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Research Article

Biological Control of Phytopathogenic Fungi Causing Chickpea Root Diseases by Means of PGPR In The Saline Soil Conditions

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ABSTRACT:

Chickpea is one of the best protein sourses among legumes however in some climatic conditions it can suffer from fungal diseases. Biological pesticides on the basis of some PGPR bacteria possessing antifungal activity can be effective against many fungal diseases. Check of pathogenicity of various phytopathogenic fungi on a chickpea, and also attempt to raise the survival rate of chickpea plants in the soil infected with fungi by means of chickpea seeds inoculation with various bacteria-antagonists and research of some mechanisms of fungi growth inhibition by effective bacteria was the objctive of our research. During research we checked antifungal activity of 13 bacterial strains from collection of Microbiology and biotechnology department of the National University of Uzbekistan. The strains *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4 were the best antagonists against variety of phytopathogenic fungi (*Fusarium oxysporum* f.sp. *ciceris*, *Fusarium verticillioides*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani*, *Fusarium solani*, *Alternaria alternata*). In the course of pot experiment the soil was specially infected with fungi and seeds were inoculated in bacterial suspensions. Coinoculation of seeds with *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4 was more efficient against fungi then at monoinoculation with one of these cultures. These 2 bacterial strains became a basis for the developed preparation "Pseudorhizobin" which showed good result in struggle with chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris*. It was revealed that strains have some antifungal properties. Thus *Pseudomonas chlororaphis*-66 produce hydrolytic enzymes (chitinase, cellulose, protease), HCN and ACC-deaminase. The strain *Mesorhizobium ciceri*-4 produced glucanase, lipase and protease which also promote inhibition of fungal activity in a chickpea rhizosphere. It is revealed that strains *M. ciceri*-4 and *P. chlororaphis*-66 possess ability to produce siderophores, thereby reducing availability of iron, necessary for fungi growth in the soil. Both strains are competitive in a chickpea roots colonization and capable to live together on roots competing with other soil microorganisms.

Keywords: chickpea, fungal diseases, biocontrol, PGPR, hydrolytic enzymes

[I] INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a legume culture widespread in Turkey, India and Central Asia. Though the potential crop of various chickpea breeds exceeds 4 t/ha, the average crop is less than 0.8 t/ha. The difference between potential and average crop takes takes place generally because of diseases and weak crop management. The most part of chickpea diseases in order of their global value: ascochitosis, wilt, blueberry blossom bligth, dry root rot, ring rot, stem rot [1-3].

Causative agents of root rots at legumes are fungi, first of all from genus *Fusarium* and a bit rare - from genera *Aphanomyces*, *Verticillium*, *Pythium*, *Rhizoctonia*, etc., sometimes bacteria. Species of *Fusarium* are propagated everywhere and cause a huge loss to crops of a chickpea, pea, lupine, bean, soybean and all other bean cultures, invoking root system rot and stem withering owing to occlusion of vascular system and intoxication. The causative agent of a chickpea wilt is a fungi *Fusarium oxysporum* f.

sp. *ciceris* [3]. This disease is a cause of loss of about 10% of a chickpea crop in India, but under certain conditions and in certain regions the loss can reach 60%. Disease became a big problem for chickpea cultivation in California, USA, and in Mediterranean area [4].

The inhibition of soil plant pathogens growth with use of microorganisms, natural or modified, genes or genetic products for reduction of effect of undesirable organisms (pests) relates to biocontrol. Some rhizobacteria inhibit growth of various pathogenic bacteria and fungi that expresses in suppression of diseases invoked by these pathogens [5- 8]. Increase of plants resistance, enhancement of organism protective functions by means of certain biological methods could substantially reduce or prevent the influence of negative factors. In this connection last years the plants growth promoting rhizobacteria (PGPR) are actively investigated [7; 9; 10].

For the effective biocontrol of plants diseases, rhizobacteria should adapt and grow in ecological conditions which include aboriginal pathogenic microorganisms. Thus, the roots colonization by rhizobacteria, as an important condition for biological control and plants growth stimulation is takes place [11; 12].

Rhizobacteria inhibit the growth of pathogenic microorganisms by means of various mechanisms: competition for nutrients and infection sites [13], siderophores production [14, 15], hydrolases production [16], secondary metabolites production [17] etc.

