

Influence of soil on the efficacy of entomopathogenic nematodes in reducing *Diabrotica virgifera virgifera* in maize

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Abstract The use of entomopathogenic nematodes is one potential non-chemical approach to control the larvae of the invasive western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) in Europe. This study investigated the efficacy of *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), *Heterorhabditis megidis* Poinar, Jackson and Klein (Rh., Heterorhabditidae) and *Steinernema feltiae* Filipjev (Rh., Steinernematidae) in reducing *D. v. virgifera* as a function of soil characteristics. A field experiment was repeated four times in southern Hungary using artificially infested maize plants potted into three different soils. Sleeve gauze cages were used to assess the number of emerging adult *D. v. virgifera* from the treatments and untreated controls. Results indicate that nematodes have the potential to reduce *D. v. virgifera* larvae in most soils; however, their efficacy can be higher in maize fields with heavy clay or silty clay soils than in sandy soils, which is in contrast to the common assumption that nematodes perform better in sandy soils than in heavy soils.

Keywords Western corn rootworm · *Heterorhabditis bacteriophora* · *Heterorhabditis megidis* · *Steinernema feltiae* · Inundative biological control · Soil texture · *Zea mays*

Introduction

The western corn rootworm, *Diabrotica virgifera* ssp. *virgifera* LeConte (Coleoptera: Chrysomelidae) is a widespread and serious pest of maize, *Zea mays* (L.). Believed to have originated in Mexico, it spread throughout parts of the United States and Canada during the twentieth century as maize production increased (Krysan and Smith 1986; Levine and Oloumi 1991). Then, over the last two decades, there were repeated accidental introductions of *D. v. virgifera* from North America into Europe (Miller et al. 2005). The pest is now an economic threat to maize production in many European countries, namely Austria, Hungary, Italy, Romania, Slovakia, Ukraine and Serbia (Sivcev and Tomasev 2002; Kiss et al. 2005).

Diabrotica v. virgifera is a univoltine species whose three larval instars feed almost exclusively on maize roots (Moeser and Vidal 2005). The feeding damage can cause plant lodging and significant yield losses. To control this pest, European farmers typically apply granular soil insecticides or insecticide-coated seeds against the larvae and foliar insecticides against the adults (Ward et al. 2004). The use of chemical pesticides in maize, however, can interfere with effective integrated pest management and biological control programmes that have been established for other European maize pests (Babendreier et al. 2006). Moreover, soil insecticides and insecticide-coated seeds can, when improperly applied, endanger honeybees (Heimbach et al. 2008; Pistorius and Bischoff 2008). Thus,

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Kuhlmann and Burgt (1998) and Babendreier et al. (2006) stressed the importance of biological control options.

For a pest like *D. v. virgifera*, whose most damaging stages are below-ground, soil-dwelling entomopathogenic nematodes are considered strong candidates for use in a biological control programme. Several field studies (e.g., Creighton and Fassuliotis 1985; Poinar et al. 1983; Kaya et al. 1989; Thurston and Yule 1990; Ellsbury et al. 1996; Jackson 1997) have shown variable efficacy of nematodes in controlling *Diabrotica* pests and have revealed a number of factors that may reduce their impact. Failed control attempts using nematodes have been attributed to (i) the use of nematode species or strains that were not adapted to the host or to local conditions (Jackson 1995; Georgis et al. 2006), (ii) a lack of alternative hosts in the soil (Brust 1991; Susurluk 2005), (iii) losses during application (Smits et al. 1994; Cabanillas et al. 2005) or, of particular interest in the current study, (iv) unfavourable soil characteristics (Kaya 1990; Koppenhöfer and Fuzy 2006a, b).

