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How maize root volatiles affect the efficacy of entomopathogenic nematodes in controlling the western corn rootworm?

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

Keywords *Heterorhabditis megidis* · *Heterorhabditis bacteriophora* · *Steinernema feltiae* · *Diabrotica virgifera virgifera* · *Zea mays* · Tritrophic interaction · Crop protection · Biological control · Root exudates · Nematode attraction

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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 arthropod pests, often causing tremendous yield losses (Oerke 2006). In nature, plants have evolved various defence strategies to fend off their herbivorous 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 Benrey 1998; Turlings and Wäckers 2004). For maize, the attractiveness of such herbivore-induced plant volatiles to natural enemies of herbivores has been demonstrated in both laboratory and field experiments (Turlings et al. 1990; Bernasconi et al. 1998; Hoballah and Turlings 2005). 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; Schnee et al. 2006).

Recently it was found that roots also are able to recruit belowground enemies of soil dwelling herbivorous insects by releasing volatile signals. These volatiles can attract entomopathogenic nematodes (EPN) (van Tol et al. 2001; Boff et al. 2001; Bertin et al. 2003; Rasmann et al. 2005; Rasmann and Turlings 2008; Degenhardt et al. 2009), predatory mites (Aratchige et al. 2004) and even parasitoids (Neveu et al. 2002). 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 (E*β*C). E*β*C 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 (Kurtz et al. 2009). Two other species of EPNs, *Heterorhabditis bacteriophora* Poinar and *Steinernema feltiae* Filipjev, are also promising candidates as biological control agents against WCR larvae (Kurtz et al. 2009; 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 E*β*C emission by WCR-damaged maize roots for the efficacy of *H. bacteriophora*, *H. megidis*, and *S. feltiae* in controlling WCR larvae under field conditions. For this purpose, the three nematode species were released at two different time points in separate, WCR-infested maize plots in Hungary. The results of the field study prompted us to also conduct additional laboratory assays to test the apparent lack of attraction of *H. bacteriophora* towards E*β*C. We discuss the control potential of the tested nematode species and the importance of choosing the right maize variety to fully exploit this potential.

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. Experimental fields were divided in two plots, the first planted with the variety Magister (UFA Semences, Bussigny, Switzerland) that emits E*β*C after WCR feeding (Hiltpold 2008) and the second with the variety Pactol (Syngenta, Budapest, Hungary) that does not emit E*β*C (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 15 cm and row spacing of 75 cm. The fields were treated once 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.

Table 1 Characteristics of the study fields in southern Hungary and the timing of EPN application

Field	A	B	C	D
Location	Northwest of Hodmezovasarhely	North of Szatymaz	North of Szatymaz	Hodmezo-vasarhely
Coordinates	N 46° 26.022	N 46° 20.945	N 46° 20.945	N 46° 25.998
	E 20° 20.143	E 20° 00.574	E 20° 00.574	E 20° 20.348
Elevation (m)	83	87	87	83
Size (ha)	0.5	0.2	0.3	0.2
Soil bulk density (g/cm^3)	1.04 ± 0.13	1.4 ± 0.13	1.7 ± 0.07	1.1 ± 0.13
Soil moisture (wt%)*	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 (H_2O)	8.3	8.4	8.4	8.3
Maize sown	25 April 2005	8 May 2005	8 May 2006	28 April 2005
EPN applications	25 April 2005	8 May 2005	8 May 2006	28 April 2005
	14 June 2005	15 June 2005	7 June 2006	14 June 2005

Significance is indicated by asterisks

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 7–9°C in darkness until use. About 2–3 h 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 diameter, 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 1 week in darkness at 22°C, mortality of 80–100% was found for all EPN batches, which was considered sufficient for use.

Diabrotica virgifera virgifera

WCR eggs were obtained from eggs laid by field-collected beetles from southern Hungary (for procedures see Singh and Moore 1985). Eggs were kept in diapause in moist sand at 6–8°C. The diapause of WCR eggs was broken in early April by transferring them to a climate chamber at 25°C for 3 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, Germany), 2 ml of the egg suspension was applied into each of two 12 cm deep holes at a distance of 5–8 cm from either side of the maize plant ($\sim$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 plots of at least 14 rows of Magister and Pactol plants. Seven groups of six to seven maize plants were randomly selected from either the 3rd, 6th, 9th or 12th row of each section, thereby ensuring buffer rows between experimental groups. 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 groups, each treated with one nematode species at one particular date, were distributed over one experimental plot. The seventh group served as control and was not treated with nematodes.

The EPN suspensions were poured by hand in a continuous stream into a 10 cm deep groove dug 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 l of water, $2.1 \times 10^5 \pm 0.07$ SD infective juvenile nematodes were applied per metre. At the later EPN application date in June, they were suspended in 0.2 l of water and $2.6 \times 10^5 \pm 0.07$ SD infective juvenile nematodes were applied per metre. All applications were carried out in the evening or during cloudy afternoons to avoid harmful UV radiation.

