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Technology of Creation of Complex Microbiological "Pseudorhizobin" Preparation Improving Chickpea Growth and Productivity in Salinity Conditions

Vyacheslav Vladimirovich Shurigin

National University of Uzbekistan named After Mirzo Ulugbek, Uzbekistan
*Corresponding author: Email: slaventus87@inbox.ru, Tel: +99893-5047522

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ABSTRACT:

We isolated a number of bacteria from a chickpea nodules and rhizosphere. Microbiologically clarified more than 20 nodule and nearby 40 rhizosphere bacteria, the majority from which related to genus *Pseudomonas*. By their screening the strain *Mesorhizobium ciceri*-4, actively forming nodules and strain *Pseudomonas chlororaphis*-66, promoting formation of symbiotic relations between a chickpea and *Mesorhizobium ciceri* - 4, and also promoting protection of a chickpea against a number of microbial diseases, characteristic for this region, were selected. Further the new, readily available economic medium on the basis of aboriginal raw materials have been developed and optimum conditions for development of these strains industrial cultivation technology for the purpose of biological preparation production on their basis are selected. As a result the biotechnology of "Pseudorhizobin" preparation reception which designed for an agricultural practice and possesses high antifungal and plant growth promoting activity has been developed. The effect of the preparation was assessed on different chickpea breeds in the soil salinity conditions of Kara-Kalpak republic.

Keywords: chickpea, growth promotion, productivity, salinity, Pseudomonas chlororaphis, Mesorhizobium ciceri, rhizobacteria

[I] INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the oldest crops grown mainly for their seed which contain 20.6% protein, 2.2% fat and 61.2% carbohydrates [1]. On the areas under crops chickpea takes third place in the world among pulse crops, conceding only to a soya and a bean. It cultivates in Turkey, Israel, Pakistan, Iran, Uzbekistan, Armenia, Kazakhstan, Azerbaijan, the countries of Africa, the North America and Southern Europe, but most of all - in India (about 10 million in hectares) [2].

Salinity is one of the major obstacles in increasing production in chickpea growing areas. Salt stress has been reported to decrease germination percentage, germination rate, shoot and root length as well as shoot and root fresh

and dry weight [3, 4, 5, 6, 7]. Ferri et. al. indicated that salt stress limits plant productivity in legumes through diminished germination, photosynthetic efficiency, nitrogen fixation and carbon metabolism. Salt in the germination medium showed a negative effect on all germination studies parameters [8].

However it is known that some PGPR (Plant Growth Promoting Rhizobacteria) can render positive effect in plants on such parameters as germination rate, tolerance to drought, crop and plant growth in soil salinity conditions [9].

Another problem in a chickpea growing is soil-borne diseases. More of them are caused by plant pathogenic fungi such as *Fusarium oxysporum*, *Fusarium solani*, *Verticillium dahlia*, *Rhizoctonia solani* and others. However some PGPR can protect plants from diseases by means of different mechanisms such as syderophore, HCN and antibiotic production [10].

Creation of the effective microbial complexes on the basis of PGPR promoting conservation of soils fertility and an intensification of a farm production, is one of actual directions of modern agricultural biotechnology. It is known, that microbiologic factors have great value in modern system of agriculture and their utilization results not only in essential growth of soils fertility of, but also stimulates realization of plants genetic potential [11]. There are constant molecular mutual relations between plants and microorganisms living in soil, in which result they interchange metabolites. During these mutual relations transformation of some metabolites is carried out also. Microorganisms promote formation in the rhizosphere zone of fund of nutrients accessible to a plant and the necessary physiologically active substances controlling metabolism and mutual relations between partners [12]. It is interesting, that in the course of microorganisms-plants interactions the integrating systems including genetic factors of partners are formed. In most cases these factors, do not work separately, but define development of adaptations which separate organisms before their affiliation have no [13].

