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SYMBIOTIC INTERACTION BETWEEN A MIXTURE OF *BRADYRHIZOBIUM JAPONICUM* STRAINS AND DIFFERENT SOYBEAN CULTIVARS

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Aim. To study the mutual relations between different soybean cultivars and two *Bradyrhizobium japonicum* strains after mixed inoculation, to evaluate the impact of the introduced microorganisms on the local rhizobial communities in soil, the formation and functioning symbiotic systems, and productivity of soybean. **Methods.** Microbiological and serological methods; field experiment, gas chromatography and mathematical-statistical methods. **Results.** A small-plot field experiment demonstrated that the use of a mixture of *B. japonicum* 46 and *B. japonicum* KB11 strains promoted a more even distribution of the local and introduced rhizobia in the nodule populations of 12 soybean cultivars of different geographic origin. There was no domination of particular strains in the nodules, and the symbiotic systems formed were more balanced than the control without inoculation. After inoculation of the above-mentioned mixture, in most cultivars there was a considerable increase in the number of nodules (10–45 % more) and their mass (11–86 % increase). There was also an increase in the level of symbiotic nitrogen fixation with 1.2–4.2 times and an increase in the weight of grain per plant by 6–29 % (depending on the cultivar). The efficiency of a mixture of *B. japonicum* 46 and KB11 strains in a preparation Rizogumin was confirmed in extensive field trials with soybean on an area of about 60 thousand ha in different regions of Ukraine. In the background of the local populations of the soybean nodulating bacteria in the areas, the application of the formulated mixture of the two rhizobia strains ensured a stable increase by 15–33 % in the soybean yield as compared with the control without the inoculation. **Conclusions.** A novel approach was investigated, which lies in the application of a mixture of *B. japonicum* 46 (genetic group USDA 6) and *B. japonicum* KB11 (genetic group USDA 123) strains for different soybean cultivars inoculation. Combining these two strains and their introduction into agrocenoses ensured the formation of a balanced symbiotic systems (without clear domination of some strains in the nodules). This was expressed in an intensified nodulation, symbiotic nitrogen fixation, and increased yield of different cultivars of soybeans by 15–33 % as compared to the control (without inoculation).

Key words: *Bradyrhizobium japonicum*, a mixture of strains, nitrogen-fixation, balanced symbiotic systems, biopreparation Rizogumin, soybean cultivars, symbiotic effectiveness.

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INTRODUCTION

Soybean *(Glycine max* (L.) Merr.) is a main leguminous plant in global agriculture and one of the strategic crops for Ukraine (Kyrychenko et al, 2016; Alkhayri et al, 2019). Similar to all legumes, soybean can enter symbiotic relation with nitrogen-fixing, nodulating bacteria, satisfying their need for nitrogen partially or completely.

Ukrainian soils do not have autochtonous populations of soybean nodulating rhizobia (Tolkachov, 1997; Krutylo, 2006), but the periodic cultivation of the crop with biopreparations in agricultural production systems resulted in the formation of local communities of bacteria which belong, as far as investigated to the species *Bradyrhizobium japonicum* (Kyrychenko et al, 2016; Kots et al, 2011; Krutylo DV et al, 2008). It is well known that the quality and quantity composition of the soil populations of these microorganisms may gradually change, and their representatives may often be competitors of the strains used in inoculum preparations (Tolkachov, 1997;

Krutylo, 2006; Kots et al, 2011; de Bruijn, 2015). Therefore, there is a persistent need for new, highly effective strains – inoculants for soybean.

Our previous studies demonstrated that in Ukrainian soil, the populations of rhizobia are represented by strains of *B. japonicum*, which differ considerably in the phenotypic and genotypic features (Krutylo et al, 2008; Krutylo, Leonova, 2016). Some 180 new strains of soybean rhizobia were isolated from soybean nodules grown on different types of soil. Special attention was paid to the study of strains that had high efficiency and increased saprophytic competence (Volkohon, Moskalenko, 2021), i.e. the ability to survive in soil outside or in absence of the host plant.

In the current global agricultural practice biopreparations designed using specific microorganisms and those obtained from combining several microorganisms from the same or different functional groups are widely used. In several cases the preparations based on mixed cultures of microorganisms showed a clear enhanced effectiveness and stability of the impact on plants (Sytnikov, 2012; Masciarelli et al, 2014; Santoyo et al, 2021). However, the results are not so unambiguous and require further research.

We selected two novel, highly effective strains of soybean rhizobia (*B. japonicum* 46 and *B. japonicum* KB11), that belong to different genetic groups (USDA 6 and (preliminary) USDA 123 respectively) (Krutylo, Leonova, 2016; Krutylo et al, 2020). Both strains are virulent, and capable of colonizing the soybean root system, also when local populations of soybean rhizobia with different density are present in the soil (VolkohonMoskalenko, 2021). In addition, the strain *B. japonicum* 46 appeared to enhance the resistance of soybean plants to bacterial and fungal diseases (Kovalevska et al, 2009). As for the strain *B. japonicum* KB11, it is remarkable for higher ability to survive in soil for a long time and dominate in the nodules, i.e. the strain appears to be more suitable for different soil and climatic conditions of Ukraine and efficiently competes with local rhizobia (Volkohon, Moskalenko, 2021).

A mixture of strains *B. japonicum* 46 and KB11 was formulated with a carrier (Krutylo DV, 2017). When this formulation was used as inoculum the selected strains mutually supplemented and enhanced the effect of each other, ensuring a reliable increase in symbiotic indices and productivity of soybean as compared to monoinoculation (Krutylo, Leonova, 2016; Krutylo, 2017; Volkohon, Moskalenko, 2021).