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

Each of the 14 experimental groups (6–7 plants) of fields A to C (because of technical problems, emergence was not assessed in field D) was covered with a fine-mesh screen cage (1.3 m height $\times$ 0.75 m width $\times$ 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 20 June and 16 August 2005 and between 27 June and 16 August 2006. Total adult emergence was normalised to 100 eggs per plant. Nematode efficacy was calculated as percentage reduction in WCR adults compared to their untreated controls (corrected efficacy% = (1 − WCR in treated plots/WCR in the control) $\times$ 100) (Abbott 1925).

Effect of application time, nematode 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 group 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 respective control groups that did not receive any nematodes (corrected efficacy % = (1 − root damage in treated plots/root damage in the control) $\times$ 100) (Abbott 1925).

Olfactometer assays

Following the methodology developed by Rasmann et al. (2005), attraction of *H. bacteriophora* was assessed in six belowground olfactometers filled with moist sand. EPNs had to choose between a Pactol maize plant damaged by four WCR larvae, a healthy Pactol maize plant and four empty control pots. After 1 day of exposure, the olfactometers were disassembled, the sand from each of the six connectors was placed in 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 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 using SAS 9.1 with 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 analysed 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 2,000 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 quasi-likelihood 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

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

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

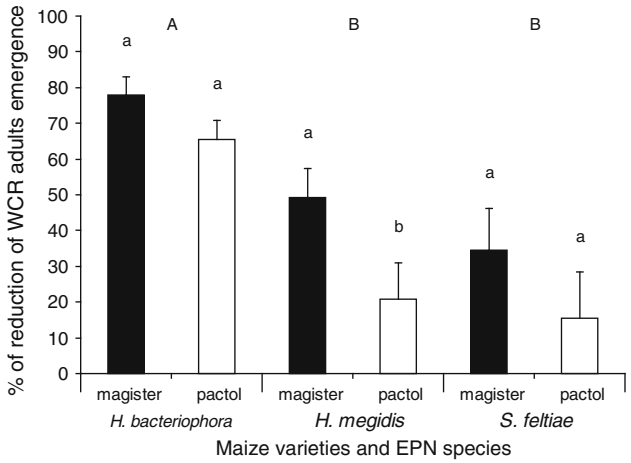

Fig. 1 Comparison of the reduction of WCR emergence relative to the untreated controls in maize fields with the EβC-emitting Magister variety and the non-emitting Pactol variety (pooled data for the two release dates). *Uppercase letters* indicate statistical differences between the three EPN species (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

Overall, WCR emergence was significantly different between the two maize varieties (Fig. 1; Table 2) with a lower emergence from rows with the EβC-emitting Magister than from rows with the non-emitting Pactol. *H. megidis* reduced WCR emergence 2.5-fold more in Magister plots than in Pactol plots ($P < 0.001$). There was no significant difference in the efficacy of *H. bacteriophora* and *S. feltiae* between the two maize varieties (Fig. 1, $P = 0.08$ and $P = 0.12$, respectively).

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

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

All tested EPN species significantly reduced root damage caused by WCR larvae, and the time of application had no

Table 2 Effects of EPN species, application period and maize variety on WCR adult emergence (% efficacy relative to control) according to the three-way ANOVA

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 × application period	0.05	2	0.02	0.30	0.735
EPN species × maize variety	0.03	2	0.02	0.23	0.795
Application period × maize variety	0.00	1	0.00	0.02	0.863
EPN species × application period × maize variety	0.04	2	0.02	0.23	0.788

Significance is indicated by asterisks

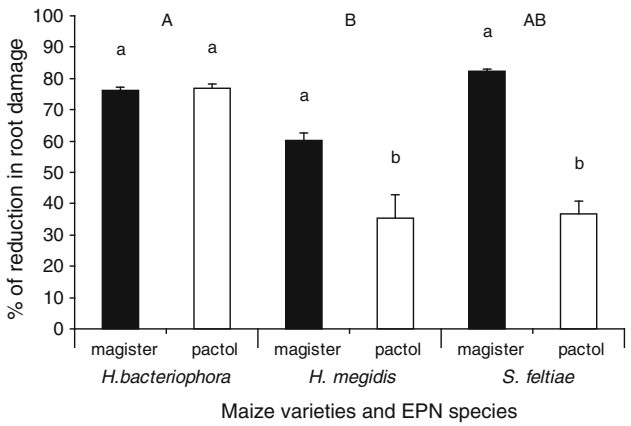

Fig. 2 Comparison of the reduction of root damage relative to the untreated controls in maize fields with the EβC-emitting Magister variety and with a non-emitting Pactol variety (pooled data for the two release dates). *Uppercase letters* indicate statistical differences between the three EPN species (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* $P = 0.480$). 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 on their efficacies (Fig. 2; Table 3). However, the efficacy of the nematodes was different for the two maize varieties (Fig. 2; Table 3). This difference was due 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 (Fig. 2; Table 3).

