DOI 10.2478/s11687-009-0003-1

HELMINTHOLOGIA, 46, 1: 14 – 20, 2009

Activity of four entomopathogenic nematode species against different developmental stages of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera, Chrysomelidae)

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Summary

Four entomopathogenic nematode species (*Steinernema feltiae*, *S. carpocapsae*, *Heterorhabditis bacteriophora*, and *H. megidis*) were tested in a laboratory bioassay for the efficacy of these pathogens in controlling the larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata*. The main aim of the study was to develop an efficient sustainable control method against the pest. With this we could develop a strategy of potato production with the intention of diminishing or even preventing the appearance of pest resistance to insecticides. The activity of these biological agents was assessed at three different temperatures (15, 20, and 25 °C) and three concentrations (200, 1000, and 2000 infective juveniles per individual). Mortality of three stages (young and old larvae and adults) was determined 2, 4, and 7 days after treatment. At 15 °C entomopathogenic nematodes showed the lowest efficacy against all insect stages. No significant differences in efficacy was determined at 20 and 25 °C as all nematodes caused prompt death of all stages. At all temperatures young larvae were most susceptible. However, when controlling overwintered adults for the purpose of preventing the mass appearance of Colorado potato beetle, we recommend an application of higher concentrations of *S. feltiae* suspension.

Key words: biological control; efficacy; laboratory conditions; *Leptinotarsa decemlineata*

Introduction

Almost 85 years after its introduction into Europe, the Colorado potato beetle (*Leptinotarsa decemlineata* Say, Coleoptera, Chrysomelidae) is still the most important potato pest in the majority of the Old Continent countries (OEPP/EPPO, 1997). Larvae and adults feed on potato leaves and hinder the normal development of the plants. The individuals of the first generation prove to be particularly damaging (Igrc-Barčić *et al.*, 1999), because the economic threshold is much lower than for the individuals of the second generation (Zehnder *et al.*, 1995). In some countries the intensive use of insecticides against the Colorado potato beetle has led to the appearance of pesticide resistance (Pap *et al.*, 1997; Stanković *et al.*, 2004). With the intention of diminishing or preventing this phenomenon, new strategies of potato production were developed in some regions that proved to be very efficient (Pruszynski & Wegorek, 2004). Since the insect is capable of gaining resistance to both chemical and biological insecticides (Loseva *et al.*, 2002), the development and optimization of new and environmentally acceptable ways of controling this pest is urgently needed.

Entomopathogenic nematodes (EPNs) are biological agents which have been tested on a wide range of plant pests under laboratory conditions (Shapiro & McCoy, 2000) and in the field (Simser, 1992; Abbas *et al.*, 2001; Susurluk, 2008). At first they were mostly known as antagonists of soil pests, but in recent years many investigations have demonstrated that they can also be effectively used against foliar pests (Arthurs *et al*., 2004).

In Europe, the efficacy of EPNs on the Colorado potato beetle was studied in the 1970s in Poland (Seryczynska & Kamionek, 1974; Lipa *et al.*, 2008). In one of the latest studies (Prishchepa *et al.*, 2000), the efficacy of the Belarus strains of *Steinernema feltiae* and *S. carpocapsae* has been proven – and the results of other studies indicate the higher efficacy of *S. carpocapsae* in comparison with strain HP 88 from the genus *Heterorhabditis* (Saringer *et al.*, 1996). A study in North America has shown that *S. carpocapsae* persists in the body of the Colorado potato beetle through both the larval-pupal and pupal-adult transitions (Stewart *et al.*, 1998). This indicates that *S. carpocapsae* could be more useful for foliar application where the nematodes are unlikely to survive long outside a host due to their sensitivity to environmental factors. Thus,