The activity, infectivity and survival of entomopathogenic nematodes can be profoundly influenced by soil composition, through its effects on moisture retention (Ellsbury et al. 1996), oxygen supply (Kaya 1990; Koppenhöfer and Fuzy 2006b) and texture (Kaya 1990). For example, survival of *Steinernema glaseri* Steiner and *S. carpocapsae* (Weiser) (both Rhabditida: Steinernematidae) was found to be lowest in clay soils followed by silty clay and sand or sandy silt (Kung et al. 1990). *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) was reported to move least in clay soil and most in fine sand or sandy silt soils (Barbercheck and Wang 1997). Kurtz et al. (2008) showed in laboratory bioassays that *H. bacteriophora*, *H. megidis* Poinar, Jackson and Klein (Rh., Heterorhabditidae) and *Steinernema feltiae* Filipjev (Rh.: Steinernematidae) were more effective in killing third instar *D. v. virgifera* larvae in sand than in non-sandy garden soil. Therefore, one could assume that *D. v. virgifera* larvae would be better controlled by nematodes in maize fields with sandy soils than in fields with heavy clay or silty soils. This, in turn, would largely reduce the potential market areas for a nematode-based biological control product.

In contrast to the high performance of nematodes observed in sandy soil, *D. v. virgifera* larval survival is reported to be lowest in such soils with high sand content (Turpin and Peters 1971; MacDonald and Ellis 1990) and economic root damage or plant lodging is more frequently reported from regions with heavy clay soils than from those with light sandy soils (I. Zseller, 2005, personal communication). Consequently, we might hypothesize nematodes to be unsuitable for the biological control of *D. v. virgifera* larvae in dense soils.

In this study, we examined the impact of soil characteristics on the efficacy of entomopathogenic nematode

species that had (a) proved virulent to *D. v. virgifera* larvae in laboratory screenings (Toepfer et al. 2005) and (b) had been successfully applied as a fluid during sowing of maize or during mechanical weed control in field experiments (Toepfer et al. 2008). The nematodes were applied to *D. v. virgifera*-infested maize pots with soils of three different compositions of clay, silt and sand. The efficacy of each nematode in controlling *D. v. virgifera* in these soils was assessed by counting the emerging *D. v. virgifera* adults in sleeve cages placed around the plants. The results of this study are a prerequisite to assess the potential market areas for a nematode-based biological control product against this invasive maize pest, prior to starting the development and implementation of such a product.

Materials and methods

Experimental setup and soil characteristics

Three entomopathogenic nematode species were applied against *D. v. virgifera* larvae in a field experiment using artificially infested maize plants potted into three different soils, referred to as soils A, B, C (Table 1). Those soils were prepared by adding different amounts of river sand (nearby river Tisza) to three batches of air dried natural gleyic Csernozom soil (IUSS 2007) of silty clay texture taken from the experimental field (Table 1). Soil and sand had been sieved through a 5-cm mesh in order to remove large pieces, prior mixing with a shovel. Five 1-l soil samples were randomly taken from each of the three prepared soil groups in order to analyse soil texture (Atterberg 1905) and pH (H₂O) (Table 1). Soil moisture was measured as w%(=grav.%) at 50–100-mm depth in two pots per soil type every 10 min (Hotdog DT1, Elpro, Switzerland). Soil temperature was measured at 100–150-mm depth in two pots per soil type every hour (Hobo data loggers, Onset Computer, Bourne, MA, USA). Soil texture, pH, moisture and temperature were compared between soil types using the non-parametric M. Whitney *U* test (Table 1).

Maize plants of the hybrid Magister (UFA Semences, Bussigny, Switzerland) were grown in plastic pots (*d* = 200 mm, *h* = 220 mm). Two fungicide-treated maize grains (fungicide Fludioxonil and Metalaxyl-M, Maxim XL 035FS, Syngenta) were sowed into each pot and, if both germinated, one plant was removed. Pots were placed into the rows of a maize field with the top of the pots at the level of the soil surface always leaving at least one maize plant as a buffer between pots (systematic block design). At the 4–6 leaf stage of maize, potted plants were infested with eight second instar *D. v. virgifera* larvae (see below). One week later, nematodes were applied (see below and Table 2). The roots of the hybrid Magister emit the

Table 1 Characteristics of the three soils of different sand content used for growing maize in pots for four plant-scale field experiments in Csongrad county, southern Hungary in 2005 and 2006 (N 46° 25' 59.54"; E 20° 20' 22.12"; 83 m elevation)

