SHORT COMMUNICATION

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Testing local isolates of entomopathogenic nematodes against the green stink bug *Nezara viridula* L.

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Abstract

Aim of the study: The green vegetable bug *Nezara viridula* L. is a polyphage that is spread all over the world, but in the last 10 years it has entered the territory of the Russian Federation. The use of biological protection against this pest is an important task in the country. We used the entomopathogenic nematodes (EPN) *Steinernema feltiae* and *Steinernema carpocapsae* to control *N. viridula*.

Area of study: Federal Research Center of Biological Plant Protection (FRCBPP), Krasnodar Krai, Russia, 2019-2020.

Material and methods: A laboratory test was carried out with adults and nymphs of *N. viridula*. Each species of EPN (*S. carpocapsae* and *S. feltiae*) was used at doses of 50, 75 and 100 individuals infective larvae per insect. The initial material for analysis was collected on soybean crops in the crop rotation of the FRCBPP. The experimental results were assessed using ANOVA.

Main results: Laboratory tests of the EPN *S. carpocapsae* and *S. feltiae* caused the death of up to 98.0% of nymphs and up to 91.4% of adults of *N. viridula*. The species *S. feltiae* turned out to be the most effective, as allowed the pathogen to develop in shorter periods of time, and caused the death of 81.9-91.4% adults and of 92.0-98.0% nymphs.

Research highlights: This study showed that during the period of growth and development of larvae, the use of EPN is more effective in nymphs than on adults.

Additional key words: isolates of entomopathogenic nematodes; biological control; *Steinernema feltiae*; *Steinernema carpocapsae*.

Abbreviations used: EPN (entomopathogenic nematodes); FRCBPP (Federal Research Center of Biological Plant Protection).

Introduction

The green stink bug *Nezara viridula* L. (Hemiptera: Pentatomidae) is a polyphagous pest that damages over 120 different crops (Esquivel et al., 2018; Harman et al., 2021). Currently, chemical methods continue to be considered the main means of protection against this species, despite the growing resistance of the stink bug to pyrethroids and organophosphates (Thrash et al., 2021). However, in recent years, information has appeared on the successful use of various entomopathogens against *N. viridula* (Pushnya et al., 2020; Portilla et al., 2022).

Table 1. Results of treatment of larvae and adults of *Nezara viridula* with entomopathogenic nematodes.

Nematode	No. of invasive larvae per insect	% dead insects day^{-1}			% of insects from which nematodes were isolated	F	F table	df
		3 days	7 days	10 days				
			Larvae					
S. carpocapsae	100	76.2	88.1	95.0	49.2	2.67	3.22	2
	75	72.5	86.7	96.2	48.3	4.29	3.22	2
	50	62.5	86.3	91.8	52.5	22.30	3.22	2
S. feltiae	100	65.3	88.0	95.3	52.5	20.23	3.22	2
	75	60.0	95.3	98.0	53.1	33.17	3.22	2
	50	56.7	80.7	92.0	436.0	15.13	3.22	2
Control		14.7	273.0	293.0	-	-	-	-
			Adults					
S. carpocapsae	100	33.3	65.7	75.2	56.5	3.96	3.15	2
	75	55.6	77.0	87.4	55.5	8.67	3.15	2
	50	45.4	69.2	85.8	59.0	18.45	3.15	2
S. feltiae	100	39.0	60.5	81.9	56.2	11.76	3.15	2
	75	41.4	64.2	84.7	58.9	12.79	3.15	2
	50	46.6	72.8	91.4	52.5	25.13	3.15	2
Control		13.6	25.7	27.8	-	-	-	-

F: actual indicator of the Fisher criterion; F table: critical indicator of the Fisher criterion; df: degrees of freedom, this is the maximum number of logically independent values that can vary in the data sample.

One of the most effective natural regulators of the number of harmful arthropods are entomopathogenic nematodes (EPN) belonging to the families Steinernematidae and Heterorhabditidae. These organisms are capable of infecting more than a thousand species of arthropods through symbiotic relationships with *Xenorhabdus* Thomas & Poinar bacteria for Steinernematidae and *Photorhabdus* Fischer-Le Saux et al. bacteria for Heterorhabditidae (Divya & Sankar, 2009; Vashisth et al., 2013; Mohan, 2015). Among biological agents produced in the world, nematode preparations are in second place after bacterial ones (Koneru et al., 2016; Shapiro-Ilan et al., 2016; Jagodič et al., 2019). EPN have the ability to independently penetrate into the victim, persist in dead insects and contribute to the invasion of other pathogens (in particular, viruses and bacteria) of entomopathogenic parasites into the body of insects. The high development rate of nematodes allows them to spread with larvae and adults of pests (Navaneethan et al., 2010; Cruz-Martínez et al., 2017; Vicente-Díez et al., 2021). There are information about the possibility of combined use of EPN with entomopathogenic bacteria (*Bacillus thuringiensis* subsp. *japonensis* (Btj)) and fungi (*Metarhizium anisopliae*), as well as with phytopathogenic bacteria *Pseudomonas* spp. (Jaffuel et al., 2019; Ogier, 2020; Ruiu et al., 2022), which creates additional opportunities for the development of new complex biological products (Vashisth et al., 2013).

