AGRICULTURAL UNIVERSITY – PLOVDIV

FACULTY OF PLANT PROTECTION AND AGROECOLOGY

DEPARTMENT OF ENTOMOLOGY

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PLANT PARASITIC NEMATODES OF THE GENUS GLOBODERA SKARBILOVICH, 1959 ON POTATOES IN BULGARIA

AUTOREFERAT

OF THE THESIS FOR AWARDING OF EDUCATION AND SCIENTIFIC DEGREE „PhD”
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The dissertation is written on 128 pages and contains 24 tables and 16 figures. The list of cited literature indicates 248 sources, of which 5 in Cyrillic and 243 in Latin.

The experimental and laboratory work was carried out in the department of Entomology and the department of Genetics and Selection at the Agricultural University - Plovdiv, Institute of Vegetable Crops „Maritsa" - Plovdiv, Institute of Plant Genetic Resources „K. Malkov"- Sadovo, Central Laboratory for Plant Quarantine - Sofia, Institute of Soil Science, Agrotechnology and Plant Protection" „Nikola Pushkarov", during the period 2017-2019.

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The defense of the dissertation will be held on ..... from ..... hours in ..... hall of the Faculty of Plant protection and Agroecology at the Agricultural University - Plovdiv - at a meeting of the scientific jury, appointed by the Rector of the Agraricultural University with order № РД 16-211/05.03.2021

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The defense materials are available on the website of the Agricultural University - Plovdiv - www.au-plovdiv.bg

1. INTRODUCTION

Potatoes are a valuable food crop and are not in vain called second bread. Their nutritional value is determined by their rich starch content, as well as the presence of many other valuable substances, such as proteins, amino acids, sugars, vitamins and others. The importance of potatoes as food for farm animals is also much greater and is determined by the fact that they are not only rich in nutrients, but are also succulent feed, especially valuable for dairy animals and pigs. Potatoes can also be a valuable technical raw material from which are obtained starch, alcohol, maltose, dextrin, rubber glue and others. It is also important to note that potatoes occupy areas that are slightly adapted to other crops.

According to the Department of Agrostatistics at the Ministry of Agriculture, Food and Forestry, the production of vegetables for the 2019 harvest amounts to 752.1 thousand tons. The largest is the production of potatoes - 197.4 thousand tons.

During the vegetation of potato plants conditions are created that are favorable for the development of various diseases and pests, including plant-parasitic nematodes. The main species of parasitic nematodes of economic importance for potatoes belong to the genera *Globodera*, *Pratylenchus*, *Meloidogyne* and *Ditylenchus*. The most important for potatoes are the parasitic nematode species of the genus *Globodera*, namely *Globodera rostochiensis* (Wollenweber, 1923) Behrens 1975 and *Globodera pallida* (Stone, 1973) Behrens 1975 (Potato Cyst Nematodes - PCN). Potato cyst nematodes are endoparasites on potato roots that are difficult to control because they form cysts containing eggs and larvae that remain viable in the soil for more than 15 years. Still the main method of pest control on cultivated plants, including nematodes, is the chemical. However, this method is unprofitable and environmentally dangerous because it contaminates the soil and plant products, and also has a negative impact on the beneficial microflora in the soil. This requires the use of other methods and approaches to control - for example, resistant or tolerant varieties of potatoes, as well as the research and application of different organic products of bacterial or botanical origin, which have a different mechanism of action against PCN.

2. AIM AND TASKS

The aim of the dissertation is to establish the species composition and distribution of cyst-forming nematodes of the genus *Globodera* in the potato plantations of the main productive areas in Bulgaria, as well as to research the possibilities for alternative means to control these parasites.

To achieve this goal, the following tasks are set:

1. Study the distribution of cyst-forming nematodes of the genus Globodera on potatoes (PCN).

2. Morphological and molecular characteristics of *Globodera* spp.

3. Determination of the reaction (resistance/susceptible) of potato varieties/lines to *Globodera* spp.

4. Selection of effective agents for biological control with PCN

a) Screening of plant extracts as possible biological control agents for *Globodera*

- Identification of the factors determining the efficacy of the selected plant extracts (*Juglans regia*, Ruta graveolens and *Plantago major*) against eggs and second stage juveniles (J_2) of *Globodera rostochiensis* and *Globodera pallida* - in vitro;
- Establishment of their metabolic profiles (available substances) of *J. regia*, *R. graveolens* and *P. major*, by gas chromatography - mass spectrometry (GC-MS).

б) Screening of rhizobacteria as possible control agents with *Globodera* spp.

- Study of the factors determining the efficacy of the selectin rhizobacterium *S. plymuthica* isolate 72 against eggs and L2 of *G. pallida* - *in vitro*;
- In vivo, establishing the efficacy of *S. plymuthica* isolate 72 against the invasion, development and reproduction of *G. pallida* on potatoes;

3. MATERIAL AND METHODS

3.1. Collection of source material

The soil samples were collected during the second half of the vegetation and at the end / after the vegetation of the potatoes and covers 15 regions on the territory of four districts - Sofia, Pazardzhik, Smolyan and Burgas.

3.2. Morphological and molecular identification of *G. rostochiensis* and *G. pallida*

3.2.1. Morphological characteristics

In identifying species of the genus *Globodera* spp. cysts and second stage juveniles were examined.

The obtained results from the morphometric characteristics were compared with the respective values for both species according to literature data (EPPO, 2013).

3.2.2. Molecular characteristics

Molecular characterization of populations was performed by the polymerase chain reaction method.

The setting of the experiment is described in detail in the relevant section of the dissertation.

3.3. Test methods for determining the reaction of potato varieties and lines to *Globodera* spp.

3.3.1. Plant material from *Solanum tuberosum* for testing

Thirteen *S. tuberosum* lines - E 1809, E 606, E 1789, E 1096, E 1811, E 1210, E 68, E 292, E 330, D 112, D 348, D 497, D 344- were screened to establish their reaction to PCN and seven commercial varieties - Cronos, Cekin, Gandawa, Gawin, Owacij, Ivetta and Desiree (control)[1]

3.3.2. Screening of potato varieties and lines to establish relative resistance / susceptibility to *Globodera* spp.

The relative resistance of each of the tested varieties / lines to PCN was calculated as % of the unstable potato variety (control) (Seinhorst method, 1986), after which the degree of resistance/suscetible was determined on a scale of 1 - 9 (OEPP / EPPO, 2006) (Table 1).

Table 1

Relative susceptible %	*scale
< 1	9
1.1 – 3	8
3.1 – 5	7
5.1 – 10	6
10.1 – 15	5
15.1 – 25	4
25.1 – 50	3
50.1 – 100	2
> 100	1

*Scale: from 1 to 9 (9 / 1 - highly resistant / highly susceptible)

3.4. Methods for determining the efficacy of products / isolates of microbial origin and products /extracts of botanical origin to *G. pallida* / *G. rostochiensis*

3.4.1. Test bacteria

The experiment included local isolates[2] of: *Pseudomonas chlororaphis* isolate 109A, *Pseudomonas chlororaphis* isolate Po4, *Bacillus amyloliquefaciens* isolate 162, *Bacillus pumilus isolate* 109, *Bacillus amyloliquefaciens* isolate 6, *Bacillus* 95, *Bacillus isolate* 132, *Bacillus subtilis* isolate 164, *Serratia plymuthica* isolate 201 and *Serratia plymuthica* isolate 72.

- Cultivation of bacterial isolates

For the purposes of the experiment, starter cultures were prepared from the respective bacterial isolates. For this purpose, 2 ml of the diluted 1/10 liquid nutrient medium is added to the corresponding bacterial inoculum. The tubes were placed in the dark in a shaker incubator at 27°C and 140 rpm for 12 hours. To prepare the test cultures, 10 µl of the corresponding starter culture was cultured in 10 ml of medium, after which the tubes were placed in the dark in a shaker incubator at 24°C and 140 rpm for 72 hours. The concentration of bacterial cells in the culture fluid was determined using a spectrophotometer.

[1]The different potato varieties and lines were provided to us, with the kind assistance of Assoc. Prof. Dr. Emilia Nacheva (IZK "Maritsa", Plovdiv) and Chief Expert Dora Bondova (Potato Experimental Station – Samokov)

[2]The test isolates of microbial origin were provided to us in the form of a culture fluid with a titer of $x10^8$ cells/ ml/cell-free filtrate, with the kind assistance of Assoc. Prof. Dr. V. Tringovska - IZK "Maritsa", Plovdiv.

The required concentration (culture fluid with a titer of $x10^8$ cells/ml) was obtained by dilution with SDW.

- Preparation of cell-free filtrates (CFF)

For this purpose, the test bacteria are cultured as specified in section 3.4.1. Then the bacterial suspension ($x10^8$ cells / ml) of the corresponding test isolate was centrifuged twice at 4000 rpm for 10 min. The extracted CFF was further filtered through a bacterial sieve with a hole diameter of 0.45 μm, in order to completely remove bacterial cells and residual particles in the filtrate. The concentrations required for the experiments were obtained by dilution with SDW.

3.4.3. Test plant extracts

- Plant material

For the purpose of the experiment, fully developed plants of *Tanacetum vulgare*, Artemisia *vulgaris*, *Allium ursinum*, *Tagetes patula*, *Yuglans regia*, *Salvia officinalis*, *Ruta graveolens*, *Plantago major* representatives of the Bulgarian flora were used[3].

- Preparation of plant extracts

Water-methanol extraction procedure: Air-dried, ground plant parts of the respective plant species was extracted by soaking in 80% methanol solution at room temperature for a certain time, depending on the plant part used, twice. After evaporation of the solvent, the resulting crude extract was subjected to further analyzes. For this purpose, each plant extract was initially prepared as a stock solution with a concentration of 10% by adding sterile distilled water followed by homogenization in an ultrasonic bath (BioLab) at 30°C. The corresponding solution was then filtered twice through filter paper. The concentrations required for the experiments were obtained by diluting the resulting stock solution with SDW.

- Gas chromatography - mass spectroscopy analysis (GC-MS)

The setting of the specific experiment is described in detail in the dissertation.

3.5. Laboratory experiments

3.5.1. CFF screening of bacterial isolates and plant extracts for their efficacy against *Globodera* spp.

Standard nematological methods were used to determine the efficacy of bacterial isolates and plant extracts against J_2 and cysts of *Globodera* spp.

With the selected bacterial isolates/plant extracts showed the best results, experiments were performed under controlled conditions adapted for *Globodera* spp. methodology of Samaliev et al. (2000): - "*in vitro*", to determine the efficacy of the concentration and exposure of the relevant bacterial strain/plant

[3] The test plant extracts were provided to us in the form of a raw extract (ml / mg), with the kind assistance of Assoc. Prof. M. Nikolova, BAS, Sofia.

extract on the hatching of J_2 and on J_2 of *G. pallida,* and - "*in vivo*", to determine the effectiveness of the selected bacterial strain/plant extract on J_2 invasion and *G. pallida* development in plant roots.

3.5.2.” *In vitro*” experiments

3.5.2.1. Determination of the effect of concentration/temperature and exposure of selected bacterial isolate (bacterial suspension of *S. plymuthica* isolate 72) on J_2 of *G. pallida*

Standard nematological methods were used to determine the efficacy of a bacterial isolate (bacterial suspension of *S. plymuthica* isolate 72) on J_2 of *G. pallida.*

The statements of the specific experiments are described in detail in the dissertation.

3.5.2.2. Determination of the effect of concentration / temperature and exposure of bacterial isolate (bacterial suspension of *S. plymuthica* isolate 72) on L2 hatching of *G. pallida*

Standard nematological methods were used to determine the efficacy of a bacterial isolate (bacterial suspension of *S. plymuthica* isolate 72) on J_2 of *G. pallida.* The hatching percentage is calculated by the formula:

$$\text{hatching rate} = \text{hatched } J_2 \times 100/\text{eggs} + J_2$$

The statements of the specific experiments are described in detail in the dissertation.

3.5.2.3. Determination of the effect of concentration / temperature and exposure of cell-free filtrate (CFF) of *S. plymuthica* isolate 72 and plant extracts of *J. regia, R. graveolens* and *P. major* on J_2 of *G. pallida*

Standard nematological methods were used to determine the efficacy of cell-free filtrate (CFF) of *S. plymuthica* isolate 72 and plant extracts of *J. regia, R. graveolens* and *P. major* on J_2 of G. pallida. The statements of the specific experiments are described in detail in the dissertation.

3.5.2.4. Determination of the effect of concentration / temperature and exposure to CFF of *S. plymuthica* isolate 72 and plant extracts of *J. regia, R. graveolens* and *P. major* on J_2 hatching of *G. pallida*

Standard nematological methods were used to determine the efficacy of CFF of *S. plymuthica* isolate 72 and plant extracts of *J. regia, R. graveolens* and *P. major* on J_2 of *G. pallida*. The hatching percentage is calculated by the formula:

$$\text{hatching rate} = \text{hatched } J_2 \times 100/\text{eggs} + J_2$$

The statements of the specific experiments are described in detail in the dissertation.

3.5.3. “*In vivo*” experiments

3.5.3.1. Influence of bacterial suspension (BS)/cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on *G. pallida* J_2 invasion in plant roots

The experiment was performed by the modified method of Philips et al. (1980).

The statements of the specific experiments are described in detail in the dissertation.

3.5.3.2. Influence of bacterial suspension (BS)/cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on the development of *G. pallida* in plant roots

The statements of the specific experiments are described in detail in the dissertation.

