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Local Entomopathogenic Nematodes (Steinernematidae and Heterorhabditidae) Against the *Cydalima perspectalis* (Lepidoptera: Crambidae) in Georgia

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ABSTRACT

Backround and Objective: *Cydalima perspectalis* is a major invasive species of *Buxus colchica* common in Georgia. It was decided to investigate the mortality of *C. perspectalis* using local entomopathogenic nematodes (EPN). **Materials and Methods:**To study the efficiency of local EPNs, was invaded the pest with the above mentioned nematodes and studied their effect on them. Use of identified effective species in the field experiments. These species of nematodes were first tested on *C. perspectalis*. The studies were carried out both in the laboratory as well in field conditions. **Results:** In the laboratory experiments, two different doses of a suspension of nematodes were used: 50 and 100 IJs/against of these five species, the highest insect mortality was due to the action of *Heterorhabditis* sp. (100%), *Steinernema gurgistana* (100%) and *Steinernema thesami* (97.4%). These three highly virulent species were used in the field trials at doses of 1000 and 2000 Ijs L^{-1} water. In this case, *Heterorhabditis* sp. was less effective (56.5%) than *S. thesami* (85.5%) and *S. gurgistana* (78.1%). **Conclusion:** Studies showed that the local species of these nematodes used in the experiments (*S. thesami* and *S. gurgistana*) are characterized by the high pathogenicity of this pest.

KEYWORDS

Buxus tree, *Cydalima perspectalis*, entomopathogenic nematodes, *Heterorhabditis*, *Steinernema*

INTRODUCTION

Cydalima perspectalis (Walker, 1859) is an invasive pest of boxwood. The larvae stage of this insect is harmful to boxwood and eats only leaves and young shoots, sometimes the bark of branches. As a result, boxwood is defoliated and dried[1,2]. Thus, *C. perspectalis* is a serious threat to boxwood both in the wild[3,4] and growing in ornamental and greenhouses in urban areas[5,6].

In Georgia, *C. perspectalis* was first observed in the Imereti lowland in 2012[2] and since 2015 it has spread to the Western and Eastern parts of Georgia. In a temperate climate, the pest gives two to three generations per year. The harm of *C. perspectalis* (along with the fungus that causes boxwood burns) led to the disappearance of the relict and endemic form of *Buxus colchica* Pojark[7]. It is included in the IUCN Red List of Georgia. The main causes of dehydration are identified: The disease the so-called

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'boxwood burn' (caused by the fungus *Calonectria pseudonaviculata*, *Cylindrocladium buxicola*)[8] and the harmful insect *C. perspectalis*, which causes significant damage both in Europe[4] and in the Caucasus and Georgia, remaining boxwood plants[7,9].

Currently, for biological control of forest and agricultural pests are mainly used entomopathogenic microorganisms such as nematodes, bacteria, fungi, protozoa and viruses[10]. Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae are effective and safe biocontrol agents for controlling both soil and above the ground a wide range of harmful insects[11]. The main interest in EPNs as biological control agents are related to their ability to search actively their host insects, where they can cause rapid host mortality within 24-48 hrs[12,13]. Nematodes kill their hosts with bacteria found in the nematode digestive tract[14]. The EPNs can effectively control several Lepidoptera species[15] but the use of the most suitable nematode with the target host is an important component for the success of any biocontrol program.

The main goal of this study was to investigate the potential of EPNs to control *C. perspectalis* larvae in laboratory conditions; compare virulence levels between five local species of nematodes to choose the most virulent species of them and optimal concentrations for nematode suspension; test the efficacy of them against this pest insect in the field trials.

MATERIALS AND METHODS

Experimental material worms of *C. perspectalis* in the third age were collected in the region of Eastern Georgia, from the cultivated plots of boxes from the private sector. Insects were collected in special 17×24 cm plastic containers. After labelling, the containers were delivered to the laboratory for further research. The material was collected from May to June of 2021 and 2022.

The reproduction of infective juveniles of experimental EPN occurred on *G. mellonella*[16] and on *Bombyx mori*[17]. Infectious larvae were collected at room temperature (24°C) according to the method of White[18]. In the experiments local isolates of EPN were used (*S. tbilisiense*, *S. thesami*, *S. borjomiense*, *S. gurgistana* and *Heterorhabditis* sp.), which were collected in previous local studies and they are currently stored in the collection fund of the Institute of Zoology of Ilia State University. The origin of the various species isolates used in the study is shown in Table 1.