It should be noted that out of many EPN, no more than 8-10 species are used in world practice. These are mainly two genera (*Steinernema* and *Heterorhabditis*), what is also explained by the complex life cycle of these pathogens and their high hygrophility (Vashisth et al., 2013; Cortés-Martínez & Chavarría-Hernández, 2020). To date, around 100 valid species of *Steinernema* and 21 species of *Heterorhabditis* have been identified from different countries of the world (Bhat & Askary, 2020). The cultivation of EPN is carried out both on artificial nutrient media and on insect hosts, as large wax moth *Galleria melonella* L. and large flour beetle *Tenebrio molitor* L. (Cortés-Martínez, Chavarría-Hernández, 2020).

There is evidence of the successful use of EPN against Hemiptera representatives (e.g. bugs *Aelia rostrata*, *Euschistus heros*, *Dichelops melacanthus* and *Dactylopius opuntiae*), which have served as the basis for the use of nematodes against other Hemiptera representatives (Lopes Nanzer et al., 2021; El Aalaoui et al., 2022; Peçen & Kepenekci, 2022).

The widespread worldwide distribution of adventitious species of stink bugs *N. viridula* and *Halyomorpha halys* has set researchers the task of finding effective biological means of protection against these pests; therefore, EPN have been actively tested against them in recent years (Pervez et al., 2014; Burjanadze et al., 2020).

The aim of this research was to study in vitro the possibility of using local strains of EPN *Steinernema feltiae* and *Steinernema carpocapsae* at different infection rates against *N. viridula* adults and nymphs.
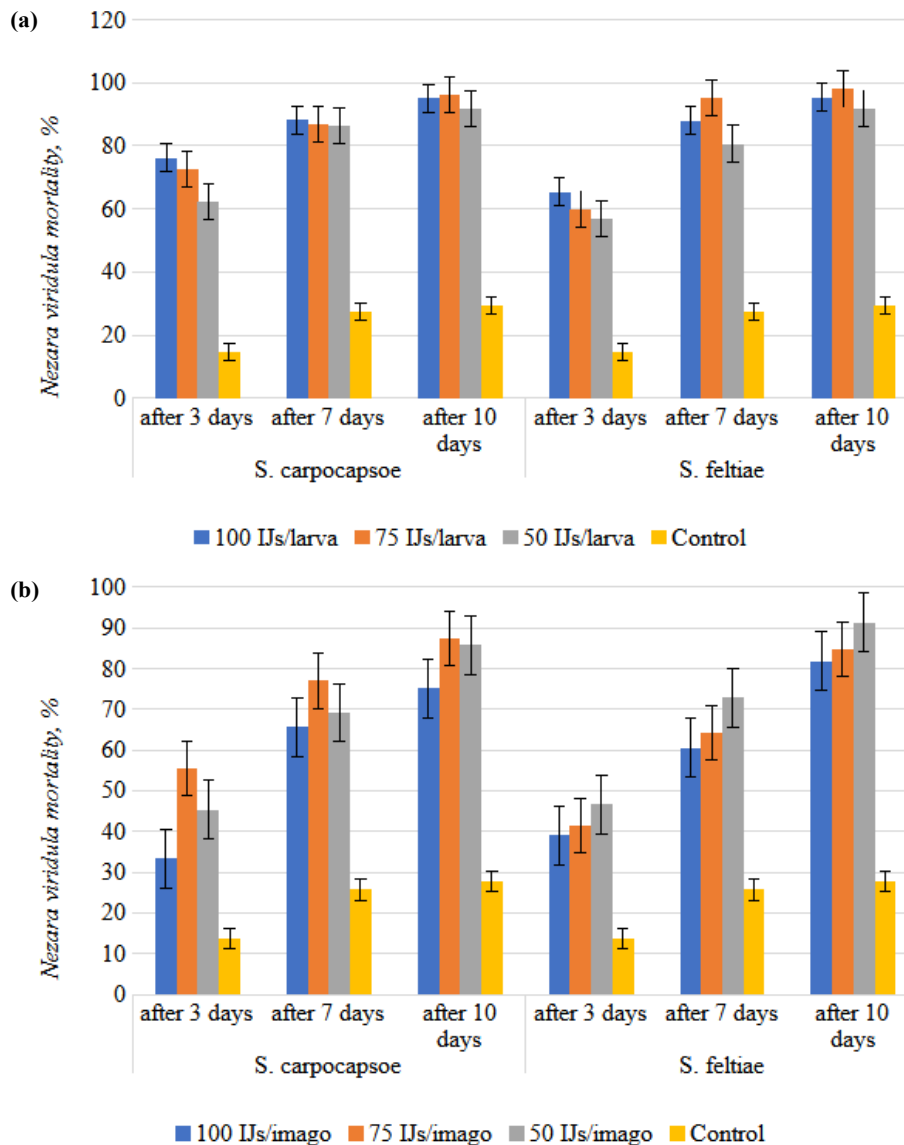
Figure 1. Relative mortality of *Nezara viridula* per day (mean ± SD): (a) nymphs, (b) adults. IJs: infective juveniles

