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SYMBIOTIC EFFICIENCY AND CYTOKININ ACTIVITY OF NEW *MESORHIZOBIUM CICERI* STRAINS

The efficiency of the introduction of nodule bacteria, microsymbionts of legumes in agrocenoses, largely depends on the activity of biologically active substances' biosynthesis by diazotrophs. Seed bacterization with effective rhizobia strains capable of synthesizing exometabolites for phytostimulating activity not only promotes the formation and functioning of symbiosis but also creates the conditions for increasing plant resistance to adverse environmental conditions. ***The aim of the work*** *was to research the symbiotic activity, efficiency and ability of chickpea rhizobia new strains to biosynthesize phytohormonal exometabolites of cytokinin nature.* ***Methods****. Microbiological, physiological, cytological, biochemical, and physicochemical.* ***Results****. New strains of Mesorhizobium ciceri ND-101 and Mesorhizobium ciceri ND-64 were shown to have different symbiotic activity. The efficiency of inoculation of Skarb chickpea seeds with bacterial suspension of Mesorhizobium ciceri ND-101 was at the same level with the industrial strain of Mesorhizobium ciceri H-12. Bacterization of Mesorhizobium ciceri ND-64 increased the chickpea roots nodules by 69%, their weight by 74%, and nitrogenase activity by 73% relative to the positive control (inoculation with Mesorhizobium ciceri H-12), as well as increased chickpeas yield by 22%. It was established that Mesorhizobium ciceri ND-64 strain exhibits the highest cytokinin activity in the bioassay. Cytokinins in the total amount of 174.94 μg/g of completely dry biomass were detected in the culture medium of Mesorhizobium ciceri ND-64, which is 53% higher than that of Mesorhizobium ciceri ND-101 strain and 99% higher than that of Mesorhizobium ciceri H-12 strain.* ***Conclusions****. Mesorhizobium ciceri ND-64 strain with high nitrogen-fixing activity and symbiotic efficiency is capable to synthesize a relatively high amount of extracellular cytokinins. The high concentration of cytokinins indicates their important role in the formation and functioning of nodules, as they stimulate the proliferation of root tissues and, in this way, have a positive effect on the chickpea productivity.*

Keywords*: legume-rhizobia symbiosis, nodule bacteria, Mesorhizobium ciceri, chickpea, auxins, cytokinins, gibberellins.*

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Among the beneficial soil microorganisms, bacteria as sources of natural biologically active substances that are regulators of key components of crop metabolism are of particular importance [1, 2]. A striking example of such organisms is nodule bacteria, which in symbiosis with legumes fix the molecular nitrogen of the atmosphere and convert it into compounds that are easily digested by plants. Rhizobia also synthesize various physiologically active substances, which, in turn, increase the resistance of plants to adverse environmental conditions, increase plant yields, and improve the quality of agricultural products [3—5].

Some studies have shown that nodule bacteria of legumes in pure culture can produce auxins, cytokinins, gibberellins, and other phytohormonal substances [6, 7]. Here is information about direct lines correlations between symbiotic activity and the ability of bacteria to actively synthesize cytokinins [8].

Recently, due to climate changes and a lack of moisture, the area of chickpea crops in Ukraine is constantly growing [9], therefore, it is relevant to study the diversity of bacteria — microsymbionts of this culture, and to search for strains of *Mesorhizobium ciceri* effective for certain soil and climatic conditions with a complex of useful properties that can significantly improve the productivity of this agricultural crop.

The aim of the work was to research the symbiotic activity, efficiency and ability of chickpea rhizobia new strains to biosynthesize phytohormonal exometabolites of cytokinin nature.

Materials and methods. The objects of the research were: the industrial strain of chickpea nodule bacteria *M. ciceri* H-12 [10] and new promising strains *M. ciceri* ND-101 and *M. ciceri* ND-64 [11]. Bacteria were isolated in 2016 from the nodules of chickpea plants (cultivars Skarb and Pamyat of chickpea) grown on the experimental fields of the Plant Breeding and Genetics Institute, the National Centre of Seed and Cultivar Investigation of the National Academy of Agrarian Sciences (NAAS) of Ukraine, and deposited in the Collection of Beneficial Soil Microorganisms of the Institute of Agricultural Microbiology and Agroindustrial Manufacture of NAAS of Ukraine.