Check of pathogenicity of various phytopathogenic fungi on a chickpea, and also attempt to raise the survival rate of chickpea plants in the soil infected with fungi by means of chickpea seeds inoculation with various bacteria-antagonists and research of some mechanisms of fungi growth inhibition by effective bacteria was the objctive of our research.

[II] MATERIALS AND METHODS

As a plant object of research, chickpea seeds of breed *Xalima* (*Cicer arietinum* L) from ICARDA collection were used. As a fungi causing chickpea root diseases, *Fusarium oxysporum* f. sp. ciceris, *Fusarium oxysporum* f. sp. *vasinfectum*, *Fusarium verticillioides*, *Rhizoctonia solani*, *Fusarium solani*, *Alternaria alternata* were used. To check the antagonistic effect against phytopathogenic fungi, bacterial strains: KR 083, KR 076, 3612, Ep 14, Tivi 7, Rif ep 17, Rube 1326, BB-135, TSAU-20, *Mesorhizobium ciceri*-4, *Rhizobium sp*.-6, *Rhizobium sp*.-9, *Pseudomonas chlororaphis*-66 from collection of Microbiology and biotechnology department of the National University of Uzbekistan were used.

2.1. Determination of *in vitro* antagonistic activity of bacteria against phytopathogenic fungi (plate experiment)

Bacterial strains are checked *in vitro* on presence of antagonistic activity against fungi mentioned earlier with use of plate method. The fungi were cultivated on agar Chapek medium with addition of 1.5% NaCl 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 solid LC medium (H_2O - 1 l, tripton - 10 g, yeast extract - 5 g, NaCl - 10 g, agar - 18 g) or on YMA medium (for rhizobia): H_2O - 1 l, mannitol - 10 g, $MgSO_4$ x 7 H_2O - 0.2 g, NaCl - 0.1 g, K_2HPO_4 - 0.25 g, KH_2PO_4 - 0.25 g, $CaCO_3$ - 1 g, yeast extract - 3 g, agar - 15 g passaged to test plates with the same medium perpendicularly to fungi. The plates incubated at 28°C within 7 days until the fungi covered control plates without bacteria. Antifungal activity fixed and measured as width of inhibition zone between fungi and test bacteria.

2.2. Determination of bacteria strains ability to biological control of phytopathogenic fungi causing chickpea root diseases (pot experiment)

Approximately one third of 7-day fungi culture 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 filtered from the nutrient medium through paper filters and suspended in sterile water (200 ml). Received suspension was stirred with the saline

soil from calculation of 100 ml/1 kg of soil. Chickpea seeds of breed «*Xalima*» 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 inoculated with bacteria by their wetting in bacterial suspension with concentration of bacteria - $1x10^8$ CFU/ml [18], whereas control seeds kept in sterile liquid LC medium within 15 minutes. For each combination of bacteria and fungi used 50 chickpea seeds.

For experiment the soil from one of the salinized cotton fields of Saykhunabad district of the Surkhan-Darya region (EC - 635 mSm/m) was used.

In each plastic pot (diameter - 9 cm, depth - 15 cm) sowed 2 seeds on 2 cm in depth. Each pot contained 300 g of saline soil. Plants cultivated in open natural conditions at 21-24°C. The watering was carried out when required. The quantity of survived plants was defined when 95-100% of plants in control without bacteria were affected by disease, in 4 weeks after sowing. Plants were taken out from soil, flushed and examined on presence of root rot symptoms. Roots without any symptoms of diseases were considered as healthy.

2.3. Determination of *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4 strains ability to biological control of chickpea wilt

Bacteria *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4 became a basis for the new biological preparation «Pseudorhizobin» [19].

For check of «Pseudorhizobin» preparation efficiency against chickpea wilt, the field experiment at the Uzbek Scientific Research Institute of Plants Protection was carried out.

90 visually healthy chickpea seeds of breed «*Nurli kuyosh*» were chosen for experiment. The first part of chickpea seeds (30) was inoculated with biological preparation «Pseudorhizobin» from calculation of 70 kg/t seeds. The second part of seeds (30) was treated with chemical preparation «Vitavaks 200 FF 34% v.s.k.» - 2.5 l/t as etalon. The third part of seeds (30) was not treated - control. After seeds inoculation they were sowed and watered. The soil was saline (EC-524 mSm/m) and specially infected by fungi *Fusarium oxysporum* f. sp. *ciceris*, invoking chickpea wilt. Dimension of each plot was 0.1 ha and each variant of treatment is carried out in 3 replications. The sowing was carried out on March 22, 2015. Three months later biological efficiency of preparations was counted up and compared.