4. RESULTS AND DISCUSSION

4.1. PCN *Globodera* spp. in the agrocenosis of potatoes

4.1.1. Distribution and species composition of *Globodera* spp.

The districts and settlements from which samples were taken to study and establish the distribution of potato cyst nematodes on potatoes are presented in Table 2 and Figure 1. The results show that of the total 199 samples studied from the 15 regions of Pazardzhik, Sofia, Smolyan and Burgas districts, PCN were established in 15 of them (Table 2; Fig. 1), the prevalence of potato cyst nematodes in the potato plantations of the studied areas was 100%.

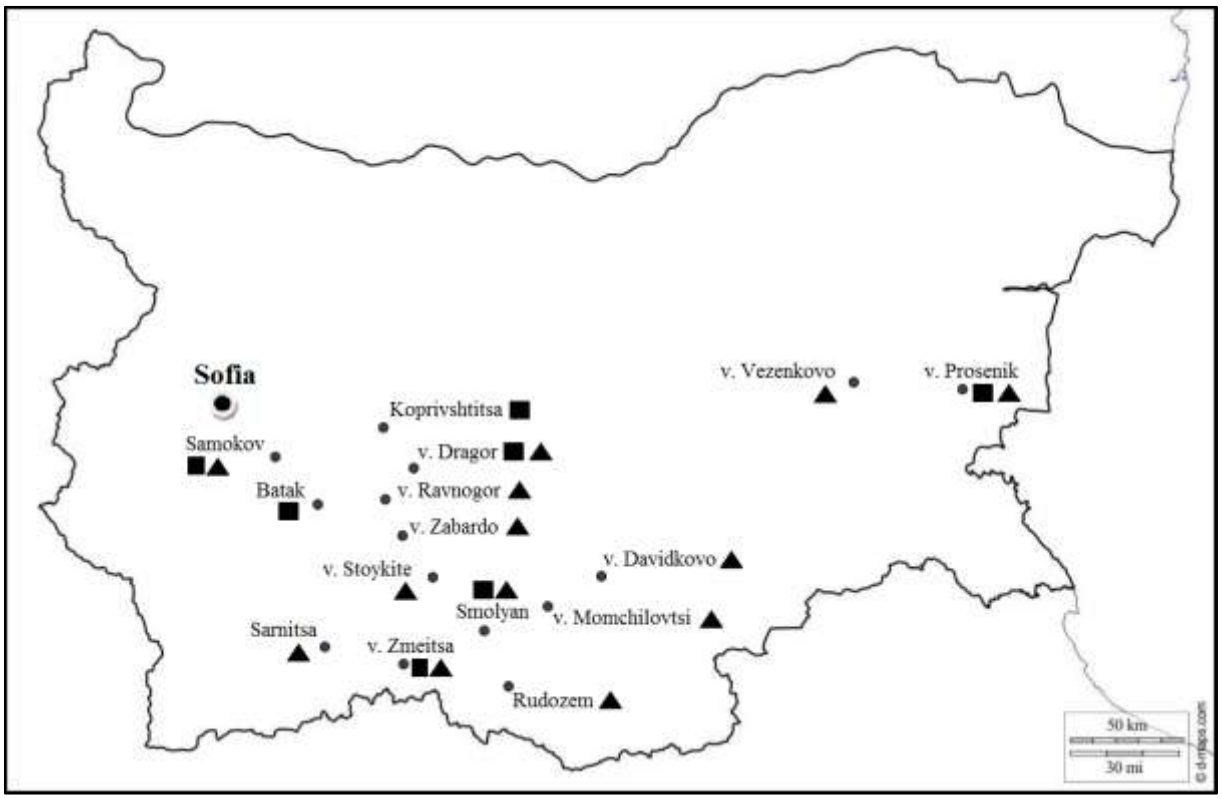

Figure 1. Research region in the four districts - Sofia, Pazardzhik, Smolyan and Burgas;
■ - *G. rostochiensis*; ▲- *G. pallida*; 2017-2019

Table 2. Distribution, species composition and population density of potato cyst nematodes /PCN/ of the genus *Globodera* in Pazardzhik, Sofia, Smolyan and Burgas districts for the period 2017-2019.

Populated location	Number of samples	Potato Cyst Nemtodes species		Density (Nambur of cysts/100 g soil)
		G.rostochiensis	*G. pallida*	
Pazardzik district				
Ravnogor	20	-	+	610
Dragor	12	+	+	252
Sarnitsa	18	-	+	470
Batak	15	+	-	252
Sofia district				
Koprivshtitsa	18	+	-	450
Samokov	14	+	+	275
Smolyan district				
Smolyan	17	+	+	18
Momchilovtsi	13	-	+	4,5
Davidkovo	12	-	+	138
Rudozem	9	-	+	15
Stoikite	3	-	+	78
Zaburdo	18	-	+	158
Zmeitsa	22	+	+	275
Burgas district				
Prosenik	5	+	+	5
Vezenkovo	3	-	+	3.5

The results in Table 2 show that the number of cysts extracted from the soil samples varies from 3.5 to 610. The highest density was reported in the areas of Ravnogor (610 cysts/100 g soil) and the lowest (3.5 cysts/100 g soil).) in Vezenkovo.

With regard to the species composition, it was found that in the studied region are distributed both species of PCN - *G.rostochiensis* and *G. pallida*. *G. pallida* was identified in 13 region and *G. rostochiensis* in 7.

The two species are distributed alone or in association as follows: *G. pallida* was found in nine of the plantations alone, in five - together with *G. rostochiensis*. In five of the regions *G. rostochiensis* was found in association with *G. pallida*, and *G. rostochiensis* was independently found in two of the regions (Batak and Koprivshtitsa) of the studied potato-producing regions (Table 2).

4.1.2. Morphological and molecular identification of *G. rostochiensis* and G. *pallida*

4.1.2.1. Morphological characteristics

The results obtained from the morphological identification of cysts and L2 showed that the latter belong to the genus *Globodera*.

Samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 15 were morphologically identified as *Globodera pallida* (Tables 3 and 4).

The average values for J_2 measured by us, such as body length 469.83 μm (395.22 - 519.85) and stylet length 23.04 μm (20.44 - 25.29), are in the range compared to the values of the reference data (Table 3). The values of the other characteristics of J_2, such as the tail length and the hyaline region of the tail,

have small differences compared to their values given in the literature data (EPPO 2013). The measured mean values for cysts as anus-fenester distance 48.22 μm (36.58 - 74.22) and number of cuticular ridges 8.38 (7.00 - 11.00) are in the range compared to their values in the reference data (Table 3). The mean values of the other characteristics for cysts - Granek's ratio have small differences compared to their values given in the literature (EPPO 2013).

The other two samples, 13 and 14, were morphologically identified as *Globodera rostochiensis* (Tables 3 and 4). The average values of the main features for J_2 - body length 451.07μm (427.25 - 474.89); tail length 43.65μm (39.44 - 47.77); the hyaline region of the tail 24.81 μm (21.52 - 28.10) and the length of the stiletto 21.80 μm (21.12 - 22.48) are within the limits of their reference values according to literature data (Table 3). The measured mean values for cysts as the number of cuticular ridges 18.00 (18.00 - 18.00) are in the range compared to their values in the reference data (Table 3). The mean values of the other characteristics for cysts - anus-fenester distance and Granek's ratio vary the most compared to their values given in the literature (EPPO 2013).

Table 3. Morphological and morphometric characteristics of second-stage juveniles and cysts from Bulgarian populations compared to characteristics of OEPP/EPPO Buletin (2013). Measurements of second-stage juveniles and cysts from Bulgarian populations are in **μ**m and represented as mean ± s.d. (range)

	*G. pallida** (n = 13)	*G. rostochiensis** (n = 2)	*G. pallida***	*G. rostochiensis***
Second-stage juveniles				
Body lenght	469.83 ± 29.02 (395.22 - 519.85)	451.07 ± 33.68 (427.25 - 474.89)	452- 486	392- 468
Tail lenght	44.98 ± 8.96 (30.91 – 59.01)	43.65 ± 5.89 (39.44 – 47.77)	50-53	44 - 51
Hyaline region	23.13 ± 3.11 (17.29 - 28.10)	24.81 ± 4.65 (21.52 - 28.10)	26-27	20-27
Stylet lenght	23.04 ± 1.38 (20.44 - 25.29)	21.80 ± 0.96 (21.12 - 22.48)	23-24	20-22
Cysts				
Fenestra to anus	48.22 ± 10.33 (36.58 - 74.22)	106.56 ± 4.28 (103.53 - 109.59)	48-54	51-70
Fenestra diam.	23.98 ± 4.86 (18.60 - 36.53)	16.70 ± 3.74 (14.05 - 19.35)	***	***
Granek’s ratio	2.12 ± 0.65 (1.38 -3.93)	6.58 ± 1.73 (5.35 - 7.80)	2.1-2.5	3.0-4.5
No. cuticular ridges	8.38 ± 1.19 (7.00 – 11.00)	18.00 ± 0.00 (18.00 – 18.00)	12	17-20

*values from Bulgarian populations of *G. pallida* and *G. rostochiensis*, **according to OEPP/EPPO Bulletin (2013), ***no data

Table 4. Morphological and morphometric measurements of *Globoderarostochiensis* and *Globodera pallida* cysts and second-stage juveniles from samples of Bulgarian populations. All measurements are in **µ**m

Samples number n*	**Second-stage juveniles**				**Cysts**				**Species**
	Body lenght	Tail lenght	Hyaline region	Stylet lenght	Fenestra to anus	Fenestra diam.	Granek's ratio	No. cuticular ridges	
1. Ravnogor	491.75	59.01	25.29	22.48	36.81	20.51	1.79	9	*G. pallida*
2. Dragor	463.65	53.39	28.10	25.29	38.74	21.82	1.77	8	*G. pallida*
3. Sarnitsa	491.75	50.58	22.48	22.48	39.34	25.29	1.55	8	*G. pallida*
4. Samokov	519.85	50.58	19.67	25.29	50.58	36.53	1.38	9	*G. pallida*
5. Smolyan	476.38	53.55	25.64	21.74	50.90	20.25	2.51	8	*G. pallida*
6. Momchilovtsi	455.88	49.13	26.03	22.76	36.58	18.60	1.97	8	*G. pallida*
7. Davidkovo	454.18	44.39	23.62	22.62	43.78	22.43	1.95	8	*G. pallida*
8. Rudozem	465.21	45.14	22.66	22.82	49.86	23.84	2.09	9	*G. pallida*
9. Stoikite	469.85	32.45	19.62	24.64	56.42	20.16	2.79	11	*G. pallida*
10. Zaburdo	483.77	35.30	22.28	22.82	46.73	23.63	1.98	7	*G. pallida*
11. Zmeitsa	395.22	35.26	17.29	20.44	46.73	23.63	1.98	7	*G. pallida*
12.Prosenik	457.06	45.11	24.83	23.76	74.22	24.12	3.07	10	*G. pallida*
13. Batak	474.89	47.77	28.10	22.48	109.59	14.05	7.80	18	*G. rostochiensis*
14.Koprivshtitsa	427.25	39.44	21.52	21.12	103.53	19.35	5.35	18	*G. rostochiensis*
15. Vezenkovo	483.32	30.91	16.86	22.48	56.20	30.91	1.82	7	*G. pallida*
GRO**	392-468	44-51	20-27	20-22	51-70	***	3.0-4.5	17-20	
GPA**	452-486	50-53	26-27	23-24	48-54	***	2.1-2.5	12	

4.1.3. Molecular characteristics

For the purpose of the study, a set of gene-specific primers was used (Table 5), in order to establish species affiliation.

Table 5. Gene-specific primers for establishing species affiliation in members of the genus *Globodera*

Species	Primers	Product (bp)
G. rostochiensis	**UNI–**5'-GCAGTTGGCTAGGGATCTTC-3'	**239**
	GPA1–5'-GGTGACTCGACGATTGCTGT-3'	
G. pallida	**GRO5A–** 5'-ATGTTGTAC GTGCCGTACCTT-3'	**391**

The application of the multiplex PCR method with the primers UNI, GPA1 and GRO1 allows to distinguish *G. rostochiensis* and *G. pallida* (Fig. 2), based on the obtained fragments of 391 bp for *G. pallida* and 239 bp for *G. rostochiensis*, respectively, and also allows for the accurate identification of each Globodera species in the analysis of mixed populations in a single reaction (Fig. 3). Figure 2 presents electrophoretic profiles by multiplex PCR of the analyzed cyst-forming nematode samples. The three primers amplify products from a small region between the internal transcribed spacer 1 (ITS1) and the 5.8 S ribosomal RNA gene region, which is extremely indicative through its constitutive presence. This region has been identified as suitable for classification, molecular diagnostics and phylogenetic reconstructions of different organisms and at different taxonomic levels, including plant-parasitic nematodes (Marek et al., 2010; Douda et al., 2013). The method of multiplex PCR analysis is reliable and easily applicable for rapid identification of different PCNs in the laboratory, which successfully complements the basic morphological method for distinguishing the two types. Based on the combined data from the morphological characteristics and the applied molecular methods, the potato cyst-forming nematodes found in Bulgaria contain G. pallida as the most common type of PCN in the studied regions.

