni*, et al.* (in prep.). "Selection for enhanced responsiveness of entomopathogenic nematodes to a volatile root signal can help to control the western corn rootworm."

Hiltpold, I., S. Toepfer*, et al.* (in prep.). "How maize root volatiles influence the efficacy of entomopathogenic nematodes against the western corn rootworm."

Huey, R. B. and E. R. Pianka (1981). "Ecological consequences of foraging mode." Ecology **62**(4): 991-999.

Jackson, J. J. (1996). "Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae)." Journal of Economic Entomology **89**(2): 366-372.

Jackson, J. J. and M. A. Brooks (1995). "Parasitism of western corn rootworm larvae and pupae by *Steinernema carpocapsae*." Journal of Nematology **27**(1): 15-20.

Jackson, J. J. and N. C. Elliot (1988). "Temperature dependent development of immature stages of western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)." Environmental Entomology **17**: 166-171.

Jansson, H. B. and B. Nordbringhertz (1979). "Attraction of nematodes to living mycelium of nematophagous fungi." Journal of General Microbiology **112**(MAY): 89-93.

Janzen, D. H. (1966). "Coevolution of mutualism between ants and acacias in Central America." Evolution **20**(3): 249-&.

Journey, A. M. and K. R. Ostlie (2000). "Biological control of the western corn rootworm (Coleoptera: Chrysomelidae) using the entomopathogenic nematodes, *Steinernema carpocapsae*." Environmental Entomology **29**(4): 822-831.

Kappers, I. F., A. Aharoni*, et al.* (2005). "Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis." Science **309**(5743): 2070-2072.

Karban, R., A. A. Agrawal*, et al.* (1997). "The benefits of induced defenses against herbivores." Ecology **78**(5): 1351-1355.

Karban, R. and I. Baldwin (1997). Induced responses to herbivory. Chicago, University Press of Chicago.

Kaya, H. K. (1990). Soil Ecology. Entomopathogenic nematodes in biological control. Boca Raton, FL, CRC Press**:** 93-115.

Kaya, H. K. and R. Gaugler (1993). "Entomopathogenic nematodes." Annual Review of Entomology **38**: 181-206.

Kessler, A. and I. T. Baldwin (2001). "Defensive function of herbivore-induced plant volatile emissions in nature." Science **291**(5511): 2141-2144.

Kirk, V. M. (1981). "Corn Rootworm: Population reduction associated with the ant, *Lasius neoniger*." Environmental Entomology **10**: 966-967.

Kiss, J., J. Komáromi*, et al.* (2005). Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and the crop rotation system in Europe. Western Corn Rootworm: Ecology and Management. S. Vidal, U. Kuhlmann and C. R. Edwards. Wallingford, Oxon, CABI Publishing**:** 320.

Krueger, S. R. and D. W. Roberts (1997). "Soil treatment with entomopathogenic fungi for Corn Rootworm (*Diabrotica* spp.) larval control." Biological Contronl **9**(1): 67-74.

Krysan, J. L. (1999). Selected topics in the biology of *Diabrotica*. Advances in Chrysomelidae Biology. M. L. Cox. Leiden, The Netherlands, Backhuys Publisher. **1:** 479-513.

Krysan, J. L. and T. A. Miller (1986). Methods for study of pest *Diabrotica*. New-York, Springer-Verlag.

Kuhlmann, U. and W. A. C. M. van der Brugt (1998). «Possibilities for biological control of the western

corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe." Biocontrol **19**: 59N-68N.

Lacey, L. A., R. Frutos*, et al.* (2001). "Insect pathogens as biological control agents: do they have a future?" Biological Control **221**: 230-248.

Laczynski, A., A. De Moor*, et al.* (2006). "The effect of hydraulic agitation on the viability of the nematode *Heterorhabditis bacteriophora*." Crop Protection **25**(10): 1135-1141.

Larsen, C. S. (2003). "Animal source foods and human health during evolution." Journal of Nutrition **133**(11): 3893S-3897S.

Lei, Z., T. A. Rutherford*, et al.* (1992). "Heterorhabditid behavior in the presence of the cabbage maggot, *Delia radicum*, and its host plants." Journal of Nematology **24**(1): 9-15.

Levine, E. and H. Oloumi-Sadeghi (1991). "Management of Diabroticite rootworms in corn." Annual Review of Entomology **36**: 229-255.

Levine, E. and S. H. Oloumi (1996). "Western corn rootworm (Coleoptera: Chrysomelidae) larval injury to corn grown for seed production following soybeans grown for seed production." Journal of economic entomology **89**(4): 1010-1016.

Levine, E., S. H. Oloumi*, et al.* (1992). "Discovery of multiyear diapause in Illinois and South Dakota northern corn rootworm (Coleoptera: Chrysomelidae) eggs and incidence of the prolonged diapause trait in Illinois." Journal of economic entomology **85**(1): 262-267.

Lewis, E. E. (2002). Behavioural Ecology. Entomopathogenic Nematology. R. Gaugler, CABI International**:** 205-223.

Lewis, E. E., J. Campbell*, et al.* (2006). «Behavioral ecology of entomopathogenic nematodes.» Biological Control **38**(1): 66-79.

Lewis, E. E., R. Gaugler*, et al.* (1992). "Entomopathogenic nematode host finding - response to host contact cues by cruise and ambush foragers." Parasitology **105**: 309-315.

Lewis, E. E., R. Gaugler*, et al.* (1993). "Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues." Canadian Journal of Zoology-Revue Canadienne De Zoologie **71**(4): 765-769.

McCoy, C. W., R. J. Stuart*, et al.* (2002). «Field efficacy of two commercial preparations of entomopathogenic nematodes against larvae of *Diaprepes abbreviatus* (Coleoptera:

Curculionidae) in ALFISOL type soil." Florida Entomologist **85**: 537-544.

McLaughlin, R. L. (1989). "Search modes of birds and lizards - Evidence for alternative movement patterns." American Naturalist **133**(5): 654-670.

Miller, N., A. Estoup*, et al.* (2005). "Multiple transatlantic introductions of the western corn rootworm." Science **310**(5750): 992-992.

Moellenbeck, D. J., M. L. Peters*, et al.* (2001). "Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms." Nature Biotechnology **19**(7): 668-672.

Mortimer, S. R., W. H. van der Putten*, et al.* (1999). Insect and nematode herbivory below ground: interactions and role in vegetation succession. Herbivores: between plants and predators. H. Olff, V. K. Brown and R. H. Drent. Oxford, Blackwell Science Ltd.**:** 205-238.

Neveu, N., J. Grandgirard*, et al.* (2002). "Systemic release of herbivore-induced plant volatiles by turnips infested by concealed root-feeding larvae *Delia radicum* L." Journal of Chemical Ecology **28**(9): 1717-1732.

Nishimatsu, T. and J. J. Jackson (1998). "Interaction of insecticides, entomopathogenic nematodes, and larvae of the Western Corn Rootworm (Coleoptera: Chrysomelidae)." Journal of Economic Entomology **91**(2): 410-418.

Nowatzki, T. M., X. Zhou*, et al.* (2006). "Effect of *Bacillus thuringiensis* Cry3Bb1 protein on the feeding behavior and longevity of adult western corn rootworms (Coleoptera : Chrysomelidae)." Journal of Economic Entomology **99**(3): 927-930.

O'Neil, M. E., C. D. DiFonzo*, et al.* (2002). "Western corn rootworm (Coleptera: Chrysomelidae) feeding on corn and soybean leaves affected by corn phenology." Environmental Entomology **31**(2): 285-292.

Paré, P. W. and J. H. Tumlinson (1999). "Plant volatiles as a defense against insect herbivores." Plant Physiology **121**: 325-331.

Peters, A. (2005). Insect based bioassays. Quality control of entomopathogenic nematodes. J. M. Grunder, European Commission Publication COST819, Brussels**:** 55-67.

Peters, A. and R. U. Ehlers (1994). "Susceptibility of leatherjackets (*Tipula paludosa* and *Tipula oleracea*, Tipulidae, Nematocera) to the entomopathogenic nematode *Steinernema feltiae*." Journal of Invertebrate Pathology **63**(2): 163-171.

Pianka, E. R. (1966). “Convexity desert lizards and spatial heterogeneity.” Ecology **47**(6): 1055-&.

Pickett, J. A., T. J. A. Bruce*, et al.* (2006). Plant volatiles yielding new ways to exploit plant defence. Chemical ecology: from gene to ecosystem. M. Dicke and W. Takken, Springer.

Pilz, C., R. Wegensteiner*, et al.* (2008). «Natural occurrence of insect pathogenic fungi and insect parasitic nematodes in *Diabrotica virgifera virgifera* populations.” Biocontrol **53**(2): 353-359.

Pimentel, D. (1997). Techniques for reducing pesticide use: economic and environmental benefits, John Wiley and Sons, New York.

Piperno, D. R. and K. V. Flannery (2001). “The earliest archaeological maize (*Zea mays* L.) from highland Mexico: New accelerator mass spectrometry dates and their implications.” PNAS **98**(4): 2101-2103.

Poinar, G. O. (1990). Taxonomy and biology of Steinernematidae and Heterorhabditidae. Entomopathogenic nematodes in biological control. R. Gaugler and H. K. Kaya. Boca Raton, CRC.

Poinar, G. O., G. Thomas*, et al.* (1980). “Growth and luminescence of the symbiotic bacteria associated with the terrestrial nematode, *Heterorhabditis bacteriophora*.” Soil Biology & Biochemistry **12**(1): 5-10.

Price, P. W., C. E. Bouton*, et al.* (1980). “Interactions among 3 trophic levels - influence of plants on interactions between insect herbivores and natural enemies.” Annual Review of Ecology and Systematics **11**: 41-65.

Puza, V. and Z. Mracek (2005). “Seasonal dynamics of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* as a response to abiotic factors and abundance of insect hosts.” Journal of Invertebrate Pathology **89**(2): 116-122.

Rasmann, S., T. G. Köllner*, et al.* (2005). “Recruitment of entomopathogenic nematodes by insect-damaged maize roots.” Nature **434**(7034): 732-737.

Rasmann, S. and T. C. J. Turlings (2008). “First insights into specificity of below ground tritrophic interactions.” Oikos **117**: 362-369.

Root, R. B. (1973). “Organization of a plant-arthropod association in simple and diverse habitats - fauna of collards (Brassica-Oleracea).” Ecological Monographs **43**(1): 95-120.

San-Blas, E., S. R. Gowen*, et al.* (2008). “Steinernema feltiae: Ammonia triggers the emergence of

their infective juveniles." Experimental Parasitology **119**(1): 180-185.

Sattaur, O. (1989). «The shrinking gene pool.» New Scientist **123**(1675): 37-41.

Schnee, C., T. G. Köllner*, et al.* (2006). «The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores.» Proceedings of the National Academy of Sciences USA **103**(4): 1129-1134.

Schoener, T. W. (1971). "Theory of feeding strategies." Annual Review of Ecology and Systematics **2**: 369-404.

Schoonhoven, L. M., T. Jermy*, et al.* (1998). Insect-plant biology, from physiology to evolution. Lodon, Chapman & Hall.

Sivcev, I., B. Manojlovic*, et al.* (1994). "Distribution and harmful effect of *Diabrotica virgifera* LeConte (Coleoptera, Chrysomelidae), a new maize pest in Yugoslavia." Zastita Bilja **45**: 19-26.

Sluyter, A. and G. Dominguez (2006). "Early maize (*Zea mays* L.) cultivation in Mexico: Dating sedimentary pollen records and its implications." PNAS **103**(4): 1147-1151.

Stapley, L. (1998). "The interaction of thorns and symbiotic ants as an effective defence mechanism of swollen-thorn acacias." Oecologia **115**(3): 401-405.

Stoewen, J. F. and C. R. Ellis (1991). "Evaluation of a technique for monitoring predation of western corn rootworm eggs, *Diabrotica virgifera virgifera* (Coleoptera, Chrysomelidae)." Proceedings of the Entomological Society of Ontario **122**: 27-33.

Strauch, O., S. Stoessel*, et al.* (1994). "Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*." Fundamental and Applied Nematology **17**(6): 575-582.

Strnad, S. P. and M. K. Bergman (1987). "Movement of 1st-instar western corn rootworms (Coleoptera, Chrysomelidae) in soil." Environmental Entomology **16**(4): 975-978.

Sundermeier, A. and B. Eisley (2001). Corn rootworm insecticide comparison. Special Circular - Ohio Agricultural Research and Development Center.

Thaler, J. S. (1999). "Jasmonate-inducible plant defences cause increased parasitism of herbivores." Nature **399**(6737): 686-688.

Thompson, J. N. and B. M. Cunningham (2002). "Geographic structure and dynamics of coevolutionary selection." Nature **417**(6890): 735-738.

Thurston, G. S. and W. N. Yule (1990). "Control of larval northern corn rootworm (*Diabrotica barberi*)

with two steinernematid nematode species." Journal of nematology **22**(1): 127-131.

Toepfer, S., C. Gueldenzoph*, et al.* (2005). "Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe." Bulletin of Entomological Research **95**(1-10).

Toepfer, S. and U. Kuhlmann (2005). Natural mortality factors acting on western corn rootworm populations: a comparison between the United States and Central Europa. Western corn rootworm Ecology and managment. S. Vidal, U. Kuhlmann and C. R. Edwards, Cabi Publishing.

Toepfer, S., F. Zhang*, et al.* (2001). Biological control of corn rootworm, *Diabrotica virgifera virgifera*. Delémont (CH), CABI Bioscience Switzerland Centre.

Tollefson, J. J. (1988). A pest insect adapts to the cultural control of crop rotation. Brighton Crop Protection Conference. Pests and Diseases.

Tonhasca, A. and D. N. Byrne (1994). "The effects of crop diversification on herbivorous insects - a meta-analysis approach." Ecological Entomology **19**(3): 239-244.

Torr, P., S. Heritage*, et al.* (2004). "Vibrations as a novel signal for host location by parasitic nematodes." International Journal For Parasitology **34**(9): 997-999.

Trenczek, T. (1998). "Endogenous defense mechanisms of insects." Zoology-Analysis of Complex Systems **101**(4): 298-315.

Turlings, T. C. J. and B. Benerey (1998). "Effects of plant metabolites on the behavior and development of parasitic wasps." Ecoscience **5**(3): 321-333.

Turlings, T. C. J. and J. Ton (2006). "Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests." Current Opinion in Plant Biology **9**(4): 421-427.

Turlings, T. C. J., J. H. Tumlinson*, et al.* (1990). "Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps." Science **250**(4985): 1251-1253.

Turlings, T. C. J. and F. Wäckers (2004). Recruitment of predators and parasitoids by herbivore injured-plants. Advances in Insect Chemical Ecology. C. U. Press.

van Tol, R. W. H. M., A. T. C. van der Sommen*, et al.* (2001). "Plants protect their roots by alerting the enemies of grubs." Ecology Letters **4**(4): 292-294.

Vaughn, T., T. Cavato*, et al.* (2005). "A method of controlling corn rootworm feeding using a *Bacillus thuringiensis* protein expressed in transgenic maize." Crop Science **45**(3): 931-938.

Viana, P. A. and E. A. I. Marochi (2002). “Chemical control of *Diabrotica* spp. larvae damaging maize under no-tillage system.” Revista Brasileira de Milho e Sorgo **1**(2): 1-11.

Walsh, G. C. (2003). “Host range and reproductive traits of *Diabrotica speciosa* (Gemar) and *Diabrotica viridula* (F.) (Coleoptera: Chrysomelidae), two species of South America pest Rootworms, with notes on other species of Diabroticina.” Environmental Entomology **32**(2): 276-285.

Wang, J. X. and R. A. Bedding (1996). “Population development of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in the larvae of *Galleria mellonella*.” Fundamental and Applied Nematology **19**(4): 363-367.

Wang, Y., J. F. Campbell*, et al.* (1995). “Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera, Scarabaeidae) larvae.” Journal of Invertebrate Pathology **66**(2): 178-184.

Webster, J. M., G. Chen*, et al.* (2002). Bacterial metabolites. Entomopathogenic Nematology. R. Gaugler, CABI International**:** 99-114.

Zhang, F., S. Toepfer*, et al.* (2004). “Reproductive biology of *Celatoria compressa* (Diptera: Tachinidae), parasitoid of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae).” Biocontrol Science And Technology **14**(1): 5-16.

CHAPTER I

Belowground chemical signalling in maize: when simplicity rhymes with efficiency

Journal of Chemical Ecology 34:628-635

Ivan Hiltpold and T. C. J. Turlings

2008

J Chem Ecol (2008) 34:628–635
DOI 10.1007/s10886-008-9467-6

Belowground Chemical Signaling in Maize: When Simplicity Rhymes with Efficiency

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Received: 14 December 2007 / Revised: 13 March 2008 / Accepted: 18 March 2008 / Published online: 29 April 2008

Abstract Maize roots respond to feeding by larvae of the beetle *Diabrotica virgifera virgifera* by releasing (*E*)-β-caryophyllene. This sesquiterpene, which is not found in healthy maize roots, attracts the entomopathogenic nematode *Heterorhabditis megidis*. In sharp contrast to the emission of virtually only this single compound by damaged roots, maize leaves emit a blend of numerous volatile organic compounds in response to herbivory. To try to explain this difference between roots and leaves, we studied the diffusion properties of various maize volatiles in sand and soil. The best diffusing compounds were found to be terpenes. Only one other sesquiterpene known for maize, α-copaene, diffused better than (*E*)-β-caryophyllene, but biosynthesis of the former is far more costly for the plant than the latter. The diffusion of (*E*)-β-caryophyllene occurs through the gaseous rather than the aqueous phase, as it was found to diffuse faster and further at low moisture level. However, a water layer is needed to prevent complete loss through vertical diffusion, as was found for totally dry sand. Hence, it appears that maize has adapted to emit a readily diffusing and cost-effective belowground signal from its insect-damaged roots.

Keywords Belowground tritrophic interactions · (*E*)-β-caryophyllene · Roots · Diffusion · Entomopathogenic nematodes · Indirect plant defense · Plant–insect interactions

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Introduction

Plants defend themselves against herbivores either directly with the use of toxins, repellents, or morphological structures (Karban et al. 1997; Karban and Baldwin 1997; Schoonhoven et al. 1998; Agrawal and Rutter 1998; Baldwin and Preston 1999; Dicke et al. 2003), or indirectly by attracting the enemies of herbivores (Dicke and Sabelis 1988; Agrawal 1998; Dicke and Hilker 2003; Turlings and Wäcker 2004). The role of volatiles in the attraction of such natural enemies was first brought to light in studies on the interactions between arthropods and plant leaves. Results revealed that herbivore attack induces emissions of volatile organic compounds (VOC) attractive to parasitoids and predators (Dicke and Sabelis 1988, Turlings et al. 1995; De Moraes et al. 1998), and this role of induced volatiles in tritrophic interactions has also been demonstrated under realistic field conditions (Bernasconi et al. 1998; De Moraes et al. 1998; Thaler 1999; Kessler and Baldwin 2001). While the aboveground portion of a plant is obviously essential for photosynthesis and reproduction, the root system is also of vital importance. Little is known about possible indirect defense mechanisms against root feeders, but several studies now have demonstrated that roots also are able to recruit enemies of herbivores by releasing chemical cues into soil. These chemicals can attract entomopathogenic nematodes (Boff et al. 2001; van Tol et al. 2001; Bertin et al. 2003), predatory mites (Aratchige et al. 2004), and even parasitoids (Neveu et al. 2002). In *Zea mais* L., feeding by larvae of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), results in the release of a few sesquiterpenoids, dominated by (*E*)-β-caryophyllene (EβC), which is attractive to the entomopathogenic nema-

tode (EPN) *Heterorhabditis megidis* Poinar (Rhabditida: Heterorhabditidae) (Rasmann et al. 2005).

Western corn rootworm is the most destructive pest of maize in the United States and its introduction in Europe (Miller et al. 2005) also has generated concern among maize growers in the Old World (Vidal et al. 2005). Several strategies have been used worldwide to control WCR populations, of which crop rotation has thus far been the most effective (Levine et al. 1992). However, certain US populations have developed an extended diapause (Tollefson 1988; Levine et al. 1992) or the ability to develop on soybean roots (O'Neil et al. 2002), and other control measures are being considered. Some potential biological control agents have been identified (Toepfer and Kuhlmann 2004). Field results with such agents have been variable (Jackson 1996; Journey and Ostlie 2000; McCoy et al. 2002), but EPNs are the most promising (Gaugler et al. 1997; Kuhlmann and Burgt 1998). EPNs are obligate parasites that kill insect hosts with the aid of mutualistic bacteria (Forst and Nealson 1996; Burnell and Stock 2000). The effectiveness of *H. megidis*, a promising nematodes against WCR, is strongly correlated with the emission of EβC (Rasmann et al. 2005). A good understanding of the mechanism by which EβC attracts EPNs could help to improve their efficacy.

The release by the roots of only one dominant compound in response to herbivory contrasts strongly with insect-damaged maize leaves, which release a complex blend of green leaf volatiles, aromatic compounds, and various terpenoids (Degen et al. 2004). We hypothesized that this difference between roots and leaves is due to the chemical properties of EβC, which might make it particularly suitable for belowground diffusion. To test this, we compared the diffusion of EβC with those of other typical maize volatiles that the roots could potentially emit.

Materials and Methods

Experiments were carried out in clean sand that had been passed through a 2mm sieve and autoclaved to obtain a homogeneous, air-dried, and VOC-free medium. Deionized water was added in precise quantities to obtain specific humidity levels. Humidity was 10% in all initial experiments, and was obtained by adding 50ml deionized water to 450g of sand in a Teflon-box (12 × 10 × 4cm; 480cm^3, internal dimensions), maintaining constant porosity at about 90%. The box was put on a thermal tray, maintaining the temperature at 12°C. A 0.2mm diameter cylinder made of ultra-fine metal mesh (2300 mesh; Small Parts Inc., USA) was inserted into the sand, thereby creating a hole in which a solid-phase microextraction (SPME) fiber could be safely inserted. Automated sampling was performed with a 100μm polydimethylsiloxane SPME fiber (Supelco, Buchs, Switzerland) within 12h with a multipurpose sampler (MPS2, Gerstel GmbH & Co. KG, Germany) (Koziel et al. 2000; Gorecki and Namiesnik 2002; Vas and Vekey 2004). At 30min intervals, the adsorbed compounds were analyzed by retracting the fiber from the sand and inserting it for 3min in the injector of an Agilent 6890 Series gas chromatograph heated at 230°C (G1530A) coupled to a quadrupole-type mass-selective detector (Agilent 5973;

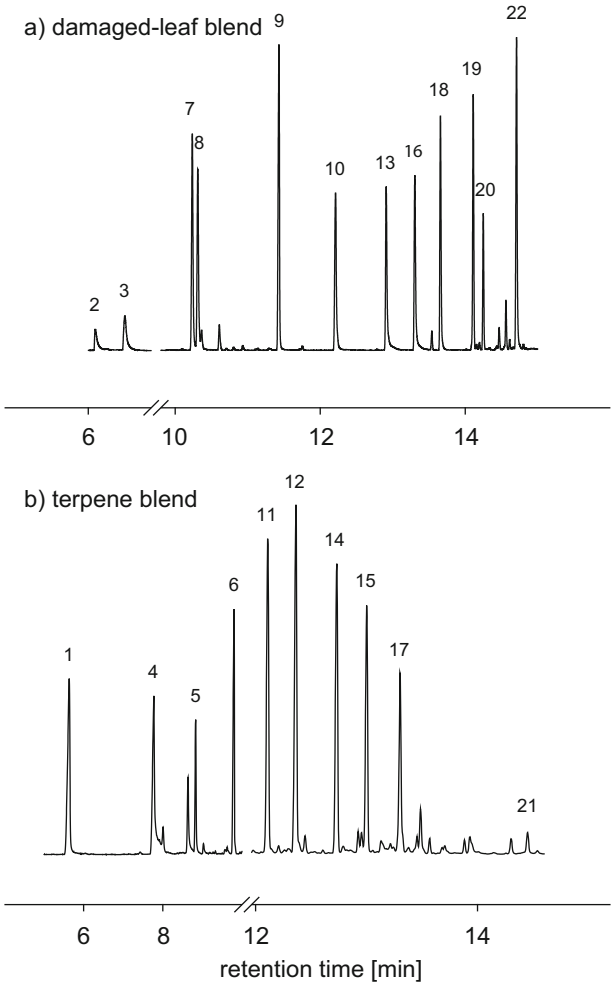

Fig. 1 Representative GC-MS chromatograms obtained by sampling just above the two synthetic blends that were used for the diffusion experiments. **a** analysis after collection with a 100μm polydimethylsiloxane SPME fiber of a synthetic mix of typical leaf volatiles. **b** analysis after collection with a 75μm corboxen™-polydimethylsiloxane SPME fiber of a blend of selected terpenoids. Labeled peaks are as follow: (*1*) (+)-α-pinene, (*2*) (E)-2-hexenal, (*3*) (Z)-3-hexenol, (*4*) β-myrcene, (*5*) cis-ocimene, (*6*) and (*9*) linalool, (*7*) β-myrcene, (*8*) (Z)-3-hexenyl acetate, (*10*) methyl salicylate, (*11*) (−)-α-cubebene, (*12*) (−)-α-copaene, (*13*) indole, (*14*) and (*19*) EβC, (*15*) α-humulene, (*16*) methyl anthranilate, (*17*) valencene, (*18*) geranyl acetate, (*20*) (E)-β-farnesene, (*21*) caryophyllene oxide and (*22*) (+/−)-trans-nerolidol

transfer line 230°C, source 230°C, ionization potential 70eV). The desorbed volatiles were separated on a polar column (HP1-MS, 30m, 0.25mm ID, 0.25μm film; Agilent Technologies, USA) using helium as a carrier gas (constant pressure of 127.9kPa). Following injection, the column temperature was maintained at 40°C for 1min and then increased 20°C min^{-1} to 250°C, where it was held for another 12min. After the first 30min sampling period, a synthetic mixture of typical caterpillar-induced leaf volatiles (0.2μg for each compound) (Turlings and Ton 2006) dissolved in 5μl of pentane (>99%; Acros Organic) (Fig. 1a) was injected 3cm into the sand. The injection site was located 0.5cm from the edge of the tray, opposite the fiber insertion site. Measurements with the fiber were done at four distances (1.5, 3, 6, and 10cm) from the injection point, and were replicated 5 times for each distance.

The results of the first experiment prompted an additional similar diffusion test with a blend that mainly comprised terpenoids (Fig. 1b). The set-up was the same as described above except that we sampled only at 10cm from the source and a 75μm Corboxen™-polydimethylsiloxane SPME fiber was used, because of its higher affinity for terpenes.

Diffusion also was assessed in a standard soil (type 5M, LUFA Speyer, Germany) that was first autoclaved. Experiments were conducted with the two synthetic blends described above (Fig. 1) following the same experimental set-ups as for the sand experiments, but sampling was only done at 10cm of the release point.

The effect of moisture level on EβC diffusion was determined by measuring horizontal diffusion of a synthetic version injected at 0.2μg/5μl pentane. Porosity of sand was maintained constant by adding a fixed mass of medium (500g) into the Teflon box. The ratio between sand and water was adjusted to obtain moisture levels of 0%, 1%, 5%, or 10% water.

All experiments were replicated five times. Chromatograms were analyzed with ChemStation (version D.00.00.38, Agilent Technologies), and peak areas of VOCs were compared with analysis of variance (ANOVA) and *t*-test. Differences among compounds and humidity levels were determined by using a Bonferroni post hoc test. All analyses were run on SigmaStat (version 2.03, Access Softek Inc.).

Results

Diffusion of Typical Maize Leaf Volatiles At concentrations of 0.2μg/5μl, the amount and number of VOCs adsorbed onto the SPME fiber decreased drastically with sampling distance (Fig. 2). At 10cm, only three terpenes EβC, β-myrcene, and (*E*)-β-farnesene were detected (Fig. 3). Of these, EβC diffused best (one-way ANOVA, $N = 5$, $F_{2,14} = 6.95$, $P = 0.01$) and was detected longer after the injection.

Diffusion of Terpenoids in Sand All terpenoids, with the exception of caryophyllene oxide diffused readily through sand and were detected 10cm from the source, but the amounts detected were considerably different for the different compounds. Data shown in Fig. 4 include only the four terpenes that are emitted by WCR-damaged maize roots (Rasmann et al. 2005). Of these, β-copaene diffused best, followed by EβC, which diffused equally well as the structurally similar α-humulene (one-way ANOVA, $N = 5$, $F_{2,14} = 21.57$, $P < 0.001$). As we did not detect any diffusion of caryophyllene oxide, we omitted this compound from the statistical analyses.

Diffusion of Terpenes in Soil Differences in how the terpenes diffused in soil were similar to those in sand, but the amount of each chemical recovered at 10cm from the source was considerably lower (Fig. 4). Again, β-copaene

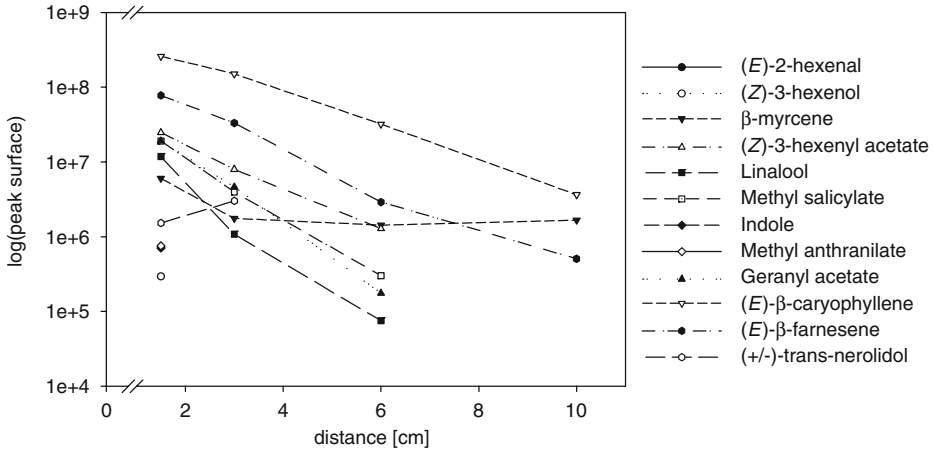

Fig. 2 Diffusion of a synthetic blend along 10cm in a sand medium. VOCs were sampled 1.5, 3, 6, and 10cm from the odor source. Only terpenes were diffused as far as 10cm. All the other compounds were not detected at distances of more than 6cm. Trans-nerolidol, methyl anthranilate and indole were detected only 1.5cm from the source. Detection directly at the release point was not possible because of fiber saturation

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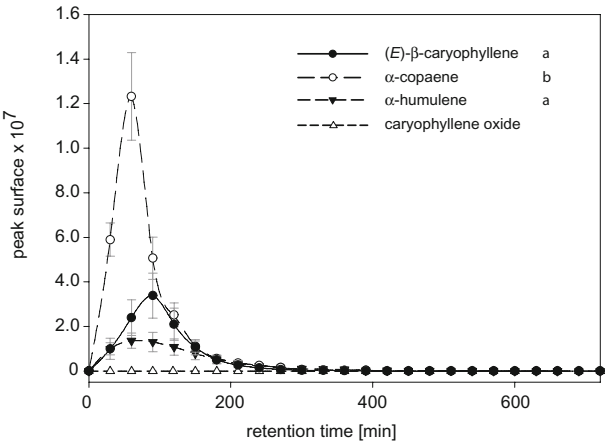

Fig. 3 Diffusion of an authentic terpenoids blend measured at 10 cm from the source. GC-MS peak surfaces of EβC, α-copaene and α-humulene reached their maximum at 72, 30, and 128 min (mean time) after injection, respectively. Maximum GC-MS peak surfaces differed significantly (one-way ANOVA, N=5, $F_{2,14}$=6.95, P=0.01). Letters next to the compound names indicate significant differences between compounds (Bonferroni posthoc test)

diffused better than EβC (t-test, N = 5, P = 0.039). α-Humulene and caryophyllene oxide were not detected in soil.

Moisture Level and EβC Diffusion The amount of water present in the sand strongly affected EβC diffusion. An almost twofold larger amount of EβC was detected at a humidity level of 1% than at 10% humidity. However, a water layer is needed to avoid loss by vertical diffusion: no EβC was detected when the sand was completely free of water (Fig. 5, one-way ANOVA, N = 5, $F_{2,14}$ = 21.47, P < 0.001). As we did not detect EβC in dry sand, results for this treatment were omitted from the statistical analyses.

Discussion

The results support our hypothesis that EβC is particularly well-suited as a belowground signal because of its diffusion properties. The only other maize-produced compound that diffused better than EβC was α-copaene. All other compounds were consistently detected at lower amounts away from the release point or not detected at all. The limited detection of these other compounds could be explained by early evaporation (vertical diffusion), which is likely to be the case for compounds with low molecular weight (Lowell and Eklund 2004) such as (*E*)-2-hexanal and (*Z*)-3-hexanol. Besides vertical diffusion, adsorption onto colloids (polar particles) within the media could slow down or stop both vertical and horizontal diffusion. Although adsorption is expected to be relatively low in sand (Ruiz et al. 1998), silicates on the surface of sand particles, and also the aqueous phase of the medium, could adsorb many of the compounds that were tested. This was evident from a study in which a blend of induced maize volatiles was pushed through a silica-filter, and several of the volatiles that did not diffuse in the current study were found trapped on the filter (D'Alessandro and Turlings 2005). As adsorption does not alter chemical properties, adsorption of VOCs onto static silicates could enhance the establishment of a chemical gradient over a longer period of time (McGechan and Lewis 2002), thus allowing EPNs to follow a chemical trail towards potential hosts.

Terpenes were the best diffusing compounds, possibly explaining why WCR-damaged maize roots exude mainly terpenes, even though maize is able to synthesize many other VOCs (Figs. 2 and 4). EβC clearly diffuses better than α-humulene, the other sesquiterpene released by maize roots, but we detected up to two times more β-copaene in

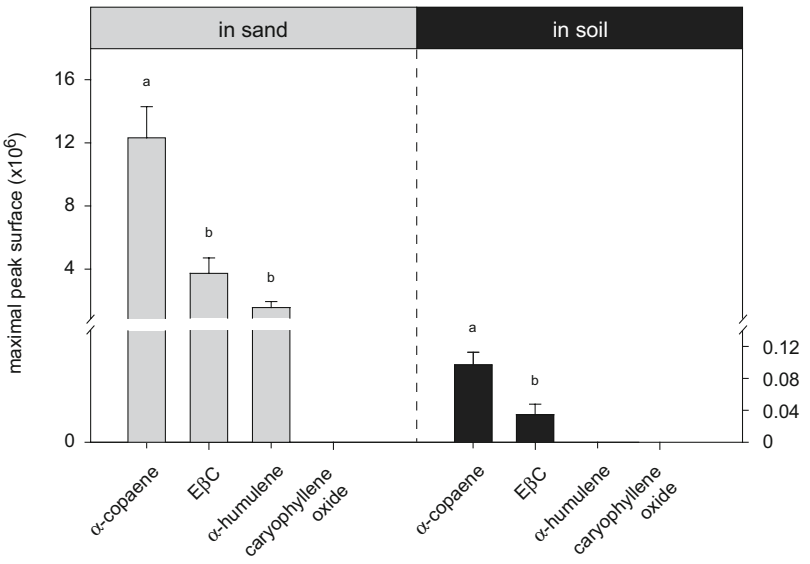

Fig. 4 Diffusion of terpenoids in sand and in soil. VOCs were sampled 10 cm from the odor source. In sand, α-copaene diffused significantly better than the other compounds (one-way ANOVA, N=5, $F_{2,14}$=21.47, P<0.001). The same pattern of diffusion was observed in soil (t-test, N=5, P=0.039). Undetected compounds were excluded from the statistic

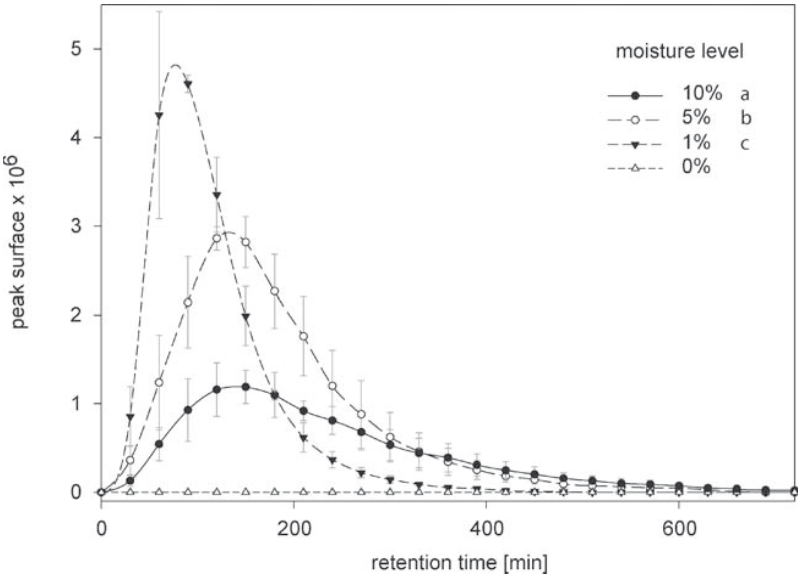

Fig. 5 Diffusion of EβC under different moisture levels measured at 10 cm from the source. Peak surfaces of EβC with 1%, 5%, and 10% of water in sand reached their maximum at 80, 140, and 160 min after injection, respectively. Diffusion velocity tended to increase with lower moisture levels, but there was no statistical difference among the three moisture levels (data not shown) (one-way ANOVA, N=5, $F_{2,14}$=2.17, P=0.19). Maximum GC-MS peak surfaces differed significantly (one-way ANOVA, N=5, $F_{2,14}$=21.47, P<0.001). *Letters* indicate significant differences between compounds (Bonferroni post hoc test). As no diffusion was detected in dry sand (0%), it was excluded from the statistical analyses

sand 10cm from the source than EβC, and this difference was similar in soil (Fig. 4, in soil). That β-copaene diffuses better than EβC (Fig. 4) may reflect their respective adsorption strengths onto the substrate. The most likely explanation why roots emit EβC rather than β-copaene as a belowground signal comes from what is known about their respective biosyntheses. While there is much speculation and some disagreement about the evolution and function of VOCs (Firn and Jones 2000; Peñuelas and Llusia 2004; Owen and Penuelas 2005, 2006; Firn and Jones 2006), researchers agree on at least one principle: production of such herbivore-induced plant volatiles can require considerable resource investment. It has been reported that some plants may allocate up to 10% of their carbon for the production of VOCs (Firn and Jones 2006). Considering this potential cost, plants can be expected to have adapted the energetically cheapest solutions. The precursor for the maize sesquiterpenes is the same, farnesyl diphosphate (FPP). When WCR larvae feed on roots, this triggers a cascade of reactions that lead to the production of two different enzymes. The first reacts with FPP to catalyze the production of EβC and α-humulene, and the second reacts with the same precursor, but forms β-copaene and approximately 50 other compounds (T. Köllner, personal communication), (Fig. 6). Because of the production of these additional compounds, a plant produces nine times more EβC than β-copaene with the same number of FPP molecules. Hence, even if EβC diffusion is half as

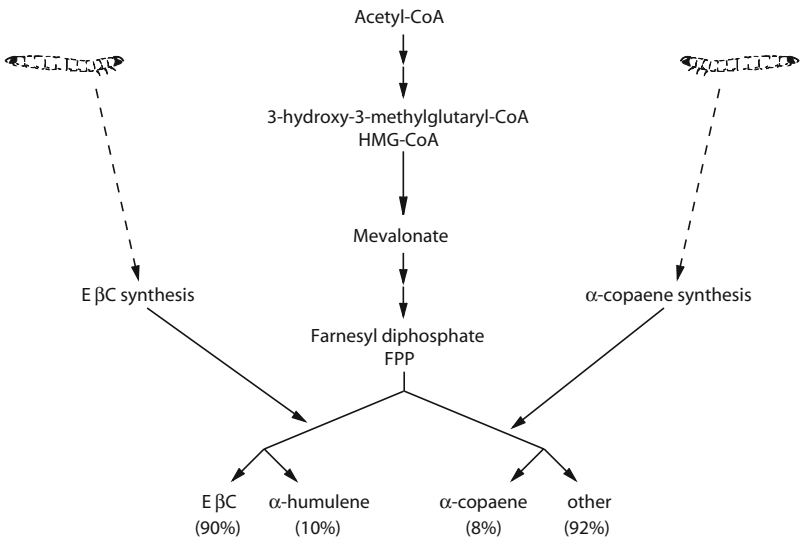

Fig. 6 Schematic model of the metabolic pathway for EβC synthesis. When WCR larvae feed on roots this triggers a cascade of reactions resulting in the production of two different enzymes that lead to two separate pathways of volatile synthesis. The ratio in which the volatiles are produced from these pathways implies a much more cost-effective production of EβC than of β-copaene (T. Köllner, personal communication)

efficient as β-copaene, the fact that it is far less expensive for the plant to produce should counterbalance this difference.