Material and methods

Maintenance of a green stink bug in laboratory

Imago of *N. viridula* were collected on soybean plants var. 'Velana' in 2019-2020, located in the crop rotation of the Federal Research Center of Biological Plant Protection (FRCBPP), Krasnodar Krai, Krasnodar, Russian Federation (45°03'19.5"N 38°52'07.1" E). A total of 1050 individuals of nymphs and adults were collected.

The bugs were grown year-round on soaked and germinated seeds of mung bean (*Vigna radiata* (L.) R. Wilczek) according to the method described in Pushnya et al. (2020), used to obtain coeval nymphs and/or adults. The laboratory population was kept in a Binder-320 climate chamber (± 2 °C, 75% RH, 14L:10D). Adults of different sexes and nymphs of the fourth age at the age of 1–2 days were used for the experiments after molting.

Cultivation of entomopathogenic nematodes

Populations of the EPN *S. feltiae* and *S. carpocapsae* were obtained from the Laboratory of Phytosanitary Monitoring of Agroecosystems (FRCBPP), Krasnodar Krai, Krasnodar, and multiplied in the greater wax moth *Galleria mellonella* and large flour beetle *Tenebrio molitor* at 25 ± 0.5 °C (Cortés-Martínez & Chavarría-Hernández, 2020). After cultivation, the nematodes were stored at + 8°C. Insects were infected with nematodes in Petri dishes with filter paper placed at the bottom. The larvae were irrigated from a pipette containing a nematode suspension with 50-100 infective larvae – infective juveniles (IJs) below. To isolate invasive nematode larvae, dead larvae were transferred to nematode traps 7–10 days after the death. The principle of the "trap" device is based on the hydrotaxis of nematodes. One of its variants is that half of the Petri dish was placed upside down in a larger ves-

sel filled with saline solution (6.5 g NaCl L^{-1} of distilled water) at the level of half the height of the Petri dish. Dead larvae were laid out on the surface of the dish. The trap was covered with a lid, moisture builds up inside and the nematodes slide down the wet glass into the saline solution. Migration of invasive larvae usually begins 10-12 days after the death of the infected insects. Migrating nematodes, together with the liquid, were poured into a collection vessel and stored in a refrigerator at 2-5 °C for one year.

Laboratory testing of entomopathogenic nematodes

Adults and nymphs of *N. viridula* were treated with invasive EPN larvae at a dose of 50, 75, and 100 individualsper insect. In each experimental option, 150 individuals were tested. Food for bedbugs (germinated mung bean seeds) was placed in Petri dishes on wet cotton swabs. The death of insects was determined after 1, 3 and 7 days. In the control, adult bugs were treated with distilled water.

Statistical analyses

To test the significance of mortality from nematodes *S. carpocapsae* and *S. feltiae* one-way analysis of variance (ANOVA) was applied using SAS software (vers. 9.4, SAS Institute, Cary, NC, USA, 2018). Mortality for each variant was adjusted for control replicates using Abbot's formula (Abbot, 1925). The experiment was organized according to a randomized design with a factorial treatment consisting of two nematode species and three application rates.

Results and discussion

Table 1 indicates that, when bug adults were treated, the maximum mortality of insects (91.4%) was observed after 10 days when using the nematode *S. feltiae* (F = 15.13, df = 2, p < 0.05) at the lowest infection dose of 50 individuals infective larvae per individual. When treating nymphs of IV age, the greatest death of insects (98.0%) was observed after 10 days also when using the nematode *S. feltiae* (F = 25.13, df = 2, p < 0.05) at an infection dose of 75 individuals infective larvae per individual.