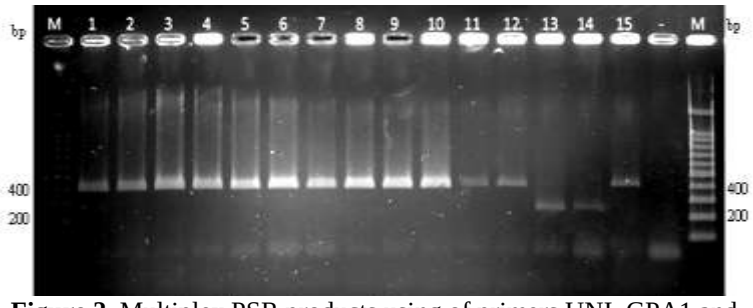

Figure 2. Multiplex PSR products using of primers UNI, GPA1 and GRO1; line M – 100bp molecular marker; rows 1-15-PCN samples; line – negative control

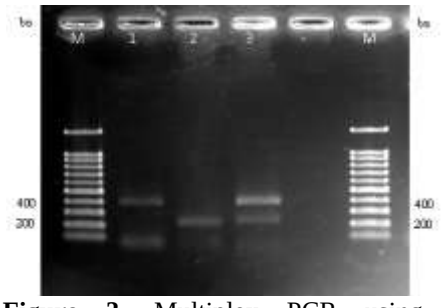

Figure 3. Multiplex PCR using primers UNI, GPA1 and GRO1; line M-100 bp molecular marker; line 1-*G. palldia*; line 2-*G.rostochiensis*; line 3-mixed population (*G. palldia/ G.rostochiensis*); - negative control

4.2. Screening of potato varieties and lines to establish relative resistance/susceptibility to *Globodera* spp.

G. rostochiensis

The data presented in Table 6 show that the tested potato varieties/lines for resistance to *G. rostochiensis* react differently.

It was found that 13 out of 13 tested potato varieties/lines are resistant to *G. rostochiensis* - resistance index from 5 to 9.

G. pallida

The results show that 5 of a total of 13 tested potato varieties/lines are high suscetible to *G. pallida* - index from 1 to 2. Two of the tested varieties Cronos and Ivetta, as well as line E 1096 have been found resistant to *G. pallida* resistance index - 5. From the tested lines E 1811, E 1809, E 606 and E 68 tolerance to *G. pallida* was established (index - 3 and 4).

Table 6. Relative susceptibility of different potato varieties/lines to *G. rostochiensis* (Koprivshtitsa population) and *G. pallida* (Ravnogor population)

No	Varieties / Lines	Resistance to *Globodera*	Index resistance*	
			G. rostochiensis	*G. pallida*
Varieties				
1	Desiree контрола	Suscetible	1	1
2	Cronos	Ro1,Ro4; Pa2, Pa3	8	5
3	Chekin	Ro1,Ro4; Pa3	8	1
4	Gandawa	Ro1,Ro3, Ro4; Pa3;	9	1
5	Gawin	Ro1,Ro3,Ro4; Pa3	7	2
6	Owacij	Ro1,Ro3,Ro4; Pa3	8	2
7	Ivetta	Ro1,Ro3; Pa2,Pa3	9	5
Lines				
1	E 1789	Unestablished	7	
2	E 606	Unestablished	7	
3	E 1096	Unestablished	8	
4	E 1809	Unestablished	8	
5	Д 344	Unestablished	7	
6	Д 497	Unestablished	6	
7	Д 348	Unestablished	7	
8	E 1811	Unestablished		3
9	E 1809	Unestablished		4
10	E 1096	Unestablished		5
11	E 606	Unestablished		4
12	Д 348	Unestablished		2
13	E 68	Unestablished		3
14	Desiree (контрола)	Suscetible	1	1

*Scale: from 1 to 9 (1- high suscetible-2-3 Suscetible; 4-5-tolerance; 6-8-resistance; 9-high resistance)

4.3. "*In vitro*" laboratory tests

4.3.1. CFF screening of bacterial isolates and plant extracts for their efficacy against *Globodera* spp.

The results of the screening of various bacterial isolates and plant extracts show that all 12 tested CFF of bacterial isolates and 8 plant extracts show a nematicidal effect against J_2 of *Globodera* spp. (Tables 7 and 8).

Table 7. CFF efficacy of bacterial isolates and plant extracts against J_2s of *G. rostochiensis* and *G. pallida* after 72 hours of exposure at 22±1°C

Variants	*G. rostochiensis*		*G. pallida*	
	Number of living	Mortality (%)*	Number of living	Mortality (%)*
CFF of bacterial isolate**				
Serratia plymuthica 72	14.00g***	86.00	16.00f	84.00
Serratia plymuthica 201	38.00f	62.00	46.00d	54.00
Bacillus pumilus 109	58.00c	42.00	60.00c	40.00
Bacillus megaterium 95	70.00b	30.00	75.00b	25.00
Bacillus subtilis 132	46.00e	54.00	52.00d	48.00
Bacillus amyloliquefaciens 162	60.00c	40.00	64.00c	36.00
Bacillus subtilis 164	82.00a	18.00	90.00a	10.00
Bacillus megaterium 174	85.00a	15.00	88.00a	12.00
Bacillus amyloliquefaciens 185	19.00g	81.00	20.00ef	80.00
Bacillus amyloliquefaciens 186	90.00a	10.00	87.00a	13.00
Pseudomonas chlororaphis *109A*	50.00de	50.00	59.00c	41.00
Pseudomonas fluorescens Po4	20.00g	80.00	26.00e	74.00

*%compared to control; **concentration 5%; ***a,b,c,….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

Table 8. Efficacy of plant extracts against J_2s of *G. rostochiensis* and *G. pallida* after 72 hours of exposure at 22±1°C

Variants		*G. rostochiensis*		*G. pallida*	
		Number of living	Mortality (%)*	Number of living	Mortality (%)*
Plants extracts**					
plant species	plant part				
Tanacetum vulgare 21	aerial parts - flowers	58.00d	42.00	60.00de	40.00
Allium ursinum 30	aerial parts - leaves	76.00b	24.00	77.00b	23.00
Tagetes patula 11	aerial parts - flowers	70.00c	30.00	67.00c	33.00
Artermisia absinthium 10	aerial parts – leaves and flowers	62.00d	38.00	66.00cd	34.00
Yuglans regia 16	aerial parts – small green fruits	20.00g	80.00	22.00g	78.00
Salvia officinalis 50	arial parts - leaves	81.00a	19.00	88.00a	12.00
Ruta graveolens	aerial parts – leaves and flowers	31.00f	69.00	33.00f	67.00
Plantago major	arial parts - leaves	53.00e	47.00	58.00e	42.00

*%compared to the control; **concentration 5%; ***a,b,c,…. grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The results of the primary screening show that of the bacterial isolates - *Serratia plymuthica* 72 demonstrated the highest efficacy compared to the tested species PCN (Table 7), and of plant extracts - *Yuglans regia* 16, followed by *Ruta graveolens* and *Plantago major* (Table 8).

The obtained initial results give us grounds to continue further "in vitro" and "in vivo" experiments to establish the efficacy of bacterial isolate *Serratia plymuthica* 72 and plant exarcates *Yuglans regia* 16, *Ruta graveolens* and *Plantago major* as control agents for *Globodera* spp. with the species PCN *G. pallida.*

4.3.2. Determination the influence of the effecacy of concentration/temperature and exposure of plant extracts of *J. regia, R. graveolens* and *P. major* on J_2 of *G. pallida*

4.3.2.1. Determination the influence of the efficacy of the concentration and exposure of plant extracts of *J. regia, R. graveolens* and *P. major* on J_2 of *G. pallida*

The results of the experiments presented in Table 9 show that all tested plant extracts showed a nematicidal efficacy on J_2 of *G. pallida*.

The nematicidal effect of plant extracts on J_2 of *G. pallida* is different at different concentrations and exposures. With increasing concentration and exposure of J_2 in the solutions of plant extract, an increase in their mortality compared to the control was reported.

Table 9. Efficacy of plant extracts against J_2s of *G. pallida* at different concentrations and exposure at 22±1ºC

Plant extracts		
Variants/ Exposure*	*Globodera pallida*	
	Number living	Mortality (%)**
	2.5%***	
1	**2**	**3**
Juglans regia		
24 hours	85.25a****	14.75
48 hours	74.25c	25.75
72 hours	63.00ef	37.00
Ruta graveolens		
24 hours	87.25a	12.75
48 hours	79.25b	20.75
72 hours	66.50de	33.50
Plantago major		
24 hours	88.25a	11.75
48 hours	80.25b	19.75
72 hours	68.25d	31.75
	5.0%	
Juglans regia		
24 hours	67.25de	32.75
48 hours	41.25k	58.75
72 hours	21.00mn	79.00
Ruta graveolens		
24 hours	79.25b	20.75
48 hours	67.25de	32.75
72 hours	34.25l	65.75
Plantago major		
24 hours	80.25b	19.75
48 hours	68.25d	31.75
72 hours	57.25gh	42.75
	10.0%	
Juglans regia		

Table 9 (continue)

1	2	3
24 hours	51.25i	48.75
48 hours	30.25l	69.75
72 hours	18.25n	81.75
Ruta graveolens		
24 hours	54.25hi	45.75
48 hours	46.25j	53.75
72 hours	24.00m	76.00
Plantago major		
24 hours	73.25c	26.75
48 hours	59.25f	40.75
72 hours	39.75k	60.25

*exposure of plant extracts to J_2; **% compared to the control; *** concentration of plant extracts; **** a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The highest mortality percent of J_2 was reported at 72 hours of exposure at 10.0% concentration, with the number of dead J_2 in the respective plant extracts being as follows *G. pallida* - 81.75% (*J. regia*), 76.00% (*R. graveolens*) and 60.25% (*P. major*). The highest mortality percent of J_2 was reported for plant extract *J. regia* at all tested concentrations and exposures. The lowest J_2 mortality percent was reported for *P. major* plant extract at all tested concentrations and exposures. No dead J_2s were reported in the controls.

4.3.2.2. Determination the influence on temperature and exposure of plant extracts of *J. regia*, *R. graveolens* and *P. major*, on *G. pallida* J_2s

The results described in Table 10 show that the efficacy of the plant extracts *J. regia*, *R. graveolens*, *P. major*, also depends on both exposure and temperature. At all tested temperature values and exposures, a nematicidal efficacy of the tested extracts was reported.

Table 10. Efficacy of plant extracts of *J. regia*, *R. graveolens*, *P. major*, (concentration 5%) against J_2s of *G. pallida* at different exposures and temperatures

Variants/ Exposure*	*Globodera pallida*	
	Number living	Mortality (%)**
	temperature 14±1°C	
1	**2**	**3**
Juglans regia		
24 hours	81.50b***	18.50
48 hours	75.50c	24.50
72 hours	64.50fg	35.50
Ruta graveolens		
24 hours	88.00a	12.00
48 hours	81.50b	21.50
72 hours	69.25e	30.75
Plantago major		
24 hours	89.75a	10.25
48 hours	82.75b	17.75
72 hours	75.25cd	24.75
	temperature 19±1°C	
Juglans regia		
24 hours	69.75e	30.25
48 hours	48.50ij	51.50
72 hours	24.75l	75.25

Table 10 (continue)

1	2	3
Ruta graveolens		
24 hours	80.50b	19.55
48 hours	70.75de	29.25
72 hours	44.50j	55.50
Plantago major		
24 hours	82.75b	17.25
48 hours	71.75cde	28.25
72 hours	61.00g	39.00
	temperature 24±1°C	
Juglans regia		
24 hours	52.75hi	47.25
48 hours	37.25k	62.75
72 hours	22.25l	77.75
Ruta graveolens		
24 hours	68.50ef	31.50
48 hours	53.25h	46.75
72 hours	39.00k	61.00
Plantago major		
24 hours	74.75cd	25.25
48 hours	60.25g	39.75
72 hours	46.00j	54.00

*exposure of plant extracts on J_2; **% compared to the control; ***a,b,c…. grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

As the temperature increased, an increase in the effect of plant extracts on the mortality of J_2 was found. High mortality against J_2 *G. pallida* was reported at temperatures: 19±1°C and 24±1°C, in the variants with plant extracts *J. regia* and *R. graveolens*. The *P. major* plant extract variants reported the lowest J_2 mortality for all exposures and temperatures compared to the *J. regia* and *R. graveolens* plant extract variants. The highest mortality of J_2 was reported in the variant with plant extract *J. regia* at a temperature of 24±1°C and exposure for 72 hours, respectively 77.75% (Table 10).

The lowest J_2 mortality of *G. pallida* at 72 hours of exposure was reported at 14±1°C for all tested plant extracts, with the best effect at this temperature value found for plant extract *J. regia* (35.50%), followed by *R. graveolens* (30.75%) and *P. major* (24.75%).