The moisture level of the substrate strongly affected EβC diffusion (Fig. 5). The detected abundance and the velocity of horizontal diffusion were negatively correlated with the water volume present in the sand. Porosity was maintained constant. Therefore, the addition of water reduced the gaseous phase volume. Thus, the results confirm the notion that the diffusion of EβC occurs in the gaseous phase, which was expected because of its poor solubility in water. Low moisture levels enhance horizontal diffusion of EβC, but a complete lack of water dramatically reduces it. This is best explained by a need for a thin layer of water to avoid quick vertical evaporation of the sesquiterpene.

EβC is a common compound and has been identified from various plant species (e.g., Rodriguez-Saona et al. 2001; Tholl et al. 2005; Calyecac-Cortero et al. 2007; Cheng et al. 2007; Helmig et al. 2007). Its function, as for most plant volatiles, remains unclear. Sesquiterpenes in general may have anti-microbial or insecticidal effects (e.g., Erasto et al. 2006; Liu et al. 2006; Sabulal et al. 2006; Ji et al. 2007), but at the dose emitted by maize roots it is unlikely to be effective against insects (Rasmann et al. 2005). Interestingly, EβC is also emitted from maize silk and has been implicated in the attraction of adult *Diabrotica* beetles (Hammack 2001), but recent behavioral assays suggest that other plant compounds are considerably more important for adult attraction (Tòth et al. 2007). Belowground sesquiterpenes (strigolactone) also have been found to play a role in the symbiosis between roots and arbuscular mycorrhiza as fungi branching factor (Akiyama et al. 2005) and in germination of *Striga*, *Alectra*, and *Orobanche* species (Butler 1995). Hence, it is important to stress that multiple functions and modes of selection for EβC or other sesquiterpenes must be considered. The recently identified sequiterpene-synthase gene TPS23, which is responsible for EβC production in maize (Köllner et al. 2008), was found to be highly conserved, implying an important function for the plant. There is little information about herbivore-induced root volatiles from other plants. A comparison among the emissions from maize, cotton, and cowpea roots in response to feeding by *Diabrotica balteata* larvae has revealed the typical release of EβC by maize roots, a more complex mixture of terpenoids emitted from cotton roots, including α-copaene, but no detectable amounts of EβC, whereas no volatiles were detected from attacked cowpea roots (Rasmann and Turlings 2008). Nematodes are most attracted to damaged maize roots and far less to damaged cowpea roots (Rasmann and Turlings 2008), which is in accordance with an important role for EβC in the attraction.

This study set out to explain why the induced emission of volatiles in maize roots is basically limited to just one dominant sesquiterpene, EβC. The hypothesized explanation that this compound would be particularly suited as a belowground signal because of its diffusion properties was confirmed. The only maize-produced compound that appeared to be even better at diffusing in the substrates tested was β-copaene, another sesquiterpene. A plausible reason why the plant has evolved to emit EβC is that β-copaene is more costly to produce. Given the logic behind these explanations, we should perhaps now turn the question around and wonder why the leaves emit such complex blends if they could be so much simpler.

Acknowledgments We thank all the members of the E-vol lab at the University of Neuchâtel for their support, in particular Matthias Held, Russell E. Naisbit, and Sarah Kenyon. We also thank Jean-Michel Gobat for advice on the experimental design, Violaine Jourdie for stimulating discussions and Marie-Eve Wyniger for assistance with the chemical analyses. This project was funded by the Swiss Confederation's innovation promotion agency (CTI project no. 7487.1 LSPP-LS).

References

AGRAWAL, A. A., and RUTTER, M. T. 1998. Dynamic anti-herbivore defense in ant-plants: the role of induced responses. *Oikos* 83:227–236.

AGRAWAL, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 29:1201–1202.

AKIYAMA, K., MATSUZAKI, K., and HAYASHI, H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435:824–827.

ARATCHIGE, N. S., LESNA, I., and SABELIS, M. W. 2004. Belowground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. *Exper. Appl. Acarology* 33:21–30.

BALDWIN, I. T., and PRESTON, C. A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137–145.

BERNASCONI, M. L., TURLINGS, T. C. J., AMBROSETTI, L., BASSETTI, P., and DORN, S. 1998. Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis*. *Entomol. Exp. Appl.* 87:133–142.

BERTIN, C., YANG, X., and WESTON, L. A. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256:67–83.

BOFF, M. I. C., ZOON, F. C., and SMITS, P. H. 2001. Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. *Entomol. Exp. Appl.* 98:329–337.

BURNELL, A. M., and STOCK, S. P. 2000. *Heterorhabditis*, *Steinernema* and their bacterial symbionts—lethal pathogens of insects. *Nematology* 2:31–42.

BUTLER, L. G. 1995. Chemical communication between the parasitic weed Striga and its crop host—a new dimension in allelochemistry. pp. 158–168, in KINDERJIT , and FAEINHELLIG (eds.). Insights into Allelopathy, ACS Symposium SeriesACS Books, Washington, DC.

CALYECAC-CORTERO, H. G., CIBRIAN-TOVAR, J., SOTO-HERNANDEZ, M., and GARCIA-VELASCO, R. 2007. Isolation and identification

of *Physalis philadelphica Lam.* volatiles. *Agrociencia* 41:337–346.

CHENG, A. X., XIANG, C. Y., LI, J. X., YANG, C. Q., HU, W. L., WANG, L. J., LOU, Y. G., and CHEN, X. Y. 2007. The rice (*E*)-beta-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes. *Phytochemistry* 68:1632–1641.

D'ALESSANDRO, M., and TURLINGS, T. C. J. 2005. In Situ modification of herbivore-induced plant odors: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem. Senses* 30:739–753.

DEGEN, T., DILLMANN, C., MARION-POLL, F., and TURLINGS, T. C. J. 2004. High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135:1928–1938.

DE, MORAES, C. M., LEWIS, W. J., PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DICKE, M., and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M., and HILKER, M. 2003. Induced plant defences: from molecular biology to evolutionary ecology. *Basic Appl. Ecol.* 4:3–14.

DICKE, M., VAN POECKE, R. M. P., and DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl. Ecol.* 4:27–42.

ERASTO, P., GRIERSON, D. S., and AFOLAYAN, A. J. 2006. Bioactive sesquiterpene lactones from the leaves of *Vernonia amygdalina*. *J. Ethnopharmacol.* 106:117–120.

FIRN, R. D., and JONES, C. G. 2000. The evolution of secondary metabolism - a unifying model. *Mol. Microbiol.* 37:989–994.

FIRN, R. D., and JONES, C. G. 2006. Do we need a new hypothesis to explain plant VOC emissions? *Trends Plant. Sci.* 11:112–113.

FORST, S., and NEALSON, K. 1996. Molecular biology of the symbiotic pathogenic bacteria *Xenorhabdus* spp and *Photorhabdus* spp. *Microbiol. Rev.* 60:21–43.

GAUGLER, R., LEWIS, E., and STUART, R. J. 1997. Ecology in the service of biological control: The case of entomopathogenic nematodes. *Oecologia* 109:483–489.

GORECKI, T., and NAMIESNIK, J. 2002. Passive sampling. *Trends Analyt. Chem.* 21:276–291.

HAMMACK, L. 2001. Single and blended maize volatiles as attractants for diabroticite corn rootworm beetles. *J. Chem. Ecol.* 27:1373–1390.

HELMIG, D., ORTEGA, J., DUHL, T., TANNER, D., GUENTHER, A., HARLEY, P., WIEDINMYER, C., MILFORD, J., and SAKULYANONTVITTAY, T. 2007. Sesquiterpene emissions from pine trees - Identifications, emission rates and flux estimates for the contiguous United States. *Environ. Sci. Technol.* 41:1545–1553.

JACKSON, J. J. 1996. Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 89:366–372.

JI, Z. Q., WU, W. J., YANG, H., SHI, B. J., and WANG, M. G. 2007. Four novel insecticidal sesquiterpene esters from *Celastrus angulatus*. *Nat. Prod. Res.* 21:334–342.

JOURNEY, A. M., and OSTLIE, K. R. 2000. Biological control of the western corn rootworm (Coleoptera: Chrysomelidae) using the entomopathogenic nematodes, *Steinernema carpocapsae*. *Environ. Entomol.* 29:822–831.

KARBAN, R., AGRAWAL, A. A., and MANGEL, M. 1997. The benefits of induced defenses against herbivores. *Ecology* 78:1351–1355.

KARBAN, R., and BALDWIN, I. 1997. Induced Responses to Herbivory. University Press of Chicago, Chicago.

KESSLER, A., and BALDWIN, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291:2141–2144.

KÖLLNER, T., HELD, M., LENK, C., HILTPOLD, I., TURLINGS, T. C. J., GERSGENZON, J., and DEGENHARDT, J. 2008. A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *Plant Cell* 20:482–494.

KOZIEL, J., JIA, M. Y., and PAWLISZYN, J. 2000. Air sampling with porous solid-phase microextraction fibers. *Anal. Chem.* 72:5178–5186.

KUHLMANN, U., and van der BURGT, W. A. C. M. 1998. Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. *Biocontrol* 19:59N–68N.

LEVINE, E., OLOUMI, S. H., and FISHER, J. R. 1992. Discovery of multiyear diapause in Illinois and South Dakota northern corn rootworm (Coleoptera: Chrysomelidae) eggs and incidence of the prolonged diapause trait in Illinois. *J. Econ. Entomol.* 85:262–267.

LIU, C. H., MISHRA, A. K., and TAN, R. X. 2006. Repellent, insecticidal and phytotoxic activities of isoalantolactone from *Inula racemosa*. *Crop Prot.* 25:508–511.

LOWELL, P. S., and EKLUND, B. 2004. VOC emission fluxes as a function of lateral distance from the source. *Environ. Prog.* 23:52–58.

MCCOY, C. W., STUART, R. J., DUNCAN, L. W., and NGUYEN, K. 2002. Field efficacy of two commercial preparations of entomopathogenic nematodes against larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in ALFISOL type soil. *Fla. Entomol.* 85:537–544.

MCGECHAN, M. B., and LEWIS, D. R. 2002. Transport of particulate and colloid-sorbed contaminants through soil, part 1: General principles. *Biosystems Engineering* 83:255–273.

MILLER, N., ESTOUP, A., TOEPFER, S., BOURGUET, D., LAPCHIN, L., DERRIDJ, S., KIM, K. S., REYNAUD, P., FURLAN, L., and GUILLEMAUD, T. 2005. Multiple transatlantic introductions of the western corn rootworm. *Science* 310:992–992.

NEVEU, N., GRANDGIRARD, J., NENON, J. P., and CORTESERO, A. M. 2002. Systemic release of herbivore-induced plant volatiles by turnips infested by concealed root-feeding larvae *Delia radicum* L. *J. Chem. Ecol.* 28:1717–1732.

O'NEIL, M. E., DIFONZO, C. D., and LANDIS, D. A. 2002. Western corn rootworm (Coleptera: Chrysomelidae) feeding on corn and soybean leaves affected by corn phenology. *J. Econ. Entomol.* 31:285–292.

OWEN, S. M., and PEÑUELAS, J. 2005. Opportunistic emissions of volatile isoprenoids. *Trends Plant Sci.* 10:420–426.

OWEN, S. M., and PENUELAS, J. 2006. Response to Firn and Jones: Volatile isoprenoids, a special case of secondary metabolism. *Trends Plant Sci* 11:113–114.

PEÑUELAS, J., and LLUSIÀ, J. 2004. Plant VOC emissions: making use of the unavoidable. *Trends Ecol. Evol.* 19:402–404.

RASMANN, S., KÖLLNER, T. G., DEGENHARDT, J., HILTPOLD, I., TOEPFER, S., KUHLMANN, U., GERSHENZON, J., and TURLINGS, T. C. J. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434:732–737.

RASMANN, S., and TURLINGS, T. C. J. 2008. First insights into specificity of belowground tritrophic interactions. *Oikos* 117:362–369.

RODRIGUEZ-SAONA, C., CRAFTS-BRANDER, S. J., PARE, P. W., and HENNEBERRY, T. J. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27:679–695.

RUIZ, J., BOLBAO, R., and MURILLO, M. B. 1998. Adsorption of different VOC onto soil minerals from gas phase: Influence of mineral, type of VOC, and air humidity. *Environ. Sci. Technol.* 32:1079–1084.

SABULAL, B., DAN, M., ANIL, J. J., KURUP, R., PRADEEP, N. S., VALSAMMA, R. K., and GEORGE, V. 2006. Caryophyllene-rich

rhizome oil of *Zingiber nimmonii* from South India: Chemical characterization and antimicrobial activity. *Phytochemistry* 67:2469–2473.

SCHOONHOVEN, L. M., JERMY, T., and VAN, LOON, J. J. A. 1998. Insect-Plant Biology: From Physiology to Evolution. Chapman and Hall, New York.

THALER, J. S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399:686–688.

THOLL, D., CCHEN, F., PETRI, J., GERSHENZON, J., and PICHERSKY, E. 2005. Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers. *Plant J.* 42:757–771.

TOEPFER, S., and KUHLMANN, U. 2004. Survey for natural enemies of the invasive alien chrysomelid, *Diabrotica virgifera virgifera*, in Central Europe. *Biocontrol* 49:385–395.

TOLLEFSON, J. J. 1998. A pest insect adapts to the cultural control of crop rotation; Brighton Crop Protection Conference. *Pests and Diseases* 3:1029–1033.

TÒTH, M., VUTS, J., SZARUKÁN, I., JUHÁSZ, I., and MANAJLOVICS, F. 2007. Preliminary study of female-targeted semiochemical baits for the western corn rootworm in Europe. *J. Appl. Entomol.* 131:416–419.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parsitic wasps. *Proc. Natl. Acad. Sci. USA* 92:4169–4174.

TURLINGS, T. C. J., and TON, J. 2006. Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Curr. Opin.Plant Biol.* 9:421–427.

TURLINGS, T. C. J., and WACKER, F. 2004. Recruitment of predators and parasitoids by herbivore injured-plants.. pp. 21–75, in R. T. CARDÉ, and J. G.MILLAR (eds.). Advances in Insect Chemical EcologyCambridge University Press, Cambridge.

VAN TOL, R. W. H. M., VAN DER SOMMEN, A. T. C., BOFF, M. I. C., VAN BEZOOIJEN, J., SABELIS, M. W., and SMITS, P. H. 2001. Plants protect their roots by alerting the enemies of grubs. *Ecology Lett.* 4:292–294.

VAS, G., and VEKEY, K. 2004. Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. *J. Mass Spectrom.* 39:233–254.

VIDAL, S., KUHLMANN, U., and EDWARDS, R. 2005. Western Corn Rootworm: Ecology and Management. p. 324. CABI, Wallingford, United Kingdom.

CHAPTER II

Restoring the communication between maize roots and insect-killing nematodes boosts the control of a major pest

Submitted to Science

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2008

When attacked by herbivorous insects, plants emit volatiles that attract natural enemies of the insects. Here we demonstrate for the first time that such volatile signals can be enhanced to improve crop protection. Maize roots normally emit (*E*)-β-caryophyllene in response to feeding by the Western corn rootworm, resulting in the attraction of entomopathogenic nematodes that infect and kill the voracious root pest. Most North American maize varieties have lost the ability to emit the signal and consequently receive little protection by the nematode. To restore the signal, a non-emitting maize line was transformed with a (*E*)-β-caryophyllene synthase gene from oregano, resulting in constitutive emissions of this bicyclic sesquiterpene olefin. In rootworm-infested field plots in which nematodes were released, transformed plants received significantly less root damage and had 60% fewer adult beetles emerge than control lines. Improving plant signals can be an ecologically sounds strategy to fight a variety of pests.

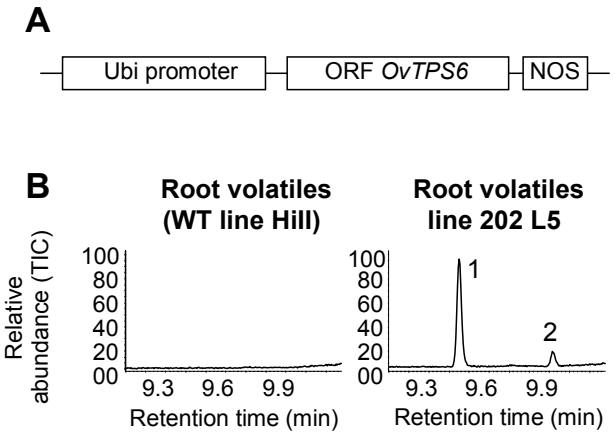
Plants synthesize and emit blends of volatile organic compounds in response to being damaged by herbivorous arthropods (*1*). The induced volatiles have been proposed to serve a variety of physiological and ecological functions (*2*), including an indirect defence function through the attraction of natural enemies of herbivores (*3-6*). It has been confirmed that plant volatiles attract natural enemies under field conditions (*3, 7*) and thus can help plants to reduce the damage inflicted by herbivores (*8*). Advances in genomics should allow us to manipulate the plant's volatiles signals to at last conclusively demonstrate the importance of specific volatile signals for these tritrophic interactions (*9*). Moreover, various researchers have proposed that enhancing specific volatile signals in crop plants may help to increase the effectiveness of natural enemies as biological control agents (*10-14*). Laboratory studies with model plants whose volatile emissions have been genetically modified provided the first evidence that this approach has potential (*15-18*).

One volatile signal that appears to be particularly important for attraction of natural enemies is (*E*)-β-caryophyllene (EβC), a sesquiterpene that is emitted by maize (*Zea mays* L.) roots in response to feeding by larvae of the Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte

(Coleoptera: Chrysomelidae) (*19*). EβC diffuses readily in air, sand and soil (*20*) and is highly attractive to the entomopathogenic nematode *Heterorhabditis megidis* (Poinar) (Rhabditida: Heterorhabditidae), which parasitizes and kills WCR larvae within a few days (*19, 21*). In contrast to the much more complex volatile blends released from other tissues (*22*), maize roots produce almost exclusively EβC and its emission appears essential for nematode attraction (*19*). WCR is the most severe pest of maize in the United States and was introduced about fifteen years ago into Europe, where it is a serious problem in the Balkan region and is rapidly spreading to other countries (*23, 24*). Controlling WCR is exceedingly difficult and pesticide applications are relatively expensive, environmentally unfriendly and not always effective (*25*). The use of genetically modified (GM) maize lines that carry bacterial-derived genes coding for Bt toxins shows promise (*26*), but resistance traits are likely to develop and in Europe the use of GM maize is still highly controversial (*27*). Biological control of WCR with nematodes could be a more acceptable alternative.

Previous attempts to control WCR with nematodes have been largely ineffective or required extraordinary large numbers of nematodes (*28-30*). A possible explanation for these failures is the inability of most North American maize lines to emit EβC (*19, 31*), the first example of an indirect defence trait lost through crop breeding. We recently identified the caryophyllene synthase gene *tps23*, which is responsible for EβC production in maize. North American maize lines possess a fully functional *tps23*, but EβC production is absent due to the lack of *tps23* transcript (*32*). The absence of EβC seems to dramatically reduce attraction of nematodes, as was apparent from a field experiment in which we found a five-fold higher infection by nematodes near a variety producing EβC than near a variety without this signal (*19*). To confirm this function of EβC and to test if manipulation of the signal can help to enhance the control of WCR we attempted to “restore” the EβC signal to a maize variety that normally does not emit this compound by transforming it with a gene from oregano (*Origanum vulgare*). We then compared WCR-inflicted root damage and beetle survival on transformed and non-transformed lines in the presence and absence of entomopathogenic nematodes.

The maize variety HiII was transformed



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Line	(*E*)-β-Caryophyllene ng/g root fresh weight
HiII	0
OE 201 L1	19.4 ± 3.1
OE 202 L2	126.5 ± 32.4
OE 202 L5	131.1 ± 54.4

Figure 1 Insertion of a (E)-β-caryophyllene synthase gene from Origanum vulgare *L. in maize variety HiII resulted in a constitutive production of EβC. (A) Terpene synthase overexpression construct (see Method section for details). (B) A typical chromatogram obtained for the volatiles emitted by roots of the hybrid variety HiII line alongside a chromatogram for one of the transformed lines. Peak 1 is EβC and peak 2 is α-humulene, a side product of (E)-β-caryophyllene synthase. (C) Average quantities of EβC present in the roots of the untransformed HiII and the three transformed lines that were used in the field experiments (n=8).*

with a (*E*)-β-caryophyllene synthase gene from *Origanum vulgare* L. (Crocoll et al., in preparation) under control of a maize ubiquitin promoter (Fig. 1a) (*33*). The transformation resulted in plants that produced EβC constitutively (Fig. 1b), supporting the observation that loss of EβC production in most American maize lines is due to the loss of (*E*)-β-caryophyllene synthase activity (*32*). We selected three independently transformed lines with EβC concentrations in their roots that were either similar (line 201 L1) or six-fold higher (lines 202 L2, 202 L5) than what is typically present in our model European maize line Delprim (*19*) (Fig. 1c). Since sesquiterpene hydrocarbons like EβC appear to be emitted from maize by passive diffusion (*34*), the roots of transgenic maize containing higher concentrations of (*E*)-β-caryophyllene can be expected to release proportionally higher levels of this compound. For the following field experiments, selfed T1 generations of the three transformed lines were tested. As control plants we used F1 progeny of the selfed non-transformed HiII, as well as the T1 progeny of a transgenic line (197 L1) with no expression of the EβC synthase gene to provide very high similarity to the transformed plants.

Since the successfully transformed lines segregated 3:1 for the transgene, a quarter of the progeny did not produce EβC. Therefore all transformed plants used in field experiments were tested for EβC production in order to identify the plants emitting EβC and accurately evaluate the results (see below).

Field tests were conducted to determine

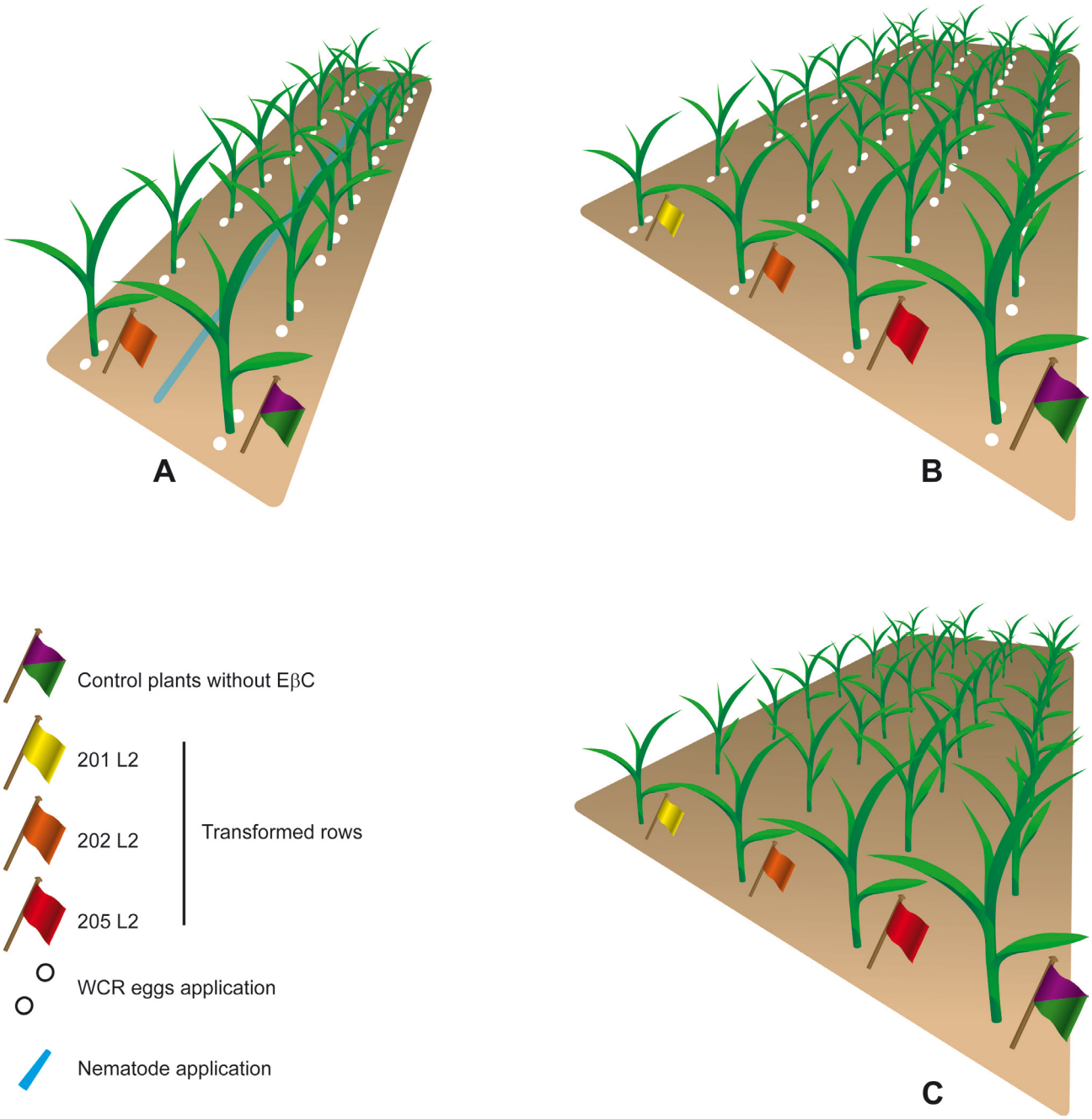


Figure 2 Design of the field plots where transformed lines were tested alongside control lines. Plants were grown in plots with two or four rows with eight plants per row. Four plants of each row were used for the root damage rating and the four remaining plants received emergence cages to monitor emergence of WCR adult. (A) One row of a control line was plant alongside a row of a transformed maize line. Each plant was infested with WCR eggs and nematodes were applied in a trench in the middle of the two rows (n=30). (B) As a first control plot, a row of eight control plants was planted alongside three rows with each eight plants of a transformed line. These plants were also infested with WCR eggs, but no nematodes were applied (n=5). (C) For the second type of control plot design was the same as for (B), but no WCR eggs or nematodes were applied (n=5).

whether the EβC emission of the transgenic plants enhanced the ability of *H. megidis* to find and kill WCR under realistic conditions and so reduce plant damage. In a 20 by 70 meter maize field thirty experimental plots were planted, each consisting of one row with eight plants of a transformed line alongside a row with eight plants of a control line (Fig. 2a). Two weeks after planting, all plots were infested with WCR eggs and another three weeks later, about 600,000 *H. megidis* were applied between the two rows of each plot. In addition to these two-row experimental plots, we planted two types of control plots that did not receive nematodes, but half of these plots did received WCR eggs (Fig. 2b, c) (*33*). The effectiveness of the EPN in controlling WCR was evaluated by measuring root damage and emergence of WCR beetles.

Damage to roots in experimental plots without WCR was minimal, but considerable root damage was found in plots that had received only WCR eggs (Fig. 3a, b). Without nematodes, there was no difference in WCR-caused root damage between transformed plants and the control plants. In accordance, the number of emerging WCR beetles (approximately four per plant) was the same for transformed and control plants (Fig. 4a). There was no WCR emergence in the plots that were not infested with WCR eggs, but eight *Diabrotica barberi* Smith and Lawrence (northern corn rootworm) beetles were captured in the emergence cages within these plots (data not shown).

In plots in which we had released *H. megidis* nematodes, the transformed plants received significantly less root damage than the controls (Fig. 3c), as would be expected from the attractiveness of EβC release. In accordance, adult WCR emergence per plant was reduced to less than half with transformed plants as compared to the control plants (Fig. 4b).

Because all seeds used for the experiments were produced by selfed plants the transformed lines segregated 3:1 for the transgene and therefore a quarter of the plants did not produce EβC. This provided us with an additional built-in, fully blind control experiment. We checked for EβC emission from clippings of leaves obtained from all plants the day before harvesting the plants that were used for the evaluation of root damage (*33*). The results for the transformed plants were corrected by discarding the plants that did not produce EβC (26.8%) from the data analyses. This correction had no effect on the average for root damage, but the average

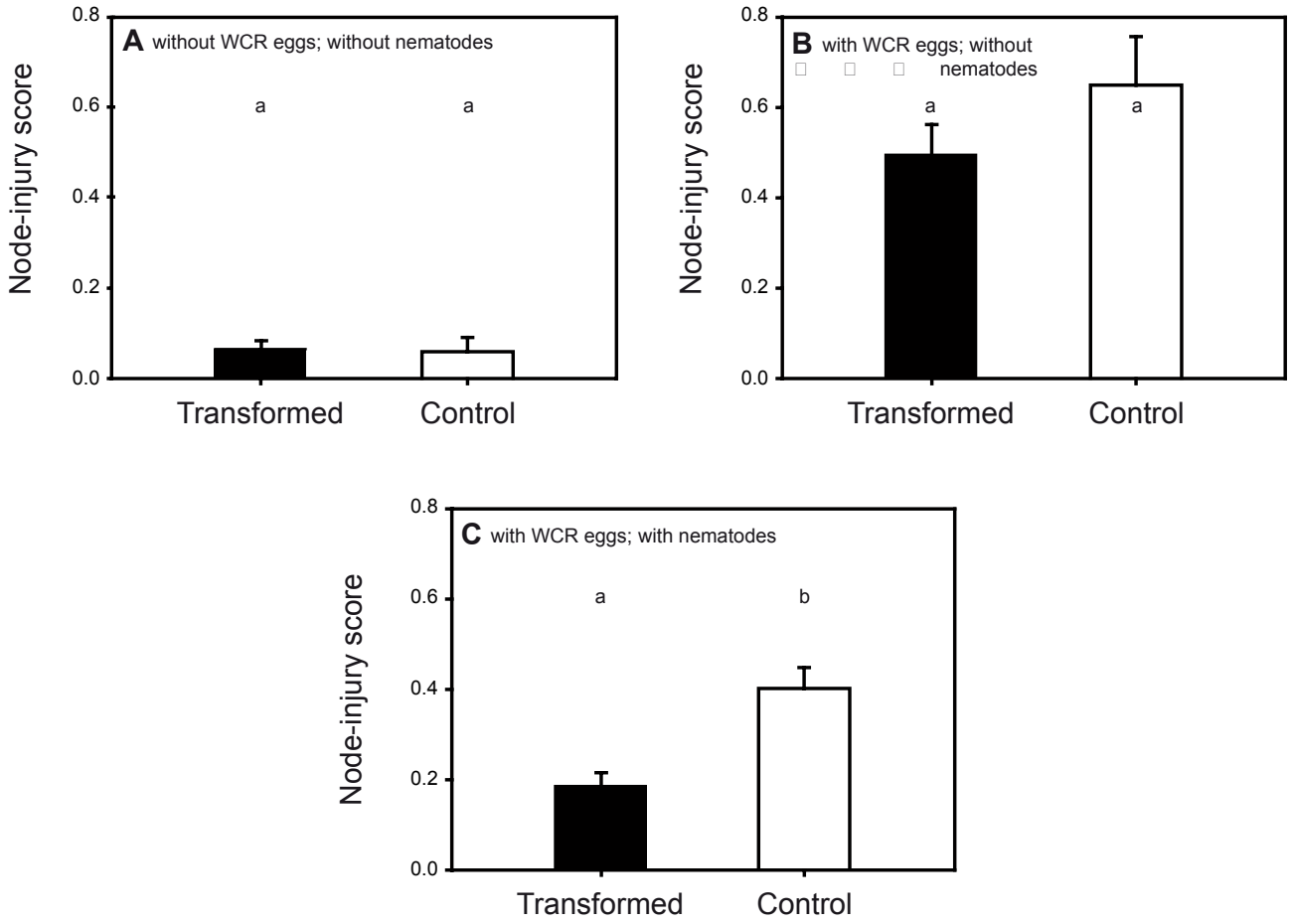


Figure 3. Root damage. (A) Root damage measured on plants that had received neither WCR eggs nor nematodes was minimal and there was no difference between transformed and non-transformed plants (n = 5, P=0.87). (B) Root damage on plants that received only WCR eggs, but no nematodes was substantial. Again, no significant difference was found between the transformed and non-transformed plants (n = 5, P=0.18). (C) In plots that received WCR eggs and H. megidis*, roots from transformed plants had significantly less damage than roots from control lines (n = 30, P=0.007). Approximately one quarter of the transformed plants were found not to emit EβC. Removing these plants from the statistical analysis did not significantly affect the results. Letters above bars indicate significant differences within a graph. Error bars indicate standard errors.*

adult emergence near EβC-emitting plants was reduced by another 5%, making it 60% less than near non-transformed plants (Fig. 4b). Moreover, there was a striking difference in the average number of adults that emerged near EβC-producing plants compared to the quarter of their sister plants that did not produce EβC in the same rows (1.79 vs. 3.80 beetles per plant, fig. 4c).

For each emerging beetle, the head capsule width and dry weight were determined, but no differences for these parameters were found among treatments (Fig. S1). Emergence of beetles started in mid July and lasted until

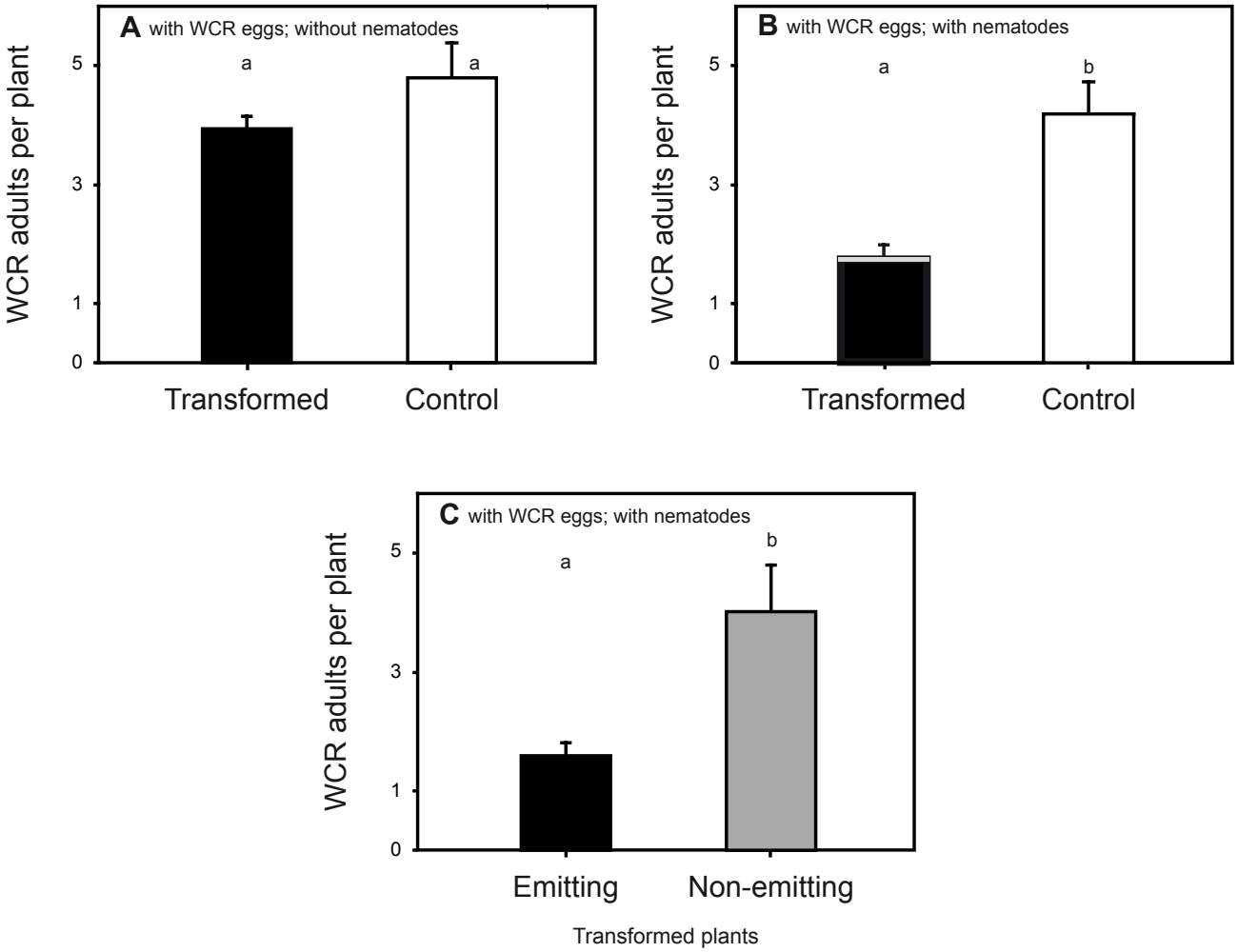


Figure 4 Beetles emergence. (A) Adult emergence for plants from the plots that received WCR eggs only, but no nematodes. No significant difference was found between the transformed and non-transformed plants (n = 5, P=0.47). (B) Adult emergence for plants that received both WCR eggs and nematodes was significantly different between transformed plants and control plants (n = 30, P<0.0001). Approximately one quarter of the transformed plants were found not to emit EβC. Removing these plants from the statistical analysis slightly reduced the average emergence near the transformed plants (black bar) and increased the difference with untransformed control plants to 60%. (C) Significantly fewer adults emerged near EβC producing transformed plants (black bar) than near transformed plants that were not emitting EβC (cross-hatched bar, P = 0.023). Letters above bars indicate significant differences within a graph. Error bars indicate standard errors. No WCR adults were recovered from plots that did not receive WCR eggs (data not shown).

mid August (Fig. S2). Comparison of the emergence over time between plots with and without *H. megidis* shows that the nematode mostly suppressed WCR emergence at the end of the season (Fig. S2). This late effect is likely due to increasing numbers of *H. megidis*, as a new generation of infective juveniles will emerge within two weeks after infection, allowing the nematode to multiply over several generations during the season.

The data are consistent with the hypothesis that the transformation with (*E*)-β-caryophyllene resulted in increased attraction of applied *H. megidis* and thereby enhanced WCR mortality and root protection. However, we considered and tested two additional explanations for increased root protection: that EβC attracted native nematodes which protected the plants or that the EβC-releasing transformants had a direct negative effect on the performance of WCR larvae.

In order to evaluate the presence of naturally occurring entomopathogenic nematode populations in the experimental field, soil samples were collected from the plots before and after the application of *H. megidis* (*33*). Waxmoth larvae (*Galleria mellonella* L., Lepidoptera: Galleridae) were incubated in these soil samples and nematode infection was determined five days later. In soil collected before *H. megidis* release, 4.9 % of the larvae were infected, whereas in soil from after release 59.2 % were infected. These results imply that there was indeed a small effect of native entomopathogenic nematodes on WCR infection rates during the experiments. Interestingly, the tendency of reduced beetle emergence near transgenic plants in the control plots was only apparent at the end of the season (Fig. S2a), when the native EPN population can be expected to have had built up its numbers.