Three days after treatment, the largest percentage (76.2%) of dead individuals was noted when using the species *S. carpocapsae* (F = 2.67, df = 2, p < 0.05) when treating nymphs at an infection dose of 100 invasive larvae per individual, when adults were treated, the percentage of deaths from this EPN species was also higher and amounted up to 55.6% (F = 3.96, df = 2, p < 0.05). Seven

days after treatment, except for the variant with the use of *S. feltiae* against nymphs at a dose of 75 invasive larvae per individual, where the percentage of death was 95.3% (F = 12.79, df = 2, p < 0.05). The total death of insects on the tenth day was higher in the species *S. feltiae*, both for adults and nymphs (Table 1). For nymphs (Fig. 1a), when using *S. feltiae* the Fisher criterion was F = 20.23, F = 33.17, F = 15.13, at doses of 50, 75, and 100 individuals per insect, respectively, df = 2, p < 0.05); while for *S. carpocapsae* these differences were not so significant (F = 2.67, F = 4.29, F = 22.30, df = 2, p < 0.05). The same regularity was noted when processing *N. viridula* adults (Fig. 1b), when using *S. feltiae* the Fisher criterion was F = 11.76, F = 12.79, F = 25.13 respectively, df = 2, p < 0.05; when using *S. carpocapsae*: F = 3.96, F = 8.67, F = 18.45, df = 2, p < 0.05. It can be concluded that the stage of development of the insect used in the experiments, the species of nematode used, and the dose of infection had the greatest influence on the death of insects.

S. feltiae nematodes were isolated: 50.00 ± 1.27 individuals of dead nymphs and 56.50 ± 1.04 individuals of green stink bug adults. This circumstance is the evidence that the death of insects in the experiment took place solely due to the action of the pathogen, and not caused by random factors. At the same time, the outcome of *S. carpocapsae* was observed from 19.73 ± 3.07 individuals of dead nymphs and 55.87 ± 1.85 individuals of green stink bug adults. The number of invasive larvae isolated from one individual of *N. viridula* increased depending on the age of the insects used and was higher in treated adults than in larvae.

As evidenced by the data presented in this work, as well as in articles by other researchers (Pervez et al., 2014; Guide et al., 2016; Burjanadze et al., 2020; Pushnya et al., 2020), EPN are promising agents for inclusion in biological control systems against harmful members of the Pentatomidae family (*N. viridula, H. halys, D. melacanthus*, etc.).

In general, studies show that the overall mortality of insects (adults and larvae) was higher in the nematode *S. felliae* than in *S. carpocapsae*. This is also reported in several pest species, e.g., in adults of *Tribolium confusum* (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae). When treating the EPN pest of corn *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) and *Dysmicoccus* sp. (Hemiptera: Pseudococcidae), the best results were found in the species *S. carpocapsae* and *Heterorhabditis bacteriophora* (Robert et al., 2013; Guide et al., 2016). Our *S. feltiae* strain has properties as a biological pest control agent: high biotic potential and relatively short life cycle for EPN, which allows several generations to develop in short periods of time (Rahoo et al., 2017). The issue of application of *H. bacteriophora* against *N. viridula* is quite controversial, since in earlier studies (Pushnya et al., 2020), the pathogen isolation from dead individuals infected with this species of invasive larvae wasn't observed.

When studying the use of different age stages of the bug, we considered that there was evidence of the absence of EPN effects on adults of a number of pests (Guide et al., 2016; Rahoo et al., 2017; Javed et al., 2020); however, in our studies, both adults and bug nymphs were susceptible to infection, although the percentage of death of insects was higher in nymphs.

Nematodes caused the death of insects at all used doses of infection. Some authors, such as Guide et al. (2016), consider that the higher the dose of invasive larvae used for infection, the higher the insect mortality. According to Rahoo et al. (2017), the likelihood of EPN infection may be reduced due to the number of nematodes applied, since higher concentrations increase the likelihood of infection and, as a result, a higher mortality rate. However, it was found that at doses of 50, 75 and 100 individuals of invasive larvae per insect, the mortality of *N. viridula* was similar in all variants. According to Selvan et al. (1993) and Gaugler et al. (1994) a minimum number of pathogens is required to reduce insect immune responses, providing conditions for the development of nematodes. However, when this amount is significantly exceeded (at very high EPN concentrations), intraspecific competition may develop, preventing the effective reproduction of nematodes (Guide et al., 2016).

In conclusion, our laboratory tests of entomopathogenic nematodes *S. carpocapsae* and *S. feltiae* against adults and nymphs of the green stink bug *N. viridula* showed that the used pathogens caused the death of up to 98.0% of nymphs and up to 91.4% of adults. Higher indicators of insect death were found for the species *S. feltiae*, as it has several properties that allow the pathogen to develop in shorter periods of time. In general, EPN can be considered as promising bioagents in plant protection against *N. viridula*; however, extensive field screening is required to confirm this hypothesis.

Authors' contributions

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Writing - Review & Editing: M. V. Pushnya, E. Y. Rodionova, I. V. Balakhnina

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