4.3.3. Determination the influence of concentration/temperature and exposure of plant extracts of *J. regia*, *R. graveolens* and *P. major* on the hatching of *G. pallida* J_2s

4.3.3.1. Determination of the influence of the concentration and exposure of plant extracts of *J. regia*, *R. graveolens* and *P. major* on the hatching of *G. pallida* J_2s

Table 11. Efficacy of plant extracts *J. regia*, *R. graveolens*, *P. major*, on hatching (%) of *G. pallida* J_2s at different concentrations and exposures and 4 weeks after cysts transfered in PRD at a temperature of 22±1ºC

Exposure/ weeks*	Hatching J_2s (%) /Concentration			
	2.5%	**5.0%**	**10.0%**	**control****
Juglans regia				
1	65.25c***	56.50d	34.00f	74.41b
2	54.25d	46.25e	27.25 gh	81.81a
3	44.75e	33.00fg	23.50hi	85.18a
6	42.55e	31.25fg	20.75i	83.65a
Ruta graveolens				
1	72.21bc	69.00c	59.00e	73.50b
2	69.75c	62.50d	51.50f	79.95a
3	47.25f	54.50f	44.75fg	83.34a
6	45.10fg	52.00f	42.45g	82.20a
Plantago major				
1	80.25bc	76.00cd	69.50ef	76.52c
2	71.00de	71.00de	63.50gh	80.21bc
3	66.00efg	63.50gh	58.25hi	86.23a
6	65.00fg	61.75ghi	57.10i	85.00ab

*exposure of plant extracts on cysts; **control – sterile distilled water; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The lowest inhibitory effect was observed in all variants of *Plantago major* plant extract (Table 11). The highest inhibitory effect was observed in all variants of the plant extract of *J. regia* (see Table 11). With increasing exposure and concentration of tested plant extracts *J. regia*, *R. graveolens*, *P. major*, was established a decrease in the percentage of hatched J_2 from cysts. At a concentration of 2.5% for all variants of the tested plant extracts *J. regia*, R. *graveolens* and *P. major* the lowest inhibitory effect was reported. At a concentration of 10.0% for all variants of the tested plant extracts *J. regia*, *R. graveolens* and *P. major*, the highest inhibitory effect on hatching of second-stage from cysts was reported.

4.3.3.2. Determining the influence of temperature and exposure of plant extracts *J. regia*, *R. graveolens* and *P. major* on the hatching of *G. pallida* J_2s.

Data on the inhibitory effect of plant extracts *J. regia*, *R. graveolens* and *P. major* on the hatching of second-stage juveniles of *G. pallida* are presented in Table 12. From the obtained results it was established that the tested plant extracts show different inhibitory effect about to the hatching of invasive larvae second age from cysts, and that the suppression effect is dissimilar at different temperature.

Table 12. Influence of plant extracts *J. regia*, *R. graveolens* and *P. major* on hatching of *G. pallida* J_2s at different temperature and exposure and 4 weeks after cysts transfered in PRD

Ezposure/ weeks*	**Hatching J_2s (%) /Temperature**			
	14±1°C	**19±1°C**	**24±1°C**	**control****
Juglans regia				
1	60.75c	56.50cd	52.00de	75.65b
2	56.60cd	46.25fg	42.00g	82.51a
3	52.25de	33.00h	31.25h	84.30a
6	49.45ef	31.25h	29.75h	83.65a
Ruta graveolens				
1	75.75bc	71.00cd	57.75e	79.00ab
2	72.00cd	67.25d	50.00f	82.85a
3	53.75ef	59.00e	46.25fg	84.64a
6	50.00f	55.25ef	43.30g	83.00a
Plantago major				
1	77.00cd	70.25ef	65.80fg	80.82bc
2	73.33de	68.00fg	65.00gh	81.00bc
3	71.00ef	60.65hi	52.50j	83.50ab
6	70.00efg	60.00i	51.75j	87.75a

*exposure of plants extracts on cysts; **control – sterile distilled water; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

With increasing exposure and concentration of tested plant extracts *J. regia*, *R. graveolens* and *P. major*, was established a decrease in the percentage of hatched J_2s from cysts.

In the variants with a temperature value of 14±1ºC for all variants of the tested plant extracts *J. regia*, *R. graveolens* and *P. major*, was reported the lowest inhibitory effect. At temperatures of 19±1ºC and 24±1ºC for the *J. regia* plant extract variants, the results obtained were similar and had a small statistical difference from each other. The lowest inhibitory effect at all variants and temperatures was reported for *P. major* plant extract. (Table 12). The highest inhibitory effect at all variants and temperatures was established for plant extract *J. regia* (see Table 12).

For all three plant extracts (*J. regia*, *R. graveolens* and *P. major*) used in the "*in vitro*" experiments, were performed additional studies by gas chromatography - mass spectrocopy (GC-MS) to establish their metabolic profiles (available substances).

The large number of peaks in the chromatograms indicates the presence of many substances (Fig. 6).

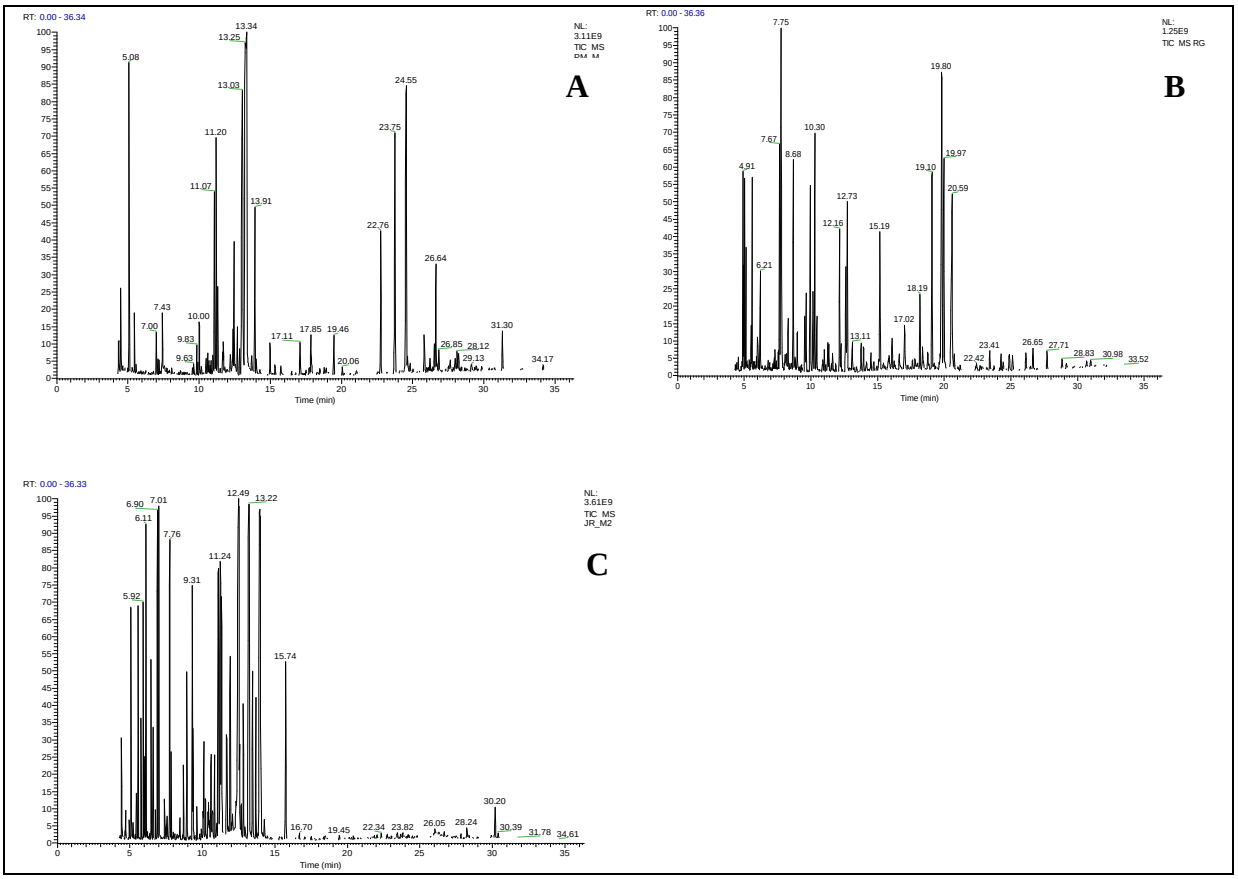

Figure. 6. GC-MS chromatograms of the studied species: **A** - *Ruta graveolens*; **B** - *Plantago major;* **C** - *Juglans regia*;

The best presented metabolic group is carbohydrates. Isomers of fructose, glucose and many monosaccharides, disaccharides and trisaccharides were detected. Wide range of organic, phenolic, amino and fatty acids were determined also. Additionally alkaloids, coumarins and sterols were identified of the extract of *Ruta graveolens*. The identified compounds are in acondance with previously preportrd data concerning chemical composition of *R. graveolens* (Ekiert and Kisiel, 1997; Kostova et al., 1999; Arora and Tandon 2015). The main peak of the chromatogram of *P. Major* extract was identified as sorbitol (polyhydric alcohol). In the *J. regia* extract, besides carbohydrates, a wide variety of organic and phenolic acids were identified. Malic and threonic acids were determined as the most abundant. Phosphoric and gallic acids were well present also (see Table 13).

Table 13. Metabolites identified in the methanolic extracts of the studied species by GC-MS

Compounds	RI	Studied plant extracts*		
		Juglans regia	Plantago major	Ruta graveolens
1	2	3	4	5
Organic acids				
Phosphoric acid	1267	1.70		
Succinic acid	1311	0.35	0.93	0.04
Glyceric acid	1324	0.13		0.06
Fumaric acid	1347	0.88		
Malic acid	1482	4.46		0.61
Threonic acid	1551	5.07	0.10	0.18
Ribonic acid	1757	0.23	0.22	0.21
Quinic acid	1855	2.06		0.33
Ascorbic acid	1943	0.02		
Phenolic acids				
3.5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	1896	0.40		
3.4.5-Trihydroxybenzoic acid (Gallic acid)	1951	1.02		
3.5-Dihydroxybenzoic acid	2007		0.01	
trans -3.4-Dihydroxycinnamic acid (Caffeic acid)	2134		0.11	
Polyols				
Glycerol	1269		5.64	
Meso-erythritol	1493		0.25	
2-Hydroxyglutaric acid	1566		0.04	
Arabinonic acid. 1.4-lactone	1630	0.84	0.10	0.02
Sorbitol	1943		34.76	
Myo-Inositol	2088	2.37	0.22	0.09
Galactosylglycerol	2313	0.07	1.01	0.03
2-Hexadecanoyl glycerol	2584		0.16	
Carbohydrates				
Arabinose	1677	3.56		
Ribose	1701	0.37	0.20	0.06
Monosaccharide 1	1738	0.08		
Fructose 1	1803	2.40	2.64	1.13
Fructose 2	1810	3.32	5.03	1.73
Fructose 3	1817	4.92	1.70	0.97
Monosaccharide 2	1844	0.05		
Monosaccharide 3	1847		0.08	0.01
Glucose	1891	13.17	2.64	83.65
Monosaccharide 4	1893	4.86		
Monosaccharide 5	1903	0.54	1.25	0.02
Glucose 2	1936	12.59		
Galactose	1965		0.34	
Monosaccharide 6	1978		3.74	
Monosaccharide 7	1979	14.55		0.20

Table 13 (continue)

1	2	3	4	5
Monosaccharide 8	1983	15.78		
Disaccharide 1	2522		3.34	2.01
Sucrose	2637	0.03	2.76	2.98
Disaccharide 2	2639		8.11	
Disaccharide 3	2641		15.06	
Threhalose	2740	0.09	0.11	
Disaccharide 4	2819		0.06	
Disaccharide 5	2859		0.31	
Trisaccharide 1	2874		0.10	
Trisaccharide 2	2883		0.38	
Trisaccharide 3	2901		0.37	
Trisaccharide 4	2907		0.06	
Sugar derivative 1	2786		2.76	
Sugar derivative 2	3134		1.23	
Amino acids				
Valine	1217		0.07	
Serine	1259		0.10	
Threonine	1295		0.08	
Phenylalanine	1365.0			0.02
Pyroglutamic acid	1522	0.13	1.09	
Alanine	1556		0.10	
Sterols				
Megestrol acetate	2659			1.83
Campesterol	3245			0.20
β-Sitosterol	3352		0.20	0.13
Alkaloids				
Furoquinoline alkaloid (skimmianine)	2299			0.07
Dictamine 6.7 dimethoxy (kokusaginin)	2382			0.21
Coumarinsfurocoumari				
Xanthotoxin (methoxsalen)	1935			0.48
Bergaptenn	1955			0.34
Fatty acids				
Octanoic acid (Caprylic acid. C8:0)	1534		0.09	0.62
Hexadecanoic acid (Palmitic acid. C16:0)	1922		0.94	1.28
Octadecadienoic acid (Linoleic acid. C18:2)	2089			0.05
Octadecatrienoic acid (Linolenic acid. C18:3)	2120		1.03	0.13
Octadecanoic acid (Stearic acid. C18:0)	2186			0.15

*Data are expressed as percentage of the total peak area of identified compounds [%]

The present GC-MS analysis of the J. regia extract showed the presence of phenolic acids (Table 13).

The present GC-MS analysis of the *J. regia* extract showed the presence of phenolic acids (Table 13). There is data that the nematicidal activity of *J. regia* is due to monohydroxy, dihydroxy and trihydroxy phenolic acids

(Mahajan et al., 1985). Mahajan et al., 1992 reported that several phenolic acids - caffeic, 2,6-dihydroxybenzoic and p-methoxycanelene showed high nematicidal activity against *Meloidogyne incognita*.

For the plant extract *R. graveolens*, the results of the GC-MS analysis showed the presence of a high content of alkaloids, coumarins and sterols (Table 13). Similar to our results, Sasanelli, 1992 reported as active components of the extract of the leaves of *R. graveolens* alkaloids, terpenes and coumarins (xanthotoxin) for nematicidal activity against the plant parasitic nematode *Xiphinema index* "*in vitro*". In the GC-MS analysis for plant extract *P. major*, the results show that it is rich in carbohydrates, especially with the sugar alcohol - orbitol (Table 13). There are no literature data on the nematicidal activity of substances in this metabolic group with the exception of chitosan polysaccharide (Khalil and Badawy, 2012).

