doi: https://doi.org/10.15407/microbiolj82.04.041

ENDOPHYTES FROM MEDICINAL PLANTS AS BIOCONTROL AGENTS AGAINST *FUSARIUM* CAUSED DISEASES

Vyacheslav Shurigin[1], Dilfuza Egamberdieva[1], Sitora Samadiy[1], Gulsanam Mardonova[1], Kakhramon Davranov[2]

[1] National University of Uzbekistan,
4 University Str., Almazar district, Tashkent, 100174, Uzbekistan
[2] Institute of Microbiology of AS RUz,
7B Kadyriy Str., Tashkent, 700128, Uzbekistan
e-mail:slaventus87@inbox.ru

Objective. *The objective of this research was to reveal bacterial endophytes isolated from some medicinal plants, which have biocontrol activity against phytopathogenic fungi Fusarium solani and Fusarium oxysporum.* ***Methods.*** *The bacterial endophytes were isolated from shoots and roots of medicinal sage (Salvia officinalis L.), fennel (Foeniculum vulgare Mill.), tansy (Tanacetum vulgare L.), medicinal calendula (Calendula officinalis L.), pale yellow iris (Iris pseudacorus L.), horseradish (Armoracia rusticana G. Gaertn., B. Mey. & Scherb.). The endophytes were isolated after grinding of the surface sterilized roots or shoots, dilution of the cell juice in phosphate buffered saline and spreading the suspension on Tryptic Soy Agar. The grown colonies were picked up in 4 days. The endophytes were checked for in vitro antagonistic activity against phytopathogenic fungi Fusarium solani and F. oxysporum on Petri dishes using Chapek medium. The endophytes were examined for the ability to inhibit the same phytopathogenic fungi in pot experiment. The soil was infected with fungi and the seeds of cotton and cucumber were inoculated with endophytes. 4 weeks after sowing the number of survived plants was defined. The bacterial isolates were identified using 16S rRNA gene analysis. For DNA extraction the heat treatment method was used. Extracted DNA was used as template for 16S rRNA gene analysis. Sequencing was performed using ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The 16S rRNA sequences were identified using BLAST and comparisons with the GenBank nucleotide data bank from the NCBI. The strains were checked for production of HCN, hydrolytic enzymes (chitinase, lipase and protease) and siderophores. All experiments were conducted in 3 replications.* ***Results.*** *In total 151 isolates were obtained from 6 medicinal plants. Only 20 isolates inhibited fungi Fusarium oxysporum, Fusarium solani or both of them. HRT18, AST3, BST3 and FST7 bacterial isolates were the most effective in biocontrol activity against disease caused by Fusarium oxysporum on a cotton plants in pot experiments. The most effective bacterial isolates having biocontrol activity against disease caused by Fusarium solani on a cucumber were KRT5, FRT13, FRT12, CST6 and BST10. In total 20 bacterial isolates were identified using 16S rRNA gene analysis as follows: Serratia ficaria KRT5, Raoultella ornithinolytica KRT12, Agrobacterium tumefaciens HRT9, Klebsiella oxytoca BST12, Pseudomonas putida FRT13, Pseudomonas kilonensis FRT12, Pseudomonas lini FRN1, Agrobacterium vitis HRT14, Bacillus toyonensis HRT5, Pseudomonas azotoformans HRT18, Pseudomonas syringae AST3, Klebsiella pneumoniae BST3, Xanthomonas translucens HRT10, Enterobacter ludwigii KRT16, Paenibacillus typhae KRN1, Pseudomonas extremaustralis CST6, Pseudomonas oryzihabitans FST7, Pseudomonas chlororaphis BST10, Agrobacterium vitis KRN2, and Pseudomonas jessenii BRT3. It was revealed that all strains having biocontrol properties could produce at least one of the tested substances (HCN, siderophores, chitinase, protease and lipase), which are considered as antifungal and by means of which the biological control can be realized.* ***Conclusions.*** *The strains possessing high biocontrol activity towards phytopathogenic fungi can be proposed for usage as a part of biofungicides after field experiments.*

Keywords: medicinal plants, endophytes, bacteria, Fusarium oxysporum, Fusarium solani, growth inhibition.

From the ancient times medicinal plants are being used all over the world as remedies against various diseases, such as respiratory, gastrointestinal, urinary, skin disorders etc. [1]. It is known that medicinal plants harbor the endophytic communities by synthesizing diverse bioactive compounds that can help plants under stressful conditions [2].

Endophytes are microbial communities which dwell in the healthy plant tissues such as stems, roots, leaves, flowers and seeds without affecting physiological plant functions and not causing any disease symptoms to the plant tissues. Under normal conditions, endophytes have important roles in host plant growth either by secondary metabolite or nutrient assimilation or by preventing induction of plant disease symptoms by different pathogens [3, 4]. There are many reports about different bacterial endophytic strains such as *Bacillus subtilis* isolated from *Speranskia tuberculata* (Bge.) Baill which has antagonistic effect *in vitro* against pathogen *Botrytis cinerea,* which cause rotting of tomato fruits during storage [5]. The endophytes, such as *Bacillus cepacia* and *Burkholderia pyrrocinia* JK-SH007*,* were used in biocontrol study against poplar canker [6].

Thus, medicinal plants are potential source of endophytes with antifungal activity against phytopathogenic fungi. The endophytes having such activity can be used as potential biocontrol agents like biofungicides.

The purpose of this work was to reveal bacterial endophytes isolated from some medicinal plants which has biocontrol activity against phytopathogenic fungi *Fusarium solani* and *Fusarium oxysporum*.

Materials and Methods

Isolation of the endophytic bacteria. The bacterial endophytes were isolated from shoots and roots of medicinal sage (*Salvia officinalis* L.), fennel (*Foeniculum vulgare* Mill.), tansy (*Tanacetum vulgare* L.), medicinal calendula (*Calendula officinalis* L.), pale yellow iris (*Iris pseudacorus* L.), horseradish (*Armoracia rusticana* G .Gaertn., B.Mey. & Scherb.).

