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THE EFFECT OF BACTERIZATION AND PRE-SOWING SEED TREATMENT WITH BENORAD ON THE GROWTH OF SOYBEAN PLANTS AND THE REALIZATION OF THE SYMBIOTIC POTENTIAL OF PESTICIDE RESISTANT RHIZOBIA

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Background. Bacterial fertilizers based on active strains of nitrogen-fixing microorganisms and fungicides for pre-sowing seed dressing are increasingly used in soybean cultivation technologies. Given the usefulness of combining the specified processes, the selection of chemical and biological preparations for the combined processing of seed material is relevant.

Materials and Methods. In vegetation experiments, the effect of treatment of soybean seeds with benorad and inoculation with nodule bacteria resistant to fungicides in pure culture, obtained by the methods of analytical selection and transposon mutagenesis, on plant growth, the number and weight of root nodules and their nitrogenase activity was studied. Physiological, microbiological, statistical methods and gas chromatography were used.

Results. Under the complex application of inoculants and benorad, we recorded a decrease in the weight of the aerial part of soybeans by 8.7–20.9 % and the weight of roots by 4.8–16.8 % during the growing season compared to control plants (regardless of the strain of rhizobia used for bacterization). In the case of seeds dressing, the dynamics of the formation of the number of root nodules by the *B. japonicum* B78 differed from other inoculant strains that were used in the research. This indicator decreased by 20.6 and 16.3% at the stage of three true leaves and budding–beginning of flowering and increased (by 28.0 %) at the stage of pods formation compared to control plants. The negative effect of seed dressing on the nitrogenase activity of symbiotic systems was



observed after the application of benorad, at the stage of three true leaves and budding–beginning of flowering was noted. The degree of inhibitory effect of the chemical preparation on intensity of N₂ assimilation depended on the properties of microsymbionts. In the stage of pods formation, the level of N₂ fixation by soybean root nodules formed by the *B. japonicum* PC07 and B144 during seed dressing exceeded the corresponding level in the control plants by 32.2 and 45.7 %, respectively.

Conclusions. The use of microbial preparations for inoculation of soybean seeds, made on the basis of nodule bacteria strains with high resistance to chemical plant protection agents, allows for a gradual reduction of the toxic effect of artificially synthesized compounds on the formation and functioning of symbiotic systems.

Keywords: soybean, fungicides, seed dressing, nodule bacteria, number and weight of nodules, nitrogenase activity

INTRODUCTION

Soybean is the leading high-protein crop in the world's crop production, which is one of the most widespread legumes and oil crops and is important in agriculture, technical industry, and medicine (Shea, Singer, & Zhang, 2020). Its unique chemical composition is complemented by an important biological feature, which consists in the effective fixation of atmospheric nitrogen in symbiosis with nodule bacteria (Ohya, 2017). As a result, there is an increased interest in biological preparations based on selected nitrogen-fixing microorganisms in the world.

Scientists in many countries are actively working on improving the productivity of symbiosis, reducing the anthropogenic load on ecosystems, and obtaining ecologically clean products of agro-industrial production using the selection of legumes and rhizobia (Kots *et al.*, 2011; Igiehon, & Babalola, 2018). However, it should be taken into account that soybean is becoming infected by many diseases of fungal, bacterial and viral origin (Whitham *et al.*, 2016; Petrychenko *et al.*, 2016). The greatest danger for this culture are fusarium (*Fusarium* spp.), phomopsis (*Phomopsis sojae*), downy mildew (*Peronospora manshurica*), sclerotiniosis (*Sclerotinia sclerotiorum*), cotyledon bacteriosis and viral diseases. Root rot is becoming more and more widespread in various soil and climatic zones of our country. Seedling diseases, which lead to thinning of crops, are particularly harmful. Soybean plants are mostly infected by several phytopathogens at the same time, which reduces their yield by 15–30 %, protein content by 4–5 %, and oil content by 3–7 % (Borzykh, 2015).