2.4. Determination of biocontrol properties of *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4

Production of hydrolytic enzymes and HCN

For check of HCN production the strains cultivated on King B medium (for *Pseudomonas* sp.-66) or on YMA medium (for *Rhizobium* sp.-4). Sterile filter paper saturated with solution of 1% picric acid and 2% sodium carbonate sticked to inner surface of Petri plate cover. Petri plate covered with parafilm and incubated at 34°C (for *Pseudomonas* sp.-66) and at 30°C (for *Rhizobium* sp.-4) within 3 days. The change of paper colour from yellow to dark blue indicated on HCN release [20].

For determination of chitinolytic activity strains 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 strain *Pseudomonas* sp.-66 cultivated at 34°C and *Rhizobium* sp.-4 at 30°C. Efficiency of chitin hydrolysis defined on magnitude of ratio of clarification zone diameter around the colony to colony diameter [21].

The presence of lipase activity at bacterial strains checked with Tween lipase indicator. Bacterial strains cultivated on LC agar (for *Pseudomonas* sp.-66) and on YMA medium (for *Rhizobium* sp.-4) with addition of 2% Tween 80 at 34°C (for *Pseudomonas* sp.-66) and at 30°C (for *Rhizobium* sp.-4) [22]. In 5 days, Tween destruction was found in the form of pure ring around bacterial colony that indicated on lipase activity at strain.

Protease production found at strains cultivation on TSA/20 (1/20 part of tripsin 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 on presence of extracellular protease [23].

Glucanase activity found at use of lichen glucan substrate, and formation of pure zones indicated on substrate destruction [24].

Cellulase activity found at use of carboxymethylcellulose (CMC) as substrate [25].

The strains ability to produce siderophores

60,5 mg of dye Chrome Azurol Sulphonate (CAS) 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 [26]. 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 with a pH 6.8. After cooling to 50°C we added 30 mg of peptone as specific source of carbon for *Pseudomonas*, in case of *Mesorhizobium* added 30 mg of tryptone. Dye solution added lengthway on a glass wall with slow stirring for foaming prevention.

Bacteria strains removed from slopes and transferred to 50 ml flasks with 25 ml of physiological solution from calculation 1 slope/25 ml. Received suspension carefully stirred. For CAS analysis on cups with blue agar we made holes where poured 200 µl of bacterial suspension. Cups incubated in darkness at 28°C within 48 hours and checked on growth presence and formation of orange rings around colonies. CAS analysis on siderophores production compared with formation of similar zones in pure culture of siderophore-producing strain *R. meliloti-NUU5* which was used as standard for comparative analysis.

Study of strains competitiveness in chickpea rots colonization

Pseudomonas chlororaphis-66.

For check and assessment of strain *P. chlororaphis*-66 competitiveness in a chickpea roots colonization used rifampicin-resistant (200 µ/ml) strain *P. chlororaphis*-66R obtained from *P. chlororaphis*-66.

Strain *P. chlororaphis*-66R cultivated for 2 days on King B medium. 1 ml of 2 days culture deposited by centrifuging (5000 rpm) and supernatant deleted. Cells washed off with 1 ml of saline phosphate buffer and diluted with the same buffer. Cells suspension adjusted (by dilution) to OD_{620}=0.1, that corresponds to density about $1x10^8$ cells/ml. As the control competitive roots colonizer used strain *P. fluorescens* WCS365 [27] with the same cells density in suspension.

Bacterial suspensions with two strains mixed in equal proportions. As plant object used 10 sterile emerged chickpea seeds. The seeds inoculated in the mixture of bacterial suspensions for 10 min. Inoculated seeds sowed to sterile Erlenmeyer flasks (1000 ml) with sand as described by Simons [28]. Sand moistured with plants nutrition solution [29], with addition of 1.5% NaCl. Flasks randomized in 10 replications. Plants grew for 15 days in the special growth chamber with 16 dayligth hours at 26±1°C and 16±1°C at night. In 15 days of plants growth the 1-cm root tips were cut off. Bacteria cells were deleted from roots by roots stirring on vortex in the saline phosphate buffer and transferred in equal volumes (0.5 ml) on King B medium, containing 200 µg/ml of rifampicin for separation of studied strain from control, and on the same medium without rifampicin for growth of both strains. On the medium with rifampicin grew only *P. chlororaphis*-66R, and on the medium without rifampicin - both strains. Calculation of CFU of both strains made by residue of the colonies grown on medium with rifampicin from total colonies grown on medium without rifampicin.