4.3.4. Determination the influence of concentration / temperature and exposure to bacterial suspension (BS) of *S. plymuthica* isolate 72 on *G. pallida* J_2s

4.3.4.1. Determination the influence of the concentration and exposure of bacterial suspension (BS) of *S. plymuthica* isolate 72 on *G. pallida* J_2s

The results showed that the bacterial suspension (BS) of *S. plymuthica* 72 showed a nematicidal efficacy against of J_2s *G. pallida* (Table 14).

The nematicidal efficacy of BS *S. plymuthica* 72 on J_2 of *G. pallida* is dissimilar at different concentrations and exposures. With increasing concentration and time of contact of J_2 with BS, their mortality compared to the control is increased.

Table 14. Efficacy of BS *S. plymuthica* 72 against J_2 of *G. pallida* at different concentrations and exposures, temperature 22±1ºC

Variants/ Exposure*	*Globodera pallida*	
	Number living	Mortality (%)**
	10^6cells/ml*	
24 hours	78.00a****	22.00
48 hours	67.00b	33.00
72 hours	52.50c	47.50
	10^7cells/ml	
24 hours	61.75b	38,25
48 hours	51.00c	49.00
72 hours	38.00d	62.00
	10^8cells/ml	
24 hours	39.00d	61.00
48 hours	26.75e	79.25
72 hours	13.25f	86.75

*exposure of BS *Serratia plymuthica* isolate 72 on J_2s**% compared to the contol ***concentration BF *Serratia plymuthica* isolate 72; ****a,b,c,….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The results in Table 14 of the tested BS of *S. plymuthica* isolate 72 show that a nematicidal effect was observed at all tested concentrations and exposures. The highest mortality of J_2s were reported at 72 hours of exposure, respectively -

86.75% (10^8 cells / ml), 62.00% (10^7 cells/ml) and 47.50% (106 cells/ml). No dead J_2s were reported in the controls.

Most rhizobacteria known to be harmful to plant parasitic nematodes act through a secondary metabolic product, enzymes and toxins. Their effects include, suppression of nematode reproduction, egg hatching and juveniles survival, as well as direct killing of nematodes (Zuckerman & Jasson, 1984; Siddiqui & Mahmood, 1999). Commonly studied species of rhizobacteria are *Bacillus* spp., *Pseudomonas* spp. (Becker et al., 1988; Sikora, 1992; Tian et al., 2007). For example, from *Bacillus* spp., the species *Bacillus subtilis* exhibits a broad spectrum of activity against phytonematodes. The strain produces hydrolytic enzymes such as protease, lipase, b-glucose and cellulase, which act on the orientation of the juveniles to the host plant.

Representatives of *Pseudomonas* spp. produce a wide range of bioactive metabolites (antibiotics, siderophores, volatiles and growth promoters). Secondary metabolites such as 2.4-diacetylfluoroglucinol (DAPG), produced by *Pseudomonas fluorescens*, which controls the larvae of cyst-forming nematodes, have been reported (Cronin et al., 1997; Siddiqui & Shaukat, 2003).

4.3.4.2. Determination the influence of temperature and exposure to bacterial suspension (BS) of *S. plymuthica* isolate 72 on *G. pallida* J_2s

The results described in Table 15 show that the efficacy of BS *S. plymuthica* isolate 72 (concentration 10^8 cells/ml) depends on both exposure and temperature. At all tested temperature values and exposures, a nematicidal effect was reported.

Table 15. Efficacy of BS *S. plymuthica* (1.7 x10^8 cells/ml) 72 against J_2 of *G. pallida* at different exposures and temperature

Variants/ Exposure*	*Globodera pallida*	
	Number living	Mortality (%)**
	temperature 14±1°C	
24 hours	87.50a***	12.50
48 hours	83.50a	16.50
72 hours	77.25b	22.75
	temperature 19±1°C	
24 hours	64.50c	35.50
48 hours	55.00d	45.00
72 hours	31.75e	68.25
	temperature 24±1°C	
24 hours	34.75e	65.25
48 hours	26.25f	73.75
72 hours	11.50g	88.50

*exposure of BS *Serratia plymuthica* isolate 72 on J_2s; **% compared to the control; ***a,b,c,….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

With increasing the temperature, an increasing the efficacy of BS on J_2 mortality. In the variants with temperatures: 19±1°C and 24±1°C the action of BS of the bacterium was manifested very quickly, as at 72 hours of exposure, the mortality of of *G. pallida* J_2s increased respectively - 68.25% (19±1°C) and

88.50% (24±1°). At a temperature value of 14±1°C the action of BS of *S. plymuthica* isolate 72 is slower and less pronounced, as the reported mortality of J_2 was 22.75%.

4.3.5. Determination the influence of concentration/temperature and exposure to bacterial suspension (BS) of *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s

4.3.5.1. Determination the influence of the concentration and exposure of bacterial suspension (BS) of *S. plymuthica* isolate 72 on the hatching of *G. pallida* J_2s

Data on the efficacy of *S. plymuthica* BS isolate 72 on the hatching of *G. pallida* J_2s are presented in Table 16. At all tested concentrations and exposures, bacterial growth was found to inhibit J_2 hatching by cysts compared to the controls.

Table 16. Efficacy of BS *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s at different concentration and exposure and 4 weeks after cysts transfered in PRD at temperature 22±1ºC

Exposure/ weeks*	**Hatching J_2s (%) / Bacterial concentration cells/ml**			
	0**	**10^6**	**10^7**	**10^8**
1	57.89c	38.63d	28.26e	11.11gh
2	66.47b	29.83e	14.90g	7.50hi
3	73.50a	22.00f	10.94gh	5.50i
6	75.25a	23.50f	11.75gh	4.25i

*exposure of BS *Serratia plymuthica* isolate 72 on cysts; **control - sterile distilled water; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

With increasing concentration and exposure to BS S. plymuthica isolate 72, a decrease in the number of L2 hatched cysts was observed compared to the control. The highest percentage of efficacy was reported at a concentration of 108 cells / ml for all exposure variants, with hatching reduced as follows: 80.80, 88.71, 92.51 and 94.35%. The lowest percentage of efficacy was reported at a concentration of 106 cells / ml for all exposure variants, 33.26, 55.12, 70.06 and 68.77%, respectively (Table 16). We assume that the inhibitory effect of the bacterium is due to the release of secondary metabolites with unclear composition during growth in a nutrient medium.

Similar to our experiment, Mendoza et al., (2008) reported nematicidal activity of secondary metabolites secreted by *Bacillus firmus* during growth in nutrient medium, significantly reducing egg hatching from *Meloidogyne incognita* egg sacs.

Basyony and Abo-Zaid, (2018) also reported an inhibitory effect of bacterial suspensions of *Bacillus subtilis* isolate B10 and B8 on egg hatching from *Meloidogyne incognita* egg sacs.

4.3.5.2. Determination the influence of temperature and exposure to bacterial suspension (BS) of *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s

Data on the efficacy of BS *S. plymuthica* isolate 72 (concentration 10^8 cells/ml) on the hatching of second-stage juveniles of *G. pallida* are presented in Table 17. The results show that the inhibitory efficacy of BS *S. plymuthica* isolate 72 depends significantly on temperature.

Table 17. Efficacy of BS *S. plymuthica* (1.7 x10^8 cells/ml) 72 on hatching of *G. pallida* J_2s at different temperature and exposure and 4 weeks after cysts transfered in PRD

Exposure/ weeks*	Hatching J_2s (%) / Temperature					
	14±1°C		19±1°C		24±1°C	
	10^8	*0***	*10^8*	*0*	*10^8*	*0*
1	58.51c***	68.00b	11.11c	77.51b	8.85c	77.97b
2	54.12d	73.09a	7.50d	81.81a	8.25c	82.92a
3	27.39e	74.70a	5.50d	80.72ab	6.33c	85.18a
6	14.61f	76.47a	7.25d	79.64	8.00c	82.82a

*exposure of BS *Serratia plymuthica* isolate 72 on the cysts at different temperature; ** control - sterile distilled water; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

At temperatures of 14±1°C, a reduction in the number of hatched J_2s was observed compared to the control variant, but the percentage was relatively lower. We assume that this is due to the low temperature at which the development of the bacterium is slowed down.

At temperature values: 19±1°C and 24±1°C, a satisfactory inhibitory efficacy was reported (Table 17). In all variants with increasing exposure of BS *S. plymuthica* isolate 72 to cysts, the inhibitory efficacy increases, respectively decreases the percentage of hatched second-stage juveniles compared to the control.

The inhibitory effect of BS *S. plymuthica* in the present experiment is most likely due to various volatile organic compounds (VOCs) that inhibit the growth of various microorganisms and nematodes (Kai et al., 2007; Dandurishvili et al., 2011). Very few scientists have tested volatile organic compounds (VOCs) derived from bacterial isolates against their negative efficacy on nematodes. Several tests have been performed primarily "*in vitro*", assessing the motility or mortality of second-stage juveniles (J_2s), and their hatching from cysts as a result of VOCs exposure (Campos et al., 2010). Huang et al., (2010) found that volatile organic compounds released by *Bacillus megaterium* caused a high mortality rate (100%) of J_2s of *Meloidogyne incognita* and strongly inhibited their hatching from egg sacs.

4.3.6. Determination the influence of concentration/temperature and exposure to cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on J_2s of *G. pallida*

4.3.6.1. Determination the influence of concentration and exposure of cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on J_2s of *G. pallida*

The results of the experiments showed that the BF of *S. plymuthica* 72 showed a nematicidal efficacy against J_2s of *G. pallida* (Table 18). The nematicidal efficacy of CFF of *S. plymuthica* 72 on J_2s of *G. pallida* is different at different concentrations and exposures. With increasing concentration and exposure of J_2s in CFF solutions, an increasing their mortality compared to the control.

Table 18. Efficacy of CFF *S. plymuthica* 72 against J_2 of *G. pallida* at different concentrationsand exposure at temperature 22±1ºC

Variants/ Exposure*	*Globodera pallida*	
	Number living	Mortality (%)**
	2.5%***	
24 hours	80.25a****	19.75
48 hours	69.25b	30.75
72 hours	54.75c	45.25
	5.0 %	
24 hours	36.75d	63.25
48 hours	18.50e	81.50
72 hours	14.25e	85.75
	10.0%	
24 hours	34.50d	65.50
48 hours	16.75e	83.25
72 hours	13.50e	86.50

*exposure of CFF *Serratia plymuthica* isolate 72 on J_2s; **% compared to the control ***concentration of CFF *Serratia plymuthica* isolate 72; ****a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The highest mortality of J_2s was reported at 72 hours of exposure - 86.50% (10.0%), 85.75% (5.0%) and 45.25% (2.5%). No dead J_2s were reported in the controls.

Our results correspond to those obtained by Nandeesha and Ravindra (2020) in "*in vitro*" CFF experiments of the rhizobacteria *Pseudomonas fluorescens* and *Bacillus subtilis*, in which the authors reported moderate to high mortality at 72 hours of exposure to second-stage juveniles of *M. incognita*. In "*in vitro*" experiments with rhizobacteria *Bacillus* spp., Tadigiri et. al., (2020), reported high toxicity of second-stage juveniles of *M. incognita* at 72 hours of exposure, which results correspond to the results obtained from our experiments. Soliman et. al. (2019) also reported high J_2s mortality and inhibition of *M. incognita* hatching in "*in vitro*" CFF experiments on some bacteria of the genus *Bacillus*.

4.3.6.2. Determination the influence of temperature and exposure to cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on J_2s of *G. pallida*

The results described in Table 19 show that the efficacy of BF *S. plymuthuca* isolate 72 (5% concentration) depends on both exposure and temperature. At all tested temperature values and exposures, a nematicidal efficacy of CFF *S. plymuthica* isolate 72 was reported. In contrast to the bacterial suspension, the action of CFF was more efficient even at the tested low temperature values.

Table 19. Efficacy of CFF *S. plymuthica* isolate 72 (concentration 5%) against J_2 of *G. pallida* at different exposure and temperature

Variants/ Exposure*	*Globodera pallida*	
	Number living	Mortality (%)**
	temperature 14±1°C	
24 hours	70.00a	30.00
48 hours	57.50b	42.50
72 hours	42.75c	57.25
	temperature 19±1°C	
24 hours	36.75d	63.25
48 hours	18.50f	81.50
72 hours	10.75g	89.25
	temperature 24±1°C	
24 hours	32.25e	67.75
48 hours	16.75f	83.50
72 hours	7.75g	92.25

*exposure of CFF *Serratia plymuthica* isolate 72 on J_2s; **% compared to the control; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

With the temperature increasing, the efficacy of CFF on the mortality of J_2s also increasing, and in contrast to the BS of *S. plymuthica* isolate 72, the fluctuations of the obtained result depend to a lesser extent on the temperature values. In the variants with temperatures: 19±1°C and 24±1°C the action of CFF of the bacterium was manifested very quickly, as at 72 hours of exposure, the mortality rate of J_2s of *G. pallida* was increased respectively - 89.25% (19±1°C) and 92.25 % (24±1°C). In the variants with a low temperature value of 14±1°C, a nematicidal efficiency was also observed, but the CFF efficacy of *S. plymuthica* isolate 72 was less pronounced compared to the J_2s mortality rate. However, the action of CFF is more effective even at this low temperature, in contrast to the bacterial suspension. At 14±1°C and 72 hours exposure, the reported *G. pallida* J_2s mortality rate was 71.50%.

The highest nematicidal efficacy of CFF *S. plymuthica* isolate 72 was reported in the variant with a temperature of 24±1°C, and the lowest nematicidal efficacy was reported in the variant with a temperature of 14±1°C (Table 19).