To limit the development of pathogens of various etiologies, the chemical method of plant protection is most often used. It is based on the use of preparations toxic to pathogenic microbiota that include artificially synthesized substances. The pre-sowing treatment of seeds with fungicides has become particularly popular (Lamichhane *et al.*, 2020; Sagitov *et al.*, 2020). It is known that seed material can be a reservoir of many pathogens. A hidden form of seed infection, which does not outwardly appear, has the greatest influence on the potential productivity of plants. It can appear only under the influence of a set of conditions during storage or after sowing (Petrychenko *et al.*, 2016, Gaur *et al.*, 2020).

According to Fostolovich (Fostolovich, 2013), pre-sowing treatment of seeds with maxim XL and benorad poisons with the sequential use of a microbial preparation of nodule bacteria provided a reduction in the development of pathogens of septoriosis,

downy mildew and rust by 5–6 % compared to inoculated plants (without the use of fungicides). The author noted that the protective effect of pre-sowing treatment does not remain throughout the growing season.

For effective protection against seed and soil infection, it is important to choose a drug that would act on the entire complex of pathogens contained in seed and soil (Lamichhane *et al.*, 2020; Gaur *et al.*, 2020).

As a result of our laboratory experiments, artificial simulation of the effect of different rates of fungicides maxim XL, standak top, fever, acanto plus and benorad on nodule bacteria obtained by various selection methods made it possible to determine the degree of their resistance to the specified preparations and to identify the most resistant rhizobia for the purpose of their further use in soybean cultivation technologies. It was found that among the chemical plant protection products involved in the work, it was benorad (based on benomyl) that was characterized by a high level of toxicity to a wide range of strains of soybean nodule bacteria (Kukol, Vorobey, & Kots, 2019; Vorobey, Kukol, & Kots, 2020).

Therefore, the aim of our work was to investigate the effect of pre-sowing treatment of seeds with benorad on plant growth and the processes of formation and functioning of soybean symbiotic systems with the participation of fungicide-resistant nodule bacteria *Bradyrhizobium japonicum*.

MATERIALS AND METHODS

Vegetation experiment was conducted with soybean plants (*Glycine max* (L.) Merr.) of the Almaz variety, which has been included in the Register of Plant Varieties of Ukraine since 2007. It is early-ripening and recommended for cultivation in the Forest-Steppe zone (State register of plant varieties suitable for dissemination in Ukraine in 2023). In the study, a systemic fungicide benorad with benomyl as the active substance (500 g/kg, benzimidazole class) was used. The consumption rate of the preparation for treating soybean seeds is 3 kg/1 ton of seeds (Yashchuk *et al.*, 2022).

Before sowing, fungicide seed treatments were inoculated for 1 h with a cell suspension (10^8 cells/mL) of fungicide resistance *B. japonicum* strains PC07, PC09 (analytical selection) and Tn5 mutants B78, B144. It was obtained by the method of transposon mutagenesis of *B. japonicum* 646 with *Escherichia coli* S17-1 containing the plasmid pSUP5011::Tn5 *mob*.

The scheme of the experiment included the following variants for pre-sowing seed treatment: 1) *B. japonicum* PC07; 2) *B. japonicum* PC07 + benorad; 3) *B. japonicum* PC09; 4) *B. japonicum* PC09 + benorad; 5) *B. japonicum* B78; 6) *B. japonicum* B78 + benorad; 7) *B. japonicum* B144; 8) *B. japonicum* B144 + benorad.

B. japonicum strains PC07, PC09 and Tn5 mutants B78, B144 used in the research are stored in the collection of nitrogen-fixing microorganisms of the Institute of Plant Physiology and Genetics NAS of Ukraine.