Mesorhizobium ciceri-4.

For check and assessment of *M. ciceri*-4 competitiveness in chickpea roots colonisation against strain *P. chlororaphis*-66, used rifampicin-resistant strain *P. chlororaphis*-66R (200 µg/ml).

Strain *P. chlororaphis*-66R cultivated for 2 days on King B medium and *M. ciceri*-4 cultivated on

YEM medium for 3 days. 1 ml of each culture deposited by centrifuging (5000 rpm) and supernatant deleted. Cells washed off with 1 ml saline phosphate buffer solution (20 mmol sodium phosphate, 50 mmol NaCl, pH 7.4) and diluted with the same buffer. Cells suspension adjusted (by dilution) to OD_{620}=0.1 that corresponds to cells density about 10^8 cells/ml. For a joint inoculation, cultural liquids with two strains mixed in equal proportions. As plant object used 10 sterile emerged chickpea seeds. The seeds inoculated in the mixture of bacterial suspensions for 10 min. Inoculated seeds sowed to sterile Erlenmeyer flasks (1000 ml) with sand as described by Simons [28]. Sand moistured with plants nutrition solution [29], with addition of 1.5% NaCl. Flasks randomized in 10 replications. Plants grew for 15 days in the special growth chamber with 16 dayligth hours at 26±1°C and 16±1°C at night. In 15 days of plants growth the 1-cm roots tips were cut off. Bacteria cells were deleted from roots by roots stirring on vortex in the saline phosphate buffer. Homogenates serially diluted to cells concentrations 10^{-3} and 10^{-4}, and transferred into Petri plates with agar. For count of rifampicin-resistant strain *P. chlororaphis*-66R cells used solid King B medium with addition of rifampicin (200 μg/ml). Calculation of *M. ciceri*-4 carried out on YMA medium with addition of Congo red, that allowed to distinguish rhizobia colonies from pseudomonades according to distinctions in colour and form. After incubation at 30°C pseudomonades colonies counted up on solid King B medium in 2 days, and rhizobia colonies on YMA medium in 3 days. The number of bacteria counted up as CFU/1 cm of root.

[III] RESULTS AND DISCUSSION

3.1. In vitro antagonistic activity of bacteria against phytopathogenic fungi (plate experiment)

The ability of investigated bacteria to growth inhibition of phytopathogenic fungi on Petri plates **[Table-1]** is checked.

It is apparently from table 1 that all studied strains possess more or less expressed antagonistic activity against various species of fungi.

Some bacterial strains possess high antagonistic activity against certain fungi. For example, strain *Rhizobium* sp.-9 inhibited *F. oxysporum* f. sp. *vasinfectum* growth and formed clear zone with radius 35 mm. Strain *Tivi* 7 inhibited growth of *R. solani* with radius of fungi growth inhibition zone - 35 mm.

Strains *M. ciceri*-4 and *P. chlororaphis*-66 are the most evident. These strains possess antagonism to all investigated species of fungi. Strain *P. chlororaphis*-66 is the best antagonist of *F. oxysporum* f. sp. *ciceris* (radius of fungi growth inhibition zone is 32 mm) and *F. solani* (33 mm). Strain *M. ciceri*-4 showed the strongest antagonism towards *F. verticillioides* (radius of fungi growth inhibition zone is 30 mm) and *A. alternata* (34 mm).

Bacterial strains	**Fungi**					
	Fusarium oxysporum* f.sp. *ciceris	***Fusarium verticillioides***	***Fusarium oxysporum* f. sp. *vasinfectum***	***Rhizoctonia solani***	***Fusarium solani***	***Alternaria alternata***
KR 083	-	20±1.79	18±0.89	32±2.68*	-	23±1.79
M. ciceri-4	28±1.55	30±2.37*	31±2.37*	16±1.79	29±3.22*	34±2.37*
3612	16±1.55	29±2.37*	32±2.37	-	31±2.37	30±2.37*
Ep 14	-	16±1.55	26±1.55	15±0.89	30±2.37	31±2.37*
Tivi 7	-	18±1.79	33±2.37*	35±2.37	-	25±1.55
KR 076	-	14±0.89	-	30±2.37	27±1.79	29±2.37*
Rhizobium sp.-6	-	23±2.37*	23±1.55	22±1.55	14±0.89	23±1.55
Rif ep17	16±1.79	-	22±1.79	22±1.79	25±1.55	-
Rhizobium sp.-9	25±1.55	15±0.89	35±2.37*	26±1.79	26±1.79	5±0.89
Rube 1326	18±1.55	-	20±1.55	24±1.55	22±1.55	17±1.79
P. chlororaphis-66	32±2.37*	23±1.55	34±2.37*	24±1.79	33±2.37	29±2.37*

