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Parasitology

Effects of Entomopathogenic Nematodes *Steinernema tbilisiensis*, *Steinernema thesami* and *Heterorhabditis bacteriophora* with Combination of Bacterial Insecticides - Bitoxybacillin (Btb) and *Bacillus thuringiensis* (Bt) against *Lymantria dispar* (L.) and *Operophtera brumata* (L.)

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(Presented by Academy Member Irakli Eliava)

This study investigated the Biological control efficacy of two isolates of the native entomopathogenic nematodes (EPN) species *Steinernema tbilisiensis, Steinernema thesami* as well as *Heterorhabditis bacteriophora* against pest insects - *L. dispar* and *O. brumata.* During field experiments small, young oak trees were chosen. Soil analysis for field was conducted and data of soil temperature and moisture during the study were recorded: Humidity - 1.3, pH 6.8, Organic matter % - 2.65, Sand - 19.4, Silt - 20.4, Clay - 23.3. The experiments were conducted in laboratory and in field conditions. For the increase mortality of insects by used only entomopathogenic nematodes, there were added combination with bacterial insecticides - Bitoxybacillin (Btb) and *Bacillus thuringiensis* (Bt). The concentration of three species of EPNs, used for laboratory conduction has been 500-1000 IJs/ml separately and added bacterial insecticides - Bitoxybacillin (Btb) (1000 + 0.3%) and *Bacillus thuringiensis* (Bt) (1000 + 0.3 - %). The corrected mortality ranges per larvae of *L. dispar* and *O. brumata* was 72.1 - 68.5% from *S. tbilisiensis*; 65.5 - 57.6% ranged from *S. thesami,* while mortality range of *H. bacteriophora* was 58.1- 47,1%. All nematode species showed statistically more mortality than control. Also for field experiments, were used concentration of EPNs 1500-3000 with combining of Bitoxybacillin (Btb 0.5%) and *Bacillus thuringiensis* (Bt) (0.5%). No significant was observed between *S. thesami* (36.4 - 32.2%; 30.4- 28.0%) and *H. bacteriophora* (30.1-28.5%; 26.5-20.1%) by combining EPNs + Btb and Bt 0.5%, P>0.05. Significant difference was observed between *S. tbilisiensis* (47.2-53.1%; 49.0-50.1%) and *H. bacteriophora* (30.1-28.5%; 26.5-20.1%) P < 0.05. Insect mortality was checked every 3, 5 and 7 days and the presence of nematodes inside the insects served as the indicator of nematode infection. Preceding from the results of the experiments presented in this paper we can conclude that the maximum effectiveness of the nematode suspension considerably increases in case of using them in combination with bacterial insecticides against pest insects *L. dispar* and *O. brumata.*

Entomopathogenic nematodes, *Lymantria dispar*, *Operophtera brumata*, Bitoxybacillin, *Bacillus thuringiensis*

Biological control with entomopathogenic nematodes (EPNs) can provide a good control of pests and reduce the population of pest insects. Phytophagous insects occupy a significant position among the living organisms that cause much damage to cultivated plants and forest species. Nowadays, the use of entomopathogenic - EPNs as a biological control agent is a key component in IPM (Integrate pest management).

Bitoxybacillin (Btb) has been widely used for a long period of time in forestry and agriculture all over the world. The active components of the (Btb) are beta-exotoxin and crystal endotoxin. It is highly effective against Colorado beetle, boll worm, winter moth larvae, *L. dispar, O. brumata* etc. [1]. *Bacillus thuringiensis* (Bt) is a naturally occurring gram-positive, spore forming, facultative aerobic, rod-like, motile and soil bacterium that is found worldwide. *B. thuringiensis* var. kurstaki has been used to effectively control numerous defoliators in forests [2].

Two native species of entomopathogenic nematodes - S. *tbilisiensis*, *S. thesami* and *H. bacteriophora* Poinar (Heterorhabditidae: Rhabditida) [3] were carried out to evaluate the biological control potential of entomopathogens against harmful insect's gypsy moth, *Lymantria dispar* (L.) & winter moth, *Operophtera brumata* (L.) etc [4,5]. The European gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) is native to temperate forests in western Europe [6]. In Georgia this moth is a serious forest pest capable of causing severe damage to hardwood trees, especially oaks, as the gypsy moth larvae defoliate entire stands of trees. The winter moth, *Operophtera brumata* (L.) (Lepidoptera: Geometridae) is a moth of the family Geometridae as well in Georgia, for evaluating insect population dynamics [7].