Soybeans were grown on a sandy substrate (15 kg washed river sand, 10 plants in each pot on) with the introduction of Hellriegel nutrient mixture with 0.25 of nitrogen norm (1 norm was 708 mg Ca (NO₃)₂·4H₂O per kg of sand). River sand is the sand extracted from riverbeds, which is characterized by a high degree of purification and the absence of foreign inclusions. The pots with plants were placed on a specially equipped site of the Institute of Plant Physiology and Genetics NAS of Ukraine under conditions of natural light, temperature and artificial controlled irrigation.

Bacterization and treatment of soybean seeds with a fungicide was carried out at intervals of 1 hour on the day of sowing. In the control, inoculated soybean seeds were not treated with the fungicide. Repeatability of the experimental variants was 6 times.

Nitrogenase activity, the number and weight of root nodules were determined at the three true leaves stage, budding–beginning of flowering stage and pods formation. Determination of the weight of the above-ground part of plants, roots and nodules was repeated in ten times. Nitrogenase activity was determined by acetylene method in terms of acetylene regeneration activity by root nodules of soybean (Hardy *et al.*, 1968). Repeatability of nitrogenase activity determination was 6-fold.

The obtained data were processed by generally accepted methods of variation statistics using Microsoft Excel. The significance of the difference between treatments were evaluated using ANOVA. The results were presented in the form of mean values and standard error ($x \pm SE$). Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of the study revealed that the indicators of the above-ground weight of soybean plants during the growing season in variants with the use of benorad were lower compared to control plants (without seed dressing), regardless of the strain of rhizobia. In the stage of three true leaves, the toxic effect of the chemical preparation led to a decrease in the weight of the aerial part of plants inoculated with analytically selected strains of *B. japonicum* PC07 and PC09 by 8.7 and 17.9 %. In soybean plants bacterized with transposon mutants of *B. japonicum* B78 and B144 it was 11.3 and 12.8 %, respectively. In the stage of budding – the beginning of flowering of soybeans, under the action of benorad, a decrease in the above-ground weight of plants after inoculation with the rhizobia by 9.8–20.9 % was noted. In the case of using the inoculant based on *B. japonicum* B144 combined with the action of the fungicide, the above-ground weight of plants at the stage of pods formation was at the level of the control plants, and in the conditions of a combined treatment with the fungicide and rhizobia of the PC07 strain, an increase by 10.4 % was noted.

The results show that during the pre-sowing treatment of chickpeas with benorad, the viability of the seed material decreased. The authors found a significant retardation of seedling development and a decrease in seed quality with full control of seed infection (Bushulyan, & Bushulyan, 2013). The highest level of fungicidal activity when adding 0.1% of captan, benomyl, carboxin, thiram and mancozeb to the nutrient culture medium for the growth of pure cultures of such pathogenic micromycetes as *Alternaria alternata*, *Macrophomina phaseolina*, *Drechslera specifera*, *Fuzarium oxysporum* and *Rhizoctonia solani* was found in benomyl (Nasreen, 2003).

During the growing season of the plants, the inhibitory effect of benorad on the weight of soybean roots was noted, with a tendency to a significant weakening of its effect during the stage of pods formation. The weight of the roots decreased with the use of nitrogen-fixing microorganisms in combination with the fungicide compared to the control plants by 10.7–16.8 % in the stage of three true leaves, 7.8–15.1 % in the stage of budding–beginning of flowering and 4.8–6.2 % in the stage of pods formation. Under the complex application of the strain *B. japonicum* PC07 and benorad, the activation of rhizogenesis in soybeans by 5.4 % in the stage of pods formation, compared to bacterization without fungicide was found (**Table 1**).