it is preferable that the endoparasite can enter the host at all stages and stay within the host. However, one study has shown relative inefficiency of *S. carpocapsae* in controlling the Colorado potato beetle (Thurston *et al.*, 1994). Thus, with some studies providing positive results and others providing weaker results for the use of this entomopathogenic species, we were interested in reassessing *S. carpocapsae* in comparison to other EPN species.
In the country where this research was performed (Slovenia), only the use of *S. feltiae* and *S. carpocapsae* is now allowed, since they recently became an indigenous species (Laznik *et al.*, 2008ab). But all preliminary research on this agent has been conducted under laboratory conditions (Trdan *et al.*, 2006, Trdan *et al.*, 2008). We have now investigated the ability of EPNs to infect larvae and adults of the Colorado potato beetle in a rearing chamber. The aim of this research was to determine how the factors of environmental temperature and EPN concentration, both important factors with impact on the control efficacy (Kreutz *et al.*, 2004; Athanassiou *et al.*, 2006) - influence the efficacy against different developmental stages of the pest.

Materials and methods

Entomopathogenic nematodes and Colorado potato beetles
The laboratory investigation was carried out in the Entomological Laboratory of the Chair of Phytomedicine, Agricultural Engineering, Crop Production, Grassland and Pasture Management (University of Ljubljana, Biotechnical Faculty, Department of Agronomy) in Ljubljana, Slovenia. The following four species of EPNs were tested: *Steinernema feltiae* (Filipjev) and *S. carpocapsae* (Weiser) (both Rhabditida: Steinernematidae); and *Heterorhabditis bacteriophora* Poinar and *H. megidis* Poinar, Jackson & Klein (both Rhabditida: Heterorhabditidae). Commercial products from Koppert B. V. (Berkel en Rodenrijs, The Netherlands) were supplied by air-mail and used within 6 weeks of their receipt. Once received, the nematode preparations were stored in the dark in a refrigerator (2 – 4 °C). Before each use, the quality of the nematodes was checked. Larvae (L1/L2 and L3/L4) and adults of the Colorado potato beetle were collected on potato plants, cv. Kondor, from a test plot of the Biotechnical Faculty in Ljubljana. Adults and larvae of both generations were used for the laboratory research**.** Young larvae (L1/L2) were collected in the first half of June and in the first half of August and older larvae (L3/L4) in the second half of June and in the second half of August. Adults were collected in mid- July and in mid- September. Individuals were hand picked in the early afternoon, placed in plastic container, and transported to the laboratory where they were exposed to EPNs.

Laboratory bioassay
The efficacy of EPNs was tested at three concentrations: 200, 1000, and 2000 infective juveniles (IJs) per individual or 2000, 10 000 and 20 000 IJs in 1 ml of water per Petri dish. The main reason for the use of relatively high concentrations of nematode suspensions is the fact, that the nematodes were also tested against the Colorado potato beetle adults. The adults are known as less susceptible to nematodes attack (Svendsen & Steenberg, 2000), therefore higher concentrations of nematode suspensions were also used in many of previous studies on the efficacy of these biological control agents (Lacey *et al.*, 1993; Trdan *et al.*, 2008).
About five potato leaves were placed in each of several 14 cm (diameter) Petri dishes lined with filter paper disks. Ten L1/L2 Colorado potato beetle larvae were then put in each Petri dish. The same was done also with L3/L4 larvae and adult Colorado potato beetles. Suspensions of nematodes were prepared in glass jars, and each Petri dish was given 1 ml of suspension. The Petri dishes were then closed. Suspensions were added to the potato leaves with a pipette whose tip was changed after every treatment. In this way we simulated the foliar application of the nematodes. The fifth treatment was a control with 1 ml distilled water added to each Petri dish instead of nematode suspension. This type of testing is widely used in the field of entomopathogenic nematology, the most actively with the steinernematids (Dolinski *et al.*, 2006), but also with heterorhabditids (Rosa & Simões, 2004).
The Petri dishes were put in a RK-900 CH type rearing chamber provided by Kambič Laboratory equipment (Semič, Slovenia) with a working capacity of 0,868 m^3 (width x height x depth = 1000 x 1400 x 620 mm). Each treatment was done in 10 replicates. Dishes were kept in the dark at three different temperatures (15, 20, and 25 °C) at a relative humidity of 95 %. The number of dead individuals was determined at 2, 4, and 7 days after treatment (DAT). Because of the large quantity of the results, only the data acquired 7 DAT are presented in this paper. Offspring of *Steinernema* nematodes appeared inside the dead insects after 8 days, while emergence of *Heterorhabditis* nematodes was observed after 14 days. Nematode-caused morality of the Colorado potato beetles was thus confirmed.