The rational use of the possibilities of legume-rhizobial symbiosis requires paying attention not only to the effectiveness of new strains and their combinations but also knowing under which conditions the strains-inoculants colonize the root system of plants successfully and preserve the symbiotic activity (Kots et al, 2011). There exists inter- and intracultivar variability of legumes towards different symbiotic strains of the same and different species (Abbasi et al, 2008; Muthuri et al, 2014; Yang et al, 2018; Mortuza et al, 2022; Nakei et al, 2022).The hypothesis for our study was that the inoculation of soybean seeds with two strains with an additive effect may promote the increase in the total effectiveness of symbiosis and, thus, its productivity.

Taking the abovementioned into consideration, the aim of our work was to study the mutual relations between different soybean cultivars and two *Bradyrhizobium japonicum* strains after mixed inoculation, to evaluate the impact of the introduced microorganisms on the local rhizobial communities in soil, the formation and functioning symbiotic systems, and productivity of soybean under experimental small- (plot) and full-scale field conditions.

MATERIALS AND METHODS

The objects of the study. The objects of the study were the strains of soybean rhizobia *B. japonicum* 46 (IMV B-7200) and KB11 (IMV B-7435) and their mixture; antisera to active strains of *B. japonicum* 46, KB11, 634b, and M8; a peat form of the biopreparation for soybean, Rizogumin (TUU 24.1-00497360-003:2007) based on the mixture of strains of *B. japonicum* 46 + *B. japonicum* KB11, as well as soybean cultivars (*Glycine max* (L.) Merr.) of different ecologic-geographic origin: Ustia (Ukraine), Suziria (Ukraine), Sito (Germany), Shara (Belarus), Beihudo (China), Heihe 6 (China), Lambert (USA), Corada (Canada), Lybid (Canada), IC–14 (Hungary), Voyva (Lithuania), Proteinka (Serbia). The soybean seeds were provided by the NSC Institute for Agriculture and the Institute of Feed Research and Agriculture of Podillia, the NAAS.

Field studies. The response of different cultivars of soybean on the inoculation using the mixture of *B. japonicum* 46 + *B. japonicum* KB11 strains was studied under field conditions in the Polissia zone of Ukraine (the experimental field of the Institute of Agricultural Microbiology and Agroindustrial Manufacture, the NAAS – IAMAM NAAS). Cultivation of nodule bacteria was carried out in flasks (750 ml) in liquid medium (for 72 hours), containing (g/l): K_2HPO_4 – 0.5, KH_2PO_4 – 0.5, $(NH_4)_2SO_4 \cdot 7H_2O$ – 1.0, $MgSO_4 \times$

× $7H_2O$ – 0.2, NaCl – 0.2, $CaCO_3$ (sterile) – 0.1, sucrose – 2.0, mannitol – 3.0, glucose – 10.0, broth of peas (peas seeds – 50 g per 1 liter of water) – 100.0 ml/l; pH 7,0–7,2. The titre of bacteria was $2\cdot10^9$ CFU/ml. The strains *B. japonicum* 46 and *B. japonicum* KB11 were mixed in a flask before sowing in the 1 : 1 ratio. The inoculation dose was $2–3\cdot10^5$ cells per seed. The soil was peat-podzolic. Twelve cultivars of soybean were grown (Ustia, Suziria, Sito, Shara, Beihudo, Heihe 6, Lambert, Corada, Lybid, IC–14, Voyva, Proteinka). The experiment had four repeats. The area of the registration plot was 4 m^2. The plots were randomly located.

The presence of a specific strain in nodule populations of soybean rhizobia was determined using agglutination (Volkohon, 2010) with specific antisera (46, M8, KB11, 634b) and nodule homogenates. For this purpose, the nodules were extracted from soybean roots in the flowering stage and washed thoroughly with running water (48 nodules from each variant were analyzed, the average sample contained 15 plants). Each nodule was crushed in the glass vial with 1–2 ml of the physiological saline solution. The physiological saline + antigen (nodule homogenate) served as a negative control and antiserum + saline diluted antigen (a pure culture of nodule bacteria, the antiserum against which was obtained) as positive control. The diversity of rhizobia in the nodules was estimated by Shannon=Wiener diversity index (H), calculated according to the formula (MacArthur, 1965; Pielou, 1969; Saeki, Shiro, 2014):

$$H = -\sum P_i \ln P_i,$$

where P_i – relative incidence of *i*-th strain, calculated as (*ni/N*), where *N* – total number of nodules, formed by different strains of soybean nodule bacteria, and *ni* – number of nodules, formed with the involvement of the rhizobia strain of a specific serogroup.

Shannon index was used to calculate Pielou index of evenness (E):

$$E = H/\ln(S),$$

where *H* – Shannon index, *S* – number of rhizobia strains of different serogroup.

The effectiveness of legume-rhizobial symbiosis was evaluated by the following indices: the nodulating capacity, nitrogen fixation activity, and impact on the productivity of plants. The activity of the symbiotic nitrogen fixation was determined by the acetylene-ethylene method (Hardy et al, 1968). Twelve plants were selected from each variant. The roots with nodules were placed into vials, filled with acetylene (10 % from the vial volume) and kept for 1 h. The amount of formed ethylene was determined using the gas chromatograph "Chrom-4" (Laboratorni Pristroji, Czechoslovakia) with the flame ionization detector (column with β-β′-oxydipropionitrile). The thermostat temperature was 50 °C. The use of gases: hydrogen – 30 ml/min, nitrogen – 100 ml/min, air – 500 ml/min. The experiment had four repeats.