Table 1. Above-ground fresh weight and root fresh weight (g/plant) of soybean plants grown under the action of benorad and inoculated with pesticide resistant strains of nodule bacteria *Bradyrhizobium japonicum*

Treatment	Stage of ontogenesis:					
	3 true leaves		budding–beginning of flowering		pods formation	
	above-ground weight	root weight	above-ground weight	root weight	above-ground weight	root weight
PC07	3.90±0.19	2.56±0.11	4.97±0.24	2.74±0.12	6.37±0.31	4.07±0.17
PC07 + benorad	3.56*±0.15	2.25*±0.09	3.93*±0.19	2.41*±0.10	7.03±0.34	4.29±0.19
PC09	3.40±0.16	2.02±0.08	5.05±0.23	3.18±0.16	8.33±0.40	3.60±0.18
PC09 + benorad	2.79*±0.14	1.68*±0.07	4.46±0.21	2.70*±0.15	7.66±0.35	3.39±0.14
B78	3.36±0.14	2.14±0.10	5.40±0.25	2.43±0.12	8.23±0.40	4.40±0.20
B78 + benorad	2.98*±0.13	1.91*±0.09	4.65*±0.22	2.24±0.11	7.44±0.36	4.19±0.16
B144	3.45±0.14	1.78±0.07	4.51±0.20	2.09±0.10	5.45±0.24	3.38±0.15
B144 + benorad	3.01*±0.15	1.53*±0.06	4.07±0.18	1.86±0.08	5.36±0.26	3.17±0.13

Note: $x \pm$ standard error, $n = 10$; * significant difference compared to the inoculation of seeds with the same strain without benorad at $P < 0.05$ within one column

As a result of the research, it was found that the toxic effect of benorad on the number of nodules formed on the roots depended on the properties of the inoculant strain (*B. japonicum* PC07, PC09, B78 and B144) and the stage of development of plants *Glycine max* (L.) Merr. In the inoculated of soybean seeds with RS07 rhizobia in the stage of budding–beginning of flowering and formation of beans, the number of root nodules was 22.0 and 16.8 % higher compared to the similar data of control plants.

Under the action of benorad, the virulence of rhizobia of RS09 and B144 strains was significantly weakened, which led to a decrease in the number of formed root nodules during the growing season of soybeans by 27–42.9 % and 13.3–23.1 %, respectively, compared to the control plants.

In variants with seed dressing with benorad, the dynamics of the formation of the number of root nodules with the participation of *B. japonicum* B78 differed from other inoculant strains used in the research. There was a decrease in the number of nodules by 20.6 and 16.3 % in the stage of three true leaves and budding–beginning of flowering, and a significant increase (by 28.0 %) in the stage of pods formation compared to control plants.

An increase in the weight of active root nodules is one of the determining indicators of the formation and functioning of an effective legume-rhizobial symbiosis (Igiehon,

& Babalola, 2018). The weight of nodules was reduced by 13.7 % when using the preparation based on *B. japonicum* PC07 and benorad only in the stage of three true leaves of soybean. In the stage of budding–beginning of flowering and the formation of beans, these values exceeded the corresponding values of the control plants by 1.6 and 1.4 times. The effect of the combined use of Tn5-mutant B78 and fungicide caused a decrease in the weight of root nodules in soybean by 34.2 % in the stage of three true leaves, by 9.3 % in the stage of budding–beginning of flowering, and an increase by 12.8 % in the stage of pods formation (**Table 2**).

Table 2. The number and weight of root nodules in soybeans of the Almaz variety after seed treatment with benorad and inoculation with pesticide resistant strains of nodule bacteria (per 1 plant)