BB-135	25±2.37*	-	22±1.55	19±1.55	16±1.79	22±1.55
TSAU-20	21±1.79	-	21±1.79	22±1.79	32±2.37	24±1.79

* - statistically significant at p≤0,05

Table: 1. Growth inhibition of phytopathogenic fungi by bacteria on plates (radius of inhibition zone, mm)

3.2. Bacteria strains ability to biological control of phytopathogenic fungi causing chickpea root diseases (pot experiment)

Results of the next experiment **[Table-2]** show the quantity of chickpea plants of breed «*Xalima*» (age 1 month), persisted in saline soil infected with various species of phytopathogenic fungi (6-days culture), after seeds inoculation with studied bacteria-antagonists (bacteria concentration - $1x10^8$ CFU/ml bacterial suspension).

From the results **[Table-2]** it is visible, that at soil infection with fungi *F. oxysporum* f. sp. *ciceris* any chickpea plants in the control did not survive. However at presowing inoculation of chickpea seeds with various bacterial strains - fungi antagonists, survival rate of plants raised to some extent. Survival rate of plants after seeds inoculation with strains *P chlororaphis*-66 (number of survived plants - 85%) and *M. ciceri*-4 (77%) is especially high. The best effect was observed after joint seeds inoculation with strains *P. chlororaphis*-66 and *M. ciceri*-4 (survival rate - 91 %) **[Figure-1]**.

After soil infection with *F. verticillioides* the number of survived plants was 65%. Strains *P. chlororaphis*-66 and *M. ciceri*-4 showed the highest antagonistic activity against this fungi and increased plants survival rate to 82% after *P. chlororaphis*-66 application and 79% after *M. ciceri*-4 application. However the best effect noticed after seeds coinoculation with these strains - 87%.

After soil infection with *F. oxysporum* f. sp. *vasinfectum* the number of survived plants was 17%. The best effect against this fungi showed strains *Tivi* 7 (plants survival rate - 94%) and *Rhizobium* sp.-9 (plants survival rate - 96%). Seeds coinoculation with strains *P. chlororaphis*-66 and *M. ciceri*-4 increased chickpea survival rate up to 89%.

Bacterial strains	**Fungi**					
	Fusarium oxysporum* f.sp. *ciceris	***Fusarium verticillioides***	***Fusarium oxysporum* f.sp. *vasinfectum***	***Rhizoctonia solani***	***Fusarium solani***	***Alternaria alternata***
Control (without bacterial inoculation)	0	65±3.90	17±1.55	0	0	33±3.22
KR 083	8±0.89	67±4.10	32±3.22	83±4.73	45±3.90	50±3.22
Mesorhizobium ciceri-4	77±4.73*	79±4.73*	85±5.59*	79±4.65	86±5.59*	86±5.59*
3612	25±2.37	73±4.10	84±5.59*	35±3.22	82±4.98	82±4.98*
ep 14	23±2.37	70±4.10	69±3.90	54±3.22	83±4.73	87±4.73
Tivi 7	18±1.79	70±4.10	94±5.59*	84±5.59*	67±4.10	65±3.90
KR 076	0	69±3.90	19±1.55	82±4.98*	81±4.73	80±4.98*
Rhizobium sp.-6	22±1.55	73±3.90	68±3.90	67±3.90	33±3.22	65±4.10
Rif ep17	35±3.22	69±4.10	53±3.22	51±3.22	55±3.22	36±3.22
Rhizobium sp.-9	66±3.90	68±3.90	96±3.90	94±5.44*	71±4.73	52±4.10
Rube 1326	32±3.22	63±3.22	64±4.10	71±3.90	69±4.73	42±4.10
Pseudomonas chlororaphis-66	85±5.44*	82±4.73*	86±5.59*	66±3.90	88±5.59*	92±5.59*
BB-135	70±4.10	68±3.90	65±3.90	48±3.22	52±3.22	54±3.90
TSAU-20	53±3.22	65±4.10	59±3.58	58±3.22	71±4.73	57±3.90
Mesorhizobium ciceri-4 + *Pseudomonas chlororaphis*-66	91±5.87*	87±5.59*	89±4.98*	82±4.98*	92±4.73	95±4.65