Soil characteristics	Soil A	Soil B	Soil C
Sand content (%)	14 ^a	47 ^b	63 ^c
Silt content (%)	44 ^b	17 ^a	16 ^a
Clay content (%)	42 ^c	36 ^b	21 ^a
PH (H ₂ O)	8.3 ^a	8.2 ^a	8.7 ^a
Humus (%)	2–4 ^a	2–3 ^a	1–3 ^a
Soil moisture at 50–100-mm depth (w% = grav.%)	24 ^b	23 ^b	19 ^a
Mean soil temperature (° C) at 100–150-mm depth	19.4 ^a	19.1 ^a	18.7 ^a
Min soil temperature (° C) at 100–150-mm depth	13.3 ^a	13.8 ^a	14.1 ^a
Max soil temperature (° C) at 100–150-mm depth	29.4 ^a	29.5 ^a	29.8 ^a

Soils were prepared by adding different amounts of river sand to natural gleyic Csernozom soil of silty clay texture (soil A) taken from the experimental field. The average soil moisture from May to June 2006 is shown as well as the average soil temperature from August to September 2006; *letters* beside values indicate significant differences between soils according to the non-parametric M Whitney *U* test at *P* < 0.05

nematode—attracting volatile β-caryophyllene in response to *D. v. virgifera* larval feeding (Rasmann et al. 2005). Eleven to twenty-two replicates were organised for each treatment group (soil type and nematode species) and for the controls (Table 2). At the 6–8 leaf stage of maize (see dates in Table 2), gauze sleeve cages (approximately 1.5-m height) were placed over the potted and *D. v. virgifera*-infested plants in order to capture emerging adults. The experiment was repeated four times.

Diabrotica v. virgifera source and infestation of maize

Diabrotica v. virgifera eggs were obtained from a laboratory culture of field-collected beetles in southern Hungary (25°C day, 15–20°C night, 14L:10D, 40–60% r.h.; for procedures see Singh and Moore 1985). The eggs were overwintered at 6–8°C in moist sand, and diapause was broken in early April by transferring them to 25°C for 14 days. Approximately 200–300 maize grains of the hybrid Magister were washed with soap to remove fungicides and planted in a plastic tray (300 × 450 mm) with moist potting soil (Garri Plusz, Garri Company, Budapest, Hungary). Five days after planting, ready-to-hatch eggs were placed into these plastic trays, which were then stored in the dark at 25°C (approximately

5,000 eggs per tray). One week after larval hatching (when most larvae were in late first instar stage), the soil containing the larvae was put onto a new tray with new maize plants to provide more food for further larval development. After another week, late second instars were obtained by manually crumbling the soil and maize roots. Using a moist paintbrush, larvae were transferred into several Petri dishes (45-mm diameter) containing a small amount of soil and then taken to the field. The potted maize plants were infested with eight *D. v. virgifera* larvae by emptying the contents of the Petri dishes (larvae and soil) into two 100–140-mm deep holes in the soil at a distance of 50–80 mm from both sides of the maize plant. About 8–10 third-instar larvae per plant root are often estimated to cause economic damage to a plant, depending on local conditions and maize prices (C. R. Edwards, personal communication, 2004); occasionally already two larvae per plant are considered sufficient (Reed et al. 1991).

Nematode sources and application

Infective juveniles of three nematode species were used in this study: (1) a hybrid of European and US strains of *H. bacteriophora* Poinar provided from liquid culture by

Table 2 Experimental time table for applying three nematode species against *D. v. virgifera* larvae into three different soils of potted maize plants in Csongrad county, southern Hungary in 2005 and 2006

