ЕФИКАСНОСТ НА БЪЛГАРСКИ ИЗОЛАТИ НА ЕНТОМОПАТОГЕННИТЕ НЕМАТОДИ *HETERORHABDITIS BACTERIOPHORA* (RHABDITIDA: HETERORHABDITIDAE), *STEINERNEMA ARENARIUM* И *S. FELTIAE* (RHABDITIDA: STEINERNEMATIDAE) СРЕЩУ ЯБЪЛКОВИЯ ПЛОДОВ ЧЕРВЕЙ *CYDIA POMONELLA* (LEPIDOPTERA: TORTRICIDAE) В ЛАБОРАТОРНИ УСЛОВИЯ

EFFICACY OF BULGARIAN ISOLATES OF THE ENTOMOPATHOGENIC NEMATODES *HETERORHABDITIS BACTERIOPHORA* (RHABDITIDA: HETERORHABDITIDAE), *STEINERNEMA ARENARIUM* AND *S. FELTIAE* (RHABDITIDA: STEINERNEMATIDAE) AGAINST THE CODLING MOTH, *CYDIA POMONELLA* (LEPIDOPTERA: TORTRICIDAE) UNDER LABORATORY CONDITIONS

Елена Петрова[1]*, Елена Ташева-Терзиева[2], Денис Градинаров[2]
Elena Petrova[1]*, Elena Tasheva-Terzieva[2], Denis Gradinarov[2]

[1]Институт по почвознание, агротехнологии и защита на растенията "Н. Пушкаров"
Селскостопанска академия, ул. „Банско шосе" № 7, 1331 София
[2]Софийски университет „Св. Климент Охридски", Биологически факултет
Бул. "Драган Цанков" № 8, 1164 София
[1]Institute of Soil Science, Agrotechnology and Plant Protection *Nikola Pushkarov*
Agricultural Academy, 7 *Bansko Shose* Str., Sofia 1331, Bulgaria
[2]Sofia University *St. Kliment Ohridski*, Faculty of Biology
8 *Dragan Tsankov* Blvd., Sofia 1164, Bulgaria

***E-mail: angellore81@abv.bg**

Abstract

The aim of the study was to determine the potential of three Bulgarian isolates of entomopathogenic nematodes, *Heterorhabditis bacteriophora, Steinernema arenarium* and *S. feltiae,* respectively, for the biological control of the codling moth *Cydia pomonella*, a common economically important pest in apple orchards. A laboratory trial was set up to evaluate the susceptibility of fifth instar larvae of the insect to equal concentrations of infective juveniles of the three nematode isolates over a 72-hour period and the mortality was recorded at three-hour intervals.

The overall statistical analysis of the obtained data showed that the nematode species factor had a significant effect on the parameters of life duration and mortality rate, and the survival curves of *C. pomonella* larvae after exposure to infective juveniles. *S. feltie* was most effective in controlling the codling moth with lowest average life duration of the larvae after exposure and 100% mortality as early as the 42nd hour of the experiment.

The Kaplan-Meier Survival Analysis and the subsequent all pairwise multiple comparison procedures following the method of Holm-Sidak showed significant differences between the survival curve of larvae parasitized with *S. feltiae* and the curves for the other two variants. Nevertheless, *H. bacteriophora* and *S. arenarium* also performed well with 97% and 83% larval mortality, respectively, for the full duration of the trial. The results demonstrate the suitability of Bulgarian isolates of entomopathogenic nematodes for biological control of *C. pomonella*.

Key words: biological pest control, *Heterorhabditis bacteriophora, Steinernema arenarium, Steinernema feltiae, Cydia pomonella.*

INTRODUCTION

The codling moth *Cydia pomonella* (Linnaeus, 1758) (Lepidoptera: Tortricidae) is a common economically important pest of apple, pear and walnut in most countries where these crops are grown (Barnes, 1991). In Bulgaria it has traditionally been controlled using organophosphates and pyrethroids. Along with the associated environmental risks, the frequent use of broad spectrum insecticides leads to the development of resistant populations (Charmillot et al., 2007). The need for alternative approaches has motivated the search for suitable biological control agents.

