Assessment of establishment and persistence of entomopathogenic nematodes for biological control of western corn rootworm

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Ms. received: December 19, 2006; accepted: April 21, 2007

Abstract: The use of entomopathogenic nematodes (EPN) is potentially one ecological approach to control the invasive alien western corn rootworm (Diabrotica virgifera virgifera LeConte, Col., Chrysomelidae) in Europe. This study investigated the establishment and the short- and long-term persistence of Heterorhabditis bacteriophora Poinar (Rh., Heterorhabditidae), Heterorhabditis megidis Poinar, Jackson and Klein (Rh., Heterorhabditidae) and Steinernema feltiae Filipjev (Rh., Steinernematidae) in three maize fields in southern Hungary, using the insect-baiting technique. All three EPN species equally established and persisted in maize fields. The timing of application (April or June) did not influence the establishment of EPN species. EPNs persisted for 2–5 months, i.e. they survived up to and throughout D. v. virgifera larval occurrence in the soil. Results demonstrate that D. v. virgifera larvae can potentially be controlled by EPNs during the same year of EPN application but no long-term control effect is expected under intensive maize cultivation practices.

Key words: Diabrotica virgifera virgifera, Heterorhabditis bacteriophora, Heterorhabditis megidis, Steinernema feltiae

1 Introduction

The western corn rootworm, Diabrotica virgifera virgifera LeConte (Col., Chrysomelidae) is the most destructive pest of maize, Zea mays L., in North America, hypothesized to have originated in Mexico (Krysan and Smith 1987). D. v. virgifera became an economically important maize pest in North America from the 1950s onwards because of increased maize cultivation and the practice of continuous maize planting without crop rotation (Steffey et al. 1999). D. v. virgifera was accidentally introduced into Europe several times from North America between the late 1980s and the early 2000s (Miller et al. 2005). The western corn rootworm has invaded almost all maize cultivation areas of the European Community (Kiss et al. 2005). The majority of yield loss attributed to this univoltine pest species, with eggs that overwinter in the soil, is due to larval feeding on the roots of maize, which results in plant lodging leading to economic loss. Adults emerge between mid-June and early August and can occasionally reduce yields because of intensive silk feeding.

In 2003, the Commission of the European Community reacted to this upcoming alien pest problem by releasing a decision (2003/766/EC) on emergency measures to prevent the spread of D. v. virgifera within the European Community (Byrne 2003). In 2006, this decision was amended to update monitoring, containment and control measures of D. v. virgifera. The following control measures are common practice in Europe today: (1) crop rotation, (2) use of soil insecticides (active ingredients: Tefluthrin, Carbafuran), (3) use of seed treatments (such as imidacloprid), and (4) occasional use of foliar application of insecticides or semiochemical-based insecticide baits (K. van Rozen and A. Ester, pers. comm. 2006). Chemical-based control measures against D. v. virgifera might interfere with integrated and biological-based management options already in place against other maize pests, such as the European corn borer [Ostrinia nubilalis Hübner (Lep., Pyralidae)], cotton bollworm [Helicoverpa armigera Hübner (Lep., Noctuidae)], Mediterranean corn stalk borer [Sesamia nonagrioides Lefebvre (Lep., Noctuidae)], and click beetles [Agriotes spp. (Col., Elateridae)] (Toepfer and Kuhlmann 2004).

In order to prevent additional insecticide usage for this new European invader, Kuhlmann and Burgt (1998) recommended that biological control options be considered. The first option is a classical biological control approach that offers the opportunity to introduce specific natural enemies from the area of origin of this pest (Kuhlmann et al. 2005). The second option, to be implemented either alone or in combination with the first option, is an inundative biological control approach using native and commercially available...
natural enemies, such as entomopathogenic nematodes (EPNs) (Kuhlmann and Burg 1998).