* - statistically significant at p≤0,05

Table: 2. Quantity of chickpea survived in the soil, infected with various phytopathogenic fungi after seeds inoculation with various bacteria-antagonists (%), (pot experiment)

After soil infection with *Rhizoctonia solani* any plant did not survive. The most efficient against *R. solani* was strain *Rhizobium* sp.-9 which raised chickpea survival rate up to 94%. Strains *M. ciceri*-4 and *P. chlororaphis*-66 were not so effective against this fungi - 79 and 66% accordingly. However

after seeds coinoculation with them survival rate increased up to 82% **[Figure-1]**.
Strains *P. chlororaphis*-66 and *M. ciceri*-4 showed high antagonistic activity against *F. solani* and raised plants survival rate up to 88 and 86 % accordingly, and after seeds coinoculation with them survival rate raised up to 92 %.
After soil infection with *Alternaria alternata* in control survived only 33% of plants. The most active against this fungi was strain *P. chlororaphis*-66 because after seeds inoculation with it, survived 92% of plants, that in 2.8 times is more than in control. On the second place was strain *Ep* 14 with 87% of survived plants, on the third - strain *M. ciceri*-4 - 86%. Seeds coinoculation with strains *P. chlororaphis*-66 and *M. ciceri*-4 increased survival rate up to 95% **[Figure-1]**.
It is obvious from experiment results that joint application of strains *M. ciceri*-4 and *P.chlororaphis*-66 for seeds inoculation promotes effective protection of a chickpea against root diseases caused by phytopathogenic fungi in soil salinity conditions.

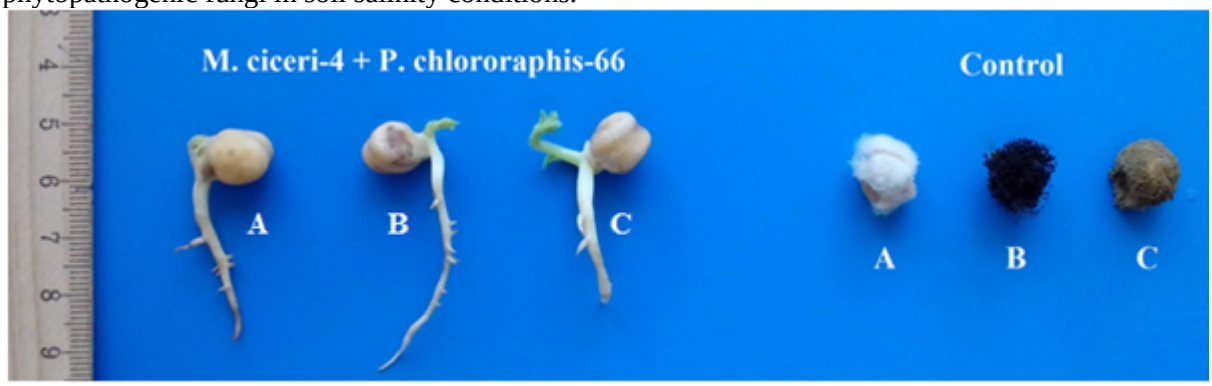

Fig: 1. Chickpea seeds from the soil infected with: a) *F. oxysporum* f. sp. *ciceris;* b) *Alternaria alternata*; c) *Rhizoctonia solani*. First 3 seeds were coinoculated with *M. ciceri*-4 and *P. chlororaphis*-66; other 3 seeds are control (without treatment).

3.3. *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4 strains ability to biological control of chickpea wilt

Bacteria *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4 became a basis for the new biological preparation «Pseudorhizobin» [19]. The biohumus modified by addition of some nutrient salts and molasses is a part of this biological preparation. Thus the biohumus is a substrate for growth of these two bacteria strains. Concentration of *Pseudomonas chlororaphis*-66 in a preparation is 4 billion cells/g, and *Mesorhizobium ciceri*-4 – 3.7 billion cells/g.
Chickpea wilt is one of the most widespread and hazardous chickpea diseases [3, 30]. To check the efficiency of «Pseudorhizobin» against chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* the field experiment was carried out at Uzbek Scientific Research Institute of Plants Protection **[Table-3]**.