The aim of our investigation was to estimate last instar of two insect's *Lymanrtia dispar* L. and *Operophtera brumata* L. by usage of EPNs as well as combination with bacterial insecticides *Bitoxibacilline* (Btb) and *Bacillus thuringiensis* (Bt) in laboratory and field conditions.

Materials and Methods

Experimental insect. Starting in the middle of May to the end of early July the last instar larvae of *L. dispar* and *O. brumata* were collected every morning and evening in Lagodekhi region. For

Table 1. The amount of the larvae of pest insects on the plot within the period before using EPNs and bacterial insecticides. Within the pre-treatment period, an average number of larvae of both insects within 1 m² experimental site was the following:

Sites and plots	Used EPNs and Bact - insecticides	*L. dispar*	*O. brumata*
Site №1, Test plot #1	*S. tbilisiensis* -1500 IJs/1 ml	56.27	89.53
Site №1, Test plot #2	*S. tbilisiensis* -3000 IJs/1 ml	76.67	79.71
Site №1, Test plot #3	*S. tbilisiensis* 3000 IJs + (Btb) 0.5%	117.06	115.72
Site №1, Test plot #4	*S. tbilisiensis* 3000 IJs + Thur. (Bt) 0.5%	88.45	99.64
Control	Water		
Site №2, Test plot #1	*S. thesami* 1500 IJs/1ml	62.05	91.31
Site №2, Test plot #2	*S. thesami* -3000 IJs/1ml	64.17	65.38
Site №2, Test plot #3	*S. thesami* -3000 IJs + (Btb) 0.5%	100.64	98.31
Site №2, Test plot #4	*S. thesami* -3000 IJs +Thur. (Bt) 0.5%	76	86.33
Control	Water		
Site №2, Test plot #1	*H. bacteriophora* - 1500 IJs/1 ml	70.8	118.61
Site №2, Test plot #2	*H. bacteriophora* – 3000 IJs/1 ml	75.51	108.68
Site №2, Test plot #3	*H. bacteriophora* - 3000 IJs + (Btb) 0.5%	65.96	49.67
Site №2, Test plot #4	*H. bacteriophora* - 3000 IJs +Thur. (Bt) 0.5%	75.57	78.28
Control	Water		

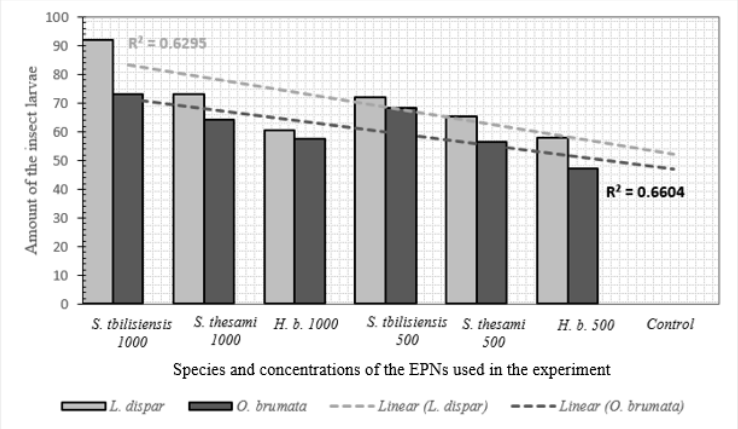

Fig. 1. Correlation between the effect of invasion of the insect species (*L. dispar, O. burnata*) by EPNs indicated in particular species (*S. tbilisiensis, S. thesami* and *H. bacteriophora*) and their concentration in the suspension used in the experiment.

experiments small, young oak trees were chosen for experiment. Soil analysis for field was conducted and data of soil temperature and moisture during the study were recorded. Humidity - 1.3, pH 6.8, Organic matter % - 2.65, Sand - 19.4, Silt - 20.4, Clay - 23.3. The last instar larvae of *L. dispar* and *O. brumata* were collected in special begs and transferred to the laboratory.

