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INVASIVENESS AND REPRODUCTION OF THE *Steinernema feltiae* FROM SELECTED AGROCOENOSE LOCATED IN WIELUN

INWAZYJNOŚĆ I ROZRODCZOŚĆ *Steinernema feltiae* Z WYBRANEJ AGROCENOZY WIELUNIA

Abstract: Faunistic studies to find the presence of nematodes of the family *Steinernematidae* were performed. Soil samples were collected from pasture area located in the municipality of Wielun (Lodz Province). Latitude and longitude of the sampling site was determined with the Gnomon software. Physico-chemical properties of samples were analyzed in the Analytical Centre of the Warsaw Agricultural University. Total lead content was analyzed with the flame atomic absorption spectrophotometry and soil pH – potentiometrically. Nematodes were isolated from the soil samples in the laboratory conditions, using method described by Bedding and Akhurst in 1975. *S. feltiae* (Filipjev 1934) were determined using the key for the determination of the species (Lacey 1997) and by molecular analysis. It was shown that nematodes from the investigated area were characterized by higher biological activity (the intensity of the invasion, the degree of migration) and longer duration of development (time of killing the host and the time needed to start migration), in comparison with nematodes from commercial biopreparation.

Keywords: entomopathogenic nematodes, *Steinernema feltiae, Galleria mellonella*

Entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* are used in biological plant protection. The animals are closely associated with bacteria of the genera *Xenorhabdus* sp. and *Photorhabdus* sp. The nematodes are characterized by a great fertility and a broad host spectrum [1–3]. Invasive larvae of the animals, under optimum conditions, are able to persist in soil and to actively search, infect and kill the insect host. They are now massively reproduced on artificial media and processed to produce bioinsecticides [4, 5].

Entomopathogenic nematodes occurring in the soil often are exposed to natural environmental resistance limiting their biological activity. The ability of the invasion of nematodes and their effectiveness in reducing insect populations is influenced by many biotic and abiotic factors [6, 7].

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Important abiotic factors are: soil structure, soil pH and heavy metals. An example is the lead (II), whose harmful effects on entomopathogenic nematodes proven in many studies [8–10]. Both lead and nematodes gather in the upper soil layers. Lead accumulates there and is not washed out downwards. Therefore, its harmful impact may last for years [11].

On the biological activity of nematodes also influences soil pH [12]. Optimum soil pH for many soil organisms is in the range 5.5–7.2 [13]. Research has shown that low soil pH a negative effect on survival and invasive larvae of nematodes [12, 14].

Soil structure also can have a big impact on entomopathogenic nematodes. In sandy substrates, and sand-clay recorded the highest survival and activity of these animals [7].

Scientific data suggest that the selection of indigenous breeds of nematodes is more beneficial than the use of commercial preparations based on nematodes. Such investigations are carried out in many countries around the world, including Hungary [15]. In Poland, however, lack of detailed studies of biogeographical data, a collection of useful species and breeds entomopathogenic nematodes effectively limiting the populations of harmful insects.

The aim of this study was to analyze insecticidal properties of nematodes isolated from natural environment in comparison with those from commercial biopreparation.

Materials and methods

Acquirement of samples from environment. Studies were carried out in the summer season of the years 2010–2011. Fifty soil samples were taken with the Egner's sampler from a soil layer 0–25 cm in selected study area (natural meadows) located in the municipality of Wielun (Lodz Province). The method ensures complete and even sampling which allows obtaining the actual spatial distribution of environmental pollution by lead and of nematode densities in the soil.

Latitude ($51^{o}14'24''$) and longitude ($18^{o}40'36''$) the geographical position of the species was determined with the Gnomon 3.3. software.

Soil samples, were examined in terms of physico-chemical industries in Analytical Center SGGW. Total lead content in soils was determined by flame atomic absorption spectrometry (FAAS) and soil pH was determined by potentiometry [16, 17].