C. pomonella undergoes most of its development in cryptic habitats. Mature larvae exit the fruit and move to protected sites to spin their cocoons e.g. under and within tree bark, cracks in wooden supports and leaf litter (Georgis et al., 2006). Fifth-instar larvae exiting the fruit in late summer and early autumn overwinter in these habitats and pupate in the following spring. In Bulgaria *C. pomonella* develops two generations per year (Angelova et al., 2006). It is most practical to control the overwintering fifth instar, as in the fall, winter and early spring diapausing larvae represent the entire codling moth population (Lacey and Unruh, 2005)

Research on entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae has shown their potential as agents for biological control of many harmful insects (Koppenhöfer, 2000; Grewal et al., 2005; Georgis et al., 2006) including orchard pests (Shapiro-Ilan et al., 2005). Their success is largely due to their symbiosis with insect-pathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus,* respectively. Both stenernematids and heterorhabditids have a single free-living stage, the infective juvenile (IJ), that carries the bacteria in its intestine. On encountering a suitable host the IJs generally enter the body either through a natural opening such as the mouth, anus or spiracles, or directly through the cuticule and migrate to the haemocoel. There they release their associated bacteria which proliferate in the haemolymph and digest the host tissues. Normally this process causes death of the insect within 24–48 hours. The IJs feed on the metabolized material and the bacteria themselves, molt through fourth to adult stage and reproduce. If resources are available, more than one generation may develop. IJs from the next generations actively exit the host and enter the soil. They do not feed until invading a new host (Griffin et al., 2005; Adams and Nguyen, 2002).

Research on EPNs in Bulgaria started in the 1990s (Shishiniova et al., 1997; Samaliev, 1997). Faunistic studies report seven species of genus *Steinernema*: *S. kraussei* (Steiner, 1923), *S. feltiae* (Filipjev, 1934), *S. affine* (Bovien, 1937), *S. carpocapsae* (Weiser, 1955), *S. arenarium* (Artyukhovsky, 1967), *S. intermedium* (Poinar, 1985) and *S. bicornutum* Tallosi, Peters & Ehlers, 1995 (Shishiniova et al., 2000, Vega et al., 2000, Gradinarov et al., 2011), and two species of genus *Heterorhabditis: H. bacteriophora* Poinar, 1976 and *H. megidis* Poinar, Jackson & Klein, 1988. The identity of *H. megidis* needs further confirmation with molecular methods (Gradinarov et al., 2012). Most systematic investigations were carried out in natural habitats and have established a number of patterns in the vertical distribution and preferences to plant communities of the reported EPN species (Shishiniova et al., 2000). Meanwhile the data on the distribution and species composition of EPNs in Bulgarian agricultural systems are limited. Samaliev (1997) reports *Steinernema* sp. in soil in potato, fruit tree and vegetable plantations in the regions of Smolyan and Velingrad. Vega et al. (2000) find *S. carpocapsae* in *C. pomonella* larvae in apple orchards in the vicinity of Kiustendil. Gradinarov (2003) reports the isolation of *H. bacteriophora* from a larva and a pupa of *Drasterius bimaculatus* (Rossi, 1790) (Coleoptera: Elateridae) in strawberry plantings near Kostinbrod.

Even though research worldwide has shown that local isolates of EPNs are most suitable for control of insect pests in agriculture in the particular area, the studies on such practical application of Bulgarian EPN isolates in our country are scarce.

MATERIALS AND METHODS

Nematodes

The nematodes used for experimental infection were isolated from soil samples collected from different locations in Bulgaria using the "nematode bait" method (Bedding and Akhurst, 1975). The isolates of *S. arenarium* (culture BG_Ks-4/2001, GenBank accession numbers HM160092 to HM160095) and *H. bacteriophora* (culture BGKB-1/2007, GenBank accession numbers JX993984 and JX992985) were obtained and identified as described in detail in previous publications (Gradinarov et al., 2011; Gradinarov et al., 2012). The *S. feltiae* isolate was recovered in March, 2001 from alluvial soil in a poplar forest on the bank of Struma river (DMM: 42 23.52N; 22 42.55E) at 500 m altitude. It was morphologically identified using characters of infective juveniles (IJs) and male individuals of the first parasitic generation (Adams and Nguyen, 2002). The identity of the species was confirmed by molecular analysis of the rDNA ITS-regions and the D2D3 expansion segment of the 28S rRNA gene using the method described by Gradinarov et al., 2011 (GenBank accession numbers KT879187, KT879188). Live nematode cultures were maintained in laboratory conditions and reared on larvae of *G. mellonella* to obtain IJs for experimental infection.