Soil-dwelling EPNs have demonstrated great potential as biological control agents of many arthropod pests (Ehlers 2003). EPNs may also be used and applied efficiently against D. v. virgifera larval populations using the following two application strategies: (1) EPNs may be curatively applied when second instar pest larvae occur in June (Jackson and Brooks 1995), which would require EPN persistence of only a few days for an immediate control effect; and (2) EPNs may be preventively applied at the time of maize sowing in April, which is considered to be less cost-intensive but would require EPN persistence of at least 2–3 weeks until pest larval emergence (Thurston and Yule 1990). Both applications could potentially lead to long-term control of D. v. virgifera as long as EPNs could persist in maize fields for 1 or 2 years.

Persistence of EPNs in the soil can vary depending on the type of habitat and the EPN species or strain (Susurluk 2005). In order to determine the most suitable EPN application strategy to suppress D. v. virgifera larvae, we studied the establishment and the short- and long-term persistence of three promising EPN species, Heterorhabditis bacteriophora Poinar (Rhabditida, Heterorhabditidae), Heterorhabditis meghidis Poinar, Jackson and Klein (Rh., Heterorhabditidae) and Steinernema feltiae Filipjev (Rh., Steinernematidae) in three maize fields in southern Hungary. All three EPN species have demonstrated a high virulence against D. v. virgifera in a laboratory study (Toepfer et al. 2005) and are commercially available (Ehlers 2001).

### 2 Materials and Methods

#### 2.1 Study sites, host and nematode species and sources, and nematode applications

This study was carried out in three maize fields, referred to as field A, B and C, in Csongrad County in southern Hungary from 2005 to 2006 (table 1). All fields consisted of a 0.1 ha experimental section that had previously been planted with non-host plants of D. v. virgifera to ensure the absence of the pest in the field plots. Fungicide-treated maize grains (middle–late silage or grain maize hybrid Magister, UFA Semences, Bassigny, Switzerland) were sown between 25 April and 8 May 2005. Individual maize grains were sown every 150 mm in rows that were 750 mm apart. Fields were treated once with 0.16 l of herbicide Merlin SC (75% Ioxaflutol, Bayer CropScience, Monheim, Germany) per hectare at maize leaf stage 3 to 5. No insecticides were applied. All fields were ploughed and tillaged between cropping cycles. Soil temperature was measured at a depth of 15–20 cm using Hobo data loggers (Onset Computer, Bourne, MA, USA).

In order to infest maize plants with D. v. virgifera, eggs were obtained from a laboratory-rearing of field-collected beetles in southern Hungary (described by Singh and Moore 1999). Eggs were allowed to hibernate in moist sand at a temperature of 6–8°C. Diapause of D. v. virgifera eggs was broken in early April by transferring the eggs to 25°C. For egg recovery, the sand was sieved through a 250 μm mesh. Recovered eggs were mixed into a solution of water and 0.15% agar. Maize plants in each field were infested twice in early May (one- to three-leaf stage) with 75 viable and ready-to-hatch eggs (in 2 ml water–agar) using a standard pipette (Eppendorf, Hamburg, Germany). The eggs were applied into 12-cm-deep holes at a distance of 5–8 cm from both sides of the maize plant. A portion of eggs was kept at 25°C in the laboratory to assess percentage emergence (about 80% hatched). In the field, larvae were expected to emerge between the middle and end of May and second instar larvae were expected in June, according to Toepfer and Kuhlmann (2006).

Three EPN species were used in this study: (1) a hybrid of European and US strains of H. bacteriophora provided from liquid culture by e-nema GmbH (Raisdorf, Germany), (2) a Swiss NL-HW79 strain of H. meghidis provided from a solid culture by Andermatt Biocontrol (Grossdietstwil, Switzerland), and (3) a hybrid of European strains of S. feltiae provided from liquid culture by e-nema GmbH. H. bacteriophora and S. feltiae were shipped in clay and plastic bags to the experimental sites, and H. meghidis was shipped in vermiculite material. All EPNs were stored in their shipping material at 7–9°C in darkness prior to the experiments. Approximately 2–3 h before application, EPNs together with the carrier material were diluted with tap water to the required concentration.