For the studies caterpillars of greater wax moth (*Galleria mellonella* L.) with an average body weight of 187 mg were used as a host. Larvae of entomopathogenic nematodes *Steinernema feltiae* investigated in this study were taken from the soil samples in the laboratory by insect trap method (Bedding and Akhurst) [18]. Well mixed soil samples were placed in plastic boxes of a volume of 250 cm^3 together with two trap insects (caterpillars of *Galleria mellonella* L.). Samples were then placed in the POL-EKO ST1 incubator at temperatures 20 ^{o}C for 16 days. Every second day, dead insects were isolated and replaced with fresh living ones. Infected larvae were placed on the migratory sponges and stored at temperature of 20 ^{o}C for the time necessary to obtain nematode larvae. Once nematodes left the host cavity, they were regularly picked up to tissue culture bottles and stored at 4 ^{o}C. Nematode species was determined using the key to the determination [19] and by molecular analysis (sequencing of the PCR product with primers complementary to the ribosomal RNA genes of *S. feltiae*).

Genetical analysis. Total DNA from pure culture of some isolations of selected nematodes were purified using Genomic mini column kit (A&A Biotechnology). Purified DNA isolates were analyzed via gel electrophoresis in 1 % agarose, Than DNA were used as a template in PCR with primers SF18SL and SF18SR designed by author and synthesized in Institute of Biochemistry and Biophysics PAS. Primers allow to amplify fragment started at the terminal part of 18S rRNA gene and contains ITS1 region, 5.8S RNA gene, ITS 2 region and finished at the beginning part of 23S rRNA gene. Sequences of primers are presented in Table 1.

Table 1

Primers used in PCR with *S. feltiae* DNA

Name	Sequence
SF18SL	GTACACACCGCCCGTCGCTGC
SF18SR	AAATCCTAGTTAGTTTCTTTTCCTCCGC

PCR reactions were performed in 0.2 cm^3 tube. For each reaction 22 mm^3 of PCR MasterMix (A&A Biotechnology) supplemented with both primers (each concentration 0.2 μM) was mixed with 10 mm^3 of nematode DNA template and finally 18 mm^3 of ultrapure water was added. Mastercycler PRO (Eppendorf) was used for PCR. Condition of PCR is presented in Table 2.

Table 2

PCR condition for *S. feltiae* analysis

Phase	Temperature [^{o}C]	Time [min]	No. of cycles
Initial denaturation	94	3	1
Denaturation	94	0.5	35
Annealing	66	0.5	35
Extension	72	0.5	35
Final extension	72	5	1

Small piece of PCR products (10 mm^3) were analyzed by electrophoresis in 1 % agarose gel. Than the rest of products were purified using Clean-Up kit (A&A Biotechnology) and sequenced (CoreLab of Medical University in Lodz).

Analysis of sequences was performed using BioEdit 5.0.6. software. For alignment ClustalX 2.012 [20] was used.

S. feltiae from a biological preparation "Owinema" were used as a control.

Insecticidal property analysis. The experiments were performed in Petri dishes in the POL-EKO ST1 incubator at 20 ^{o}C. For infection of insect the dose of 50 infective juveniles (IJs) nematodes per insect were used. It was the optimal dose to obtain the maximum number of larvae of nematodes [21]. The same doses were used to investigate the mortality of insects, extensiveness and intensity of invasion. Dead insects were dissected two days after their death.

In the experiment, the about 400 caterpillars of *G. mellonella* were applied. There were used to isolation of nematodes from soil samples (200 caterpillars), for dissection during analysis of the intensity of the invasion (150 caterpillars) and for the analysis of nematode reproduction (50 caterpillars). This parameter was tested during five consecutive days after the first appearance invasive larvae in an artificial environment outside (Petri dish). Total number of larvae that managed to leave the host body for the day and the number of live larvae (capable of following an invasion) was assessed.

For the statistical analysis used analysis of variance of one variable (UNIANOVA). The experiment was repeated twice.

Results and discussion

The analysis of physico-chemical soil samples showed a natural content of lead (II) (6.86 ppm) and acidic (5.62). Granulometric soil subgroup was defined as sandy clay. Above parameters are characteristic for the environments appropriate to the occurrence of entomopathogenic nematodes. This is also acknowledgement by the results received of other researchers [7, 8, 14].

In the analyzed soil samples found to larvae of entomopathogenic nematodes and morphometric measurements of all development stages of nematodes and comparison with the key shown that the analyzed nematodes belonged to species *S. feltiae*. The obtained results were also confirmed using the PCR reaction. Species caught in one of the largest populations of nematodes occurring in Polish [6, 22].

During analysis of the biological activity of nematodes has been shown that the total mortality insects, nematodes infecting subjects from habitat, remained at the same level as a control (nematodes from biopreparation). Extensiveness of invasion was slightly low compared with the control sample, but this result still indicates a high efficiency of occurring naturally in the habitat nematodes against insects (Table 3).