Insects

Mature (fifth-instar) larvae of the second generation of the codling moth *C. pomonella* were collected in September - October, 2011 from the apple orchard of ISSAPP using corrugated cardboard bands wrapped around the tree trunks.

Bioassays

The susceptibility of the larvae to equal rates of infective juveniles (100 IJs per individual) recovered from the three nematode cultures was evaluated over a 72-hour period under controlled conditions (T = 25±3°C). The three host-parasite pairs were tested against an untreated control group. For each pair and for the control group 30 Petri dishes (a total of 120), d = 9 cm were lined with double filter paper and a single *C. pomonella* larva was placed in each dish. The IJs were introduced in 2 ml of water per dish and 2 ml of pure water were added on the 36th hour of the trial to keep the filter paper moist. Only pure water was added to the control group. Insect mortality was recorded every 3 hours for the whole duration of the experiment.

Biostatistical analysis

The main statistics, i.e. mean, standard error of mean (SEM), standard deviation (STD), coefficient of variation (CV) and median, of the survival time of the codling moth larvae after exposure to the IJs were calculated. The average life duration of the insects infected with the three nematode species respectively was compared using Kruskal-Wallis ANOVA on ranks, followed by Tukey test for multiple comparison. Mortality data were summarized by the number of the dead larvae and cumulative mortality [%] over time. In addition, Kaplan-Meier (K-S) survival analysis was performed. The difference between the survival curves was checked by Log Rank test and Holm-Sidak method as Post Hoc procedure. The statistical software SigmaPlot 12 for Windows was used for calculations and construction of graphs.

RESULTS

The results concerning the life duration of the larvae of *C. pomonella* exposed to the IJs of the three nematode species are presented on Table 1 and Figure 1. The infection with *S. feltiae* led to lowest average survival time of the larvae of 27.7 hours (*Me* = 27 h) from the start of the experiment. In the trial with *S. arenarium* the average life duration was 36.4 hours (*Me* = 30 h). It took longer for *H. bacteriophora* to kill the host with average survival time of the larvae 43.4 hours (*Me* = 42 h). All individuals in the control group survived for the whole duration of the experiment. Kruskal-Wallis ANOVA on ranks determined statistically significant differences between the three host-parasite pairs (H = 31.2, $d.f.$ = 2, P < 0.001).

Table 1. Summary statistics of the life duration [h] of the larvae of *Cydia pomonella* after exposure to IJs of *Steinernema arenarium*, *S. feltiae* and *Heterorhabditis bacteriophora*

EPN species	n	Min	Max	Mean	SEM	STD	CV	Median
S. arenarium	30	15	72	36.4	3.36	18.41	50.6	30
S. feltiae	30	21	39	27.7	0.87	4.77	17.2	27
H. bacteriophora	30	24	72	43.3	1.75	9.61	22.2	42