4.3.7. Determination the influence of concentration/temperature and exposure to cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s

4.3.7.1. Determination the influence of concentration and exposure of cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s

Data on the efficacy of CFF *S. plymuthuca* isolate 72 on hatching of *G. pallida* J_2s are presented in Table 20. An inhibitory efficacy on J_2s hatching from cysts was observed at all tested concentrations and exposures.

Table 20. Efficacy of CFF *S. plymuthica* изолат 72 on hatching of *G. pallida* J_2s at different concentration and exposure and 4 weeks after cysts transfered in PRD at temperature 22±1ºC

Exposure/ weeks*	**Hatching J_2s (%) /Concentration**			
	*control***	*2.5%*	*5.0%*	*10.0%*
1	60.52d***	31.00e	21.45fg	5.26ij
2	66.84c	25.52f	11.94h	0.00j
3	75.43b	19.04g	7.91hi	0.00j
6	81.50a	17.65g	6.55hi	0.00j

*exposure of CFF *Serratia plymuthica* isolate 72 on the cysts; **control - sterile distilled water; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

With an increasing the exposure and CFF concentration of *S. plymuthica* isolate 72, the number of hatched J_2s from the cysts decreased. The lowest percentage of efficacy was observed at a concentration of 2.5% for all exposure variants, 69.00, 74.48 and 80.96%, respectively, however, the efficacy of CFF was significant compared to the control. The highest efficacy rate was reported at a concentration of 10.0% for all exposure variants. At this concentration for 2 and 3 weeks of exposure, hatching J_2 is completely suppressed.

4.3.7.2. Determination the influence of the temperature and exposure to cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s

Data on the efficacy of CFF *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s are presented in Table 21. In contrast to the bacterial suspension, the efficacy of CFF *S. plymuthica* isolate 72 on hatching of *G. pallida* J_2s depends a less of the temperature.

Table 21. Efficacy of CFF *S. plymuthica* isolate 72 (concentration 5%) on hatching of *G. pallida* J_2s at different temperature and exposure and 4 weeks after cysts transfered in PRD

Exposure/ weeks*	**Hatching J_2s (%) /Temperature**					
	14±1°C		**19±1°C**		**24±1°C**	
	5.0%	*0***	*5.0%*	*0*	*5.0%*	*0*
1	41.30c***	66.79b	21.45c	76.23b	19.95c	78.20b
2	32.56d	69.88b	11.94d	79.00ab	9.25d	82.89a
3	24.70e	75.13a	7.91e	82.20a	0.00e	84.10a
6	19.25f	77.46a	3.10f	81.57a	0.00e	83.79a

*exposure of CFF *Serratia plymuthica* isolate 72 on the cysts; **control - sterile distilled water; ***a,b,c….grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

At temperatures of 19±1 and 24±1°C, a medium to high reduction in J_2s hatching was observed compared to the control variants. The hatching rate

decreases with increasing exposure. At 24±1°C, exposure for 3 and 6 weeks, complete suppression of J_2s hatching was observed.

At a temperature of 14±1°C, the efficacy of CFF was slower, but a reduction in the percentage of J_2s hatching compared to the control was also observed (Table 21).

4.4. Laboratory experiments "*in vivo*"

4.4.1. Influence of bacterial suspension (BS)/cell-free filtrate (CFF) of *S. plymuthica* isolate 72 at different temperatures on the invasion of *G. pallida* J_2s in plant roots

4.4.1.1. Influence of bacterial suspension (BS) of *S. plymuthica* isolate 72 at different temperatures on the J_2s invasion of *G. pallida* in plant roots

The obtained results show that the bacterial suspension (BS) of *S. plymuthica* isolate 72 reduces the number of penetrated J_2s of *G. pallida* into the roots of potato plants at all three experimental temperatures (14±1, 19±1 and 24±1°C) (Fig. 7 /**A**, **B**, **C**/). The results of Figure 7 (**A**) from the tested BS of *S. plymuthica* isolate 72 at a temperature of 14±1 show that it reduces the invasion of J_2s in the roots of potato plants compared to the controls in all variants. The highest efficacy of BS in relation to the invasion of J_2s in the roots was reported in the infection of plants on the seventh day after application of BS in the rhizosphere of plants, with 23.00% penetrated J_2s, and in the control, significantly more - 54.00% (Fig.7 /**A**/). The lowest efficacy of BS in relation to the invasion of J_2s was reported in the infection of plants on the twenty-seventh day after application of BS in the rhizosphere of plants as 41.75 % of penetrated J_2s was reported, and in the control variant 54.00% (Fig. 7 /**A**/). The percentage of J_4s (female and male) growing in the treated plants was significantly lower than in the untreated variants. The action of BS of *S. plymuthica* isolate 72 is similar to that of J_2s penetrated into the roots of potato plants (Fig. 7 /**A**/).

The results of Figure 7 (**B**) from the tested BS of *S. plymuthica* isolate 72 at a temperature of 19±1 show that it significantly reduces the invasion of J_2s in the roots of potato plants compared to the controls in all variants. The highest efficacy of BS against the invasion of J_2s in the roots was reported when infecting plants on the seventh day after introduction of BS into the rhizosphere of plants, with 15.25% infiltrated J_2s, and in the control, significantly more - 51.00% (Fig. .7 /**B**/). The lowest efficacy of BS in relation to the invasion of J_2 was reported in the infection of plants on the twenty-seventh day after introduction of BS in the rhizosphere of plants, as 34.00% of penetrated J_2 was reported, and in the control variant 51.00% (Fig. 7 /**B**/). The percentage of J_4s (female and male) developing in the treated plants was significantly lower compared to the untreated variants. In variants when the plants were infected with J_2s up to 14 and 7 days after application of BS to *S. plymuthica* isolate 72, no percentage of nematodes reached the stage of J_4s development was reported (Fig. 7 /**B**/).

The results of Figure 7 (**C**) from the tested BS of *S. plymuthica* isolate 72 at a temperature of 24±1, show that they reduce the invasion of J_2s in the roots of potato plants compared to the controls in all variants. The highest efficacy of BS in relation to the invasion of J_2s in the roots was reported in the infection of plants on the seventh day after introduction of BS in the rhizosphere of plants, with 16.90% penetrated J_2s, and in the control, significantly more - 52.00% (Fig. 7 /**C**/).

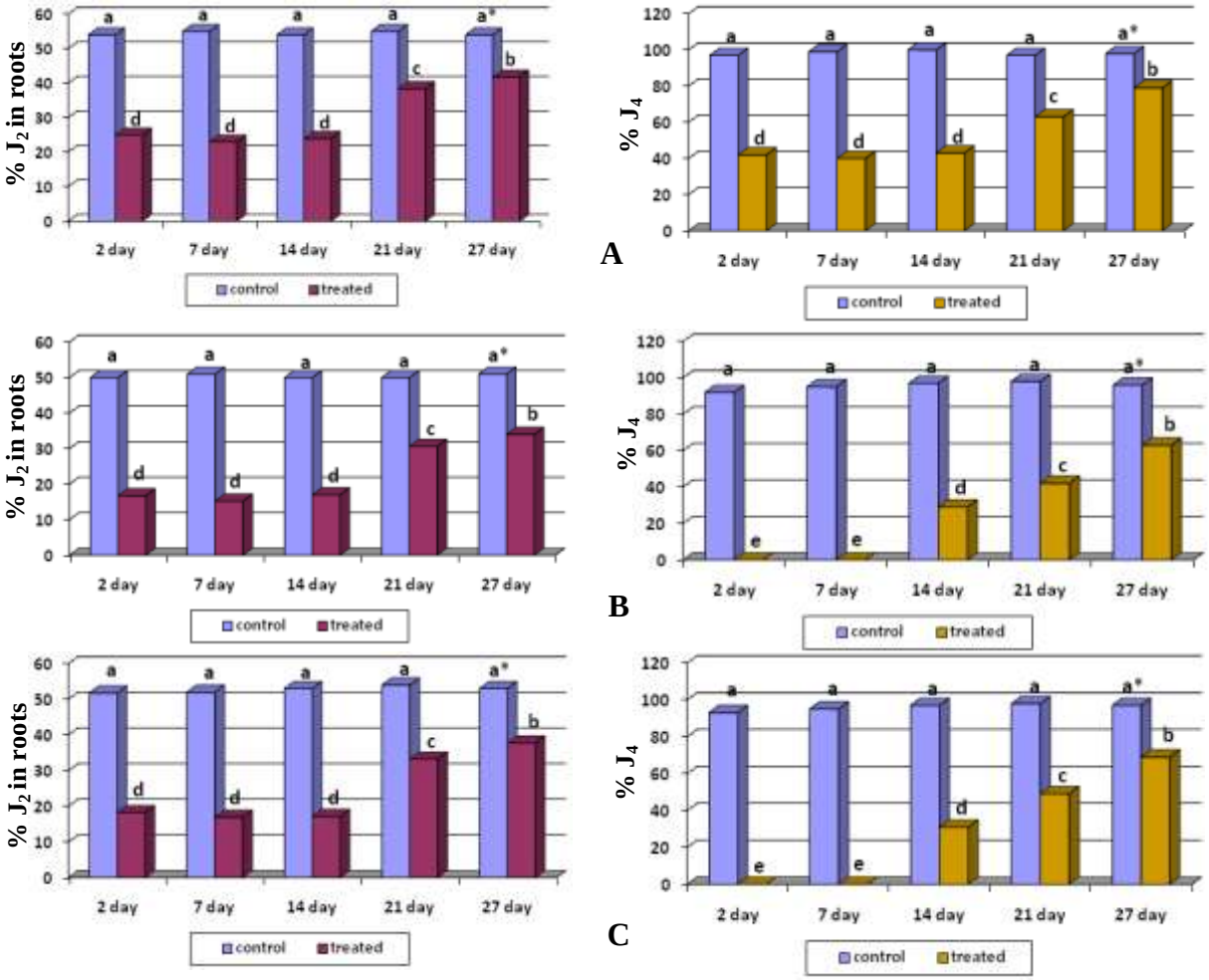

Figure 7. Percentage J_2s and J_4s of *G. pallida* penetrated into the roots of potatoes at: **A** 14±1ºC; **B** 19±1°C; C 24±1°C 65, 31 and 21 days after infection with 1000 second-stage juveniles after different periods of exposure with bacterial suspension *S. plymuthica* isolate 72 ($1.7x10^8$ cells / ml). The percentage of J_4 is calculated as % of infiltrating nematodes; a, b, c…. grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The lowest efficiency of BS in relation to the invasion of J_2s was reported in the infection of plants on the twenty-seventh day after introduction of BS in the rhizosphere of plants, as 37.75% of penetrated J_2s was reported, and in the control variant 53.00% (Fig. 7 /**C**/). In variants when the plants were infected with L2 up to 14 and 7 days after application of BS to *S. plymuthica* isolate 72, no percentage of nematodes reached the stage of J_4s development was reported (Fig. 7 /**C**/).

When treating plants with *S. plymuthica* BS, the efficacy of the bacterium is more pronounced by day 14. This allows the bacterium to grow in the

rhizosphere of plants to optimally prevent invasion of J_2s into the roots. On the 21st and 27th day of infection of the treated plants, the BS of S. *plymuthica* isolate 72 had a nematicidal effect. The results also show that the secondary metabolites released by the bacterium during the growth in the soil act against J_2s, directly killing them or affecting their normal movement and preventing them from penetrating the roots of potato plants. Similar to the results obtained in our experiment, Mochamedova et al., (2016) reported a significant reduction in the number of J_2s penetrates of other nematode species (*M. javanica*) in the roots of eggplant plants treated with the rhizobacter *B. subtilis*.

4.4.1.2. Influence of cell-free filtrate (CFF) of *S. plymuthica* isolate 72 at different temperatures on the J_2s invasion of *G. pallida* in plant roots

The results of the experiments showed that the cell-free filtrate (CFF) of *S. plymuthica* isolate 72 also reduced the number of J_2 *G. pallida* penetrates in the roots of potato plants at all three experimental temperatures (14±1, 19±1 and 24±1°C) (Fig. 8 /**A**, **B**, **C**/).

The results presented in Figure 8 (**A**) of the tested CFF of *S. plymuthica* consisting only of metabolites of the bacterium, at a temperature of 14±1, show that it also significantly reduces the invasion of J_2s in the roots of potato plants compared to controls in all variants. In contrast to BS, the efficacy of the cell-free filtrate of *S. plymuthica* is better expressed compared to the bacterial suspension at the same temperature conditions. The highest efficacy of CFF against the invasion of J_2s in the roots was reported when infecting plants on the seventh day after CFF application into the rhizosphere of plants, as the percentage of penetrated J_2s was 16.20%, and in the control - 56.50% (Fig. 8 /**A**/). The lowest efficacy of CFF in relation to the invasion of J_2s was reported in the infection of plants on the twenty-seventh day after application of CFF in the rhizosphere of plants, as the percentage of penetrated J_2s is 40.50%, and in the control variant respectively 57.00 % (Fig. 9). . The percentage of J_4s (female and male) growing in the treated plants was significantly lower than in the untreated variants. The CFF action of *S. plymuthica* isolate 72 is similar to that of J_2s penetrated into the roots of potato plants (Fig. 8 /**A**/).