Experiment	Dates			<i>n</i>
	Infestation with 8 <i>D. v. virgifera</i> larvae	Nematode application	<i>D. v. virgifera</i> adult emergence	
1	Early Sept 2005	Mid Sept 2005	Early Oct–late Oct 2005	19–22
2	4 May 06	15 May 2006	Late May–early July 2006	11–13
3	30 May 06	8 June 2006	Mid June–mid July 2006	17–19
4	15 Mid Aug 2006	22 Aug 2006	Early Sept–mid Oct 2006	11–13

n = number of plants (=pots) assessed per treatment group (soil type and nematode species) and control

e-nema GmbH, Schwentimental, Germany, (2) the Dutch NL-HW79 strain of *H. megidis* Poinar, Jackson and Klein re-isolated from Swiss soils and provided from a liquid culture by Andermatt Biocontrol, Grossdietwil, Switzerland, and (3) a hybrid of European strains of *S. feltiae* (Filipjev) provided from liquid culture by e-nema GmbH. These nematodes were known to be effective against second and third instars from previous laboratory bioassays (70–100% mortality; Rasmann et al. 2005; Toepfer et al. 2005). *Heterorhabditis bacteriophora* and *S. feltiae* were shipped in clay in a cool box from the producer to the experimental site, and *H. megidis* was shipped in vermiculite. All nematodes were stored in their shipping material at 7–9°C in darkness until use. Approximately 2–3 h before application, the infective juveniles were diluted with the carrier material in tap water to the required concentration. Using a pipette, 2,000 infective juveniles in 1.2–2-ml tap water were injected twice (in the late evening and following morning) 100 mm into the soil at distances of 150 mm from the plant, totalling 4,000 juveniles per pot (=13 juveniles per cm² or 1.3×10^9 juveniles per hectare). These injections simulated the commonly practised application of nematodes as a fluid into soil during sowing of maize or during mechanical weed control (Toepfer et al. 2008, 2010).

To evaluate the quality of each of the nematode shipments prior to application (Kaya and Stock 1997), 100 infective juveniles were added to three plastic cups ($d = 40$ mm, $h = 60$ mm) containing 200 g of moist, sterilised sand and five larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae). Mortality of *G. mellonella* larvae was assessed after 1 week in darkness and at 22°C. Mortality of 70–100% of *G. mellonella* was found for all nematode batches, indicating that the test material was of sufficient quality for subsequent applications and analyses.

Data analyses

Emerged adults were removed weekly from the sleeve cages during their period of emergence (Table 2) and counted. Emergence of adults was compared among soils, nematode species and controls in each experiment (Fig. 1) using the non-parametric M. Whitney *U* test. In order to pool data from the four experimental repetitions, the mean weighted efficacy of each nematode species in each of the three soils was then calculated as the reduction of *D. v. virgifera* relative to the untreated controls (corrected efficacy % = $(100 - (\text{beetles in treated pots} \times 100/\text{beetles in the control}))$ (Fig. 2). A comparison among efficacies of nematode species in the three different soils was conducted using the Bonferroni post hoc test following an ANOVA (Kinnear and Gray 2000).

The influence of soil characteristics on nematode efficacy in reducing *D. v. virgifera* was tested with between-

subjects ANOVA (GLM procedure) in cases where a certain soil characteristic was proven significantly different between the soil types according the M Whitney *U* test (see Table 1). Linear associations between soil characteristics and nematode efficacy were determined by the Pearson correlation coefficient after visually consulting scatter plots for linearity (Kinnear and Gray 2000).

Results

In most experiments nematodes reduced *D. v. virgifera* larvae, regardless of the soil they were applied to (Fig. 1). None of the three nematode species consistently reduced *D. v. virgifera*, i.e. *H. bacteriophora* reduced *D. v. virgifera* in eight out of eleven cases, and *H. megidis* and *S. feltiae*, each reduced *D. v. virgifera* in five out of 11 cases. Soil type generally influenced the efficacy of nematodes in the reduction of *D. v. virgifera* (one-way ANOVA: $F_{(2,717)} = 13.4$, $P < 0.001$).