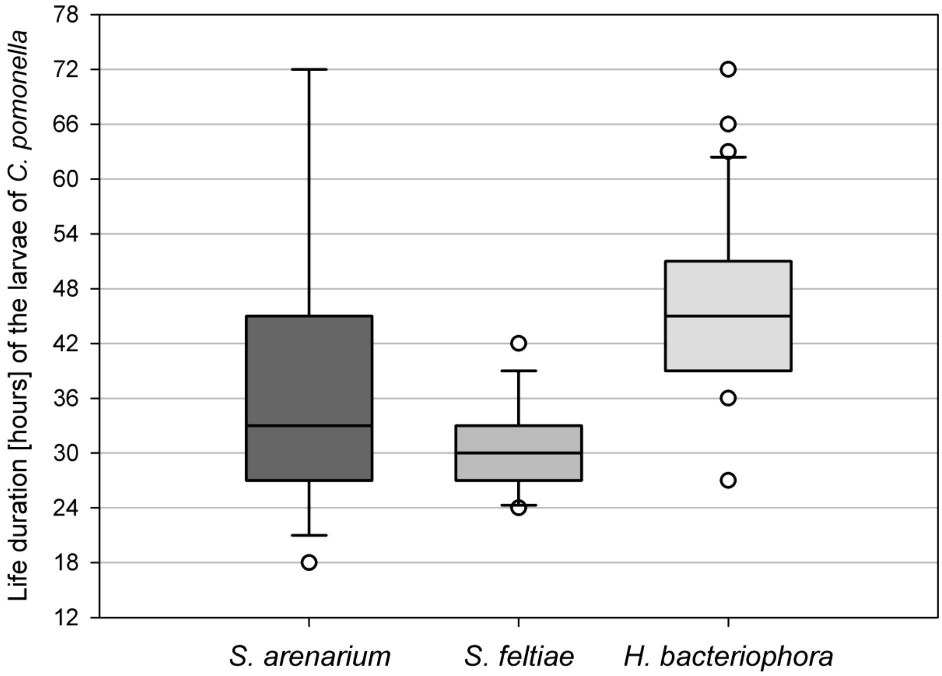

Fig. 1. *Comparison between the life duration of the larvae of C. pomonella after exposure to IJs of Steinernema arenarium, S. feltiae and Heterorhabditis bacteriophora. The Box Plots display the median, the 10th, 25th, 75th and 90th percentiles and the outliers*

The data obtained on the mortality rate of the larvae showed that *S. feltiae* caused 100% mortality by the 42nd hour after exposure. In the trial with *S. arenarium*, the mortality reached 83.3% by the end of the experimental period, while *H. bacteriophora* caused higher mortality of 96.7%. Peak mortality intervals were recorded on the 27th hour for *S. feltiae,* the 30th hour for *S. arenarium* and the 39th hour for *H. bacteriophora*.

The larvae of *C. pomonella* demonstrated different survival pattern depending on the EPN species (Figure 2). The first dead larvae were observed after infection with *S. arenarium*, but the mortality rate was relatively low. The K-S survival curve in the case of *S. feltiae* rapidly descends to zero as all larvae in the group die within 18 hours. In comparison, the shape of the curve of *H. bacteriophora* is similar but demonstrates a delayed pathogenic effect.

Statistical differences between the survival curves of the codling moth larvae in the trials were established by means of the Log Rank test (χ^2 = 29.8, *d.f.* = 2, *P* < 0.001). The subsequent Post Hoc analysis according to the method of Holm-Sidak indicated that the survival curve of the larvae parasitized with *S. feltiae* differs significantly from the other two.

Hence, the fifth instar of *C. pomonella* is highly but not equally susceptible to the three tested nematode isolates. The species factor has significant effect on the parameters of life duration, mortality rate and survival curves.

DISCUSSION

When considering EPNs as bioagents for control of insect pests in agriculture, one of the first steps is to select a suitable species of nematode for the purpose. Trials with different EPN-host pairs are useful in determining the efficacy of various nematode species or isolates to inform decisions on production and/or application depending on the environmental conditions. For example, *S. carpocapsae*, one of the first commercially produced nematode bioagents, was originally isolated from cocooned coddling moth larvae (Dutky & Hough, 1955) and was expected to perform well in controlling the pest. However, *S. feltiae* has been reported to be more effective than *S. carpocapsae* when the temperature is below 15°C (Lacey and Unruh, 1998). Thus in early spring and autumn, when applications against *C. pomonella* would lead to best results as its whole population is represented by diapausing larvae (Lacey and Unruh, 2005), the more cold tolerant *S. feltiae* would be a better choice of