The effectiveness of microbial preparation Rizogumin (peat form) based on the mixture of *B. japonicum* 46 + *B. japonicum* KB11 strains (the rhizobia strains were used in a ratio of 1 : 1) was studied in the small-plot field experiment in the Polissia zone of Ukraine, on leached chernozem (IAMAM NAAS). In addition to the nodule bacteria of *B. japonicum*, Rizogumin also contains a complex of biologically active substances of vermicompost: plant growth regulators, humic acids, aminoacids, vitamins, a small amount of macroelements and microelements in chelated form (Volkohon, Moskalenko, 2021). Soybean seeds of Suziria cultivar were used in the experiment. The scheme of the small-scale (plot) experiment: 1. Without treatment (control); 2. Treatment with Rizogumin (*B. japonicum* 46 + KB11). In the control plot, the seeds were treated with running water (0.8–1.0 % from the mass of the seeds). The biopreparation was used in the ratio of 200 g of Rizogumin per 100 kg of soybean seeds and suspended in the required amount of water (0.8–1.0 % from the mass of the seeds). The uniformly treated seeds were kept for 2 h in a place, protected from sunlight, and then sown into soil. No seed dressers or herbicides were used. The experiment had four repeats. The area of the registration plot was 8 m^2. The plots were randomly located.

The full-scale field trial of Rizogumin preparation, based on the mixture of strains *B. japonicum* 46 + KB11 was conducted in 2018–2021 in different regions of Ukraine, using the premises of farms of different ownership types: PAF Mriia, Hrebinkivsky district, Poltava region; PF Spas-2007, Bilopilsky district, Sumy region; PF Shliakhova, Chutyvsky district, Poltava region; PF Luhivske, Illinetsky district, Vinnytsia region. Soybeans were grown according to the technologies, developed at the farms, in the areas from 5 to 250 ha. In the control plots, the seeds were treated with running water (0.8–1.0 % from the mass of the seeds). In the experimental plots, the seeds were treated with Rizogumin biopreparation (peat form). The bacterization of 1 ton of seeds required 2 kg of Rizogumin and 8–10 l of water. The uniformly treated seeds were kept for 2 h in a place, protected from sunlight, and then sown into soil. The norm of soybean sowing was 120–150 kg per 1 ha.

Statistical analysis of the results. The statistical processing of the data was conducted using the dispersion analysis (Dospekhov, 1985) in Microsoft Office package, Excel 2010, and Statistica 6.0. The least significant difference (LSD) was used to evaluate the reliability of differences between the experimental variants. The significance level used was 0.05.

RESULTS

Field conditions were used to study the response of 12 soybean cultivars to the inoculation using the mixture of strains from different genetic groups of *B. japonicum* 46 and KB11 (USDA 6 and USDA 123 respectively).

As seen from the data in **Table 1**, during the cultivation of soybean on the peat-podzolic soil, numerous nodules (47–110 per plant) were formed on the roots

Table 1. The impact of the inoculation using the mixture of *B. japonicum* 46 + KB11 strains on the symbiotic indices of different soybean cultivars (field experiment, IAMAM NAAS, 2013)

Cultivars of soybean	Variants	Number of nodules, items/plant	% to control	Weight of nodules, g/plant	% to control
Ustia	No inoculation (control)	61.8 ± 4.66	–	0.93 ± 0.04	–
	With inoculation	75.8 ± 4.33	+23	1.11 ± 0.03	+19
	LSD_{05}	15.6		0.12	
Suziria	No inoculation (control)	81.9 ± 2.75	–	0.97 ± 0.06	–
	With inoculation	98.5 ± 5.50	+20	1.18 ± 0.06	+22
	LSD_{05}	15.0		0.21	
Sito	No inoculation (control)	46.5 ± 3.23	–	0.78 ± 0.01	–
	With inoculation	45.3 ± 1.83	–3	0.79 ± 0.08	+1
	LSD_{05}	9.1		0.19	
Shara	No inoculation (control)	76.5 ± 1.51	–	0.54 ± 0.03	–
	With inoculation	100.0 ± 7.32	+31	0.69 ± 0.02	+28
	LSD_{05}	18.3		0.09	
Beihudo	No inoculation (control)	56.8 ± 2.62	–	0.44 ± 0.03	–
	With inoculation	82.5 ± 4.11	+45	0.82 ± 0.07	+86
	LSD_{05}	11.9		0.18	
Heihe 6	No inoculation (control)	84.0 ± 7.33	–	0.94 ± 0.02	–
	With inoculation	85.0 ± 3.47	+1	0.92 ± 0.01	–2
	LSD_{05}	19.8		0.06	
Lambert	No inoculation (control)	98.6 ± 8.38	–	0.89 ± 0.06	–
	With inoculation	97.3 ± 2.89	–1	1.31 ± 0.10	+47
	LSD_{05}	21.7		0.28	
Corado	No inoculation (control)	65.0 ± 4.31	–	0.97 ± 0.02	–
	With inoculation	74.3 ± 6.95	+14	1.08 ± 0.07	+11
	LSD_{05}	20.0		0.17	
Lybid	No inoculation (control)	58.5 ± 4.54	–	0.89 ± 0.03	–
	With inoculation	64.1 ± 5.36	+10	1.21 ± 0.02	+36
	LSD_{05}	17.0		0.09	
IC-14	No inoculation (control)	88.9 ± 7.22	–	1.15 ± 0.06	–
	With inoculation	114.9 ± 4.67	+29	1.57 ± 0.07	+37
	LSD_{05}	21.0		0.23	
Voyva	No inoculation (control)	109.8 ± 7.95	–	0.64 ± 0.02	–
	With inoculation	111.5 ± 4.95	+2	1.07 ± 0.07	+67
	LSD_{05}	22.9		0.19	
Proteinka	No inoculation (control)	82.1 ± 3.87	–	0.88 ± 0.04	–
	With inoculation	84.6 ± 6.53	+3	1.07 ± 0.04	+22
	LSD_{05}	18.6		0.14	

Note. The data are presented as mean values ± standard deviation (x ± SE). LSD – the least significant difference. The significance level used was 0.05.

of non-inoculated control plants of all the investigated cultivars, which demonstrated a dense population of specific nodulating rhizobia already present in the soil. Some cultivar-specific differences were revealed in the ability of plants to enter symbiotic relations with the local rhizobial populations. High ability for the nodule formation (77–110 nodules per plant were formed) was noted for seven cultivars of soybean of different ecologic-geographic origin: Suziria, Shara, Heihe 6, Lambert, Voyva, IC-14, and Proteinka. The remaining cultivars (Ustia, Sito, Beihudo, Corado, and Lybid) were found less sensitive to being infected by the representatives of the soil population of soybean rhizobia, forming 47–65 nodules per plant.