Proceeding from resulted above postulates, we have set for ourselves a problem to create an effective complex preparation on the basis of salt tolerant PGPR capable to stimulate chickpea growth and development and protect chickpea against plant pathogenic fungi that finally will result in high chickpea productivity in soil salinity conditions of Kara-Kalpak republic.

[II] MTERIALS AND METHODS

2.1. Bacterial inoculum preparation

For bacterial inoculum preparation 2 bacterial strains were used: strain *Mesorhizobium ciceri*-4 isolated from the nodules of a chickpea cultivated in the territory of Uzbekistan, and also strain *Pseudomonas chlororaphis*-66, isolated from a chickpea rhizosphere.

These strains have been selected by screening methods on plant growth promoting and biocontrol properties in relation to plant pathogenic fungi [14, 15, 16]. And these strains have been chosen as a basis for creation of "Pseudorhizobin" microbiologic preparation for a chickpea.

Strain *Mesorhizobium ciceri*-4 were cultivated within 3 days at temperature 27^0C on slopes with YMA medium of following composition (g/l): H_2O - 1 l, mannitol - 10 g, $MgSO_4 \cdot 7\ H_2O$ – 0.2 g, NaCl – 0.1 g, K_2HPO_4 – 0.25 g, KH_2PO_4 – 0.25 g, yeastrel - 3 g, agar - 15 g. Strain *Pseudomonas chlororaphis*-66 was cultivated within 3 days at temperature 27^0C on peptone agar of following composition (g/l): H_2O - 1 l, peptone - 10 g, sucrose - 2 g, NaCl – 0.5 g, $MgSO_4 \cdot 7\ H_2O$ – 0.5 g, K_2HPO_4 – 0.5 g, agar - 15 g. Further increased bacteria *Mesorhizobium ciceri*-4, from calculation of 1slope/100 ml, transferred to preliminarily prepared flasks with the sterile leguminous broth medium of the following composition (g/l): leguminous broth - 1000 ml; sucrose – 2.0 g; KH_2PO_4 – 1.0 g; $MgSO_4 \cdot 7H_2O$; - 0.3 g; pH 7.0 – 7.2. Bacteria *Pseudomonas*

chlororaphis-66 were transferred to liquid peptone broth from calculation 1 slope/100 ml of medium. Bacteria in a liquid medium were cultivated on a temperature-controlled shaker "UVMT - 12 – 250" at 180 rpm within 3 days at 27°C. In 3 days the titre of cells *Mesorhizobium ciceri*-4 in culture fluid was $8 \cdot 10^7$ CFU/ml, and with *Pseudomonas chlororaphis*-66 – $3.6 \cdot 10^8$ CFU/ml.

2.2. Preparation of substrate

As substrate for microbiologic preparation for a chickpea the biohumus - a product of plant residues processing by red Californian worms was used. To the biohumus were added the following salts and substances: $MgSO_4 \cdot 7H_2O$ - 1%, K_2HPO_4 - 1%, $(NN_4)_6Mo_7O_{24} \cdot 4H_2O$ – 0.05%, $MnSO_4$ – 0.05%, $FeSO_4$ – 0.05%, $CaCO_3$ - 3% (for neutralization of medium acid reaction), molasses - 3-5% (as an additional source of carbon), potassium humate - 3 %. Also 1% of CMC (Carboxymethyl cellulose) was added to biohumus as agglutinant. After that biohumus packaged in 1 kg in the polypropylene packages with special cotton-gauze stoppers for ventilation and sterilized by autoclaving at 1 atm. within 1 hour.

2.3. Making of a microbiologic preparation

3 Diurnal culture fluids with *Mesorhizobium ciceri*-4 and *Pseudomonas chlororaphis*-66 bacteria sterilely filled in packages with the sterile biohumus from calculation of 20 ml/kg of the biohumus. Finally humidity of preparation constituted 50-60%, pH≈6.7. Packages densely occluded with cotton-gauze stoppers and left for the further cultivation for a week at temperature 27°C. In 2 weeks the titer of *Mesorhizobium ciceri*-4 cells in preparation constituted $8.5 \cdot 10^9$ CFU/ml, and *Pseudomonas chlororaphis*-66 - $9 \cdot 10^9$ CFU/ml. Then packages seated in a cool place with temperature 4°C for the further storage.