Before application, subsamples of EPNs were taken to determine virulence. The mortality of five Galleria mellonella L. (Lepidoptera: Pyralidae) larvae was assessed over 1 week in each of three plastic cups (diam. 40 mm, 60 mm height) per EPN batch. Each cup was filled with 200 g moist sterilized sand to which 100 infective dauer juveniles were added. After 1 week, 80–100% virulence was found for all EPN batches, and therefore establishment and persistence data from all fields (see below) were used for analyses.

Entomopathogenic nematodes were applied as row sprays in one of the following ways: about 10 cm into the soil at the time of maize sowing in 2005, or onto the soil along maize

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**Table 1. Characteristics of the three studied maize fields in Csongrad County in southern Hungary in 2005 and 2006 and the timing of EPN application**

<table>
<thead>
<tr>
<th>Field</th>
<th>Hodmezovasarhely</th>
<th>North-west of Hodmezovasarhely</th>
<th>North of Szatymaz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Location</td>
<td>Location</td>
<td>Location</td>
</tr>
<tr>
<td>Coordinates</td>
<td>46°25.998°N</td>
<td>46°26.022°N</td>
<td>46°20.945°N</td>
</tr>
<tr>
<td>Size (ha)</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Soil bulk density (g/cm³)</td>
<td>1.1 ± 0.13</td>
<td>1.04 ± 0.13</td>
<td>1.4 ± 0.13</td>
</tr>
<tr>
<td>Soil moisture (%)</td>
<td>18.5 ± 2.1</td>
<td>17.2 ± 1.1</td>
<td>11.6 ± 0.3</td>
</tr>
<tr>
<td>Sand content (%)</td>
<td>14</td>
<td>36</td>
<td>85</td>
</tr>
<tr>
<td>Loam content (%)</td>
<td>44</td>
<td>34</td>
<td>5</td>
</tr>
<tr>
<td>Clay content (%)</td>
<td>42</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>8.3</td>
<td>8.3</td>
<td>8.4</td>
</tr>
<tr>
<td>EPN applications</td>
<td>28 April 2005</td>
<td>25 April 2005</td>
<td>8 May 2005</td>
</tr>
<tr>
<td></td>
<td>13 June 2005</td>
<td>13 June 2005</td>
<td>14 June 2005</td>
</tr>
</tbody>
</table>
rows later in June 2005. In total, 200 000 EPNs were applied at a concentration of 0.21 H₂O per row metre, in four randomized plots in each of the three fields. Each plot contained seven plants (~1.3 row metres) for every EPN species and application date. All applications were carried out using watering cans. Four untreated plots served as a control in each field.

2.2 Establishment of nematodes

In order to study the establishment of the EPNs in the soil, soil samples were taken 48 h after EPN application. To detect EPNs in the soil samples, the insect-baiting technique as well as methods such as nematode flotation sieving are time-consuming and labour-intensive, because work-intensive identification of nematodes is needed (Fan and Hominick 1991).

In this study, 28 soil samples per EPN species and application date were randomly taken from the treated plots as well as from control plots (seven samples per plot randomly taken along maize rows, which equals approximately 5 samples/m²). Soil cores of 20 cm length and 2 cm diameter were collected and transferred separately into plastic cups (5 cm diameter and 15 cm height).