Production of EPN and bacterial insecticides. Production of EPN and bacterial insecticides. The native nematodes *S. tbilisiensis, S. thesami* as well as *H. bacteriophora* were reared at 25°C at last instar of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleridae), following the method of [8].

The infective juveniles (IJs) that emerged from cadavers of *G. mellonella* were recovered using modified White traps [9], and stored at 7°C for 7-14 days before use [10]. IJ were used in experiments for two weeks after emergence from dead insects. For the control of *O. brumata* and *L. dispar* was used entomopathogenic nematodes *S. tbilisiensis, S. thesami* and *H. bacteriophora* with concentration 1500 -3000 IJs/ml and solution of bacterial insecticides Bitoxibacilline (Btb) and Bacillus thuringiensis (Bt) 0.3-0.5%.

Bioassay. For laboratory experiment ten last instars of both insects - L. dispar and O. bumata were separately placed onto a wet filter paper in a 10 x10 cm² diameter Petri dishes. Suspension of 500 and 1000 IJs infective juvenile's ml-1 (dose of 50 and 100 IJs per insect) respectively, were treated in each petri dish. For the increase efficacy of entomopathogenic nematodes on the target insects, combination of EPNs with bacterial preparations Btb - 0.3 and Thuringin-2 - 0.3 were used. Insect mortality was checked every 3, 5 and 7 days and the presence of nematodes inside the insects served as the indicator of nematode infection. Each infected insect cadaver was then placed on separate white traps and incubated at 25°C until the emergence of a new generation of IJs. Nematode reproduction potential was evaluated in dead insects. The emerging IJs were harvested and counted after 11 to 18 days, until emergence ceased or was considered negligible. Total number of IJs produced per host insect was then determined. Emerging IJs from the insect cadaver were kept at 10°C and were used within one week after emergence. Death of insects sprayed with entomopathogenic nematode suspension was directly dependent on virulence of symbiotic bacteria.

Table 2. Efficacy of EPNs and their combination with bacterial preparations in devastation of the pest insects (*L. dispar* and *O. brumata*).

Microbial pesticides – *EPNs :* S. *tbilisiensis, S. thesami, H. bacteriophora* & Bacterial pesticides : (Btb) and *Turingin* (Bt)	*Lymantria dispar*			*Operophtera brumata*		
	Before treatment	After treament	Effect %	Before treatment	After treament	Effect %
I expérimental plot						
S. tbilisiensis -1500 IJs/1 ml	56.27	42.08	25.2	89.53	71.44	20.2
S. tbilisiensis -3000 IJs/1 ml	76.67	45.7	40.3	79.71	51.57	35.3
S. tbilisiensis 3000 IJs + (Btb) 0.5%	117.06	61.77	47.2	115.72	59.02	49.0
S. tbilisiensis 3000 IJs +*Thur*.0.5% (Bt)	88.45	41.57	53.1	99.64	49.82	50.1
Control (water)	(0.00)	(0.00)		(0.00)	(0.00)	
II experimental plot						
S. thesami 1500 IJs/1ml	62.05	50.57	18.5	91.31	77.06	15.6
S. thesami -3000 IJs/1ml	64.17	48.64	24.2	65.38	51.52	21.2
S. thesami 3000 IJs + (Btb) 0.5%	100.64	64.0	36.4	98.31	68.42	30.4
S. thesami 3000 IJs +*Thur.* 0.5% (Bt)	76	51.5	32.2	86.33	62.15	28.0
Control (water)	(0.00)	(0.00)		(0.00)		
III experimental plot						
H. bacteriophora - 1500 IJs/1 ml	70.8	60.10	15.1	118.61	104.7	11.7
H. bacteriophora - 3000 IJs/1 ml	75.51	58.37	22.7	108.68	88.9	18.2
H. bacteriophora -3000 IJs + (Btb) 0.5%	65.96	46.1	30.1	49.67	36.50	26.5
H. bacteriophira-3000 IJs +*Thur.* 0.5% (Bt)	75.57	54.03	28.5	78.28	62.54	20.1
Control (water)	(0.00)	(0.00)		(0.00)	(0.00)	

