Selection of entomopathogenic nematodes for enhanced responsiveness to a volatile root signal helps to control a major root pest

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SUMMARY
The efficacy of natural enemies as biological control agents against insect pests can theoretically be enhanced by artificial selection for high responsiveness to foraging cues. The recent discovery that maize roots damaged by the western corn rootworm (WCR) emit a key attractant for insect-killing nematodes has opened the way to explore whether a selection strategy can improve the control of root pests. The compound in question, (E)-β-caryophyllene, is only weakly attractive to Heterorhabditis bacteriophora, one of the most infectious nematodes against WCR. To overcome this drawback, we used a six-arm below-ground olfactometer to select for a strain of H. bacteriophora that is more readily attracted to (E)-β-caryophyllene. After six generations of selection, the selected strain responded considerably better and moved twice as rapidly towards a (E)-β-caryophyllene source than the original strain. There was a minor trade-off between this enhanced responsiveness and nematode infectiveness. Yet, in subsequent field tests, the selected strain was significantly more effective than the original strain in reducing WCR populations in plots with a maize variety that releases (E)-β-caryophyllene, but not in plots with a maize variety that does not emit this root signal. These results illustrate the great potential of manipulating natural enemies of herbivores to improve biological pest control.

Key words: entomopathogenic nematode, tritrophic interaction, artificial selection, biological control, Diabrotica virgifera virgifera, western corn rootworm.

INTRODUCTION
The idea to improve biological control by enhancing the foraging efficiency, persistence and killing potential of biological control agents has been around for a considerable time (Hoy, 1976). Attempts to improve traits such as 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 have never been put into practice (Hoy, 2000). Also considered has been enhancing the responsiveness to specific host foraging cues in predators, parasitoids (Cortesero et al., 2000) and entomopathogenic nematodes (Gaugler et al., 1989; Gaugler and Campbell, 1991; Gaugler et al., 1991; Gaugler et al., 1994), but such attempts have been largely hampered by a lack of knowledge on which cues are of key importance (D’Alessandro and Turlings, 2006). For certain entomopathogenic nematode species, we now have knowledge of such key attractants (see below). This prompted us to investigate whether selection for enhanced responsiveness to a crucial root signal can improve the efficiency of nematodes in controlling the larvae of the beetle Diabrotica virgifera virgifera LeConte [Coleoptera: Chrysomelidae, known commonly as western corn rootworm (WCR)].

WCR is the most destructive maize pest in North America (Krysan and Miller, 1986), and, since its first introduction in the early 1990s (Baca, 1994; Sivcev et al., 1994), it also has become a serious invasive pest in Europe (Miller et al., 2005; Vidal 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 WCR larvae. Besides impeding water and nutrient uptake, the destruction of the root system can result 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 et al., 2005), provoking further investigations to develop novel control strategies.

Besides conventional pest-control strategies, biological control is being considered to manage the WCR population in Europe (Kuhlmann and van der Brugt, 1998). As yet, no effective indigenous natural enemies have been found (Toepfer and Kuhlmann, 2004). The release of mass-produced entomopathogenic nematodes (EPN) as biological agents has been considered one of the most promising strategies (Kuhlmann and van der Brugt, 1998). These nematodes are obligate insect parasites in symbiosis with bacteria. After entering the host, the infective juvenile (the infective stage of EPN) releases its symbiotic bacteria, which multiply and kill the insect by septicaemia and serve as food for EPN. When nutrients are consumed and space is exhausted, a new generation of infective juveniles leave 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 highly attracted by (E)-β-caryophyllene (EβC), a sesquiterpene released by WCR-damaged maize roots (Rasmann et al., 2005; Rasmann and Turlings, 2007; Köllner et al., 2008), and the presence of this signal is essential for this nematode to be effective as a control agent (Rasmann et al., 2005; Degenhardt et al., 2009). By contrast, H. bacteriophora, one of the most virulent EPN against all WCR larval stages (Toepfer et al., 2005; Kurzt et al., 2009; Hiltpold et al., 2009), does not seem to respond well to EβC (Rasmann and Turlings, 2008; Hiltpold et...
al., 2009). Here, we explored the feasibility of enhancing this responsiveness through artificial selection. Because of their short generation time, small genome size and ease of culture, EPNs are ideal subjects for genetic improvement, and several studies have succeeded in selecting beneficial traits such as host finding (Gaugler et al., 1989; Gaugler and Campbell, 1991), virulence (Tomalak, 1994; Peters and Ehlers, 1998) and tolerance to temperature (Griffin and Downes, 1994; Grewal et al., 1996; Ehlers et al., 2005) or desiccation (Strauch et al., 2004).