Olfactometer assays

When offered a choice between volatiles emitted by a WCR-damaged Pactol maize plant or a healthy Pactol 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 were found to be repellent, evidenced by the fact that fewer nematodes were collected from arms connected to pots with healthy plants than from arms connected to the control pots with sand only.

Discussion

The results from the field experiment confirm that the choice of maize variety and/or nematode species can significantly affect the control efficacy of EPNs. Kurtz et al. (2009) had already compared the efficacy of the three nematode species against WCR in a laboratory study and also reported that WCR was most susceptible to *H. bacteriophora.* EPN persistence in the soil has been shown to rapidly decrease with time (Kurtz et al. 2007). It was, therefore, surprising to find that there was no difference in the efficacy of EPN between the two application periods (during sowing in April/May or later in June) (Tables 1, 2). Apparently some early applied nematodes persisted, probably by producing a new generation on alternative hosts that resulted in sufficiently high abundance to reduce the later hatching WCR population. For inundative biological control strategies, it remains essential to find the optimal dose and release timing (Fenton et al. 2002).

The choice of the right maize variety seems particularly important for two of the three EPN species investigated, *H. megidis* and *S. feltiae* (Figs. 1, 2). *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 mainly use the so-called ambusher (nictating) foraging strategy. Although *S. feltiae* is known to actively move through soil (Grewal et al. 1994; Lewis 2002), it never responded to any 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 (Kurtz et al. 2009). Yet, the trend of reduced of adult emergence and a significant reduction of root damage for the Magister plants

Table 3 Effects of EPN species, application period and maize variety on WCR's root damage (% efficacy relative to control) according to the three-way ANOVA

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 varieties	15.25	1	15.25	7.81	0.005**
EPN species × application period	10.04	2	5.02	0.41	0.663
EPN species × maize variety	1.56	2	1.56	2.53	0.080
Application period × maize variety	1.63	1	0.82	0.78	0.377
EPN species × application period × maize variety	0.44	2	0.22	0.11	0.894

Significance is indicated by asterisks

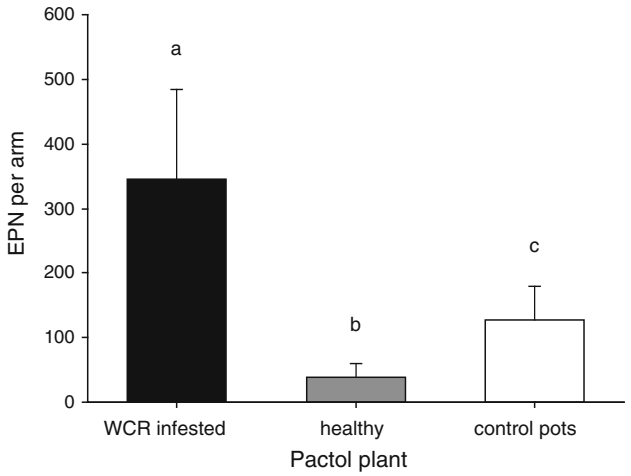

Fig. 3 *H. bacteriophora* is attracted by EβC non-emitting plants. When offered choice between a healthy, a WCR damaged Pactol plant or sand, this nematode species is significantly attracted towards the damaged plant even if no EβC is emitted (ANOVA, $F_{2,33} = 6.6$, $P < 0.001$). The healthy plant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

after *S. feltiae* application suggests that this nematode also responds to this root signal under field conditions. However, *S. feltiae* foraging efficiency might also have been affected by other aspects, such as WCR larval behaviour, affected by Magister plants. The combined results from these studies suggest that *S. feltiae* foraging behaviour is strongly determined by the media in which it has to ambush or cruise.

The effectiveness of *H. bacteriophora* was not affected by EβC emission from WCR-damaged maize roots (Figs. 1, 2) suggesting a random and uniformed spread of the nematode in the field. However, when offered a Pactol plant infested with WCR larvae (no emission of EβC) or a healthy Pactol plant, *H. bacteriophora* significantly migrated more toward the damaged plant (Fig. 3), implying that it uses other chemical cues for host location. These cues might come from the plants, but also from the hosts themselves. These and other (Rasmann and Turlings 2008) olfactometer assays show that healthy maize roots are repellent to *H. bacteriophora*. This could imply that it uses a unique and potentially highly efficient host location strategy. It remains unknown what signals allow *H. bacteriophora* to make the distinction, but it has been shown that it is sensitive to long-chain alcohols and possibly other, more insect specific, volatiles (O'Halloran and Burnell 2003).

Current WCR management strategies involve crop rotation and the use of insecticides (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), others have shown that resistance developed within three generations under greenhouse conditions (Meihls et al. 2008).

In the current study, we show 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 (Figs. 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, Kurtz et al. 2009; Toepfer et al. 2005), whereas transgenic maize seems to be efficient only against the first instar (Oyediran et al. 2005). Neonate WCR larvae may 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). In contrast, 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).

Conclusion

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. Further studies are needed to take optimal advantage of the biology and behavioural plasticity of EPN to maximise their persistence and their responses to plant-provided signals in the soil.

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