Table 3

The biological activity of *S. feltiae*

Tested samples	Control	Nematodes from investigated areas
Total mortality of insects [%]	100	100
Extensiveness of invasion [%]	100	98
Intensity of invasion [pcs.]	8^a	9^b
Time to kill the insect by the nematode [days]	2	4
The first day of the migration of invasive larvae from the insect-host to the external environment [days]	8	18

Different letters in columns denote significant differences at $p < 0.05$.

The intensity of the invasion of isolated from the habitat nematodes was a slightly higher than the control sample, and conducted analysis of variance shown important differences between the control sample, and nematodes from land (Table 4).

Table 4

Variance analysis for "invasion intensity" feature

Source of variation	Type III sum of squares	df	Mean square	F	Significance
Model adjusted	1679(a)	1	167	14	$p < 0.001$
Constant	17888	1	17888	1477	$p < 0.001$
Habitat	167	1	167	14	$p < 0.001$
Error	2883	238	12		
Total	20938	240			
Total adjusted	3050	239			

(a) – R square = 0.055 (corrected R square = 0.051).

Examining the time of death caterpillars after infection nematode (time of killing an insect – host) was in the sample of nematodes from the habitat the municipality Wielun, longer than control sample (Table 3). This time is also much more than gives other researchers [21].

A similar relationship was recorded by analyzing the time required to start the migration invasive larvae of the host body to the external environment. The first day on which the presence of larvae was observed in the external environment, have been reported at 18 days of infection, whereas for the control of nematodes to migrate there have been at day 8 of insect infestation (Table 3).

Insect killing time and time of migration may indicate that entomopathogenic nematodes are food selectivity.

During analysis of the reproduction of nematodes from the habitat and the survival of their larvae after migration, has been observed that the number of alive larvae which emerged from the host's body is higher than in the population of nematodes in the control sample (Table 5, Fig. 1).

Table 5

Nematodes migration in the first and next successive four days with specification of the number of alive larvae [pcs.]

Days	Number of migration larvae from an insect [pcs.]			
	Control		Nematodes from investigated areas	
	Altogether	Alive	Altogether	Alive
1	3829	3829	23917	23483
2	12886	11517	19286	18979
3	21795	19251	13951	13559
4	13403	12756	10167	10052
5	12096	11586	11932	11532
Total	64009	58940^{a}	79254	77605^{b}

Different letters in columns denote significant differences at $p < 0.01$.

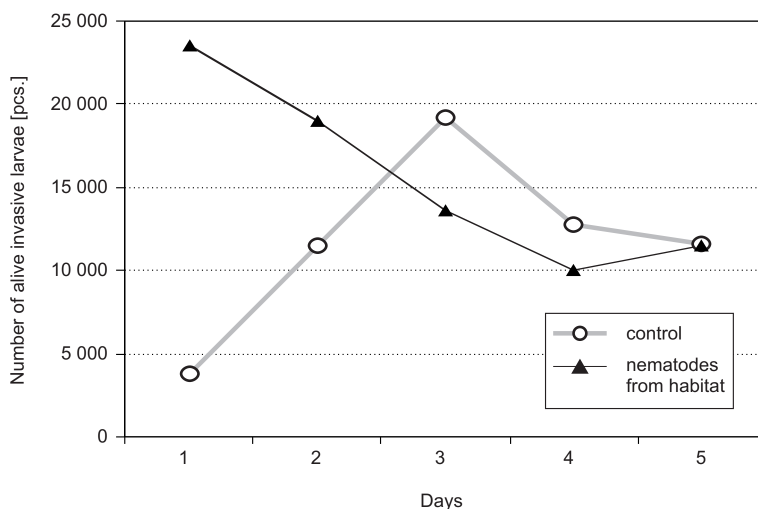

Fig. 1. Number of the alive invasive larvae of nematodes after migration

Mortality of larvae of nematodes from the area during the first five days of migration was lower compared with the control sample and was characterized by less dynamic than the mortality of larvae of nematodes from biopreparation (Table 5, Fig. 2).