For field experiments two plots with the size of 225 m² (450 m² in total), separately for *L. dispar* and *O. brumata* were allocated and set up for field experiments in the habitat of the degraded deciduous forest where Georgian oak trees dominate. Each plot consisted of fifteen sections - 15 m² each. The average number of the individuals per section - 5 pcs. The trees were young, three years of middle age. Each plot included four experimental tests and one control plot. Suspension of three species of EPNs – *S. tbilisiensis, S. thesami* and *H. bacteriophora* with the concentration of 1500-3000 IJs/ml separately as well as with combining bacterial preparations - Bitoxibacilline (Btb) (3000 IJ+ 0.5%) and *B. thuringiensis* (Bt) (3000 IJ +0.5%) were used against last instar *L. dispar* and *O. brumata* as biological control agents. The degree of infestation in all experimental plot trials was assessed in 5, 7, 10 days. Spray with the determined concentration of entomopathogens was used on the branches rather than the entire trees.

Statistical analysis. In statistical analysis, One-way ANOVA was used to compare the sites in variation of the number of the parasitic insects before and after use of insecticides. Means were compared at the P=0.05 level. Effect of the invasion of insects by EPNs and bacterial insecticides were calculated using Pearson correlation coefficient and the coefficient of the correlation of polynomial regression. Analysis of the laboratorial and field collected statistical data was done using software SPSS v. 21 [11–14].

Results and Discussion

Laboratory experiments. The mortality caused by *S. tbilisiensis* was more pathogenic against insects compared to *S. thesami* and *H. bacteriophora* depending on the low and high concentration. At the low 500 IJ concentration, no significant difference was observed between *S. thesami* and *H. bacteriophora*. However, percentage mortality was significantly different between *S. tbilisiensis* and *H. bacteriophora*.

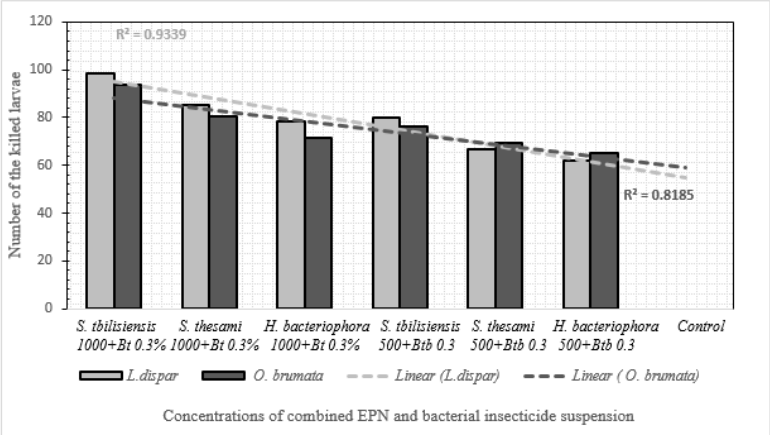

Fig. 2. The effect of the Combination of EPNs and bacterial preparations on the mortality of *L. dispar* and *O. brumata* in Laboratory conditions.

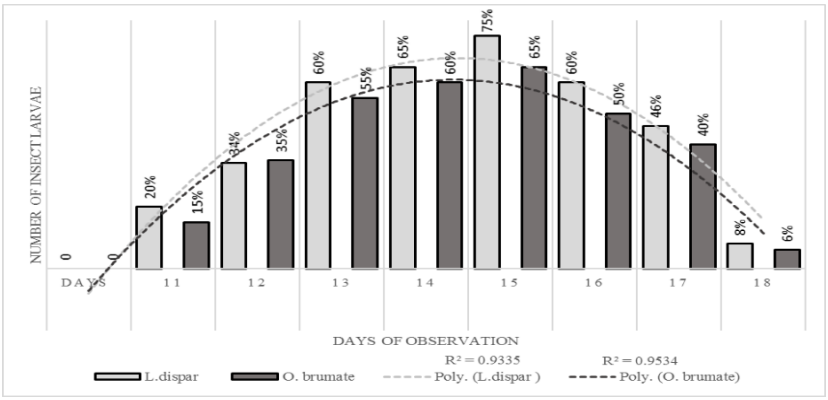

Fig. 3. Variability of average number of larvae affected by emerging IJs from cadaver. Figure shows the change in the number of insect larvae during the period of observation affected by IJs from cadaver.