After 3 months of preparation storage in a cool place again counted up quantity of viable bacterial cells in a preparation. *Mesorhizobium ciceri*-4 CFU has constituted $4.4 \cdot 10^9$, and *Pseudomonas chlororaphis*-66 - $5 \cdot 10^9$ CFU/ml.

CFU was defined by the method of limiting delutions of culture fluid with bacteria [17] and the further inoculation of fluid by 1 ml on Petri plates with YMA medium for *Mesorhizobium ciceri*-4 and with peptone agar for *Pseudomonas chlororaphis*-66. Plates seated in a thermostat for 2 days at 27°C. In 2 days we counted up quantity of grown colonies.

2.4. Chickpea seeds inoculation with preparation

Field experiment for an assessment of preparation efficacy for chickpea was carried out in Amudarya district of Kara-Kalpak republic. The soil in the field had EC - 568 mSm^{-1}. Soils with an electrical conductivity (EC) greater than 400 mSm^{-1} soil are considered saline. For the field experiment we used healthy chickpea seeds of "*Xalima*", "*Uzbekiston*-32", "*Lazzat*", "*Miroz*", "*Jahongir*", "*CIEW*-45" and "*Flip* 1-31" breeds.

Chickpea seeds were inoculated with preparation, preliminary suspended in tap water from calculation of l kg/0.5 l of water. Seeds mixed with preparation mixture within 30 minutes and then, not waiting for preparation drying on a seeds surface, were sowed into soil. After that, field watering has been carried out. Norm of the preparation expense is 2 kg on chickpea seeds hectare norm. In 75 days of chickpea cultivation the seeds yield has been counted up.

[III] RESULTS

3.1. The effect of "Pseudorhizobin" preparation on a chickpea nodules formation

We isolated a number of bacteria from a chickpea nodules and rhizosphere. Microbiologically clarified more than 20 nodule and nearby 40 rhizosphere bacteria, the majority from which related to genus *Pseudomonas*. By their screening the strain *Mesorhizobium ciceri*-4, actively forming nodules and strain *Pseudomonas chlororaphis*-66, promoting formation of symbiotic relations between a chickpea and

Mesorhizobium ciceri - 4, and also promoting protection of a chickpea against a number of microbial diseases, characteristic for this region, were selected [14, 15, 16]. Pseudomonas chlororaphis-66 had fungistatic activity to the following plant pathogenic fungi: *Fusarium oxysporum* (inhibition zone 32±3 mm), *Fusarium maniliforme* (23±2 mm), *Fusarium vasinfectum* (34±3 mm), *Rhizoctonia solani* (24±2 mm), *Fusarium solani* (33±3 mm), *Alternaria alternata* (29±3 mm) [16]. Further the new, readily available economic medium on the basis of aboriginal raw materials have been developed and optimum conditions for development of these strains industrial cultivation technology for the purpose of biological preparation production on their basis are selected. As a result the biotechnology of "Pseudorhizobin" preparation reception which designed for an agricultural practice and possesses high antifungal and plant growth promoting activity has been developed.

“Pseudorhizobin” preparation is ecologically safe and harmless to health of people, animals and plants as it is created on the basis of microorganisms and natural ingredients. The bacterial strains which are a part of a preparation are salt tolerant.

The preparation has been tested in field conditions, on experimental plot on the field of Amudarya district of Kara-Kalpak republic. Soil where chickpea was cultivated was salted (EC - 568 mSm^{-1}).

In [Figure-1] the effect of “Pseudorhizobin” preparation on nodules formation on roots of different chickpea breeds in the conditions of soil salinity is shown. “Pseudorhizobin” promotes substantial increase of nodules number on roots of all chickpea breeds.