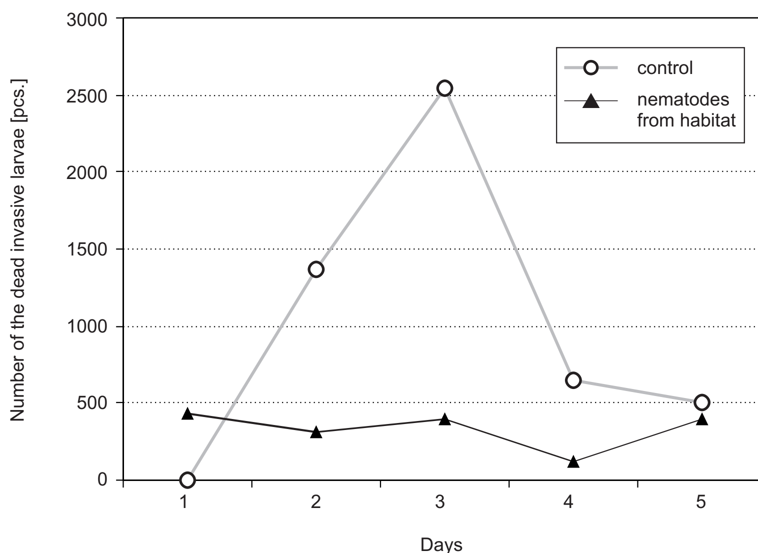

Fig. 2. Number of the dead invasive larvae of nematodes after migration

Statistical analysis of the invasive larvae, staying alive after migration from the host's body, showed the high essential difference between the number of investigated nematodes, and of the control sample (Table 6).

Table 6

Variance analysis for "nematodes reproductivity" feature

Source of variation	Type III sum of squares	df	Mean square	F	Significance
Model adjusted	712 663 381(a)	1	712 663 381	7 777	p < 0.001
Constant	37 407 552 526	1	37 407 552 526	408 227	p < 0.001
Habitat	712 663 381	1	712 663 381	7 777	p < 0.001
Error	549 806	6	91 634		
Total	38 120 765 713	8			
Total adjusted	713 213 187	7			

(a) – R square = 0.999 (corrected R square = 0.999).

Researches on the biological activity of nematodes from the habitat are still under development. It was shown that one of the most effective pathogens against pests (*eg* grubs of cockchafer) is entomopathogenic nematodes isolated from the habitat [23].

Results obtained also show that the nematodes originating from the habitat, characterized by higher reproduction and survival of invasive larvae than nematodes from biopreparation. Is important to continue and extend the research topic, which in future will enable better use of biological methods of plant protection.

Conclusions

1. Strains of nematodes isolated from the natural environment are characterized by higher biological activity (intensiveness of invasion, degree migration) than nematodes from biopreparation.

2. Development time (killing the host and the time required to migration) of nematodes isolated from natural environment is longer in comparison with nematodes from biopreparation.

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INWAZYJNOŚĆ I ROZRODCZOŚĆ *Steinernema feltiae* Z WYBRANEJ AGROCENOZY WIELUNIA

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Abstrakt: Przeprowadzono badania faunistyczne w celu stwierdzenia obecności nicieni z rodziny *Steinernematidae*. Próby glebowe pobrano z terenu użytku zielonego położonego w gminie Wieluń (województwo łódzkie). Długość i szerokość geograficzną stanowiska występowania gatunku oznaczono za pomocą programu komputerowego Gnomon. Próby zostały zbadane pod względem fizykochemicznym w Centrum Analitycznym SGGW. Oznaczono całkowitą zawartość ołowiu w glebie metodą płomieniowej absorpcyjnej spektrometrii atomowej (FAAS) oraz odczyn gleby metodą potencjometryczną. Nicienie wyizolowano z prób glebowych w warunkach laboratoryjnych, metodą Beddinga i Akhursta (1975). *S. feltiae* (Filipjev 1934) oznaczono przy pomocy klucza do oznaczania gatunku (Lacey 1997) oraz za pomocą analiz molekularnych. Wykazano, że nicienie pochodzące z badanego terenu charakteryzują się większą aktywnością biologiczną (intensywność inwazji, stopień migracji) oraz dłuższym czasem rozwoju (czas uśmiercenia żywiciela i czas niezbędny do rozpoczęcia migracji) w porównaniu z nicieniami pochodzącymi z komercyjnego biopreparatu.

Słowa kluczowe: nicienie entomopatogeniczne, *Steinernema feltiae*, *Galleria mellonella*