Table 2. The impact of the inoculation using the mixture of *B. japonicum* 46 + KB11 strains on the activity of symbiotic nitrogen fixation of different soybean cultivars (field experiment, IAMAM NAAS, 2013)

Soybean cultivars	Variants	Nitrogen fixation activity, μg N/plant per h
Ustia	No inoculation (control)	28.4 ± 2.50
	With inoculation	40.6 ± 3.63
	LSD_{05}	10.8
Suziria	No inoculation (control)	37.3 ± 0.63
	With inoculation	52.8 ± 1.94
	LSD_{05}	5.0
Sito	No inoculation (control)	25.1 ± 2.35
	With inoculation	33.6 ± 1.37
	LSD_{05}	6.7
Shara	No inoculation (control)	12.5 ± 0.38
	With inoculation	28.7 ± 1.65
	LSD_{05}	4.1
Beihudo	No inoculation (control)	10.6 ± 0.54
	With inoculation	44.5 ± 2.77
	LSD_{05}	6.9
Heihe 6	No inoculation (control)	37.6 ± 0.44
	With inoculation	51.4 ± 2.35
	LSD_{05}	5.8
Lambert	No inoculation (control)	26.4 ± 1.94
	With inoculation	54.1 ± 4.54
	LSD_{05}	12.1
Corado	No inoculation (control)	52.1 ± 1.57
	With inoculation	62.0 ± 5.02
	LSD_{05}	12.9
Lybid	No inoculation (control)	40.9 ± 3.00
	With inoculation	75.8 ± 3.47
	LSD_{05}	11.2
IC-14	No inoculation (control)	35.6 ± 1.42
	With inoculation	68.6 ± 2.75
	LSD_{05}	7.6
Voyva	No inoculation (control)	18.5 ± 1.20
	With inoculation	38.6 ± 2.18
	LSD_{05}	6.1
Proteinka	No inoculation (control)	28.4 ± 4.32
	With inoculation	61.3 ± 0.85
	LSD_{05}	10.8

Note. The data are presented as mean values ± standard deviation (x ± SE). LSD – the least significant difference. The significance level used was 0.05.

On the background of the numerous rhizobia in the soil, the inoculation of soybean seeds with the mixture of *B. japonicum* 46 + KB11 strains had a positive impact on the process of nodule formation in most cultivars, regardless of their geographic origin. For instance, in the flowering stage, the number of nodules as compared to the control (without inoculation) in the cultivars Ustia, Suziria, Beihudo, Shara, Corado, Lybid, and IC-14 increased by 10–45 %. In the cultivars Heihe 6, Voyva, Proteinka, Sito and Lambert, however, the number of nodules remained on the control level.

Another relevant index of the symbiotic interaction between legumes and rhizobia is the weight of root nodules. It was noted that soybean cultivars Heihe 6, IC-14, Lambert, Proteinka, and Suziria, which formed the largest number of nodules at the interaction with local rhizobia, also had their largest weight (0.88–1.15 g/plant). In the cultivars Beihudo, Voyva and Shara, the weight of the root nodules was the smallest (0.44–0.64 g/plant).

The use of the mixture of *B. japonicum* 46 + KB11 strains caused a considerable increase in the weight of the nodules (by 11–86 %) as compared to the control variants in ten out of twelve soybean cultivars in our small-plot experiments. The exceptions were found in cultivars Sito and Heihe 6, where the weight of the nodules practically did not change.

As for the control variants, the highest level of fixing molecular nitrogen was noted for cultivars Corado, Lybid, Heihe 6, IC-14, and Suziria, selected in Ukraine (35.6–52.1 μg N_2/plant per h) (**Table 2**). The lowest nitrogenase activity (10.6 and 12.5 μg N_2/plant per h) was registered for soybean cultivars Beihudo and Shara respectively; they also had the smallest weight of the nodules.

It should be noted that after the inoculation of soybean with the mixture of *B. japonicum* 46 + *B. japonicum* KB11 strains, all cultivars demonstrated a considerable increase in the level of nitrogen fixation activity (1.2–4.2 times, see Table 2). It is important that this index increased not only for the cultivars less sensitive to being infected with local rhizobial microbiota but also for the cultivars capable of active symbiosis with the representatives of soil populations of soybean nodule bacteria. This fact may demonstrate the highly competitive capacity of the inoculant strains and their complementarity to different soybean genotypes, which is relevant for applying the strain mixture as inoculant.

Considering the fact that the effectiveness of the functioning of legume-rhizobial symbiosis greatly depends on the competitiveness of the inoculant strains, we investigated the structure of the nodule populations of rhizobia in different soybean cultivars and evaluated the impact of the mixture of *B. japonicum* 46 + KB11 strains on the communities of specific nodule bacteria in the soil.