The results presented in Figure 8 (**B**) of the tested CFF of *S. plymuthica*, at a temperature of 19±1ºC, show that they significantly reduce the invasion of J_2 in the roots of potato plants compared to the controls in all variants. The action of the cell-free filtrate of *S. plymuthica* is characterized by a similar initial effect of BS, which is confirmed by a reduction of J_2 invasion (penetrated J_2s - 14.33%) in the roots in variant – J_2s infection on the second day after CFF application into the rhizosphere of plants. The highest efficacy of CFF against the invasion of J_2 in the roots was reported when infecting plants on the second day after CFF introduction into the rhizosphere of plants, as the percentage of penetrated J_2 was 14.33%, and in the control - 52.00% (Fig. 8 /**B**/). The lowest efficacy of CFF against J_2s invasion was reported when infecting plants on the

twenty-seventh day after BF introduction into the rhizosphere of plants, as the percentage of penetrated J_2 was 36.00% and in the control variant, respectively 53.00% (Fig. 8 /**B**/). The percentage of J_4s (female and male) development in the treated plants was significantly lower than in the untreated variants. In variants when the plants were infected with J_2 up to 14 and 7 days after application of CFF to *S. plymuthica* isolate 72, no percentage of nematodes reached the stage J_4 development was reported (Fig. 8 /**B**/).

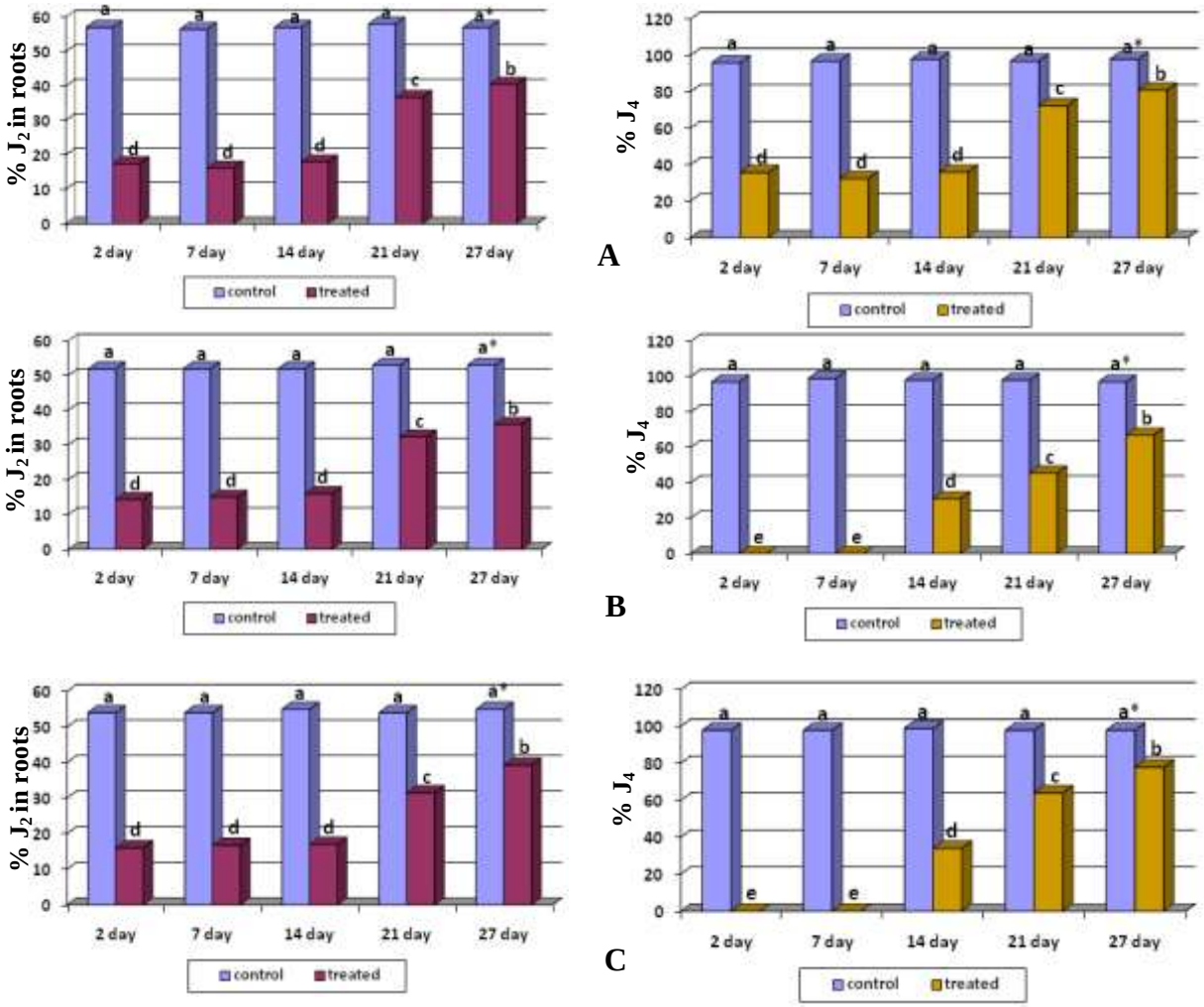

Figure 8. Percentage J_2s and J_4s of *G. pallida* penetrated into the roots of potatoes at: **A** 14±1ºC; **B** 19±1°C; C 24±1°C 65, 31 and 21 days after infection with 1000 second-stage juveniles after different periods of exposure with cell-free filtrate *S. plymuthica* isolate 72 (concentration 5%). The percentage of J_4 is calculated as % of infiltrating nematodes; a, b, c…. grade of significantly according by Duncan's Multiple Range Test $P_{0.05}$

The results presented in Figure 8 (**C**) of the tested CFF of *S. plymuthica* at a temperature of 24±1ºC, show that they reduce the invasion of J_2s in the roots of potato plants compared to the controls in all variants. The highest efficacy of CFF in relation to the invasion of J_2s in the roots was reported when infecting the plants on the second day after CFF application into the rhizosphere of the plants as the percentage of penetrated J_2s was 16.00%, and in the control - 54.00%. The lowest efficacy of CFF against J_2s invasion was reported when

infecting plants on the twenty-seventh day after CFF application into the rhizosphere of plants as the percentage of penetrated J_2s was 39.25% and in the control variant respectively 55.00% (Fig. 8 /**C**/). In variants when the plants were infected with J_2s up to 14 and 7 days after CFF application of *S. plymuthica* isolate 72 no percentage of nematodes reached the stage of J_4s development was reported (Fig. 8 /**C**/ and Fig. 9).

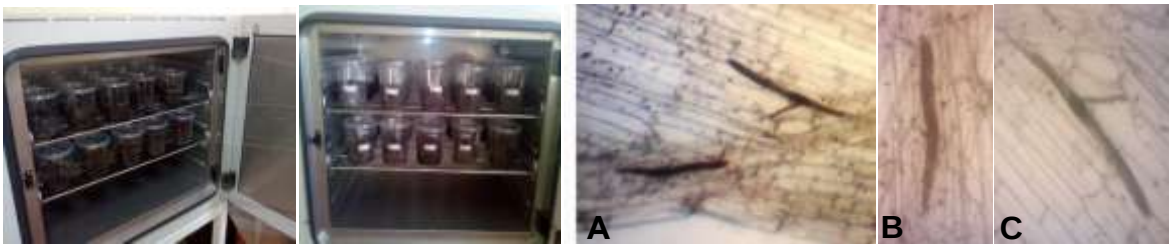

Figure 9. Experiment to determine the efficacy of BS/CFF of *S. plymuthica* isolate 72 on J_2s invasion of *G. pallida* in plant roots at different temperature in a thermostat; **A**, **B**, **C** - invasive second- stage juveniles of *G. pallida* in the tissues of the roots, orig.

4.4.2. Influence of bacterial suspension (BS)/cell-free filtrate (CFF) of *S. plymuthica* isolate 72 on the development of *G. pallida* in plant roots

The influence and effectiveness of BS / BF of *S. plymuthica* isolate 72 on the development and reproduction of *G. pallida* on the roots of potato plants are presented in Table 22. On the 6th and 12th day after infection of plants and introduction of BS/BF *S. plymuthica* isolate 72, only penetrated J_2s were reported in the roots. In the control variant on day 12, 44.8% of the penetrated juvelies passed to stage J_3. As the experiment progressed, the BS / BF efficacy of *S. plymuthica* isolate 72 decreased, with the number of different development stages gradually increasing after the 18th and 24th day of plant treatment. On the 18th day of infection and treatment of plants, 25.0% (for BS of *S. plymuthica* isolate 72) and 21.0% (for BF of *S. plymuthica* isolate 72) of the penetrated juveniles passed to stage J_3 (Table 22). On day 24 of inoculation and application of bacterial preparations into the rhizosphere, only 9.2% (for BS of *S. plymuthica* isolate 72) and 9.5% (for BF of *S. plymuthica* isolate 72) of penetrated juveniles reached to stage J_4, compared to 76.2% in the control (Table 22, Fig. 12).

Table 22. Efficacy of *S. plymuthica* isolate 72 on development of *G. pallida* in potato's roots on different time cafter inoculation with 9 cysts (*Pf* ~ 3 eggs/g soil) and application of 20 ml BS *S. plymuthica* ($1.7x10^8$ cells/ml) и 20 ml CFF *S. plymuthica* (concentration 5%).

Treatments	Reporting period (days)	Development stage of *G. pallida* (%)*			
		J_2	J_3	J_4	
				female	male
S. plymuthica BS	6	100a**	0f	0d	0d
S. plymuthica CFF		100a	0f	0d	0d
Control		100a	0f	0d	0d
S. plymuthica BS	12	100a	0f	0d	0d
S. plymuthica CFF		100a	0f	0d	0d
Control		55.2f	44.8a	0d	0d
S. plymuthica BS	18	75.0c	25.0c	0d	0d
S. plymuthica CFF		79.0b	21.0d	0d	0d
Control		18.9g	45.6a	24.5b	11b
S. plymuthica BS	24	61.3e	29.5b	5.7c	3.5c
S. plymuthica CFF		69.5d	21.0d	6.3c	3.2c
Control		10.5h	13.3e	47.5a	28.7a

* calculated as % of penetrated nematodes in the roots; ** a,b,c…values followed by different letters in the columns differ significantly according to Duncan's Multiple Range Test (P <0.05)

Hatched J_2s in the soil, determined for each sampling date calculated from the cyst count data (Fig. 11), was significantly delayed (P <0.05) in pots treated with BS/BF of *S. plymuthica* compared to control variants. The delay observed during hatching is also reflected in the number of J_2s extracted from the soil, mostly on the 6^{th} and 12^{th} day, when they were reported by 89.5% (6 days after treated with BS), 86.3% (6 day after treated with BF) and 88.7% (12 days after treated with BS), 86.7% (12 days after treated with BF), less J_2s than the control variants (Fig. 11).

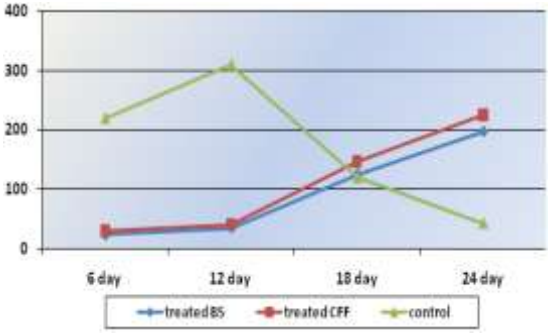

Figure 10. Number of remaining eggs / cysts of G. pallida at different times after infection with 9 cysts (Pk ~ 3 eggs / g soil) and application of 20 ml BS S. plymuthica (1.7x108 cells / ml) and 20 ml BF S. plymuthica (concentration 5%); * a, b, c… values, followed by different letters for each variant, differ significantly according to Duncan's Multiple Range Test (P <0.05).

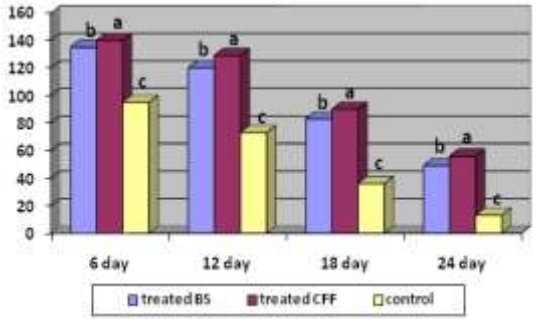

Figure 11. Number L2 of G. pallida extracted from the soil at different times after infection with 9 cysts (Pk ~ 3 eggs / g soil) and application of 20 ml BS S. plymuthica (1.7x108 cells / ml) and 20 ml BF S. plymuthica (concentration 5%).

Extracted J_2s from the soil for variants 18 and 24 days after treatment with BS/CFF of *S. plymuthica* increased sharply, indicating that the bacterium had only an inhibitory effect on eggs and inhibited J_2s hatching (Figs. 10 and 11). The results obtained from the experiments performed on the influence of

BS/CFF *S. plymuthica* isolate 72 on the development of *G. pallida* on the roots of plants most strongly affect J_2s and less J_3s. Treatment of potato plants with BS/CFF of *S. plymuthica* isolate 72, reduces the number of infested invasive second-stage juveniles in the roots and prevents their development to J_4 (males and females) for about 2 weeks. This indicates that the bacterium secretes secondary metabolites, and the cell-free filtrate, consisting only of secondary metabolites, affects the normal movement of J_2s or directly kills them. Treatment of plants with BS/BF of *S. plymuthica* isolate 72 inhibits the hatching of invasive second-stage juveniles, which reduces their number in the soil, determined after extraction during the first 6 to 12 days.

Figure 12. J_4s young females of Globodera pallida formed on the roots of potato plants[4], orig.