In field experiments, we used bacterial suspensions, which were cultured in-depth on a shaker at 220 rpm for three days in an organic (pea) liquid nutrient medium of the following composition (g/L): $(NH_4)_2SO_4$ — 1.0; K_2HPO_4 — 0.5; KH_2PO_4 — 0.5; $MgSO_4 \cdot 7H_2O$ — 0.2; $CaCO_3$ — 1.0; sucrose — 10.0; pea broth — 1 L (100 g of peas in 1 L of H_2O), pH = 6.8—7.0. The medium was sterilized at a pressure of 1.0 atm for 30 min.

Morphological features of bacterial cells were determined using an electron microscope Tesla BS 540 (Czech Republic) at an instrumental magnification of × 16,000 [12]. Bacterial cells were tested by the method of negative contrast enhancement with uranyl acetate.

Field experiments were conducted on the experimental fields of the Institute of Agricultural Microbiology and Agro-industrial Manufacture of NAASU (Region of Chernihiv), which are characterized by the following agrochemical parameters: humus content in the arable layer — 3.6%, mobile forms of phosphorus — 210—240 mg P_2O_5, exchangeable potassium (according to Kirsanov) — 160 — 170 mg K_2O per 1 kg of soil, water pH — 6.5.

The estimated area of the field experiments was 10 m^2. Experiments were repeated 4 times; placement of variants was randomized. Predecessor — spring oats. Pesticides and herbicides were not used: weeds were destroyed mechanically. When inoculating chickpea seeds, the bacterial load was 10^6 cells/ seed. The control seeds were moistened with water (1—2% by weight).

The nitrogenase activity of the nodules was determined by the acetylene method [13] at the end of the incubation period. The samples were analyzed on a gas chromatograph HP 4890A (Hewlett Packard, USA).

The ability of chickpea rhizobia to synthesize biologically active substances was studied using specific bioassays according to the guidelines for the determination of phytohormones [14] and methods for the determination of cytokinins, growth inhibitors, defoliants, and herbicides [15]. Analysis of the quantitative content of extracellular phytohormones synthesized by *M. ciceri* was performed using high-performance liquid chromatography (HPLC) [16].

To study the ability of *M. ciceri* to produce exometabolites of cytokinin substances by the method of specific bioassay and *analysis* of their quantitative and qualitative content in the culture medium of *M. ciceri*, the bacterial suspension was centrifuged at 4,000 rpm for 1 hour.

Extracellular phytohormones cytokinins were isolated from supernatants of culture medium of soil microorganisms according to [14], and their qualitative and quantitative determination was carried out by the method of HPLC using an Agilent 1200 liquid chromatograph (Agilent Technologies, USA) [8, 16].

Experimental data were processed using current mathematical statistics methods and the computer package Microsoft Excel.

Results. The cultural and morphological properties of the chickpea nodule bacteria strain *M. ciceri* H-12 (positive control) and new promising strains of *M. ciceri* ND-101 and *M. ciceri* ND-64 isolated from the nodules of chickpea cultivars contrasting in seed size and height and type of bush were tested.

According to the cultural characteristics, these strains are characterized by a moderate growth rate: they form small colonies on bean agar (1—2 mm) on the 4th—7th days of cultivation (Fig. 1).

Electron microscopic studies of strains with moderate growth rates show similar morphology of bacteria isolated from the nodules of chickpea plants of different cultivars (Fig. 2). The cells did not form spores; they were mobile, rod-shaped, and 1.0—2.0 × 0.3—0.5 μm in size.

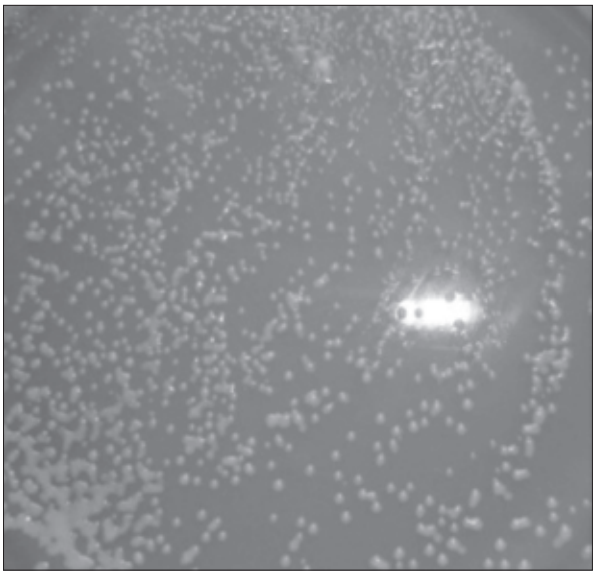


Fig. 1. Colonies of chickpea rhizobia *Mesorhizobium ciceri* ND-64 cells on bean agar on the 6th day of cultivation