Here, we used six-arm below-ground olfactometers (Rasmann et al., 2005) to select for a *H. bacteriophora* strain with enhanced responsiveness to EJC. The effectiveness of this new strain in killing WCR and protecting maize roots was then tested under field conditions. Recognizing that, because of trade-offs, artificially selecting for one trait can negatively affect other important traits (Stuart et al., 1996), we also compared overall infectiousness between the original and the selected strain.

**MATERIALS AND METHODS**

**Selection for an enhanced responsiveness of *H. bacteriophora* to EJC**

**Olfactometer assays**

The selection of a *H. bacteriophora* strain that responds well to EJC and more quickly migrates towards an EJC source was performed using six-arm below-ground olfactometers (Fig. 1). Six glass pots (5 cm diam., 11 cm deep) were each connected to a central glass pot (8 cm diam., 11 cm deep) using glass tubes (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, Miami Lakes, FL, USA), which prevented the nematodes from entering the odour source pots (for details, see Rasmann et al., 2005). Olfactometers were filled with moist sand (Migros, Switzerland, 10% water), allowing for good passive diffusion of the volatiles from the surrounding pots to the central arena (Hiltpold and Turlings, 2008). Slow-release capillaries (see below), containing either EJC, (E)-β-farnesene or α-pinene (Fluka c/o Sigma Aldrich Chemie GmbH, Switzerland), were inserted into separate olfactometer pots; the three pots with a capillary were alternated with control pots that also contained sand but no capillary.

**Slow-release capillary**

Amber glass vials (1.5 ml, Supelco c/o Sigma Aldrich Chemie GmbH, Switzerland) were half-filled with glass wool, which was first rinsed with 200 ml of CH2Cl2 and, after the solvent had evaporated, heated up to 200°C for 4 h. A volume of 200 ml of one of the selected synthetic compounds was placed on the glass wool, and each vial was 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. To ensure an airtight seal, Teflon tape was wrapped around the lid and part of the glass capillary. This device was then placed upside down, with the capillary projecting into the sand of the designated olfactometer pot (Fig. 1).

**EPN selection for responsiveness and motility towards EJC**

Batches of 10,000 *H. bacteriophora* nematodes from the foundation strain PS8 (Ehlers et al., 2005) were released in a drop of water in the central arenas of six below-ground olfactometers that were prepared as described above. This allowed them to move freely into the arms that were connected to the six outer pots. Each olfactometer was disassembled at a different time point and sand was collected to retrieve the nematodes. The intervals were 4 h, 6 h, 8 h, 12 h, 24 h and 48 h after nematode release. The sand contained in each glass connector was then placed onto a separate cotton filter disk (19 cm diam., Hoeschele GmbH, Switzerland). The disks were placed on Baermann extractors (Curran, 1992; Hass et al., 1999), and nematodes were counted the next day under a dissection microscope. When at least 500 active individuals were recovered in the EJC arm of an olfactometer (corresponding to a selection percentage of 5%), these nematodes were used to produce the next generation. This next generation was obtained by infecting *Galleria mellonella* L. larvae (Lepidoptera: Galleridae) with Ringer solution and stored in the dark at 25 ± 3°C. About 10 days later, the offspring that emerged from the cadavers were pooled and used for the next step of selection in the olfactometers. In total, six such selection steps were carried out, with the sampling schedule modified to account for the increasingly rapid response (olfactometers were disassembled and nematodes collected after 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 9 h in the final step). A seventh test was carried out using the original sampling scheme to allow a comparison of the EPN response before and after selection. The newly selected strain was named EJC-2.4.

**Testing for a possible trade-off between responsiveness and infectiousness**

Infectiousness is a measure of nematode performance, starting with host finding (over a limited distance) and ending with the death of the host, including bacteria establishment and overcoming the host immune system (Peters, 2005). To test whether the selected strain was equally infective as the original strain, plastic trays (54 cm2, 9 cm × 6 cm × 5.5 cm) were filled with 200 g sterilized sand (10% moist and sieved at 200 μm). The bottom of each tray was covered with 1 cm of wet sand. EPN strains PS8 or EJC-2.4 were applied in a drop of water in two opposite corners of the tray at a concentration of 16 EPN/cm2. Control trays were supplied with drops of water without nematodes. Next, the 20 treatments and control boxes were completely filled with the remaining moist sand. Ten WCR L2 larvae were placed on the top of the sand in each...
and mixed with 500 ml of agar solution (0.15%) in order to assure a better homogeneity of eggs in the formulation. The concentration of eggs was adjusted to 29,000 eggs per litre.

Experimental 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 holes (12 cm depth) at a distance of 5 to 8 cm from both sides of the maize plant (~200 eggs/plant).

Experimental design and EPN application

In each half of the six fields, 12 groups of six to seven plants were inoculated with WCR eggs. These groups were randomly distributed in either the third, fifth, seventh or ninth row of both Magister and Pactol plots. The edges of the Magister and Pactol plots were ‘buffered’ by at least one row of untreated maize plants.

