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

B. Kurtz · I. Hiltpold · T. C. J. Turlings · U. Kuhlmann · S. Toepfer

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*

Introduction

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

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I. Hiltpold similarly contributed to this paper as the first author.

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B. Kurtz
Institute for Phytopathology, Christian-Albrechts-University, Hermann Rodewald Strasse 9, 24118 Kiel, Germany

I. Hiltpold · T. C. J. Turlings
Laboratory of Animal Ecology and Entomology, Institute of Zoology, University of Neuchâtel, CP 2, 2007 Neuchatel, Switzerland

U. Kuhlmann
CABI Europe-Switzerland, Rue des Grillons 1, 2800 Delemont, Switzerland

S. Toepfer (✉)
c/o Plant Health Service, CABI Europe, Rarosi ut 110, 6800 Hodmezovasarhely, Hungary
e-mail: s.toepfer@cabi.org

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

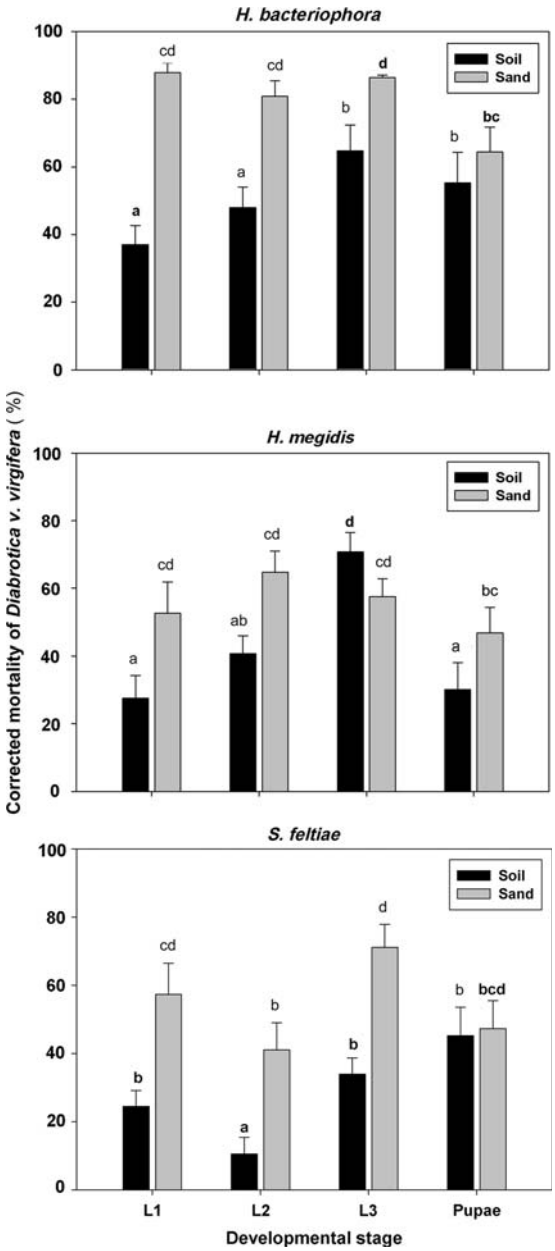

Fig. 1 Susceptibility of first instars (L_1), second instars (L_2), third instars (L_3) and pupae of WCR to infection by *Heterorhabditis bacteriophora*, *Heterorhabditis megidis* and *Steinernema feltiae* in sand- and soil-filled plastic trays. The corrected mortality was calculated as the relative 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.

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