The serological analysis of the nodules of the non-inoculated soybean demonstrated that the local community of specific nodule bacteria is heterogeneous, mainly belonging to three serogroups, namely 46, M8, and KB11. There were also bacteria in the nodule populations which did not relate to any of the investigated serogroups, the share of which varied in the range from 4.2 to 58.3 %, depending on the cultivar (**Table 3**). On the roots of most soybean cultivars, the inoculated, strain *B. japonicum* KB11 dominated (found in 45.8–75.0 % of the investigated nodules). Only in the nodules of Heihe 6 soybean cultivar, its share was smaller (29.2 %) as compared to the representatives of other serogroups. Rhizobia of serogroups 46 and M8 were found in minor quantities (4.2–29.2 % and 4.2–12.5 %, respectively).

The use of the mixture of *B. japonicum* 46 + KB11 strains promoted the correction of rhizobia in the nodule populations of different soybean cultivars. In all the cultivars, there was an increase in the share of the inoculant strain *B. japonicum* 46, a decrease or increase (depending on the cultivar) in the number of nodules, formed by the strain *B. japonicum* KB11 and non-identifiable nodule bacteria. The stimulating effect of the mixture on the local rhizobial communities promoted the occurrence of a minor representa-

Table 3. The composition of the nodule populations of rhizobia of different soybean cultivars after the treatment of the seeds with the mixture of *B. japonicum* 46 + KB11 strains (field experiment, IAMAM NAAS, 2013)

Cultivars of soybean	Variants of experiment	Share of soybean rhizobia strains in the nodules, %					Index	
		46	M8	KB11	643b	Others*	H	E
Ustia	No inoculation	8.33	0	75.00	0	16.67	0.72	0.66
	With inoculation	**47.92**	**2.08**	**39.58**	**0**	**10.42**	**1.04**	**0.75**
Suziria	No inoculation	20.83	8.33	45.83	0	25.00	1.24	0.89
	With inoculation	**22.92**	**16.67**	**39.58**	**0**	**20.83**	**1.33**	**0.96**
Sito	No inoculation	4.17	0	45.83	0	50.00	0.84	0.76
	With inoculation	**37.50**	**8.33**	**41.67**	**0**	**12.50**	**1.20**	**0.87**
Shara	No inoculation	8.33	4.17	66.67	0	20.83	0.94	0.68
	With inoculation	**16.67**	**0**	**66.67**	**0**	**16.67**	**0.87**	**0.79**
Beihudo	No inoculation	16.67	0	58.33	0	25.00	0.96	0.87
	With inoculation	**39.58**	**4.17**	**39.58**	**0**	**16.67**	**1.16**	**0.84**
Heihe 6	No inoculation	8.33	4.17	29.17	0	58.33	1.01	0.73
	With inoculation	**39.58**	**8.33**	**45.83**	**0**	**6.25**	**1.10**	**0.80**
Lambert	No inoculation	8.33	0	45.83	0	45.83	0.92	0.84
	With inoculation	**29.17**	**20.83**	**33.33**	**0**	**16.67**	**0.99**	**0.90**
Corado	No inoculation	20.83	12.50	50.00	0	16.67	1.23	0.89
	With inoculation	**43.75**	**16.67**	**27.08**	**0**	**12.50**	**1.27**	**0.92**
Lybid	No inoculation	29.17	12.50	54.17	0	4.17	1.08	0.78
	With inoculation	**50.00**	**8.33**	**33.33**	**0**	**8.33**	**1.13**	**0.81**
IC-14	No inoculation	16.67	8.33	45.83	0	29.17	1.22	0.88
	With inoculation	**33.33**	**12.50**	**41.67**	**0**	**12.50**	**1.25**	**0.90**
Voyva	No inoculation	4.17	4.17	70.83	0	20.83	0.84	0.60
	With inoculation	**22.92**	**8.33**	**37.50**	**0**	**31.25**	**1.28**	**0.92**
Proteinka	No inoculation	12.50	8.33	50.00	0	29.17	1.17	0.85
	With inoculation	**41.67**	**12.50**	**33.33**	**0**	**12.50**	**1.25**	**0.90**

Note. * – nodule bacteria are not assigned to studied serogroups; H – Shannon diversity index; E – Pielou evenness index.

tive of the soil population – strain *B. japonicum* M8 (which formed 2.1–8.3 % nodules) in the soybean nodules of cultivars Ustia, Sito, and Beihudo. It was found that the combined use of two inoculant strains *B. japonicum* 46 and *B. japonicum* KB11 ensured the formation of balanced symbiotic systems (without clear domination of some strains in the nodules) in most investigated soybean cultivars. This is demonstrated by the higher values of Shannon diversity index (H) and Pielou evenness index (E) after the inoculation with the mixture of *B. japonicum* strains as compared to the control variants. The exceptions were found in soybean cultivars Shara and Beihudo, which were notable for the decrease in indices H and E, respectively.

Table 4. The impact of the inoculation using the mixture of *B. japonicum* 46 + KB11 strains on the productivity of different soybean cultivars (field experiment, IAMAM NAAS, 2013)

Cultivars of soybean	Variants	Weight of grain, g/plant	% to control
Ustia	No inoculation (control)	2.6 ± 0.13	–
	With inoculation	3.4 ± 0.20	+28
	LSD_{05}	0.59	
Suziria	No inoculation (control)	3.7 ± 0.03	–
	With inoculation	4.3 ± 0.11	+19
	LSD_{05}	0.27	
Sito	No inoculation (control)	4.0 ± 0.06	–
	With inoculation	5.1 ± 0.33	+29
	LSD_{05}	0.81	
Shara	No inoculation (control)	3.2 ± 0.03	–
	With inoculation	3.3 ± 0.03	+6
	LSD_{05}	0.21	
Beihudo	No inoculation (control)	3.7 ± 0.14	–
	With inoculation	4.6 ± 0.24	+23
	LSD_{05}	0.01	
Heihe 6	No inoculation (control)	3.6 ± 0.07	–
	With inoculation	4.4 ± 0.47	+22
	LSD_{05}	0.04	
Lambert	No inoculation (control)	3.9 ± 0.14	–
	With inoculation	4.5 ± 0.10	+15
	LSD_{05}	0.16	
Corado	No inoculation (control)	4.2 ± 0.04	–
	With inoculation	4.9 ± 0.15	+16
	LSD_{05}	0.27	
Lybid	No inoculation (control)	3.6 ± 0.09	–
	With inoculation	3.9 ± 0.17	+9
	LSD_{05}	0.81	
IC-14	No inoculation (control)	4.1 ± 0.02	–
	With inoculation	4.7 ± 0.17	+13
	LSD_{05}	0.21	
Voyva	No inoculation (control)	2.9 ± 0.10	–
	With inoculation	3.1 ± 0.08	+7
	LSD_{05}	0.01	
Proteinka	No inoculation (control)	3.3 ± 0.09	–
	With inoculation	3.7 ± 0.01	+10
	LSD05	0.04	