Treatment	Stage of ontogenesis:					
	3 true leaves		budding–beginning of flowering		pods formation	
	number of nodules	weight of nodules	number of nodules	weight of nodules	number of nodules	weight of nodules
PC07	12.7±1.5	0.153±0.007	13.2±1.7	0.227±0.010	22.0±2.0	0.469±0.023
PC07 + benorad	12.3±1.2	0.132*±0.006	16.1±1.9	0.362*±0.016	25.7±2.5	0.637*±0.031
PC09	14.0±1.7	0.204±0.010	13.7±1.5	0.164±0.008	18.3±1.8	0.555±0.026
PC09 + benorad	8.0*±0.5	0.130*±0.005	10.0*±0.8	0.147*±0.007	12.3*±1.0	0.356*±0.017
B78	18.0±1.5	0.140±0.007	20.3±2.0	0.214±0.011	19.3±1.6	0.493±0.024
B78 + benorad	14.3*±1.5	0.092*±0.004	17.0±2.0	0.194±0.009	24.7*±2.2	0.556±0.026
B144	16.0±1.6	0.120±0.006	17.3±1.8	0.143±0.007	27.3±2.7	0.392±0.017
B144 + benorad	13.7±1.0	0.101*±0.004	15.0±1.5	0.126*±0.006	21.0*±1.8	0.269*±0.014

Note: $x \pm$ standard error, $n = 10$; * significant difference compared to the inoculation of seeds with the same strain without benorad at $P < 0.05$ within one column

Pre-sowing seed treatment had a negative effect on the nitrogenase activity of symbiotic systems formed with the participation of rhizobia strains when applying benorad, at the stages of three true leaves and budding–beginning of flowering. At the same time, the degree of inhibitory effect of the chemical preparation on the intensity of N_2 assimilation depended on the properties of the microsymbionts. In the case of the complex application of a fungicide and seed bacteriization with rhizobia of the RS07 strain, the nitrogenase activity of nodules in experimental plants compared to control plants was lower by 14.2 and 22.9 % (at the stages of three true leaves and budding–beginning of flowering, respectively) and significantly increased – by 32.2 % in the stage of pods

formation. Similar dynamics of nitrogenase activity was observed during inoculation of seeds of *Glycine max* (L.) Merr. by rhizobia strain B144 in a complex with benorad. At the stages of three true leaves and budding–beginning of flowering, the nitrogenase activity of soybean nodules was lower compared to the data of control plants by 28.9 and 13.5 %, respectively, and increased by 45.7 % in the stage of pods formation (Fig. 1).