To test for a possible direct effect of the (*E*)-β-caryophyllene synthase transformation on WCR performance, as well as to confirm enhanced attractiveness of transformed plants to *H. megidis*, we conducted an additional laboratory experiment with the use of six-arm belowground olfactometers (*19, 33*). In these olfactometers nematodes were give the choice between the odour of WCR-infested transformed plant and a similar non-transformed plant that were placed in opposite interconnected glass pots. Four remaining pots contained only moist sand. Consistent with the reported (*19, 32*) attractiveness of EβC, significantly more nematodes were recovered from the olfactometer arms connected to the pot with the WCR-infested transformed plants than from those connected to the pots with the infested non-transformed plants (Fig. 5). The transformation had no direct effect on the performance of WCR larvae. Average weight increase and survival were the same for larvae that had fed for five days on transformed plants as compared to larvae that had fed the same

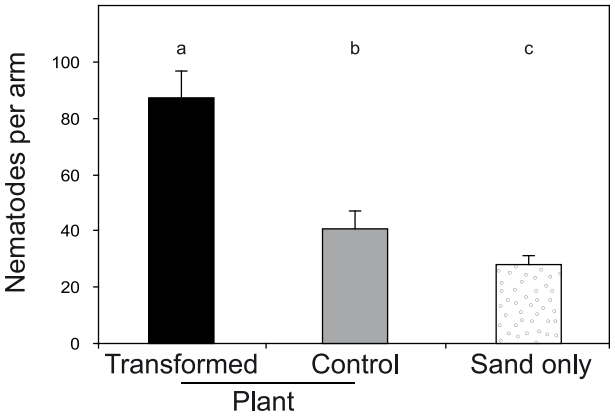


Figure 5 Comparison of attraction of H. megidis *nematodes to transformed plants versus untransformed control plants in six-arm olfactometers. Graph depicts the average number of nematodes recovered from olfactometer arms connected to pots containing either a WCR-infested transformed plant (black bar), a WCR-infested control plant (grey bar), or only moist sand (white bar). The plants were each infested with five second instar WCR larvae. For each replicate, the total number of nematodes found in the four moist sand-only control pots (white bar) were summed and divided by four. The attraction to transformed plants was significantly higher than to the control plants ($n = 11$, $P < 0.001$). Letters above bars indicate significant differences. Error bars indicate standard errors.*

period of time on non-transformed plants (Fig. 6). These results support the conclusion that the improved control of WCR near transformed plants in the field experiments was due to the enhanced attractiveness of the roots to EPN and not to a direct negative effect on the WCR larvae.

Besides irrefutably demonstrating that the presence of a specific volatile signal is of essential importance for the attraction of a major natural enemy of soil herbivores, the current study shows for the first time that plant-produced chemical signals can be modified to improve the search and killing efficiency of natural enemies of pest insects. The 60% reduction in adult WCR emergence achieved by nematodes on plants transformed to release (*E*)-β-caryophyllene approaches the efficacy of synthetic pesticides that have been employed to control this important pest (*35, 36*). Moreover, this considerable effect on the pest was accomplished with a much lower nematode dose than is commonly used (*28-30*). It should be noted that the transformed lines are not of commercial value. The variety HiII was chosen because it can be transformed and the experiments were conducted as a proof of the principle that enhancing natural defence signals is a promising strategy for ecologically sound pest control. As the EβC signal is already present in many maize lines, as well as in ancestral wild relatives, optimizing the production of EβC might be achieved through classical breeding. However, we do not rule out a transgenic approach, which could be faster and minimize changes of desirable traits in existing lines. This would also facilitate a strategy that combines direct resistance traits

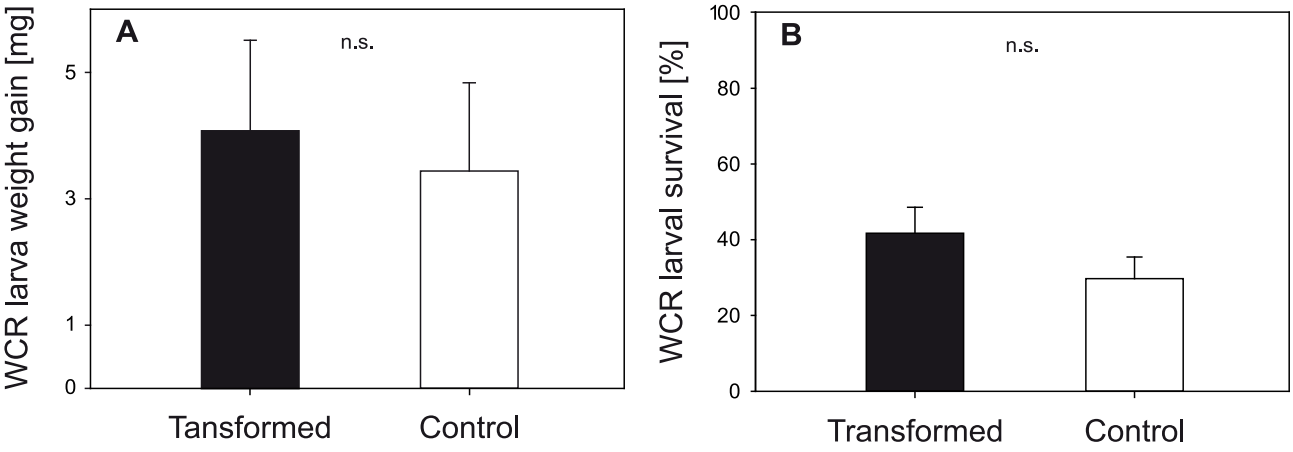


Figure 6 WCR performance on transformed and control plants. (A) Average weight gain of WCR larvae fed for five days roots. No statistical difference was found (n = 13, P = 0.75). (B) Survival of WCR larvae after five days of root feeding. No statistical difference was found (n = 13, P = 0.18). Error bars indicate standard errors.

with the indirect resistance tested here. It could be assumed that constitutive emission of EβC might be costly and might negatively affect plant performance. Although, yield measurements did not detect any such performance costs (data not shown), making the introduced gene inducible and only expressed when insects feed on the roots might be a better strategy to guide the nematodes specifically to those plants that are actually under insect attack. Studies to further optimize the system are underway and we are finding that one additional promising approach is to select for nematodes that respond better to EβC. An optimal combination of attractive maize plants, highly responsive nematodes and an efficient, cost-effective application method should make EPN a realistic alternative to other control strategies that are currently employed to combat WCR.

1. P. W. Paré, J. H. Tumlinson, *Plant Physiology* **121**, 325 (1999).

2. N. Dudareva, F. Negre, D. A. Nagegowda, I. Orlova, *Critical Reviews in Plant Sciences* **25**, 417 (2006).

3. C. M. De Moraes, W. J. Lewis, P. W. Pare, H. T. Alborn, J. H. Tumlinson, *Nature* **393**, 570 (1998).

4. M. Dicke, M. W. Sabelis, *Netherlands Journal of Zoology* **38**, 148 (1988).

5. M. Hilker, T. Meiners, *Entomologia Experimentalis Et Applicata* **104**, 181 (2002).

6. T. C. J. Turlings, J. H. Tumlinson, W. J. Lewis, *Science* **250**, 1251 (1990).

7. J. S. Thaler, *Nature* **399**, 686 (1999).

8. A. Kessler, I. T. Baldwin, *Science* **291**, 2141 (2001).

9. I. Baldwin, R. Halitschke, A. Paschold, C. C. von Dahl, C. A. Preston, *Science* **311**, 812 (2006).

10. D. G. Bottrell, P. Barbosa, *Annu. Rev. Entomol.* **43**, 347 (1998).

11. J. Degenhardt, J. Gershenzon, I. T. Baldwin, A. Kessler, *Current Opinion in Biotechnology* **14**, 169 (2003).

12. J. A. Pickett *et al.*, in *Chemical ecology: from gene to ecosystem* M. Dicke, W. Takken, Eds. (Springer, 2006) pp. 161-173.

13. A. Aharoni, M. A. Jongsma, H. J. Bouwmeester, *Trends in Plant Science* **10**, 594 (2005).

14. T. C. J. Turlings, J. Ton, *Current Opinion in Plant Biology* **9**, 421 (2006).

15. M. H. Beale *et al.*, *Proceedings of the National Academy of Sciences of the United States of America* **103**, 10509 (2006).

16. A. X. Cheng *et al.*, *Phytochemistry* **68**, 1632 (2007).

17. I. F. Kappers *et al.*, *Science* **309**, 2070 (2005).

18. C. Schnee *et al.*, *Proceedings of the National Academy of Sciences USA* **103**, 1129 (2006).

19. S. Rasmann *et al.*, *Nature* **434**, 732 (2005).

20. I. Hiltpold, T. C. J. Turlings, *Journal of Chemical Ecology* **34**, 628 (2008).

21. B. Kurzt, I. Hiltpold, T. C. J. Turlings, U. Kuhlmann, S. Toepfer, *Biocontrol* (2008).

22. S. Rasmann, T. C. J. Turlings, *Ecology Letters* **10**, 926 (2007).

23. S. Vidal, U. Kuhlmann, C. R. Edwards, *Ecology and Management of the Western Corn Rootworm* (CABI Publishers, London, 2005), pp. 310.

24. N. Miller *et al.*, *Science* **310**, 992 (2005).

25. E. Levine, H. Oloumisadeghi, *Annu. Rev. Entomol.* **36**, 229 (1991).

26. T. Vaughn *et al.*, *Crop Science* **45**, 931 (2005).

27. J. Romeis, M. Meissle, F. Bigler, *Nat Biotech* **24**, 63 (2006).

28. J. J. Jackson, *Journal of Economic Entomology* **89**, 366 (1996).

29. A. M. Journey, K. R. Ostlie, *Environmental Entomology* **29**, 822 (2000).

30. T. Nishimatsu, J. J. Jackson, *Journal of Economic Entomology* **91**, 410 (1998).

31. T. Degen, C. Dillmann, F. Marion-Poll, T. C. J. Turlings, *Plant Physiology* **135**, 1928 (2004).

32. T. G. Köllner *et al.*, *Plant Cell* **20**, 482 (2008).

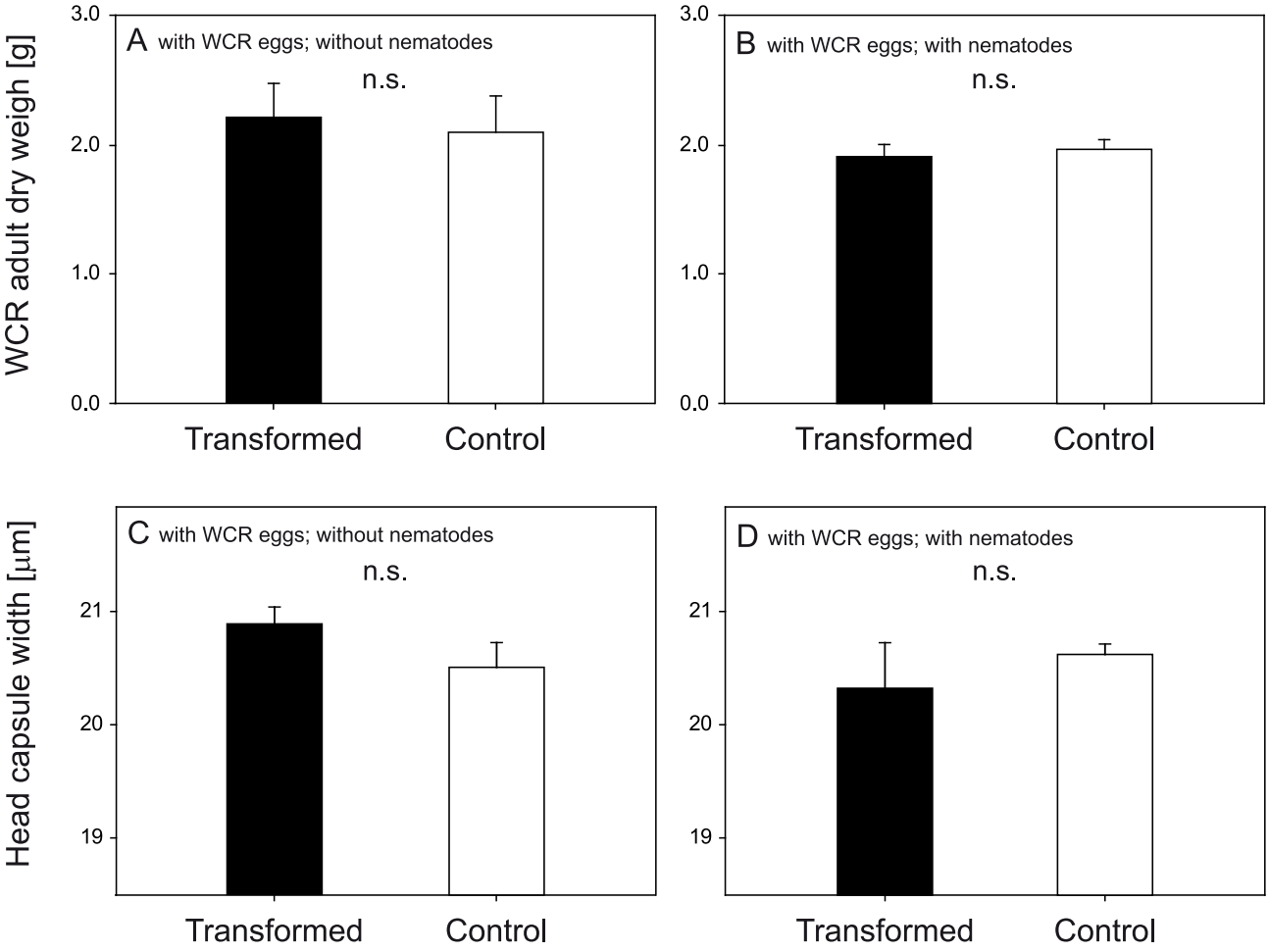
33. Materials and methods are available as supporting material on *Science* Online.

34. T. G. Köllner, C. Schnee, J. Gershenzon, J. Degenhardt, *Phytochemistry* **65**, 1895 (2004).

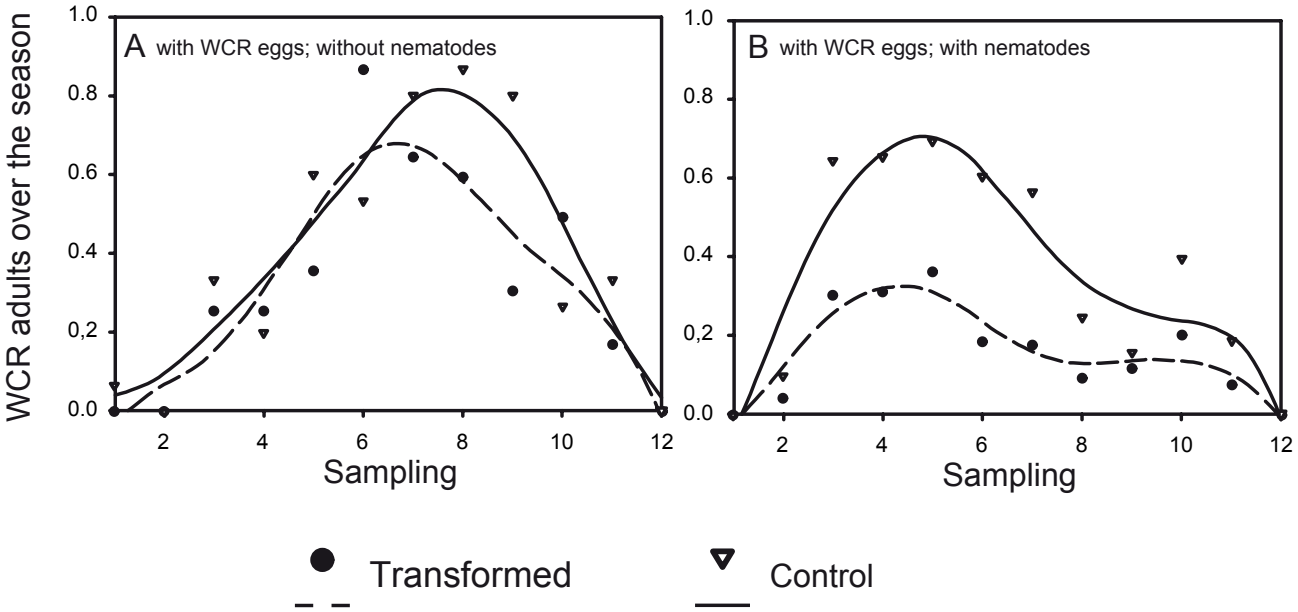
35. W. J. Cox, E. Shields, D. J. R. Cherney, J. H. Cherney, *Agronomy Journal* **99**, 1640 (2007).

36. B. W. French, L. D. Chandler, W. E. Riedell, *Journal of Economic Entomology* **100**, 1542 (2007).

37. This work was supported by the Swiss National Centre of Competence in Research “Plant Survival”, the German National Science Foundation (grant DE-837/2-2) and the Max Planck Society.



Supplementary Figure 1. WCR adult head capsule width and weight. To evaluate if there was a difference in the quality shape size of the adult beetles that emerged near transgenic and non-transgenic plants in the two plot types that had received WCR eggs, the head capsule width and dry weight of each beetle was measured. A) Average head capsule width of beetles that emerged in maize plots infested with WCR eggs without nematode application (n = 5, P = 0.57). B) Average head capsule width of beetles that emerged in maize plots infested with WCR eggs and application of nematodes (n = 30, P = 0.41). C) Average dry weight of beetles that emerged in maize plots infested with WCR eggs without nematode application (n = 5, P = 0.39). d) Average dry weight of beetles that emerged in maize plots infested with WCR eggs and application of nematodes (n = 30, P = 0.94). Letters above bars indicate significant differences. Error bars indicate standard errors.



Supplementary Figure S2. Emergence of WCR adults over the collection period. WCR adults were sampled from July 17 to August 16. The number of adults collected per sampling date was plotted for the two plot types that had received WCR eggs to visualize any time effect on the emergence. A) WCR emergence over time in maize plots infested with WCR eggs without nematode application. B) WCR emergence over time in maize plots infested with WCR eggs and application of nematodes. The late decrease of the adult emergence in plots treated with nematodes may reflect an effect of multiple generations of the nematodes. Once they reached and infected their first hosts, a new generation of infective juveniles can be expected to emerge from this host within 10 days. A minimum of three generations must have exponentially increased the nematodes population throughout the season, thereby having the most significant effect on the WCR larvae with slowest development.

Supporting Online Material

Transformation

The general procedure for the transformation of the maize line HiII has been described by Frame et al. (2002) (S*1*). The Ti-plasmid pTF101.1 was kindly provided by the Plant Transformation Facility, Iowa State University, Ames, Iowa, USA. Plasmid pTF101.1 derived from pTF102. It lacked the β-glucuronidase-reporter gene and instead expressed the phosphoinothricin acetyl transferase from *Streptomyces hygroscopicus* gene via a double Cauliflower Mosaic Virus 35S promoter. Inserted in this vector was the (*E*)-β-caryophyllene synthase *tps6* from *Origanum vulgare* under control of a maize ubiquitin promoter (S*2, S3*). The structural gene was followed by a terminator from nopaline synthase of the *Agrobacterium tumefaciens* Ti plasmid. The transgenic lines 202 L1, 202 L2 and 202 L5 were regenerated from three independent transgenic calli generated in two independent transformation experiments. Selfed T1 generations of the three transformed lines were used in the experiments. As non-transformed controls, the selfed F1 progeny of HiII as well as the T1 progeny of a transgenic line with no expression of the EβC synthase gene, 197 L1, were used.

Field experiment

All experimental plots were hand-planted on May 22 (2007) at the Bradford Research and Extension Center, Columbia, Missouri, USA. Each plot had rows of eight plants of a particular line with 43 cm spacing between plants and 76 cm spacing between rows. For the main experiment there was a total of thirty plots. Each plot had a row of control plants and a row of a transformed line (Fig. 2a). There were ten plots for each of the three transformed lines and all plots received feral WCR eggs (French Agricultural Research, MN, USA) as well as nematodes (Becker Underwood Ltd, UK). In addition, we planted ten control plots that did not receive nematodes. Half of the control plots were infested with WCR. Each control plot had four rows: three rows with each eight plants of a particular transformed line and one row with eight control plants (Fig. 2b, c). Plots were randomly distributed in the field, with each plot surrounded by two rows of maize plants of the variety Pioneer 3394, serving as a buffer between plots. The above plantings and infestation procedures resulted in three types of plots (Fig. 2): 1) thirty principal experimental plots with WCR infestation and nematode release, 2) five plots without WCR

and without nematodes, and 3) five plots that had only WCR and no nematodes.

On June 4, all plots except half (five) of the control plots were infested with WCR eggs. Eggs were mixed into a solution of water and 0.15% agar and each plant received about 400 viable eggs in two 10 cm deep holes at a distance of 5 cm from the plant (~800 eggs per plant). On June 28, about 600,000 *H. megidis* (Becker Underwood Ltd, UK) were applied in between the two rows of the thirty experimental plots. Nematodes were mixed in 0.5 l of water and the solution was poured into a 2 cm deep trench that was dug between the rows. The number of EPNs that was used is equivalent to 250,000 EPN per square meter. No nematodes were applied to the ten control plots. Plans had been made to irrigate before and after infection with EPN, but 5.9 cm of rain fell starting the day before infection, extending 3 d after infection, including 3.76 cm on the day of infection.

On July 11 root damage was assessed by digging out half of the plants in each plot (four plants per row). Plants were immediately and carefully removed from the field in order to avoid nematode and WCR contamination of the neighbouring plants and plots. The root systems were washed and damage from WCR larval feeding was rated using a 0-3 root scale (S*4*).

The 400 remaining plants stayed in the field and emergence cages (78 x 36 cm) modified after Pierce and Gray (2007) (S*5*) were placed over each plant on July 11. Traps were checked three times per week during the peak of WCR emergence and twice per week during periods when few adults were emerging. The last cage check was performed on September 7. All beetles collected were placed in individually labelled scintillation vials (J & H Berge, Inc., Plainfield, NJ USA) containing 95% ethanol and brought to laboratory for processing. Firstly, beetles were counted and sexed by emergence date. Secondly, the head capsule width of each beetle was measured using an ocular micrometer (10x/21, Wild Co., Heerbrugg, Switzerland) mounted on a microscope (M3Z, Wild Co., Heerbrugg, Switzerland). Upon these two measurements, beetles were placed in a desiccating oven (Thelco Model 16, GCA/ Precision Scientific Co., Chicago, IL) at 60 ^{o}C for 48 h. The dried beetles were then placed on an analytical scale (Model AB135-S FACT, Metler, Columbus, OH), and total dry weight was recorded.

PROC MIXED of the statistical package

SAS (SAS Institute 1990) was used for data analyses. Data were analysed as a randomized complete block with treatments arranged in a 5 × 2 factorial (5 maize genotypes × 2 experiments) as outlined in Steel et al. (1997) (S*6*). Half of the blocks contained a randomization of the main experimental plots (WCR plus nematodes) and the control plots with WCR and the other half contained a randomization of the main experimental plots and control plots without WCR. Consequently, two separate groups of analyses were performed for plant damage, adult emergence, beetle dry weight, and head capsule width. First, a comparison among the three transformed lines revealed no significant differences within the data sets. Therefore, these lines were pooled for further analyses. A separate comparison between the two isolines sources of the original non-transformed line also revealed no signif cant differences in any of the above factors and they were also pooled for subsequent analyses. Finally, selected contrasts were made between specific treatments within each type of plots following the methods outlined in Littell et al. (1998) (S7). Difference in WCR adult emergence between transformed plants producing EβC or not was analysed with SPSS 14.0. As homogeneity of variance test failed, means were compared with the nonparametric Mann-Whitney test.

Screening for the presence of native EPN

Three soil samples along the application trench were taken two days prior to (June 26) and again seven days after the nematode *H. megidis* application (July 5). Each sample contained approximately 200 g soil from an area of 5 cm2 and was kept at 4°C until it was used for the next step. Detection of nematodes was done with the *Galleria mellonella* baiting method (S*8*). Soil was homogenized by hand and each soil sample was placed in a 350 ml glass jar (7cm diam., 12cm deep) with three final instar larvae of *G. mellonella* (Pet Centre, Columbia, MO, USA). After the larvae had been added, the jars were stored upside-down at 25°C in darkness. Five days later, larvae were examined for nematode infection. Larvae with the typical red colour, indicative of the Heterorhabiditidae bacterial symbiont introduced into the host by EPN (S*9*), were recorded as infected. As other families of EPN could also have infected the larvae, the remaining cadavers were dissected and checked for EPN presence/absence.

EβC emission screens

Leaf clippings (~25 cm^2) were sampled from all plants the day before harvesting plants used for

root damage rating (on July 10). These clippings were frozen in the field by placing them in liquid nitrogen and subsequently stored in a -80$_{o}$C freezer. Before chemical analyses, individual leaf samples were ground in liquid nitrogen and about 0.3 g of leaf powder was placed in a 20 ml glass vials. Using a multi-purposesampler (MPS2, Gerstel GmbH & Co.KG, Germany) a 100 mm polydimethylsiloxane solid-phase microextraction fibre (Supelco, Buchs, Switzerland) was inserted in the vial through a septum in the lid and exposed for 60 min at 40 ^{o}C. The compounds adsorbed on the fibre were analysed with an Agilent 6890 Series (G1530A) gas chromatograph coupled to a quadrupole-type mass-selective detector (Agilent 5973; transfer line 230 $_{o}$C, source 230 ^{o}C, ionization potential 70 eV). The fibre was inserted into the injector port (230 ^{o}C, 50 mm), desorbed for 3 min and chromatographed on a DB5-MS column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness; J &W Scientific). Helium at a constant pressure of 18.55 lb in^{-2} (127.9 kPa) was used for carrier gas flow. After fibre insertion, the column temperature was maintained at 60 ^{o}C for 1 min and then increased to 270 ^{o}C at 10 ^{o}C min^{-1} and ended with a final step of 5 min at 270 ^{o}C. Chromatograms were analysed using ChemStation D.00.00.38.

Olfactometer assays

Olfactometer assays were modified after Rasmann et al. (2005) (S*10*). Olfactometers consisted of six glass pots connected via glass tubes to a central pot. Each system of seven pots was filled with 10% moist sand (Migros, Switzerland). A twelve-day old transformed plant (line 202 L2) that had been grown in a climate chamber (Weiss Technik, Switzerland, 16:8 light/dark hours, 25:15 ^{o}C day/night temperature and 60% humidity) in a mix of potted soil and sand was transplanted in sand in one of the outer pots (5 cm diam., 11 cm deep). A similar non-transformed plant (line 197 L1) was placed in another sand-filled pot. The four remaining pots only contained sand. The pots with the plants each received five second instar WCR larvae that were weighed as a group just before on a micro-scale (Mettler-Toledo GmbH, Switzerland). Two days after WCR infestation, the posts were connected to the olfactometer central pot (8 cm diam., 11 cm deep) using a glass connector (8 cm long; 24/29 male connector on both sides, all glassware from VQT-Verre Quartz Technique SA, Switzerland) and a Teflon connector (24/29 female to 29/31 male) containing an ultra-fine mesh metal screen (2300 mesh; Small Parts Inc., FL, USA), which

prevented the nematodes from entering the odour source pots. One day later, about 2,000 *H. megidis* nematodes (Becker Underwood Ltd, UK) were released in the central pot and the following day the olfactometers were disassembled. Sand contained in the glass and Teflon connectors was separately placed on cotton filters (19 cm diam., Hoeschele GmbH, Switzerland). Filters and sand were placed on a Bearmann extractor (S*11,* S*12*) and recovered nematodes were counted the following day.

To evaluate the performance of the WCR larvae on the plants, the larvae were left in the pots for two additional days. Then they were recovered from the pots by

passing sand through a 0.6 mm sieve (Eijkelkamp, The Netherlands). Survival was determined and recovered WCR larvae were again weighed.

The nematodes' choices among the arms of the olfactometers were examined with a log-linear model. The entity computing a repetition in the statistical analysis corresponds to the response of a group of nematodes released, which was shown to follow a multinomial distribution (S*13*). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasilikelihood functions to compensate for the overdispersion of nematodes within the olfactometer (S*14*). WCR larvae performances differences were tested with SPSS 14.0. As normality and homogeneity of variance tests passed, survival and weigh means were compared with a t-test.

S1. B. R. Frame *et al.*, *Plant Physiol.* **129**, 13 (May 1, 2002, 2002).

S2. A. H. Christensen, P. H. Quail, *Transgenic Research* **5**, 213 (May, 1996).

S3. F. Torney *et al.*, *Plant Cell Reports* **22**, 931 (Jul, 2004).

S4. J. D. Oleson, Y. L. Park, T. M. Nowatzki, J. J. Tollefson, *Journal of Economic Entomology* **98**, 1 (Feb, 2005).

S5. C. M. F. Pierce, M. E. Gray, *Journal of Economic Entomology* **100**, 1104 (2007).

S6. R. D. Steel, J. H. Torrie, D. A. Dickey, *Principles and procedures of statistics: a biometrical approach* (McGraw-Hill, New York, ed. 3rd, 1997), pp.

S7. R. C. Littell, P. R. Henry, C. B. Ammerman, *Journal of Animal Science* **76**, 1216 (Apr, 1998).

S8. R. A. Bedding, R. J. Akhurst, *Nematologica* **21**, 109 (1975).

S9. S. Forst, D. Clarke, in *Entomopathogenic Nematology* R. Gaugler, Ed. (CABI International, 2002) pp. 57-77.

S10. S. Rasmann *et al.*, *Nature* **434**, 732 (Apr 7, 2005).

S11. J. Curran, *Journal of Nematology* **24**, 631 (1992).

S12. B. Hass, C. T. Griffin, M. J. Downes, *Journal of Nematology* **31**, 508 (Dec, 1999).

S13. I. Ricard, A. C. Davison, *Journal of the Royal Statistical Society: Series C (Applied Statistics)* **56**, 479 (2007).

S14. T. C. J. Turlings, A. C. Davison, C. Tamo, *Physiological Entomology* **29**, 45 (Mar, 2004).

CHAPTER III

Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes

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*these authors contributed equaly to the work and have to be considered as joint first athours

2008

BioControl
DOI 10.1007/s10526-008-9156-y

Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes

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Received: 20 September 2007 / Accepted: 3 March 2008

Abstract As a first step towards the development of an ecologically rational control strategy against western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) in Europe, we compared the susceptibility of the soil living larvae and pupae of this maize pest to infection by three entomopathogenic nematode (EPN) species. In laboratory assays using sand-filled trays, *Heterorhabditis bacteriophora* Poinar and *H. megidis* Poinar, Jackson & Klein (both Rhabditida: Heterorhabditidae) caused comparable mortality among all three larval instars and pupae of *D. v. virgifera*. In soil-filled trays, *H. bacteriophora* was slightly more effective against third larval instars and pupae, and *H. megidis* against third larval instars, compared to other developmental stages. In both sand and soil, *Steinernema feltiae* (Filipjev) (Rh.: Steinernematidae) was least effective against second instars. In conclusion, all larval instars of *D. v. virgifera* show susceptibility to infection by all three nematodes tested. It is predicted that early application against young larval instars would be most effective at preventing root feeding damage by *D. v. virgifera*. Applications of nematodes just before or during the time period when third instars are predominant in the field are likely to increase control efficacy. According to our laboratory assays, *H. bacteriophora* and *H. megidis* appear to be the most promising candidates for testing in the field.

Keywords *Diabrotica virgifera virgifera* · Coleoptera · Chrysomelidae · *Heterorhabditis bacteriophora* · *Heterorhabditis megidis* · *Steinernema feltiae*

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Handling editor: Ralf-Udo Ehlers

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Introduction

Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) is one of the most serious maize pests in North America and

more recently in Europe (Miller et al. 2005). WCR is a univoltine species with eggs that overwinter in the soil and three larval instars that feed on maize roots (Krysan and Miller 1986). In central Europe, the first instars close in May and the adults emerge between mid June and early August (Toepfer and Kuhlmann 2006). The larvae can cause economic loss due to voracious root feeding and are the main target for control measures. To date, there is no commercially available biological control product against WCR. This is remarkable, as several biological-based approaches are already in practice against other maize pests and could be compromised by the chemical-based control of WCR. Biological control agents are, for example, used against the European corn borer [*Ostrinia nubilalis* Huebner (Lepidoptera: Pyralidae)], the cotton bollworm [*Helicoverpa armigera* Huebner (Lepidoptera: Noctuidae)], the Mediterranean corn stalk borer [*Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae)], and click beetles [*Agriotes* spp. (Coleoptera: Elateridae)] (Toepfer and Kuhlmann 2004). In order to avoid using insecticides against WCR, a biological control approach should be considered (Kuhlmann and Burgt 1998). The first option would be a classical biological control approach involving selection and introduction of specific natural antagonists from the area of origin (Kuhlmann et al. 2005). The second option would be an inundative biological approach using commercially available and native natural antagonists, such as entomopathogenic nematodes (EPNs) (Kuhlmann and Burgt 1998).

Nematodes have successfully been used as biological control agents against a range of different insect pests (Grewal et al. 2005), and have shown potential for controlling WCR larvae (Jackson and Brooks 1989; Jackson 1996; Toepfer et al. 2005). EPNs have several stages within their life cycle. The third stage persists in the soil, where it locates and penetrates the host. These so-called infective dauer juveniles enter the host through the mouth, anus, spiracles or thin parts of the cuticle, which can be pierced by a tooth located in the mouth region of Heterorhabditidae (Adams and Nguyen 2002; Koppenhöfer et al. 2007). Having reached the haemocoel of the insect, the juvenile releases symbiotic bacteria that propagate and kill the host (Byron and Khuong 2002). The EPNs feed on the bacteria and host tissues and reproduce. Infective dauer juveniles develop and then leave the cadaver once it has been consumed.

Nematodes possess traits that make them particularly suitable as biological control agents, such as: their host finding ability (Griffin et al. 2005), specificity of strains (Jackson and Brooks 1989), compatibility with conventional agricultural spraying equipment (Wright et al. 2005), compatibility with most pesticides (Nishimatsu and Jackson 1998; Koppenhöfer and Grewal 2005) and applicability of commercial production techniques in liquid culture (Ehlers 2001). Apart from these positive traits, other factors restrict the use of EPNs, including their higher cost relative to chemical alternatives (Grewal and Peters 2005), and their susceptibility to UV-radiation (Gaugler et al. 1992), high temperatures and desiccation (Glazer 2002). Therefore, EPNs must be applied at dawn in a high volume of water. Even then, between 40% and 80% of the sprayed EPNs may die during the first few hours after application (Smits 1996). Thus, more efficient application methods are needed to maximize EPN field efficacy. One way to increase the efficacy of EPNs is to specifically apply them against the most susceptible developmental stage of the target insect (Wright et al. 1993). This is complicated by the fact that multiple stages of WCR can occur simultaneously in the field (Toepfer and Kuhlmann 2006). Therefore, it would be advantageous if it were proven that EPNs are able to infect all developmental stages to the same degree.

Several field trials have shown an effect of the host developmental stage on the efficacy of EPNs, however, such studies have not yet led to the adoption of a strategy for the use of EPNs against WCR (Thurston and Yule 1990; Jackson and Brooks 1995; Journey and Ostlie 2000). To date, information on the different susceptibilities of larval instars to EPNs has not been considered when developing control strategies against WCR using EPNs. Moreover, information is missing about their susceptibility to the most promising known species and strains of EPNs, which could be considered for use against WCR in Europe (Toepfer et al. 2005).

The three species, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), *H. megidis* Poinar, Jackson and Klein and *S. feltiae*, were chosen for this study, since they are known to kill third instar WCR larvae and are commercially available from liquid cultures (Toepfer et al. 2005). This study aimed to compare the susceptibility of each larval instar and the pupae of WCR to these promising EPN species and

strains. Standard bioassays involving EPN applications to sand- (Peters 2005) or soil-filled trays containing WCR larvae and pupae were used to assess EPN-induced mortality of the different pest life stages. The use of semi-natural conditions i.e. soil-filled trays, allowed determination of optimal EPN application timing relative to WCR phenology. This information will be critical for the development of an effective nematode-based biological control product.

Materials and methods

Source and handling of WCR

WCR eggs were obtained from a laboratory rearing of field-collected beetles in southern Hungary in 2004 and 2005 (25°C day, 15–20°C night, 14L: 10D, 40–60% r.h., procedures see Singh and Moore 1985). Eggs were overwintered in moist sieved sand (<200 µm grains) at 6–8°C. Their diapause was broken in early April of the following year by transferring eggs to 25°C for 20 days. About 200–300 maize grains of the hybrid Magister (UFA Semences, Bussigny, Switzerland) were planted in a plastic tray (300 × 450 mm) with moist potting soil (Garri Plusz, Garri Company, Budapest, Hungary). Five days after planting, eggs with broken diapause were placed into these plastic trays, which were then stored in the dark at 25°C (~5,000 eggs per tray). Larvae and pupae were recovered from the soil for experiments.

Source and handling of EPNs

Three EPN species, produced in liquid culture, were used in this study: (1) a hybrid of European and US strains of *H. bacteriophora* Poinar (Rhabditida: Heterorhabditidae) (2) the NL-HW79 strain of *H. megidis* Poinar, Jackson & Klein (Rh.: Heterorhabditidae) from the Netherlands and re-isolated from Swiss soils, and (3) a hybrid of European strains of *S. feltiae* (Filipjev) (Rh.: Steinernematidae). *Heterorhabditis bacteriophora* and *S. feltiae* were shipped in clay from e-nema GmbH (Raisdorf, Germany) to the experimental sites, and *H. megidis* was shipped in vermiculite from Andermatt Biocontrol, Switzerland. All EPNs were stored in their shipping material at 7–9°C in darkness prior to the experiments. About 2–3 h before application, EPNs together with the carrier material were diluted with tap water to the required concentration.