Note. The data are presented as mean values ± standard deviation (x ± SE). LSD – the least significant difference. The significance level used was 0.05.

The effectiveness of the use of the mixture of *B. japonicum* strains was evaluated by the index of the weight of grain per plant. In ten out of twelve soybean cultivars, this index increased considerably as compared to the control – by 9–29 % (**Table 4**). The maximal productivity was registered for soybean cultivars Sito, Ustia, Beihudo, Heihe 6, and Suziria. Only in two cultivars, Shara and Voyva, the gain in the weight of grain was lower, amounting to 6–7 %.

Taking the abovementioned into consideration, the following stage of our work was to check the effectiveness of the mixture of *B. japonicum* 46 + KB11 strains in the combination of Rizogumin, a microbial preparation, developed by us. The study was conducted on leached chernozem under field conditions in the Polissia zone of Ukraine.

The application of Rizogumin on the background of dense communities of soybean microsymbionts (represented by nodule bacteria, which belong to serogroups 46, M8, KB11, and 634b) led again to the change in the composition of nodule populations of rhizobia (**Table 5**). The treatment of the seeds with the biopreparations promoted the increase in the share of inoculant strain *B. japonicum* 46 in the nodules, from 4.2–27.1 (in the control variant) to 35.4–39.6 % (with inoculation). On the contrary, due to the regrouping of the local and introduced rhizobia in the nodules, the share of the strain *B. japonicum* KB11 decreased from 58.3–68.8 down to 35.4–39.6 %. The formed symbiotic systems were more balanced as compared to the control ones, the Shannon diversity index increased from 0.93–0.95 to 1.6–1.27, and Pielou evenness index – from 0.58–0.87 to 0.92–0.97.

The application of Rizogumin promoted the increase in the symbiotic indices and ensured a stable increase in the yield of soybean cultivar Suziria by 33 % on average for three years as compared to the control without inoculation (**Table 6**).

The effectiveness of the mixture of *B. japonicum* 46 + KB11 strains as the foundation for Rizogumin was

Table 5. The share of *B. japonicum* strains in the soybean nodules using Rizogumin based on the mixture of *B. japonicum* 46 + KB11 strains (field experiments, IAMAM NAAS, 2016, 2017)

Variants of experiment	Share of soybean rhizobia strains in the nodules, %					Index	
	46	M8	KB11	643b	Others*	H	E
2016							
No treatment (control)	4.17	2.08	68.75	4.17	20.83	0.93	0.58
Treatment with Rizogumin (*B. japonicum* 46 + KB11)	35.42	0	35.42	8.33	20.83	1.27	0.92
2017							
No treatment (control)	27.08	0	58.33	0	14.58	0.95	0.87
Treatment with Rizogumin (*B. japonicum* 46 + KB11)	39.58	0	39.58	0	20.83	1.06	0.97

Note. * – nodule bacteria are not assigned to studied serogroups; H – Shannon diversity index; E – Pielou evenness index.

Table 6. The impact of Rizogumin on the productivity of soybean cultivar Suziria (field experiments, IAMAM NAAS, 2016–2018)

Variants of experiment	Yield of grain, tons/ha				Yield gain	
	2016	2017	2018	Average	t/ha	%
Without treatment (control)	2.75 ± 0.08	2.38 ± 0.04	3.54 ± 0.07	2.89	–	100
Treatment with Rizogumin (*B. japonicum* 46 + KB11)	3.53 ± 0.08	3.08 ± 0.08	4.91 ± 0.10	3.84	+0.95	133
LSD_{05}	0.27	0.22	0.30			

Notes. The data are presented as mean values ± standard deviation (x ± SE). LSD – the least significant difference. The significance level used was 0.05.

Table 7. The impact of Rizogumin based on the mixture of *B. japonicum* 46 + KB11 strains on the yield of soybean seeds in different regions of Ukraine (industrial conditions, 2018–2021)

The place of conducting studies	Yield, tons/ha		Gain	
	No inoculation	Rizogumin	t/ha	%
2018				
PAF Mriia, Hrebinkivsky district, Poltava region (90 ha)	2.20	2.60	0.40	18
PF Spas-2007, Bilopilsky district, Sumy region (60 ha)	1.90	2.46	0.56	30
2019				
PF Shliakhova, Chutyvsky district, Poltava region (252 ha)	1.04	1.20	0.16	15
2021				
PF Luhivske, Illinetsky district, Vinnytsia region (7 ha)	3.00	3.50	0.50	17

proven by its use in practice on the area of about 60 thousand ha in different regions of Ukraine.

Under different variants of technology and the presence of the local populations of soybean nodule bacteria with different density in soil, the application of Rizogumin promoted a stable increase in soy yield by 15–30 % as compared to the control without inoculation (**Table 7**).