Before the experiment, for testing nematodes of each species for insects prepared Petri dishes of a diameter of 90 mm for four experimental and one control. In each experimental Petri dishes there have put filter paper on ten instances of the third age of the *C. perspectalis* worm and several fresh boxwood leaves for feeding the worms.

To infect experimental insects, two different concentrations of the nematode suspension were selected: 50 and 100 infective juvenile/insects. While control insects were treated only with distilled water[19]. The experiments were carried out in a laboratory at a temperature of 22-24°C and a relative humidity of

Table 1: Steinernematidae and Heterorhabditidae isolate, locality, habitat and GenBank number used in the study

Species	Isolate	Origin	Habitat	GenBank
*Steinernema tbilisiensis**	Geo-02	Tbilisi, Georgia	Oak forest	KC287225
Steinernema thesami	Geo-10	Tbilisi, Georgia	Alder forest	LT984410
Steinernema borjomiense	Geo-369	Borjomi, Georgia	Spruce forest	LT900493
Steinernema gurgistana	Geo	Tskhneti, Georgia	Oak forest	n/a
Heterorhabditis sp.	Geo	Tsalka, Georgia	Oak forest	n/a

*Gorgadze[20-22] and n/a: Not applicable

68-70%. The mortality of insect pests was recorded by the Abbott[23] formula 24, 48, 72 and 96 hrs after infection:

$$X\ (\%) = \frac{D_i - D_c}{100 - D_c} \times 100$$

Where:

D_i = Percentage of dead individuals in the experiment
D_c = Percentage of dead individuals in the control
X = Insect mortality in percentage

Microscale field experiments were conducted in 2021-2022 on boxwood from the private sector in Eastern Georgia. For the experiments selected boxwood, which was infected with the 3rd and 4th age *C. perspectalis* larvae. Three experimental and one control bushes were selected for testing each species of nematodes. The area of each bush ranged from 2.5 to 3 m^2. The number of *C. perspectalis* larvae per meter/square of each bush ranged from 35 to 70. Field experiments were carried out with three species of EPN (*S. thesami*, *S. gurgistana* and *Heterorhabditis* sp.). Two high concentrations of nematode suspensions were used in the experiments: 1000 and 2000 IJs mL^{-1} water. Suspensions of nematodes were sprayed onto experimental boxwood bushes by manual spraying, while the control was treated with distilled water.

After spraying one square centimeter leaves of boxwood, 20-35 infective juveniles were found. The experiments were carried out mainly in the first and second decades of May at temperatures of 18-26°C and 75-80% relative humidity, in the evening in cloudy weather. The death of *C. perspectalis* worms was observed on days three and five after the start of the experiment. The final accounting was performed on the 7th day according to the method of Ghavamabad *et al.*[24]:

$$\text{Mortality}\ (\%) = \frac{100}{I} - \frac{A \times C}{B \times D}$$

Where:

A = Number of live individuals after treatment in the experiment
B = Number of live individuals before processing in the experiment
C = Number of live individuals before treatment in the control
D = Number of live individuals after treatment in the control

Cydalima perspectalis, killed worms were investigated on nematodes in a laboratory under a stereo microscope. Biological Microscope, MSC-B208(Sliding) Bioevopeak Co., Ltd., Jinan, Shandong, China.

Statistical analysis: Statistical analysis[23] showed that there are no statistically significant differences between the EPNs species in terms of the effect of nematodes on pest mortality, however, it should be noted that the effectiveness of nematodes does differ depending on the species of EPNs, the concentration of the nematode suspension and $p>0.05$.

RESULTS

Laboratory experiments showed that, during the concentration of a suspension of nematodes (50 Ijs/insect), the mortality of *Cydalima percpectalis* worms varied from 34 to 80%. Nematodes of all five species of EPN (*S. thesami*, *S. borjomiense*, *S. tbilisiense*, *S. gurgistana* and *Heterorhabditis* sp.), showed results above average (>50%) insect death. Among them, the highest mortality rate (76.3%) was obtained by the action of *Heterorhabditis* sp. (Fig. 1) compared with *S. thesami*, the last one is somewhat less effective.