Root and shoots (stem and leaves) were separated with a sterile scalpel and sterilized with 99.9 % ethanol for 2 min, subsequently with 10 % NaClO and rinsed five times in sterile distilled water. The sterile leaves and root (10 g fresh weight) were cut into 3–4 cm pieces and macerated using a sterile mortar and pestle [7]. The macerated tissue were (1 g) transferred into plastic tubes with 9 ml sterile phosphate buffered saline (PBS) (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and shaken for 1 minute using Vortex Biosan B-1. A 100 µl aliquots from dilutions (10^1–10^5) were spread on Tryptic Soy Agar (TSA) (BD, Difco Laboratories, Detroit, USA) supplemented with nystatin in concentration 50 µg/ml, and plates were incubated for four days at 28° C. The content of 30 % TSA (g/l): pancreatic digest of casein – 4.5, enzymatic digest of soya bean – 1.5, NaCl – 5.0, agar – 15.0. After four days colonies with different shape, colour and consistency were picked up, and accurately transferred by streaking on nutrient agar plates and incubated for the next 72 hours to check the purity of isolates. Visually homogenous colonies of different sizes, shapes and colours were used for DNA isolation. To test the sterility of the plant parts outer surface after sterilization with ethanol, 2 uncut pieces of root and leaf were put on agar media and the absence of any colonies after 72 h confirmed that sterilization was successful.

Determination of in vitro antagonistic activity of bacteria against phytopathogenic fungi (plate experiment). As pathogenic fungi we used *Fusarium solani* and *Fusarium oxysporum* from the collection of the department of Microbiology and biotechnology. The strains of bacterial endophytes were checked *in vitro* on presence of antagonistic activity against fungi mentioned earlier using the plate method. The fungi were cultivated on agar Chapek medium at 28° C within 5–7 days. Agar disks with grown fungi culture were cut on small squares (with the side size 7–8 mm) and placed in the center on Petri plates (9 cm in diameter). Bacteria were cultivated on Peptone agar medium passaged to test plates with the same medium perpendicularly to fungi. The plates were incubated at 28° C within 7 days until the fungi covered control plates without bacteria. Antifungal activity was measured as width of growth inhibition zone between fungi and test bacteria.

Determination of bacteria strains ability for biological control of phytopathogenic fungi causing plants root diseases (pot experiment)*.* Approximately one third of 7-day fungi culture (*Fusarium solani* and *Fusarium oxysporum*) grown on Petri plates with agar Chapek medium homogenized and used for inoculation of 200 ml of liquid Chapek medium in Erlenmeyer flask (500 ml). After growth within 6 days at 28° C at aeration, the grown fungal mycelium was filtered from the nutrient medium through paper filters and suspended in sterile water (200 ml). Received suspension was separately stirred with the soil from

calculation of 100 ml/1 kg of soil. *Fusarium solani* was used for cucumber infecting and *Fusarium oxysporum* – for cotton plant infecting.

Cotton and cucumber seeds were sterilized by exposition in 70 % ethanol during 5 minutes and in 0.1 % $HgCl_2$ during 1 minute, washed 3 times in sterile water, and left for swelling for 6 hours at room temperature (28° C). Seeds were inoculated with bacteria by their wetting in bacterial suspension with concentration of bacteria – $1x10^6$ CFU/ml [8], whereas control seeds were kept in sterile liquid peptone medium within 15 minutes. 100 seeds were used for each combination of bacteria and fungi. The soil from the Botanical garden of NUUz was used for the experiment. 4 seeds were sowed into each plastic pot (diameter – 10 cm, depth – 15 cm). Each pot contained 300 g of soil. Plants were cultivated in special growth chamber at 21º C in a nighttime and at 28º C in a daytime. The watering was carried out when required. The quantity of survived plants was defined 4 weeks after sowing.

Identification of endophytes

DNA isolation. For DNA extraction the heat treatment method [9] was used. The small parts of colonies were transferred into 2 ml Eppendorf tubes with 1.5 ml of sterile MQ-water and mixed with vortex Biosan B-1 for 10 sec. The tubes were incubated at 90° C during 20 min in a Dry Block Heater (IKA Works) and centrifuged at 12,000 rpm for 5 min. The DNA-containing supernatant was taken and stored at -20°C. The presence of DNA was checked by horizontal Gel electrophoresis (0.8 % agarose) and quantified with NanoDrop™ One (ThermoFisher Scientific).

Polymerase chain reaction (PCR). Extracted DNA was used as template for 16S rRNA gene analysis. The 16S rRNA genes were amplified with PCR using the following primers: 16SF 5'-GAGTTTGATCCTGGCTCAG-3' (Sigma-Aldrich, St. Louis, MO) and 16SR 5'-GAAAGGA-GGTGATCCAGCC-3' (Sigma-Aldrich, St. Louis, MO). Each 25 µl of reaction mixture contained 1µl with 15–40 ng of DNA; 5x One Taq standard reaction buffer (BioLabs, New England) – 5 µl; 10 mM dNTP mix (Thermo Scientific) – 0.5 µl; 10 mM primer 16SF (Merck) – 0.5 µl; 10 mM primer 16SR (Merck) – 0.5 µl (25 pmol/ml); 0.1% bovine serum albumin (TaKaRa Bio Inc.) – 1 µl; One Taq polymerase (BioLabs, New England) – 0.125 µl; MQ water – 16.375 µl. The PCR was performed using PTC-200 thermocycler (BioRad). PCR program was as follows: primary heating step for 30 s at 94° C, followed by 30 cycles of denaturation for 15 s at 94° C, annealing for 30 s at 55° C, and extension for 1.5 min at 68° C, then followed by the final step for 20 min at 68° C. The PCR amplified products were examined by electrophoresis using 0.8 % agarose gel containing GelRed.

Sequencing and phylogenetic analysis. Before being sequenced, the PCR products were purified with the USB® ExoSAP-IT® PCR Product Cleanup Kit (Affymetrix, USB® Products, USA) according to the protocol of the manufacturer.

Sequencing was performed using ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) by the protocol of the manufacturer. Received data were analyzed and corrected using Chromas (v. 2.6.5) software. Corrected sequences were merged manually using EMBOSS Explorer (http://emboss.bioinformatics.nl/). The sequences were identified using the basic local alignment search tool (BLAST) and comparisons with the GenBank nucleotide data bank from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

The study of biocontrol properties of the most effective bacteria with high biocontrol activity. The isolated endophytes were checked for shape, Gram staining and motility by their observing under phase-contrast microscope Leica DM750.