DISCUSSION

It is known that many biotic, abiotic, and anthropogenic factors impact the interaction of micro- and macrosymbionts, thus, it is not always effective in the sense of agricultural usefulness/productivity (Spaink et al, 1998; Iutynskaya, Ponomarenko, 2010; Abou-Shanab et al, 2017). The negative effect on the legume-rhizobial symbiosis may be imposed by pesticides and mineral fertilizers and/or the highly competitive capacity of less effective local nodule bacteria. Successful realization of an optimal nitrogen fixation requires the constant search for highly efficient strains of nodule bacteria and the study of the specificities of introducing these microorganisms into the pre-root zone of plants (Iutynskaya, Ponomarenko, 2010; Kots et al, 2011; de Bruijn , 2015).

The selected strains of nodule bacteria, used as inoculant, can form an efficient symbiosis with one legume cultivar and have less impact on the yield of another cultivar. It is related to the complementarity phenomenon of some strains of rhizobia and cultivars of legumes. Due to this fact, many scientists have been paying attention to the selection of complementary pairs: a cultivar – a strain of nodule bacteria (Kots et al, 2011; Sichkar et al, 2014; Duchene et al, 2017; Pandey et al, 2018). However, in our opinion, these pairs are usually effective on condition of no local nodule bacteria in the soil. Under periodic cultivation of the legume crop and the application of microbial preparations in the agrosystems, there is the formation of heterogeneous populations of specific nodule bacteria, different in their density (Spaink et al, 1998; Krutylo, 2006; Kots et al, 2011; de Bruijn, 2015). In the background of such communities, the symbiotic interaction may be started by inoculant strains and local rhizobial microbiota, so the effectiveness of symbiotic "cultivar-strain" systems may decrease under these conditions (Abou-Shanab et al, 2017). This issue may be solved by searching for competitive rhizobial strains, which form the effective symbiosis with most existing cultivars of the corresponding legume crop or the application of several such strains for inoculation.

Recent technologies for cultivating legumes also use biopreparations based on specific nodule bacteria and plant growth-promoting microorganisms (PGPM). For instance, Kumawat et al demonstrated a positive impact on the yield of *Vigna radiata* (L.) Wilczek from the double bioinoculant based on *Rhizobium* sp. LSMR-32 and *Enterococcus mundtii* LSMRS-3 (Kumawat et al, 2021). This preparation allowed mitigating the impact of the salt stress and turned out to be more effective than mono-inoculation. Double inoculation of chick-pea with a mixture of *Mesorhizobium ciceri* IC-53 and *Bacillus subtilis* NUU4 had a positive effect on the formation of the symbiosis with nodule bacteria, enhanced the growth of plants and promoted the yield gain (Egamberdieva et al, 2017). With combined inoculation, the number of pods and yield of chickpeas were 39 and 13 % higher compared to the control. With mono-inoculation with *Mesorhizobium ciceri* IC-53, the increases were only 12 and 7 %, respectively. Aung et al (Aung et al, 2013) used nodule bacteria *B. japonicum* CB 1809 and *B. japonicum* USDA 110 in combination with a growth-stimulating *Azospirillum* sp., which promoted a considerable increase in the number and weight of soybean nodules, the height of plants and the weight of the aboveground parts as compared to the use of strains separately. An efficient biopreparation for the soybean and other legumes, Ekovital, was developed in the D. K. Zabolotny Institute of Microbiology and Virology, the NAS of Ukraine, based on nodule bacteria of the genera *Rhizobium*, *Bradyrhizobium* or *Sinorhizobium* in combination with a phosphorus-mobilizing *Bacillus megaterium* strain (Iutynskaya, Ponomarenko, 2010).

Contrary to the application of rhizobia together with PGP microorganisms, researchers pay less attention to the combination of several strains of nodule bacteria belonging to one of several species in the inoculum. However, Carvalho et al proved that the combined application of two species of rhizobia, *R. tropici* and *B. elkanii* ensured a reliable increase in the number (by 48–70 %) and weight (by 29–41 %) of the nodules, the weight of plants (by 29–67 %) and roots (by 29–67 %) in different cultivars of common beans as compared to the separate use of strains (de Carvalho et al, 2020). Other researchers demonstrated a positive effect of the double inoculation of common bean (*Phaseolus vulgaris* L.) with a mixture of strains of *R. tropici* and *Bradyrhizobium elkanii, R. tropici* and *Bradyrhizobium diazoefficiens* on the formation of the symbiotic apparatus and accumulation of nitrogen by plants (Jesus et al, 2018). Similar results were obtained in the experiments with cowpea while studying 11 combinations of strains of *Bradyrhizobium* sp. and *B. pachyrhizi* BR 3262, *B. yuanmingense* BR 3267 and *B. pachyrhizi* BR 3262, which turned out to be more effective than monoinoculation (do Nascimento et al, 2021). As compared to the treatment of soybean using one strain, the combined inoculation with two strains of *Bradyrhizobium* sp. CPO4.24C and *B. japonicum* USDA 110 promoted nodule formation and the activity of the symbiotic nitrogen fixation, with 5–17 and 67–79 % respectively (Vargas-Díaz et al, 2019). In the experiment with soybean on autoclaved and non-autoclaved soils, Abbasi MK *et al*. demonstrated that the use of the mixture of strains *B. japonicum* 377 and *B. japonicum* 379 was more effective than the use of strains separately (Abbasi et al, 2008). The percent increase in seed yield due to the inoculation of soybean by the mixture of two strains used in the inoculum was 21 % over control.