The corrected mortality ranges per larvae of *L. dispar* and *O. brumata* was 72.1 - 68.5% from *S. tbilisiensis*; 65.5 - 57.6% ranged from *S. thesami*, while mortality range of *H. bacteriophora* was 58.1- 47,1%. All nematode species showed statistically more mortality than control. The mortality increased with the application of a higher nematode concentration - 1000 IJs. The corrected mortality range of larvae of *L. dispar* and *O. brumata* was 92.2 – 72.3%, from *S. tbilisiensis*; 73.3- 64.3 ranged from *S. thesami*, while mortality from *H. bacteriophora* was 60.1 - 56.5%. All nematode species showed statistically more mortality than control (Fig. 1). As the results of the laboratory study show the most effective EPN in control of the populations of *L. dispar* and *O. brumata* was *S. tbilisiensis*, medium level of strength shows the use of *S. thesami* and weaker strength the use of *H. bacteriophora*. The use of EPNs is more effective against *L. dispar* than *O. brumata*. There is moderate strength of the linear (Pearson) correlation between the effect of the concentration of EPNs in the suspension used in experiment and the number of larvae of *L. dispar* (R^2=0.63; P<0.05) and *O. brumata* (R^2=0.66; P<0.05). Higher concentration of the EPNs infect and kill larger number of the larvae of the studied pest insects. There was no significant difference between *S. tbilisiensis*, *S. thesami* and *H. bacteriophora* at low concentration P>0.05,

significant difference was observed between *S. tbilisiensis* and *H. bacteriophora* at high concentration P< 0.05.

For the increase mortality of insects by used only entomopathogenic nematodes, there were added combination with bacterial insecticides - *Bitoxibacilline* (Btb) and *Thuringiensis* (Bt) 0.3% (Fig. 2). Mortality of the pest insects *L. dispar* and *O. brumata* shoved strong correlation with the concentration [*L. dispar* (R^2=0.93; P<0.05); *O. brumata* (R^2=0.82; P<0.05)] of the pesticide including the combination of EPNs and bacteria. In case of using the combination of EPNs species with dose 500 -1000 + *Bitoxybacillin* (Btb) 0.3% and *B. thuringiensis* (Bt) 0.3%, the mortality of *L. dispar* and *O. brumata* was 79.2-77.6 and 98.1-89.2 for *S. tbilisiensis*; 66.2 - 67.2 and 82.3 - 80.4 for *S. thesami;* 63.1 – 64.2 and 78.5– 73.5 for *H. bacteriophora* was marked. However, no significant difference was observed between *S. thesami* and *H. bacteriophora* at both concentrations. Significant difference was observed between *S. tbilisiensis* follow *H. bacteriophora* at low and high concentrations, P< 0.05.

Field experiment. Analysis of the field experiment data showed differences in the number of insect larvae before and after use of the pathogens containing EPN and bacterial factors of the invasion. *L. dispar* was more sensitive than *O. brumata.* On the first experimental plot the efficacy of high mortality of both insects was (1500-3000 IJs/1ml) 25.2- 20.2%; 40.3-35.3% for *S. tbilisiensis*, following second and third experimental plot 18.5-15.6% and 24.2-21.2% for *S. thesami* and 15.1-11.7% and 22.7- 18.2% for *H. bacteriophora.* No significant difference was observed between *S. tbilisiensis*, *S. thesami* and *H. bacteriophora* at low concentration (1500 IJs/1ml)**.** Significant difference was observed between *S. tbilisiensis* and *S. thesami* follow *H. bacteriophora* at high concentration (3000 IJs/1ml).

For field experiments were used combining - entomopathogenic nematodes with bacterial preparations - *Btb* and *Bt* 0.5% (Table 2). No significant difference was observed between *S. thesami* (36.4 - 32.2%; 30.4 - 28.0%) and *H. bacteriophora* (30.1-28.5%; 26.5-20.1%) by combining EPNs + *Btb* and *Bt* 0.5%, P>0.05. Significant difference was observed between *S. tbilisiensis* (47.2-53.1%; 49.0-50.1%) and *H. bacteriophora* (30.1-28.5%; 26.5-20.1%) P < 0.05. Steinernema species produced significantly more mortality than control. The emerging IJs of *S. tbilisiensis* from larvae of *L. dispar* and *O. brumata* were harvested and counted after 11 to 18 days and continued until the expiration.