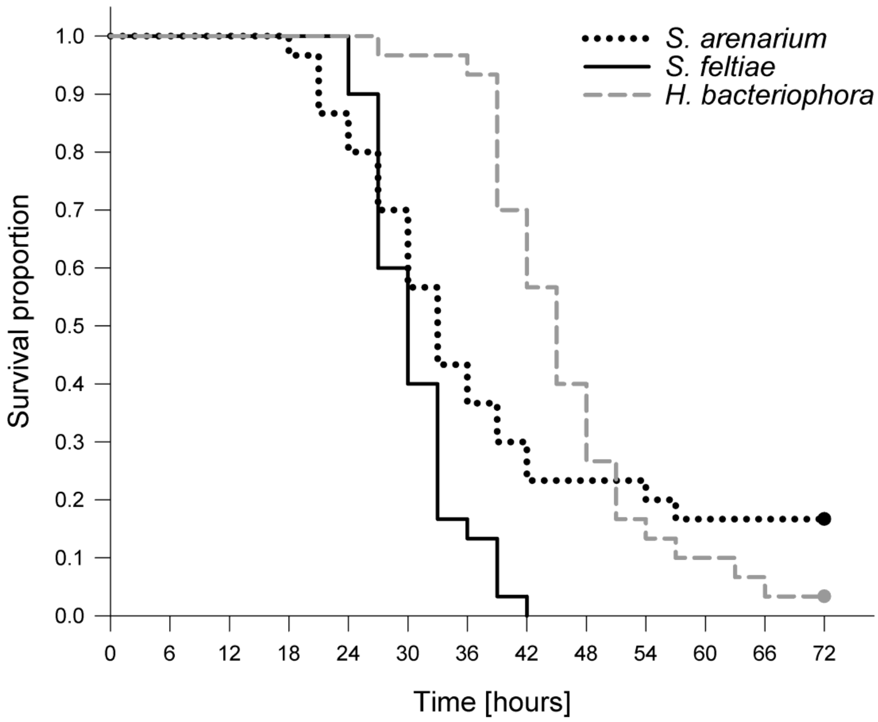

***Fig. 2.** Kaplan-Meier survival curves of the larvae of C. pomonella after exposure to IJs of Steinernema arenarium, S. feltiae and Heterorhabditis bacteriophora*

bioagent. The present study shows that the Bulgarian isolate of the species *S. feltiae* is highly pathogenic to *C. pomonella* and may have potential to control the pest under such conditions.

Another important characteristic of EPNs is the strategy that they employ in seeking their hosts (Gaugler, 1999). Nematodes with ambush strategy, such as *S. carpocapsae,* are more suitable for controlling mobile insects while cruisers, such as *H. bacteriophora* actively seek their hosts (Lewis et al., 1992). The other two species tested in the present study, *S. feltiae* and *S. arenarium*, have an intermediate strategy (Campbell and Gaugler, 1997; Andaló et al., 2012). All three studied isolates readily parasitized *C. pomonella* and offer different options for biological control of the pest, depending on the particular conditions and the desired strategy.

The species *S. feltiae* and *H. bacteriophora* used in the present study are very effective biological agents for control of insect pests of different orders on a number of agricultural crops (Georgis et al., 2006). Their suitability for control of *C. pomonella* is also demonstrated in our experiment. The species are widely distributed in natural ecosystems in Bulgaria (Shishiniova et al., 2000; Gradinarov et al., 2012; Gradinarov, 2012), a good indication that they are well adapted to the conditions of the habitats in the country. Meanwhile the species *S. carpocapsae* has not been found in Bulgaria after 2002 (Gradinarov, unpublished data, 2015). Further investigations are needed to elucidate its distribution and role in the regulation of pests in orchards. With respect to the control of *C. pomonella*, the use of alternative nematode agents with different host-seeking behaviour and confirmed efficacy may prove to be a successful strategy.

The species *S. arenarium* belongs to the *"glaseri"* group within genus *Steinernema* (Spiridonov et al., 2004). Studies suggest that the members of this group are specialized in parasitizing insects of family Scarabaeidae (Coleoptera) (Poinar and Kozodoi, 1988). The high efficacy of *S. arenarium* against *C. pomonella* observed in our experiment shows that the species is not a strict specialist. Furthermore, the culture we used has been successfully maintained on the laboratory host *G. mellonella* for more than 10 years.

Even though the three tested species caused high mortality of *C. pomonella*, laboratory results should not be directly extrapolated to field efficacy, which is a major prerequisite for commercialization. Further trials are needed to confirm whether the tested strains perform well in the orchard.

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