Galleria mellonella was reared at 25°C to the final larval stage in glass containers (8 cm diameter and 11 cm height) on an artificial diet (Singh and Moore 1999). Tenebrio molitor L. (Col., Tenebrionidae) larvae were commercially available and stored at 8°C in a 1.51 plastic container (11 cm diameter and 15 cm height) on yeast diet. These two host species were chosen based on the knowledge that penetration success by steinernematid and heterorhabditid nematodes differs between the two host species (Caroli et al. 1996). Individual Galleria or Tenebrio larvae were placed into each of the 28 plastic cups containing the soil samples. The soil in the cups was sprayed with water to ensure high moisture content. Soil samples were then stored with insect larvae in the dark at 20°C for 2 weeks to allow sufficient time for the infective dauer juveniles of EPNs to become active (Bohan and Hominick 1996) and attack the larvae. After the first and second week, the soil samples were checked for dead insect larvae, which were then placed into a nematode emergence trap (Woodring and Kaya 1988). For another 2 weeks those dead larvae were observed for propagation of nematodes, for adult nematodes under the larval skin, or for a change in colour of the larvae, the red colour indicating an infection with Heterorhabditis sp. or a greyish colour with Steinernema sp. If necessary, larvae were dissected to search for nematodes.

Establishment was defined by the percentage of soil samples that were positively tested for EPN infection 48 h after application. In order to standardize the rate of positively tested soil samples among fields, proportions of EPN infections in the treated plots compared with the control plots were calculated for each field. The influence of the two application dates and three EPN species on the establishment were tested by a factorial ANOVA after Mauchly’s test of sphericity of covariance (Kinnear and Gray 2000). Establishment was compared between EPN species and between application timing by the independent-sample t-test according to the Levene’s test for equality of variances.

2.3 Persistence of nematodes

In order to study the persistence of applied EPNs, soil samples were taken monthly from May 2005 until September 2005 and then every second month until June 2006. Soil samples were taken from treated and untreated plots as described above, a total of 11 times after application in April and nine times after application in June, equalling 3696 soil samples over the time period between April 2005 and June 2006. Soil samples were then placed into a nematode emergence trap (Woodring and Tenebrio baiting technique as described above.

Persistence was defined by the percentage of soil samples that were positively tested for EPNs on each sampling date. In order to standardise the rate of positively tested soil samples among fields, proportions of EPNs in the treated plots were calculated in relation to the control plots. The period from 0 to 20 weeks after application was considered for analyses because very few EPNs were detected beyond this period. The persistence of EPN species and the persistence after both application dates were analysed using a pairwise Bonferroni test, after having passed a within-subjects ANOVA (Kinnear and Gray 2000).

3 Results

3.1 Establishment of nematodes

All three EPN species, H. bacteriophora, H. megidis and S. feltiae, were able to establish in the soil of maize fields after application (fig. 1). No significant differences were found between the establishment of the three EPN species after each of the two application dates (t-test; d.f. = 4; April: H. bacteriophora vs. H. megidis, P = 0.8; H. bacteriophora vs. S. feltiae, P = 0.71; H. megidis vs. S. feltiae, P = 0.94; June: H. bacteriophora vs. H. megidis, P = 0.09; H. bacteriophora vs. S. feltiae, P = 0.22; H. megidis vs. S. feltiae, P = 0.75) (fig. 1). Consequently, the choice of the EPN species had no influence on establishment in the soil (factorial ANOVA: d.f. = 2, F = 0.064, P = 0.94). The timing of application, whether in April as a row spray during maize sowing or in June as a row spray along maize rows, also had no influence on the establishment of the EPN species (factorial ANOVA: d.f. = 1, F = 1.93, P = 0.19) (fig. 1). In control plots, 5.6% ± 7.2 SD of soil samples showed a native EPN population in April, and 7.7% ± 7.2 SD in June.

3.2 Persistence of nematodes

All three EPNs, H. bacteriophora, H. megidis and S. feltiae were detected in the soil of maize fields up to 5 months after application (fig. 2). The persistence of all EPN species decreased from the time of application in April or June until August. Few or no positive soil samples containing EPNs were found from September onwards until June of the following year (fig. 2).