Production of HCN. To check the HCN production, the strains were cultivated on Triptic soy agar (TSA) medium. Sterile filter paper saturated with solution of 1 % picric acid and 2% sodium carbonate were stick to inner surface of Petri dish cover. Petri dishes were covered with parafilm and incubated at 28° C within 3 days. The change of paper colour from yellow to dark blue indicated the presence of HCN [10].

Production of hydrolytic enzymes. For determination of chitinolytic activity strains were cultivated on the following medium (g/l of distilled water): yeast extract – 0.5; $(NH_4)_2SO_4$ – 1; $MgSO_4x7H_2O$ – 0.3; KH_2PO_4 – 1.36; agar – 1.5. As a carbon source we put colloidal chitin (0.5 %) into medium. The strains were cultivated at 28° C. Efficiency of chitin hydrolysis was defined by appearance of clarification zones around the colonies [11].

The lipase activity of bacterial strains was checked using Tween lipase indicator. Bacterial strains were cultivated on Tryptic Soy Agar with addition of 2 % of Tween 80 at 28° C [12]. In 5 days, Tween destruction was found in the form of pure ring around bacterial colony that indicated the

presence of lipase activity in the strain.

Protease production was detected at strains cultivation on TSA/20 (1/20 part of Tryptic Soybean broth with 1.5 % agar) with addition of skim milk to final concentration of 5 %. The ring appeared around colonies on the first-second day of cultivation and indicated the presence of extracellular protease [13].

The strains ability to produce siderophores. 60.5 mg of dye Chrome Azurol Sulphonate (CAS) were dissolved in 50 ml of distilled water and stirred with 10 ml of iron (III)-containing solution (1 mmol $FeCl_3 \cdot 6H_2O$ and 10 mmol HCl). At stirring solution was added to 72.9 mg HDTMA (hexadecyl-3-methylammonium bromide), dissolved in 40 ml of distilled water. The received dark-blue solution was sterilized by autoclaving [14]. The mixture of 750 ml distilled water and 100 ml of salts solution (Na_2HPO_4 – 60 g/l, KH_2PO4 – 30 g/l, NaCl – 5 g/l, NH_4Cl – 10 g/l, 2 ml of 1 M $MgSO_4$, 20 ml of 20 % glucose and 100 µl of 1M $CaCl_2$), 15 g of agar and 10.29 g of 0.1 M Tris-HCl mixed in solution, pH 6.8. After cooling to 50° C we added 30 mg of peptone as specific source of carbon. Dye solution was added lengthways on a glass wall with slow stirring for foaming prevention.

Bacteria strains were removed from slopes and transferred to 50 ml flasks with 25 ml of physiological solution from calculation 1 slope/25 ml. Received suspension was carefully stirred. For CAS analysis holes were made in blue agar in Petri dishes; 200 µl of bacterial suspension where poured into holes. Plates were incubated in darkness at 28° C within 48 hours and checked on growth presence and formation of orange rings around colonies.

Statistical analysis. All experiments were conducted in 3 replications. Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 2010. Mean comparisons were conducted using the least significant difference (LSD) test (P = 0.05).

Results

Isolated bacterial endophytes. In total we obtained 151 isolates from 6 medicinal plants (Table 1).

As you can see from table 1, we isolated bacteria separately from shoots (stem with leaves) and roots of the plants. In total 22 bacteria were isolated from *Salvia officinalis* L., 25 – from *Foeniculum vulgare* Mill., 19 – from *Tanacetum vulgare* L., 29 – from *Calendula officinalis* L., 34 – from *Iris pseudacorus* L., 22 – from *Armoracia rusticana* G.Gaertn., B.Mey. & Scherb.

Inhibition of phytopathogenic fungi by bacterial endophytes. All isolated bacterial endophytes were checked for their ability to inhibit the growth of two phytopathogenic fungi obtained from the Institute of Microbiology of Academy of Sciences of the Republic of Uzbekistan. Only 20 strains from all isolated endophytes inhibited fungi *Fusarium oxysporum*, *Fusarium solani* or both of them. The results presented in Table 2 and Fig. 1, 2.

Table 1

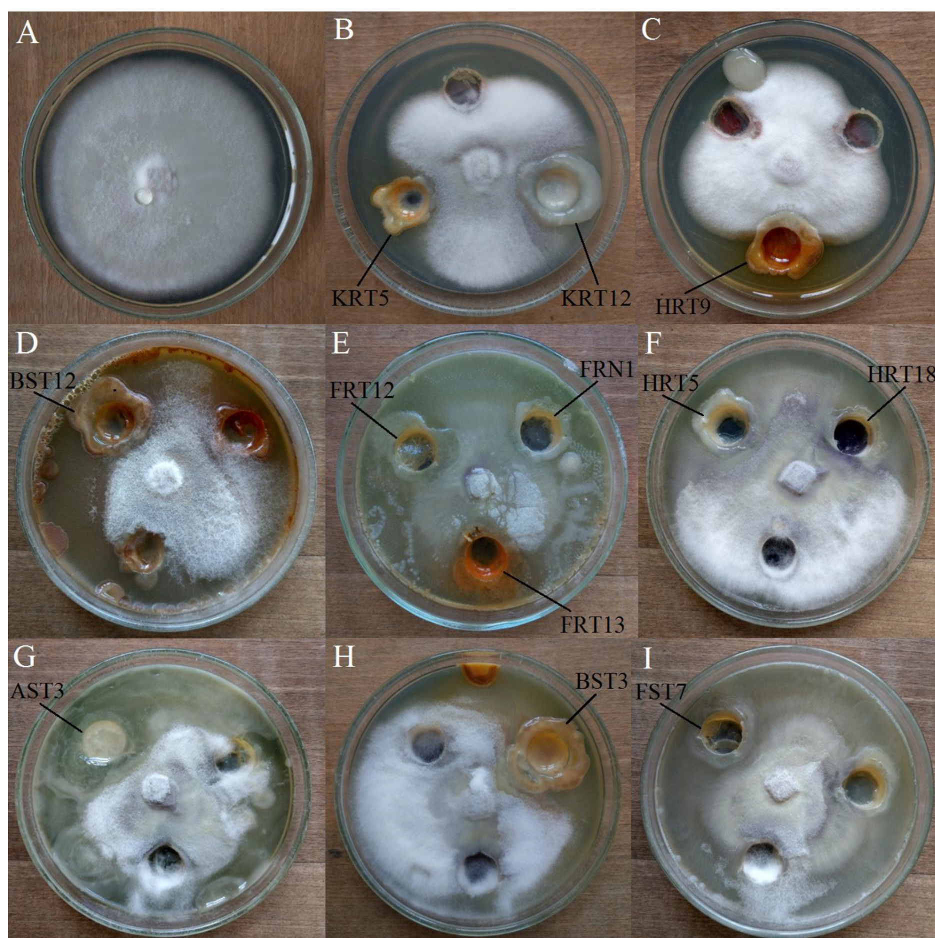
Medicinal plants and isolated endophytic bacteria