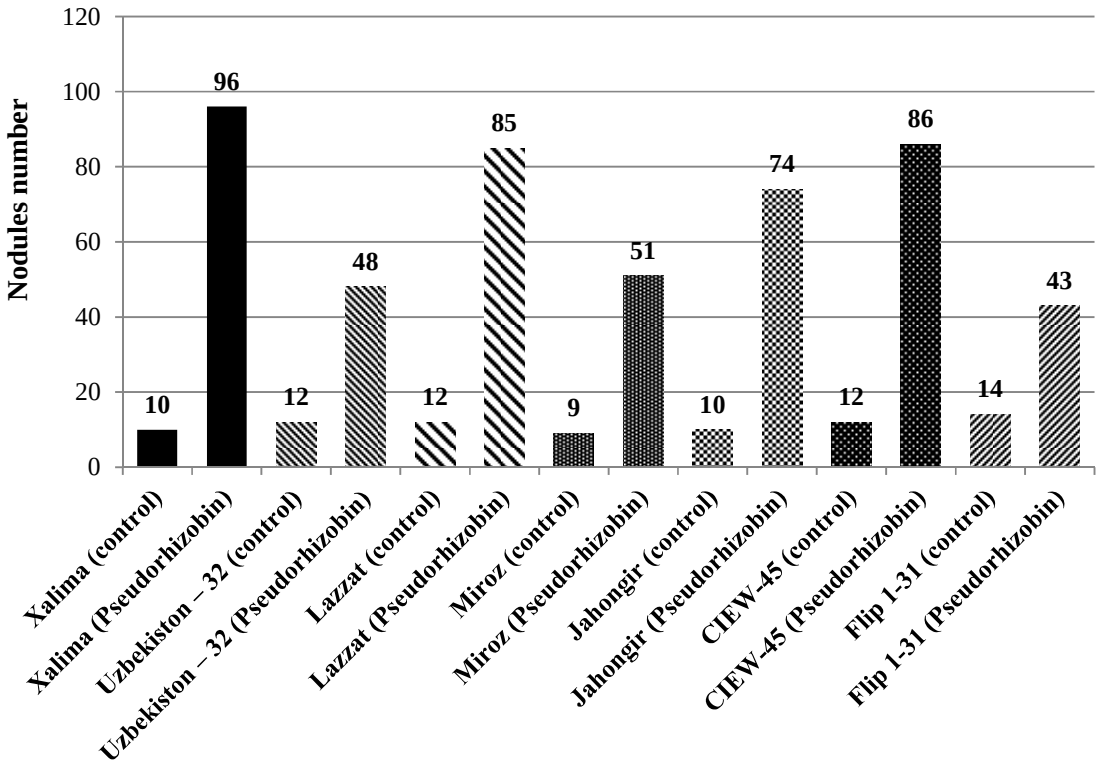

Fig: 1. The influence of a chickpea different breeds inoculation with “Pseudorhizobin” preparation on the nodules formation on chickpea roots in a soil salinity conditions.

However it is visible, that for some breeds "Pseudorhizobin" is more effective, than for others. For example, the average nodules number on control plants of "*Xalima*" chickpea breed - 10, and after application of the preparation nodules number has increased to 96, that almost in 10 times is more in comparison with the control. The nodules number on the plants roots of "*Uzbekiston*-32" chickpea breed after application of "Pseudorhizobin" preparation has increased in 4 times, at "*Lazzat*" breed - in 7 times, at "*Miroz*" breed - in 5.6 times, at "*Jahongir*" breed - in 7.4 times, at "*CIEW*-45" breed - in 7.2 times, and at "*Flip* 1-31" breed - in 3 times in comparison with control plants. The greatest effect "Pseudorhizobin" rendered on nodules formation on roots of "*Xalima*", "*Lazzat*", "*Jahongir*" and "*CIEW*-45" chickpea breeds plants.