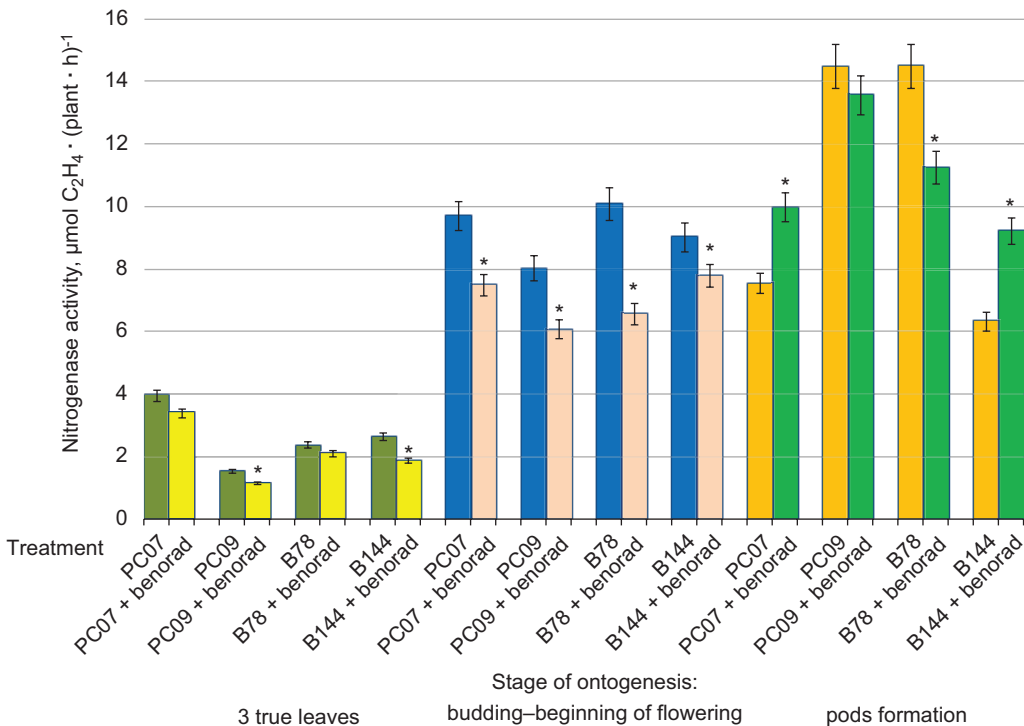


Fig. 1. Nitrogenase activity of soybean-rhizobial symbioses ($\mu\text{mol C}_2\text{H}_4 \cdot (\text{plant} \cdot \text{h})^{-1}$) after seed treatment with benorad and inoculation with pesticide resistance rhizobia

Note: $x \pm$ standard error, $n = 6$; * significant difference compared to the inoculation of seeds with the same strain without benorad at $P < 0.05$ within a pair of columns

Some studies reported the isolation and determination of biological properties of nodule bacteria with high resistance to the active substances of pesticides. Shahid and Khan (2019) researched *B. japonicum* strain RV9 in pure culture and symbiosis with mung bean (*Vigna radiata* (L.) R. Wilczek) plants under the effect of hexaconazole (triazole fungicide). The authors concluded that the use of this strain as an inoculant reduces the toxic effect of the pesticide on the macrosymbiont. After increasing the concentration of the fungicide, they noted a negative effect on the growth and development of plants, ultrastructural anatomical changes in nodules, changes in the level of proline and antioxidant enzymes.

The use of fungicides in growing technologies of other leguminous crops, in particular peas, is increasing. Shahid, Khan, & Kumar (2019) are actively working on the isolation and identification of nitrogen-fixing microorganisms, the introduction of which in the agrobiocenosis could reduce the phytotoxic effect of the active substances of pesticides

on plants. As a result of this work, the *Rhizobium leguminosarum* RP1 strain was isolated, which provided a reduction in the confirmed in vitro and in vivo toxicity of chitazine for pea plants. In the conditions of pre-sowing bacteriization with this strain, the weight of accumulated dry matter, the content of photosynthetic pigments, and the quantity and quality of grain increased compared to non-inoculated control plants. Under fungicidal stress (an increased level of chitazine in the soil), *R. leguminosarum* RP1 formed an effective symbiosis with pea plants and produced biologically active substances that stimulated the growth of aerial weight and roots, which led to an increase in grain productivity of the pea (Shahid, Khan, & Kumar, 2019).

Another study that investigated the symbiotic systems formed with the participation of soybean plants and *B. japonicum* 634b under the seed dressing with benorad 14 days before sowing and on the sowing day revealed a complete suppression of the symbiotic apparatus formation in plants during the budding and flowering stages. The nodulation ability of rhizobia was activated only at the stage of pods formation in plants of the variant with pre-treatment of the seed. The authors attribute this effect to the fact that the active substance of the preparation – benomyl is quite persistent, as a result of which it acts on the physiological processes of plants and nodule bacteria for a long time. They do not exclude the presence of an increased toxic effect of benomyl at certain stages of the nodulation process. The symbiotic apparatus was not formed at all after seed treatment with strain 634b and benorad on the day of sowing, so the plants did not assimilate N₂ (Pavlyshche, Kiriziy, & Kots, 2017).

CONCLUSION

Thus, when using strains of nitrogen-fixing microorganisms with high sensitivity to the active substance of the pesticide, there is a possibility of impact on micro- and macrosymbionts at certain stages of their interaction, as well as the risk of extremely high fungicidal stress. It may be the reason for the complete absence of biological nitrogen fixation. Application of microbial preparations based on the strains of nodule bacteria with high resistance to chemical plant protection agents for inoculation of soybean seeds allows for a gradual weakening of the toxic effect of artificially synthesized compounds on the formation and functioning of symbiotic systems.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by any of the authors.