Medicinal plants				Isolated bacteria	Total number of endophytes
No	**Latin name of a plant**	**Common name of a plant**	**Source of isolation**		
1	*Salvia officinalis* L.	Medicinal sage	Shoots	AST1-AST7	22
			Roots	ART1-ART15	
2	*Foeniculum vulgare* Mill.	Fennel	Shoots	BST1-BST15	25
			Roots	BRT1-BRT10	
3	*Tanacetum vulgare* L.	Tansy	Shoots	CST1-CST6	19
			Roots	CRT1-CRT13	
4	*Calendula officinalis* L.	Medicinal calendula	Shoots	FST1-FST16	29
			Roots	FRT1-FRT13	
5	*Iris pseudacorus* L.	Pale yellow iris	Shoots	HST1-HST16	34
			Roots	HRT1-HRT18	
6	*Armoracia rusticana* G.Gaertn., B.Mey. & Scherb.	Horseradish	Shoots	KRN1-KRN5	22
			Roots	KRT1-KRT17	

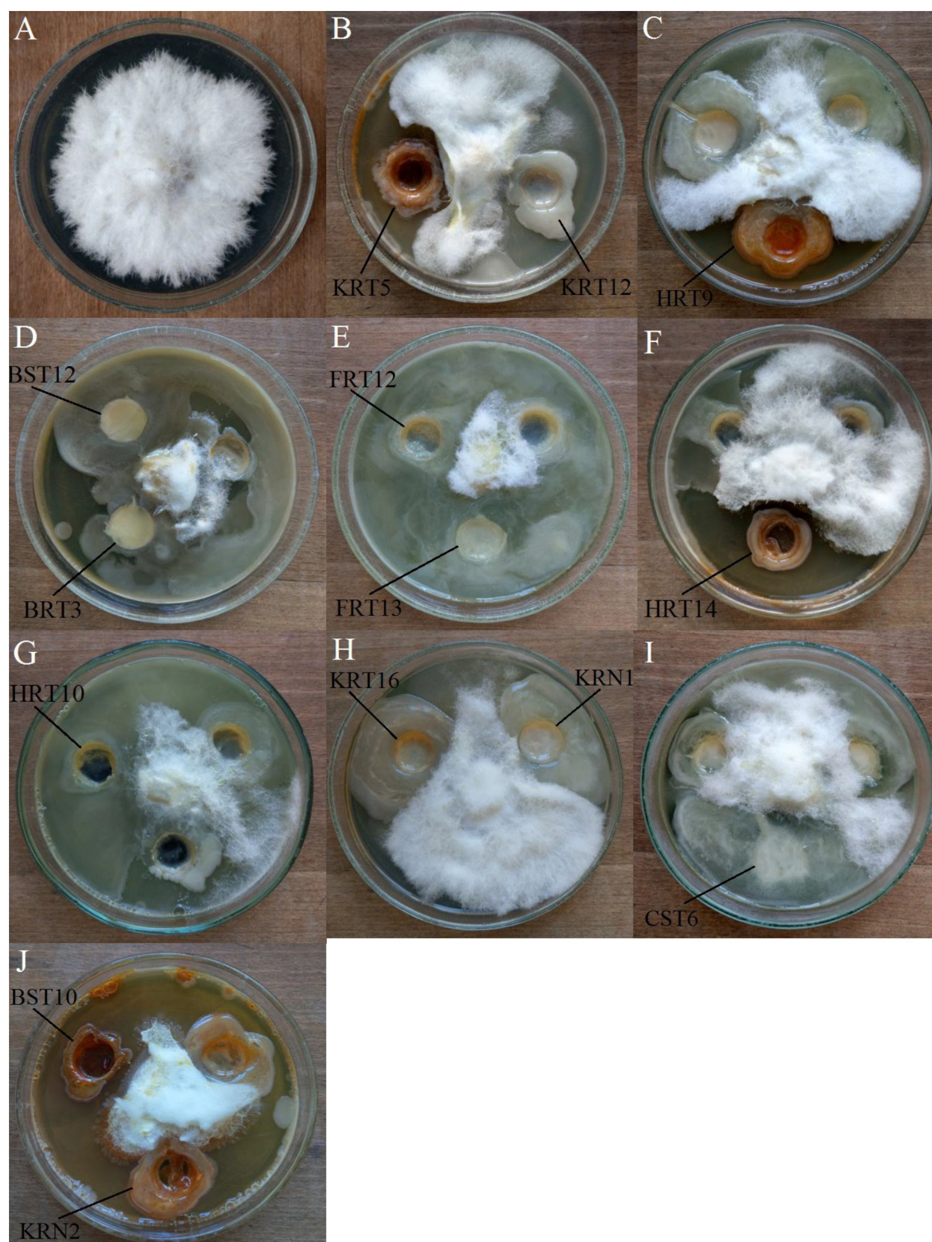
Table 2

Inhibition of phytopathogenic fungi by bacterial endophytes

No	Bacterial endophytes	Fungi growth inhibition zone (mm)	
		Fusarium oxysporum	*Fusarium solani*
1	KRT5	6	10
2	KRT12	5	7
3	HRT9	6	8
4	BST12	6	7
5	FRT13	5	10
6	FRT12	6	8
7	FRN1	4	–
8	FST7	7	–
9	HRT5	6	–
10	HRT18	8	–
11	AST3	8	–
12	BST3	10	–
13	HRT10	–	8
14	KRT16	–	4
15	KRN1	–	2
16	CST6	–	12
17	HRT14	–	6
18	BST10	–	8
19	KRN2	–	5
20	BRT3	–	5