Susceptibility of WCR to infection by EPNs

Two sets of experiments were conducted: one in trays filled with sand and the other in trays filled with sandy soil. For each set, two distinct series of experiments were conducted, i.e. with different shipments of nematodes. Plastic trays (54 cm^2; 9 × 6 × 5.5 cm) were filled with 200 ml of sterilised river sand (sieved at 200 µm, 15% soil moisture) or sandy soil (sieved at 600 µm, 15% soil moisture, neutral pH, 40–50% sand, 5–10% clay, 5–10% loam, 30–40% organic matter; black mould type potting soil of generic nature from Garri Plusz, Garri Company, Budapest, Hungary). Seeds from the maize hybrid Magister were stored on wet filter paper for three days to initiate germination. One germinated seed was then placed into each tray. Each sand- and soil-filled tray was infested with 10 larvae of either first, second or third instars or with eight pupae of WCR. One day later, infective dauer juveniles of one EPN species were applied at a concentration of 16 individuals per cm^2 equating to 864 EPN in 3 ml tap water per tray. This relatively low concentration of EPNs has been used in previous studies (Toepfer et al. 2005) and was chosen to ensure that differences in the mortality among larval instars and pupae due to EPN infection could be detected. A pipette was used to distribute half of the 3 ml EPN–water mix onto the substrate surface in one corner of the tray and the other half in the opposite corner for optimal distribution. This study therefore considers both host finding ability and pathogenicity (Peters 2000, 2005). Tap water without nematodes was applied to control trays. For the first instars, there were 8–13 replicates per EPN species (+control), per soil type and for each of the two series (Table 1). There were 10–13 replicates for the second and third instars and 12–13 replicates for the pupae (total numbers of replicates in Table 1). The trays were incubated for 1 week at 22°C in darkness to allow EPNs to infect the WCR. The living first instars were recovered by depositing the content of the trays on a Berlese screen for two days and collecting the larvae that dropped into a moist tray beneath the screen. The living and dead second and third instars and pupae were collected by sieving the sand or soil through a 600 µm mesh sieve. Although a large proportion of larvae turned red due to nematode

Table 1 Comparison of EPN species regarding their effect on three larval instars and pupae of WCR

Developmental stage of WCR	EPN species	In sand				In soil			
		% Corrected mortality ± SD	Differences			% Corrected mortality ± SD	Differences		
			P	*Z*	*n*		*P*	*Z*	*n*
First instar	*H. bacteriophora* vs. *H. megidis*	87.8 ± 12.3 vs. 52.5 ± 38.3	**0.00**	−4.73	18/23	37.0 ± 28.8 vs. 27.6 ± 31.1	0.29	−1.05	27/23
	H. bacteriophora vs. *S. feltiae*	87.8 ± 12.3 vs. 57.3 ± 37.6	**0.03**	−2.18	18/17	37.0 ± 28.8 vs. 24.5 ± 22.6	0.12	−1.56	27/24
	H. megidis vs. *S. feltiae*	52.5 ± 38.3 vs. 57.3 ± 37.6	**0.01**	−2.45	23/17	27.6 ± 31.1 vs. 24.5 ± 22.6	0.91	−0.97	23/24
Second instar	*H. bacteriophora* vs. *H. megidis*	80.9 ± 20.1 vs. 64.6 ± 29.2	**0.00**	−4.57	20/28	48.0 ± 30.3 vs. 40.7 ± 27.4	0.30	−1.03	26/28
	H. bacteriophora vs. *S. feltiae*	80.9 ± 20.1 vs. 41.1 ± 35.5	**0.001**	−3.22	20/20	48.0 ± 30.3 vs. 10.6 ± 24.1	**0.00**	−4.00	26/25
	H. megidis vs. *S. feltiae*	64.6 ± 29.2 vs. 41.1 ± 35.5	0.60	−0.51	28/20	40.7 ± 27.4 vs. 10.6 ± 24.1	**0.00**	−3.48	28/25
Third instar	*H. bacteriophora* vs. *H. megidis*	89.7 ± 3.2 vs. 57.4 ± 23.8	**0.00**	−3.72	21/20	64.7 ± 39.1 vs. 70.7 ± 29.7	0.34	−0.95	25/27
	H. bacteriophora vs. *S. feltiae*	89.7 ± 3.2 vs. 71.1 ± 30.1	**0.01**	−2.46	21/20	64.7 ± 39.1 vs. 33.9 ± 22.3	0.15	−1.39	25/25
	H. megidis vs. *S. feltiae*	57.4 ± 23.8 vs. 71.1 ± 30.1	0.09	−1.69	20/20	70.7 ± 29.7 vs. 33.9 ± 22.3	**0.00**	−3.66	27/25
Pupae	*H. bacteriophora* vs. *H. megidis*	64.4 ± 36.5 vs. 46.7 ± 38.9	0.07	−1.82	27/27	55.3 ± 46.7 vs. 30.13 ± 41.5	0.13	−1.51	27/27
	H. bacteriophora vs. *S. feltiae*	64.4 ± 36.5 vs. 47.3 ± 40.3	0.23	−1.22	27/27	55.3 ± 46.7 vs. 45.2 ± 43.3	0.28	−1.09	27/28
	H. megidis vs. *S. feltiae*	46.7 ± 38.9 vs. 47.3 ± 40.3	0.77	−0.30	27/27	30.13 ± 41.5 vs. 45.2 ± 43.3	0.80	−0.26	27/28

Mann–Whitney *U*-test at $P < 0.05$, bold if significant; SD, standard deviation; *n*, total number of assay trays from first/second experimental series in laboratory

infection, the data on infection rates were not used for analyses because many dead WCR larvae had decomposed before recovery. Therefore, the mortality was calculated and corrected by comparing proportions of dead larvae between treatments and controls (Abbott 1925). This allowed pooling of data from each of the two series. A Kolmogorov–Smirnov test (Achim and Zöfel 2000) showed the data to be non-normal (even after arcsine transformation). Therefore, the non-parametric Mann–Whitney test was used to compare the stage-specific mortality among the three EPN species and between sand and soil.

Results

Heterorhabditis bacteriophora caused the greatest mortality of WCR in both sand and soil when considering the corrected mortalities of the pooled developmental stages (*H. bacteriophora* vs. *H. megidis*: $P = 0.001$, $Z = -3.89$; *H. bacteriophora* vs. *S. feltiae*: $P = 0.04$, $Z = -2.06$). *Heterorhabditis megidis* caused higher mortality than *S. feltiae* ($P = 0.034$, $Z = -2.12$). The differences in susceptibility between each developmental stage of WCR varied depending on the EPN species tested (Table 1).

Heterorhabditis bacteriophora killed 37–90% of all WCR larval instars and pupae (Fig. 1 and Table 1), with mortality being significantly higher in sand than in soil (80.7% vs. 51.3%: $P < 0.001$, $Z = -8.53$, $n = 104$). In sand, *H. bacteriophora* caused comparable mortality among the different larval instars (Fig. 1), but pupae were slightly, but significantly, less susceptible than third instars. In soil, however, *H. bacteriophora* caused greater mortality of third instars and pupae than of first and

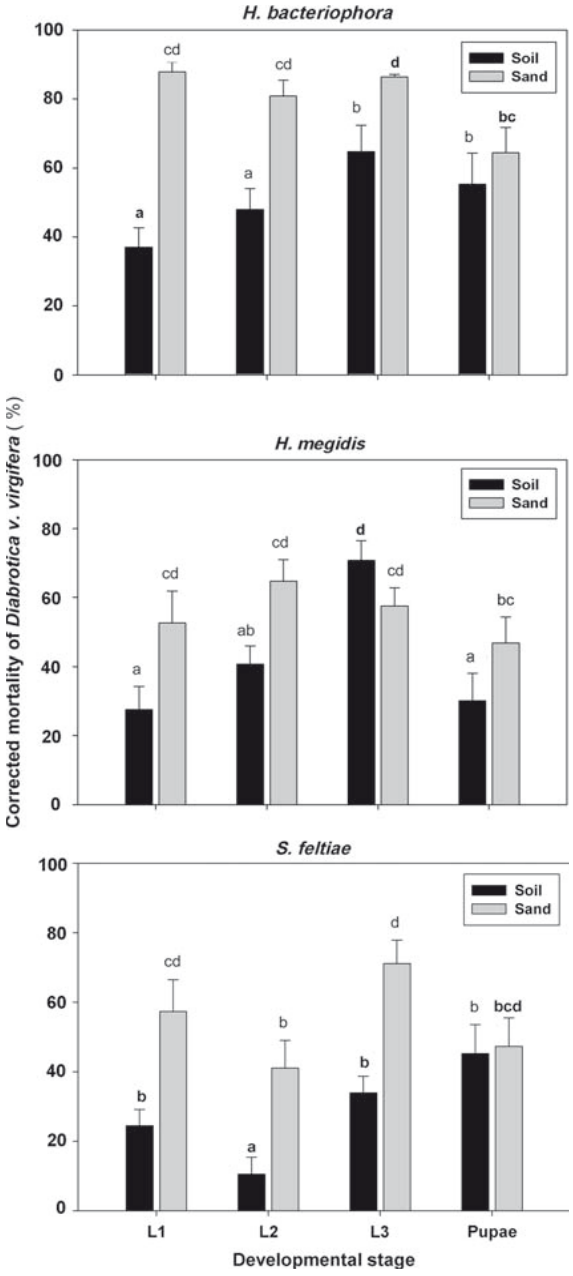

Fig. 1 Susceptibility of first instars (L_1), second instars (L_2), third instars (L_3) and pupae of WCR to infection by *Heterorhabditis bacteriophora*, *Heterorhabditis megidis* and *Steinernema feltiae* in sand- and soil-filled plastic trays. The corrected mortality was calculated as the relative number of dead WCR compared to the control. Letters on columns show significant differences at $P < 0.05$ according to the Mann–Whitney *U*-test. Error bars = standard errors

second instars (Fig. 1). No differences were found between first and second instars or between third instars and pupae.

Heterorhabditis megidis killed 28–71% of all WCR larval instars and pupae (Fig. 1 and Table 1). The mean efficacy of *H. megidis* was the same in sand and soil (51.4% vs. 42.3%: $P = 0.81$, $Z = -1.75$, $n = 105$). In sand, EPN-induced mortality was comparable among all larval instars and pupae while in soil, *H. megidis* killed significantly more third instars than the other life stages (Fig. 1).

Steinernema feltiae killed 11–71% of all WCR larval instars and pupae (Fig. 1 and Table 1). This EPN was in average more effective in sand than in soil (50% vs. 28.6%: $P < 0.001$, $Z = -4.15$, $n = 105$). *Steinernema feltiae* showed a significantly reduced efficacy against second instar larvae (Fig. 1).

The natural mortality of WCR larvae and pupae, as recorded in the control trays, was low (5.6% ± 1.6 SD of L1, 0.4% ± 0.1 L2, 0.8% ± 0.7 L3, 2.4% ± 2.2 pupae in sand; and 2.3% ± 2, 1.7% ± 1.6, 1.4% ± 0.3, 2.7% ± 0.5 in soil).

Discussion

These experiments demonstrate that *H. bacteriophora* and *H. megidis* were more effective in controlling WCR than *S. feltiae* and were able to kill all three larval instars as well as the pupae. However, particularly in soil, they caused greatest mortality of third instars. This is akin to many studies reporting that EPN efficacy can vary with host developmental stage. For example, Journey and Ostlie (2000) reported that the field efficacy of *S. carpocapsae* Weiser (Rh.: Steinernematidae) was higher against second and third instars of WCR compared to first instars. This was supported by laboratory trials of Jackson and Brooks (1995), who reported that first instar larvae and pupae of WCR were less susceptible to *S. carpocapsae* than second and third instars. In contrast, Thurston and Yule (1990) reported that the first instar of *Diabrotica barberi* Smith Lawrence was highly susceptible to *S. feltiae*. Thus, they recommended applying EPNs against the first instar of *Diabrotica barberi* to kill the larvae before they can enter the roots where they may be protected against the attack of EPNs. Also, Koppenhöfer and Fuzy (2004) reported that the efficacy of *H. bacteriophora* against *Anomala orientalis* Waterh. (Coleoptera: Scarabaeidae) decreased from first to third instar. Variations of EPN efficacy with host developmental stage may

result from different host finding ability or virulence of nematode species and strains (Peters 2000).

Nematodes orientate towards stimuli such as, carbon dioxide, long chain alcohols or thiazoles (Gaugler and Campbell 1991; O′Halloran and Burnell 2003), host excretory products (Schmidt and All 1978; Ramos-Rodríguez et al. 2007), temperature gradients (Byers and Poinar 1982) and herbivore-induced plant volatiles (Rasmann et al. 2005). The ability of EPNs to use these cues varies between species with different foraging strategies. *Steinernema feltiae* is known as an intermediate forager that responds poorly to host associated cues (Peters et al. 1996; Campbell et al. 2003). *Heterorhabditis bacteriophora* and *H. megidis* are classified as cruise foragers that respond relatively well to host-associated cues (Grewal et al. 1994). *Heterorhabditis megidis* is additionally attracted to emissions from insect damaged roots (van Tol et al. 2001). One such attractant, the sesquiterpene (E)-β-caryophyllene, is emitted by WCR-damaged maize roots (Rasmann et al. 2005). Caryophyllene diffuses faster in a sandy medium than in soil (Hiltpold, personal observation), which could explain the differences in mortality observed in this study. Volatile emissions by the roots might also vary depending on the larval instar that is feeding on them. This is already known for insects feeding on maize above-ground (Takabayashi et al. 1995). All of these factors might explain why EPNs differ in their ability to kill different larval instars.

The dispersal ability of EPNs may be restricted in dense soils like clay loam or silty clay, but strong movement has been observed in loamy sand or sandy soil (Barbercheck and Kaya 1991; Barbercheck 1992; Boff et al. 2001; Csontos 2002; Portillo Aguilar et al. 1999), similar to the substrates used in this study. Occasionally, oxygen can become a limiting factor for the survival of EPNs in soil with high organic content (Kaya 1990). However, this was not shown to have any impact on the infectivity of different nematodes, including *H. bacteriophora* (Koppenhöfer and Fuzy 2006). Finally, Koppenhöfer and Fuzy (2007) stated that moderate soil moisture, as used in this study, is optimal for nematode infectivity. Therefore, substrate characteristics can probably not explain the differences in EPN performance seen in this study.

If virulence was the main factor determining differences in stage-specific mortality, the same pattern would be expected in sand and in soil. However, in sand, no mortality differences were found among the larval instars or pupae, whereas such differences did occur in soil (Fig. 1). Moreover, the process of infection by EPNs and their pathogenesis is similar for all rootworm instars (Jackson and Brooks 1995).

In conclusion, the differences in stage-specific mortality of WCR seem to be more dependent on the host finding ability of EPNs compared to their virulence. The results imply that EPNs could be applied for field use at any time that larvae or pupae are present. Early application against young larvae should best prevent root feeding damage. Larvae surviving an early EPN treatment could be killed with a later application, especially of *H. bacteriophora*, when third instars are predominant in the field (i.e. June in Hungary). Strong efficacy can be expected against pupae as well. However, the application should not be timed too late, i.e. when adult emergence has already started, because EPNs are significantly less efficient against adult beetles than against larvae (Burgt et al. 1998; Toepfer et al. 2005). The persistence of EPNs in maize fields is generally only 3–5 months (Kurtz et al. 2007). Therefore, they may work better when regularly applied rather than a one time inoculative release (Journey and Ostlie 2000, Kurtz et al. 2007). Based on these results, *H. bacteriophora* and *H. megidis* appear to be the most promising candidates for testing in the field.

Acknowledgements This study was enabled by the support of Ibolya Zseller, Jozsef Gavallier, Kataline Buzas, Erzsebet Dormannsne, Piroska Szabo, Andras Varga and others from the Plant Health Service in Hodmezovasarhely in Hungary. We would like to thank Arne Peters (e-nema GmbH, Raisdorf, Germany), Ralf-Udo Ehlers (Institute for Phytopathology, University of Kiel, Germany) as well as Erich Franck (Andermatt BioControl, Switzerland) for providing nematodes and advice. We would also like to thank Emma Hunt and Wade Jenner (CABI) and Marianne Baroni (University of Neuchatel) for reviewing the manuscript. Funding for this project was received from the CTI Innovation Promotion Agency of the Federal Office for Professional Education and Technology, Bern, Switzerland, and from the COST 850 Action through a short term scientific mission.

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18:265–267

Achim B, Zöfel P (2000) SPSS Version 10 Einführung in die moderne Datenanalyse unter Windows. Addison Wesley, Munich

Adams BJ, Nguyen KB (2002) Taxonomy and systematics. In: Gaugler R (ed) Entomopathogenic nematology. University of Florida, Gainesville, pp 1–33

Barbercheck ME (1992) Effect of soil physical factors on biological control agents of soil insect pests. Fla Entomol 75:539–548

Barbercheck ME, Kaya HK (1991) Effect of host condition and soil texture on host finding by the entomopathogenous nematodes *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) and *Steinernema carpocapsae* (Rhabditida: Steinernematidae). Environ Entomol 20:582–589

Boff MIC, Zoon FC, Smits PH (2001) Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. Entomol Exp Appl 98:329–337

Burgt Wvd, Budai C, Fodor A, Lucskai A, Francke PJ (1998) Parasitism of western corn rootworm adults by *Steinernema* spp. and *Heterorhabditis* spp. In: Proceedings of the 9th meeting of experimental and applied entomologists, Leiden, pp 165–170, 19 December 1997

Byers JA, Poinar GO (1982) Location of insect hosts by the nematode *Neoaplectana carpocapsae* response to temperature. Behaviour 79:1–10

Byron JA, Khuong NB (2002) Taxonomy and systematics. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, Wallingford, pp 1–35

Campbell JF, Lewis EE, Stock SP, Nadler S, Kaya HK (2003) Evolution of host search strategies in entomopathogenic nematodes. J Nematol 35:142–145

Csontos AS (2002) Lateral movement of the entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* in sand at different temperatures in response to host seeking. Biocontrol Sci Technol 12:137–139

Ehlers RU (2001) Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol Biotechnol 56:623–633

Gaugler R, Campbell JF (1991) Selection for enhanced host-finding of scarab larvae (Coleoptera Scarabaeidae) in an entomopathogenic nematode. Environ Entomol 20: 700–706

Gaugler R, Bednarek A, Campbell JF (1992) Large scale inoculative release of the entomopathogenic nematode *Steinernema glaseri* assessment 50 years later. Biol Control 59:155–160

Glazer I (2002) Survival biology. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, Wallingford, pp 169–187

Grewal PS, Peters A (2005) Formulation and quality. In: Grewal PS, Ehlers RU, Shapiro DI (eds) Nematodes as biocontrol agents. CABI Publishing, Wallingford, pp 79–90

Grewal PS, Lewis EE, Gaugler R, Campbell J (1994) Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. Parasitology 108:207–215

Grewal PS, Ehlers RU, Shapiro DI (2005) Nematodes as biocontrol agents. CABI Publishing, Wallingford

Griffin CT, Boemare NE, Lewis EE (2005) Biology and behaviour. In: Grewal PS, Ehlers R-U, Shapiro-Ilan DI (eds) Nematodes as biological control agents. CABI Publishing, Wallingford, pp 47–64

Jackson JJ (1996) Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 82(4): 366–372

Jackson JJ, Brooks M (1989) Susceptibility and immune response of western corn rootworm larvae (Coleoptera: Chrysomelidae) to the entomopathogenic nematode, *Steinernema feltiae* (Rhabditida: Steinernematidae). J Econ Entomol 82:1073–1077

Jackson JJ, Brooks M (1995) Parasitism of western corn rootworm larvae and pupae by *Steinernema carpocapsae*. J Nematol 27:15–20

Journey AM, Ostlie KR (2000) Biological control of the western corn rootworm (Coleoptera: Chrysomelidae) using the entomopathogenic nematode *Steinernema carpocapsae*. Environ Entomol 29:822–831

Kaya HK (1990) Soil ecology. In: Gaugler R, Kaya HK (eds) Entomopathogenic nematodes in biological control. CRC, Boca Raton, pp 281–324

Koppenhöfer AM, Fuzy EM (2004) Effect of white grub developmental stage on susceptibility to entomopathogenic nematodes. J Econ Entomol 97:1842–1849

Koppenhöfer AM, Fuzy EM (2006) Effect of soil type on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *S. glaseri*, *Heterorhabditis zealandica* and *H. bacteriophora*. J Invertebr Pathol 92:11–22

Koppenhöfer AM, Fuzy EM (2007) Soil moisture effects on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *S. glaseri*, *Heterorhabditis zealandica* and *H. bacteriophora*. Appl Soil Ecol 35:128–139

Koppenhöfer AM, Grewal PS (2005) Compatibility and interactions with agrochemicals and other biocontrol agents. In: Grewal PS, Ehlers RU, Shapiro DI (eds) Nematodes as biocontrol agents. CABI Publishing, Wallingford, pp 363–381

Koppenhöfer AM, Grewal PS, Fuzy EM (2007) Differences in penetration routes and establishment rates of four entomopathogenic nematode species into four white grub species. J Invertebr Pathol 94:184–195

Krysan JL, Miller TA (1986) Methods for study of pest *Diabrotica*. Springer, New York

Kuhlmann U, Burgt WACM (1998) Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. Biocontrol News Inf 19:59–68

Kuhlmann U, Toepfer S, Zhang F (2005) Is classical biological control against western corn rootworm in Europe a potential sustainable management strategy? In: Vidal S, Kuhlmann U, Edwards CR (eds) Western corn rootworm ecology and management. CABI Publishing, Wallingford, pp 263–284

Kurtz B, Toepfer S, Ehlers RU, Kuhlmann U (2007) Assessment of establishment and persistence of entomopathogenic nematodes for biological control of western corn rootworm. J Appl Entomol 131(6):420–425

Miller N, Estoup A, Toepfer S, Bourguet D, Lapchin L, Derridj S, Kim KS, Reynaud P, Furlan L, Guillemaud T (2005) Multiple transatlantic introductions of the western corn rootworm. Science (Washington) 310:992

Nishimatsu T, Jackson JJ (1998) Interaction of insecticide, entomopathogenic nematodes, and larvae of the western corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 91:410–418

O′Halloran DM, Burnell AM (2003) An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*. Parasitology 127:375–385

Peters A (2000) Insect based assay for entomopathogenic nematode infectiousness: definitions, guidelines, problems. IOBC/WPRS Bull 23(2):109–114

Peters A (2005) Insect based bioassays. In: Grunder JM (ed) Quality control of entomopathogenic nematodes—COST Action 819. Agroscope FAW Wädenswil, Switzerland, pp 55–72

Peters A, Huneke K, Ehlers RU (1996) Host finding by the entomopathogenic nematodes *Steinernema feltiae*. IOBC/WPRS Bull 19:99–102

Portillo Aguilar C, Villani MG, Tauber MJ, Tauber CA, Nyrop JP (1999) Entomopathogenic nematode (Rhabditida: Heterorhabditidae and Steinernematidae) response to soil texture and bulk density. Environ Entomol 28:1021–1035

Ramos-Rodríguez O, Campbell JF, Christen JM, Shapiro-Ilan DI, Lewis EE, Ramaswamy SB (2007) Attraction behaviour of three entomopathogenic nematode species towards infected and uninfected hosts. Parasitology 134:729–738

Rasmann S, Köllner D, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TCJ (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732–737

Schmidt J, All JN (1978) Chemical attraction of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae) to insect larvae. Environ Entomol 7:605–607

Singh P, Moore RF (1985) Handbook of insect rearing. Elsevier Science, Amsterdam

Smits PH (1996) Post-application persistence of entomopathogenic nematodes. Biocontrol Sci Technol 6:379–387

Takabayashi J, Takahashi S, Dicke M, Posthumus MA (1995) Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore induced synomone by corn plants. J Chem Ecol 21:273–287

Thurston GS, Yule WN (1990) Control of larval northern corn rootworm (*Diabrotica barberi*) with two steinernematid nematode species. J Nematol 22:127–131

Toepfer S, Kuhlmann U (2004) IPM strategies for the western corn rootworm (Coleoptera: Chrysomelidae): current status and potential adaptation to the European maize production systems. Mitt Dtsch Ges Allg Angew Entomol 14:391–398

Toepfer S, Kuhlmann U (2006) Constructing life-tables for the invasive alien maize pest *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. J Appl Entomol 130(4):193–205

Toepfer S, Gueldenzoph C, Ehlers RU, Kuhlmann U (2005) Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. Bull Entomol Res 95:473–482

van Tol RWHM, van der Sommen ATC, van Bezooijen J, Smits PH (2001) Olfactory attraction of *Heterorhabditis megidis* to roots and vine weevil feeding induced root semiochemicals. In: Griffin CT, Burnell AM, Downes MJ, Mulder R (eds) Development in entomopathogenic nematode bacteria research. Office for Official Publications of the EC, Luxembourg, pp 156–163

Wright RJ, Witowski JF, Echtenkamp G, Georgis R (1993) Efficacy and persistence of *Steinernema carpocapsae* (Rhabitidae: Steinernematidae) applied through a center-pivot irrigation system against larval corn rootworms (Coleoptera: Chrysomelidae). J Econ Entomol 86(5):1348–1354

Wright DJ, Peters A, Schroer S, Fife JP (2005) Application technology. In: Grewal PS, Ehlers RU, Shapiro DI (eds) Nematodes as biocontrol agents. CABI Publishing, Wallingford, pp 91–106

CHAPTER IV

How maize roots volatiles influence the efficacy of entomopathogenic nematodes in locating the western corn rootworm?

Chemoecology, in prep

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2008

Abstract

Because the ferocious maize pest *Diabrotica virgifera virgifera* LeConte is adapting or can adapt to all currently used control strategies, focus has turned to the development of more sustainable control methods such as biological control using entomopathogenic nematodes. A good understanding of the biology and behaviour of these potential control agents is essential for their successful employment. Root systems of many maize varieties emit the sesquiterpene (*E*)-β-caryophyllene (EβC) after feeding damage by *D. v. virgifera* larvae and this chemical cue has been shown to attract certain nematodes, thereby enhancing their control potential. In this study, the effect of this root volatile was tested for the field efficacy of the three entomopathogenic nematodes *Heterorhabditis bacteriophora, Heterorhabditis megidis and Steinernema feltiae* against *D. v. virgifera* larvae in southern Hungary. By comparing root damage and beetle emergence for two maize varieties, one that emits EβC and one that does not, it was found that the signal was important for the efficacy of *H. megidis* and *S. feltiae*, but not for *H. bacteriophora*. Overall, all three nematode species showed good control potential and if properly applied and in combination with the right maize line the release of these nematodes can be as effective as other control methods.

Introduction

Since the domestication of maize, *Zea mays* (L.), about 5,000 – 7,000 years ago (Piperno and Flannery 2001, Sluyter and Dominguez 2006), this crop has been targeted by a variety of pests, such as herbivorous arthropods and pathogens, which can cause tremendous yield losses (Oerke 2006). In nature, plants have evolved various defence strategies to fend off their attackers either directly (Baldwin and Preston 1999, Agrawal 1998, Dicke*, et al.* 2003, Karban*, et al.* 1997, Karban and Baldwin 1997, Schoonhoven*, et al.* 1998) or indirectly (Agrawal 1998, Dicke and Sabelis 1998, Dicke*, et al.* 2003, Turlings and Wäckers 2004). Direct defence traits of plants comprise physical or chemical barriers, whereas indirect defences consist of the attraction and maintenance of the herbivore's natural enemies by providing shelter and/or food (Janzen 1966, Stapley 1998) and/or the emission of inducible volatile organic compounds (Dicke*, et al.* 2003, Turlings and Benerey 1998, Turlings and Wäckers 2004). In maize, the attractiveness of such herbivore-

induced plant volatiles to natural enemies of herbivores has been demonstrated in both laboratory and field experiments (Bernasconi*, et al.* 1998, Fritzsche Hoballah and Turlings 2001, Hoballah*, et al.* 2002, Turlings*, et al.* 1990). For instance, green leaf volatiles, as well as terpenoids such as monoterpenes, sesquiterpenes and homoterpenes, have been found to attract parasitoids aboveground (D'Alessandro and Turlings 2005, Hoballah and Turlings 2005).

Recently it was found that roots are able to recruit belowground enemies of soil dwelling herbivorous insects by releasing chemical signals. These chemicals can attract entomopathogenic nematodes (EPN) (Bertin*, et al.* 2003, Rasmann et al. 2005 #90, Boff*, et al.* 2001, Rasmann and Turlings 2008, van Tol*, et al.* 2001), predatory mites (Aratchige*, et al.* 2004) and even parasitoids (Neveu*, et al.* 2002). In maize, terpenoids appear to be important in the root system as well as in the aboveground part of the plant. Maize roots fed upon by larvae of *Diabrotica virgifera virgifera* LeConte (western corn rootworm, WCR, Coleoptera: Chrysomelidae), one of the most destructive maize pests worldwide (Miller*, et al.* 2005, Vidal*, et al.* 2005), release the sesquiterpene (*E*)- β-caryophyllene (EβC). EβC diffuses well in soil (Hiltpold and Turlings 2008) and plays an important role in the recruitment of the EPN *Heterorhabditis megidis* Poinar (Rhabditida: Heterorhabditidae) (Rasmann*, et al.* 2005), which is highly virulent to WCR larvae (Kurzt*, et al.* 2008). Two other species of EPNs, *Heterorhabditis bacteriophora* Poinar and *Steinernema feltiae* Filipjev, are also promising as biological control agents against WCR larvae in Europe (Kurzt*, et al.* 2008, Toepfer*, et al.* 2005), but it is unknown if their host finding ability is also improved by attraction to belowground signals.

The aim of the current study was to determine the relative importance of EβC emission by WCR-damaged maize roots for the efficacy of *H. bacteriophora*, *H. megidis*, and *S. feltiae* in controlling WCR larvae under field conditions. We further examined whether the timing of EPN application was related to control efficacy. The importance of choosing the right maize variety when using nematodes as biological control agents is discussed for each of the tested EPN species. Furthermore, the results prompted us to test in the laboratory the attraction of *H. bacteriophora* towards EβC non-emitting plants.

Materials and Methods

Field sites and maize varieties

The study was carried out in four maize fields (referred to as fields A to D) in Csongrad County in southern Hungary in 2005 and 2006 (Table 1). All fields contained an experimental section that had been planted with non-host plants of WCR the year before to ensure the initial absence of this pest in the experimental plots. One half of each experimental field was planted with the variety Magister (UFA Semences, Bussigny, CH) that emits EβC after WCR feeding (data not shown) and the other half with the variety Pactol (Syngenta, Budapest, HU) that does not emit EβC (Rasmann*, et al.* 2005). The seeds were sown between late April and early May (Table 1). All maize seeds were sown in rows with plant spacing of 150 mm and row spacing of 750 mm. The fields were treated once

Table1. Characteristics of the study fields in southern Hungary and the timing of EPN application (Average soil moisture from May to July of the study year shown).

Field	A	B	C	D
Location	Northwest of Hodmezovasarhely	North of Szatymaz	North of Szatymaz	Hodmezo - vasarhely
Coordinates	N 46° 26.022 E 20° 20.143	N 46° 20.945 E 20° 00.574	N 46° 20.945 E 20° 00.574	N 46° 25.998 E 20° 20.348
Elevation (m)	83	87	87	83
Size (ha)	0.5	0.2	0.3	0.2
Soil bulk density (g/cm3)	1.04 ± 0.13	1.4 ± 0.13	1.7 ± 0.07	1.1 ± 0.13
Soil moisture (w %)	17.2 ± 1.1	11.6 ± 0.3	7.1 ± 2.5	18.5 ± 2.1
Sand content (%)	36	85	85	14
Loam content (%)	34	5	5	44
Clay content (%)	30	10	10	42
pH (H2O)	8.3	8.4	8.4	8.3
Maize sown	25 April 2005	8 May 2005	8 May 2006	28 April 2005
EPN appl ications	25 April 2005 14 June 2005	8 May 2005 15 June 2005	8 May 2006 7 June 2006	28 April 2005 14 June 2005

with 0.16 l of the herbicide Merlin SC (75% Izoxaflutol, Bayer Crop Science) per hectare when maize was at the 3-5 leaves stage. No insecticides were applied.

Entomopathogenic nematodes

Three EPN species were used in this study: (1) a cross of European and US strains of *Heterorhabditis bacteriophora* Poinar provided from liquid culture by e-nema GmbH (Raisdorf, DE), (2) the NL-HW79 strain of *H. megidis* Poinar, Jackson & Klein from the Netherlands, re-isolated from Swiss soils and provided from a semi-liquid culture by Andermatt Biocontrol AG (CH), and (3) a cross of European strains of *Steinernema feltiae* Filipjev provided from liquid culture by e-nema GmbH. *H. bacteriophora* and *S. feltiae* were shipped in clay from the producer to the experimental sites, and *H. megidis* was shipped in vermiculite. All EPNs were stored in their shipping material at 7 to 9 °C in darkness until use. About 2 to 3 hours prior to application, EPNs together with the carrier material were diluted in tap water. Before application, aliquots of EPNs were taken to determine the quality of the shipment batches. For this purpose *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were exposed to nematodes in plastic cups (40 mm diam, 60 mm height). Each cup was filled with 200 g of 10% moist sterilised sand to which five larvae and 100 infective juvenile nematodes were added. Three replicates per EPN shipment batch were used for this assay. After one week in darkness at 22°C, mortality of 80 to 100% was found for all EPN batches, which was considered sufficient for use.

Diabrotica v. virgifera

WCR eggs were obtained from eggs laid by of field-collected beetles from southern Hungary (for procedures see (Singh and Moore 1985). Eggs were kept in diapause in moist sand at 6 to 8°C. The diapause of WCR eggs was broken in early April by transferring them to a climate chamber at 25°C for three weeks. The sand was sieved through a 250 μm mesh to recover the eggs. The eggs were then mixed into a solution of water and 0.15% agar in order to obtain an egg suspension of 38 eggs/ml. Maize plants of each field were infested in early May (1 - 3 leaf stage) with the suspension of viable and ready-to-hatch eggs. Using a standard pipette (Eppendorf Company, Hamburg, DE), 2 ml of the egg suspension were applied into each of two 12 cm deep holes at a distance of 5 to 8 cm from either side of the maize plant (approx. 150 eggs/plant). The larvae were

expected to hatch by mid- to late May and to reach the second larval instar in June (Toepfer and Kuhlmann 2006).

Experimental setup and EPN application

Each of the four fields contained two sections of at least 14 rows of Magister or Pactol maize varieties, respectively. Seven plots of six to seven maize plants were randomly assigned to either the 3rd, 6th, 9th or 12th row of each section, thereby ensuring buffer rows between experimental plots. The three different entomopathogenic nematode species were applied at two different times (early: during sowing in April/May; late: in June, see Table 1). Thus six rows, each containing one nematode species applied at one particular date, were distributed in one experimental block. The seventh row served as control and was not treated with any nematodes.

EPNs were poured by hand in a continuous stream at about 10 cm depth into the soil directly along each row. When applied at the earlier date in April/May, this was done at the same time as maize was hand-sown. Suspended in 0.2 litres of water, 2.1x105 ± 0.07 SD infective juvenile nematodes were applied per meter. At the later EPN application date in June, they were suspended in 0.2 litres of water and 2.6x105 ± 0.07 SD infective juvenile nematodes were applied per meter. All applications were carried out in the evening or during cloudy afternoons to avoid damage to the EPNs from UV radiation.

Effects of EPN application, EPN species, and maize variety on WCR adult emergence

Each 14 experimental plots (6-7 plants) of fields A to C (because of technical problems, emergence was not assessed in field D) were covered with fine-mesh screen cages (1.3 m height x 0.75 m width x 1.5 m length, maize plants had been cut to a height of 1 m). WCR adult emergence within these cages was recorded weekly between June 20 and August 16, 2005 and between June 27 and August 16, 2006. Total adult emergence was normalised to100 eggs per plant. The efficacy of EPN was calculated as percentage reduction in WCR adults compared to their untreated controls (corrected efficacy % = (1- WCR in treated plots / WCR in the control) * 100) (Abbott 1925).

Effect of EPN application, EPN species, and maize variety on root damage by D. v. virgifera

In mid-September, after adult emergence was completed, field cages were removed and

all plants of each plot were dug up. Plants from field D were also used for this part of the experiment. Soil and other particles were removed from the roots using a high-pressure water sprayer. Damage was rated according to Oleson's Node Injury Scale from 0.00 to 3.00 with 0.00 being no damage and 3.00 being three or more damaged root nodes (Oleson, *et al.* 2005).

The efficacy of EPNs was calculated as percentage reduction in root damage compared to the overall controls (corrected efficacy % = (1- root damage in treated plots / root damage in the control) * 100) (Abbott 1925)

Olfactometer assays

Following the methodology developed by (Rasmann, *et al.* 2005), the attraction of the *H. bacteriophora* was tested in six belowground olfactometers filled with moist sand. EPNs had to choose between three treatments, a Pactol maize plant damaged by four WCR larvae, a healthy Pactol maize plant and a control treatment consisting of four empty pots. After three days of exposure, the olfactometers were disassembled, the sand from the connectors was placed on a Baermann extractor (Hass, *et al.* 1999), and the next day, nematodes were counted under a microscope on a counting plate.

Statistical analyses

The effect of the tested parameter (EPN species, application periods and maize varieties) on reduction of WCR emergence and root damage was analysed comparing EPN average efficacies using a three-way ANOVA. Then EPN species, maize varieties and application periods were compared using Tukey's post-hoc tests.

All statistical tests of field data were performed in SAS 9.1. Comparisons were done using a three-way ANOVA (GLM procedure) with EPN species, application period, maize variety, EPN species*application period, EPN species*maize variety, application period*maize variety and EPN species*application period*maize variety as independent variables and WCR emergence (relative to control) and node injury rate (relative to control) as dependent variables. Differences were analyzed using LSMEANS with Tukey–Kramer adjustments for the *p-values* (SAS 9.1.).

The nematodes' behavioural responses in the six-arm olfactometer were tested with a log-linear model. The entity computing a repetition in the statistical analysis corresponds to the response of a group of 2000 nematodes

released, which was shown to follow a multinomial distribution. As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasilikelihood functions to compensate for the over dispersion of nematodes within the olfactometer (Turlings*, et al.* 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings*, et al.* 2004).

Results

Effectof EPN application, EPN species, and maize variety on WCR adult emergence

All tested EPN species were able to significantly reduce the percentage of emerging *D. v. virgifera*, and the time of application had no major effect on their respective efficacies (Fig. 1, Table 2).

The choice of the maize variety, i.e. an EβC-emitting or non-emitting variety, had a significant effect on the adult emergence (Fig. 1, Table 2) with a lower emergence of WCR from rows planted with the EβC-emitting Magister than with the non-emitting Pactol. Particularly, *H. megidis* reduced WCR 2.5-fold more in Magister plots than in Pactol plots. No significant differences in the efficacy of *H. bacteriophora* and *S. feltiae* were detected between the two maize varieties (Fig. 1, Table 2, 3-way ANOVA).

On average, the reduction of WCR emergence was higher from plots treated with

Table 2 Effects of EPN species, application period and maize variety on WCR adult emergence (% efficacy relative to control) according to the 3-way ANOVA. Significance is indicated by an asterisk.

Factor	Sum of squares	DF	Mean of squares	F	*p*
EPN species	2.10	2	1.05	14.41	<0.001***
Application period	0.02	1	0.02	0.32	0.567
Maize variety	0.55	1	0.55	7.57	0.007**
EPN species x application period	0.05	2	0.02	0.30	0.735
EPN species x maize variety	0.03	2	0.02	0.23	0.795
Application period x maize variety	0.00	1	0.00	0.02	0.863
EPN species x application period x maize variety	0.04	2	0.02	0.23	0.788

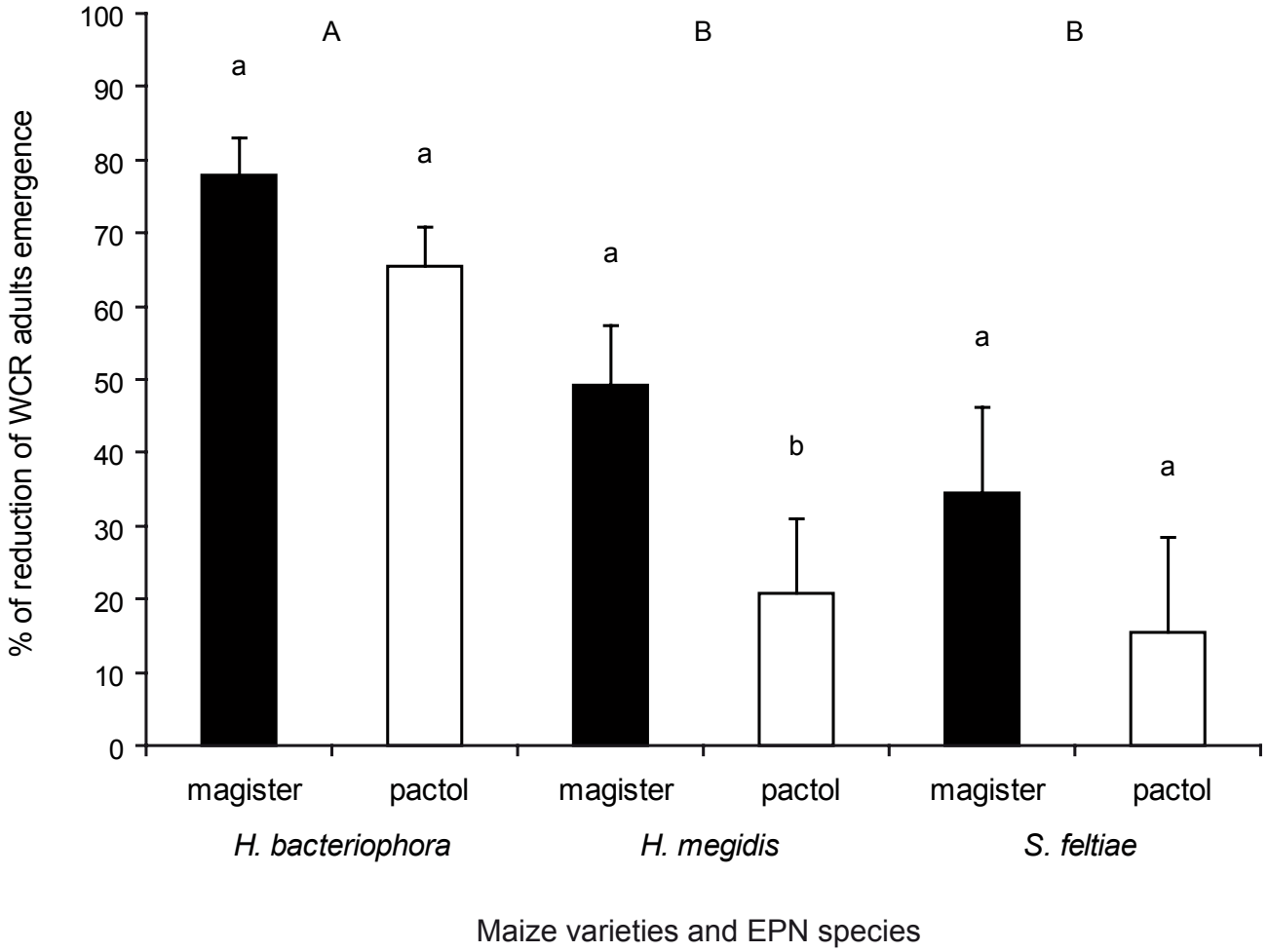

*Figure 1 Comparison of the reduction of WCR emergence after early EPN application relative to the untreated controls in maize fields with the E*β*C-emitting Magister variety and the non-emitting Pactol variety. Uppercase letters indicate statistical differences between the three EPN species (Tukey post-hoc test, H.* bacteriophora *vs.* H. megidis *p<0.001,* H. bacteriophora *vs.* S. feltiae *P<0.001 and* H. megidis *vs.* S. feltiae *p=0.70). Lowercase letters above bars indicate statistical differences between maize varieties within each EPN species (Tukey post-hoc test,* H. bacteriophora *Magister vs. Pactol p=0.08,* H. megidis *Magister vs. Pactol p<0.001,* S. feltiae *Magister vs. Pactol p=0.12). Error bars represent the standard error of mean.*

H. bacteriophora than from plots treated with either *H. megidis* or *S. feltiae* (Fig. 1, Table 2, 3-way ANOVA). This was reflected in an average WCR emergence per plant, 0.65 and 0.75 adult WCR per 100 eggs from the *H. megidis* and *S. feltiae* treated rows respectively, versus 0.28 adults in the *H. bacteriophora* treated Magister rows (1.13 WCR adults emerged per plant from control rows). In Pactol rows, 0.3 WCR adults emerged when treated with *H. bacteriophora*, while on average 0.8 WCR adults emerged when treated with *H. megidis* or with *S. feltiae* (1.60 WCR adults emerged per plant from control rows).