At the time of D. v. virgifera larval occurrence in the field, in May, June and July, no differences in the persistence could be found among the different EPN species, whether applied in April or in June (post hoc Bonferroni test after ANOVA: April: H. bacteriophora vs. H. megidis P = 0.53, H. bacteriophora vs. S. feltiae
Fig. 1. Establishment of entomopathogenic nematodes in three maize fields in southern Hungary after two different application dates: (a) In April as row spray into the soil at the time of maize sowing; (b) in June as row spray onto the soil along maize rows. Relative number of positive soil samples in comparison to the control are presented (mean % EPNs in control plots in April: 5.6 ± 7.2; and in June 7.7 ± 7.2). Presented numbers are mean values from three different fields (table 1). Twenty-eight soil samples were taken 48 hours after application of 200,000 H. bacteriophora, H. megidis or S. feltiae per row metre and from untreated controls. EPNs were collected using the insect baiting technique. Letters on columns indicate significant differences among EPN species at P < 0.05 for the independent sample t-test according to the Levene’s test for equality of variances. No influence of application date on establishment was found (factorial ANOVA: d.f. = 2, F = 0.064, P = 0.94)

P = 1.00, H. megidis vs. S. feltiae P = 0.59; June: H. bacteriophora vs. H. megidis P = 0.22, H. bacteriophora vs. S. feltiae P = 1.00, H. megidis vs. S. feltiae P = 0.79).

The timing of application, April or June, significantly influenced EPN persistence (within-subject ANOVA: d.f. = 1, F = 9.24, P = 0.01). Persistence was longer after applications in April compared with applications in June (fig. 2). H. bacteriophora disappeared within 20 weeks after both application dates. S. feltiae and H. megidis were found for 16 weeks after their application in April but both disappeared within 12 weeks after application in June. Soil temperature measurements at a depth of 15–20 cm revealed that the mean temperature was 14.6°C (max = 24°C, min - 10.6°C) between 25 April and the end of May 2005; 20.5°C (max = 23°C, min = 15.6°C) in June 2005; and 21.8°C (max = 25.6, min = 19.8°C) in July 2005. Between 28 July and 4 August 2005, the mean soil temperature was 24.5°C (max = 25.6°C, min - 23.6°C).

4 Discussion

Results of this study demonstrate that each of the three EPN species equally established and persisted in maize.

As these species/strains were also shown to be highly virulent against D. v. virgifera larvae (Toepfer et al. 2005), it is suggested that they could potentially control the pest during the same year of inundative EPN application. However, no long-term control effect is expected at least under current intensive maize cultivation practices.

Successful establishment and a sufficient persistence of EPNs are important requirements for control of soil-dwelling pests such as D. v. virgifera larvae. EPNs have been found to persist in several habitats for months or even years. For example, H. megidis (strain

