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Intraspecific variability of *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) as biological control agent of rice weevil (*Sitophilus oryzae* [L.], Coleoptera, Curculionidae) adults

Žiga LAZNIK[1], Stanislav TRDAN[2]

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ABSTRACT

The efficacy of three strains (B30, B49 in 3162) of *Steinernema feltiae* to control adults of rice weevil (*Sitophilus oryzae*) was tested in a laboratory experiment in 2009. The activity of entomopathogenic nematodes was assessed at five different concentrations (125, 250, 500, 1000 and 2000 infective juveniles/individual) and four different temperatures (15, 20, 25 and 30 ºC). Results demonstrated that all strains acted most effective at 25 ºC and at highest concentration of nematode suspension, meanwhile the lowest mortality of rice weevil adults was attained at 30 ºC. The results of our research showed that at high concentrations entomopathogenic nematodes are an effective biological agent for controling the studied primary stored products pest.

Key words biological control, entomopathogenic nematodes, *Steinernema feltiae*, *Sitophilus oryzae*, strains, laboratory experiment, stored products pest

IZVLEČEK

ZNOTRAJVRSTNA VARIABILNOST ENTOMOPATOGENE OGORČICE *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) KOT BIOTIČNEGA AGENSA ZA ZATIRANJE ODRASLIH OSEBKOV RIŽEVEGA ŽUŽKA (*Sitophilus oryzae* [L.], Coleoptera, Curculionidae)

V letu 2009 smo pri laboratorijskem poskusu preizkušali učinkovitost treh ras (B30, B49 in 3162) entomopatogene ogorčice *Steinernema feltiae* na odrasle osebke riževega žužka (*Sitophilus oryzae*). Delovanje entomopatogenih ogorčic smo preizkušali pri petih različnih koncentracijah (125, 250, 500, 1000 in 2000 infektivnih ličink/osebek) in štirih različnih temperaturah (15, 20, 25 in 30 ºC). Rezultati so pokazali, da so vse rase najbolj učinkovito delovale pri 25 ºC in najvišji koncentraciji suspenzije ogorčic, medtem ko smo najmanjšo smrtnost odraslih osebkov riževega žužka ugotovili pri 30 ºC. Rezultati naše raziskave so pokazali, da so v visokem številu entomopatogene ogorčice učinkovit biotični agens za zatiranje preučevanega primarnega skladiščnega škodljivca.

Key words: entomopatogene ogorčice, *Steinernema feltiae*, *Sitophilus oryzae*, biotično varstvo, laboratorijski poskus, skladiščni škodljivec

1 INTRODUCTION

On stored agricultural products, especially cereal grains and legume seeds, several pests appear which can cause a large amount of damage. While they feed on stored grains, the yield quantity is reduced and the pests especially lower product quality (Neethirajan *et al*., 2007). Annual damage due to storage pests on stored products amounts to from 1.25 to 2.5 billion USD (Schöller *et al*., 2006). Storage pest insects cause stored

[1] Young researcher, Teach. Assist., B. Sc., Jamnikarjeva 101, SI-1111 Ljubljana, e-mail: ziga.laznik@bf.uni-lj.si

[2] Assoc. Prof., Ph. D., ibid.

products to heat up or that their moisture level increases with the consequence of grain infection due to micro-organisms (Rees, 1998).

We also consider the rice weevil (*Sitophilus oryzae* [L.], Curculionidae) to be among the most important pests regarding stored grain in Europe in recent years (Stejskal *et al*., 2003). It is a known fact that the insect developed a resistance to the insecticide malation already in the 1960s (Haliscak & Beeman, 1983; van Graver & Winks, 1994). Only four years passed from the appearance of resistance until the total ineffectiveness of malation in controlling the rice weevil (van Graver & Winks, 1994). Despite data which shows that the usage of the insecticide in controlling storage pest is not notably hazardous to consumers, researchers are still of the opinion that insecticide residues in food are harmful to human health, as are insecticide residues in feed to animal health and indirectly as well to human beings (Acuff, 1993; South, 1993; Bryne *et al*., 1994). Consumers seem willing to pay more for food produced without the use of chemical products (Ott, 1990; Brewer *et al*., 1994).