Animal Studies: This article does not include animal studies.

AUTHOR CONTRIBUTIONS

Conceptualization, [K.P.;N.A.]; methodology, [P.P.;K.P.;N.A.]; validation, [K.P.;S. Ya.]; formal analysis, [P.P.;K.P.;S.Ya.]; investigation, [P.P.;K.P.;N.A.;S.Ya.]; resources, [P.P.;K.P.]; data curation, [K.P.;N.A.;S.Ya.]; writing – original draft preparation, [K.P.;N.A.;S. Ya.]; writing – review and editing, [P.P.;K.P.;S.Ya.]; visualization, [P.P.;K.P.;N.A.;S.Ya.] supervision, [S.Ya.].

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ВПЛИВ БАКТЕРИЗАЦІЇ ТА ПЕРЕДПОСІВНОЇ ОБРОБКИ НАСІННЯ БЕНОРАДОМ НА РІСТ РОСЛИН СОЇ ТА РЕАЛІЗАЦІЮ СИМБІОТИЧНОГО ПОТЕНЦІАЛУ РИЗОБІЙ, СТІЙКИХ ДО ПЕСТИЦИДІВ

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Вступ. У технологіях вирощування сої значного поширення набуло застосування бактеріальних добрив, виготовлених на основі активних штамів азотфіксуювальних мікроорганізмів і фунгіцидів для передпосівного протруювання насіння. Беручи до уваги доцільність суміщення вказаних процесів, актуальним є добір хімічних і біологічних препаратів для сумісної обробки посівного матеріалу.

Матеріали й методи. У вегетаційних дослідах вивчали вплив обробки насіння сої бенорадом та інокуляції стійкими у чистій культурі до фунгіцидів бульбочковими бактеріями, отриманими методами аналітичної селекції і транспозонового

мутагенезу на ріст рослин, кількість і масу корневих бульбочок і їхню азотфіксувальну активність. Застосовували фізіологічні, мікробіологічні, статистичні методи та газову хроматографію.

Результати. За комплексного застосування інокулянтів та бенораду зафіксовано зниження маси надземної частини сої на 8,7–20,9 % та маси коренів на 4,8–16,8 % упродовж вегетації порівняно з контрольними рослинами незалежно від використаного для бактеризації штаму ризобій. На тлі протруювання посівного матеріалу відрізнялась від інших штамів-інокулянтів, використаних у роботі, динаміка формування кількості корневих бульбочок за участю *V. japonicum* B78. Зокрема, відмічено зменшення цього показника на 20,6 та 16,3 % у фази трьох справжніх листків і бутонізації–початку цвітіння та істотне його збільшення (на 28,0 %) у фазу утворення бобів порівняно з контрольними рослинами. У результаті аналізу азотфіксувальної активності симбіотичних систем, сформованих на тлі застосування бенораду, відмічено негативний вплив протруювання на досліджуваний показник у фази трьох справжніх листків і бутонізації–початку цвітіння. За такої умови ступінь інгібувальної дії хімічного препарату на інтенсивність асиміляції N_2 залежав від властивостей мікросимбіонтів. Водночас у фазу утворення бобів рівень фіксації N_2 корневими бульбочками сої, сформованими за участю *V. japonicum* PC07 та B144 на тлі протруювання, перевищував контрольні рослини на 32,2 та 45,7 % відповідно.

Висновки. Застосування для інокуляції насіння сої мікробних препаратів, виготовлених на основі штамів бульбочкових бактерій з високою резистентністю до хімічних засобів захисту рослин дає змогу забезпечити поступове ослаблення токсичного впливу штучно синтезованих сполук на формування та функціонування симбіотичних систем.

Ключові слова: соя, фунгіциди, протруювання, бульбочкові бактерії, кількість і маса бульбочок, азотфіксувальна активність