F i g. 1. The inhibition of *Fusarium oxysporum* by endophytic bacteria: a) control (without bacteria), b) KRT5 and KRT12, c) HRT9, d) BST12, e) FRT12, FRT13 and FRN1, f) HRT5 and HRT18, g) AST3, h) BST3, i) FST7



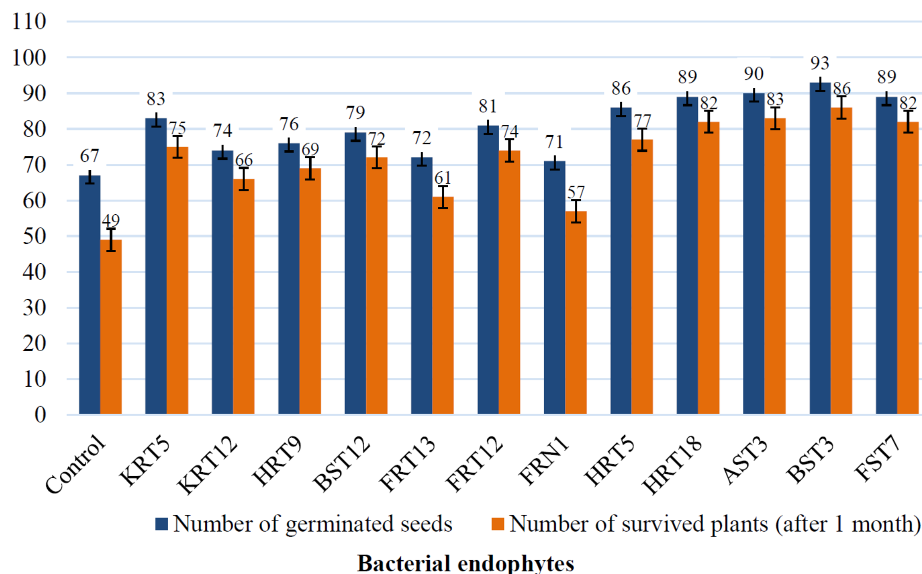
F i g. 2. The inhibition of *Fusarium solani* by endophytic bacteria: a) control (without bacteria), b) KRT5 and KRT12, c) HRT9, d) BST12 and BRT3, e) FRT12 and FRT13, f) HRT14, g) HRT10, h) KRT16 and KRN1, i) CST6, j) BST10 and KRN2

Biological control of Fusarium caused diseases in cotton and cucumber plants using bacterial endophytes. The bacterial isolates according to their ability to inhibit phytopathogenic fungi *in vitro* were checked for biocontrol activity against these fungi in pot experiment. The soil was infected with the fungi and the seeds were inoculated with bacterial isolates in the form of bacterial suspension in a culture liquid in concentration of $1x10^6$ CFU/ml.

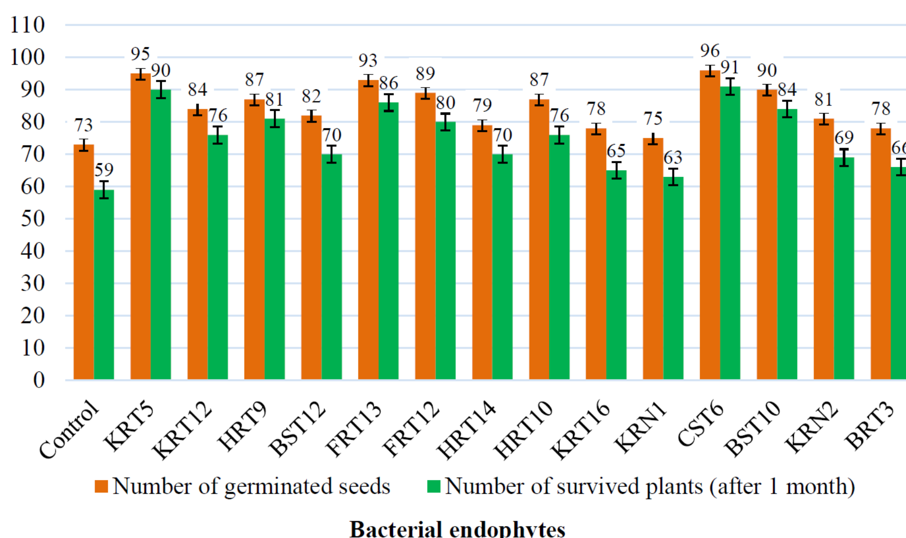
We used cotton plant seeds as a potential susceptible to *Fusarium oxysporum*, as far as this fungi cause Fusarium wilt disease. The results are shown in the Fig. 3.

The most effective bacterial isolates with biocontrol activity against disease caused by *Fusarium oxysporum* on a cotton plant were HRT18, AST3, BST3, and FST7. These bacteria increased the number of germinated seeds from 67 in control (without inoculation) up to 89–93 after bacterial inoculation. The number of survived plants increased from 49 in control up to 82–86 after bacterial inoculation.

We used cucumber seeds as a potential plant susceptible to *Fusarium solani*, as far as this fungi cause *Fusarium* foot rot. The results are shown in the Fig. 4.



F i g. 3. Biological control of *Fusarium oxysporum* infecting cotton plants by bacterial endophytes



F i g. 4. Biological control of *Fusarium solani* infecting cucumber plants by bacterial endophytes

The most effective bacterial isolates having biocontrol activity against disease caused by *Fusarium solani* on a cucumber were KRT5, FRT13, FRT12, CST6 and BST10. These bacteria increased the number of germinated seeds from 73 in control (without inoculation) up to 89–96 after bacterial inoculation. The number of survived plants increased from 59 in control up to 80–91 after bacterial inoculation.

Results of the endophytic bacteria identification. In total 20 bacterial isolates having biocontrol activity against phytopathogenic fungi were identified using 16S rRNA gene analysis (Table 3).

As a result of analysis all 16S rRNA gene sequences were compared with bacterial strains registered in GenBank using BLAST alignment tool and identified as follows: *Serratia ficaria* KRT5, *Raoultella ornithinolytica* KRT12, *Agrobacterium tumefaciens* HRT9, *Klebsiella oxytoca* BST12, *Pseudomonas putida* FRT13, *Pseudomonas kilonensis* FRT12, *Pseudomonas lini* FRN1, *Agrobacterium vitis* HRT14, *Bacillus toyonensis* HRT5, *Pseudomonas azotoformans* HRT18, *Pseudomonas syringae* AST3, *Klebsiella pneumoniae* BST3, *Xanthomonas translucens* HRT10, *Enterobacter ludwigii* KRT16, *Paenibacillus typhae* KRN1, *Pseudomonas extremaustralis* CST6, *Pseudomonas oryzihabitans* FST7, *Pseudomonas chlororaphis* BST10,

Agrobacterium vitis KRN2 and *Pseudomonas jessenii* BRT3.