Statistical analysis
A multifactor analysis of variance (ANOVA) was conducted to determine the differences in mortality (%) between the larvae (L1/L2, L3/L4) and adults of the Colorado potato beetle. Before the analysis, each variable was tested for homogeneity of treatment variances. The mortality data were corrected according to Abbott's formula (Abbott, 1925) and normalized using the arcsine square-root transformation. Duncan's multiple range test ($P \leq 0.05$) was used to separate mean differences among the parameters in all the treatments. LC_{50} and LC_{90} values (numbers of IJs/individual causing 50 % and 90 % mortality) were estimated. All statistical evaluations were performed with Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Manugistics, Inc., Maryland, USA). Data are presented as untransformed means ± SE.

Results

Analysis of pooled results

The percentage mortality of the Colorado potato beetle was significantly influenced by temperature ($F = 37.98$; df = 2, 3219; $P < 0.001$), EPN species ($F = 25.47$; df = 3, 3219; $P < 0.001$), nematode concentration ($F = 40.93$; df = 2, 3219; $P < 0.001$), DAT ($F = 25.10$; df = 2, 3219; $P < 0.001$), and developmental stage ($F = 203.73$; df = 2, 3219; $P < 0.001$). All interactions were non-significant. In all nematode treatments, the total mortality was significantly higher than the mortality in the control treatment. Corrected mortality was therefore calculated.

The highest mortality of the Colorado potato beetle was recorded at 20 °C (56.79 ± 2.54) and 25 °C (61.29 ± 2.33) with *S. feltiae* (60.07 ± 2.08) and *S. carpocapsae* (61.66 ± 2.20) at 2000 IJs/adult (62.15 ± 1.84) and at 7 DAT (70.66 ± 2.23). The lowest mortality was seen at 15 °C (36.37 ± 1.57), with *H. megidis* (41.87 ± 1.88) and *H. bacteriophora* (42.35 ± 1.96), at 200 IJs/adult (37.13 ± 1.54), and at 2 DAT (30.91 ± 1.71). Young larvae were the most susceptible developmental stage of the pest (79.24 ± 1.81), while adults showed the highest tolerance (18.86 ± 1.08) to EPNs infection.

Individual analysis

At 15 and 20 °C, a significant influence of EPN species, nematode concentration, and the interaction between EPN species and nematode concentration on mortality of the young larvae was assessed seven days after treatment (Table 1). At 25 °C, the influence of both parameters and their interaction was non-significant.

In the 15 and 25 °C conditions, significant influences were found on the mortality of old larvae seven days after treatment for EPN species, nematode concentration, and the interaction between EPN species and nematode concentration, while at 20 °C none of the parameters or their interaction had such influence.

At 15 °C, a significant effect of EPN species, nematode concentration and interaction between both factors was ascertained for adult mortality seven days after treatment, while at 25 °C only significant influence for nematode concentration and the interaction between EPN species and nematode concentration was approved. Just the same as for the old larvae, at 20 °C none of the parameters or their interaction had significant influence on the adult mortality.

Dose effect of EPNs

LC_{50} and LC_{90} values calculated from the bioassay at the seventh day after treatment are summarized in Table 2. At 15 °C the lowest LC values are those of *S. feltiae* (LC_{50} = 484 IJs/young larvae and LC_{90} = 1025 IJs/old larvae). The highest values at the same temperature are attributed to *S. carpocapsae* (LC_{50} = 2111 IJs/adult, and LC_{90} = 3000 IJs/adult). At 20 °C the lowest LC_{50} values are associated with *S. carpocapsae* (LC_{50} = 463 IJs/adult) and *H. megidis* (LC_{90} = 664 IJs/old larvae), while the highest values belong to *H. megidis* (LC_{50} = 1375 IJs/adult) and *S. feltiae* (LC_{90} = 1992 IJs/old larvae). At the highest temperature, *S. carpocapsae* (LC_{50} = 541 IJs/old larvae) and *H. bacteriophora* (LC_{90} = 1057 IJs/adult) proved to be the most effective against the pest, while *S. feltiae* (LC_{50} = 1250 IJs/adult) and *H. bacteriophora* (LC_{90} = 1964 IJs/old larvae) showed the lowest efficacy.