Table 3 Effects of EPN species, application period and maize variety on WCR's root damage (% efficacy relative to control) according to the 3-way ANOVA. Significance is indicated by an asterisk.

Factor	Sum of squares	DF	Mean of squares	F	*p*
EPN species	16.13	2	8.07	4.07	0.017*
Application period	0.00	1	0.00	0.00	0.957
Maize variety	15.25	1	13.25	7.81	0.005**
EPN species x application period	10.04	2	5.02	0.41	0.663
EPN species x maize variety	1.56	2	1.56	2.53	0.080
Application period x maize variety 1	1.63		0.82	0.78	0.377
EPN species x application period x maize variety	0.44	2	0.22	0.11	0.894

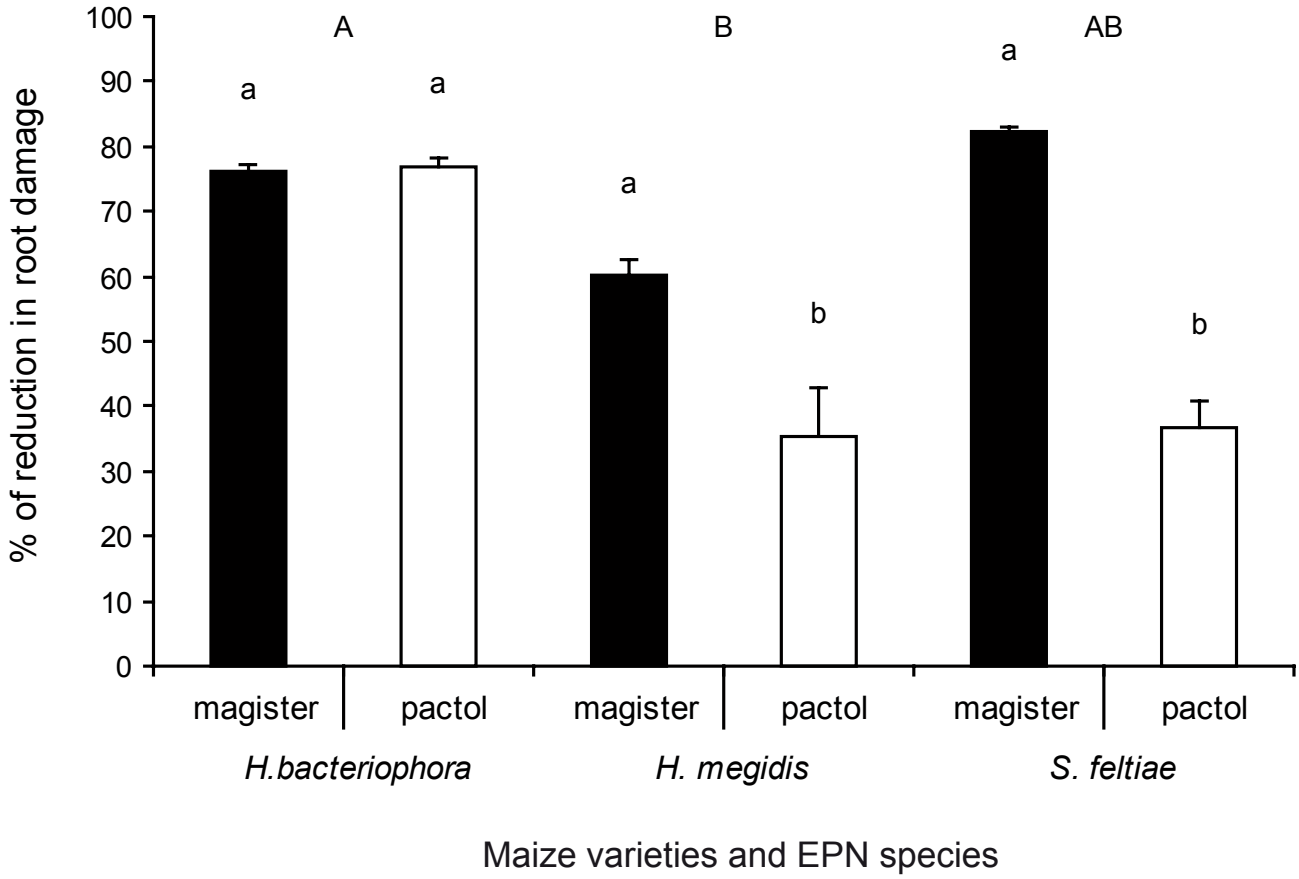

Figure 2 Comparison of the reduction of root damage after late EPN application relative to the untreated controls in maize fields with the EβC-emitting Magister variety and with a non-emitting Pactol variety. Uppercase letters indicate statistical differences between the three EPN species (Tukey post-hoc test, H. bacteriophora *vs.* H. megidis *p=0.012,* H. bacteriophora *vs.* S. feltiae *p=0.226 and* H. megidis *vs.* S. feltiae *p=0.480). Lowercase letters above bars indicates statistical differences between maize varieties within each EPN species (Tukey post-hoc test,* H. bacteriophora *Magister vs. Pactol p=1.00,* H. megidis *Magister vs. Pactol p=0.042,* S. feltiae *Magister vs. Pactol p=0.024). Error bars represent the standard error of mean.*

Effect of EPN application, EPN species, and maize variety on root damage by D. v. virgifera

All tested EPN species were able to reduce the root damage caused by WCR larvae, and the time of application had no effect on their efficacies (Fig. 2, Table 3). However, the type of maize variety influenced the efficacy of the nematodes (Fig. 2, Table 3). This was mainly attributed to *H. megidis* and *S. feltiae*, which reduced root damage to a higher degree in rows with the EβC-emitting Magister than in rows with the non-emitting Pactol when applied in June (Fig. 2, Table 3, 3-way ANOVA).

Olfactometer assays

When offered choice between volatiles emitted by a WCR damaged Pactol maize plant or a healthy plant, *H. bacteriophora* preferred the arm with the pest feeding on the roots (Fig. 3, ANOVA, $F_{2,33}$=6.6, p<0.001). Surprisingly, the healthy plants appear to be repellent compared to the control pots with sand only.

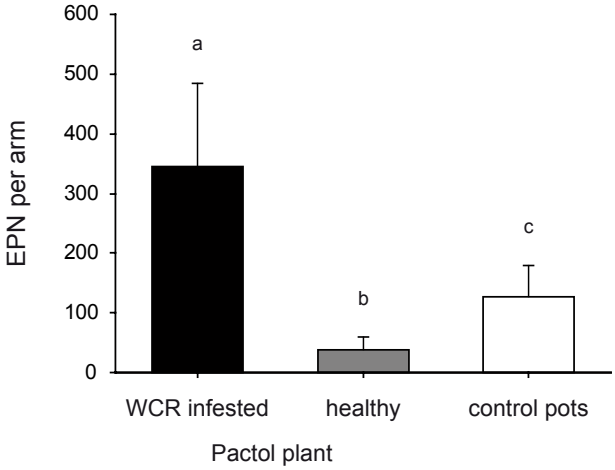

Figure 3 H. bacteriophora *is attracted by EβC non-emitting plants. When offered choice between a healthy, a WCR damaged Pactol plant or sand, this nematode species is significantly attracted towards the damaged plant even if no EβC is emitted (ANOVA, $F_{2,33}$=6.6, p<0.001). The healthy plant seems to repel* H. bacteriophora *compared to the control pots filled with sand only. These results let speculate the emission of other volatile attractants, remaining unidentified, beside EβC. Letters indicate statistical differences. Error bars represent the standard error of mean.*

Discussion

The findings of this study demonstrate that the choice of maize variety and/or nematode species can significantly affect the control efficacy of EPNs in the field. In the laboratory, the efficacy of the three nematode species against WCR was already shown by Kurzt*, et al.* (2008) and the field results confirm the superior WCR susceptibility to *H. bacteriophora* that was found in the laboratory. Surprisingly, the application period, i.e. during sowing in April/May or later in June, did not influence the efficacy of any EPN species (Table 1 & 2). EPN persistence has been shown to rapidly decrease with time (Kurtz*, et al.* 2007), but apparently some early applied nematodes persisted and were still abundant enough to reduce the later hatching

WCR population. A possible explanation is that some early nematodes first found alternative hosts and produced a new generation that later attacked the WCR larvae. For inundative biological control strategies, it will be essential to find the optimal dose and release timing (Fenton*, et al.* 2002).

The choice of the right maize variety appears to be particularly important for two of the three EPN species investigated, *H. megidis* and *S. feltiae* (Fig. 1 & 2). That *H. megidis* was more effective near the EβC-emitting Magister variety was expected from the results of previous studies (Rasmann*, et al.* 2005, Rasmann and Turlings 2007). However, that this was also the case for *S. feltiae* was surprising*,* as *S. feltiae* is considered to use the so-called ambusher (nictating) strategy, but is also known to actively movement through soil (Grewal*, et al.* 1994, Lewis 2002). *S. feltiae* never responded to any investigated cues in olfactometer experiments ((Rasmann and Turlings 2008); personal observations), suggesting that they were not very mobile or not responding to the compounds tested*. S. feltiae* has been shown to be effective against WCR (Kurzt*, et al.* 2008) and from our results it appears that they also react to root signals under field conditions. Following these data, it appears that *S. feltiae* foraging behaviour is highly influenced by the media in which it has to ambush or cruise.

Control effectiveness of *H. bacteriophora* was apparently not affected by EβC emission from WCR-damaged maize roots (Fig. 1 & 2). Similar results were obtained in laboratory bioassays and *H. bacteriophora* appears to use other root signals. When offered a Pactol plant infested with WCR larvae (no emission of EβC) or a healthy Pactol plant, *H. bacteriophora* migrated more toward the damaged plant (Fig. 3). Moreover, several olfactometer assays indicate that healthy maize roots are repellent to *H. bacteriophora* ((Rasmann and Turlings 2008), Fig. 3). It remains unknown what signals allow *H. bacteriophora* to make this distinction, but it has been shown that it is sensitive to long-chain alcohols and possibly other insect-specific volatiles (O'Halloran and Burnell 2003). We also have found that *H. bacteriophora* can be selected to respond to sesquiterpenes (Hiltpold*, et al.* in prep.), which may help them to be more efficient against WCR feeding on EβC-emitting maize varieties.

Currently used WCR management strategies are crop rotation and chemical control (Levine and Oloumi-Sadeghi 1991),

but WCR has shown the ability to evolve resistance to both these methods (Ball and Weekman 1962, Meinke*, et al.* 1998, Zhou*, et al.* 2002) (Levine*, et al.* 2002, O'Neal*, et al.* 2001). Moreover, soil insecticides that are still effective pose environmental and human health risks. Recently, genetically modified maize expressing Cry3 proteins, a *Bacillus thuringiensis* toxin against WCR larvae, has become available on US market (Moellenbeck*, et al.* 2001). Bt maize appears to be effective against WCR, reducing populations of by 80-96% in the field/lab (Siegfried*, et al.* 2005, Storer*, et al.* 2006, Vaughn*, et al.* 2005). This high but incomplete efficacy can be expected to lead to rapid resistance to Bt-maize in WCR populations. While some models estimate that resistance will not occur until at least 20 years after farmers start growing Bt maize with 5-10% refuge (Storer*, et al.* 2006), other models have shown that resistance developed within three generations under greenhouse conditions (Meihls*, et al.* 2008).

In the current study we have shown that the synergetic effect of using the appropriate EPN species combined with attractive maize varieties can result in a control of WCR that is almost as effective as the use of pesticides or Bt maize (Fig. 1 &2). WCR populations are unlikely to be able to develop resistances against EPNs. Moreover, EPNs are able to infect and kill all the larval instars of WCR (Jackson and Brooks 1995, Kurzt*, et al.* 2008, Toepfer*, et al.* 2005), whereas transgenic maize seems to be efficient only against the first instar (Oyediran*, et al.* 2005). Neonate WCR larvae appear can survive on neighbouring weed roots and as second instar larvae could move back to the Bt maize roots on which they can survive (Moeser and Vidal 2004, Oyediran*, et al.* 2005). EPN will also be effective against WCR larvae on roots of other plants (Christen*, et al.* 2007, Gaugler and Campbell 1991, Rae*, et al.* 2006, Ramos-Rodriguez*, et al.* 2007).

In conclusion, the efficacy of the tested EPN species in controlling WCR populations is promising. Based on our findings, it should be possible for farmers to match their crops with the most effective nematode. As the application period of EPN does not seem to influence their efficacy, for convenience, farmers could apply EPN even during sowing. Further studies are needed to take optimal advantage of the biology and behavioural plasticity of EPN to maximize their persistence and their responses to chemical signals in the soil.

Acknowledgements

This work was possible due to the hospitality of the Plant Health Service in Hodmezovasarhely in Hungary, offered by Ibolya Zseller, Jozsef Gavallier, Kataline Buzas, Erzsebet Dormannsne, Piroska Szabo, Andras Varga and others. We would like to also thank our summer students Bobe Kovacs, Benedikt Kurtz, and Ferenc Koncz for their help in field work; Arne Peters (e-nema GmbH, Germany) as well as Erich Frank (Andermatt Biocontrol, Switzerland) for providing nematodes. This study was funded by the CTI Innovation and Technology Fund of Switzerland in collaboration with Landi REBA Basel and e-nema GmbH, Raisdorf, Germany.

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18: 265-267

Agrawal AA (1998) Induced responses to herbivory and increased plant performance. Science 29: 1201-1202

Aratchige NS, Lesna I , Sabelis MW (2004) Below-ground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. Experimental and Applied Acarology 33: 21-30

Baldwin IT , Preston CA (1999) The eco-physiological complexity of plant responses to insect herbivores. Planta 208: 137-145

Ball HJ , Weekman GT (1962) Insecticide resistance in the adult western corn rootworm in Nebraska. Journal of Economic Entomology 55: 439-441

Bernasconi ML, Turlings TCJ, Ambrosetti L, Bassetti P , Dorn S (1998) Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis*. Entomologia Experimentalis et Applicata 87: 133-142

Bertin C, Yang X , Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. Plant and Soil 256: 67-83

Boff MIC, Zoon FC , Smits PH (2001) Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. Entomologia Experimentalis et Applicata 98: 329-337

Christen JM, Campbell JF, Lewis EE, Shapiro-Ilan DI , Ramaswamy SB (2007) Responses of the entomopathogenic nematode, *Steinernema riobrave* to its insect hosts, *Galleria mellonella* and *Tenebrio molitor*. Parasitology 134: 889-898

D'Alessandro M , Turlings TCJ (2005) *In Situ* modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. Chem Senses 30: 739-753

Dicke M , Sabelis MW (1998) How plants obtain predatory mites as bodyguards. Netherlands Journal of Zoology 38: 148-165

Dicke M, van Poecke RMP , de Boer JG (2003) Inducible indirect defence of plants: from mechanisms to ecological functions. Basic And Applied Ecology 4: 27-42

Fenton A, Gwynn RL, Gupta A, Norman R, Fairbairn JP , Hudson PJ (2002) Optimal application strategies for entomopathogenic nematodes: integrating theoretical and empirical approaches. Journal of Applied Ecology 39: 481-492

Fritzsche Hoballah ME , Turlings TCJ (2001) Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. Evolutionary Ecology Research 3: 553-565

Gaugler R , Campbell JF (1991) Selection for enhanced host-finding of scarab larvae (Coleoptera, Scarabaeidae) in an entomopathogenic nematode. Environmental Entomology 20: 700-706

Grewal PS, Lewis EE, Gaugler R , Campbell JF (1994) Host finding behavior as a predictor of foraging strategy in entomopathogenic nematodes. Parasitology 108: 207-215

Hass B, Griffin CT , Downes MJ (1999) Persistence of *Heterorhabditis* infective juveniles in soil: Comparison of extraction and infectivity measurements. Journal of Nematology 31: 508-516

Hiltpold I, Baroni M, Toepfer S, Kuhlmann U , Turlings TCJ (in prep.) Selection for enhanced responsiveness of entomopathogenic nematodes to a volatile root signal can help to control the western corn rootworm.

Hiltpold I , Turlings TCJ (2008) Belowground chemical signalling in maize: when simplicity rhymes with efficiency. Journal of Chemical Ecology 34: 628-635

Hoballah ME , Turlings TCJ (2005) The role of fresh versus old leaf damage in the attraction of parasitic wasps to herbivore-induced maize volatiles. Journal of Chemical Ecology 31: 2003-2018

Hoballah MEF, Tamo C , Turlings TCJ (2002) Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: Is quality or quantity important? Journal of Chemical Ecology 28: 951-968

Jackson JJ , Brooks MA (1995) Parasitism of western corn rootworm larvae and pupae by *Steinernema carpocapsae*. Journal of Nematology 27: 15-20

Janzen DH (1966) Coevolution of mutualism between ants and acacias in Central America. Evolution 20: 249-&

Karban R, Agrawal AA , Mangel M (1997) The benefits of induced defenses against herbivores. Ecology 78: 1351-1355

Karban R , Baldwin I (1997) Induced responses to herbivory. Chicago: University Press of Chicago

Kurtz B, Toepfer S, Ehlers RU , Kuhlmann U (2007) Assessment of establishment and persistence of entomopathogenic nematodes for biological control of western corn rootworm. Journal of Applied Entomology 131: 420-425

Kurzt B, Hiltpold I, Turlings TCJ, Kuhlmann U , Toepfer S (2008) Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes. Biocontrol online:

Levine E , Oloumi-Sadeghi H (1991) Management of Diabroticite rootworms in corn. Annual Review of Entomology 36: 229-255

Levine E, Spencer JL, Isard SA, Onstad DW , Gray ME (2002) Adaptation of the western corn rootworm, *Diabrotica vigifera virgifera* LeConte (Coleoptera: Chrysomelidae), to crop rotation: evolution of a new strain in response to a cultural management practice. Am Entomol 48: 94-107

Lewis EE (2002) Behavioural Ecology. P^Pp 205-223 in Gaugler R (ed)^(eds) Entomopathogenic Nematology: CABI International

Meihls LN, Higdon ML, Siegfried BD, Spencer TA, Miller NK, Sappington TW, Ellersieck MR , Hibbard BE (2008) Increased survival of western corn rootworm, *Diabrotica virgifera virgifera* LeConte, on transgenic corn in three generations of on-plnat greenhouse selection. PNAS in press:

Meinke LJ, Siegfried BD, Wright RJ , Chandler LD (1998) Adult susceptibility of Nebraska western corn rootworm (Coleoptera : Chrysomelidae) populations to selected insecticides. Journal of Economic Entomology 91: 594-600

Miller N, Estoup A, Toepfer S, Bourguet D, Lapchin L, Derridj S, Kim KS, Reynaud P, Furlan L , Guillemaud T (2005) Multiple transatlantic introductions of the western corn rootworm. Science 310: 992-992

Moellenbeck DJ, Peters ML, Bing JW, Rouse JR, Higgins LS, Sims L, Nevshemal T, Marshall L, Ellis RT, Bystrak PG, Lang BA, Stewart JL, Kouba K, Sondag V, Gustafson V, Nour K, Xu DP, Swenson J, Zhang J, Czapla T, Schwab G, Jayne S, Stockhoff BA, Narva K, Schnepf HE, Stelman SJ, Poutre C, Koziel M , Duck N (2001) Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. Nature Biotechnology 19: 668-672

Moeser J , Vidal S (2004) Do alternative host plants enhance the invasion of the maize pest *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae, Galerucinae) in Europe? Environmental Entomology 33: 1169-1177

Neveu N, Grandgirard J, Nenon JP , Cortesero AM (2002) Systemic release of herbivore-induced plant volatiles by turnips infested by concealed root-feeding larvae *Delia radicum* L. Journal of Chemical Ecology 28: 1717-1732

O'Halloran DM , Burnell AM (2003) An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*. Parasitology 127: 375-385

O'Neal ME, Gray ME, Ratcliffe S , Steffey KL (2001) Predicting western corn rootworm (Coleoptera : Chrysomelidae) larval injury to rotated corn with pherocon AM traps in soybeans. Journal of Economic Entomology 94: 98-105

Oerke EC (2006) Crop losses to pests. Journal of Agricultural Science 144: 31-43

Oleson JD, Park YL, Nowatzki TM , Tollefson JJ (2005) Node-injury scale to evaluate root injury by corn rootworms (Coleoptera : Chrysomelidae). Journal of Economic Entomology 98: 1-8

Oyediran IO, Hibbard BE , Clark TL (2005) Western corn rootworm (Coleoptera: Chrysomelidae) beetle emergence from weedy Cry3Bb1 rootworm-resistant transgenic corn. Journal of Economic Entomology 98: 1679-1684

Piperno DR , Flannery KV (2001) The earliest archaeological maize (*Zea mays* L.) from highland Mexico: New accelerator mass spectrometry dates and their implications. PNAS 98: 2101-2103

Rae RG, Robertson JF , Wilson MJ (2006) The chemotactic response of *Phasmarhabditis hermaphrodita* (Nematoda : Rhabditida) to cues of *Deroceras reticulatum* (Mollusca : Gastropoda). Nematology 8: 197-200

Ramos-Rodriguez O, Campbell JF, Christen JM, Shapiro-Ilan DI, Lewis EE , Ramaswamy SB (2007) Attraction behaviour of three entomopathogenic nematode species towards infected and uninfected hosts. Parasitology 134: 729-738

Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J , Turlings TCJ (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434: 732-737

Rasmann S , Turlings TCJ (2007) Simultaneous feeding by aboveground and belowground

herbivores attenuates plant-mediated attraction of their respective natural enemies. Ecology Letters 10: 926-936

Rasmann S , Turlings TCJ (2008) First insights into specificity of below ground tritrophic interactions. Oikos 117: 362-369

Schoonhoven LM, Jermy T , Loon JJAv (1998) Insect-plant biology, from physiology to evolution. Lodon: Chapman & Hall

Siegfried BD, Vaughn TT , Spencer T (2005) Baseline susceptibility of western corn rootworm (Coleoptera : Crysomelidae) to Cry3Bb1 *Bacillus thuringiensis* toxin. Journal of Economic Entomology 98: 1320-1324

Singh P , Moore RF (1985) Handbook of Insect Rearing. Elsevier Science Lt

Sluyter A , Dominguez G (2006) Early maize (*Zea mays* L.) cultivation in Mexico: Dating sedimentary pollen records and its implications. PNAS 103: 1147-1151

Stapley L (1998) The interaction of thorns and symbiotic ants as an effective defence mechanism of swollen-thorn acacias. Oecologia 115: 401-405

Storer NP, Babcock JM , Edwards JM (2006) Field measures of western corn rootworm (Coleoptera: Chrysomelidae) mortality caused by Cry34/35Ab1 proteins expressed in maize event 59122 and implications for trait durability. Journal of Economic Entomology 99: 1381-1387

Toepfer S, Gueldenzoph C, Ehlers RU , Kuhlmann U (2005) Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. Bulletin of Entomological Research 95:

Toepfer S , Kuhlmann U (2006) The life-table of the invasive alien *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Central Europe. Journal of Applied Entomology 1033:

Turlings TCJ , Benerey B (1998) Effects of plant metabolites on the behavior and development of parasitic wasps. Ecoscience 5: 321-333

Turlings TCJ, Davison AC , Tamò C (2004) A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiological Entomology 29: 45-55

Turlings TCJ, Tumlinson JH , Lewis WJ (1990) Exploitation of herbivore-induced plant odors

by host-seeking parasitic wasps. Science 250: 1251-1253

Turlings TCJ , Wäckers F (2004) Recruitment of predators and parasitoids by herbivore injured-plants. P^Pp in Press CU (ed)^(eds) Advances in Insect Chemical Ecology:

van Tol RWHM, van der Sommen ATC, Boff MIC, van Bezooijen J, Sabelis MW , Smits PH (2001) Plants protect their roots by alerting the enemies of grubs. Ecology Letters 4: 292-294

Vaughn T, Cavato T, Brar G, Coombe T, DeGooyer T, Ford S, Groth M, Howe A, Johnson S, Kolacz K, Pilcher C, Purcell J, Romano C, English L , Pershing J (2005) A method of controlling corn rootworm feeding using a *Bacillus thuringiensis* protein expressed in transgenic maize. Crop Science 45: 931-938

Vidal S, Kuhlmann U , Edwards R (2005) Western corn rootworm: ecology and management. CABI publishers, Wallingford, United Kingdom

Zhou XG, Scharf ME, Parimi S, Meinke LJ, Wright RJ, Chandler LD , Siegfried BD (2002) Diagnostic assays based on esterase-mediated resistance mechanisms in western corn rootworms (Coleoptera: Chrysomelidae). Journal of Economic Entomology 95: 1261-1266

CHAPTER V

Selection of entomopathogenic nematodes for a better responsiveness to a root volatile signal can help to control the western corn rootworm

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2008

Abstract

The efficacy of natural enemies as biological control agents against insect pests can be enhanced by breeding for improved responsiveness to foraging cues. The recent identification of a specific root-produced volatile serving as a key-attractant for entomopathogenic nematodes has opened the way to explore if such selection can improve the control of the important maize pest *Diabrotica virgifera virgifera*. Previously effective methods against the root-feeding larvae of this beetle are no longer management options in certain areas because of resistant variants of the insect. Among considered alternatives is the release of entomopathogenic nematodes showing high potential in killing *D. v. virgifera* larvae. Induced maize roots damaged by these larvae emit (*E*)-β-caryophyllene, a sesquiterpene highly attractive to some species of entomopathogenic nematodes. Among available nematode species, *Heterorhabditis bacteriophora* is the most infectious against this pest larva. However, the highly variable attraction of *H. bacteriophora* towards (*E*)-β-caryophyllene could compromise its effectiveness as a control agent. To overcome this drawback, we used a six-arm belowground olfactometer to select for a *H. bacteriophora* strain more readily attracted towards (*E*)-β-caryophyllene. After six generations of selection, the selected strain moved twice as rapidly towards (*E*)-β-caryophyllene in the belowground olfactometers than the original. This new strain was tested in the field and was significantly more effective in reducing pest larvae impacts in plots with a maize hybrid that releases (*E*)-β-caryophyllene than the original strain. As expected, there was no difference between the strains in controlling *D. v. virgifera* larvae in plots with a maize variety that doesn't emit (*E*)-β-caryophyllene. These results illustrate the high potential of manipulating entomopathogenic nematodes to improve their effectiveness as biological control agents against insect pests.

Introduction

The idea to improve biological control by enhancing the foraging efficiency, persistence and killing power of biological control agents has been around for some time (Hoy 1976). Attempts to improve traits like temperature tolerance, host range, sex ratio or resistance to pesticides (Beckendorf and Hoy 1985) through

selection and even genetic manipulation have in some cases been successful, but never been put to practice (Hoy 2000). It has also been suggested to enhance the responsiveness to specific host foraging cues in predators, parasitoids (Cortesero, Stapel *et al.* 2000) and entomopathogenic nematodes (Gaugler, Campbell *et al.* 1989; Gaugler and Campbell 1991; Gaugler, Campbell *et al.* 1991; Gaugler, Glazer *et al.* 1994), but such attempts have been largely hampered by a lack of knowledge on which cues are of key importance. For certain entomopathogenic nematodes we now have such knowledge (Rasmann *et al*. 2005), prompting us to investigate if selection for enhanced responsiveness to such signals can improve the efficiency of nematodes in controlling the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), the most destructive maize pest in North America (Krysan and Miller 1986).

Since its first introduction in the early 1990s (Baca 1994; Sivcev, Manojlovic *et al.* 1994), WCR has also become a serious invasive pest in Europe (Miller, Estoup *et al.* 2005; Vidal, Kuhlmann *et al.* 2005). Most of the yield losses attributed to this pest are the result of damage to the maize roots caused by the soil-dwelling larvae. The destruction of the root system sometimes results in plant lodging (Krysan 1999). Occasionally, WCR adults also contribute to yield loss by intensive feeding on maize silks (Chiang 1973). By 2004, WCR had invaded most of the European Community (Kiss, Edwards *et al.* 2005), provoking investigations to develop novel control strategies.

Besides conventional pest control strategies, biological control is being considered to manage WCR population in Europe (Kuhlmann and van der Brugt 1998). As yet no effective indigenous natural enemies have been found (Toepfer and Kuhlmann 2004). An inundative approach, using entomopathogenic nematodes (EPN) as biological agents, is considered as one of the most promising strategies (Kuhlmann and van der Brugt 1998). These nematodes are obligate insect parasites in symbiosis with bacteria. Once introduced into the host, the infective juvenile (the infective stage of EPN) releases its symbiotic bacteria, which multiply and kill the insect and serve as food for EPN. When nutrients are consumed and space is exhausted, a new generation of infective juveniles leaves the cadaver and search for a new host (Kaya and Gaugler 1993). Among the

species available on the market, *Heterorhabditis megidis* Poinar (Rhabditida: Heterorhabditidae) exhibits behavioural traits highly interesting for biological control. Indeed, infective juveniles of *H. megidis* are attracted by (*E*)-β-caryophyllene (EβC), a sesquiterpene released by WCR damaged maize roots (Rasmann, Köllner *et al.* 2005; Rasmann and Turlings 2007; Köllner, Held *et al.* 2008) and the presence of this signal is essential for this nematode to be effective as a control agent (Rasmann, Köllner *et al.* 2005). *H. bacteriophora*, one of the most virulent EPN against all WCR larval stages (Toepfer, Gueldenzoph *et al.* 2005; Kurzt, Hiltpold *et al.* 2008; Hiltpold, Toepfer *et al.* in prep.), does not seem to respond well to EβC (Rasmann and Turlings 2008; Hiltpold, Toepfer *et al.* in prep.). Here we explored the possibility to enhance this responsiveness through artificial selection. Because of their short generation time, small genome size and ease of culture, EPN are ideal subjects for genetic improvement and several studies have succeeded in selecting beneficial traits such as host finding (Gaugler, Campbell *et al.* 1989; Gaugler and Campbell 1991), virulence (Tomalak 1994; Peters and Ehlers 1998), and tolerance to temperature (Griffin and Downes 1994; Grewal, Gaugler *et al.* 1996; Ehlers, Oestergaard *et al.* 2005) or desiccation (Strauch, Oestergaard *et al.* 2004). However, artificially selecting for one trait can negatively affect another beneficial trait (for references see (Stuart, Lewis *et al.* 1996)).

Here we used six-arm belowground olfactometers to select for a *H. bacteriophora* strain with enhanced responsiveness to EβC. The effectiveness of this new strain in killing WCR and protecting maize roots was then tested under field conditions.

Material and methods

Selection for an enhanced responsiveness of H. bacteriophora to EβC

Olfactometer assays

The selection of a *H. bacteriophora* strain that responds well to EβC and quickly migrates towards a EβC source was performed with the use of a six-arm belowground olfactometer. Six glass pots (5 cm diam., 11 cm deep) were each connected to a glass central pot (8 cm diam., 11 cm deep) using glass connectors (8 cm long; 24/29 male connector on both sides, all glassware from VQT-Verre Quartz Technique SA, Switzerland) and a Teflon connector (24/29 female to 29/31 male) containing an ultra-fine mesh metal screen (2300 mesh; Small Parts

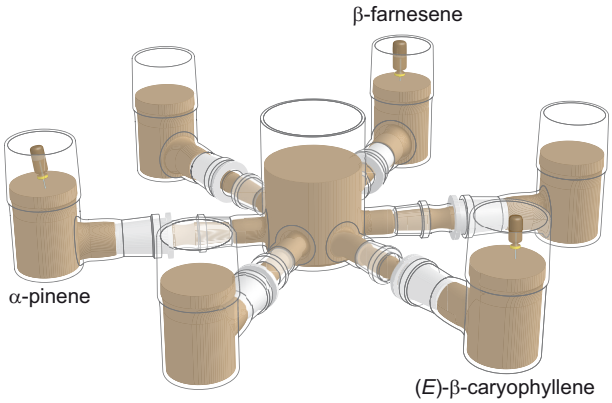

Figure 1 Drawing of the six-arm belowground olfactometer adapted for the selection of H. bacteriophora. *The slow diffusing capillaries were inserted upside down in three of the conected pot. Remaining pots served as controls.*

Inc., Miami Lakes, FL, USA), which prevented the nematodes from entering the odour source pots (for details see (Rasmann, Köllner *et al.* 2005)). Olfactometers were filled with moist sand (10% water) (Migros, Switzerland), allowing for good passive diffusion of the volatiles from the surrounding pots to the central arena (Hiltpold and Turlings 2008). Slow-diffusion capillaries (see below), containing respectively synthetic EβC, (*E*)-β-farnesene and α-pinene (Fluka c/o Sigma Aldrich Chemie GmbH, Switzerland), were inserted each in a separate olfactometer pot. The three pots with a capillary were alternated with sand-filled pots without a capillary, serving as controls.

Slow-diffusion capillary

Amber glass vials (1.5 ml, Supelco c/o Sigma Aldrich Chemie GmbH, Switzerland) were half-filled with glass wool. A volume of 200 ml of one of the selected synthetic compounds was added and the vials closed with an open screw cap with a septum. A 100 µl capillary (Hirschmann Laborgeräte GmbH & Co., Germany) was inserted through the septum into the saturated headspace of the vial. The vial was then placed upside down with the capillary projecting into the sand of the designated olfactometer pot.

EPN selection for sensitivity and motility towards EβC

Batches of 10'000 *H. bacteriophora* nematodes from the original strain PS8 (Ehlers, Oestergaard *et al.* 2005) were released in a drop of water in the center of the central arenas of six belowground olfactometers that were prepared as described above. They were then exposed and allowed to move towards the three different odour sources. Each olfactometer was disassembled at a different time period either 4h, 6h, 8h, 12h, 24h and 48h after nematode release. The sand contained in each glass connector was then placed onto separate cotton filter disk (19 cm diam., Hoeschele GmbH, Switzerland). The disks were deposited on Bearmann extractors (Curran 1992; Hass,

Griffin *et al.* 1999) and nematodes were counted the next day under a binocular. The time period at which at least 500 active EPN (~5% of the total applied) were recovered in the arm treated with EβC was chosen for the selection. The nematodes from this arm were allowed to infect *Galleria mellonela* L. (Lepidoptera: Galleridae) larvae in order to reproduce and form the next generation of infective juveniles for selection. Each larva was infected with 15 infective juveniles in 10% moist sand and stored in the dark at 25 (±3) °C. Four days after nematode infection, *G. mellonela* larvae were placed on white traps (Kaya and Stock 1997) with Ringer solution and stored in the dark at 25 (±3)°C. After about 10 days, the emerging offspring from the cadavers were pooled and used for the next round of selection. In total, six such selection steps were carried out, with the sampling schedule modified to account for the increasingly rapid response (taking samples after 3h, 4h, 5h, 6h, 7h, 8h and 9h in the final round). A seventh test was carried out using the original sampling scheme to allow a comparison of the EPN response before and after selection. The new selected strain was called EβC-2.4.

Comparison of the infectiousness of the selected and original H. bacteriophora strains

Infectiousness is defined by all steps from host finding (over a limited distance) to the death of the host (Peters 2005). To test if the selected strain was equally infective as the original strain, plastic trays (54 cm^2, 9cm×6cm×5.5cm) were filled with 200g sterilized sand (10% moist and sieved at 200μm). The bottom of each tray was covered with 1 cm of sand. EPN strains PS8 or EβC-2.4 were applied in a drop of water in two opposite corners of the tray in a concentration of 8 or 16 EPN/cm^2. Controls trays were supplied with only drops of water. Boxes were then completely filled with the remaining moist sand. Ten WCR L2 larvae were laid on the top and boxes were placed at 22°C for seven days. Following this incubation period, WCR larvae were recovered from each tray by sieving sand through a 500 μm sieve. The number of dead larvae infected by nematodes was recorded and infectiousness was calculated as a percent of dead larvae out of the initial ten larvae.

Evaluation of the EPN strains ability to control WCR in the field

Field experiments were carried out in Hodmezovasarhely, Szeged and Szatymaz (southern Hungary) during the summer of 2007. Half of each experimental field was

sowed with maize seeds of variety Magister (UFA Semences, Bussigny. Switzerland) which emits EβC and half with seeds of variety Pactol (Syngenta, Budapest, Hungary), a variety that does not emit EβC (Fig. 2).

In Hodmezovasarhely and Szatymaz, seeds of both varieties were hand-planted 10 cm deep, with 15 cm plant spacing and 75 cm row spacing on April 18th and on May 2nd, respectively. In Szeged, maize was planted on April 24th with 20 cm plant spacing, 80 cm row spacing and 10 cm deep using a sowing machine.

WCR handling and application

WCR eggs (eastern Europe population) were obtained from a laboratory colony founded with field-collected beetles in southern Hungary (for procedures see Singh and Moore (1985)). Eggs were kept in diapause in moist sand at 6 to 8°C. The diapause of WCR eggs was broken end of April by exposing them to a temperature of 25 ± 2°C. After three weeks, eggs were ready to hatch and recovered by sieving the sand (250 μm sieve). Recovered eggs were diluted in water and stored overnight at 6 to 8°C. The following day, 500ml of the egg solution was poured in a 1000 ml beaker and mixed with 500 ml of Agar solution (0.15%) in order to assure a better homogeneity of eggs in

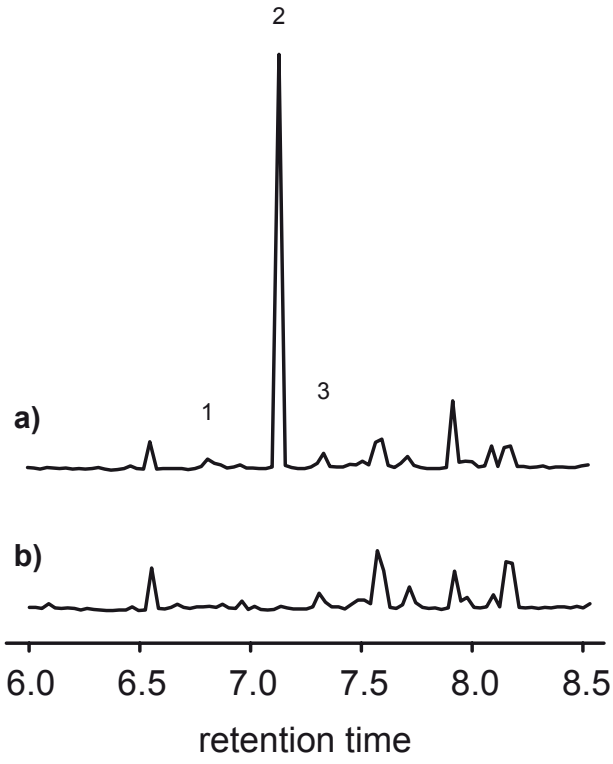

Figure 2 Chromatographic spectra of SPME analysis of maize roots of the variety Magister. a) volatile emission from Magister root system induced by four larvae of D. v. virgifera*. b, volatile emission from an uninduced Magister root system. Labelled compounds are: (1) α-copaene, (2) (*E*)-β-caryophyllene and (3) α-humulene. For details on volatile collection see Rasmann* et al. *(2005).*

the formulation. Number of eggs per litre was adapted to 29'000 eggs.