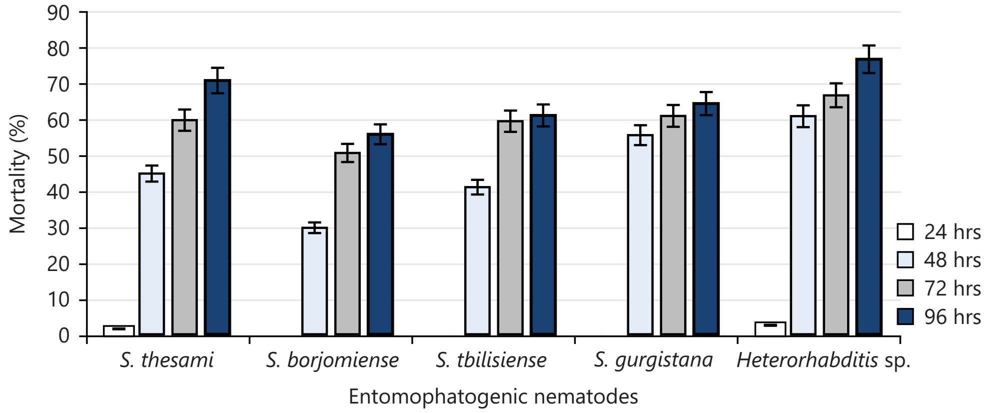

Fig. 1: Laboratory bioanalysis showing percentage mortality (mean+standard error) at the third age of the larvae of *Cydalima perspectalis* inoculated with 50 IJs individuals of various EPN nematode isolates by the hours

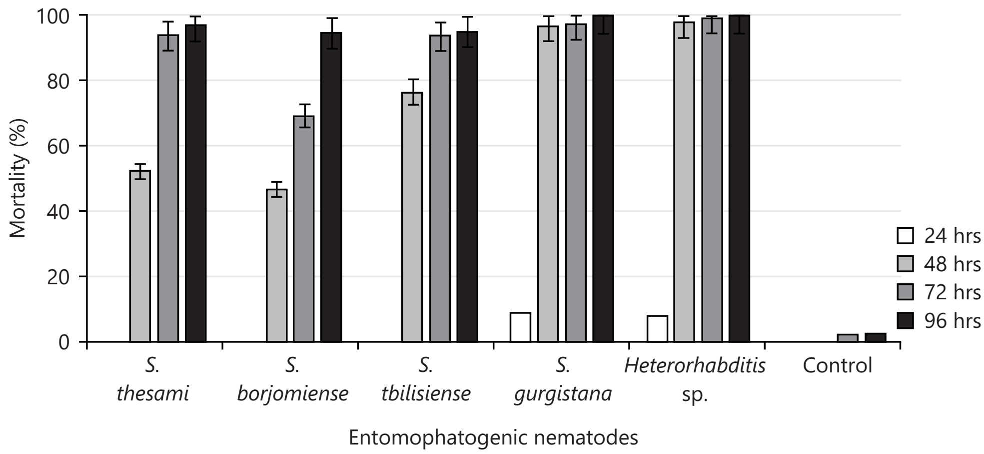

Fig. 2: Laboratory bioanalysis showing percentage mortality (mean+standard error) at the third age of the larvae of *Cydalima perspectalis* inoculated with 100 IJs individuals of various EPN isolates by the hours

Regarding the results obtained using *S. borjomiense*, *S. tbilisiense* and *S. gurgistana*, compared *S. thesami* and *Heterorhabditis* sp., slight differences were observed (4-5%). When using a high (100 IJs/insect) dose of a nematode suspension, a high percentage of insect mortality was observed (minimum 94.7% and maximum 100%) (Fig. 2).

Infective juveniles of *S. gurgistana* and *Heterorhabditis* sp. (Fig. 3a-b) caused 100% insect death (within 96 hrs). The next high result (97.4%) was shown by *S. thesami* nematodes. *Steinernema borjomiense* (94.7%) and *S. tbilisiense* (95.6%) also showed similar and high results. It should be noted that between the local species of nematodes used in laboratory experiments, according to the results, there was no sharp difference. Consequently, the most effective nematode species, such as *S. thesami*, *S. gurgistana* and *Heterorhabditis* sp., were selected for field trials against *C. percpectalis*.