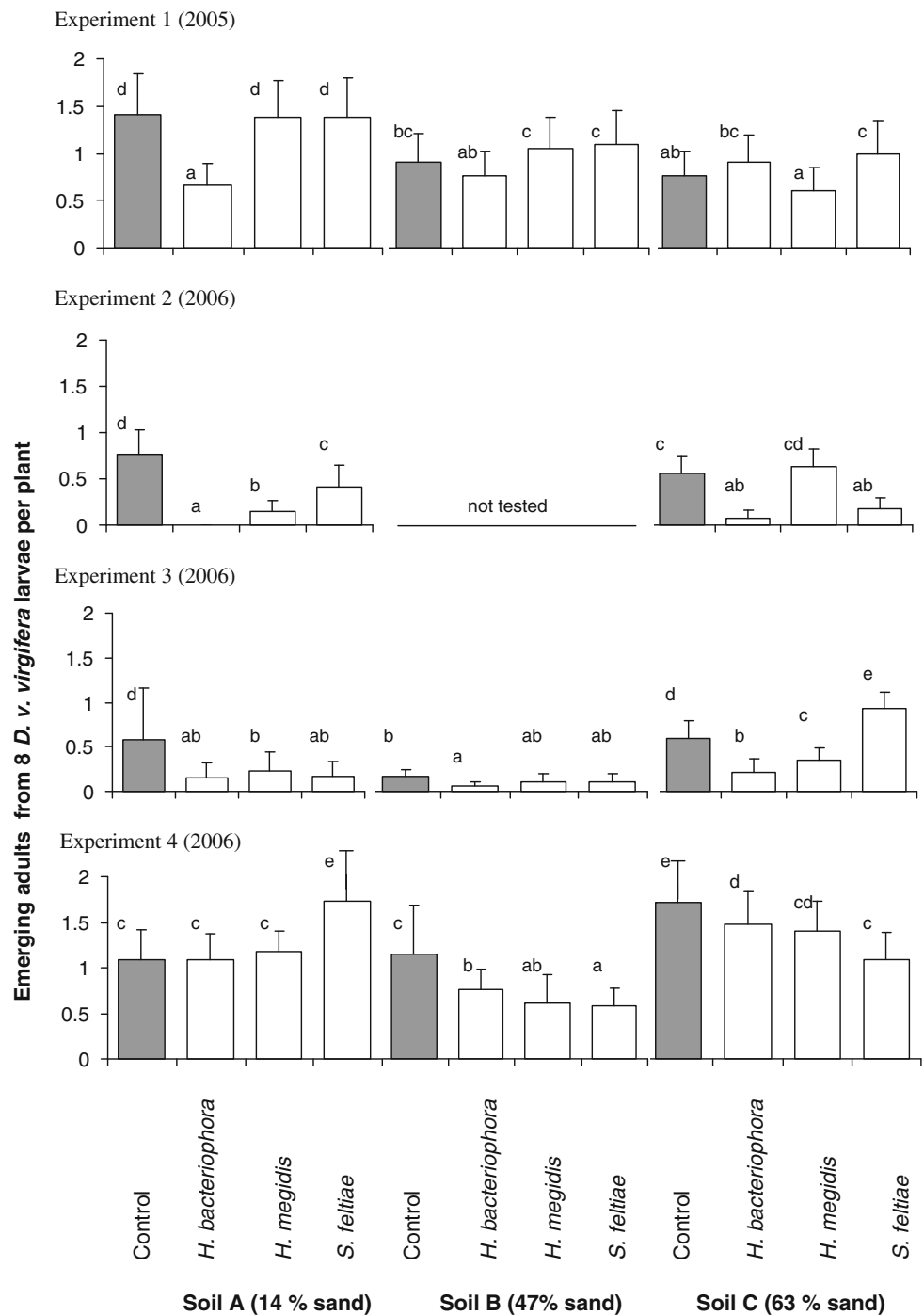
Heterorhabditis bacteriophora and *H. megidis* were nearly double as effective in soil A as in soil B or C (soil A being the soil with the lowest sand and highest silt and clay content) (Fig. 2). In soil A, *H. bacteriophora* reduced *D. v. virgifera* by $58 \pm 33\%$ SD, and *H. megidis* reduced *D. v. virgifera* by $33 \pm 37\%$. No difference in the efficacy of both nematodes was found in soils B and C. Among the soil factors shown in Table 1, low sand content and high silt content were the factors best correlated with the efficacy of *H. bacteriophora* ($r = -0.311$ and 0.309 , both $P < 0.001$; both had higher r values than other factors). High silt content was the factor best correlated with the efficacy of *H. megidis* ($r = 0.23$, $P = 0.001$).