Variants	Rate of application	The number of sowed seeds	The number of germinated seeds	The number of infected plants	Biological efficiency, %
Chemical preparation "Vitavaks" 200 FF 34% v.s.k. – (etalon)	2.5 l/t	30.0	29.0	0.5	86.1
Biopreparation "Pseudorhizobin" (*P. chlororaphis*-66-4 billion CFU/g, *M.ciceri*-4 – 3,7 billion CFU/g)	70.0 kg/t	30.0	28.0	0.6	83.3
Control (without treatment)	-	30.0	19.0	3.6	-

Table: 3. Biological efficiency of biopreparation «Pseudorhizobin» against chickpea wilt (breed "*Nurli kuyosh*")

Biological efficiency of biopreparation «Pseudorhizobin» in a saline soil against chickpea wilt at norm 70.0 kg/t seeds in the end of vegetation was 83.3% **[Figure-2]**. Biopreparation showed small lag in efficiency as compared with etalon - chemical preparation «Vitavaks 200 FF 34% v.s.k.» which biological efficiency was 86.1%. The control from the very beginning of observation invariably showed progress of disease development.

Thus, biological preparation «Pseudorhizobin» showed high biological efficiency that allows to call it perspective in chickpea wilt control.

Fig: 2. Experimental plot with chickpea growing in soil infected with *Fusarium oxysporum* f. sp. *ciceris*: a) «Vitavaks 200 FF 34% v.s.k.» (etalon); b) Control; c) «Pseudorhizobin»

3.4. Biocontrol properties of *Pseudomonas chlororaphis*-66 and *Mesorhizobium ciceri*-4

It is apparently from the obtained results that bacterial strains *P. chlororaphis*-66 and M. *ciceri*-4 possess high antifungal activity against all range of phytopathogenic fungi checked in this research. Therefore we decided to study bacterial factors realizing biological control of phytopathogenic fungi. Such biocontrol properties, as hydrolytic enzimes, HCN and siderophores production, and competition in roots colonisation are studied.

Hydrolytic enzymes and HCN production

Bacterial strain *P. chlororaphis*-66 is checked on production of volatile HCN and extracellular enzymatic activity

Time of cultivation, days	Efficiency of chitin hydrolysis
4	1.6
6	2.8
7	3.5
8	5.8

- ratio of diameter of clarification zone of the cloudy medium (containing chitin) around colony to diameter of colony, mm/mm

Table: 4. Chitin hydrolysis by strain *Pseudomonas chlororaphis*-66

Strain *P. chlororaphis*-66 produced some hydrolytic enzimes (chitinase, cellulase and protease), and also HCN. Results presented in **Table-4** are indicate on presence of chitinolytic activity at this strain.

It is known that secretion of these enzymes and also HCN by microorganisms can result in inhibition of plants pathogens action [31].

Nielsen and Sorensen [32] reported that *P.fluorescens* showed antagonism to *R. solani* and *P. ultimum*, producing hydrolytic enzymes. Strain *P. chlororaphis-66*, producing enzymes destroying fungal cell wall (chitinase, protease) showed antagonistic activity against all investigated phytopathogenic fungi and can protect plant from root diseases caused by them.

Cellulase production by strain *P. chlororaphis*-66 at growth in a chickpea rhizosphere promotes partial dissolution of root hairs cellular wall and due to this the penetration of nodule bacteria into root hairs become easier, thus the number of formed nodules increases.

Bacterial strain *P. chlororaphis*-66 is capable to use ACC as a nitrogen source that indicate on presence of ACC-deaminase which plays an

important role in lowering of ethylene level in plants in stress conditions.

Fungal pathogens are the cause of ethylene synthesis by plants [33] and the most part of the damage suffered by plants infected with phytopathogens, takes place as a result of plant response reaction to increase of ethylene concentration [34]. It is known, that external ethylene often increases gravity of infection with pathogenic fungi while some inhibitors of ethylene synthesis considerably reduce the seriousness of fungal infection [35, 36]. It is revealed, that many PGPR stimulating plants roots growth, contain enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which hydrolyzes ACC - the precursor of ethylene to ammonium and α-ketobutyrate and as a result reduces ethylene biosynthesis by plants [37-39]. Biocontrol strains of bacteria containing ACC-deaminase, are more effective, than strains which are not possess this enzyme [40].