The application of entomopathogenic nematodes (EPNs) as biological control agents in controlling pest insects is well documented (Kaya & Gaugler, 1993; Helyer *et al*., 1995; Trdan *et al*., 2007). EPNs live in symbiosis with bacteria which after infection are released by nematodes into the hemolymph system of the host (Gaugler, 2002). The ability to infect the host holds only for infective juveniles (IJs) which carry mutualistic bacteria in special intestine vesicles (Kaya, 2000). A host in which the nematodes manage to penetrate usually die due to septicemia or failure of some insect organs in 24 to 72 hours after infection (Smart, 1995; Forst & Clarke, 2002).

The results of many research studies have demonstrated that EPNs at high concentrations together with favourable abiotic factors (high humidity, optimal temperature) can be effective biological agents in controlling adults from the order Coleoptera (Journey & Ostlie, 2000; Trdan *et al*., 2006). Recent research has confirmed their efficacy in controlling adults of the Japanese beetle *Popillia japonica* Newman (Lacey *et al*., 1993; Grewal *et al*., 2002; Koppenhöfer & Fuzy, 2007), the western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (van der Burgt *et al*., 1998; Toepfer, 2005), the hairy fungus beetle (*Typhaea stercorea* [L.]) (Svendsen & Steenberg, 2000), the granary weevil (*Sitophilus granarius* [L.]) (Trdan *et al*., 2006), the sawtoothed grain beetle (*Oryzaephilus surinamensis* [L.]) (Trdan *et al*., 2006), the flea beetle (*Phyllotreta* spp.) (Trdan *et al*., 2008), and some others which belong to the order Coleoptera.

In previous years many research studies were carried out in connection with EPNs ability to control storage pests (Trdan *et al*., 2006; Ramos-Rodriguez *et al*., 2007; Athanassiou *et al*., 2007), yet data on their effectiveness in controlling the rice weevil are limited (Ramos-Rodriguez *et al*., 2006). Because in Europe EPNs have not yet been tested for controlling the rice weevil - which appears to be an increasingly harmful species in grain warehouses (Hamel, 2007) - we decided to study the activity of one of the generally most effective species for controlling pest insects, the nematode *Steinernema feltiae* (Filipjev) (Scheepmaker *et al*., 1998; Cuthbertson *et al*., 2003) with regard to the above mentioned storage pest. The aim of our research was to determine which of three tested strains (B30, B49, and 3162) is the most effective and how its effectiveness is related to temperature and suspension concentration. Namely, the most efficient strain could possibly be applied in the near future for practical purposes, i.e. for controlling this pest in grain storehouses.

2 MATERIALS AND METHODS

2.1 Sitophilus oryzae and entomopathogenic nematodes

In this experiment we studied nematode pathogenicity against adults of the rice weevil (*Sitophilus oryzae*). Adults of the mentioned insect had been in culture at the Chair of Phytomedicine, Agricultural Engineering, Crop Production, Grassland and Pasture Management (University of Ljubljana, Biotechnical Faculty, Dept. of Agronomy) in Ljubljana, Slovenia. If required, we added undamaged wheat grain in a two week intervals in a rearing vessels to provide optimal conditions for the studied insect species to feed and multiply. The rearing vessels were stored in darkness and at room temperature.

We included three strains of *Steinernema feltiae* EPNs in the experiment. We isolated strains B30 and B49 in Slovenia (Laznik *et al*., 2008), meanwhile strain 3162 was isolated in Hungary (Toth, 2006). The two Slovenian strains were tested for the first time in this experiment, while strain 3162 has been proven to be very effective in many laboratory experiments (Lakatos, unpubl.). All EPN strains were reared using late instar larvae of *Galleria mellonella* L. (Bedding and Akhurst, 1975). We used only infective juveniles which were less than 2 weeks old. During the experiment, which was repeated three times, we stored the infective juveniles at 4 ºC.