Analyzing the results of field trials, a relatively high dose of nematodes (2000 Ijs mL^{-1}) caused high mortality of *C. percpectalis*. From the nematodes used in field experiments, *S. thesami* is the most virulent (85.5±4.7%) against harmful insects (Fig. 4). Slightly lower results (78.1±3.7%) were observed with *S. gurgistan*. *Heterorhabditis* sp., among these three species of nematodes used in field experiments, caused the lowest mortality rate (56.4±4.5%) of insects.

Fig. 3(a-b): Worms *Cydalima perspectalis* infection with infectious larvae of (a) *Steinernema gurgistana* and (b) *Heterorhabditis* sp.

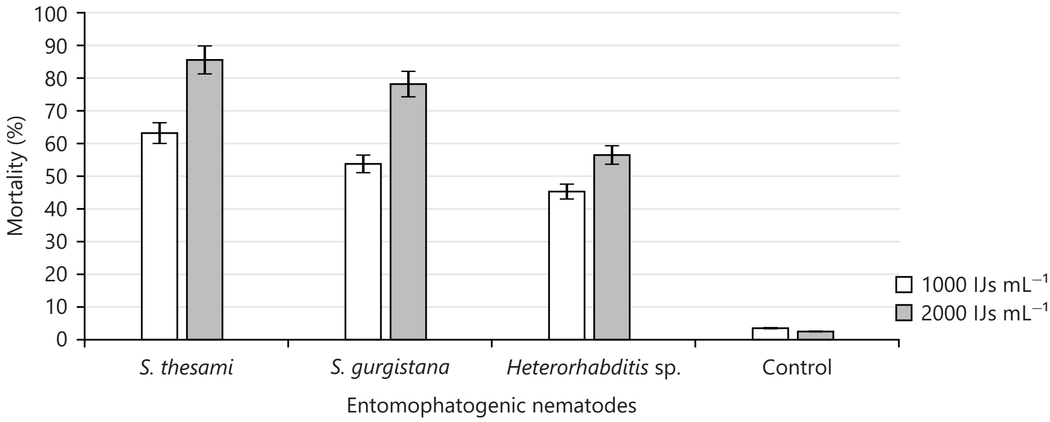

Fig. 4: Bioeffectiveness of two concentrations (1000 and 2000 IJs mL^{-1}) of three types of EPN (*S. thesami*, *S. gurgistana* and *Heterorhabditis* sp.) against 3rd, 4rd instar *Cydalima perspectalis* larvae in the field trials

DISCUSSION

Studies show that only limited species of entomopathogenic nematodes cause *C. perspectalis* mortality, among them the most promising nematode species were *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. As already noted in the literature[25], only a limited species of entomopathogenic nematodes can affect *C. perspectalis*, among them, as in this case, the most promising nematode species were *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. Based on the results, it can be concluded that *Heterorhabditis* sp., in the laboratory experiments, is characterized by high pathogenicity against *C. percpectalis*; however, in the field experiments it is less effective than *S. thesami* and *S. gurgistana*. It should be noted that the less efficiency *Heterorhabditis* sp., in field experiments should be due to its cruise lifestyle.

In laboratory experiments with two different doses (50 and 100 IJs/insect), the highest mortality of *C. perspectalis* was shown in *Heterorhabditis* sp. (76.3-100%), followed by *S. gurgistana* (64.0-100%) and *S. thesami* (70.4-97.4%); *S. tbilisiense* killed 60.7-97.6% of insects and *S. borjomiense* - 55.5-94.7%. Using *S. borjomiense* against *C. perspectalis*[25] with concentrations of EPNs from 500 to 300 IJ/1insect, the mortality of the larvae was 100%. The third and fourth-generation of Iranian isolates *S. borjomiense* used against *C. perspectalis* showed a mortality rate of 70 and 65%, respectively[25].