Strain *M. ciceri*-4 does not able to produce HCN, ACC-deaminase, chitinase and cellulase. However, the strain produces glucanase, lipase and protease which also promote inhibition of fungal activity in a chickpea rhizosphere.

Siderophores production

Strains *P. chlororaphis*-66 and *M. ciceri*-4 are checked on siderophores production by means of CAS (chrome azurol sulfonate) analysis and compared to type strain *R. meliloti*. At strains cultivation on plates with CAS agar, slow growth of colonies and formation of orange rings (diameter - 1.67 cm) surrounding colonies was observed. 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.

When orange ring regions of strains *P. chlororaphis*-66 and *M. ciceri*-4 were compared to control strain *R. meliloti*, it appeared that diameter of orange rings of investigated strains was a little bit lower, than at control strain, as shown in **Table-5**.

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 siderophores synthesis. At this is noted not only suppressing effect of siderophores on phytopathogenes, but also stimulating influence on plants [41].

Bacterial strains	CAS analysis	Rings diameter (cm)
P. chlororaphis-66	+	1.67±0.2
M. ciceri-4	+	1.53±0.4
R. meliloti-*NUU5*	+	1.70±0.3

Note: + means that CAS analysis was positive and orange rings formed on a blue agar

Table: 5. CAS analysis on siderophores production by strains

It is revealed that strains *M. ciceri*-4 and *P. chlororaphis*-66 possess ability to produce siderophores, thereby reducing availability of iron, necessary for fungi growth in the soil. This ability is one of the most important bacterial factors inhibiting the growth of phytopathogenic fungi in soil.

Competitiveness in a chickpea roots colonization

Pseudomonas chlororaphis-66. PGPR *Pseudomonas* can render positive impact on a plant only at successful colonization of its rhizosphere. In case of use of PGPR *Pseudomonas* as plants protection means from phytopathogens it is a question of these microorganisms introduction to this environmental niche [41].

Rifampicin-resistant strain *P. chlororaphis*-66R was checked on competitiveness in a chickpea roots colonization against studied roots colonizer *P. fluorescens* WCS365 [27]. It was found that in presence of 1.5% NaCl strain *P. chlororaphis*-66R colonizes chickpea roots better than control strain **[Table-6]**. It is obvious from table 6 that an index of roots colonization by *P. chlororaphis*-66R is $10.5x10^3$ CFU/cm and at control strain *P. fluorescens* WCS365 - $9,3x10^3$ CFU/cm.

Bacterial strains	CFU/cm root
P. fluorescens WCS365	9.3 x10^3
P.chlororaphis-66R	10.5 x 10^3

Table: 6. The ability of *P. chlororaphis*-66R to compete with *P. fluorescens* WCS365 in a chickpea roots colonization

Mesorhizobium ciceri-4. Strain *M. ciceri*-4 was checked on competitiveness in a chickpea roots colonization against strain *P. chlororaphis*-66R which showed the ability to colonize chickpea roots **[Table-7]**.

Bacterial strains	CFU/cm root
M. ciceri-4	8.9x10^3
P. chlororaphis-66R	11.4 x10^3

Table: 7. The ability of *Mesorhizobium ciceri*-4 and *Pseudomonas chlororaphis*-66R to compete with each other in a chickpea roots colonization

In the presence of 1.5% NaCl strain *M. ciceri*-4 is capable to compete in a chickpea root colonization and CFU of this strain is just a little bit lower than at *P. chlororaphis*-66R. It means that both strains are competitive in a chickpea roots colonization and capable to live together on roots competing with other soil microorganisms.

[IV] CONCLUSION

As a results of research the strains *M. ciceri*-4 and *P. chlororaphis*-66 appeared to be the most active antagonists against checked phytopathogenic fungi caused chickpea diseases. It was revealed that strains have some antifungal properties. Thus *P. chlororaphis*-66 produce hydrolytic enzymes (chitinase, cellulose, protease), HCN and ACC-deaminase. The strain *M. ciceri*-4 produced glucanase, lipase and protease which also promote inhibition of fungal activity in a chickpea rhizosphere. It is revealed that strains *M. ciceri*-4 and *P. chlororaphis*-66 possess ability to produce siderophores, thereby reducing availability of iron, necessary for fungi growth in the soil. Both strains are competitive in a chickpea roots colonization and capable to live together on roots competing with other soil microorganisms. Biological preparation "Pseudorhizobin" based on these two strains is an effective mean to struggle with a chickpea wilt.

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