2.2 Laboratory bioassay

We tested the efficacy of the EPNs in controlling adults of the rice weevil *Sitophilus oryzae* by exposing individuals to either 0, 250, 500, 1000, or 2000 IJ/adult. We determined the number of infective juveniles in a previously prepared unknown concentration of nematode suspension by counting the number of such in droplets (5 µl x 5) and by diluting (adding M9 solution) or by concentrating (reduction to an adequate volume with the assistance of centrifugation). In this manner we obtained the selected concentrations of nematode suspensions (0, 2500, 5000, 10000, and 20000 IJ/ml).

We carried out the experiment according to the procedure described in the paper of Trdan *et.al.*, 2006. The following procedure was performed with a time interval in three replications. We placed adults in glassy Petri dishes (diameter = 9 cm) with each containing 10 adults. Prior to this, we put filter paper into each Petri dish (the same diameter as the former Petri dish) and 30 wheat grains. Each treatment in the experiment was repeated 10 times. The assigned nematode concentration was added to the filter paper with a pipette (1 ml). If needed, we additionally moistened the filter paper every second day of the experiment. The Petri dishes were put in a rearing chamber (type: RK-900 CH, producer: Kambič Laboratory equipment, Semič, Slovenia) with a volume of 0.868 m^3 (width x height x depth = 1000 x 1400 x 620 mm). We tested the efficacy at four different temperatures (15, 20, 25, and 30 ºC) and at a relative humidity of 80 %. The number of dead adults of *S. oryzae* was determined 4, 6, and 8 days after treatment. The dead individuals were dissected to determine if the nematodes were present. In such a manner we wanted to prove that the insects died due to the EPNs' activity.

2.3 Statistical analysis

A multifactor analysis of variance (ANOVA) was conducted to determine the differences in mortality rates (%) between the adults of *S. oryzae* reared in 48 different treatments (three strains of *S. feltiae* – each with four different concentrations at four different temperatures). Before the analysis, the mean mortality was tested for the homogeneity of treatment variances. Mortality rate data were corrected for control mortality, using Abbott's formula (Abbott, 1925). The arcsine square-root was transformed before this analysis. Duncan's multiple range test ($P \leq 0.05$) was used to separate mean differences among the parameters in all the treatments. All statistical analyses were performed with Statgraphics Plus for Windows 4.0 (Manugistics, Rockville, MD, USA) and the figures were created with MS Office Excel 2003. The data are presented as untransformed means ± SE.

3 RESULTS

3.1 Group analysis

Group analysis indicated that the mortality percentage of granary weevil (*Sitophilus oryzae*) adults was statistically significantly influenced by the concentration of the nematode suspension (F= 151.09; df= 4, 720; P<0.0001), temperature (F= 61.43; df= 3, 720; P<0.0001), DAT (F= 54.75; df= 2, 720; P<0.0001), nematode strain (F=4.15; df=2, 720; P=0.0162), interaction between DAT and the concentration of the nematode suspension (F=7.23; df=8, 720; P<0.0001), interaction between the concentration of the nematode suspension and the strain (F=5.33; df=8, 720; P<0.0001), interaction between the concentration of the nematode suspension and temperature (F=14.57; df=12, 720; P<0.0001), interaction between the strain and temperature (F=9.77; df=6, 720; P<0.0001), interaction between DAT, strain, and temperature (F=2.12; df=12, 720; P=0.0142) and interaction between the concentration of the nematode suspension, strain, and temperature (F=2.95; df=24, 720; P<0.0001). Interaction between DAT and strain (F=1.03; df=4, 720; P=0.3893), interaction between DAT and temperature (F=1.45; df=6, 720; P=0.1920), interaction between DAT, the concentration of the nematode suspension, and the strain (F=0.32; df=16, 720; P=0.9951), interaction between DAT, the concentration of the nematode suspension, and temperature (F=1.08; df=24, 720; P=0.3587), and interaction between DAT, the concentration of the nematode suspension, strain, and temperature (F=0.45; df=48, 720; P=0.9995) did not statistically significantly influence beetle mortality. In all treatments total mortality was significantly different from the control treatment. Corrected mortality was therefore calculated.