However, the combination of two strains of nodulating bacteria in the inoculum is not always effective. Thus, Gitonga NM and co-authors (Gitonga et al, 2021) investigated the use of bradyrhizobia in the cultivation of three soybean varieties in organic farming. The experimental treatments included native bradyrhizobia, commercial *B. japonicum* strain USDA 110 and a mixture of native bradyrhizobia and strain *B. japonicum* USDA 110. The control was a variant without inoculation. The largest increases in the number and mass of nodules (by 43.7 and 17.0 times, respectively), the above-ground mass of plants (by 1.9 times) and the mass of seeds (by 2.1 times) compared to the control were obtained by mono-inoculation of soybeans with the strain *B. japonicum* USDA 110. The increase of these indicators in the variant with a mixture of two strains of nodule bacteria was higher than when using native bradyrhizobia, but lower than when treated with *B. japonicum* strain USDA 110 alone. The data obtained by these authors confirm the thesis of the need for careful selection of strains for their effective combined use.

We proposed a slightly different approach to increase the efficiency of the symbiotic systems of soybeans, which consisted in combining inoculum of two strains of nodule bacteria from one species – *B. japonicum* 46 and *B. japonicum* KB11 (a strain mixture), but different genetic groups (USDA 6 and USDA 123, respectively) (Krutylo, 2017; Volkohon, Moskalenko, 2021). In functional terms, the selected strains mutually supplemented and enhanced the action of each other (additive effect) which allows them to realize their symbiotic potential more fully (Krutylo, 2017). The result of this interaction is a reliable increase in the symbiosis indices and productivity of soybean compared to the monoinoculation (Krutylo, Leonova, 2016; Volkohon, Moskalenko, 2021).

In this study it was proven in the series of small- and full-scale field experiments with different soybean cultivars that the introduction of the mixture of competitive *B. japonicum* 46 + KB11 strains in a peat formulated carrier ensures the formation of balanced symbiotic systems of the host plant with several complementary but serologically different strains of rhizobia of the same species. This approach creates a possibility of intensifying the nodule formation process, enhancing the level of nitrogen fixation (1.2–4.2 times), and increasing the yield of soybean by 15–33 %.

The high effectiveness of the mixture of our combined strains as compared to the monoinoculation may also be related to achieving balance in supplying plants with phytohormonal substances. Our previous studies demonstrated that these rhizobia differ in the level of synthesis of exogenous phytohormones: the strain *B. japonicum* 46 produces a larger amount of auxins (1.4 times), whereas a strain *B. japonicum* KB11 largely prevails in the amount of cytokinins (2.5 times) (Krutylo DV, Leonova NO, 2016). Based on our earlier and present studies, we confirm the advantages of the application of the mixture of *B. japonicum* 46 + + KB11 strains as the basis for the microbial preparation Rizogumin.

The results of this work demonstrate that further study of the symbiotic interaction of soybean cultivars and the involved nodule rhizobial strains of different genetic groups, is important and promising from both scientific and practical standpoint.

CONCLUSIONS

In this study the positive effects of an application of a formulated mixture of nitrogen fixing *B. japonicum* 46 (genetic group USDA 6) and *B. japonicum* KB11 (preliminarily placed in genetic group USDA 123) strains, on different soybean cultivars are reported. Combining these two strains with their apparent additive effect in the inoculum in small- and full-scale field experiments lead to ensured the formation of balanced and more productive symbiotic systems. This was expressed in an intensified nodulation (by 10–45%), symbiotic nitrogen fixation (with 1.2–4.2 times), and increased yield of different cultivars of soybeans by 15–33 % as compared to the control (without inoculation).

Adherence to ethical principles. All experiments described in this paper did not involve the use of animals.

Conflict of interest. The author declares no conflict of interest.

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Симбіотична взаємодія суміші штамів *Bradyrhizobium japonicum* із соєю різних сортів

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Мета. Вивчити взаємовідносини сої різних сортів з двома штамами *Bradyrhizobium japonicum* за умов сумісної інокуляції, оцінити вплив інтродукованих мікроорганізмів на місцеві ризобіальні угруповання в ґрунті, формування та функціонування симбіотичних систем і продуктивність сої. **Методи.** Мікробіологічні та серологічні; польовий дослід, газова хроматографія та математично-статистичні. **Результати.** У дрібноділянковому польовому досліді показано, що використання суміші штамів *B. japonicum* 46 та *B. japonicum* КВ11 сприяло більш рівномірному розподілу місцевих та інтродукованих ризобій у бульбочкових популяціях 12 сортів сої різного географічного походження. У бульбочках не спостерігалося домінування окремих штамів, а сформовані симбіотичні системи були більш збалансованими, ніж у контролі. За інокуляції згаданою сумішшю у більшості сортів спостерігалося значне збільшення кількості бульбочок (на 10–45 %) та їх маси (на 11–86 %). Також відмічено підвищення рівня симбіотичної азотфіксації в 1,2–4,2 раза та збільшення маси зерна з однієї рослини на 6–29 % (залежно від сорту). Ефективність суміші штамів *B. japonicum* 46 і КВ11 як основи мікробного препарату Різогуміну підтверджена у польових випробуваннях із соєю на площі близько 60 тис. га у різних регіонах України. На фоні місцевих популяцій бульбочкових бактерій сої застосування суміші двох штамів ризобій забезпечувало стабільне підвищення врожаю сої на 15–33 % порівняно з контролем без інокуляції. **Висновки.** Запропоновано новий підхід, який полягає у застосуванні суміші штамів *B. japonicum* 46 (генетична група USDA 6) та *B. japonicum* КВ11 (генетична група USDA 123) для інокуляції сої різних сортів. Поєднання цих двох штамів та їх інтродукція в агроценози забезпечували формування збалансованих симбіотичних систем (без явного домінування окремих штамів у бульбочках). Це проявлялося у посиленні бульбоутворення, симбіотичної азотфіксації та збільшенні врожайності різних сортів сої на 15–33 % порівняно з контролем (без інокуляції).

Ключові слова: *Bradyrhizobium japonicum*, суміш штамів, азотфіксація, збалансовані симбіотичні системи, біопрепарат Ризогумін, сорти сої, симбіотична ефективність.

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