3.2. The effect of "Pseudorhizobin" preparation on a chickpea shoots and roots growth and pods formation

In **[Table-1]** the effect of "Pseudorhizobin" preparation on a chickpea growth and development in the conditions of soil salinity is shown.

After application of "Pseudorhizobin" preparation the shoots length of "*Xalima*" chickpea breed increased by 41.6% and

[Table-1].

Chickpea breeds	Shoot length (cm)	Root length (cm)	Shoot fresh mass (g)	Root fresh mass (g)	Shoot dry mass (g)	Root dry mass (g)	Pods number
Xalima (control)	36±3	22±3	77.56±5.25	5.83±0.98	18.29±1.02	1.68±0.23	31±3
Xalima (Pseudorhizobin)	51±6	31±6	112.14±6.81	8.12±1.07	21.46±1.11	2.11±0.24	45±4
Uzbekiston – 32 (control)	33±3	25±3	68.14±4.52	5.97±1.03	17.05±1.09	1.69±0.26	16±3
Uzbekiston – 32 (Pseudorhizobin)	45±5	33±4	96.71±4.37	7.94±1.11	20.27±1.14	1.98±0.09	25±3
Lazzat (control)	33±2	21±3	73.42±4.83	5.18±0.71	17.63±1.14	1.63±0.34	30±3
Lazzat (Pseudorhizobin)	46±4	27±4	95.36±4.67	7.57±1.09	20.13±1.13	1.94±0.12	43±4
Miroz (control)	32±3	24±3	65.45±4.11	5.72±0.88	16.21±0.12	1.67±0.49	17±3
Miroz (Pseudorhizobin)	39±4	30±5	85.17±5.74	6.92±0.94	18.07±1.04	1.78±0.21	24±3
Jahongir (control)	30±2	24±4	67.15±4.29	6.03±0.72	17.02±0.07	1.72±0.05	26±4
Jahongir (Pseudorhizobin)	41±4	33±4	88.23±6.21	7.09±1.15	18.65±1.16	1.81±0.37	38±4
CIEW-45 (control)	32±3	21±3	86.54±6.52	6.23±0.73	18.31±0. 85	1.75±0.21	38±4
CIEW-45 (Pseudorhizobin)	48±4	29±5	119.74±7.14	8.25±1.15	21.72±1.42	2.14±0.37	54±4
Flip 1-31 (control)	33±3	25±4	69.59±5.35	6.17±0.85	17.37±1.21	1.72±0.34	17±3
Flip 1-31 (Pseudorhizobin)	43±4	30±5	93.48±4.11	7.63±1.14	19.65±1.13	1.96±0.12	26±3

Table: 1. The influence of a chickpea different breeds inoculation with "Pseudorhizobin" preparation on a chickpea growth and development in a soil salinity conditions.

constituted 51 cm, at "*Uzbekiston*-32" breed - by 36.4% with 45 cm, at "*Lazzat*" breed - by 39.4% with 46 cm, at "*Miroz*" breed - by 21.9% with 39 cm, at "*Jahongir*" breed - by 36.7% with 41 cm, at "*CIEW*-45" breed - by 50 % with 48 cm, at "*Flip* 1-31" breed - by 30.3% with 43 cm.

The highest shoots appeared at "*Xalima*" breed - 51 cm, however the strongest effect "Pseudorhizobin" rendered for shoot length of "*CIEW*-45" breed which has increased by 50% in comparison with the control.

Application of "Pseudorhizobin" preparation promoted increase in roots length. For example, at "*Xalima*" breed the roots length has increased by 41%, at "*Uzbekiston*-32" breed - by 32%, at "*Lazzat*" breed - by 28.6%, at "*Miroz*" breed - by 25%, at "*Jahongir*" breed - by 37.5%, at "*CIEW*-45" breed - by 38%, at "*Flip* 1-31" breed - by 20% as compared to control plants. The strongest effect "Pseudorhizobin" rendered for roots length of "*Xalima*", "*CIEW*-45" and "*Jahongir*" chickpea breeds.