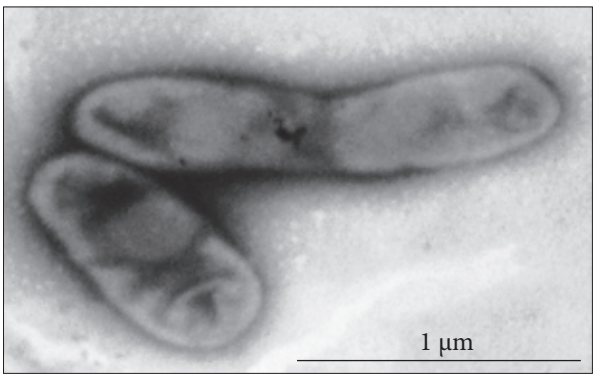


Fig. 2. Morphology of chickpea rhizobia Mesorhizobium ciceri ND-64 cells (magnification 1 × 16,000)

The following results were obtained when studying the effect of inoculation on the symbiosis formation using bacterial suspensions of *M. ciceri* and chickpea seeds of the Skarb cultivar, zoned for the Forest-Steppe zone. Thus, it was shown that *M. ciceri* ND-101 and *M. ciceri* ND-64 strains exhibited different symbiotic activity. For example, inoculation of seeds with bacterial suspension of *M. ciceri* ND-101 did not provide a significant increase in nodules on the roots of

chickpeas relative to positive control (inoculation with *M. ciceri* H-12), their weight increased by only 5% and nitrogenase activity — by 12.5% (Table 1). The use of *M. ciceri* ND-64 for bacterization of chickpea seeds contributed to the formation of the largest number of nodules (69% higher than in the positive control variant), an increase in their weight by 74% and in nitrogenase activity by 73% (Table 1, Fig. 3).

It was shown that the efficiency of chickpea seeds inoculation with bacterial suspension of *M. ciceri* ND-101 was at the same level as in industrial strain of *M. ciceri* H-12 (Table 2). At the same time, pre-sowing bacterization with *M. ciceri* ND-64 contributed to the greatest increase in yield. For example, the increase was 22% compared to the variant with the industrial strain of *M. ciceri* H-12 (Table 2).

The following results were obtained during the study of the culture medium for the presence of specific cytokinin activity at certain dilutions on the Dzherelo cultivar cucumber cotyledons. For example, at high concentrations of bacterial suspension (1:10 to 1:50), all studied strains inhibited the growth of ethylated cotyledon leaves (Fig. 4) suggesting the presence of large amount of exometabolites in the culture medium of rhizobia. Higher dilutions of rhizobia culture medium (1:500 and 1:1.000) resulted in manifestation of stimulatory activity, and a difference between the studied strains was revealed. Thus, during the incubation of cucumber cotyledons with the culture medium of the strain *M. ciceri* ND-101 (dilution 1:1,000), the increase was higher than in the positive control by 12.5% (Fig. 4). The highest rate of manifestation of cy-