Maize plants of each field were individually infested in early May with the suspension of viable and ready-to-hatch eggs. Using a standard pipette (Eppendorf Company, Hamburg, Germany), 2 ml of the egg suspension was applied into each of two 12 cm deep holes at a distance of 5 to 8 cm from both sides of the maize plant (~200 eggs/plant). Experimental

plots were constituted of six to seven plants.

Experimental set-up and EPN application

In each of the three fields, seven fine-mesh screen cages (1.3 m height x 0.75 m width x 1.5 m length, maize plants had been cut to a height of 1 m) were randomly placed over plants in either the 3rd, 5th, 7th or 9th row of both Magister and Pactol maize variety plots, to ensure buffer rows. On the edge of both Magister and Pactol sections, a frame of at least one untreated row of maize was kept as buffer. The two different EPN strains, H. bacteriophora PS8 and H. bacteriophora EβC-2.4, were used to inoculate the cages, each cage with only one of the two stains. Four cages per EPN strain were distributed in one experimental plot. Four additional cages, where no EPN but water only was applied, served as control.

In order to get enough individuals for field application the *H. bacteriophora* strains PS8 and EβC-2.4 were reared on *G. mellonella* using the standard method described in (Kaya and Stock 1997). On June 11th (28 days after egg application), a suspension of newly emerged nematodes was applied by hand with a core spray at a 40 cm height directly on the maize line. Application was performed in late afternoon to avoid UV radiation (Gaugler, Bednarek *et al.* 1992). Experimental plots were sprayed with 0.6 liters of water containing approximately 0.36 $x10^6$ EPN (~ $0.3x10^6$ EPN/ m^2) while control plots were sprayed with the same amount of water only

To assess the quality of the EPN applied in the field, one batch of each nematode formulations described above were sampled and stored for four days in the lab. Five *G. mellonella* larvae were placed in a plastic cup (diam. 4.5 mm, 60 mm high) filled with 150 g of 10% moist sand. Per cup, 100 EPN were applied with matched controls with water only. Per treatment, 16 boxes were stored for seven days in dark at 22°C after which survival of larvae was recorded.

Influence of EPN strains on WCR survival

As described above, experimental plots (6-7 plants) of the three fields were covered with fine-mesh screen cages three days after nematode application (on June 14th). WCR adult emergence within these cages was recorded weekly between June 20th and August 15th. WCR survival was calculated by dividing the number of emerged adults per cage by the approximate number of eggs applied.

Statistical analyses

Selection for an enhanced responsiveness of H. bacteriophora to EβC

Attraction over time of *H. bacteriophora* PS8 and EβC-2.4 strains was tested using a one way repeated measures ANOVA. Differences between periods of sampling or chemical cues were tested using a Tukey post-hoc test. A Friedman Repeated Measures Analysis of Variance on Ranks was used to test the attraction of EPN after 8h of exposure to the chemical signal through the 6 selection steps. To detect differences between selection steps or arm treatments, a post-hoc test was run based on the SNK method. Infectiousness for both EPN concentrations was tested with an ANOVA on Ranks. The impact of *H. Bacteriophora* strain on this EPN trait was compared using a Kruskal-Wallis post-hoc test. Statistics described above were conducted in SigmaStat Version 2.03.

Evaluation of the EPN strains ability to control WCR in the field

The ability of EPN strains to control WCR populations was tested for each maize variety using a two-way ANOVA (GLM procedure) in SAS 9.1. EPN strain, field and EPN strain*field were considered as independent variables and lnWCR survival (a natural log transformation was performed to obtain a normal distribution) as dependent variable. Differences were analyzed using LSMEANS with Tukey–Kramer adjustments for the *p-values* (SAS 9.1.).

Results

Selection for an enhanced responsiveness of H. bacteriophora to EβC

The *H. bacteriophora* PS8 strain responded weakly to and moved slowly towards all chemical cues offered (Fig. 3a). Indeed, there were no statistical differences between the number of EPN recovered in either EβC, (*E*)-β-farnesene, α-pinene or empty treated arms (RM ANOVA, $F_{5,15}$=1.95, p=0.165). However, even if the response was slow, nematodes were moving in the olfactometers and after 48 hours, significantly more EPN were recovered (RM ANOVA, $F_{6,30}$=10.29, p<0.001) (Fig. 3a). The number of EPN required for the next selection round, 5% of the total applied, was reached after 12 hours (Fig. 3a). After six rounds of selection, the new *H. bacteriophora* strain, EβC-2.4, was responding better and faster to some of the offered chemical signals (Fig. 3b). EPN were more attracted towards the sesquiterpenes EβC or (*E*)-β-farnesene

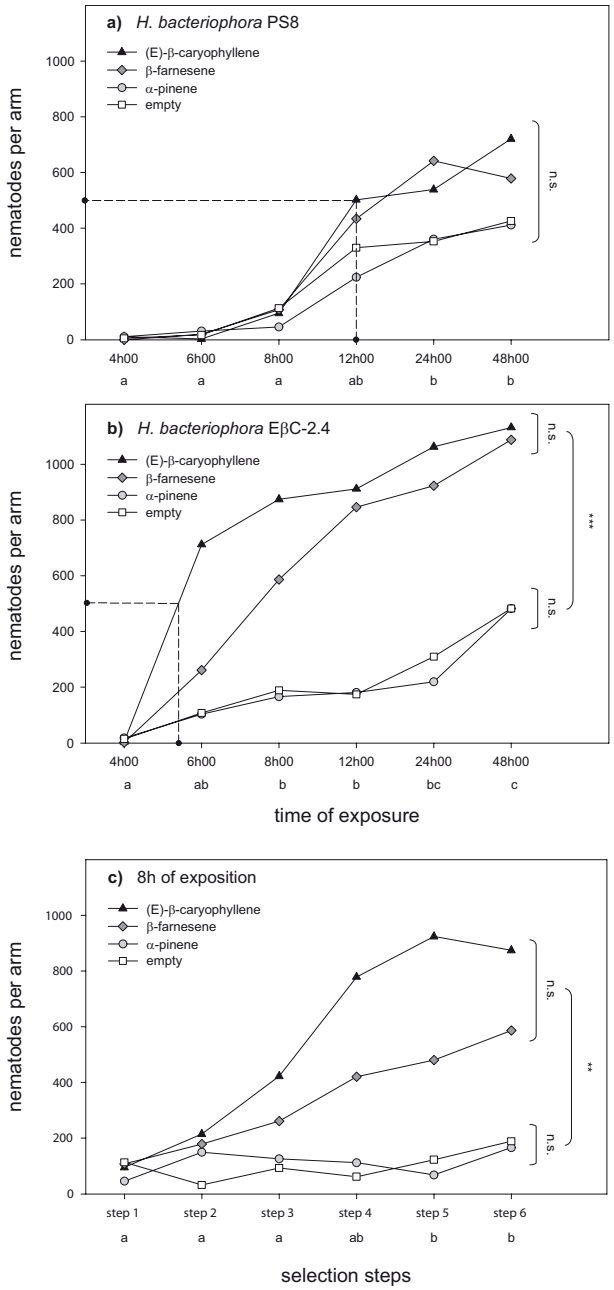

Figure 3 Selection of a H. bacteriophora *strains responding well to EβC led to increased responsiveness of the nematodes over six generations. a) Responsiveness to chemical signals of the original strain PS8. EPN number recovered in the olfactometer arms was increasing with time of exposure. However, there was no difference in the attraction of the nematodes to any of the signals offered or the control arms. Dashed line indicates the time of exposure needed to recover 5% of the injected EPN in the EβC arm. b) Responsiveness to chemical signals newly selected strain EβC-2.4. Compared to the original strain, the number of EPN recovered in the arm with EβC doubled. Dashed line indicates the time of exposure needed to recover 5% of the injected EPN in the EβC arm. c) Responsiveness of the H. bacteriophora over the six selection steps and 8 h of exposure to the offered signals. Attraction towards arms treated with EβC and β-farnesene increased over the selection process while the attraction to a-pinene or the empty arms remained stable. Letters along the X-axis indicate significant differences between times of exposure or selection steps. Statistical differences between arm treatments in are indicated either by n.s. (not significant) or asterisks.*

than towards α-pinene and the empty arms (RM ANOVA, $F_{5,15}$=16.74, p<0.001) (Fig. 3b). However, no statistical differences between EβC and β-farnesene or between α-pinene and the empty arms were measurable even if both "groups" were significantly different (Fig. 3b). EβC-2.4 nematodes were reacting faster (RM ANOVA, $F_{6,30}$=16.15, p<0.001). Migration of this strain toward EβC was over twofold faster than with *H. bacteriophora* PS8 and over 5% of the total applied EPN were sampled after 6 hours of exposure (Fig. 3b).

Figure 3c shows the response of the *H. bacteriophora* over the six selection

steps after 8 h of exposure to the offered signals. Migration velocity and EPN reactivity significantly increased through selection steps (Friedman RM ANOVA on Ranks, P = 0.039). A plateau of selection was reached after four selection steps and selection appears to be stable as sensitivity or migration velocity did not significantly increase during the three last selection rounds (Fig. 3c). Again, no statistical differences between EβC and β-farnesene or between α-pinene and the empty arms were measured but both "groups" were different from each others (Freidman RM ANOVA on Ranks, P = 0.012) (Fig. 3c).

Infectiousness of the H. bacteriophora strains

The application of nematodes dramatically influenced the survival of the WCR larvae (ANOVA on Ranks, H = 49.61; P<0.001). When used with a concentration of 8 EPN/ cm^2, the original strain PS8 performed slightly better in infecting and killing WCR larvae than the selected strain EβC-2.4. Such a difference was not true for the second concentration (Fig. 4).

Evaluation of the EPN strains ability to control WCR in the field

Treatments in Magister plots had a significant impact on the WCR survival (Two Way ANOVA, $F_{2,24}$=42.7, p<0.001). Survival in plots where EPN from the strain PS8 were applied was twofold lower compared to control plots. *H. bacteriophora* EβC-2.4 was significantly more effective in reducing WCR survival compared to the original strain PS8. In other words, when EβC-2.4 was applied, the WCR survival was reduced sevenfold more compared to control plots (Fig. 5a). Significant impact of EPN application was also found in Pactol

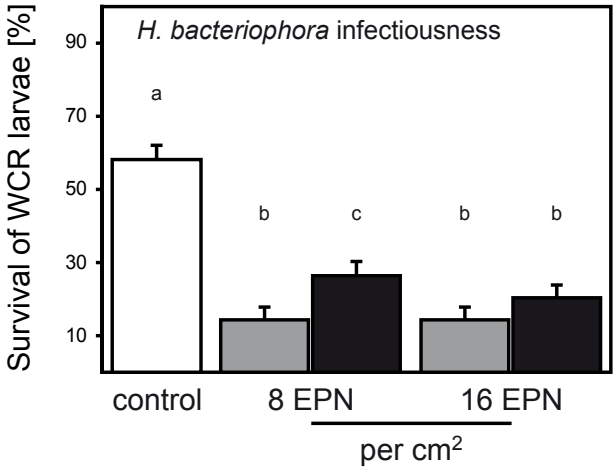

Figure 4 H. bacteriophora *strain infectiousness against WCR tended to be lower after selection. In laboratory assays, the infectiousness of the strain EβC-2.4 was significantly lower when applied at a concentration of 8 EPN/cm^2. However, there was no significant difference when the higher nematode dose was tested. The white bar represents mean survival when only water was added. Mean survivals in bioassays with EPN application are in grey and black, corresponding to H. bacteriophora strain PS8 and EβC-2.4 respectively. Error bars indicate standard error. Letters indicate statistical differences.*

plots (Two Way ANOVA, $F_{2,24}$=9.19, p=0.001). Nonetheless, no statistical differences were found between the two tested EPN strains (Fig. 5b)

EPN applied in the field were of good quality. In cups with either *H. bacteriophora* PS8 or EβC-2.4 strains application, 91% of the *G. mellonela* larvae died while only 20% did not survive in the control.

Discussion

We show that selective breeding of *H. bacteriophora* for increased responsiveness to damaged plant signals is a feasible approach. Before selection, the original strain did not exhibit any clear preferences for any of the three chemical signal offered (Fig. 3a). Selection of *H. bacteriophora* for responsiveness to one of these signals, EβC, resulted in a stable strain with enhanced responsiveness to the root signal. This is an important development, as *H. bacteriophora* already exhibits high virulence and infectiveness against WCR (Kurzt, Hiltpold *et al.* 2008) and exhibits high motility (O'Halloran and Burnell 2003). The selection process resulted in a new strain, which not only entered the olfactometer arm with the EβC

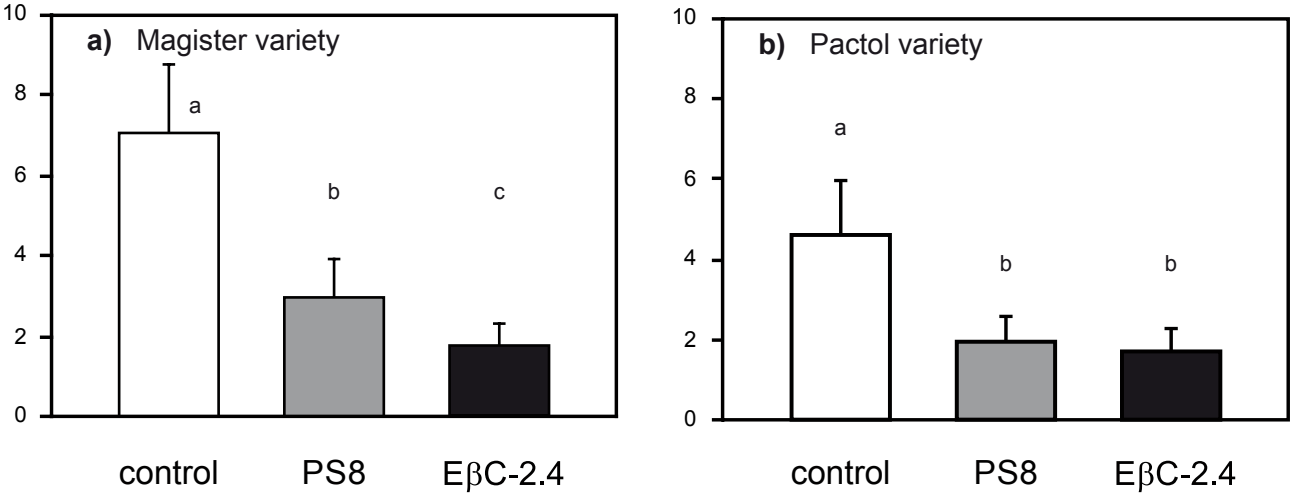

Figure 4 The emission of EβC by the maize plant significantly influenced the capacity of the tested EPN strains to control WCR population in the field. a. Survival of WCR adult in plots with the maize variety Magister was significantly lower when treated with nematodes. Additionally, the strain EβC-2.4, responding to the plant signal, was significantly more efficient in controlling WCR than the foundation line. b. Again, the application of nematode had a significant impact on the WCR survival. However, as the maize variety Pactol was not emitting EβC in soil, there was no statistical difference between the strains EβC-2.4 and its foundation strain PS8. White bars represent mean survival when only water was added. Mean survivals in plots with EPN application are in grey and black, corresponding to H. bacteriophora strain PS8 and EβC-2.4 respectively. Error bars indicate standard error. Letters indicate statistical differences.

twice as fast as the original strain (Fig. 3a & b), but also many more nematodes responded, i.e. 8 h after release in the olfactometers the number of EPN recovered from the EβC arm was eightfold higher for the selected strain (Fig. 3c). The responsiveness was not specifically increased only for EβC, but also for the other sesquiterpene (*E*)-β-farnesene (Fig. 3b & c). This similarity in responsiveness to similar compounds is in agreement with the results of other studies. For instance, long-chain alcohols are in general attractive to *Heterorhabditis bacteriophora* (O'Halloran and Burnell 2003) and various terpenes are known to attract the phytopathogenic nematode *Bursaphelancus xylophilus* (Zhao, Wei *et al.* 2007).

Field tests confirmed that the application of selected EPN as bio-control agents is highly promising. The impact of EPN application was evident for both the maize varieties that were tested (Fig. 5). Furthermore, WCR survival in plots with the EβC-releasing Magister variety was significantly lower when treated with the selected strain EβC-2.4 than with the original strain PS8 (Fig 5a).

The apparent cost of selection in terms of reduced infectiousness was low, but significant (Fig. 4). Studies have previously shown that enhancing beneficial traits through selective breeding can negatively alter other traits in the selected strain, such as storage stability (Gaugler, Campbell *et al.* 1990) or the capacity of EPN in killing their hosts (Stuart, Lewis *et al.* 1996; Wang and Grewal 2002). In our study, the infectiousness in the laboratory was reduced by 1.6, but in the field WCR survival in Magister field plots was twofold lower when *H. bacteriophora* EβC-2.4 was applied than when plots were treated with *H. bacteriophora* PS8 (Fig. 5a). Thus, in this case, the benefits derived from enhanced responsiveness outweighed any costs resulting of selection. Nevertheless, other important traits, like infectiveness, should be taken into account in further selection efforts.

Previous studies already showed that artificial selection of EPN can improve traits such as host finding (Gaugler, Campbell *et al.* 1989; Gaugler and Campbell 1991), virulence (Tomalak 1994; Peters and Ehlers 1998) and tolerance to temperature (Griffin and Downes 1994; Grewal, Gaugler *et al.* 1996; Ehlers, Oestergaard *et al.* 2005) or desiccation (Strauch, Oestergaard *et al.* 2004). Additional traits such as UV tolerance or persistence in soil after application should also be considered for further selection

studies. Crossing such selectively bred strains could potentially provide EPN strains that are exceptionally effective against WCR or other soil dwelling pests and might facilitate field scale application by farmers. The plant signals that help to recruit EPN can be enhanced as well (Degenhardt, Hiltpold *et al.* in prep.). By combining these strategies, a synergistic effect can be expected resulting in a drastic improved control of soil dwelling pests by EPN.

Acknowledgments

We thank all the members of the *FARCE* and e-vol labs at the University of Neuchâtel for support, in particular Matthias Held, Claudia Zwahlen and Sarah Kenyon. We also thank Marie-Eve Wyniger and Sergio Rasmann for advice and help in developing the experimental set-ups. The field work was possible due to the hospitality of the Plant Health Service in Hodmezovasarhely in Hungary, offered by Ibolya Zseller, Jozsef Gavallier, Kataline Buzas, Erzsebet Dormannsne, Piroska Szabo, Andras Varga and others. We would like to thank Hungarian summer students for their help in field work. The original strain *H. bacteriophora* PS8 was kindly provided by Prof. Dr. R. U. Ehlers (Christian-Albrechts Universität, Kiel, DE). This study was funded by the CTI Innovation and Technology Fund of Switzerland (CTI project no. 7487.1 LSPP-LS).

References

Baca, F. (1994). “New member of the harmful entomofauna of Yugoslavia *Diabrotica virgifera virgifera* LeConte (Coleoptera, Chrysomelidae).” Zastita Bilja **45**: 125-131.

Beckendorf, S. K. and M. A. Hoy (1985). Biological Control in Agricultural IPM Systems. M. A. Hoy and D. C. Herzog, Academic, Orlando, FL**:** 166-187.

Chiang, H. C. (1973). “Bionomics of the northern and western corn rootworms.” Annual Review of Entomology **18**: 47-72.

Cortesero, A. M., J. O. Stapel*, et al.* (2000). “Understanding and Manipulating Plant Attributes to Enhance Biological Control.” Biological Control **17**(1): 35-49.

Curran, J. (1992). “Influence of application method and pest population size on the field efficacy of entomopathogenic nematodes.” Journal of Nematology **24**: 631-6.

Degenhardt, J., I. Hiltpold*, et al.* (in prep.). “Restoring the communication between maize roots and entomopathogenic nematodes helps to control a major pest insect.”

Ehlers, R. U., J. Oestergaard*, et al.* (2005). “Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode-bacterium complex *Heterorhabditis bacteriophora-Photorhabdus luminescens*.” Biocontrol **50**(5): 699-716.

Gaugler, R., A. Bednarek*, et al.* (1992). “Ultraviolet inactivation of Heterorhabditid and Steinernematid nematodes.” Journal of Invertebrate Pathology **59**(2): 155-160.

Gaugler, R. and J. F. Campbell (1991). “Selection for enhanced host-finding of scarab larvae (Coleoptera, Scarabaeidae) in an entomopathogenic nematode.” Environmental Entomology **20**(2): 700-706.

Gaugler, R., J. F. Campbell*, et al.* (1991). “Characterization and basis of enhanced host-finding in a genetically improved strain of *Steinernema carpocapsae*.” Journal of Invertebrate Pathology **57**(2): 234-241.

Gaugler, R., J. F. Campbell*, et al.* (1989). “Selection for host-finding in *Steinernema feltiae*.” Journal Of Invertebrate Pathology **54**(3): 363-372.

Gaugler, R., J. F. Campbell*, et al.* (1990). «Fitness of a genetically improved entomopathogenic

nematode.» Journal of Invertebrate Pathology **56**(1): 106-116.

Gaugler, R., I. Glazer*, et al.* (1994). "Laboratory and field evaluation of an entomopathogenic nematode genetically selected for improved host-finding." Journal of Invertebrate Pathology **63**(1): 68-73.

Grewal, P. S., R. Gaugler*, et al.* (1996). "Rapid changes in thermal sensitivity of entomopathogenic nematodes in response to selection at temperature extremes." Journal of Invertebrate Pathology **68**(1): 65-73.

Griffin, C. T. and M. J. Downes (1994). Selection of *Heterorhabditis* sp. for improved infectivity at low temperatures. Genetics of entomopathogenic nematode-bacterium complexes. A. M. Burnell, R. U. Ehlers and J. P. Masson, European Commission Publication EUR 15681 EN, Luxembourg**:** 120-128.

Hass, B., C. T. Griffin*, et al.* (1999). «Persistence of *Heterorhabditis* infective juveniles in soil: Comparison of extraction and infectivity measurements." Journal of Nematology **31**(4): 508-516.

Hiltpold, I., S. Toepfer*, et al.* (in prep.). "How maize root volatiles influence the efficacy of entomopathogenic nematodes against the western corn rootworm."

Hiltpold, I. and T. C. J. Turlings (2008). "Belowground chemical signalling in maize: when simplicity rhymes with efficiency." Journal of Chemical Ecology **34**(5): 628-635.

Hoy, M. A. (1976). "Genetic Improvement of Insects - Fact or Fantasy." Environmental Entomology **5**(5): 833-839.

Hoy, M. A. (2000). "Transgenic arthropods for pest management programs: Risks and realities." Experimental and Applied Acarology **24**(5-6): 463-495.

Kaya, H. K. and R. Gaugler (1993). "Entomopathogenic nematodes." Annual Review of Entomology **38**: 181-206.

Kaya, H. K. and S. P. Stock (1997). Techniques in insect nematology. Manual of Techniques in Insect Pathology**:** 281-323.

Kiss, J., C. R. Edwards*, et al.* (2005). Monitoring of western corn rootworm (*Diabrotica v. virgifera* LeConte) in Europe 1992–2003. Western corn rootworm: ecology and management. S. Vidal,

U. Kuhlmann and C. R. Edwards. Wallingford, Oxon, CABI Publishing.

Köllner, T., M. Held*, et al.* (2008). "A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties." Plant Cell **20**: 482-494.

Krysan, J. L. (1999). Selected topics in the biology of *Diabrotica*. Advances in Chrysomelidae Biology. M. L. Cox. Leiden, The Netherlands, Backhuys Publisher. **1:** 479-513.

Krysan, J. L. and T. A. Miller (1986). Methods for study of pest *Diabrotica*. New-York, Springer-Verlag.

Kuhlmann, U. and W. A. C. M. van der Brugt (1998). «Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe." Biocontrol **19**: 59N-68N.

Kurzt, B., I. Hiltpold*, et al.* (2008). "Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes." Biocontrol **online**.

Miller, N., A. Estoup*, et al.* (2005). "Multiple transatlantic introductions of the western corn rootworm." Science **310**(5750): 992-992.

O'Halloran, D. M. and A. M. Burnell (2003). "An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*." Parasitology **127**: 375-385.

Peters, A. (2005). Insect based bioassays. Quality control of entomopathogenic nematodes. J. M. Grunder, European Commission Publication COST819, Brussels**:** 55-67.

Peters, A. and R. U. Ehlers (1998). Evaluation and selection for enhanced nematode pathogenicity against *Tipula* spp. Pathogenicity of entomopathogenic nematodes vesus insect defense machanisms: impact on selection of virulent strains. N. Simoes, N. Boemare and R. U. Ehlers, European Commission Publication COST819, Brussels**:** 225-241.

Rasmann, S., T. G. Köllner*, et al.* (2005). "Recruitment of entomopathogenic nematodes by insect-damaged maize roots." Nature **434**(7034): 732-737.

Rasmann, S. and T. C. J. Turlings (2007). "Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies." Ecology

Letters **10**(10): 926-936.

Rasmann, S. and T. C. J. Turlings (2008). "First insights into specificity of below ground tritrophic interactions." Oikos **117**: 362-369.

Singh, P. and R. F. Moore (1985). Handbook of Insect Rearing, Elsevier Science Lt.

Sivcev, I., B. Manojlovic*, et al.* (1994). «Distribution and harmful effect of *Diabrotica virgifera* LeConte (Coleoptera, Chrysomelidae), a new maize pest in Yugoslavia." Zastita Bilja **45**: 19-26.

Strauch, O., J. Oestergaard*, et al.* (2004). "Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding." Biological Control **31**(2): 218-226.

Stuart, R. J., E. E. Lewis*, et al.* (1996). "Selection alters the pattern of emergence from the host cadaver in the entomopathogenic nematode, Steinernema glaseri." Parasitology **113**: 183-189.

Toepfer, S., C. Gueldenzoph*, et al.* (2005). "Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe." Bulletin of Entomological Research **95**(1-10).

Toepfer, S. and U. Kuhlmann (2004). "Survey for natural enemies of the invasive alien chrysomelid, *Diabrotica virgifera virgifera*, in Central Europe." Biocontrol **49**(4): 385-395.

Tomalak, M. (1994). "Selective breeding of *Steinernema feltiae* Filipjev (Nematoda, Steinernematidae) for improved efficacy in control of a mushroom fly, *Lycoriella solani* Winnertz (Diptera, Sciaridae)." Biocontrol Science and Technology **4**(2): 187-198.

Vidal, S., U. Kuhlmann*, et al.* (2005). Western corn rootworm: ecology and management, CABI publishers, Wallingford, United Kingdom.

Wang, X. D. and P. S. Grewal (2002). "Rapid genetic deterioration of environmental tolerance and reproductive potential of an entomopathogenic nematode during laboratory maintenance." Biological Control **23**(1): 71-78.

Zhao, L. L., W. Wei, et al. (2007). "Chemotaxis of the pinewood nematode, Bursaphelenchus xylophilus, to volatiles associated with host pine, Pinus massoniana, and its vector Monochamus alternatus." Journal of Chemical Ecology 33(6): 1207-1216.

Conclusions

&

Outlook

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2008

Conclusions

The experiments conducted and described in the different Chapters above demonstrate that manipulations of tritrophic interaction aiming to achieve a better control of the belowground insect pest *Diabrotica v. virgifera* is feasible. However, one important question remains unanswered; does the manipulation of both first and third trophic levels at the same time result in a synergistic effect?

Questions asked in the outline are briefly answered in the following paragraphs:

Why is (E)-β-caryophyllene such a good belowground signal? (Chapter I)

EβC was found to be a good horizontally diffusing signal. However, some other compounds produced by maize roots diffused even better either in sand or in soil. Nevertheless, taking into account the production costs of these compounds, EβC appears to be the best candidate for a belowground signal. Its biosynthesis cost is relatively low and its diffusion abilities are good.

Is manipulation of trophic levels feasible in the context of belowground tritrophic system exploitation in a bio-control strategy? (Chapters II & V)

Manipulating signal and response in a tritrophic system enhanced the efficiency of EPN. Both genetic manipulation of a maize plant (Chapter II) and selective breeding of EPN (Chapter V) resulted in a better control of WCR populations in the field. The number of WCR emerging from plants that were genetically transformed to emit EβC was reduced by 60% compared to the EβC non-emitting controls (Chapter II). Similar results were obtained with a new EPN strain selected for a better responsiveness to EβC. When applied in plots with a maize variety emitting this sesquiterpene, the survival of WCR was cut to half compared to when the original strain was applied. EPN used in plot where a non-emitting maize variety was planted had an impact on WCR survival, but no differences between the two strains were found (Chapter V).

Is the virulence of EPN affected by the different WCR development stages? (Chapter III)

Akin to previous studies on other species, the virulence of the three EPN species was only slightly influenced by WCR development stages. Nevertheless, particularly in sand, the three species tend to be more effective against the third larval instar. Overall, *H. bacteriophora* and *H. megidis* were

more effective in killing WCR than *S. feltiae*.

How do different species of nematodes react to different belowground signal blends in the field? (Chapter IV)

Among the three EPN species (*H. Bacteriophora, H. megidis* and *S. feltiae*) tested, only *H. bacteriophora* did not respond to the WCR-damaged root signal EβC. After treatment with the two responding species, WCR adult emergence was higher in fields were an EβC non-emitting variety of maize than in fields planted with an emitting variety. This difference was also evident from the root damage measurements. Results from olfactometer experiments suggest that *H. bacteriophora* responds to other root cues and that it is curiously repelled by cues from healthy roots. All three species showed a good ability to control WCR. In some trials this was almost equivalent to the efficacy reported for pesticides.

Outlook

Based on the results obtained reported in this thesis and sum up above, new question or perspectives arise

- The genetic manipulation of the maize plant resulted in a constitutive production of EβC. This continuous production in all the plant's vegetative tissues might have ecological drawbacks such as recruiting pests (Hammack 2001). Making the inserted gene inducible and only be expressed when insects feed on the roots might be a better strategy to guide the nematodes specifically to those plans that are actually under insect attack.

- Apart from their role in indirect induced defence, VOCs might also enhance the level of resistance in neighbouring plants. Since the first experimental indications of plant-plant communication using VOCs (Baldwin and Schultz 1983), confirmatory results have been published from studies under both laboratory and field conditions (e.g. Engelberth, Alborn *et al.* 2004; Ruther and Furstenau 2005; Ruther and Kleier 2005; Baldwin, Halitschke *et al.* 2006; Ton, D'Alessandro *et al.* 2007). Plant-plant communication may not be limited to the aboveground plant parts and indeed recent studies have shown a belowground information exchange in beans (Dicke and Dijkman 2001; Chamberlain, Guerrieri *et al.* 2001; Guerrieri, Poppy *et al.* 2002; Guerrieri, Lingua *et al.* 2004). Having such

plant-plant interaction in maize might be of great interest for the use of EPN in a biological control strategy.

- The EPN *H. bacteriophora* has been shown to react poorly to EβC (Chapter IV). Rasmann and Turlings (2008) demonstrated that this EPN seems to be repelled by healthy plants. We also found repulsion of *H. bacteriophora* by healthy root system compared to controls (Chapter IV). Astonishingly, in the six-arm belowground olfactometer, *H. bacteriophora* is attracted by the maize variety Pactol which has been previously shown to not attract EPN under laboratory conditions (Chapter IV and Rasmann, Köllner *et al.* 2005). By identifying the compounds involved in these newly described interaction could lead to a more complete understanding of belowground interaction complexity and thus to enlarge their field of application in biological crop.

- Application of EPN in the field remains the weakness of this method of control. These organisms are UV sensitive (Gaugler, Bednarek *et al.* 1992) and not tolerant for desiccation (Georgis 2002), which are conditions are often combined in the field. In addition, even if feasible, applying EPN through an irrigation system consumes a large volume of water (Toepfer, Burger *et al.* in prep.). Developing an efficient strategy of application is crucial and might lead to a more widespread use of EPN in biological control. Among possibilities, the development of a slow dissoluble capsules containing quiescent EPN is promising. The capsules might be mixed with maize seed and applied in the field while sowing as an “in the bag” solution. Timing of dissolving could be calculated in a way that EPN are released from the capsules when WCR eggs hatch and first instar larvae start to feed on roots.

References

Baldwin, I. T., R. Halitschke, et al. (2006). «Volatile signaling in plant-plant interactions: «Talking trees» in the genomics era.» Science 311(5762): 812-815.

Baldwin, I. T. and J. C. Schultz (1983). «Rapid changes in tree leaf chemistry induced by damage - evidence for communication between plants.» Science 221(4607): 277-279.

Chamberlain, K., E. Guerrieri*, et al.* (2001). “Can aphid-induced plant signals be transmitted aerially and through the rhizosphere?” Biochemical Systematics and Ecology **29**(10): 1063-1074.

Dicke, M. and H. Dijkman (2001). “Within-plant circulation of systemic elicitor of induced defence and release from roots of elicitor that affects neighbouring plants.” Biochemical Systematics and Ecology 29(10): 1075-1087.

Engelberth, J., H. T. Alborn, et al. (2004). “Airborne signals prime plants against insect herbivore attack.” PNAS 101(6): 1781-1785.

Gaugler, R., A. Bednarek, et al. (1992). “Ultraviolet inactivation of Heterorhabditid and Steinernematid nematodes.” Journal of Invertebrate Pathology 59(2): 155-160.

Georgis, R. (2002). The biosys experiment: an insider’s perspective. Entomopathogenic Nematology. R. Gaugler, CABI Publishingm, Oxon, UK: 357-372.

Guerrieri, E., G. M. Poppy*, et al.* (2002). “Plant-to-plant communication mediating in-flight orientation of *Aphidius ervi*.” Journal of Chemical Ecology **28**(9): 1703-1715.

Guerrieri, E., G. Lingua*, et al.* (2004). “Do interactions between plant roots and the rhizosphere affect parasitoid behaviour?” Ecological Entomology **29**(6): 753-756.

Hammack, L. (2001). “Single and blended maize volatiles as attractants for diabroticite corn rootworm beetles.” Journal of Chemical Ecology 27(7): 1373-1390.

Rasmann, S., T. G. Köllner, et al. (2005). “Recruitment of entomopathogenic nematodes by insect-damaged maize roots.” Nature 434(7034): 732-737.

Rasmann, S. and T. C. J. Turlings (2008). “First insights into specificity of below ground tritrophic interactions.” Oikos 117: 362-369.

Ruther, J. and B. Furstenau (2005). “Emission of herbivore-induced volatiles in absence of a herbivore - Response of Zea mays to green leaf volatiles and terpenoids.” Zeitschrift Fur Naturforschung C-A Journal Of Biosciences 60(9-10): 743-756.

Ruther, J. and S. Kleier (2005). “Plant-plant signaling: Ethylene synergizes volatile emission in Zea mays induced by exposure to (Z)-3-Hexen-1-ol.” Journal Of Chemical Ecology 31(9): 2217-2222.

Toepfer, S., R. Burger, et al. (submitted). “Best techniques to control Diabrotica virgifera virgifera larvae with entomopathogenic nematodes in maize.” Journal of Applied Entomology.

Ton, J., M. D'Alessandro, et al. (2007). «Priming by airborne signals boosts direct and indirect resistance in maize.» Plant Journal 49(1): 16-26.

Annex

A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties

The Plant Cell 20: 482-494

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2008

The Plant Cell, Vol. 20: 482–494, February 2008, www.plantcell.org © 2008 American Society of Plant Biologists

A Maize (*E*)-β-Caryophyllene Synthase Implicated in Indirect Defense Responses against Herbivores Is Not Expressed in Most American Maize Varieties [W][OA]

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The sesquiterpene (*E*)-β-caryophyllene is emitted by maize (*Zea mays*) leaves in response to attack by lepidopteran larvae like *Spodoptera littoralis* and released from roots after damage by larvae of the coleopteran *Diabrotica virgifera virgifera*. We identified a maize terpene synthase, Terpene Synthase 23 (TPS23), that produces (*E*)-β-caryophyllene from farnesyl diphosphate. The expression of TPS23 is controlled at the transcript level and induced independently by *D. v. virgifera* damage in roots and *S. littoralis* damage in leaves. We demonstrate that (*E*)-β-caryophyllene can attract natural enemies of both herbivores: entomopathogenic nematodes below ground and parasitic wasps, after an initial learning experience, above ground. The biochemical properties of TPS23 are similar to those of (*E*)-β-caryophyllene synthases from dicotyledons but are the result of repeated evolution. The sequence of TPS23 is maintained by positive selection in maize and its closest wild relatives, teosinte (*Zea* sp) species. The gene encoding TPS23 is active in teosinte species and European maize lines, but decreased transcription in most North American lines resulted in the loss of (*E*)-β-caryophyllene production. We argue that the (*E*)-β-caryophyllene defense signal was lost during breeding of the North American lines and that its restoration might help to increase the resistance of these lines against agronomically important pests.

INTRODUCTION

In natural ecosystems, plants are usually part of a complex web of interactions with other organisms that may influence their growth and survival. To be successful in such an environment, plants have to respond correctly to a multitude of different herbivores, pathogens, competitors, and mutualists. Much plant biology research today is devoted to unraveling the molecular and biochemical processes that provide plants with flexible and appropriate responses to these various enemies and friends. Plant responses to herbivory often include the formation of secondary metabolites, especially phenolic and terpene compounds that act as toxins and feeding deterrents when ingested by the herbivore (Karban and Baldwin, 1999). Another defense tactic involves the recruitment of natural enemies of herbivores with induced volatiles. This so-called indirect defense has already been identified in >10 plant species (Dicke, 1999; Dicke and van Loon, 2000; Meiners and Hilker, 2000; Kessler and Baldwin, 2002).

A well-studied example of indirect defense is found in maize (*Zea mays*), in which foliar damage by lepidopteran larvae results in the release of a complex volatile mixture containing indole, lipoxygenase pathway products, and a variety of monoterpene and sesquiterpene olefins. These volatiles attract parasitic wasps like *Cotesia marginiventris* to the site of damage, where they oviposit in the lepidopteran larvae (Turlings et al., 1990). Parasitized lepidopteran larvae feed less than unparasitized larvae and die upon emergence of the adult wasp, which can result in a considerable reduction in damage to the plant (Hoballah et al., 2002, 2004). The identification of the precise volatile compound(s) that attracts the wasp to the plant for oviposition is a complex and difficult task (Turlings et al., 1991; D'Alessandro and Turlings, 2005). We previously isolated the gene responsible for the biosynthesis of (*E*)-β-farnesene and (*E*)-α-bergamotene (Schnee et al., 2006), the predominant sesquiterpenes released upon caterpillar attack by maize (Köllner et al., 2004a). Overexpression of this gene in *Arabidopsis thaliana* demonstrated that the parasitic wasp *C. marginiventris* can use these sesquiterpenes for host finding after an initial learning experience (Schnee et al., 2006).