Steinernema feltiae was most effective in soils A and B (Fig. 2), i.e. in soils with low or medium sand content. In those soils, *S. feltiae* reduced *D. v. virgifera* by $20 \pm 46\%$ and $30 \pm 6\%$, respectively. However, it rarely reduced *D. v. virgifera* in soil C with high sand content (Fig. 1). Among the soil factors shown in Table 1, high clay content was the factor best correlated with the efficacy of *S. feltiae* ($r = 0.246$, $P = 0.01$).

The choice of a nematode species influenced the reduction of *D. v. virgifera* adult emergence (between-subject ANOVA: $F_{(11,717)} = 23.8$, $P < 0.001$). *Heterorhabditis bacteriophora* reduced *D. v. virgifera* by 33–58% on average across soils, which was significantly more than *H. megidis* (16–33%) and *S. feltiae* (0–20%) (Fig. 2).

Adult emergence of *D. v. virgifera* from untreated control pots varied among experiments and soils, i.e. between 0.08 and 1.8 emerging adults from the eight larvae used to infest each maize plant (Fig. 1). On average, 0.99 ± 0.35 adults emerged from soil A, 0.58 ± 0.45 from soil B and 0.88 ± 0.47 from soil C. Overall, high sand

Fig. 1 Adult emergence from eight second instar *D. v. virgifera* larvae per maize plant from three different soils either treated with entomopathogenic nematodes or left untreated (potted maize plants in a maize field in southern Hungary; soils were prepared by adding different amounts of river sand to natural gleyic Csernozsem soil of silty clay texture (soil A) taken from the experimental field; 11–22 potted maize plants were allocated to every treatment and control group in each of four experiment repetitions; error bars = SEM; letters above bars indicate significant differences according to M Whitney *U* test at $P < 0.05$)



content influenced *D. v. virgifera* emergence negatively ($r = -1.5$, $P = 0.037$), whereas high silt content influenced *D. v. virgifera* emergence positively ($r = 0.21$, $P = 0.005$).

Discussion

This study showed that nematodes can reduce *D. v. virgifera* emergence in all three of the soil types tested.

However, the higher efficacy in soils with high clay and silt content was surprising because several previous studies suggested that sandy soils provide good conditions for nematode survival, movement, host finding behaviour and infectivity (e.g., Kung et al. 1990; Barbercheck 1992). Our results suggest the opposite is true for *D. v. virgifera* control in maize fields. Only Barbercheck and Wang (1997) also found that *H. bacteriophora* killed more larvae of *Diabrotica undecimpunctata* Barber in loam than in sand. Our study does not provide much insight into which

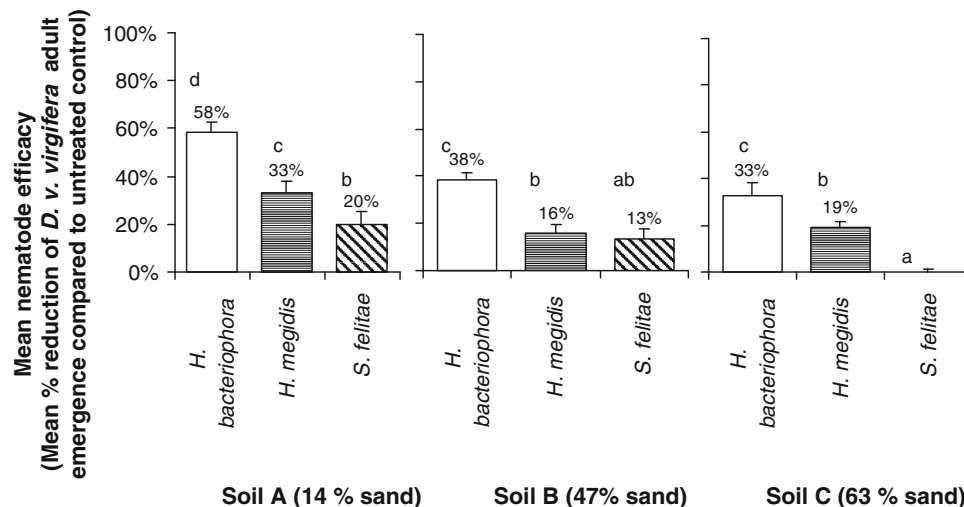


Fig. 2 Mean percent reduction of *Diabrotica v. virgifera* emergence due to applications of entomopathogenic nematodes into three different soils. Mean weighted reduction of adult emergence shown in comparison to the controls (=corrected efficacy); potted maize plants in a maize field in southern Hungary; soils were prepared by adding different amounts of river sand to natural gleyic Csernozem

factors might have prevented the nematodes from being more effective in the sandy soils.