In experiments against *C. perspectalis* at concentrations of 25-200 EPN by Göttig and Herz[15] (2018) obtained good results when using *S. feltiae* (46-100%) and *S. carpocapsae* (85-100%) and low (8-15%)

using *H. bacteriophora*. The opposite is the other author's[1]; he notes that in experiments against Japanese worms *C. perspectalis* (10 to 80 EPN versus 1 worm in the range), experiments using *H. bacteriophora* and *S. carpocapsae* resulted in equally high mortality (100%). Compared with young larvae, infection of the larvae, which received further development, occurred faster, after three days.

It is known that the size of insects, as well as the size of nematodes, can affect the potential of EPN species to penetrate into potential host species because they most often penetrate through openings in the body. Further developed larvae have large openings or probably fed more leaves or had greater contact with the surface[26]. According to Göttig and Herz[15] in field experiments using application of nematodes *S. carpocapsae* suspensions (10-200 EPN/mL), demonstreited about 10-75% mortality of *C. perspectalis* 2nd larval instars and 45-100% mortality of 4th larval instars was obtained; the mortality rate in the field and the successful penetration of nematodes into host species are closely related to weather conditions, application technique and easy host access to plant foliage, unlike application in the soil. Factors such as ultraviolet radiation and temperature, as well as relative humidity, play a decisive role in the use of leaves and the preservation of EPN in the field[27]. Many benefits also relate to EPN. They are partially able to actively search for their master, they are practically harmless to target organisms that are easily produced in droves, have a wide range of hosts and can quickly kill their hosts[14]. The use of EPN in biological pest control is very suitable for small scale control of boxwood plantations widely used worldwide as an ornamental shrub, in Georgia it is also used as a spice. Therefore, the fight against its pests is very significant.

Successful control of *C. perspectalis* with EPN depends on abiotic factors, like moisture and temperature, of the pest stage and the species of EPNs. The motility of nematodes and the likelihood of their success can be further increased by selecting highly mobile species for biocontrol, such as cruisers of the genus Steinernematidae. They are partially able to actively search for their hosts-insects. They are practically harmless to target organisms that are easily produced in droves, have a wide range of hosts and are able to quickly kill their hosts[28].

The results of providing research study showed that the local isolates of entomopathogenic nematodes used in the experiments (*S. thesami*, *S. borjomiense*, *S. tbilisiense*, *S. gurgistana* and *Heterorhabditis* sp.) are characterized by the high pathogenicity of *C. perspectalis*. High pathogenicity of EPNs is also due to the ability to actively feed *C. perspectalis* larvae.

As a consequence of the research and the results obtained, it is possible to recommend natural remedies for the prevention of *Buxus colchica* Pojark pests such as EPNs *Heterorhabditis* sp., *S. gurgistana* and *S. thesami*. The pathogenicity of which in field conditions is quite high and using a suspension with a concentration of 2000 μg mL^{-1}, larval mortality averaged 62.5%.

CONCLUSION

Studies have shown that using nematode suspension of all five EPNs (*S. thesami*, *S. borjomiense*, *S. tbilisiense*, *S. gurgistana* and *Heterorhabditis* sp.) with a concentration of (100 Ijs/insect) in laboratory conditions, has reached a high percentage of larval mortality *C. percpectalis* from 94-100%. While in the field, when using the same suspension, but with a concentration of 2000 IJs mL^{-1}, the mortality rate of larvae averaged 62.5%. The conducted studies made it possible to identify more effective species of nematodes that cause the death of *C. perspectalis*, in contrast to the known experiments. Further field studies are needed to improve results and to commercialize more effective species of EPNs.

SIGNIFICANCE STATEMENT

The monophagous pest-insect *Cydalima perspectalis* is a highly invasive species of boxwood. In Georgia, it was first observed in the Imereti Lowland in 2012, since 2015 it has spread to the Western and Eastern parts of Georgia. In a temperate climate, the pest gives two to three generations per year. The main reason for the boxwood decrease was identified as the disease so-called ‘boxwood burn’ and the pest-insect *C. perspectalis*, which causes significant damage in Europe, Caucasus and Georgia. Studies of local EPNs’ effectiveness against *C. perspectalis*, showed that the highest insect mortality was affected by the *Heterorhabditis* sp., *S. gurgistana* and *S. thesami*. Studies showed that the local EPNs used in the experiments are characterized by the high pathogenicity of this pest.

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