An additional sesquiterpene often emitted after herbivore damage is (*E*)-β-caryophyllene. This volatile compound has been found in response to herbivore damage in several wild relatives of maize (Gouinguené et al., 2001) and in cultivated maize lines from European breeding programs, but it is absent from maize lines originating from North American breeding programs (Degen et al., 2004). Below ground, (*E*)-β-caryophyllene has been found to serve as an important signal in the attraction of enemies to another maize herbivore, the root-feeding pest western maize rootworm (*Diabrotica virgifera virgifera*) (Rasmann

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jörg Degenhardt (degenhardt@ice.mpg.de).
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www.plantcell.org/cgi/doi/10.1105/tpc.107.051672

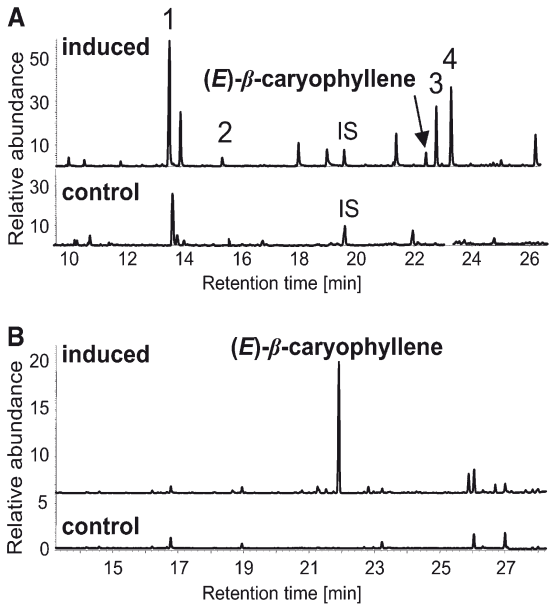

Figure 1. (*E*)-β-Caryophyllene Is Emitted in Response to Both Damage of the Leaves by *S. littoralis* and Attack of the Roots by *D. v. virgifera*.

(A) Volatiles from control leaves and leaves damaged by *S. littoralis* were collected and separated by gas chromatography. The major terpene compounds were identified as linalool (peak 1), 4,8-dimethylnona-1,3,7-triene (peak 2), (*E*)-α-bergamotene (peak 3), and (*E*)-β-farnesene (peak 4). Depicted are traces of the total ion current detector. IS, internal standard (nonylacetate).

(B) Volatiles from control roots and roots damaged by *D. v. virgifera*.

et al., 2005). In contrast with the complex volatile blend emitted by caterpillar-damaged leaves, maize roots only release (*E*)-β-caryophyllene upon damage by *D. v. virgifera*, which attracts entomopathogenic nematodes (Rasmann et al., 2005).

In the biosynthesis of terpenes, the large class of terpene synthase enzymes converts linear prenyl diphosphate precursors into the large diversity of terpene skeletons encountered in plants. For example, the sesquiterpene synthases convert the C_{15} farnesyl diphosphate to sesquiterpene olefin products. A characteristic feature of terpene synthases is the formation of multiple terpenes from a single substrate (Gershenzon and Kreis, 1999). In the course of our work on maize terpene biosynthesis, we have characterized the multiproduct sesquiterpene synthases TPS1, TPS4, TPS5, and TPS10, which contribute to the overall terpene composition of the aboveground plant parts (Schnee et al., 2002, 2006; Köllner et al., 2004b). However, no gene has been described yet for the biosynthesis of (*E*)-β-caryophyllene in maize.

The bicyclic sesquiterpene (*E*)-β-caryophyllene is of particular interest in maize interactions with other organisms since it is released both above and below ground (Turlings et al., 1998; Rasmann et al., 2005). A gene encoding its biosynthesis would provide a useful tool to study its regulation and function. Here, we describe the properties of maize Terpene Synthase 23 (TPS23), which catalyzes the cyclization of farnesyl diphosphate to (*E*)-β-caryophyllene and the complex regulation of the *tps23* gene in leaves and roots in response to damage by different herbivores. We also show that the TPS23 product, (*E*)-β-caryophyllene, can function as a signal both above and below ground, thereby contributing to the plant's defense against herbivores with completely different sites and modes of attack. Finally, we demonstrate that the ability to produce (*E*)-β-caryophyllene is under positive selection pressure among the wild relatives of maize but was lost during the breeding of most North American maize varieties, not because of direct mutation of the *tps23* gene itself but due to alteration of the regulatory network that results in its transcription.

RESULTS

Caryophyllene Can Attract Two Types of Herbivore Enemies, Entomopathogenic Nematodes and Parasitic Wasps

In our attempt to identify the maize volatiles that are responsible for interactions with other organisms, we previously identified (*E*)-β-farnesene and (*E*)-α-bergamotene as major constituents of a blend used by parasitic wasps to find their lepidopteran hosts (Schnee et al., 2006). Many maize lines, especially those originating from European breeding programs, also emit the sesquiterpene (*E*)-β-caryophyllene after damage by lepidopterans (Degen et al., 2004). The hybrid line Delprim, which is of European origin, also emits high concentrations of this compound from leaves and roots after herbivore induction (Figure 1). In roots, (*E*)-β-caryophyllene is the sole compound released in significant amounts after damage by the herbivore *D. v. virgifera* (Rasmann et al., 2005). To test whether this amount of (*E*)-β-caryophyllene attracts the entomopathogenic nematode *Heterorhabditis megidis* Poinar, we applied (*E*)-β-caryophyllene from a volatile source in one arm of the six-arm olfactometer (Figure 2). Approximately twice as many nematodes were recovered on average from the arm of the olfactometer spiked with biologically

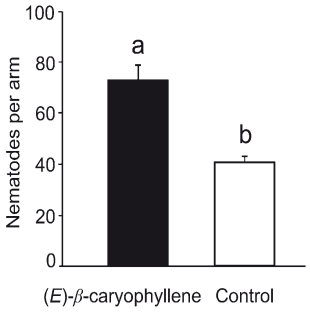

Figure 2. (*E*)-β-Caryophyllene Attracts Nematodes.

The attractiveness of (*E*)-β-caryophyllene to the entomopathogenic nematode *H. megidis* was demonstrated in six-arm olfactometers filled with moist sand. Of the nematodes that were released in the centers of the olfactometers, a significantly larger number was recovered from the arm connected to a pot spiked with 0.2 μL of (*E*)-β-caryophyllene than from each of the five control arms ($P < 0.0001$; $n = 12$). Different letters above the bars indicate a significant difference. Means and SE are shown.

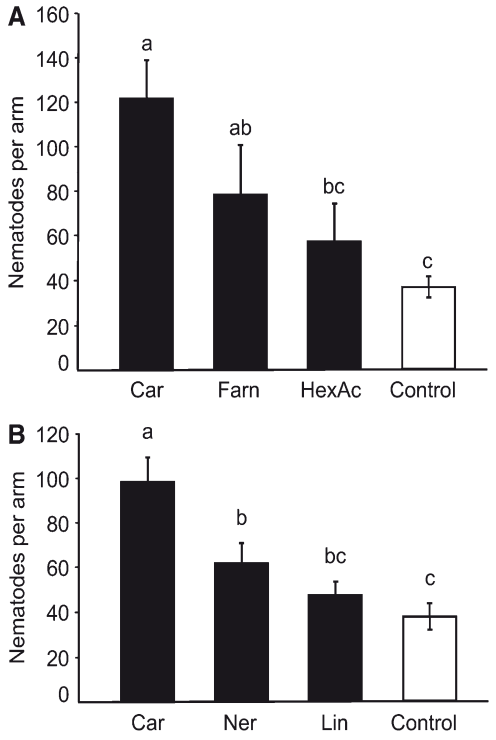

Figure 3. Nematode Attraction Is Specific to (*E*)-β-Caryophyllene.

The attractiveness of plant volatile compounds to the entomopathogenic nematode *H. megidis* was demonstrated in six-arm olfactometers filled with moist sand. The nematodes were released in the center of the olfactometer and chose between arms in which 0.2 μL of (*E*)-β-caryophyllene (Car), (*E*)-β-farnesene (Farn), and (*Z*)-3-hexenyl acetate (HexAc) **(A)** or (*E*)-β-caryophyllene, (*E*)-nerolidol (Ner), and linalool (Lin) **(B)** were added and a sand-only control. Different letters above the bars indicate significant differences at $P < 0.05$. Means and SE of $n = 8$ **(A)** or $n = 12$ **(B)** repetitions are shown.

relevant amounts of authentic caryophyllene compared with the average for the five remaining arms that were not spiked ($F_{1,34} = 13.13$; $P < 0.0001$).

To test whether the attraction of nematodes is specific to (*E*)-β-caryophyllene, we compared it with another sesquiterpene olefin, (*E*)-β-farnesene, the sesquiterpene alcohol (*E*)-nerolidol, the monoterpene alcohol linalool, and a common volatile originating from the lipoxygenase pathway, (*Z*)-3-hexenyl acetate (Figures 3A and 3B). With the exception of (*E*)-β-farnesene, the remaining plant volatile compounds were less attractive [$F_{3,44} = 9.39$; $P < 0.001$ for (*E*)-β-farnesene and (*Z*)-3-hexenyl acetate, $F_{3,68} = 8.26$; $P < 0.001$ for (*E*)-nerolidol and linalool] than (*E*)-β-caryophyllene, indicating that (*E*)-β-caryophyllene is particularly effective at promoting plant–nematode interaction.

To test for a possible aboveground role of (*E*)-β-caryophyllene in attracting the parasitic wasp *C. marginiventris*, we used the pure compound for bioassays in a four-arm olfactometer. Naive wasps without any oviposition experience were not attracted ($F1.82 = 0.98$; $P = 0.32$). However, wasps preferred air containing (*E*)-β-caryophyllene to pure air ($F1.82 = 52.06$; $P < 0.0001$) after they had experienced laying eggs in host larvae while perceiving (*E*)-β-caryophyllene (Figure 4). This effect of associative learning was reflected in a significant treatment effect ($F1.166 = 34.04$; $P < 0.0001$) and a significant treatment–experience interaction ($F1.164 = 13.49$; $P < 0.001$). These results confirm that (*E*)-β-caryophyllene by itself is a key attractant for the nematodes, whereas above ground this bicyclic sesquiterpene olefin is a component of a blend of leaf volatiles produced after herbivore damage that can be perceived and learned as a host location cue by herbivore parasitoids, as has been found for several other volatiles within this blend (D'Alessandro et al., 2006; Schnee et al., 2006).

Cloning of the Maize Terpene Synthase Gene *tps23*

To find the terpene synthase gene(s) producing (*E*)-β-caryophyllene from farnesyl diphosphate in maize, we screened a public maize genome database (http://maize.tigr.org/) for sequences with similarity to known terpene synthases. One of the resulting fragments, AZM4_53695, contained the two exons flanking the last intron of a putative sesquiterpene synthase gene. The 5′ end of this fragment was extended using a cDNA library from herbivore-induced leaves of the maize cultivars Graf and Delprim to obtain the complete open reading frame (ORF). Both cDNAs contained an identical ORF of 1644 bp designated as *tps23* that encodes a protein with a predicted molecular mass of 63.6 kD. Numerous amino acid motifs throughout its sequence are highly conserved among plant terpene synthases (Figure 5). The most characteristic element is an Asp-rich DDxxD motif in the C-terminal part that was implicated in the binding of the divalent metal

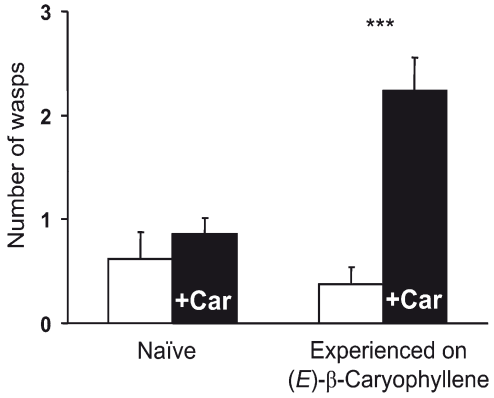

Figure 4. The Parasitic Wasp *C. marginiventris* Is Attracted by (*E*)-β-Caryophyllene after Previous Oviposition Experience.

Responses of the parasitic wasp *C. marginiventris* to (*E*)-β-caryophyllene. The attraction of parasitoid females to a pure standard of (*E*)-β-caryophyllene was tested in a four-arm olfactometer. Two groups of parasitoids were tested: naive wasps and wasps with a previous oviposition experience on host larvae in the presence of (*E*)-β-caryophyllene. The parasitoids were tested in groups of six ($n = 14$). The asterisks indicate a significant preference ($P < 0.0001$) of experienced wasps for the odor of (*E*)-β-caryophyllene (black bars) versus pure air (white bars show average preference for one arm). Naive wasps did not show this preference. Means and SE are shown.

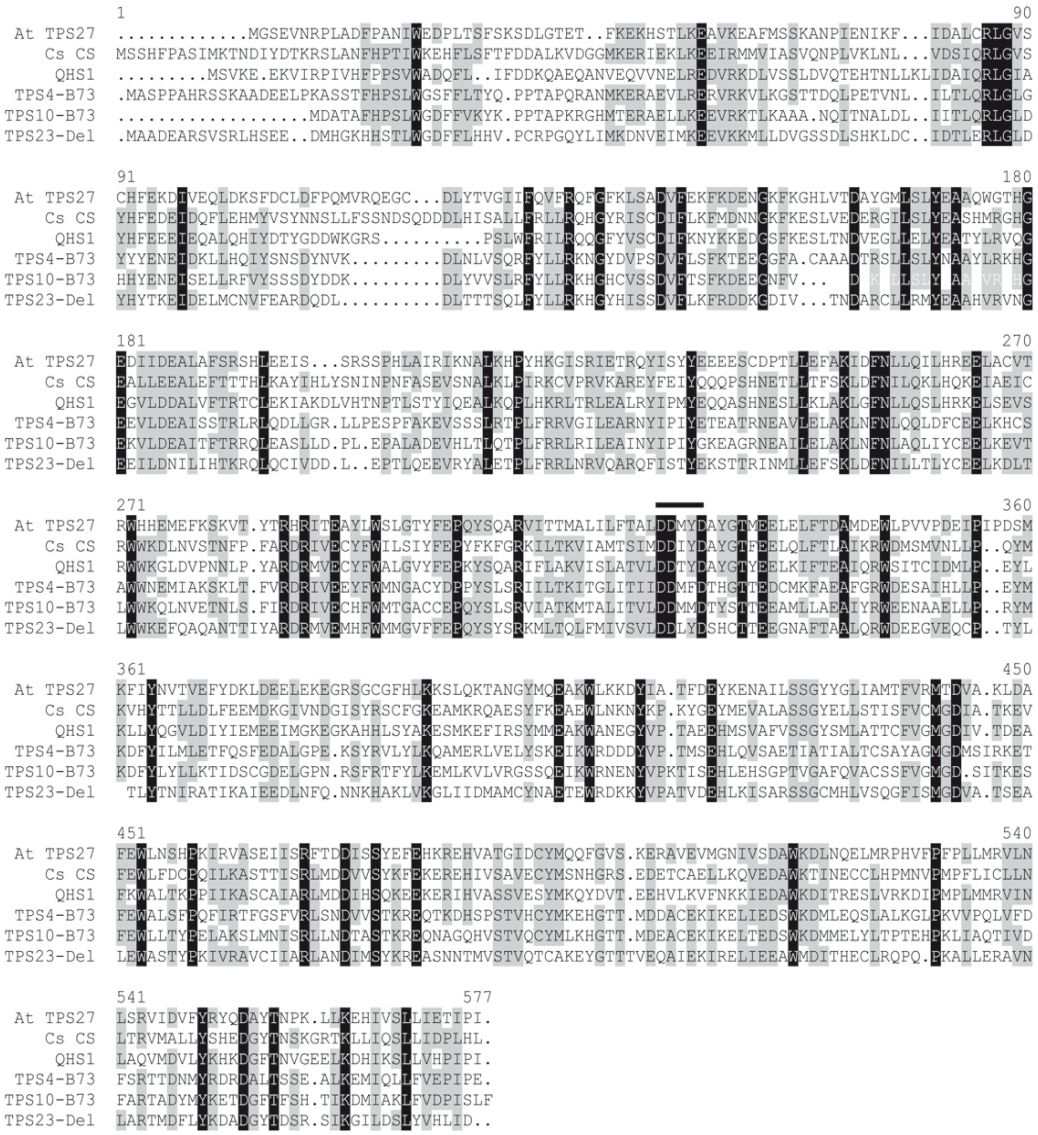

Figure 5. Comparison of the Deduced Amino Acid Sequence of TPS23-Del with Other Terpene Synthases.

The sequence of TPS23 was compared with sequences of (*E*)-β-caryophyllene synthases from other plants (*Arabidopsis* At TPS27, *C. sativus* Cs CS, and *A. annua* QHS1) and two sesquiterpene synthases from maize (TPS10-B73 and TPS4-B73). Amino acids identical in all six proteins are indicated by black boxes. Amino acids identical in at least four proteins or representing conservative changes are highlighted with gray boxes. The highly conserved DDxxD region is marked with a bar.

cofactor in a sesquiterpene synthase from tobacco (*Nicotiana tabacum*) (Starks et al., 1997). The deduced amino acid sequence of TPS23 shows similarities to sequences of other terpene synthases from maize, for example, 40.5% amino acid identity with TPS10 (Schnee et al., 2006) and 37.8% amino acid identity with TPS4 (Köllner et al., 2004b). Among maize terpene synthases, the sequences are most highly conserved in the regions encoding the active site, which are situated toward the C terminus (Figure 5). Considerable effort was expended to search for maize terpene synthase sequences with higher similarity by repeated PCR with maize cDNA and rapid amplification of cDNA ends (RACE) libraries as well as searching of all available maize genomic databases. However, no genes encoding proteins with a sequence identity of >50% were found, which was further supported by DNA hybridization analysis with *tps23* as a probe under low-stringency conditions (data not shown).

tps23 Encodes an (*E*)-β-Caryophyllene Synthase

Since the product specificity of a putative terpene synthase cannot be predicted from its amino acid sequence, we cloned

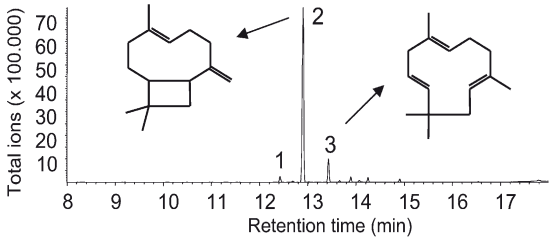

Figure 6. Sesquiterpene Products of TPS23.

The enzyme was expressed in *Escherichia coli*, extracted, partially purified, and incubated with the substrate (*E*,*E*)-FPP. The resulting terpene products were collected with a solid-phase microextraction (SPME) fiber and analyzed by gas chromatography–mass spectrometry. The products were identified as δ-elemene (peak 1), (*E*)-β-caryophyllene (peak 2), and α-humulene (peak 3) by comparison of their retention times and mass spectra with those of authentic standards.

tps23 into a bacterial expression system and incubated the recombinant protein with the potential substrates geranyl diphosphate (GPP; C_{10}), farnesyl diphosphate (FPP; C_{15}), and geranylgeranyl diphosphate (GGPP; C_{20}). The enzyme did not accept GPP or GGPP as a substrate (data not shown), converting only FPP to terpene products (Figure 6). The major product formed from FPP was identified as (*E*)-β-caryophyllene by mass spectrometry and cochromatography with an authentic standard, and the two minor products were α-humulene and δ-elemene. A similar spectrum of terpene products was observed from Os TPS3, an (*E*)-β-caryophyllene synthase from rice (*Oryza sativa*) (Cheng et al., 2007). Although this enzyme has 50% amino acid identity to TPS23, the present genomic information on rice and maize is not sufficient to establish homology between these two species. Caryophyllene synthases identified from dicotyledonous plants, including those from *Arabidopsis* (At TPS27), *Cucumis sativus* (Cs CS), and *Artemisia annua* (Aa QHS1), show only low amino acid identity to TPS23: 33.9, 30.3, and 35.7%, respectively. A dendrogram analysis demonstrates that TPS23 is much more closely related to the functionally unrelated terpene synthases of maize than to terpene synthases of similar function in other plant species, suggesting a repeated evolution of the ability to make (*E*)-β-caryophyllene in monocotyledonous grasses and dicotyledonous plants (Figure 7; see Supplemental Data Set 1 online).

Biochemical Characterization of TPS23

The biochemical properties of TPS23 were determined with purified enzyme and tritium-labeled FPP substrate. The enzyme exhibited a broad catalytic optimum from pH 8.0 to 9.5 but still showed substantial activity at typical cytoplasmic pH conditions (Figure 8). A divalent metal ion cofactor was required for enzyme activity (Figure 9). A range of potential cofactor species were tested at different concentrations, with Mg^{2+} ions at a concentration of 10 mM and Mn^{2+} ions at a concentration of 0.25 mM giving maximal activities. The K_m values were 183 ± 34 and 28 ± 6 μM for Mg^{2+} and Mn^{2+}, respectively (Table 1). Both the K_m and the k_{cat} values for the FPP substrate were similar to those of most characterized plant sesquiterpene synthases identified to date (Chen et al., 1996; Crock et al., 1997; Picaud et al., 2005, 2006).

Herbivory above and below Ground Induces Transcript Levels of *tps23* Independently

To determine whether TPS23 is involved in the herbivore-induced synthesis of (*E*)-β-caryophyllene, we measured the transcript levels of *tps23* in response to feeding damage by *S. littoralis* and *D. v. virgifera* and compared them with the expression pattern of *tps10*, a gene known to be involved in aboveground, herbivore-induced sesquiterpene synthesis in maize (Schnee et al., 2006). The (*E*)-β-caryophyllene–producing line Graf accumulated transcripts of *tps23* in the leaves only after leaf damage by *S. littoralis*, but no transcripts were detectable in roots after *S. littoralis* leaf damage (Figure 10). Conversely, root damage by *D. v. virgifera* resulted in the accumulation of *tps23* transcripts in the roots but not in the shoots. The high transcript levels in the roots of Graf correlated with the production of more (*E*)-β-caryophyllene in this line (Rasmann et al., 2005). Simultaneous feeding by both herbivores resulted in the accumulation of *tps23* transcripts in both leaves and roots. The inbred line B73, which does not emit (*E*)-β-caryophyllene, had no detectable levels of *tps23* transcript throughout the plant, indicating the lack of *tps23* transcription or low transcript stability as the cause of the lack of (*E*)-β-caryophyllene production. As expected, the transcript of the leaf-specific terpene synthase *tps10* accumulated only in response to the aboveground damage by *S. littoralis* but was not induced by belowground attack by *D. v. virgifera*. Transcripts of *tps10* are also present in caryophyllene-producing and non-caryophyllene-producing plants alike, consistent with earlier reports on the emission of TPS10 volatiles by line B73 (Köllner et al., 2004a).

Maize *tps23* and Its Teosinte Orthologs Are Maintained by Positive Selection

The appearance of (*E*)-β-caryophyllene in herbivore-induced volatiles of many grasses related to maize suggests that this

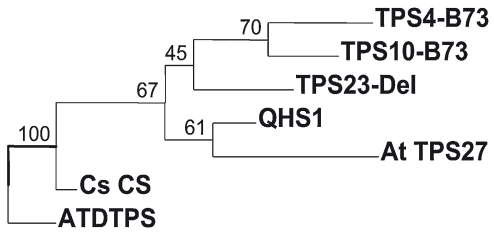

Figure 7. TPS23 Shows Low Sequence Identity to Other (*E*)-β-Caryophyllene Synthases.

Dendrogram analysis of TPS23 with two closely related maize sesquiterpene synthases (TPS10-B73 and TPS4-B73) and several (*E*)-β-caryophyllene synthases from other plants (*Arabidopsis* At TPS27, *C. sativus* Cs CS, and *A. annua* QHS1). The analysis was conducted using a neighbor-joining algorithm. Bootstrap values are shown in percentage and were generated with a sample of $n = 1000$. ATDTPS indicates ent-kaurene synthase from *Arabidopsis*, a functionally different diterpene synthase that was used as an outgroup.

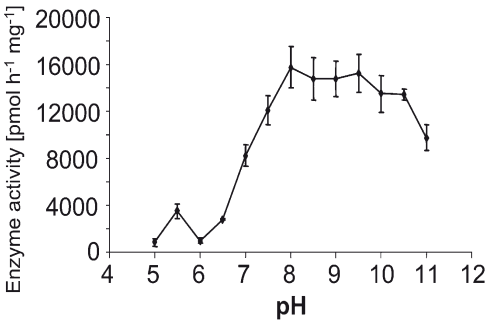

Figure 8. pH Dependence of the Enzymatic Activity of TPS23.

The catalytic activity of the purified enzyme was measured in the presence of 10 mM Mg^{2+}. The pH values were adjusted with the following buffers: pH 5.0 and 5.5, acetate buffer (100 mM); pH 6.0, MES buffer (100 mM); pH 6.5 to 9.5, bis-Tris-propane buffer (100 mM); pH 10.0 to 11, CAPS buffer (10 mM). Means ± SE of triplicate assays are shown.

compound has a widespread role in indirect defense (Gouinguené et al., 2001; Degen et al., 2004). To learn more about the evolution of (*E*)-β-caryophyllene formation, we isolated the apparent orthologs of *tps23* from six teosinte (*Zea* sp) taxa utilizing PCR (Figure 11A; see Supplemental Figure 1 and Supplemental Data Set 2 online). After expression in a bacterial system, all *tps23* apparent orthologs produced the (*E*)-β-caryophyllene main product as well as the characteristic by-products α-humulene and δ-elemene (Figure 11B; see Supplemental Figure 2 online), demonstrating complete functional conservation of *tps23* among maize and its close relatives. A dendrogram analysis of *tps23* apparent orthologs followed the phylogeny generally observed among the teosinte species (Buckler et al., 2006) and showed high levels of amino acid identity (Figure 11A). A positive selection pressure for the maintenance of (*E*)-β-caryophyllene synthase function is evident from the high average number of synonymous nucleotide changes relative to nonsynonymous changes among *tps23* from maize and its teosinte apparent orthologs (dS/dN = 6.88).

Reduced Transcription of *tps23* Prevents (*E*)-β-Caryophyllene Formation in Most North American Maize Lines

Initial studies by Degen et al. (2004) and Rasmann et al. (2005) suggested that maize lines originating from North American breeding programs have largely lost the ability to produce the (*E*)-β-caryophyllene signal. To determine the extent to which this defense trait was lost during domestication, we studied (*E*)-β-caryophyllene production in a set of 24 inbred founder lines assembled to reflect ~85% of the polymorphisms in North American maize (Liu et al., 2003). Of these 24 lines, only 2, NC358 and CML322, were found to produce (*E*)-β-caryophyllene in response to herbivore damage, suggesting that this trait is indeed largely absent from North American breeding lines (Figure 12A). The two (*E*)-β-caryophyllene–producing lines displayed high concentrations of transcripts of *tps23*, while very low levels or no *tps23* transcripts were observed in all other lines except one (Figure 12B). This exceptional line, B97, accumulated high concentrations of *tps23* transcripts despite no (*E*)-β-caryophyllene production. Sequencing of the *tps23-B97* allele, however, showed a 2-bp insertion at position 315, which results in a frameshift that prevents the correct translation of the protein and thereby blocks (*E*)-β-caryophyllene production (Figure 12C).

To understand how domestication and breeding may have caused the loss of this defense signal, we compared the *tps23* alleles of six (*E*)-β-caryophyllene–producing lines (the hybrids Graf and Delprim and the inbred lines F2, F476, Du101, and W401) with four nonproducing lines (the hybrid Pactol and the inbred lines B73, F7001, and F670). All lines tested contained an active *tps23* allele, indicating that the lack of transcript in some of the lines is not due to differences in the ORF (Table 2). Next, the genomic structure of *tps23* was determined by analysis of the alleles *tps23-B73* and *tps23-F2*. The structure of both alleles consists of seven exons and is generally similar to that of other terpene synthases from maize (Shen et al., 2001; Köllner et al., 2004b) and class III terpene synthases from other plants (Trapp and Croteau, 2001). Unlike other terpene synthases, however, the first intron is very large and contains transposon sequences, indicating that this intron was enlarged by transposon insertion from a size of ~121 bp, usually observed in terpene synthases, to 5.6 kb (Figure 13). Since this insertion is observed in the *tps23* alleles of all lines, regardless of (*E*)-β-caryophyllene production, it too is not likely to be responsible for the inactivation of the gene in the nonproducing lines. Furthermore, we tested a 1.8-kb promoter fragment for specific differences that might regulate the transcriptional activity of *tps23* in the different maize lines. Two types of promoter sequence were found, which are distinguished by 18 single base pair changes throughout the fragment. A single base pair change that created an *Eco*RI restriction site was located 425 bp upstream of the transcription start. Among the hybrid lines, Pactol has only promoter type 1, Graf has only type 2, and Delprim has both. However, since all inbred lines had type 1 promoters regardless of their ability to produce (*E*)-β-caryophyllene, the changes in the 1.8-kb fragment did not account for the differences in the transcriptional activity of *tps23*. Similarly, the 880-bp 3′ untranslated region of all alleles was shown to be identical and therefore cannot cause differences in transcriptional activity or changes in mRNA stability.

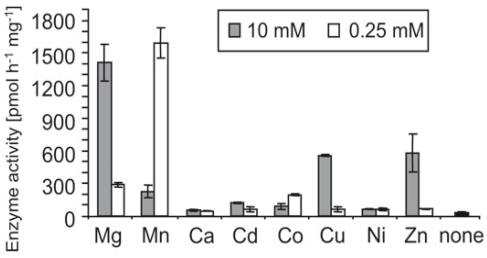

Figure 9. Metal Cofactors Affect the Enzymatic Activity of TPS23.

The catalytic activity of the purified enzyme was measured in the presence of various divalent metal ions at concentrations of 10 and 0.25 mM. Means ± SE of triplicate assays are shown.

Table 1. Kinetic Constants for TPS23-Del Heterologously Expressed in *E. coli*

Constant	FPP (10 mM $MgCl_2$)	FPP (0.25 mM $MnCl_2$)	Mg^{2+}	Mn^{2+}
K_m (μM)	3.7 ± 0.5	1.1 ± 0.3	183 ± 34	28 ± 5
k_{cat} (s^{-1})	(1.91 ± 0.09) × 10^{-3}	(1.13 ± 0.07) × 10^{-3}		

DISCUSSION

TPS23 Provides a Signal for the Attraction of Herbivore Enemies Both above and below Ground

Plants produce a large arsenal of terpenes, phenolics, and other presumed defensive metabolites whose exact functions are largely unknown. Here, we confirm that the sesquiterpene olefin (*E*)-β-caryophyllene can play a role in two spatially separate modes of induced defenses against herbivores: the attraction of parasitic wasps above ground that oviposit on lepidopteran larvae, such as *S. littoralis*, and the attraction of nematodes below ground that can attack larvae of the beetle *D. v. virgifera* (Rasmann et al., 2005). Both volatile signals are produced by a single enzyme, the terpene synthase TPS23. The attraction of the nematode to (*E*)-β-caryophyllene is innate (Rasmann et al., 2005) (Figure 2), whereas females of the parasitic wasp are attracted only after associative learning (Figure 4). (*E*)-β-Caryophyllene is one of many volatiles released by maize leaves that can serve as cues for parasitoids to find their herbivore hosts, but this specific compound is essential for attracting nematodes. A comparison with authentic versions of volatiles that are typically released from maize leaves has revealed that none diffuses as readily in sand and soil as (*E*)-β-caryophyllene (Hiltpold and Turlings, 2008), providing a possible reason why this substance is so much more attractive to the nematode than other typical maize volatiles (Rasmann et al., 2005).

(*E*)-β-Caryophyllene is also suitable for defense signaling above ground, as it diffuses rapidly in the air. However, compared with most other volatile monoterpenes and sesquiterpenes, (*E*)-β-caryophyllene is unstable in the atmosphere, reacting readily with ozone and other reactive oxygen species (Grosjean et al., 1993). Thus, (*E*)-β-caryophyllene may be diagnostic as a short-range cue for host or prey location. (*E*)-β-Caryophyllene also has antimicrobial activity (Sabulal et al., 2006) and might have been initially selected as a direct defense against pathogen attack. Its signaling function in indirect defense could have evolved secondarily.

Maize (*E*)-β-Caryophyllene Synthase Is a Product of Convergent Evolution

The properties of maize TPS23 are similar to those of other plant caryophyllene synthases from *A. annua* (Cai et al., 2002), *C. sativus* (Mercke et al., 2004), and *Arabidopsis* (Tholl et al., 2005) in kinetic parameters and cofactor requirement. These enzymes also have a common reaction mechanism, as indicated by the formation of the same minor products, α-humulene and δ-elemene.

Despite these similarities, sequence comparisons indicate that the (*E*)-β-caryophyllene synthases are products of convergent or repeated evolution (Pichersky and Gang, 2000). TPS23 is more closely related to other maize terpene synthases than to (*E*)-β-caryophyllene synthases isolated from dicotyledons. One such closely related maize terpene synthase is TPS10, which might share with TPS23 a common ancestor involved in indirect defense. The mechanism to produce (*E*)-β-caryophyllene may subsequently have been acquired by TPS23. Such convergent evolution is probably facilitated by the ability of terpene synthases to alter product specificity in response to only a few amino acid changes (Köllner et al., 2004b, 2006; Yoshikuni et al., 2006).

Regulation of *tps23* Transcript Allows Independent Expression in Different Organs

Enzyme activities in plants are often controlled by differential regulation of the members of a gene family. In terpene biosynthesis, for example, enzymes catalyzing important regulatory steps, such as 3-hydroxy-3-methylglutaryl-CoA synthase (Enjuto et al., 1995; Daraselia et al., 1996; Korth et al., 1997), 1-deoxy-xylulose phosphate synthase (Walter et al., 2002), and isoprenyl diphosphate synthases (Cunillera et al., 1997; Okada et al., 2000), are encoded by small gene families with differential expression. However, *tps23* provides an example of a single gene with two distinct expression patterns in different organs. The aboveground induction of *tps23* is similar to that of *tps10*, a maize terpene synthase gene that is activated after herbivory by lepidopteran

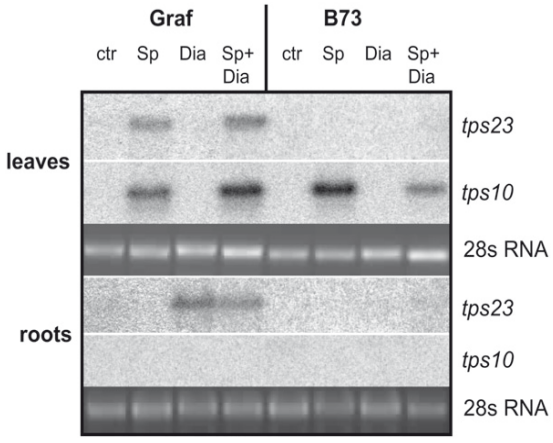

Figure 10. *tps23* Is Selectively Induced by Herbivory of *S. littoralis* and *D. v. virgifera*.

The transcript levels of *tps23* and *tps10* were determined in leaves and roots of the maize cultivars Graf and B73 after feeding of *S. littoralis* (Sp), *D. v. virgifera* (Dia), and *S. littorals* plus *D. v. virgifera* (Sp+Dia) or in undamaged controls (ctr). RNA isolated from 2-week-old plants was hybridized with probes specific for *tps23* or *tps10*. The bottom panels show the 28S RNA band of the ethidium bromide–stained RNA gels.

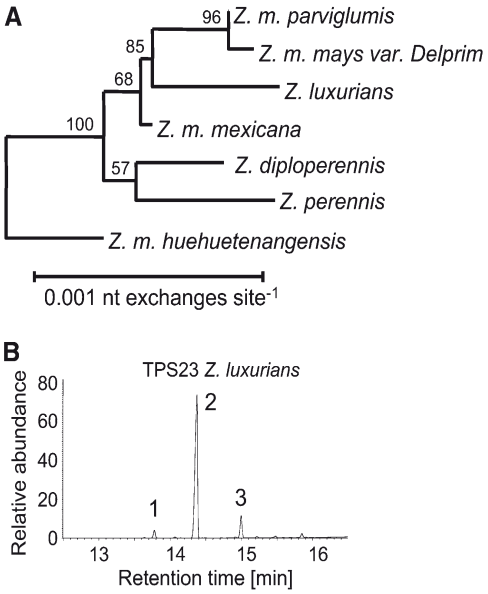

Figure 11. TPS23 Is Functionally Conserved among Relatives of Maize.

(A) Dendrogram analysis of TPS23 from maize (*Z. m. mays*) and its teosinte orthologs from *Z. m. parviglumis*, *Z. luxurians*, *Z. m. mexicana*, *Z. diploperennis*, *Z. perennis*, and *Z. m. huehuetenangensis*. The analysis was conducted as described in Methods. The teosinte *Z. m. huehuetenangensis* was the designated outgroup due to its overall distance from the other teosinte varieties used in the analysis.

(B) Sesquiterpene products of a putative TPS23 ortholog from *Z. luxurians*. The enzyme was expressed in *E. coli*, extracted, partially purified, and incubated with the substrate (*E,E*)-FPP. The resulting terpene products were collected with a SPME fiber and analyzed by gas chromatography–mass spectrometry. The products were identified as δ-elemene (peak 1), (*E*)-β-caryophyllene (peak 2), and α-humulene (peak 3) by comparison of their retention times and mass spectra with those of authentic standards.

larvae on leaves and produces most of the herbivore-induced sesquiterpene hydrocarbon volatiles of maize (Schnee et al., 2006). By contrast, only *tps23*, and not *tps10*, is activated below ground. It is conceivable that the *tps23* and *tps10* genes share the same regulatory mechanism for aboveground induction but that *tps23* contains an additional promoter element that activates the gene in the root after herbivory. We will test this hypothesis of a modular regulatory system by comparing the promoters of *tps23* and *tps10*.

(*E*)-β-Caryophyllene Emission Was Lost Due to a Decrease in *tps23* Transcription during the Breeding of North American Maize Lines

(*E*)-β-Caryophyllene is emitted from all tested maize lines from European breeding programs and from species of teosinte, the closest wild relative of maize. On the other hand, a range of inbred lines that represent ~85% of the genetic diversity of North American maize lines showed (*E*)-β-caryophyllene production in <10% of the lines. Therefore, we assume that this defensive trait was largely lost during the breeding of North American maize lines. All lines that did not produce (*E*)-β-caryophyllene show very low or no *tps23* transcript (with one easily rationalized exception), indicating that the (*E*)-β-caryophyllene polymorphism results from differences in transcription. Differences in transcript stability can be ruled out, since the hypothetical *tps23* transcript is identical in all maize lines analyzed regardless of (*E*)-β-caryophyllene production. The strongly reduced transcription in most North American lines might be due to the inactivation of a transcription factor or the corruption of an enhancer element outside of the assayed promoter region. Whatever the identity of this factor or enhancer element, it is clearly not necessary for the activation of *tps10*, which has a very similar expression profile to that of *tps23* in leaves after *S. littoralis* attack.

The loss of defensive traits during crop domestication has frequently been postulated, but the genetic basis of this process, such as the inactivation of *tps23* expression described here, has rarely been elucidated (Sotelo, 1997). The loss of *tps23* expression might be ascribed to several causes. First, a null allele of a required transcription factor may be closely linked to a trait that is known to differ between North American and European maize lines, like flowering time. Breeding efforts to alter this trait could then have resulted in the accumulation of the null allele for (*E*)-β-caryophyllene production. Alternatively, the release of (*E*)-β-caryophyllene might be disadvantageous under conditions specific to North American agriculture and therefore may have been selected against. A possible disadvantage of (*E*)-β-caryophyllene release in this scenario could be its reported attractiveness to adult females of *D. v. virgifera* (Hammack, 2001).

The use of natural enemies, such as entomopathogenic nematodes, is an important component of many integrated pest control programs and could reduce damage by *D. v. virgifera*, an economically important maize pest that causes extensive yield losses. The failure of past efforts to control this pest with nematodes in North America (Ellsbury et al., 1996; Jackson, 1996) may be due to the lack of (*E*)-β-caryophyllene release from maize lines under cultivation. (*E*)-β-Caryophyllene release is correlated with increased nematode attraction to maize in the field (Rasmann et al., 2005). The identification of *tps23* provides a molecular tool to devise alternative strategies for *D. v. virgifera* control. The restoration of (*E*)-β-caryophyllene production in nonproducing maize lines should enhance their attractiveness to nematodes and thus increase *D. v. virgifera* mortality. We are currently transforming a non-(*E*)-β-caryophyllene–producing maize line with an (*E*)-β-caryophyllene synthase and will evaluate its performance in an agronomical setting.