Among the individual strains of EPNs (3162, B49, and B30) we did not observe statistically significant differences in pathogenicity, while the average values of beetle mortality treated with them were 17.71 ± 1.08, 16.60 ± 0.79, and 15.33 ± 0.77 %, respectively. But we confirmed statistically significant differences between different temperatures. The studied strains were the most active at 25 ºC (22.34 ± 1.19 %), while no difference was observed at 15 and 20 ºC (15.75 ± 0.98 in 18.33 ± 1.11 %). The lowest efficacy was noted at 30 ºC (9.75 ± 0.52 %) (Figure 1). Statistically significant differences were confirmed also between the concentration of the nematode suspension. At the highest concentration of the suspension beetle mortality was the highest (31.79 ± 1.53 %), less effective were nematodes at both lower concentrations (8.8 ± 0.66 and 11.19 ± 0.63 %, respectively).

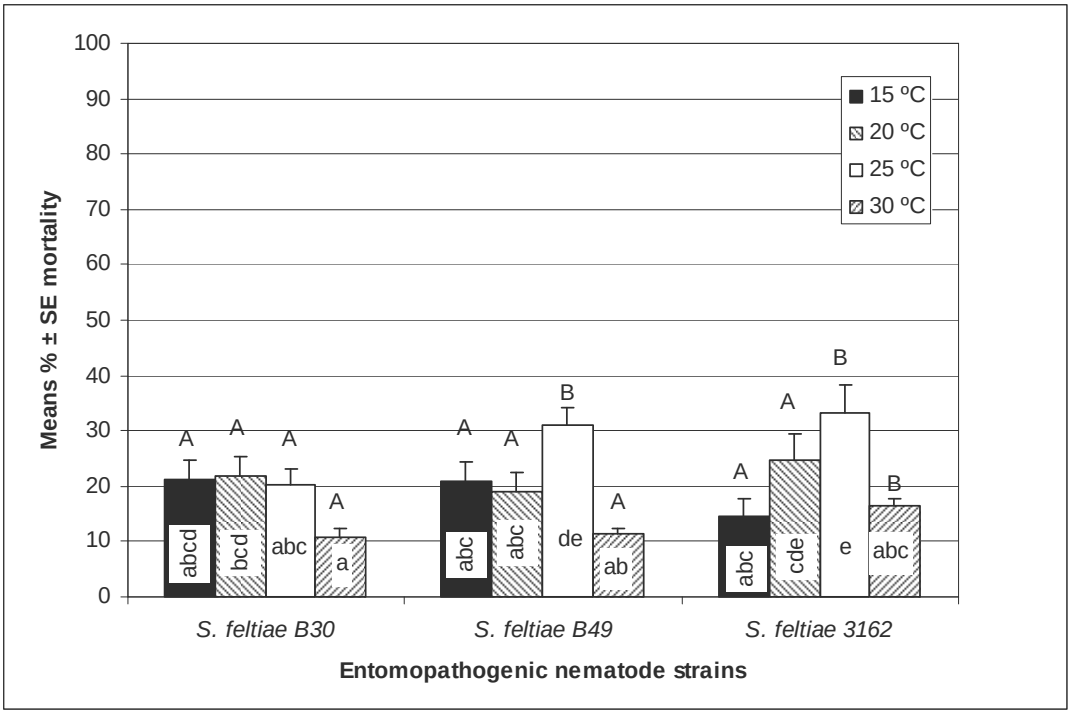

Fig. 1: Mean adult mortality of *Sitophilus oryzae* treated with three different strains of the entomopathogenic nematode *Steinernema feltiae* depending on rearing temperature. The data shown are corrected for control mortality and analyzed by multifactor ANOVA. Capital and lower-case letters correspond to the grouping of means by Duncan's multiple range test ($P \leq 0.05$) for EPN strains and temperature, respectively. The same letters do not differ significantly.