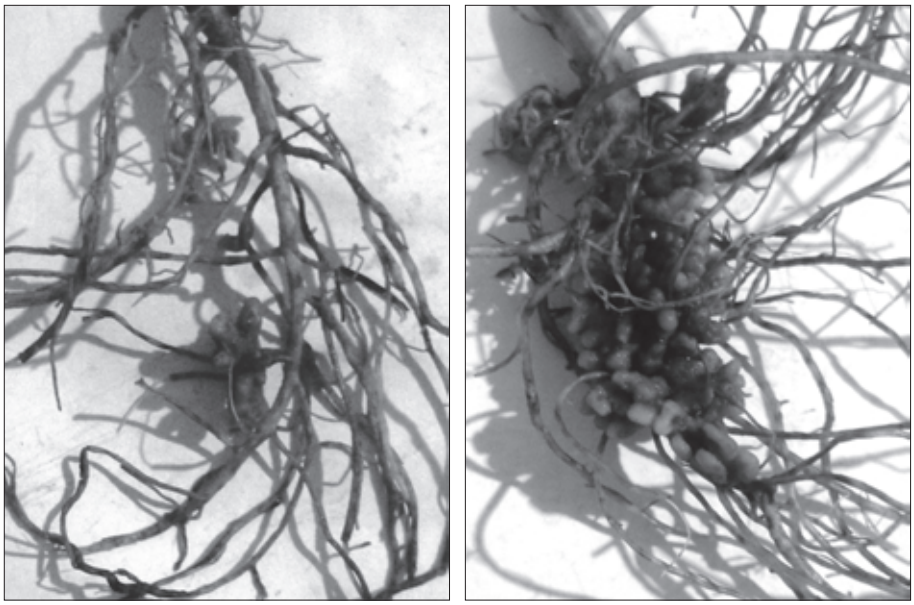
Table 1. **Symbiotic nitrogen fixation activity of Skarb cultivar chickpea plants upon inoculation with *Mesorhizobium ciceri* in the flowering phase (leached chornozem, 2019)**

Variant	Amount of nodules, pcs/plant	Weight of nodules, mg/plant	Nitrogenase activity, nmol C_2H_4 per plant per hour
Control (without inoculation)	8.4 ± 2.7	204.4 ± 9. 6	1,276.2 ± 44.0
Inoculation with *Mesorhizobium ciceri* H-12	20.8 ± 3.0	495.6 ± 4.8	1,850.0 ± 56.0
Inoculation with *Mesorhizobium ciceri* ND-101	22.4 ± 2.3	518.0 ± 9.0	2,082.0 ± 61.0
Inoculation with *Mesorhizobium ciceri* ND-64	35.1 ± 2.3	863.6 ± 4.0	3,206.0 ± 73.0

Table 2. **Influence of seed bacterization with *Mesorhizobium ciceri* strains on the yield of Skarb cultivar chickpea (leached chornozem, 2019)**

Variants of experiment	Yield, t/ha	Increase	
		t/ha	%
Control (without inoculation)	1.25	—	—
Inoculation with *Mesorhizobium ciceri* H-12	1.43	0.18	14.4
Inoculation with *Mesorhizobium ciceri* ND-101	1.44	0.19	15.2
Inoculation with *Mesorhizobium ciceri* ND-64	1.75	0.50	40.0
LSD_{05}	0.06	—	—

Note: LSD_{05} — the least significant difference



a *b*

Fig. 3. Nodules on chickpea plant roots (flowering phase): *a* — control (without inoculation), *b* — *Mesorhizobium ciceri* ND-64