One explanation for the apparently high efficacy of nematodes in non-sandy soils might be that the nematodes were applied too close to maize roots and *D. v. virgifera* (here 150 mm) to detect a reduction in nematode movement in heavy soils with low sand content. It is known that movement of nematodes can be restricted in dense soils such as clay or silty clay; whereas, strong movement can usually be observed in loamy sand or sandy soil (Barbercheck and Kaya 1991; Barbercheck 1992; Portillo Aguilar et al. 1999; Boff and Gandin 1992). Alekseev et al. (2006) reported that the mobility of *S. carpocapsae* was greater in marine sand than in sandy loam soil, and Koppenhoefer and Fuzy (2006a) showed that the infectivity of *S. scarabaei* slightly declined from sandy soils to fine clay soils, whereas no such effects were reported for *H. bacteriophora* and *H. zealandica*. Despite the advantages of sandy soils, nematodes may still find their hosts easier in clay soils than in sandy soils when moving along cracks formed by plant roots or the host larvae. In sand, however, they will disperse more equally and the net-movement towards the host larvae may therefore be less directed.

Another possible explanation for our results is that the survival of *D. v. virgifera* larvae is usually high in heavy soils (Turpin and Peters 1971; Beckler et al. 2004), such as clay, and would therefore provide most host larvae for nematode attack and propagation. Gaugler (2002) reported that nematodes usually have an advantage over insecticides in that they propagate within the pests and thus can react to high pest densities. This was also suggested for *H. megidis*

soil of silty clay texture (soil A) taken from the experimental field; 11–22 potted maize plants were allocated to every treatment and control group for each of four experiment repetitions; error bars = SEM; letters above bars indicate significant differences of efficacies between soils and between nematode species according to the Bonferroni post hoc test at $P < 0.05$ following an ANOVA

when applied against *D. v. virgifera* in maize fields in the USA (I. Hiltbold 2008, personal communication). However, in our study, the time period required for nematode propagation (1–2-week period) would hardly be enough to infest more larvae because the applied larvae already started to pupate and emerge as adults (Toepfer and Kuhlmann 2006). Moreover, our results suggest that high sand content, in contrast to above mentioned papers, only slightly reduced *D. v. virgifera* survival, measured in the untreated controls. We found approximately 12% survival from late second instar to the adult stage in the silty clay with low sand content and 8% in the sandiest soil. However, the negative influences of sand to first instar larvae, such as through coarse texture or fast desiccation of sandy soils (Gustin and Schumacher 1989; Macdonald and Ellis 1990) were excluded in this study through the infestation of plants with later less sensitive instars. Such mortality factors of first instars might be the reason that greater damage from *D. v. virgifera*, particularly plant lodging, are more often reported from regions with heavy and dense soils than from regions with light sandy soils (I. Zseller, 2008, personal communication). Another reason might be that population pressure of *D. v. virgifera* is usually higher in regions with heavy and dense soils due to higher intensity of maize growing when compared to regions with sandy soils considered suboptimal for maize production due to soil aridity (I. Zseller, 2008, personal communication).

In conclusion, the efficacy data presented here suggest that nematodes might be suitable biological control agents for managing *D. v. virgifera* in most soils, including heavy non-sandy soils, such as those found in the intensive maize

production areas of Central Europe. On average across soils, *H. bacteriophora* was more effective at controlling *D. v. virgifera* larvae (43%) than *H. megidis* (23%) and *S. feltiae* (11%). This should encourage and support the development of a biological control product against this invasive alien maize pest in Europe.

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