Table 1: Mean adult mortality (± SE) of *Sitophilus oryzae* treated with five different concentrations of three entomopathogenic nematode *Steinernema feltiae* strains at 15, 20, 25, and 30 ºC at 8 DAT. The data shown are corrected for control mortality.

Tempe-rature (ºC)	*S. feltiae* strain	Nematode concentration (IJs/adult) 125	250	500	1000	2000
15	B30	6.80 ± 3.39	14.89 ± 4.75	10.63 ± 4.25	36.16 ± 7.52	38.29 ± 6.20
	B49	10.86 ± 4.06	15.21 ± 5.32	13.04 ± 3.43	23.91 ± 9.09	41.30 ± 8.13
	3162	5.95 ± 4.96	5.52 ± 2.48	14.04 ± 5.28	16.16 ± 8.20	31.91 ± 7.21
20	B30	16.66 ± 4.65	14.58 ± 3.89	11.24 ± 4.77	16.66 ± 3.29	49.99 ± 6.91
	B49	8.69 ± 4.34	8.69 ± 2.66	19.56 ± 2.66	15.21 ± 2.17	43.47 ± 7.98
	3162	3.26 ± 1.99	14.69 ± 4.88	18.36 ± 3.22	22.44 ± 2.50	65.30 ± 6.12
25	B30	11.81 ± 3.89	11.36 ± 2.27	11.81 ± 3.89	20.45 ± 3.59	45.45 ± 4.25
	B49	24.44 ± 2.22	22.22 ± 3.51	17.77 ± 2.72	48.88 ± 4.44	42.21 ± 5.44
	3162	11.81 ± 5.29	20.45 ± 5.08	20.45 ± 3.59	40.90 ± 9.77	72.72 ± 5.79
30	B30	9.78 ± 3.19	6.37 ± 2.12	10.63 ± 2.60	10.63 ± 4.25	17.01 ± 2.12
	B49	6.37 ± 2.12	8.50 ± 2.60	10.63 ± 4.25	14.89 ± 4.75	17.01 ± 3.98
	3162	10.20 ± 2.04	16.32 ± 2.04	18.36 ± 3.22	20.40 ± 2.04	16.32 ± 3.81

We determined statistically significant differences also between days after treatment (DAT). At 8 DAT we confirmed the significantly highest beetle mortality (20.49 ± 1.00 %), and 6 and 4 DAT gave the significantly lowest mortality rate of rice weevil adults (17.21 ± 0.91 and 11.93 ± 0.66 %, respectively). Between all three time treatments there were statistically significant differences.

3.2 Individual analysis

At 15 ºC, 8 DAT, and the highest concentration of the nematode suspension, strains B49 and B30 demonstrated the highest mortality (41.30 ± 8.13 % and 38.29 ± 6.20 %, respectively). Similar effectiveness was attained for strain B30 at a lower concentration of the nematode suspension (1000 IJ/adult), namely (36.16 ± 7.52 %). At the lowest concentrations (125 IJ/adult and 250 IJ/adult) of the nematode suspension the best pathogenicity was noted in strain B49 (10.86 ± 4.06 %; 15.21 ± 5.32%, respectively). The results showed that at the lowest temperatures the weakest efficacy was for strain 3162 at all concentrations of the nematode suspension (from 5.52 ± 2.48 to 31.91 ± 7.21) (8 DAT) (Table 1).

At 20 ºC, 8 DAT, and at the highest concentration of the nematode suspension, the highest mortality rate was noted in strain 3162 (65.30 ± 6.12 %). Also strains B30 and B49 were satisfactorily effective at the highest concentration of the nematode suspension (49.99 ± 6.91 %; 43.47 ± 7.98 %, respectively) (8 DAT). At the lower concentration of the nematode suspension their pathogenicity was less satisfying (from 3.26 ± 1.99 to 22.44 ± 2.50 %).