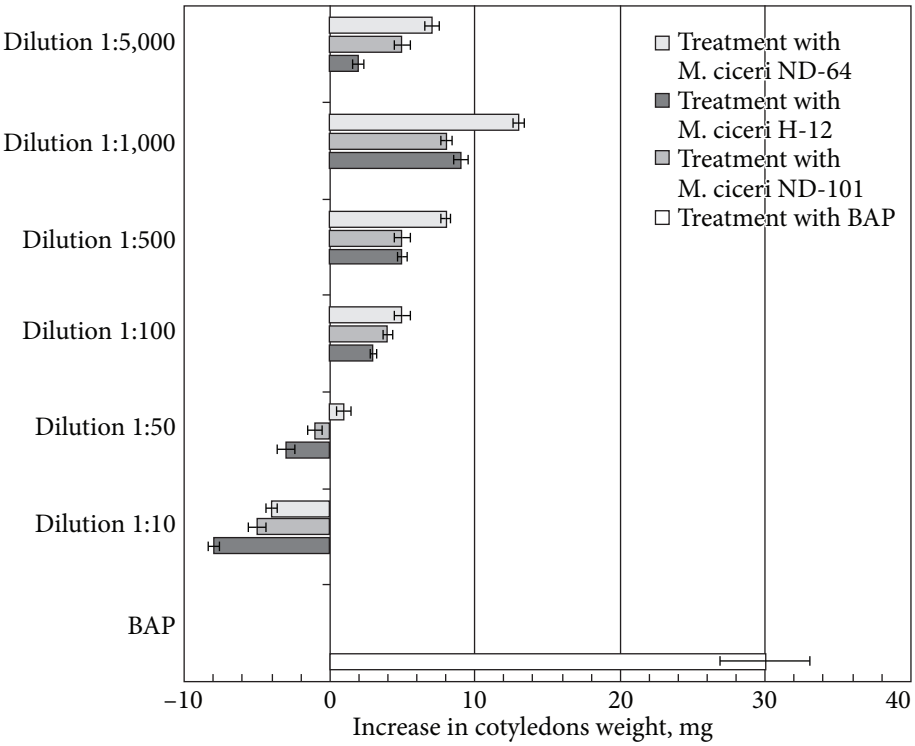


Fig. 4. Cytokinin activity of *Mesorhizobium ciceri* (bioassay — cotyledons of Dzherelo cultivar cucumber)

Table 3. **Quantitative content of extracellular cytokinins synthesized by *Mesorhizobium ciceri***

Cytokinins	Concentration of extracellular cytokinins, μg/ADB		
	Mesorhizobium ciceri H-12	*Mesorhizobium ciceri* H-64	*Mesorhizobium ciceri* H-101
Kinetin	28.51	—	—
Zeatin	—	—	—
Zeatin riboside	59.43	174.94	114.37
Zeatin glucoside	—	—	—
Isopentenyladenine	—	—	—
1-Isopentenyladenosine	—	—	—
Total amount	87.94	174.94	114.37

Note: «—» was not defined

tokinin activity was observed at 1:1,000 dilution of *M. ciceri* ND-64 culture medium. It exceeded the version with exometabolites of *M. ciceri* ND-101 strain by 44% and *M. ciceri* H-12 by 62.5% (Fig. 4).

Therefore, according to the specific biotest results, nodule bacteria *M. ciceri* are capable of synthesizing extracellular cytokinins. *M. ciceri* H-12 and *M. ciceri* ND-101 strains, which had lower indicators of symbiotic activity and efficiency than *M. ciceri* ND-64, showed a lower cytokinin activity as well.

Further, the quantitative and qualitative composition of phytohormonal exometabolites of cytokinin nature synthesized by *M. ciceri* strains was studied by HPLC. The largest concentration of synthesized cytokinins, 174.94 μg/g of absolutely dry biomass, was found in the culture medium of *M. ciceri* ND-64, which was 53% higher than that for *M. ciceri* ND-101 strain and 99% higher than for *M. ciceri* H-12 (Table 3). Differences in the qualitative composition of cytokinins were also observed. Only zeatin riboside was detected in the culture medium of the new strains of *M. ciceri* ND-64 and ND-101, while kinetin was also detected in the culture medium of the reference strain *M. ciceri* H-12. However, it is the ability to synthesize extracellular zeatin and its derivatives that plays an important role in characterizing the symbiotic efficiency of the inoculant strain [1].

Discussion. Cytokinins are a class of stimulating phytohormones that are derivatives of adenine. These compounds are similar in structure, but have different biological activity: they affect cell division, stimulate the formation and activity of meristems of lateral shoots, increase the attractiveness of tissues, delay the aging process of leaves, inhibit the growth and branching of roots, and together with auxins participate in the regulation of morphogenesis, chloroplast formation, and stomatal regulation [17]. In legume roots, cytokinins play an important role in the reactivation of the cell cycle, which contributes to the formation and growth of root nodules due to the activation of genes associated with it [18, 19].

It is known that rhizobia strains are capable of synthesizing extracellular cytokinins involved in the regulation of cell division processes, which initiates the formation of root nodules [1—8, 20].

Among domestic and foreign scientists, there is no consensus on the direct relationship between the ability of nodule bacteria to synthesize cytokinin exometabolites and their symbiotic effectiveness [6—8, 21, 22]. Studies of domestic scientists indicate that the ability of

microorganisms to produce zeatin riboside (the transport form of cytokinins) plays a key role in the formation of an effective symbiosis between soybean plants and *Bradyrhizobium japonicum* [6—8, 23].