Another strategy is the use of the *tps23* promoter to control the expression of toxins, such as the *Bacillus thuringensis* Cry3 Bb 1 protein, that are effective against *D. v. virgifera*. This could provide the plant with an efficient, timely, and well-localized defense against this pest.

METHODS

Plant and Insect Material

Plants of the maize (*Zea mays*) varieties B73 (KWS Seeds), Graf (Landi), Delprim (Delley Samen und Pflanzen), and Pactol (Syngenta) were kindly provided by their respective breeders. The inbred lines F2, F476, Du101,

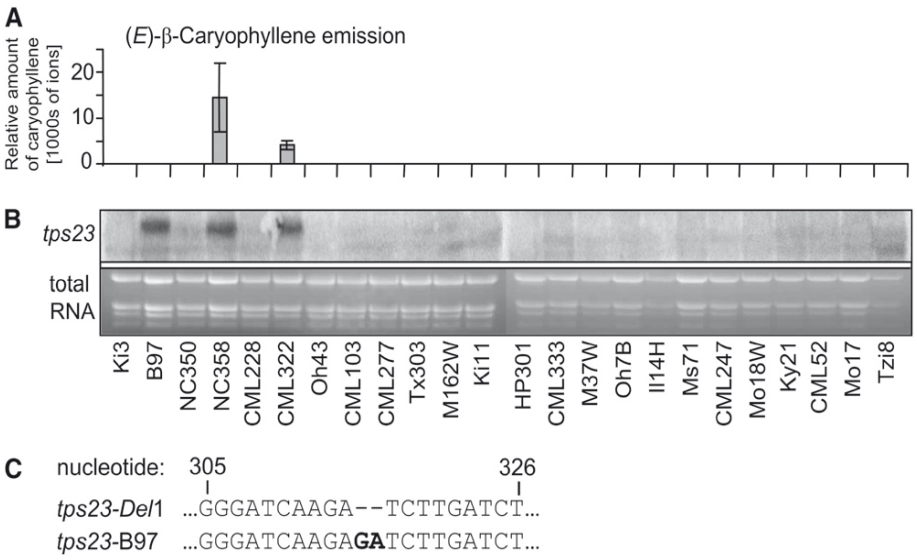

Figure 12. Most Maize Lines of North American Origin Do Not Produce (*E*)-β-Caryophyllene.

(A) A set of 24 inbred lines was tested for (*E*)-β-caryophyllene production in herbivore-damaged leaves. The averages ± SE of triplicate measurements of the (*E*)-β-caryophyllene amounts are shown.

(B) Accumulation of *tps23* transcript in herbivore-damaged leaves. The bottom panel shows the total RNA on the ethidium bromide–stained gel as a loading control.

(C) The *tps23* allele of the inbred line B97 contains a 2-bp insertion at nucleotide 315 compared with *tps23-Del*, which results in an inactive enzyme.

W401, F7001, and F670 and seeds of the teosinte (*Zea* sp) species were a gift from the Station de Génétique Végétale, Institut National de la Recherche Agronomique, and the 24 North American inbred lines (small diversity panel) was supplied by the National Germplasm System of the USDA Agricultural Research Service. The plants were grown in commercially available potting soil in a climate-controlled chamber with a 16-h photoperiod, 1 mmol·m^{-2}·s^{-1} photosynthetically active radiation, a temperature cycle of 22/18°C (day/night), and 65% RH. Twelve- to 15-d-old plants (20 to 30 cm high, four to five expanded leaves) were used in all experiments. Eggs of *Spodoptera littoralis* (Lepidoptera: Noctuidae) were obtained from Aventis and were reared on an artificial wheat germ diet (Heliothis mix; Stonefly Industries) for ~10 to 15 d at 22°C under an illumination of 750 μmol·m^{-2}·s^{-1}. For the *S. littoralis* treatments, three third instar larvae were enclosed on the middle portion of each plant for 20 h in a cage made out of two halves of a Petri dish (9 cm diameter) with a circle cut out of each side and covered with gauze to allow for ventilation (Röse et al., 1996). Larvae of *Diabrotica virgifera virgifera* were obtained from CABI BioSience, and nematodes of the species *Heterorhabditis megidis* were supplied by Andermatt Biocontrol. For the *D. v. virgifera* treatment, each maize plant was subjected to four second instar or third instar larvae for 2 d. The solitary endoparasitoid *Cotesia marginiventris* that was used in the experiments originated from the USDA Agricultural Research Service, Biological Control and Mass Rearing Research Unit. For the rearing of parasitoids, 25 young caterpillars (3 to 4 d old) were offered to a single mated female (4 to 7 d old) for 3 h in a plastic box (9.5 cm diameter, 5 cm high). The caterpillars were further reared on artificial diet in an incubator (25°C, 16 h of light/8 h of dark) until cocoon formation. Cocoons were kept in Petri dishes until adult emergence. Emerging adults were sexed and kept in cages (30 × 30 × 30 cm) at a male:female ratio of 1:2, with distilled water on cotton and honey as a food

Table 2. Properties of the *tps23* Alleles and Their Promoters in the Hybrid Lines Delprim, Graf, and Pactol and the Inbred Lines B73, F2, F467, Du101, W401, F670, and F7001

Line	Caryophyllene	5.6-kb Intron	Promoter Allele 1	Promoter Allele 2	ORF Allele 1	3′ Untranslated Region Allele 1
Graf	+	+	−	+	+	+
Delprim	+	+	+	+	+	+
F2	+	+	+	−	+	+
F476	+	+	+	−	Not tested	+
Du101	+	+	+	−	Not tested	+
W401	+	+	+	−	Not tested	+
F670	−	+	+	−	Not tested	+
F7001	−	+	+	−	Not tested	+
B73	−	+	+	−	+	+
Pactol	−	+	+	−	+	+

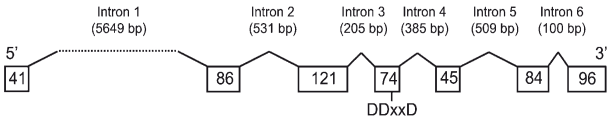

Figure 13. Exon–Intron Structure of *tps23*.

The seven exons are represented by boxes showing the number of amino acids they contain. The first intron is enlarged by the insertion of a transposon-like sequence element of ~5.4 kb. DDxxD marks the position of the Asp-rich region in the active center of the protein. The dotted line marks the transposon-like sequence included in the first intron.

source. The cages were kept in the laboratory under ambient light and temperature conditions.

Bioassays

Attraction of the nematode *H. megidis* toward (*E*)-β-caryophyllene was tested with belowground six-arm olfactometer assays (Rasmann et al., 2005). The apparatus consisted of a central glass chamber with six evenly distributed side arms that connect it to six glass pots. The entire system was filled with moist sand (10% water). To test the attractiveness of (*E*)-β-caryophyllene, amber glass vials (1.5 mL; Supelco and Sigma-Aldrich) were half filled with glass wool and 200 mL of authentic (*E*)-β-caryophyllene was added. Vials were closed with an open screw cap containing a septum, through which a 100-μL capillary (Hirschmann Laborgeräte) was inserted into the vial's saturated head space. The vial was then placed upside down with the capillary projecting into the sand in one of the outer pots of the six-arm olfactometer, ensuring a constant release of (*E*)-β-caryophyllene into the sand.

To compare the attractiveness of (*E*)-β-caryophyllene with that of other substances, an aliquot of 0.2 μL of authentic (*E*)-β-caryophyllene (98% pure; Sigma-Aldrich), (*E*)-β-farnesene (Bedoukian), linalool (95% pure; Sigma-Aldrich), nerolidol (98% pure; Sigma-Aldrich), or (*Z*)-3-hexenyl acetate (98% pure; Sigma-Aldrich) was injected into one of the glass pots, and the five untreated pots were used as controls. About 2000 *H. megidis* were released in a drop of water in the center of the central arena. Ultrafine screens at the end of each olfactometer arm prevented the nematodes from entering the pots. These arms consist of detachable parts from which nematodes can be recovered (for details, see Rasmann et al., 2005). Our study used six belowground olfactometers simultaneously. Twenty-four hours after *H. megidis* release, the belowground olfactometers were disassembled and the sand from each arm was placed on separate cotton filter discs (Hoeschele) in Bearmann extractors (Curran, 1992; Hass et al., 1999). The next day, recovered nematodes were counted.

To test the aboveground role of (*E*)-β-caryophyllene in attracting herbivore enemies, *S. littoralis* caterpillars and the solitary endoparasitoid wasp, *C. marginiventris*, were reared as described above. We tested mated, 2- to 5-d-old females, both naive and experienced individuals. Experienced females were obtained by placing them in a tube containing 20 *S. littoralis* larvae on top of a vessel that was connected via a glass capillary to a 2-mL glass vial filled with 300 μL of synthetic (*E*)-β-caryophyllene. The release rate was calibrated to the range of (*E*)-β-caryophyllene concentrations that are released by maize plants. The wasps were released in the tube one at a time and removed after three to five ovipositions. For each replicate, six wasps were provided with this oviposition experience.

The attractiveness of the (*E*)-β-caryophyllene to *C. marginiventris* females was tested in a four-arm olfactometer as described by D'Alessandro and Turlings (2005). In all experiments, the (*E*)-β-caryophyllene–releasing device was installed in the airflow of one of the four olfactometer arms. Cleaned and humidified air entered each vessel at 1.2 L/min carrying the volatiles via the arms to a central cylinder. Simultaneously, 0.6 L/min air was pulled out through volatile collection traps containing the adsorbent Super Q (80/100 mesh; Alltech), which were connected to a port at the top of each vessel. Wasps of the same experience type were released in groups of six into the central glass cylinder and could choose to enter one of the four arms. After entering an arm, their passage was blocked by a stainless steel screen and eventually they oriented toward a light source into glass bulbs, where they were counted and removed. Wasps that did not enter an arm within 30 min were considered as having made no choice, while wasps that chose an arm were considered responsive. A total of four groups of six wasps were tested during a 3-h period, alternating between the two experience types [naive or experienced with (*E*)-β-caryophyllene]. The experiment was repeated seven times.

The behavioral responses of parasitoids and entomopathogenic nematodes to (*E*)-β-caryophyllene were analyzed with a log-linear model. The data did not conform to the simple variance assumptions implied in using the multinomial distribution. Therefore, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer and for the fact that not all of the wasps made a choice during the 30 min of the trial. The adequacy of the model was assessed through likelihood ratio statistics and examination of residuals in the software package R (R Foundation of Statistical Software, version 2.4.0; www.r-project.org) (Turlings et al., 2004; Ricard and Davison, 2007). We tested treatment effects (i.e., odor sources) for naive and experienced wasps individually. In addition, we tested for a significant effect of the experience and an interaction between treatment and experience.

Volatile Collection

For the analysis of volatile terpenes, leaf material was frozen in liquid nitrogen and pulverized in a mortar. An aliquot of 0.2 g of plant powder was placed in a glass vial with a septum in the lid. A 100-μm PDMS SPME fiber (Supelco) was inserted through the septum and exposed for 60 min at 40°C. The compounds adsorbed onto the fiber were analyzed by gas chromatography–mass spectrometry.

cDNA Library Construction

Ten-day-old maize plants of cv Delprim were subjected to herbivory by *S. littoralis* for 4 h. One gram of leaf material was ground in a mortar to a fine powder in liquid nitrogen and added to 10 mL of Trizol reagent (Gibco BRL). The mixture was treated with a Polytron (Kinematika) for 1 min and incubated for 3 min on ice. Total RNA was isolated according to the manufacturer's instructions. From ~80 μg of total RNA, the mRNA was isolated using poly(T)-coated ferromagnetic beads (Dynal). The mRNA was transcribed into cDNA, and a Marathon RACE library was constructed according to the manufacturer's instructions (Clontech).

Isolation of *tps23* cDNA from Maize and Teosinte

Sequences with high similarity to plant terpene synthases were identified in BLAST searches of The Institute for Genomic Research Maize Database (http://maize.tigr.org/). One of these fragments (AZM4_53695) was cloned, sequenced, and extended toward the 5′ end by the Marathon RACE procedure (Clontech) from a cDNA library of herbivore-induced leaves of the maize cultivars Graf and Delprim. The complete sequence, amplified with the primers BH3fwd (5′-ATGGCAGCTGATAGGCAAGATCCG-3′) and BH2rev (5′-TTAGTCTATTAGATGCACATACAATG-3′) from a cDNA and introduced into the sequencing vector pCR4-TOPO (Invitrogen), contains an ORF of 1644 bp. The apparent orthologs of *tps23* from the teosinte species were cloned using cDNA from herbivore-induced leaves of each of the teosinte species and the primers mentioned above. The genomic clones from *tps23* were cloned from genomic DNA of the respective maize varieties using the same set of primers. To avoid sequence errors, all putative orthologs were cloned from two independent

amplification reactions. No evidence for additional genes with high sequence identity was found in any of the teosinte species or maize varieties.

Phylogenetic Analysis

Sequence analysis was performed with the DNAstar suite of programs (Lasergene), and nucleotide substitution rates were determined with the program Syn-SCAN (http://hivdb.standford.edu/pages/synscan.html) according to the method of Nei and Gojobori (1986). For dendrogram analysis, the ORFs of (*E*)-β-caryophyllene synthases (Figure 5) and ORFs of putative *tps23* orthologs (see Supplemental Figure 1 online) were aligned with DNAstar utilizing a ClustalW algorithm (matrix, PAM250; gap penalty,10; gap length, 0.2; delay divergent sequence, 20; DNA transition weight, 0.5) with no additional adjustment. The dendrograms were created using the TREECON 1.3b software package (Van de Peer and De Wachter, 1994) using a neighbor-joining algorithm with bootstrap values from 1000 trials.

Isolation of the 5′ and 3′ Flanking Regions and Intron 1 of *tps23*

For each of the maize lines assayed, genomic DNA was prepared with the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The Universal GenomeWalker kit (Clontech) was used to isolate a 1.8-kb DNA fragment upstream of the *tps23* open reading frame. To compare promoter alleles from different maize cultivars, the promoter fragments were amplified from genomic DNA by nested PCR using the primers BH13fwd (5′-GTTAGTCCAATATTTGTGTTGGGC-3′), BH12rev (5′-GACGGATCTTGCCTCATCAGCTGCC-3′) and BH14fwd (5′-TTCAACCACCAAAATTAATACTGGG-3′), BH11rev (5′-GTATACTAGCTAGCTACTCTCCTGC-3′), respectively, cloned into the sequencing vector pCR4-TOPO (Invitrogen), and fully sequenced. To analyze the downstream region of *tps23*, a 1.5-kb fragment containing 880 bp of the untranslated 3′ region was amplified with the primers BH31fwd (5′-GTGCTATAATGCCGAGACAGAATGGCGTGACAAG-3′) and BH32rev (5′-CAATTCATGTGGATTGGGTAGGATTGAGTGGGTTTC-3′), cloned, and sequenced as described above. To test for the presence of the unusually large intron 1, PCR was performed with the gene-specific primer BH3fwd (5′-ATGGCAGCTGATAGGCAAGATCCG-3′) located on exon 1 and the intron-specific primer BH26rev (5′-GATCTAAGGCCGTGTTTTATTCGC-3′). The resulting 600-bp fragment was cloned and sequenced. The complete intron 1 was isolated from the inbred lines B73 and F2 using nested PCR with the primers BH3fwd, BH22rev (5′-AGTAACATTTTCTTCACCTCCTCC-3′) and BH27fwd (5′-CACAGTGAGGAGGACATGCATGGG-3′), BH21rev (5′-ATTTCGACGTTATCCTTCATAATC-3′), respectively.

Heterologous Expression of Terpene Synthases

For expression with an N-terminal 8× His tag, the ORF of *tps23* was amplified with the primers BH8fwd (5′-ATTGCCATGGCGCAGCTGATGAGGCAAGATCC-3′) and BH9rev (5′-ATTAGAATTCTTAGTCTATTAGATGCACATAC-3′) and cloned as a *Nco*I-*Eco*RI fragment into the expression vector pHIS8-3. The construct was introduced into the *Escherichia coli* strain BL21 (DE3) and fully sequenced to avoid errors introduced by DNA amplification. Liquid cultures of the bacteria harboring the expression constructs were grown at 37°C to an OD_{600} of 0.6. Then, isopropyl-β-thiogalactopyranoside was added to a final concentration of 1 mM, and the cultures were incubated for 20 h at 18°C. The cells were collected by centrifugation and disrupted by a 4 × 30 s treatment with a sonicator (Bandelin UW2070) in chilled extraction buffer (50 mM MOPSO, pH 7.0, with 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM DTT, and 10% [v/v] glycerol). The cell fragments were removed by centrifugation at 14,000*g*, and the supernatant was desalted into assay buffer (10 mM MOPSO, pH 7.0, 1 mM DTT, and 10% [v/v] glycerol) by passage through an Econopac 10DG column (Bio-Rad). For kinetic studies, the His-tagged enzyme was further purified on a nickel-nitrilotriacetate agarose column (Qiagen) according to the manufacturer's instructions.

Assay for Terpene Synthase Activity

To determine the catalytic activity of the terpene synthase TPS23, enzyme assays containing 50 μL of the bacterial extract and 50 μL of assay buffer with 10 μM (*E*,*E*)-FPP, 10 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.2 mM $NaWO_4$, and 0.1 mM NaF in a Teflon-sealed, screw-capped 1-mL gas chromatograph glass vial were performed. A SPME fiber consisting of 100-μm polydimethylsiloxane (Supelco) was placed into the head space of the vial for a 1-h incubation at 30°C. For analysis of the adsorbed reaction products, the SPME fiber was inserted directly into the injector of the gas chromatograph.

For the determination of metal ion cofactors, K_m values, and effects of pH, an assay containing 1 μM purified TPS23 protein, 10 μM [1-^{3}H](*E*,*E*)-FPP (37 GBq/mol; American Radiolabeled Chemicals), and 10 mM $MgCl_2$ in 100 μL of assay buffer was used. The assay was overlaid with 1 mL of pentane to trap volatile products and incubated for 20 min at 30°C. The reaction was stopped by mixing, and 0.5 mL of the pentane layer was taken for the measurement of radioactivity by liquid scintillation counting in 2 mL of Lipoluma cocktail (Packard Bioscience) using a Packard Tricarb 2300TR liquid scintillation counter (^{3}H efficiency = 61%).

The pH optimum was determined in buffers from pH 5.0 to 11.0. Assay results are reported as means of three independent replicate assays, and each experiment was repeated two to three times with similar results. The K_m values were determined using seven substrate concentrations with four repetitions each. The enzyme activity was stable for at least 1 month when stored at −80°C. The concentration of the purified protein was determined by the method of Bradford (1976) using the Bio-Rad reagent with BSA as a standard.

Gas Chromatography–Mass Spectrometry

A Hewlett-Packard model 6890 gas chromatograph was employed with the carrier gas He at 1 mL/min, splitless injection (injector temperature of 220°C), a Chrompack CP-SIL-5 CB-MS column [5% (phenyl)-methylpolysiloxane, 25 m × 0.25 mm i.d. × 0.25 μm film thickness; Varian], and a temperature program from 40°C (3-min hold) at 5°C/min to 240°C (3-min hold). The coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector, transfer line temperature of 230°C, source temperature of 230°C, quadrupole temperature of 150°C, ionization potential of 70 eV, and a scan range of 40 to 350 atomic mass units. Products were identified by comparison of retention times and mass spectra with authentic reference compounds.

RNA Hybridization

Plant RNA was prepared with the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. A 400-bp fragment containing the first two exons of *tps23* was used as a probe, generated by linear PCR with the primer 5′-GAACTTCAAAAATACATCAGA-3′ and the complete ORF as a template. The probe was labeled with [^{32}P]ATP using the Strip-EZ PCR procedure (Ambion). Blotting on a Nytran-Plus nylon membrane (Schleicher and Schuell), hybridization, and washing were performed (Sambrook, 1989). The blots were scanned with a Storm 840 PhosphorImager (Molecular Dynamics). All RNA hybridization experiments were performed in two biological replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database. The cDNA sequences for *tps23* alleles and its promoters from the different maize lines were deposited in GenBank (http://www.ncbi.

nlm.nih.gov) with the accession numbers EU259634 (genomic sequence from inbred line B73), EU259632 (coding sequence *tps23-Graf1*), EU259633 (coding sequence *tps23-Del1*), EU259635 (coding sequence *tps23-Del2*), EU259636 (1.8-kb promoter fragment *tps23-Del1*), and EU259637 (1.8-kb promoter fragment *tps23-Del2*). The apparent orthologs of *tps23* in teosinte species have the accession numbers EU259638 (*Z. diploperennis*), EU259639 (*Z. m. huehuetenangensis*), EU259640 (*Z. luxurians*), EU259641 (*Z. m. mexicana*), EU259642 (*Z. m. parviglumis*), and EU259643 (*Z. perennis*). The accession numbers for the maize terpene synthases TPS10 and TPS4 are AAS88571 and AAX99146, respectively. The accession numbers of (*E*)-β-caryophyllene synthases from other plants are AAO85539 (*Arabidopsis* At TPS27), AAL79181 (*Artemisia annua* QHS1), AAU05952 (*Cucumis sativus*), and ABJ16553 (*Oryza sativa* Os TPS3), and the accession number of the *Arabidopsis* diterpene synthase At DTPS is NP_178064.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Amino Acid Alignment of the Putative TPS23 Orthologs from Maize and the Teosinte Species *Z. parviglumis*, *Z. luxurians*, *Z. mays mexicana*, *Z. diploperennis*, *Z. perennis*, and *Z. huehuetenangensis*.

Supplemental Figure 2. Sesquiterpene Products of the Putative TPS23 Orthologs.

Supplemental Data Set 1. Amino Acid Alignment Used to Produce the Dendrogram Presented in Figure 7.

Supplemental Data Set 2. Amino Acid Alignment Used to Produce the Dendrogram Presented in Figure 11.

ACKNOWLEDGMENTS

We thank Jens Wurlitzer for excellent technical assistance. We are also indebted to KWS Seeds for maize B73 seeds and Syngenta for *Spodoptera littoralis*. The inbred lines were kindly provided by the Station de Génétique Végétale, Institut National de la Recherche Agronomique Ferme du Moulon, and the National Germplasm System of the USDA Agricultural Research Service. This work was supported by the German National Science Foundation (Grant DE-837/2-2) and the Max Planck Society and in part by the Swiss National Center of Competence in Research, Plant Survival.

Received March 15, 2007; revised December 17, 2007; accepted February 6, 2008; published February 22, 2008.

REFERENCES

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

Buckler, E.S., Goodman, M.M., Holtsford, T.P., Doebley, J.F., and Sanchez, J. (2006). Phylogeography of the wild subspecies of *Zea mays.* Maydica **51:** 123–134.

Cai, Y., Jia, J.W., Crock, J., Lin, Z.X., Chen, X.Y., and Croteau, R. (2002). A cDNA clone for β-caryophyllene synthase from *Artemisia annua.* Phytochemistry **61:** 523–529.

Chen, X.J., Wang, M., Chen, Y., Davisson, V.J., and Heinstein, P. (1996). Cloning and heterologous expression of a second (+)-δ-cadinene synthase from *Gossypium arboreum.* J. Nat. Prod. **59:** 944–951.

Cheng, A.-X., Xiang, C.-Y., Li, J.-X., Yang, C.-Q., Hu, W.-L., Wang, L.-J., Lou, Y.-G., and Chen, X.-Y. (2007). The rice (*E*)-β-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes. Phytochemistry. **68:** 1632–1641.

Crock, J., Wildung, M., and Croteau, R. (1997). Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha* × *piperita*, L.) that produces the aphid alarm pheromone (*E*)-β-farnesene. Proc. Natl. Acad. Sci. USA **94:** 12833–12838.

Cunillera, N., Boronat, A., and Ferrer, A. (1997). The *Arabidopsis thaliana fps*1 gene generates a novel mRNA that encodes a mitochondrial farnesyl-diphosphate synthase isoform. J. Biol. Chem. **272:** 15381–15388.

Curran, J. (1992). Influence of application method and pest population size on the field efficacy of entomopathogenic nematodes. J. Nematol. **24:** 631–636.

D'Alessandro, M., Held, M., Triponez, Y., and Turlings, T.C.J. (2006). The role of indole and other shikimic acid derived maize volatiles in the attraction of two parasitic wasps. J. Chem. Ecol. **32:** 2733–2748.

D'Alessandro, M., and Turlings, T.C.J. (2005). In situ modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. Chem. Senses **30:** 739–753.

Daraselia, N.D., Tarchevskaya, S., and Narita, J.O. (1996). The promoter for tomato 3-hydroxy-3-methylglutaryl coenzyme A reductase gene 2 has unusual regulatory elements that direct high-level expression. Plant Physiol. **11:** 727–733.

Degen, T., Dillmann, C., Marion-Poll, F., and Turlings, T.C.J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. Plant Physiol. **135:** 1928–1938.

Dicke, M. (1999). Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? Entomol. Exp. Appl. **91:** 131–142.

Dicke, M., and van Loon, J.J.A. (2000). Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomol. Exp. Appl. **97:** 237–249.

Ellsbury, M.M., Jackson, J.J., Woodson, W.D., Beck, D.L., and Stange, K.A. (1996). Efficacy, application distribution, and concentration by stemflow of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) suspensions applied with a later-move irrigation system for corn rootworm (Coleoptera: Chrysomelidae) control in maize. J. Econ. Entomol. **89:** 74–81.

Enjuto, M., Lumbreras, V., Marín, C., and Boronat, A. (1995). Expression of the Arabidopsis *HMG2* gene, encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, restricted to meristematic and floral tissues. Plant Cell **7:** 517–527.

Gershenzon, J., and Kreis, W. (1999). Biosynthesis of monoterpenes, sesquiterpenes, diterpenes, sterols, cardiac glycosides and steroid saponins. In Biochemistry of Plant Secondary Metabolism, Annual Plant Reviews, Vol. 2, M. Wink, ed (Sheffield, UK: Sheffield Academic Press), pp. 222–299.

Gouinguené, S., Degen, T., and Turlings, T.C.J. (2001). Variability in herbivore-induced odour emissions among maize cultivars and their wild ancestors (teosinte). Chemoecology **11:** 9–16.

Grosjean, D., Williams, E.L., Grosjean, E., Andino, J.M., and Seinfeld, J.H. (1993). Atmospheric oxidation of biogenic hydrocarbons: Reaction of ozone with β-pinene, D-limonene and *trans*-caryophyllene. Environ. Sci. Technol. **27:** 2754–2758.

Hammack, L. (2001). Single and blended maize volatiles as attractants for diabroticite corn rootworm beetles. J. Chem. Ecol. **27:** 1373–1390.

Hass, B., Griffin, C.T., and Downes, M.J. (1999). Persistence of *Heterorhabditis* infective juveniles in soil: Comparison of extraction and infectivity measurements. J. Nematol. **31:** 508–516.

Hiltpold, I., and Turlings, T.C.J. (2008). Belowground chemical signaling in maize: When simplicity rhymes with efficiency. J. Chem. Ecol., in press.

Hoballah, M.E., Köllner, T.G., Degenhardt, J., and Turlings, T.C.J. (2004). Costs of induced volatile production in maize. Oikos **105:** 168–180.

Hoballah, M.E., Tamò, C., and Turlings, T.C.J. (2002). Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: Is quality or quantity important? J. Chem. Ecol. **28:** 951–968.

Jackson, J.J. (1996). Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. **89:** 366–372.

Karban, R., and Baldwin, I.T. (1999). Induced Responses to Herbivory. (Chicago: University of Chicago Press).

Kessler, A., and Baldwin, I.T. (2002). Plant responses to insect herbivory: The emerging molecular analysis. Annu. Rev. Plant Biol. **53:** 299–328.

Köllner, T.G., O'Maille, P.E., Gatto, N., Boland, W., Gershenzon, J., and Degenhardt, J. (2006). Two pockets in the active site of maize sesquiterpene synthase TPS4 carry out sequential parts of the reaction scheme resulting in multiple products. Arch. Biochem. Biophys. **448:** 83–92.

Köllner, T.G., Schnee, C., Gershenzon, J., and Degenhardt, J. (2004a). The sesquiterpene hydrocarbons of maize (*Zea mays*) form five groups with distinct developmental and organ-specific distributions. Phytochemistry **65:** 1895–1902.

Köllner, T.G., Schnee, C., Gershenzon, J., and Degenhardt, J. (2004b). The variability of sesquiterpenes emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. Plant Cell **16:** 1115–1131.

Korth, K.L., Stermer, B.A., Bhattacharyya, M.K., and Dixon, R.A. (1997). HMG-CoA reductase gene families that differentially accumulate transcripts in potato tubers are developmentally expressed in floral tissues. Plant Mol. Biol. **33:** 545–551.

Liu, K., Goodman, M., Muse, S., Smith, J.S., Buckler, E., and Doebley, J. (2003). Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. Genetics **164:** 2117–2128.

Meiners, T., and Hilker, M. (2000). Induction of plant synomones by oviposition of a phytophagous insect. J. Chem. Ecol. **26:** 221–232.

Mercke, P., Kappers, I.F., Verstappen, F.W.A., Vorst, O., Dicke, M., and Bouwmeester, H.J. (2004). Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. Plant Physiol. **135:** 2012–2024.

Nei, M., and Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:** 418–426.

Okada, K., Saito, T., Nakagawa, T., Kawamukai, M., and Komiya, Y. (2000). Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in Arabidopsis. Plant Physiol. **122:** 1045–1050.

Picaud, S., Brodelius, M., and Brodelius, P.E. (2005). Expression, purification and characterization of recombinant (*E*)-β-farnesene synthase from *Artemisia annua.* Phytochemistry **66:** 961–967.

Picaud, S., Olsson, M.E., Brodelius, M., and Brodelius, P.E. (2006). Cloning, expression, purification and characterization of recombinant (+)-germacrene D synthase from *Zingiber officinale.* Arch. Biochem. Biophys. **452:** 17–28.

Pichersky, E., and Gang, D.R. (2000). Genetics and biochemistry of secondary metabolites in plants: An evolutionary perspective. Trends Plant Sci. **5:** 439–445.

Rasmann, S., Köllner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J., and Turlings, T.C.J. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature **434:** 732–737.

Ricard, I., and Davison, A.C. (2007). Statistical inference for olfactometer data. Appl. Stat. **56:** 479–492.

Röse, U.S.R., Manukian, A., Heath, R.R., and Tumlinson, J.H. (1996). Volatile semiochemicals released from undamaged cotton leaves. A systemic response of living plants to caterpillar damage. Plant Physiol. **111:** 487–495.

Sabulal, B., Dan, M., Anil, J.J., Kurup, R., Pradeep, N.S., Valsamma, R.K., and George, V. (2006). Caryophyllene-rich rhizome oil of *Zingiber nimmonii* from South India: Chemical characterization and antimicrobial activity. Phytochemistry **67:** 2469–2473.

Sambrook, J. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schnee, C., Köllner, T.G., Gershenzon, J., and Degenhardt, J. (2002). The maize gene terpene synthase 1 encodes a sesquiterpene synthase catalyzing the formation of (*E*)-β-farnesene, (*E*)-nerolidol, and (*E,E*)-farnesol after herbivore damage. Plant Physiol. **130:** 2049–2060.

Schnee, C., Köllner, T.G., Held, M., Turlings, T.C.J., Gershenzon, J., and Degenhardt, J. (2006). The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. Proc. Natl. Acad. Sci. USA **103:** 1129–1134.

Shen, B., Zheng, Z., and Dooner, H.K. (2001). A maize sesquiterpene cyclase gene induced by insect herbivory and volicitin: Characterization of wild-type and mutant alleles. Proc. Natl. Acad. Sci. USA **97:** 14801–14806.

Sotelo, A. (1997) Constituents of wild food plants. In Functionality of Food Phytochemicals, T. Jones and J. Romeo, eds (New York: Plenum Press), pp. 89–111.

Starks, C.M., Back, K., Chappell, J., and Noel, J.P. (1997). Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. Science **277:** 1815–1820.

Tholl, D., Chen, F., Petri, J., Gershenzon, J., and Pichersky, E. (2005). Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from Arabidopsis flowers. Plant J. **42:** 757–771.

Trapp, S.C., and Croteau, R. (2001). Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics **158:** 811–832.

Turlings, T.C.J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G., and Dorn, S. (1998). The induction of volatile emissions in maize by three herbivore species with different feeding habits: Possible consequences for their natural enemies. Biol. Control **11:** 122–129.

Turlings, T.C.J., Davison, A., and Tamò, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiol. Entomol. **29:** 45–55.

Turlings, T.C.J., Tumlinson, J.H., Heat, R.R., Proveaux, A.T., and Doolittle, R.R. (1991). Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. J. Chem. Ecol. **17:** 2235–2251.

Turlings, T.C.J., Tumlinson, J.H., and Lewis, W.J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Science **250:** 1251–1253.

Van de Peer, Y., and De Wachter, Y. (1994). TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. **10:** 569–570.

Walter, M.H., Hans, J., and Strack, D. (2002). Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: Differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J. **31:** 243–254.

Yoshikuni, Y., Ferrin, T.E., and Keasling, J.D. (2006). Designed divergent evolution of enzyme function. Nature **440:** 1078–1082.

Curriculum Vitae

I. Hiltpold

2008

Ivan Hiltpold; biologist

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Switzerland
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Swiss, born in Neuchâtel
09/07/1980
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curriculum vitae

education

2005 - 2008	**PhD, laboratory of fundamental and applied research in chemical ecology, Manipulation of tritrophic interactions: a key for belowground biological control?** (FARCE lab, supervised by Prof. T. C. J. Turlings, University of Neuchâtel, Switzerland, www.unine.ch/leae)	*Neuchâtel/CH*
2000 - 2005	**Master of Science in Behavioral Ecology and Evolution, Orientation of entomopathogenic nematodes towards *Diabrotica virgifera virgifera* infested maize roots** (FARCE lab, supervised by Prof. T. C. J. Turlings, University of Neuchâtel, Switzerland, www.unine.ch/leae) *CUM LAUDE*	*Neuchâtel/CH* *Hvasarhely/Hungary*
1999	**High school degree in economics** (Lycée Jean-Piaget, Neuchâtel, Switzerland (Ecole sup. de commerce))	*Neuchâtel/CH*

complementary education

2005 to present — **Graduate School of the National Center of Competence in Research «Plant Survival»** (completed the following courses) — *Neuchâtel/CH*

- Intellectual Property: must scientists care?
- How to make scientific presentations and posters interesting.
- Interaction of plants with other organisms: friends or foes?
- Effective public speaking
- Chemical identification: linking chromatography with mass spectrometry
- Deontology and ethics in science
- The rhizosphere
- An introduction to the practice of statistics in R
- Planning a career strategy - job finding methodology and networking
- Peer review and writing manuscripts

Ivan Hiltpold; biologist(2)

professional experience

teaching

2004 to present

supervision

co-advisor of a master student, Uni Neuchâtel

lectures

pest control in agriculture (2005, master level); nematodes and their impact on soil ecosytems (2006-2008, bachelor level)

practical courses / laboratory

invertebrate biology (2006-2008, course leader, bachelor level)
introduction to parametric statistic (2005, bachelor level)
entomology and systematics of insects (2004, bachelor level)

workshop

plant-insect interactions

field work

2002 to present

Turkey-Georgia-Letonia-Ukrania

field collection of pollinasators and plant in a evolutionary biology project in 2008 (1 month, help in a PhD project)

USA

field experiments in collaboration with Ass. Prof. Bruce Hibbard at the University of Missouri (Columbia, MO) in 2007 (3 months, part of the PhD)

Mexico

field collection of parasitoids of insect pests of maize (2006, 2 months, part of the PhD) and beans (2005, 2 months, help in a master project)

Hungary

field experiments in collaboration with Dr. S. Töpfer at the Plant Health Station of Hodmesovasarhely in 2004 (5 months, field work during the master thesis) and 2005 (1 month, part of the PhD)

Switzerland

biodiversity and conservation studies for the cantonal office for nature conservation in 2002 (3 months, internship)

popularization

2007

botanical garden of Neuchâtel

co-organisation of the exhibition «Bzzzzz...» on insect-plant interactions

other

2005 to present

webmaster

responsibale for creation and updates of the FARCE and e-vol laboratory websites (www2.unine.ch/leae)

meeting organisation

co-organiser of the meeting «Conflicts of interest in mutualistic interactions» at the University of Neuchâtel (September 2006)

Ivan Hiltpold; biologist(3)

languages

French - mother tongue

English - excellent knowledge (written and oral, work language)

German - basic knowledge (written and oral)

computer knowledge

considerable practice in the use of Microsoft Office software (Word, Excel and PowerPoint)

much experience using graphical software including Adobe Illustrator, Adobe InDesign and Adobe Photoshop

good practice of statistical and graphical software such as SigmaPlot, SigmaStat, SPSS, R, SAS, and GIS software (ESRI, Arc Info, Arc View GIS)

personal skills

independence in work

due to considerable experience with lab and field work, I am able to find original and ingenious ways to solve scientific questions

organisation and planning

as a result of the responsabilities I was given in the Swiss national boy scout movement (MSdS) I acquired skills in event organisation that I can apply to other situations such as work planning

chemical analyses

during my Master and PhD I used many different volatile compound analysis methods (GC, GC-MS, PTRMS, HPLC, SPME)

oral presentation

over the years, by attending and participating at international meetings and other events, I acquired excellent skills in oral presentation

design

I had many occasions to exercise my intuitive knowledge in designing posters and oral presentations

social skills

having had good human contacts and long experience in various types of communities, I am able to easily integrate into a group and rapidly understand the underlying rules of a community

Ivan Hiltpold; biologist(4)

grants

2008 **NCCR «Plant survival» travel grant (1000 CHF)**

2008 **Swiss Zoological Society travel grant (1000 CHF)**

publications

2008 Meissle M. Hiltpold I. *et al*., Belowground volatile emission of Bt maize after induction of plant defence , **IOBC wprs Bulletin** 33 85-92

2008 Hiltpold I. and Turlings T., Belowground chemical signaling in maize: When simplicity rhymes with efficiency, **Journal of Chemical Ecology** 34:5, 628-635

2008 Kölner T., Held M., Lenk C., Hiltpold I. *et al.*, A maize (E)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties, **The Plant Cell** 20 482-494

2008 Kurtz B., Hiltpold I., *et al*., Comparison of the infectiousness of entomopathogenic nematodes among larval instars and pupae of the western corn rootworm, **Biocontrol** (in press, available online)

2005 Rasmann S., Köllner T., Degenhardt J., Hiltpold I. *et al.*, Recruitment of entomopathogenic nematodes by insect-damaged maize roots, **Nature** 434: 732 - 737

meetings

invited presentation

4th International Symposium «Entomopathogenic nematodes and symbiotic bacteria» - June 2005 - Salzau, Germany - Influence of insect-damaged roots on the host-finding behavior of the entomopathogenic nematode *Heterorhabditis megidis* (invited speaker)

oral presentations

25th meeting of the International Society of Chemical Ecology - July 2008 - State College, PA, USA - Tritrophic interactions manipulation: a key to belowground biological control?

Diabr'Act meeting - May 2008 - Göttingen, Germany - Tritrophic interactions manipulation: a key to belowground biological control?

experts meeting on *Diabrotica* ecology - March 2007 - Nice - France

22nd Conference of the International Working Group on *Ostrinia* and other maize pests (IWGO) - November 2006 - Vienna, Austria - Influence of insect-damaged roots on the host-finding behavior of the entomopathogenic nematode *Heterorhabditis megidis*

Ivan Hiltpold; biologist(5)

poster presentations

13rd Symposium on Insect-Plant Relationships - July 2007 - Uppsala, Sweden - Belowground plant VOC - opening a new can of worms? (poster)

NCCR Plant Survival, Review Pannel Meeting - May 2005 - Neuchâtel, Switzerland - Nematode attraction: Is (E)-BETA-caryophyllene an ideal belowground signal? (poster)

references

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