The two H. bacteriophora strains, PS8 or EβC-2.4, were used each to inoculate four groups per plot. The remaining four groups in each plot received only water, without EPN, and served as a control.

In order to get sufficient 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 (Kaya and Stock, 1997). On 11 June (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 next to the maize plants.

Application was performed in the late afternoon to reduce the effects of UV radiation (Gaugler et al., 1992). Experimental groups were sprayed with 0.6 litre of water containing approximately 0.36 × 10^6 EPN (~0.3 × 10^6 EPN/m²), whereas control groups were sprayed with the same amount of water without EPNs.

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

Effect of EPN strains on WCR survival

Each experimental group of plants was covered with a fine-mesh screen cage (1.3 m height × 0.75 m width × 1.5 m length; plants had been cut to a height of ~1 m) three days after nematode application (on 14 June). WCR adult emergence within these cages was recorded weekly between 20 June and 15 August. 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’s post-hoc test. A Friedman repeated measures analysis of variance on ranks was used to test the attraction of EPNs after 8 h of exposure to the chemical signal through the six 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. Comparison between the strains for this trait was done using a
Evaluation of the ability of the EPN strains to control WCR in the field

A difference in ability of the two 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 x field were considered as independent variables, and lnWCR survival (a natural log transformation was performed to obtain a normal distribution) as a 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 only slowly into the arms of, the olfactometers (Fig. 3A). There were no statistical differences between the number of EPN recovered in arms with either EβC, (E)-β-farnesene, α-pinene or empty arms (RM ANOVA, F_{5,15}=1.95, P=0.165). However, many of the nematodes did move out of the central pot, and, after 48 h, significantly more EPN were recovered in the arms than earlier on during the bioassays (RM ANOVA, F_{6,30}=10.29, P<0.001) (Fig. 3A). In the first selection step, the number of EPN in the EβC arm required for the next step, 5% of the total applied, was reached after 12 h (Fig. 3A).

After six rounds of selection, the new H. bacteriophora strain, EβC-2.4, responded considerably better and moved faster towards the chemical signals (Fig. 3B). EPN were more attracted towards the sesquiterpenes EβC and (E)-β-farnesene than towards α-pinene and the empty arms (RM ANOVA, F_{5,15}=16.74, P=0.001) (Fig. 3B). No statistical differences between EβC and β-farnesene or between α-pinene and the empty arms were measurable (Fig. 3B). The selected strain moved significantly faster into the arms (RM ANOVA, F_{6,30}=16.15, P<0.001) and in particular into the EβC arms, which contained 5% of the total applied EPN after less than 6 h. This level of accumulation took the original strain more than twice as long to attain (Fig. 3A versus Fig. 2B).

Fig. 3C shows the distribution of the nematodes after 8 h of exposure to the chemical signals for each of the six selection rounds. Migration velocity and EPN survival significantly increased after selection (Friedman RM ANOVA on ranks, P<0.039). A plateau of responsiveness was reached after four selection steps as sensitivity or migration velocity did not increase significantly during the last three steps of selection (Fig. 3C). For the selection curves, there were no statistical differences between responses to EβC and β-farnesene or between responses to α-pinene and the empty arms, but these two ‘groups’ were different from each other (Friedman RM ANOVA on ranks, P=0.012) (Fig. 3C).

Infectiousness of the H. bacteriophora strains

The application of nematodes dramatically reduced the survival of the WCR larvae in the laboratory experiment (ANOVA on ranks, H=49.61; P<0.001). When used with a concentration of 8 EPN/cm², the foundation strain was slightly but significantly better at infecting and killing WCR larvae than the selected strain. No such difference was found for the higher concentration of 16 EPN/cm² (Fig. 4).

Ability of EPN strains to control WCR in the field

There were major differences between the field sites, with overall poor survival of WCR in Satzynaz, intermediate survival in Hodmezovasarhely, and good survival in Szeged. In all cases, these plot effects were significant. Treatment with EPN significantly
reduced WCR survival in both the Pactol and Magister plots (two-way ANOVA, Pactol: \(F_{1,20}=9.19, P<0.001\), \(F_{1,20}=55.56, P<0.001\), Magister: \(F_{1,20}=4.2, P<0.053\)) that the selection for enhanced responsiveness to \(E\) resulted in a stable foundation strain. In Satzymaz, WCR emergence was much lower, but there was still a clear trend (\(P=0.053\)) that the selected strain reduced WCR survival more than that obtained by treatment with the foundation strain. On average, the selected strain reduced survival in the Magister plots by more than 70% compared to the original strain (Fig. 5B).

In the laboratory, it was confirmed that the EPN applied in the field were of good quality. In cups with application of either the \(H.\) bacteriophora PS8 or \(E\)-B strain, 91% of the \(G.\) mellonella larvae died, whereas only 20% did not survive in the control cups.