Biocontrol properties of bacterial endophytes. It is apparently from the obtained results (Table 2, Fig. 1, 2) that bacterial endophytes possess antifungal activity against phytopathogenic fungi *Fusarium oxysporum* and *Fusarium solani*. Therefore we decided to study bacterial factors (hydrolytic enzymes, HCN and siderophores production) realizing biological control of phytopathogenic fungi (Table 4).

Hydrolytic enzymes and HCN production. Bacterial strains were checked for production of volatile HCN and extracellular enzymatic activity (Table 4). Bacterial strains produced HCN and different hydrolytic enzymes: lipase, protease and chitinase.

HCN producing strains: AST3, BST12, BST10, CST6, FRT13, FRT12, FST7, HRT9, HRT5, HRT18 and KRT12. Lipase producing strains: BST12, BST3, FRT13, FRT12, FST7, HRT9, HRT14, HRT18, HRT10, KRT5 and KRT16. All strains excluding HRT14 and KRN1 produced protease. Chitinase producing strains: AST3, BST12, BST3, BST10, CST6, FRT13 and KRT12.

Siderophores production. The endophytes were checked for siderophores production by means of chrome azurol sulfonate (CAS) analysis (Table 4). At strains cultivation on plates with CAS agar, the growth of colonies and formation of orange rings surrounding colonies was observed, indicating production of siderophores. It was found that at presence of HDTMA, CAS is competitive in metal chelation at pH below neutral while iron hydroxide has higher stability at pH above 7.

The following siderophores-producing strains were detected: AST3, BRT3, BST3, BST10, CST6, FRT12, FRT13, FST7, FRN1, HRT14, HRT18, KRN2, KRT5, KRT12 and KRN1.

Discussion. All isolates were checked for *in vitro* fungi growth inhibition. FST7, HRT18, AST3 and BST3 strains had the highest growth inhibition activity against *Fusarium oxysporum*. KRT5, HRT9, FRT13, FRT12, HRT10, CST6 and BST10 strains had the highest growth inhibition activity against *Fusarium solani*. Some strains could inhibit both *Fusarium oxysporum* and *Fusarium solani:* KRT5, KRT12, HRT9, BST12, FRT13 and FRT12.

Table 3

Sequence similarities of the isolated endophyte bacteria with sequences registered in GenBank

No	Isolated endophytes	Closest match among bacteria (16S rRNA genes) (GenBank)		
		Species	Accession number	Identity
1	FRT13	*Pseudomonas putida*	CP000949.1	99.9
2	FRT12	*Pseudomonas kilonensis*	MH165354.1	99.7
3	FRN1	*Pseudomonas lini*	MH165352.1	99.9
4	AST3	*Pseudomonas syringae*	AB680547.1	99.9
5	HRT18	*Pseudomonas azotoformans*	MN758765.1	99.9
6	CST6	*Pseudomonas extremaustralis*	KY939745.1	99.8
7	FST7	*Pseudomonas oryzihabitans*	LC015573.1	99.9
8	BST10	*Pseudomonas chlororaphis*	JX477174.1	99.9
9	BRT3	*Pseudomonas jessenii*	EU019982.1	99.6
10	BST12	*Klebsiella oxytoca*	MK212915.1	99.6
11	BST3	*Klebsiella pneumoniae*	MN691776.1	99.9
12	KRT12	*Raoultella ornithinolytica*	CP038281.1	99.7
13	HRT9	*Agrobacterium tumefaciens*	CP042275.1	99.8
14	HRT14	*Agrobacterium vitis*	MH197382.1	99.9
15	KRN2	*Agrobacterium vitis*	KC196472.1	99.5
16	KRN1	*Paenibacillus typhae*	MH165369.1	99.9
17	HRT5	*Bacillus toyonensis*	MH197375.1	99.9
18	KRT5	*Serratia ficaria*	MH165373.1	99.9
19	HRT10	*Xanthomonas translucens*	KX507148.1	99.4
20	KRT16	*Enterobacter ludwigii*	MN641475.1	99.9

Table 4

Biocontrol properties of endophytes

No	Bacterial endophytes	HCN	Side-rophores	Lipase	Protease	Chitinase
1	*Pseudomonas syringae* AST3	+	+	–	+	+
2	*Pseudomonas jessenii* BRT3	–	+	–	+	–
3	*Klebsiella oxytoca* BST12	+	–	+	+	+
4	*Klebsiella pneumoniae* BST3	–	+	+	+	+
5	*Pseudomonas chlororaphis* BST10	+	+	–	+	+
6	*Pseudomonas extremaustralis* CST6	+	+	–	+	+
7	*Pseudomonas putida* FRT13	+	+	+	+	+
8	*Pseudomonas kilonensis* FRT12	+	+	+	+	–
9	*Pseudomonas oryzihabitans* FST7	+	+	+	+	–
10	*Pseudomonas lini* FRN1	–	+	–	+	–
11	*Agrobacterium tumefaciens* HRT9	+	–	+	+	–
12	*Agrobacterium vitis* HRT14	–	+	+	–	–
13	*Bacillus toyonensis* HRT5	+	–	–	+	–
14	*Pseudmonas azotoformans* HRT18	+	+	+	+	–
15	*Xanthomonas translucens* HRT10	–	–	+	+	–
16	*Agrobacterium vitis* KRN2	–	+	–	+	–
17	*Serratia ficaria* KRT5	–	+	+	+	+
18	*Raoultella ornithinolytica* KRT12	+	+	–	+	+
19	*Enterobacter ludwigii* KRT16	–	–	+	+	–
20	*Paenibacillus typhae* KRN1	–	+	–	–	–

Legends: “+” positive; “–“ negative”.

The next step was to check biocontrol activity of bacterial endophytes against fusarium-caused disease. The most effective bacterial isolates with biocontrol activity against disease caused by *Fusarium oxysporum* on a cotton plant were HRT18, AST3, BST3 and FST7. The most effective bacterial isolates having biocontrol activity against disease caused by *Fusarium solani* on a cucumber were KRT5, FRT13, FRT12, CST6 and BST10.