At 25 ºC and 8 DAT the effectiveness of all studied concentrations of the nematode suspension was the highest. Strain 3162 pathogenicity at the highest concentration led to 72.72 ± 5.79 % mortality of the studied insect. Less effective were strains B30 and B49 (45.45 ± 4.25 %; 42.21 ± 5.44 %, respectively). Also the lowest concentration (1000 IJ/adult) of strain B49 demonstrated satisfactory pathogenicity (48.88 ± 4.44 %). At all studied concentrations strain 3162 was the most effective (from 11.81 ± 5.29 to 72.72 ± 5.79 %), except for the better performance at the lowest concentration of strain B49 (24.44 ± 2.22 %) (Table 1).

At 30 ºC and 8 DAT the effectiveness of all studied strains was the lowest (from 6 to 20 %). The highest mortality was reached by strain 3162 at a suspension concentration of 1000 IJ/adult (20.40 ± 2.04 %), while the lowest mortality was attained by strain B30 at a suspension concentration of 250 IJ/adult (6.37 ± 2.12 %). Strain 3162 demonstrated the best infectivity at this temperature (from 10.20 ± 2.04 to 20.40 ± 2.04 %), while strain B30 was the least infective (from 6.37 ± 2.12 to 17.01 ± 2.12 %) (Table 1).

Strain 3162 (5.95 ± 4.96) and strain B30 (6.80 ± 3.39) at the lowest concentration of the nematode suspension (125 IJ/adult) and at 15 ºC and 8 DAT caused statistically significantly lower mortality of rice weevil adults, while strain B49 (6.37 ± 2.12) acted as weakly at 30 ºC as at the lowest concentration of the nematode suspension (Table 1). The highest mortality of *Sitophilus oryzae* adults was noted in strain 3162 (72.72 ± 5.79) at 25 ºC and the highest concentration of the nematode suspension (2000 IJ/adult) (8 DAT), which holds true also for strain B49 (48.88 ± 4.44). Strain B30 reached the highest mortality of the studied insect (49.99 ± 6.91) at 20 ºC and at the highest concentration of the nematode suspension (2000 IJ/adult) (8 DAT).

4 DISCUSSION

The results of our research showed that the mortality of rice weevil (*Sitophilus oryzae*) adults is mostly affected by temperature in connection with the concentration of the nematode suspension, the nematode strain, and DAT. All three studied strains (B30, B49, and 3162) caused the highest mortality of the storage pests 8 days after treatment at 25 ºC (45 %; 48 %, and 72 %, respectively) and at the two highest concentrations of the nematode suspension (1000 and 2000 IJ/adult). The lowest mortality rate was recorded at 30 ºC, at which none of the strains with none of the concentrations of the nematode suspension exceeded 20 % efficacy. Sufficient efficacy at the lowest temperature (15 ºC) was reached only by strain B49 (41 %), while the pathogenicity of strain 3162 at this temperature was less satisfactory (from 6 to 31 %). One of the reasons for such ineffectiveness of this strain may lie in the origin of this strain, which was not isolated in Slovenia. Namely, this strain originates from Hungary, where the climate is typically continental and the average temperatures higher than in Slovenia (Peel *et al*., 2007). Confirmation of the above mentioned thesis is also demonstrated in that strain 3162 was the best (72 %) of all the studied strains. Also, the calculated LC_{50} value achieved was the lowest when compared at the highest temperature. A similar conclusions was also arrived at by Hazir *et al*. (2001), when they established that EPNs

which were isolated from areas of warmer climate had better pathogenicity at higher temperatures.