**DISCUSSION**

This first study to selectively breed a biological control agent for increased responsiveness to a foraging cue demonstrates that this approach has great potential to improve pest control. By comparing mortality of the pest on a maize variety that emits the nematode attractant \(E\)-B with a variety that does not emit \(E\)-B, it can be deduced that the superior control efficiency of the selected EPN strain was due to improved response to the signal. The foundation strain of \(H.\) bacteriophora did not exhibit any attraction to the three chemical signals that were offered in an olfactometer (Fig. 3A). Yet, selection for enhanced responsiveness to \(E\)-B resulted in a stable responsive strain after only six rounds of selection. Compared with other EPN species, \(H.\) bacteriophora exhibits high virulence and infectiveness against WCR (Kurzt et al., 2009) and shows high motility (O’Halloran and Burnell, 2003). In addition to these favourable traits, the selection process resulted in a strain that not only entered the olfactometer arm with the \(E\)-B twice as fast as the foundation strain (Fig. 3A), but also many more nematodes responded to the signal: 8h after release in the olfactometers, the number of EPN recovered from the \(E\)-B arm was eightfold higher for the selected strain than for the foundation strain (Fig. 3C). The responsiveness was not specifically increased only to \(E\)-B but also to the other sesquiterpene offered, \((E)-\beta\)-farnesene (Fig. 3B,C). This similarity in responsiveness to structurally analogous compounds is in agreement with the results of other studies. For instance, long-chain alcohols are in general attractive to \(H.\) bacteriophora (O’Halloran and Burnell, 2003), and various terpenes are known to attract the phytopathogenic nematode \(Bursaphelenus xylophilus\) (Zhao et al., 2007). Hence, \(H.\) bacteriophora can be expected to have generalist receptors reacting to groups of similar chemicals instead of molecule-specific receptors.

Field tests confirmed that the application of selected EPNs as bio-control agents is highly promising. The impact of EPN application on WCR mortality was evident for both the maize varieties that were tested (Fig. 5). Furthermore, overall WCR survival in plots with the \(E\)-B-releasing Magister variety was significantly lower when treated with the selected strain than with the foundation strain (Fig. 5B). However, this was not the case for the Satzymaz site where WCR survival was very low. The differences between the sites are best explained by differences in soil characteristics. Satzymaz soil is much more sandy, and we had already observed poor WCR survival in this location (unpublished...
data). Indeed, studies on the effects of soil texture correlate a high degree of sandiness with poor WCR performance (Turpin and Peters, 1971; Beckler et al., 2004). With such poor WCR survival in Satzmyaz, the possible differences between nematodes strains are hard to detect. Soil characteristics might also explain differences between Hodmezovasarhely and Szeged. Indeed, Szeged soil contains twice as much clay as Hodmezovasarhely soil (unpublished data). This possible correlation between WCR survival and soil composition emphasizes the importance of conducting future studies that take into account soil physics and/or chemistry as factors that determine WCR and/or EPN performance.

The apparent cost of selection in terms of reduced infectiosity was low but significant (Fig. 4). This reflects a classical trade-off between life-history traits, as defined by Stearns (Stearns, 1992), which has also been found in studies on foraging efficiency and resource exploitation in other organisms (Kraaijeveld and Godfray, 1997; Chittka et al., 2003). For EPN too, previous studies have shown that enhancing beneficial traits through selective breeding can negatively alter other traits in the selected strain, such as storage stability (Gaugler et al., 1990) or the capacity of EPN to kill their hosts (Stuart et al., 1996; Wagel and Grewal, 2002). In our study, the infectiosity in the laboratory was reduced by a factor of 1.6, but, in the field, the positive effect on host location ability easily outweighed the negative effect on infectiosity – WCR survival in Magister field plots was twofold lower when the selected H. bacteriophora strain βEβC-2.4 was applied than when Magister plots were treated with the foundation strain (Fig. 5B). Nevertheless, possible effects on important traits, such as infectiosity, should be taken into account in future selection efforts.

Previous studies have shown that an approach through artificial selection of EPNs can improve traits such as host finding (Gaugler et al., 1989; Gaugler and Campbell, 1991), virulence (Tomalak, 1990; Gaugler and Campbell, 1991; McGuire et al., 1999; Griffin and Downes, 1994; Peters and Ehlers, 1998) and tolerance to extreme temperature (Tomalak and Campell, 1991), virulence (Tomalak, 2005). Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode-bacterium complex Heterorhabditis bacteriophora-Photorhabdus luminescens was low but significant (Fig. 4). This reflects a classical trade-off in the identification of volatiles that mediate interactions among plants and arthropods. Analysis 131, 24-32.


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Selection of a biocontrol agent