The highest numbers of IJs emerging from the grub cadavers were obtained on the day 13 to 16 post-inoculation days (Fig.3). There is strong correlation between the time (amount of the days of the effect) and control efficacy of emerging IJs from cadaver expressed in percentage of the decrease of the number of larvae of *L. dispar* (R^2 of polynomial regression=0.93) and *O. brumata* (R^2 of polynomial regression=0.95).

Counts of harvested IJs from White traps revealed the effect of high concentration 100 IJs/ per both larvae. S. *tbilisiensis* produced the highest number of infective juveniles – 75% per cadaver on the fifteenth day. For all species of EPNs the maximum larval mortalities in average ranged from 20-25%. Whereas high mortality was observed at 96 hr. So, the mortality rate changed depending on time and dose.

პარაზიტოლოგია

ენტომოპათოგენური ნემატოდების – *Steinernema tbilisiensis, Steinernema thesami, Heterorhabditis bacteriophora* ბაქტერიულ ინსექტიციდებთან – Bitoxybacillin (Btb), *Bacillus thuringiensis* (Bt) კომბინაციის ეფექტი მავნე მწერების – *Lymantria dispar* (L.), *Operophtera brumata* (L.) წინააღმდეგ

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ნაშრომში მოცემულია ტყის მავნებელი მწერების ***Lymantria dispar*** და ***Operopthera brumata***-ის წინააღმდეგ გამოყენებული ენტომოპათოგენური ნემატოდების ***Steinernema tbilisiensis, Steinernema thesami, Heterorhabditis bacteriophora*** და ასევე ბაქტერიული ინსექტიციდების – **Bitoxybacillin (Btb)** და ***Bacillus thuringiensis* (Bt)** ეფექტურობა. ***Lymantria dispar*** და ***Operophtera brumata*** განიხილება როგორც ტყის, ხილისა და დეკორატიული ხეების ერთ-ერთი ყველაზე მნიშვნელოვანი მავნებლები და იწვევენ ფართო ფოთოლცვენას, რის გამოც ხდება ხის ზრდის შემცირება. ორივე მწერი ევროპის ბევრ ქვეყანაში არის გავრცელებული, მათ შორის საქართველოში და პასუხისმგებელია ხეების ფართო ფოთოლცვენაზე. ექსპერიმენტები ჩატარდა ლაბორატორიულ და საველე პირობებში. ლაბორატორიული ექსპერიმენტებისთვის გამოყენებული იყო განსხვავებული კონცენტრაციის **500-1000** (ნემ/1 მლ წყალში) ინვაზიური ლარვები როგორც ცალკე, ისე ბაქტერიულ ინსექტიციდებთან – **Bitoxybacillin (Btb) (1000 + 0,3%)** და ***Bacillus thuringiensis* (Bt) (1000 + 0,3%)** ერთად. საველე ექსპერიმენტებისათვის ორივე მწერის მასობრივი გამრავლების დროს შეიძლება მნიშვნელოვანი ზიანი მიადგეს ტყეს. აქედან გამომდინარე, გამოყენებული იყო ენტომოპათოგენების ინვაზიური ლარვების მაღალი კონცენტრაცია **1500-3000** (ნემ/1 მლ წყალში) და ასევე ბაქტერიული ინსექტიციდების **Bitoxybacillin (Btb) (0,5%)** და ***Bacillus thuringiensis*-ის (0,5%)** კომბინაცია. ნაშრომში წარმოდგენილი ექსპერიმენტების შედეგებიდან გამომდინარე, შეგვიძლია დავასკვნათ, რომ ენტომოპათოგენური ნემატოდების მაღალი კონცენტრაციის და ბაქტერიული ინსექტიციდების გამოყენების შემთხვევაში მნიშვნელოვნად იზრდება მავნე მწერების სიკვდილიანობა.

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