Fig. 2. Persistence of entomopathogenic nematodes in the soil of three maize fields in southern Hungary after two different application dates: (a) In April as row sprays at the time of maize sowing; (b) in June as row core sprays onto the soil along maize rows. The relative numbers of infected soil samples in comparison to the control are shown as mean values of three different fields. Twenty-eight soil samples were collected per month for each EPN species and control, application timing and field. EPNs were collected using the insect baiting technique. In order to test for differences during week 0 to week 20 after application, pairwise comparisons were used with post hoc Bonferroni test at P < 0.05 after within-subject ANOVA: April: H. bacteriophora vs. H. megidis, P = 0.53; H. bacteriophora vs. S. feltiae, P = 1; H. megidis vs. S. feltiae, P = 0.59; June: H. bacteriophora vs. H. megidis, P = 0.22; H. bacteriophora vs. S. feltiae, P = 1; H. megidis vs. S. feltiae, P = 0.79.
NLH-E87.3) and H. bacteriophora (Oswego strain) have been reported to persist for 2 years after their application on golf courses (Ferguson et al. 1995) and in alfalfa fields (Smits 1996). Kaya (1993) reported that S. feltiae (strain 27) persisted for 550 days after their application in turf infested with larvae of the grub Cyclocephala hirta LeConte. In contrast to golf courses or turf, Warshaw (1992) stated that maize is a poorly suited habitat for EPN persistence. The study presented here demonstrates that H. bacteriophora, H. megidis and S. feltiae cannot persist in the soil of maize fields for longer than 5 months. A possible reason for their comparably short persistence in this habitat is the absence of suitable alternative hosts (Brust 1991). Maize is considered to be an intensively cultivated, alien field crop in Europe, serving only a small number of potential hosts (Journey and Ostlie 2000). Moreover, intensive tillage and ploughing between cropping cycles, as well as intensive weed control practices, destroy the soil biota, including host species or EPNs themselves (Brust 1991). Furthermore, it can be argued that the target host larvae of D. v. virgifera are of small body size and have a fragile integument that quickly decomposes when larvae are killed. Therefore, Jackson and Brooks (1995) suggested that D. v. virgifera larvae can hardly support the propagation of an EPN population, even though a single D. v. virgifera larva can potentially propagate a few thousand infective dauer juveniles, as shown under sterile conditions by Toepfer et al. (2005). In addition, high soil temperatures might have played a role in reducing the persistence of EPNs in this particular study since it was conducted in southern Hungary, a region with a continental dry climate and warm summers. In our study fields, soil temperature was nearly continuously above 24°C at a depth of 15 to 20 cm between 28 July and 4 August 2005. Infectivity and vitality of EPNs can be reduced at temperatures between 20 and 25°C. For example, Boff et al. (2000) reported a reduction in infectivity at 20°C for H. megidis. Also, persistence of H. bacteriophora in soil samples from oil seed rape and potato fields was up to 60% lower at 25°C than at 15°C (Susurluk 2005). This temperature–persistence relationship is likely to be the reason for the slightly higher persistence of EPNs applied in April in this study than those applied in June. Consequently, persistence of EPNs may be higher in D. v. virgifera-invaded regions with colder summers, where soil temperatures remain consistently below 20°C at depths of 15–20 cm. The lack of long-term persistence of EPNs in maize over several cropping cycles will limit the development of a cost-effective biological control strategy against D. v. virgifera larvae. From this study it can be concluded that conservation of EPNs after inundative applications is unlikely to be feasible under the current intensive tillage and weed control practices in the European maize agro-ecosystem. Nonetheless, all EPN species tested in this study persisted at least until and throughout the time period of D. v. virgifera larvae occurrence in the soil, during the same year of EPN application (up to 140 days persistence, mean 67 days ± 28 SD). This is consistent with the results of Kaya (1990), and Klein (1990) who reported a persistence of H. bacteriophora of at least 140–150 days, even without suitable hosts. Based on our findings on the known high virulence of the species/strains (Toepfer et al. 2005), an inundative biological control approach could be developed, applying EPNs annually. We recommend that two different inundative biological control approaches should be tested further. The first method involves EPNs being applied preventively at the time of maize sowing. This method would help reduce application costs for farmers as well as potentially ensure a higher persistence of EPNs in the soil. The second method involves making a curative application of EPNs when larval stages of D. v. virgifera are present in the field. Although this method would require a cost-intensive application in June when farmers do not usually expend resources on pest management, it would ensure that the highest possible proportion of EPNs is applied onto the target.

Acknowledgements

This work was possible due to the kind hospitality of the Plant Health Service in Hodmezovasarhely in Hungary, offered by Ibolya Zseller, Jozsef Gavallier, Kataline Buzas, Erzsebet Dormannsne, Piroska Szabo, Andras Varga and others. We like to thank our summer students Bobe Kovacs and Ference Koncz for their help during field work; Arne Peters (e-nema GmbH, Raisdorf, Germany) and Erich Frank (Andermatt Biocontrol, Grossdietwil, Switzerland) for their advice and for providing nematodes, as well as Ivan Hilti (University Neuchatel, Switzerland) and L. Kunos (Plant Protection Service, Szolnok, Hungary) for analysing the soil. We like to thank Emma Hunt for reviewing the manuscript. This study was funded by the COST 850 Action via short-term scientific mission, and by the CTI Innovation Promotion Agency of Switzerland.

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