Table 1: ANOVA results for corrected mortality of different developmental stages of Colorado potato beetle at three different temperatures 7 days after treatment

Temperature	Source	Young larvae			Old larvae			Adults		
		F	df	*P*	*F*	df	*P*	*F*	df	*P*
15º C	EPN species	22.03	3. 44	<0.001*	7.00	3.44	<0.001*	101.86	3.44	<0.001*
	Nematode concentration	166.58	2.44	<0.001*	7.33	2.44	0.016*	40.55	2.44	<0.001*
	EPN x nematode concentration	22.03	6.44	<0.001*	6.15	6.44	0.021*	30.37	6.44	<0.001*
20º C	EPN species	37.24	3.44	<0.001*	3.05	1.20	0.0962	2.96	1.20	0.1010
	Nematode concentration	31.73	2.44	<0.001*	1.08	2.20	0.3576	1.42	2.20	0.2646
	EPN x nematode concentration	21.88	6.44	<0.001*	0.93	2.20	0.4102	0.06	2.20	0.9404
25º C	EPN species	25.21	3.44	0.0752	10.96	3.44	<0.001*	10.59	3.44	<0.001*
	Nematode concentration	23.12	2.44	0.0842	18.98	2.44	<0.001*	2.39	2.44	0.1035
	EPN x nematode concentration	28.21	6.44	0.0911	2.75	6.44	0.0234*	2.65	6.44	0.0279*

* Source of variation significant at $\alpha = 0.05$

Table 2: Dose effect of four different entomopathogenic species on larvae and adults of Colorado potato beetle at three different temperatures 7 days after treatment

		LC_{50}[z] (95 % CL[y])			LC_{90}[z] (95 % CL[y])		
Nematode species	Growth stage	15°C	20°C	25°C	15°C	20°C	25°C
S. feltiae		484 (60-908)	-[(4)]	-[(4)]	1232 (927-1536)	889 (435-1344)[(5)]	-[(4)]
S. carpocapsae		-	-[(4)]	-[(4)]	-	-[(4)]	-[(4)]
H. megidis	L1/L2	797 (527-1066)	312 (0-1090)[(4)]	481 (0-974)[(4)]	1357 (1084-1630)	1106 (736-1477)[(4)]	1184 (854-1514)[(4)]
H. bacteriophora		586 (200-973)	558 (116-999)	-[(4)]	1253 (945-1560)	1211 (884-1538)	-[(4)]
S. feltiae		1259 (537-1980)	865 (252-1477)	761 (99-1423)	1025 (574-1476)	1102 (670-1535)	1142 (707-1576)
S. carpocapsae		950 (672-1228)	-[(4)]	541 (0-1204)	1550 (1196-1905)	-[(4)]	1088 (706-1470)
H. megidis	L3/L4	1093 (644-1542)	-	894 (486-1302)	1218 (470-1967)	664 (0-1504)	1334 (882-1786)
H. bacteriophora		1256 (834-1678)	2570 (1453-3688)[(4)]	1204 (999-1410)	1688 (964-2412)	3939 (1842-6036)[(4)]	1964 (1623-2305)
S. feltiae		887 (672-1101)	1500 (600-2401)	1250 (865-1634)	1467 (1224-1710)	1992 (241-3743)	1872 (1130-2614)
S. carpocapsae		2111 (0-5538)	463 (0-1163)	1141 (641-1640)	3000 (0-9307)	1158 (772-1545)	1314 (381-2247)
H. megidis	Adult	1355 (685-2024)	1375 (759-1991)	1067 (549-1584)	1905 (328-3482)	1937 (575-300)	1067 (0-2251)
H. bacteriophora		1206 (0-4296)	570 (230-909)	1062 (0-2333)	1327 (0-7062)	-	1057 (0-3575)