Our findings prove that the studied strain of *M. ciceri* ND-64, which is characterized by high indicators of symbiotic efficiency and contributes to the early formation of symbiosis (compared to the industrial strain of *M. ciceri* H-12), which actively functions throughout the entire period of plant ontogenesis [24], contains a relatively high amount of exometabolites of cytokinin nature in the culture medium. In particular, the ability of *M. ciceri* ND-64 bacteria to produce zeatin riboside in the amount of 174.94 μg/g of absolutely dry biomass, which exceeds the amount of this cytokinin in the culture medium of the industrial strain *M. ciceri* H-12 by 99% and by 53% in *M. ciceri* ND-101, consistent with its high nitrogenase activity and efficiency of symbiosis with chickpea plants.

So, as a result of the research, the new strains of *M. ciceri* ND-101 and *M. ciceri* ND-64 have different symbiotic activities. The efficiency of inoculation of chickpea seeds with bacterial suspension of *M. ciceri* ND-101 was at the same level as for the industrial strain of *M. ciceri* H-12. Bacterization of *M. ciceri* ND-64 increased the number of nodules on chickpea roots (by 69% relative to the positive control), their weight by 74%, and nitrogenase activity by 73%. The yield of chickpea plants increased by 22% as a result of the bacterization of *M. ciceri* ND-64 compared to the variant of inoculation with the *M. ciceri* H-12 strain.

M. ciceri ND-64 was shown to have the highest cytokinin activity with relatively high nitrogen-fixing activity and other indicators of symbiotic efficiency. In the culture medium of *M. ciceri* ND-64, a relatively large amount of cytokinin exometabolites was found (174.94 μg/g of absolutely dry biomass), which is 53% higher than for *M. ciceri* ND-101 and by 99% — for *M. ciceri* H-12. The obtained data do not contradict the previous results regarding the increased ability of highly virulent strains to synthesize cytokinins and, in this way, influence the formation of a highly effective symbiosis with chickpea plants.

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СИМБІОТИЧНА ЕФЕКТИВНІСТЬ ТА ЦИТОКІНІНОВА АКТИВНІСТЬ НОВИХ ШТАМІВ *MESORHIZOBIUM CICERI*

Ефективність інтродукції бульбочкових бактерій, мікросимбіонтів бобових рослин, в агроценози значною мірою залежить від активності біосинтезу діазотрофами біологічно активних речовин. Бактеризація насіння ефективними штамами ризобій, що здатні до синтезу екзометаболітів з фітогормональною активністю, не лише сприяє формуванню та функціонуванню симбіозу, а й створює передумови для підвищення стійкості рослин до несприятливих умов довкілля. **Метою** роботи було дослідити симбіотичну активність, ефективність та здатність нових штамів ризобій нуту до біосинтезу фітогормональних екзометаболітів цитокінінової природи. **Методи.** Мікробіологічні, фізіологічні, цитологічні, біохімічні та фізико-хімічні. **Результати.** Показано, що нові штами *M. ciceri* ND-101 та *M. ciceri* ND-64 мають різну симбіотичну активність. Ефективність інокуляції насіння нуту сорту Скарб бактеріальною суспензією *M. ciceri* ND-101 була на рівні з виробничим штамом *M. ciceri* H-12. Бактеризація *M. ciceri* ND-64 сприяла підвищенню кількості бульбочок, збільшенню їх маси та нітрогеназної активності щодо позитивного контролю (інокуляція *M. ciceri* H-12), а також збільшенню врожайності рослин нуту на 22%. Встановлено, що штам *M. ciceri* ND-64 проявляє найвищу цитокінінову активність у біотесті. В культуральній рідині штаму *M. ciceri* ND-64 виявлено цитокініни загальною кількістю 174,94 мг/г абсолютно сухої біомаси, що на 53% перевищує даний показник штаму *M. ciceri* ND-101 та на 99% — штаму *M. ciceri* H-12. **Висновки.** Штам *M. ciceri* ND-64 з високими показниками азотфіксувальної активності та симбіотичної ефективності здатний до синтезу порівняно високої кількості позаклітинних цитокінінів. Велика концентрація цитокінінів свідчить про їх важливу роль у формуванні і функціонуванні бульбочок, оскільки вони стимулюють процеси проліферації тканин кореня і, в такий спосіб, позитивно впливають на продуктивність нуту.

Ключові слова: *бобово-ризобіальний симбіоз, бульбочкові бактерії, Mesorhizobium ciceri, нут, ауксини, цитокініни, гібереліни.*