The preparation application also increased shoots and roots fresh mass at "*Xalima*" breed - by 44.6 and 39.3%, at "*Uzbekiston*-32" breed - by 41.9 and 33%, at "*Lazzat*" breed - by 29.9 and 46.1%, at "*Miroz*" breed - by 30.1 and 21%, at "*Jahongir*" breed - by 31.4 and 17.6%, at "*CIEW*-45" breed - by 38.4 and 32.4%, at "*Flip* 1-31" breed - by 34.3 and 23.7% respectively in comparison with control plants.

From [Table-1] it is visible, that "Pseudorhizobin" preparation also influenced pods number on chickpea plants. So at "*Xalima*" breed plants the pods quantity increased by 45.1%, at "*Uzbekiston*-32" breed - by 56.3%, at "*Lazzat*" breed - by 43.3%, at "*Miroz*" breed - by 41.2%, at "*Jahongir*" breed - by 46,2%, at "*CIEW*-45" breed - by 42.1%, at "*Flip* 1-31" breed - by 52.9% in comparison with control plants. The biggest pods number was formed on plants of "*CIEW*-45" chickpea breed after preparation application - 54 pods, and rather less at "*Xalima*" breed - 45 pods.

3.3. The effect of "Pseudorhizobin" preparation on a chickpea crop

In **[Figure-2]** the influence of "Pseudorhizobin" preparation on the crop of various chickpea breeds is shown. So the crop of "*Xalima*" and "*Uzbekiston*-32" chickpea breeds increased by 21.9%, at "*Lazzat*" breed - by 18.9%, at "*Miroz*" breed - by 19.4%, at "*Jahongir*" breed - by 20.6%, at "*CIEW*-45" breed - by 22%, at "*Flip* 1-31" breed - by 18.5% in comparison with control plants. The greatest crop after preparation application was at "*CIEW*-45" chickpea breed - 18.23 centner/ha, at "*Xalima*" breed the crop was a little bit less - 17.61 centner/ha, at "*Lazzat*" breed - 17.39 centner/ha. The least crop after preparation application was at "*Miroz*" breed - 11.96 centner/ha, and without preparation application its crop was 10.02 centner/ha.

[IV] DISCUSSION

From the experiment results we can see, that "Pseudorhizobin" preparation improves growth, development and productivity of all investigated chickpea breeds, however in relation to some breeds ("*Xalima*", "*CIEW*-45") this preparation is especially effective. It is related to breed-specificity of our salt-tolerant bacteria *Mesorhizobium ciceri*-4 in relation to various chickpea breeds. Nodule bacteria are divided on wide-specific - capable to form effective symbiosis with various species of legume plants, and narrow-specific - capable to form productive symbiosis only with one species or even several breeds of one legumes species [18].