Bacterial isolated were examined for production of HCN. The following HCN-producing isolates were revealed: AST3, BST12, BST10, CST6, FRT13, FRT12, FST7, HRT9, HRT5, HRT18 and KRT12. It is known that secretion of these enzymes and also HCN by microorganisms can result in inhibition of plants pathogens action [15]. Ramette et al. [16] reported that it is often the case that biocontrol PGPB (plant growth-promoting bacteria) that can produce HCN also synthesize some antibiotics or cell wall degrading enzymes. It was revealed that the low concentration of HCN synthesized by the bacteria improves the efficacy of antifungal substances against fungal pathogens ensuring that the fungi do not get resistance to the particular antifungals. Suppression of tomato root knot disease caused by *Meloidogyne javanica* was attributed to the effect of HCN [17].

The endophytes were also examined for hydrolytic enzymes production. Lipase producing isolates: BST12, BST3, FRT13, FRT12, FST7, HRT9, HRT14, HRT18, HRT10, KRT5 and KRT16. All isolates excluding HRT14 and KRN1 produced protease. Chitinase producing isolates: AST3, BST12, BST3, BST10, CST6, FRT13 and KRT12. Nielsen and Sorensen [18] reported that *P. fluorescens* showed antagonism to *R. solani* and *P. ultimum*, by hydrolytic enzymes producing. Husson et al. [19] reported that chitinase degrades chitin, which it is a residue of β-(1, 4)-N-acetyl glucosamine polymer and an important part of the cell wall of many phytopathogenic fungi. Protease can degrade cell wall proteins and lipase can degrade cell wall-associated lipids. Each of these enzymes can lyse fungal cell walls by destruction of its components (chitin, proteins or lipids) [20, 21].

The following siderophores-producing endophytes were detected among isolates: AST3, BRT3, BST3, BST10, CST6, FRT12, FRT13, FST7, FRN1, HRT14, HRT18, KRN2, KRT5, KRT12 and KRN1. Siderophores are small peptide molecules that have side chains and functional groups to which ferric ions can bind [22]. Siderophore-producing microbes can prevent or lessen pathogen

proliferation by reducing the amount of iron that is available to a pathogen [23]. The important role of siderophores in antagonistic interactions of rhizosphere bacteria with soil phytopathogens and in plants growth stimulation is repeatedly proved at plants inoculation by siderophore-producing strains and their mutants defective on synthesis of siderophores. Not only suppressing effect of siderophores on phytopathogens is noted, but also stimulating influence on plants [24].

Conclusions. The strains possessing high biocontrol activity towards phytopathogenic fungi can be proposed for usage as a part of biofungicides after field experiments.

Financial support. This research was supported by CPEA-LT-2016/10095 grant from the Eurasia program of the Norwegian Centre for Cooperation in Education and by German Academic Exchange Service (DAAD) for Vyacheslav Shurigin.

ЕНДОФІТИ ЛІКАРСЬКИХ РОСЛИН ЯК АГЕНТИ БІОКОНТРОЛЮ ПРОТИ ХВОРОБ, ВИКЛИКАНИХ *FUSARIUM*

В'ячеслав Шуригін[1], Ділфуза Егамбердієва[1], Ситора Самадій[1], Гульсанам Мардонова[1], Кахрамон Давранов[2]

[1] Національний Університет Узбекистану, вул.Університетська, 4, Алмазарський район, Ташкент, 100174, Узбекистан

[2] Інститут Мікробіології АН РУз, вул. Кадирій, 7Б, Ташкент, 700128, Узбекистан

Резюме

Мета. Метою даного дослідження було виявлення бактеріальних ендофітів, виділених з деяких лікарських рослин, які пригнічують ріст фітопатогенних грибів *Fusarium solani* і *Fusarium oxysporum*. **Методи**. Бактеріальні ендофіти виділяли з пагонів і коренів лікарської шавлії (*Salvia officinalis* L.), фенхелю (*Foeniculum vulgare* Mill.), пижми (*Tanacetum vulgare* L.), календули лікарської (*Calendula officinalis* L.), ірису болотного (*Iris pseudacorus* L.), хрону (*Armoracia rusticana* G. Gaertn., B.Mey. & Scherb.). Ендофіти виділяли після подрібнення поверхнево стерилізованих коренів або пагонів, розведення клітинного соку в фосфатному буфері і розподілу суспензії на триптон-соєвому агарі. Вирощені колонії пересівали через 4 доби. Ендофіти перевіряли на антагоністичну активність проти фітопатогенних грибів *F. solani* і *F. oxysporum* на чашках Петрі з використанням середовища Чапека. Ендофіти були досліджені також на здатність до біологічного контролю тих же фітопатогенних грибів в горшкових експериментах. Грунт був заражений грибами, а насіння бавовни і огірка були інокулювані ендофітами. Кількість рослин, що вижили, визначали через 4 тижні після посіву. Бактеріальні ізоляти ідентифікували з використанням аналізу гена 16S рРНК. Для виділення ДНК був використаний метод теплової обробки. Отриману ДНК використовували як матрицю для аналізу гена 16S рРНК. Секвенування здійснювали з використанням готового реакційного комплекту ABI PRISM BigDye 3.1 (Applied Biosystems). Послідовності 16S рРНК були ідентифіковані з використанням BLAST і порівняні з банком даних нуклеотидів GenBank від NCBI. Штами були перевірені на продукування HCN, гідролітичних ферментів (хітинази, ліпази і протеази) і сідерофори. Всі експерименти були проведені в 3 повторностях. **Результати**. Всього нами було виділено 151 ізолят з 6 лікарських рослин. З усіх виділених ендофітів тільки 20 ізолятів інгібували гриби *F. oxysporum*, *F. solani* або їх обидва. При проведенні експериментів в горщиках найбільш ефективними бактеріальними ізолятами з біоконтролюючою активністю щодо хвороби, викликаної *F. oxysporum* на бавовнику, були HRT18, AST3, BST3 і FST7. Найбільш ефективними бактеріальними ізолятами, які проявляли біоконтролюючу активність щодо хвороби, викликаної *F. solani* на огірку, були KRT5, FRT13, FRT12, CST6, BST10. В цілому було ідентифіковано 20 бактеріальних ізолятів з використанням аналізу гена 16S рРНК: *Serratia ficaria* KRT5, *Raoultella ornithinolytica* KRT12, *Agrobacterium tumefaciens* HRT9, *Klebsiella oxytoca* BST12, *Pseudomonas putida* FRT13, *Pseudomonas kilonensis* FRT12, *Pseudomonas lini* FRN1, *Agrobacterium vitis* HRT14, *Bacillus toyonensis* HRT5, *Pseudomonas azotoformans* HRT18, *Pseudomonas syringae* AST3, *Klebsiella pneumoniae* BST3, *Xanthomonas translucens* HRT10, *Enterobacter ludwigii* KRT16, *Paenibacillus typhae* KRN1, *Pseudomonas extremaustralis* CST6, *Pseudomonas oryzihabitans* FST7, *Pseudomonas chlororaphis* BST10, *Agrobacterium vitis* KRN2, *Pseudomonas jessenii* BRT3. Було виявлено, що всі штами, які здатні пригнічувати ріст фітопатогенних грибів, можуть продукувати щонайменше одну з тестованих ре-