S. plymuthica isolate 72 was effective in preventing invasion and reducing the number of nematodes in the roots of the plant and accordingly significantly affected the final number of identified cysts/pots (Table 24, $P < 0.05$). There was clear evidence that the delayed onset affected J_2 invasion into the roots, as the number of different juvenile stages increased gradually as the experiment progressed (Table 22). Twenty-four days after inoculation, only 9.2 to 9.5% (BS and CFF, respectively) of J_2 in plant roots reached to J_4 (young female/male nematodes) in treated plants compared to 76.2% in untreated plants. Although some of the invasive second-stage juveniles can develop to mature individuals (9.2-9.5%), *S. plymuthica* isolate 72 inhibits female fertility by reducing the number of eggs recording in cysts by 40-32% (respectively in the variant with BS and CFF) in comparison with those of the untreated variants ($P<0.05$) (Table 24). Although the final population density of nematodes (*Pf*) in the bacterium-treated variants (13.0 and 13.6 eggs/g soil, respectively with BS and CFF) was 91.0 and 90.3% lower than in the untreated control (141.5 eggs/g soil).

Twenty-four days after inoculation, the weight of the vegetative part of the plants treated with BS and CFF of *S. plymuthica* was similar to those of the control without nematode infection, but the weight of their roots was ~ 11% less (Table 23). However, the weight of the vegetative part and the roots of the treated plants was significantly higher than those not treated with nematodes, respectively by 16.7 and 13.3% ($P < 0.05$). The positive results of plant growth in the first month, when they are most vulnerable, also have a positive effect on

[4] The photos were taken with a Zeiss Stemi 2000-C stereomicroscope equipped with a Zeiss Axio Cam ERc 5s

potato yield (114 days after planting), which is 1.34 and 1.31 times higher, respectively, in the plants treated with the bacterium in comparison with the untreated control (Table 24). No significant differences in tuber weight were observed between plants treated with BS and CFF of *S. plymuthica* and untreated plants without nematode infection (27.4, 27.0 and 27.6 g, respectively).

Table 23. Weight of the vegetative part and root of potato variety Nadezhda, 24 days after inoculation with cysts of *G. pallida* (*Pi* ~ 3 eggs/g soil) and application of 20 ml BS of *S. plymuthica* (~ 10^8 cells/ml) or BF of the bacterium (5%).

Variants	Vegetative part weight (g)	Root weight (g)
S. plymuthica (БС)	12.6a*	11.1a
S. plymuthica (БФ)	12.1a	11.0a
Control 1	10.5b	7.2b
Control 2	12.8a	12.5a

Control 1 - with *G. pallida*; Control 2 - no nematode infection from *G. pallida*; * a,b,c…values, followed by different letters in the columns, differ significantly according to Duncan's Multiple Range Test (P <0.05).

Table 24. Tube weight, number of cysts, number of eggs/cyst, number of eggs/g soil, *Pf* (eggs/g) and reproduction (*Pf*/*Pi*) of *G. pallida* 114 days after inoculation with cysts of G. pallida (*Pi* ~ 3 eggs/g soil) and apply to 20 ml of BS of *S. plymuthica* (~ 10^8 cells/ml) or CFF of the bacterium (5%).

Variants	Tuber weight (g)	Cysts/ pot	Eggs/ cysts	Eggs/g soil	Reduction in *Pf* (%)	*Pf*/*Pi*
S. plymuthica (БС)	27.4a*	60a	130a	13.0a	91.0	4.3
S. plymuthica (БФ)	27.0a	55a	148a	13.6a	90.3	4.5
Control 1	20.4b	393b	216b	141.5b		47.2
Control 2	27.6a	-	-	-		

Control 1 - with *G. pallida*; Control 2 - no nematode infection from *G. pallida*; * a,b,c…values, followed by different letters in the columns, differ significantly according to Duncan's Multiple Range Test (P <0.05).

Based on the first studies on the influence of *S. plymuthica* on the development and reproduction of *G. pallida* in plant roots, it was found that of all stages of development of *G. pallida* invasive second-stage juveniles and third-stage juveniles are most susceptible to the bacterium.

S. plymuthica inhibits the development of J_4 (female/male specimens in plant roots) and reduces the reproductive capacity of PCNs.

The results of the „*in vitro*" and „*in vivo*" experiments give grounds to recommend the application of *S. plymuthica* during the period of active vegetation of the plants, as the moment of application takes into account the development of PCNs, which depends from soil temperature. The best results can be expected if *S. plymuthica* is applied no later than the third-stage juveniles.

A single application of *S. plymuthica* (x10^8 cells/ml) at a dose of 20 ml/plant protects the roots from the invasion of PCNs during the first 24 days and increases the yield by about 1.9 times compared to untreated plants.

The use of the bacterial suspension of *S. plymuthica* should be limited to temperatures above 14°C. At lower temperatures as an alternative can be applying cell-free filtrate of the bacterium *S. plymuthica*.

5. CONCLUSIONS

1. The survey of the areas of the potato plantations in the districts - Sofia, Pazardzhik, Smolyan and Burgas, during the period 2017-2019, confirm the distribution of the potato cyst-forming nematodes of the genus *Globodera*.

2. The highest population density of the studied areas was found in the village of Ravnogor (610 number of cysts/100 g of soil), and the lowest in the village of Vezenkovo (3.5 number of cysts/100 g of soil).

3. Morphologically established and molecularly proven are two species of nematodes of the genus *Globodera* - *Globodera rostochiensis* (golden potato cyst nematode) and *Globodera pallida* (pale potato cyst nematode). The predominant species is *G. pallida*. The species is found in 86.6% in the studied potato production areas.

4. Four varieties (Cronos, Cekin, Gawin, Ovacij) and seven lines (E 1789, E 606, E 1096, E 1809, D 344, D497, D 348) *Solanum tuberosum* are resistant to *G. rostochiensis* (resistance index 6-8). Two varieties (Gandawa and Ivetta) show strong resistance (resistance index 9) to *G. rostochiensis*. Two of the tested varieties Cronoss and Ivetta, as well as line E 1096, E 1809 and E 606 have an established tolerance to *G. pallida* (resistance index 4-5).

5. All 8 tested plant extracts showed nematicidal action against second-stage juveniles (J_2) of *G. rostochiensis* and *G. pallida*, with three of them having the highest efficiency - *Juglans regia* 80.00 and 78.00%, *Ruta graveolens* 69.00 and 67.00% and *Plantago major* 47.00 and 42.00%.

6. „*In vitro*" experiments with plant extracts at different temperatures show that it does not have a significant effect on the inhibitory efficacy on cysts and their toxic efficacy on J_2 of *G. pallida*.

7. GC-MS analysis of plant extracts determined the composition of some of the compounds (mainly non-polar) in them.

8. Twelve isolates of rhizobacteria showed larvicidal action against *G. rostochiensis* and *G. pallida*. *Serratia plymuthica* isolate 72 shows the highest efficacy.

9. Highest degree of inhibition of hatching of J_2 in *G. pallida* (94.50 - 95.75 and 100.00%), BS and CFF of *S. plymuthica* isolate 72 caused in concentration, respectively 10^8 cells/ml and 10.0% at 3 and 6 weeks of exposure. At the same doses, *S. plymuthica* showed the best larvicidal efficacy at 72 hours of exposure - 86.75% and 86.50%, respectively.

10. In the range of 19-24°C, the BS and CFF efficacy of *S. plymuthica* isolate 72 against J_2 of *G. pallida*, at concentrations of 10^8 cells/ml and 5.0% at exposures of 72 hours, respectively, caused the highest J_2 mortality and best

expressed inhibitory efficacy on the hatching of juveniles from cysts at exposure 3 and 6 weeks.

11. At 14°C, the CFF efficacy of *S. plymuthica* on *G. pallida* is better expressed.

12. The nematicidal action of *S. plymuthica* isolate 72 against the invasion of J_2 on the roots of potato plants lasts until the 14^{th} day, and the nematostatic - until the 21^{st} day.

13. The bacterium inhibits the development of female specimens in the roots and reduces the fertility of nematodes. Invasive J_2 and third-stage juveniles (J_3) are most susceptible to *S. plymuthica* isolate 72.

14. The application of *S. plymuthica* should take place during the period of active vegetation of the plants, as the moment of application takes into account the development of PCNs. The best results can be expected if *S. plymuthica* is applied no later than the J_3.

15. Once application of *S. plymuthica* ($x10^8$ cells/ml) - BS at a dose of 20 ml/plant/CFF (5% solution with 20 ml/plant), protects the roots from the invasion of PCNs during the first 24 days and increases the yield by about 1.9 times compared to untreated plants. The use of *S. plymuthica* BS should be limited to temperatures above 14ºC. At lower temperatures as an alternative can be applying cell-free filtrate of the bacterium *S. plymuthica*.

6. CONTRIBUTIONS

I. Original contributions

1. The distribution of the potato cyst-forming nematodes (CCN) of the genus *Globodera* in the Sofia, Pazardzhik, Smolyan and Burgas potato-producing regions of Bulgaria has been studied. For the period 2017-2019, for each of the identified 15 areas infected with these parasites, the following have been identified: Globodera spp. and their population density.

2. A genetic bank of the two species of PCNs distributed in the potato-producing region of the country has been created: *Globodera rostochiensis* and *Globodera pallida*.

3. In the identification of PCNs for the first time in our country the method of polymerase chain reaction (PCR) was applied, using gene-specific primers.

4. Plant extracts and isolates of rhizobacteria as biological control agents against Globodera have been studied.

5. For the first time in the country, the optimal concentrations and temperatures have been established, in which the plant extracts *Juglans regia*, *Ruta graveolens* and *Plantago major* show the highest nematicidal activity against *G. pallida*.

6. The metabolic profiles of *J. regia*, *R. graveolens* and *P. major* were determined by gas chromatography - mass spectrometry (GC-MS).

7. For the first time were established the optimal parameters (concentration and temperature) in which the rhizobacterium *Serratia plymuthica* showed the highest efficacy against *G. pallida*.

8. The period of nematicidal and preventive action of *S. plymuthica* against the invasion of second-stage juveniles (J_2) of *G. pallida* on the roots of potato plants was established.

9. The effect of the rhizobacterium *S. plymuthica* on the development and reproduction of *G. pallida* in plant roots was determined.

10. The application of *S. plymuthica*, for the control of PCN on vegetable crops - potatoes, to be done during the period of active vegetation, in accordance with the development of J_2 - not later than the third-stage juveniles (J_3) of the parasite.

II. Contributions with a confirmatory nature

1. The studies on the morphological and morphometric characteristics of the identified two species of PCN of the genus *Globodera* are of a confirmatory nature.

7. LIST OF PUBLICATIONS ON THE DISSERTATION

1. Kostadin Trayanov, Harry Samaliev, Silvia Valcheva, Strahil Berkov, Milena Nikolova, (2018). The effect of plant extracts on egg hatching and second-stage juvenile motility of potato cyst nematode *Globodera pallida. Journal of Mountain Agriculture on the Balkans, 2018, 21 (2), 257-273*

2. Kostadin Trayanov, Dima Markova, Ivanka Tringovska, Harry Samaliev, (2019). Influence of the Temperature and the Time of Exposure on the Inhibitory Effect of *Serratia plymuthica* on the Potato Cyst Nematode *Globodera pallida. Journal of Mountain Agriculture on the Balkans, 2019, 22 (1), 348-357*

3. Trayanov, K., Samaliev, H., Kostova, M., Bojinov, B. and Besheva, A. (2020). Morphological and molecular identification of potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida* in Bulgaria. *Bulgarian Journal of Agricultural Science, 26 (No 2) 2020, 416–422*

4. Kostadin Trayanov and Milena Kostova, (2020). ISSR molecular markers for the study of the genetic diversity in bulgarian populations of PCN from genus *Globodera*. *Agricultural Sciences, Volume 12 Issue 27 2020, 25-28*

PLANT PARASITIC NEMATODES OF THE GENUS *GLOBODERA* SKARBILOVICH, 1959 ON POTATOES IN BULGARIA

Summary

Kostadin Kirilov Trayanov

Soil samples from 15 areas located in Sofia, Pazardzhik, Smolyan and Burgas potato-producing regions in Bulgaria have been investigated and in all of

them have been identified potato cyst nematodes (PCN) from genus *Globodera*, namely: *G. rostochiensis and G. pallida*. Morphological and molecular date of the two identified species of PCN have been presented.

Studies have been performed to establish the resistance of different varieties and lines (Bulgarian selection) of potatoes to location population of the two parasites. The lines *Solanum tuberosum* - E 1096, E 1809 and E 606 have a resistance to *G. rostochiensis* and partial resistance to *G. pallida*.

The influence of the rhizobacterium *Serratia plymuthica* and three plant extracts *Juglans regia, Ruta graveolens* and *Plantago major* on the potato cyst nematode *G. pallida* was investigated. *In vitro*, exposure of cysts of *G. pallida* to bacteria resulted in reduced hatching of second stage juveniles (J_2) and J_2 exposed to the bacteria or plant extracts were paralyzed or even killed as the dosage and time of exposure increased. The effect was more expressed at 24 and 19°C than at 14°C. However, cell-free filtrate of the bacterium irreversibly prevented hatching even at 14^oC. In *in vivo* experiments, in closed containers and in pots, *S. plymuthica* delayed hatching of *G. pallida* J_2 and preventing their invasion in potato roots. The application of *S. plymuthica* at the beginning of the growing season, before the formation of third stage juveniles (J_3) was increased the potato yield with 26%, in comparison with the control.