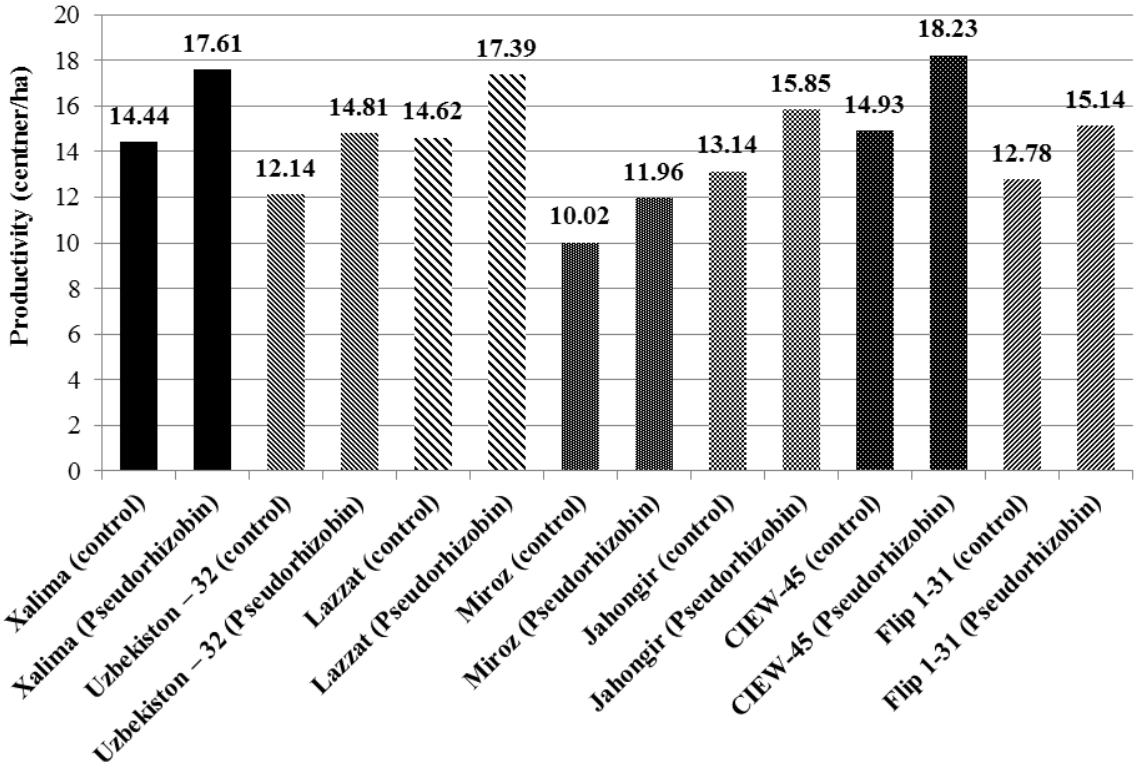

Fig: 2. The influence of a chickpea different breeds inoculation with “Pseudorhizobin” preparation on a chickpea yield in a soil salinity conditions.

Bacteria *Pseudomonas chlororaphis*-66 entering into composition of "Pseudorhizobin" preparation are capable to produce various physiologically active agents, including indolil-3-acetic acid (IAA), ACC-deaminase. It is known, that IAA is a phytohormone promoting growth of plants shoots and roots [19]. And so, thanks to IAA production by salt-tolerant bacteria *Pseudomonas chlororaphis*-66, the growth of root system in the soil salinity conditions strengthens, owing to what increases the potential quantity of sites for roots infection with nodule bacteria *Mesorhizobium ciceri*-4 [20, 21].

Also efficacy of symbiosis in many respects depends on quantity of carbohydrate-containing root exudates of this or that chickpea breed. Rhizobia synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots [22, 23, 24].

Improved mineral nutrition would explain the promotion of root and shoot growth [25, 26].

Production of ACC-deaminase by *Pseudomonas* strain make possible to reduce the level of ethylene in the roots of plants in salt stress [27]. The ACC-deaminase enzyme can cleave the ethylene precursor ACC to α-ketobutyrate and ammonium and thereby lower the level of ethylene in developing or stressed plants [28, 29, 30]. In addition *Pseudomonas chlororaphis*-66 strain is able to protect chickpea from plant pathogenic fungi, causing chickpea root diseases that increase chickpea crop too.

At application of "Pseudorhizobin" preparation, the bacteria which enter into the composition of the preparation, form "threefold" symbiosis with the chickpea plants, that is all 3 organisms

(*Mesorhizobium ciceri*-4, *Pseudomonas chlororaphis*-66 and a chickpea (*Cicer arietinum* L)) form system where each participant make special contribution to stable coexistence that as a result brings big crop.

[V] CONCLUSION

We had been created biological preparation “Psedorhizobin” on the technology described in section “materials and methods”. The received results indicate that “Pseudorhizobin” preparation can be recommended for cultivation of a chickpea in the conditions of soil salinity for the purpose of a chickpea crop increase and protection from diseases caused by plant pathogenic fungi.

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