[(4)] 100% mortality 4 DAT

Discussion

The results of the present research indicate that both the temperature and the developmental stage of the Colorado potato beetle have an important influence on the efficacy of the EPNs as pest-control agents. At 15 °C, a lower mortality was recorded than at 20 or 25 °C, thus supporting the results of our previous research (Trdan *et al.*, 2006) and research from other groups (Kaya *et al.*, 1993; Doucet *et al.*, 1996; Choo *et al.*, 2002; Belair *et al.*, 2003; Yang *et al.*, 2003). Controlling insect pests with a foliar application is becoming a more widely-used practice (Broadbent & Olthof, 1995). If this method is required for the control of the first (overwintered) adults of the Colorado potato beetle, application of *S. feltiae* suspension in higher concentrations is recommended as our research demonstrated that this species showed the highest efficacy in controlling adults at 15 °C. The first adults usually appear in the second half of May when the nights are still relatively fresh in the area in which our research took place. The early application of EPNs is a necessity as the excrements of Colorado potato beetle repel these biological agents (Thurston *et al.*, 1994). Thus the rapid application of nematodes at higher concentrations not only provides a larger number of nematodes entering exposed individuals but also limits the repellent action of this insect pest on nematodes.

At higher environmental temperatures of 20 and 25 °C, the best control of adults was obtained with *S. carpocapsae;* therefore we recommend this particular EPN in the control of the first adult generation (i.e. the offspring of overwintered adults). Control of adult chrysomelids with EPNs has not proven to be particularly successful until now. We assert this on the basis of research on *Diabrotica virgifera virgifera* LeConte (Toepfer *et al.*, 2005) and *Colaphellus bowringi* Baly (Wei *et al.*, 2000). The conclusion is also supported in part by our own data here, with adult beetles proving to be much more resistant to nematode infection than their larval counterparts.

At the lowest temperature of 15 °C, the youngest larvae were the most susceptible to nematode infection. In fact, the youngest larvae were the most susceptible at all three temperatures assessed. From this point of view, old larvae are a somewhat less effective target for EPN treatment, but nevertheless still much more susceptible than adults. It is known that EPNs are more efficient against larvae and other pre-imaginal stages of insects because they can enter their body more easily (LeBeck *et al.*, 1993). From this knowledge in combination with our present data, we might expect that the most effective EPN control would be seen in young larvae following a summer night application. In conditions of warm (above 20 °C) and humid nights, a high percentage of mortality of young larvae would be expected after just two days following application of the biological agents.

Our current aim is to repeat the present research in field conditions as soon as possible. Now, when the use of *S. feltiae* and *S. carpocapsae* is allowed - namely, they recently become an indigenous species in our country (Laznik *et al.*, 2008ab) - there are no legal obstacles for building the field experiment with these biological control agents. But it is also important to note that results from laboratory tests are not always comparable with field testing (Cantelo & Nickle, 1992) as functioning of EPNs in the open is influenced by an extensive list of factors. In one relevant study, the 100 % efficacy rate of *S. carpocapsae* in controlling Colorado potato beetle adults, pupae and larvae in the laboratory manifested in only a 31 % reduction rate of the pest population when the test was repeated outdoors (Stewart *et al.*, 1998) and a supporting dose of fenvalerate was considered. Some further results from studies of the activity of EPNs on related (Yang *et al.*, 2003) and others species of beetles (Łabanowska *et al.*, 2004) have also shown that these agents could be an effective alternative to insecticides. It is our hope that these promising studies can soon be extended to outdoor tests of foliar applications of EPNs as a control method for the Colorado potato beetle - and that these studies will further demonstrate these biological agents to be a practical and effective alternative to currently used methods for the control of this important agricultural pest.

Acknowledgements

This work was done within Horticulture No P4-0013-0481, a programme funded by the Slovenian Research Agency, and within L4-6477 and V4-0524, the programmes funded by the Slovenian Research Agency and the Ministry of Agriculture, Forestry and Food of Republic Slovenia. Tjaša Gril and Nevenka Valič are acknowledged for providing technical support.

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RECEIVED NOVEMBER 13, 2007

ACCEPTED NOVEMBER 24, 2008