The weaker efficacy of all three strains at 30 ºC can be attributed to the fact that the interval of optimal control for the majority of EPNs is between 20 and 26 ºC (Kaya *et al*., 1993; Belair *et al*., 2003; Trdan *et al*., 2008), yet this characteristic is specific to either species or strain (Grewal *et al*., 1994; Hazir *et al*., 2001). It is well known that temperatures which fall below 0 ºC or rise above 40 ºC are fatal for the majority of EPNs (Brown & Gaugler, 1996). The reason for studying the activity of EPNs also at 30 ºC is a certain characteristic of the species *Sitophilus oryzae,* which is a distinctive thermophilus insect species with the ability to multiply more successfully at higher temperatures (from 25 to 35 ºC) (Fields, 1992). It is know for the majority of storage pests that temperature has a great influence on their survival ability (Ileleji *et al*., 2004), while most of them feed and multiply less intensively at temperatures lower than 18 ºC (Howe, 1965). In a similar research study (Ramos-Rodriguez *et al*., 2006) it was determined that the EPN *Steinernema riobrave* controls storage pests at 32 ºC better than some other species of EPNs. And this is because this species tolerates very high temperatures (up to 35 ºC) and in such conditions demonstrates a very high rate of pathogenicity (Cabanillas *et al*., 1994). Such an EPN species has not been found in Europe yet. Until now it has only been found in the USA (Cabanillas *et al*., 1994).

Previous research studies showed the efficacy of the EPN *Steinernema feltiae* in controlling some other storage pests from the order Coleoptera: *Sitophilus granarius* (Trdan *et al*., 2006), *Tribolium confusum* Jacquelin du Val (Athanassiou *et al*., 2007), *Tenebrio molitor* L., *Tribolium castaneum* (Herbst), *Trogoderma variabile* Ballion, *Sitophilus oryzae* (Ramos-Rodriguez *et al*., 2006), and *Oryzaephilus surinamensis* (Ramos-Rodriguez *et al*., 2006; Trdan et al., 2006). Some authors (Chen *et al*., 2003; Arthurs, 2004; Singh-Somvanshi, 2006) established that the efficacy of EPNs depends to a large extent on temperature and the concentration of the nematode suspension, but others established that only temperature influences the efficacy of EPNs (Trdan *et al*., 2008).

Research studies have also shown that EPNs can more or less effectively control some other beetles (Jaworska & Ropek, 1996; Trdan *et al*., 2006, 2008), while their control of larvae is much more substantial (Pezowicz, 1992; Nadasy *et al*., 1999; Arthurs *et al*., 2004). All hitherto research has demonstrated that the sensitivity of insects to EPN activity is a complex process which does not depend only on the momentary vitality of the insect, but also on the developmental stage, size, and aggressiveness of the nematode species (Jian *et al*, 2002). Several insects developed mechanisms during their evolution which serve as protection against natural enemies, including EPNs. In this way it is known that some larvae from the family Scarabaeidae lower the possibility of infection from EPNs by more frequent excreta secretion, which lessens the ability of infective juveniles to enter through the anus. It is characteristic of some representatives from the order Diptera and Lepidoptera that the anus is closed by a muscle and the spiracles are covered by setae or are too narrow for the EPNs to enter. Some insects do not activate their defence mechanisms until the nematode has already entered into their body. They start to form hoops of melanin around the EPN in order to isolate it before it releases mutualistic bacteria into the host's hemolymph. In this way the isolated nematode decays and the insect eliminates it from its body (Koppenhöffer, 2000). Hitherto no indication of a similar defence mechanism in *Sitophilus oryzae* has been reported by any researcher, however we can attribute the lower efficacy of the studied strains of EPNs in our experiment also perhaps to special defence mechanisms of the studied insect.

In Slovenia only the EPNs *Steinernema feltiae* (Laznik *et al*., 2008a) and *Steinernema carpocapsae* (Laznik *et al*., 2008b) have the status of indigenous species, while other discovered species of EPNs in Slovenia are for the present in the process of being removed from the national list of exotic species. That means that other species of EPNs in Slovenia for the time being can only be tested under laboratory conditions. But it is important to have in mind that results obtained from laboratory research can not be uncritically transferred to natural conditions, especially because of the many limiting factors which affect the survival of EPNs (Berry, 1993). In the future we will use the *Steinernema feltiae* 3162 strain in combination with biotechnical methods (Trdan *et al*., 2006) also in a trial in a grain warehouse, as the above mentioned strain demonstrated the greatest efficacy among the studied strains in our laboratory bioassay.

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