човин – HCN, сідерофори, хітиназу, протеазу і ліпазу, які розглядають як протигрибкові і за допомогою яких може бути реалізований біологічний контроль. **Висновки**. Штами, які мають високу біоконтролюючу активність щодо фітопатогенних грибів, можуть бути запропоновані для використання у складі біофунгіцидів після польових експериментів.

Ключові слова: лікарські рослини, ендофіти, бактерії, *Fusarium oxysporum*, *Fusarium solani*, інгібування росту.

1. Cushnie TT, Cushnie B, Lamb AJ. Alkaloids: an overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents. 2014; 44:377–386.
2. Bajguz A. Metabolism of brassinosteroids in plants. Plant Physiol Biochem. 2007; 45:95–107.
3. Passari AK, Mishra VK, Singh G, Singh P, Kumar B, Gupta VK, Sarma RK, Saikia R, Donovan AO, Singh BP. Insights into the functionality of endophytic actinobacteria with a focus on their biosynthetic potential and secondary metabolites production. Sci Rep. 2017; 7:11809.
4. Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau China. Curr Microbiol. 2011; 62:182–190.
5. Wang S, Hu T, Jiao Y, Wei J, Cao K. Isolation and characterization of *Bacillus subtilis* EB-28, an endophytic bacterium strain displaying biocontrol activity against *Botrytis cinereal* Pers. Front Agric China. 2009; 3(3):247–252.
6. Ren JH, Ye JR, Liu H, Xu XL, Wu XQ. Isolation and characterization of a new *Burkholderia pyrrocinia* strain JK SH007 as a potential biocontrol agent. World J Microbiol Biotechnol. 2011; 27(9):2203–2215.
7. Mora-Ruiz MDR, Font-Verdera F, Díaz-Gil C, Urdiain M, Rodríguez-Valdecantos G, González B, Orfila A, Rosselló-Móra R. Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily Salicornioideae (Amaranthaceae). Syst Appl Microbiol. 2015; 38:406–416.
8. Leeman M, van Pelt JA, den Puden FM, Heinsbroek M, Bakker PAHM, Schippers B. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to *Fusarium* wilt, using a novel bioassay. Eur J Plant Pathol. 1995; 101:655–664.
9. Dashti AA, Jadaon MM, Abdulsamad AM, Dashti HM. Heat Treatment of Bacteria: A simple method of DNA extraction for molecular techniques. Kuwait Med J. 2009; 41:117–122.
10. Castric PA. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can J Microbiol. 1975; 21:613–618.
11. Moreal J, Relse ET. The chitinase of *Serratia marcescens*. Can J Microbiol. 1969; 15:689–696.
12. Howe TG, Ward JM. The utilization of tween 80 as carbon source by *Pseudomonas*. J Gen Microbiol. 1976; 92:234–235.
13. Brown MR, Foster JH. A simple diagnostic milk medium for *Pseudomonas aeruginosa.* J Clin Path. 1970; 23:172–177.
14. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem. 1987; 160:45–46.
15. Voisard C, Keel C, Haas D, Defago G. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. The EMBO Journal. 1989; 8 (2):351–358.
16. Ramette A, Moënne-Loccoz Y, Défago G. Genetic diversity and biocontrol potential of fluorescent pseudomonads producing phloroglucinols and hydrogen cyanide from Swiss soils naturally suppressive or conducive to *Thielaviopsis basicola*-mediated black root rot of tobacco. FEMS Microbiol Ecol. 2006; 55:369–381.
17. Siddiqui IA, Shaukat SS, Sheikh IH, Khan A. Role of cyanide production by *Pseudomonas fluorescens* CHA0 in the suppression of root-knot nematode, *Meloidogyne javanica* in tomato. World J Microbiol Biotechnol. 2006; 22:641–650.
18. Nielsen P, Sorensen J. Multi-target and medium independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus*

pumilus strains from barley rhizosphere. FEMS Microbiol and Ecol. 1997; 22:183–192.

19. Husson E, Hadad C, Huet G, Laclef S, Lesur D, Lambertyn V, Jamali A, Gottis S, Sarazina C. Nguyen Van Nhien A. The effect of room temperature ionic liquids on the selective biocatalytic hydrolysis of chitin via sequential or simultaneous strategies. Green Chem. 2017.
20. Friedrich N, Hagedorn M, Soldati-Favre D, Soldati T. Prison break: pathogens' strategies to egress from host cells. Microbiol Mol Biol Rev. 2012; 76:707–720.
21. Gortari MC, Hours RA. Fungal chitinases and their biological role in the antagonism onto nematode eggs: a review. Mycol Prog. 2008; 7:221–238.
22. Goswami D, Thakker JN, Dhandhukia PC, Tejada Moral M. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric. 2016; 2:1127500.
23. Shen X, Hu H, Peng H, Wang W, Zhang X. Comparative genomic analysis of four representative plant growth-promoting rhizobacteria in *Pseudomonas*. BMC Genomics. 2013; 14:1471–2164.
24. Boronin AM. Rhizosphere bacteria of genus *Pseudomonas* promoting growth and development of plants. Soros Educational Journal. 1998; 10:25–31.

Received 10.04.2020