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Effects of soybean co-inoculation with plant growth promoting rhizobacteria in field trial

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Abstract

The aim of this study was to investigate the possibility of improving the microbiological fertilizer for soybean by co-inoculation with PGPR strains. Experiment was carried out under field conditions, on a chernozem soil, using soybean cultivar Galina and five inoculation treatments: B. japonicum; B. japonicum + A. chroococcum + humic acid; B. japonicum + A. chroococcum; B. japonicum + B. subtilis; B. japonicum + B. megaterium. Bacterial treatments had a positive effect on activity and number of investigated microbial groups in rhizosphere, nitrogen fixation parameters and yield of soybean. Inoculation, on average, increased the number of azotobacters, total microbial number, ammonifiers, N-fixing bacteria, actinomycetes, dehydrogenase activity, as well as the mass and number of nodules, nitrogen content in plants and seeds, number of poods and seeds, dry seed mass, and yield of soybean. Co-inoculation had a better effect on the number of microorganisms, dehydrogenase activity and yield of soybean, while the best effect on nitrogen fixation and yield parameters was achieved by single inoculation.

Keywords: *Azotobacter*, *Bacillus*, *Bradyrhizobium*, microbial activity, nitrogen fixation

1. Introduction

Soybean (*Glycine max* L.) is one of the most important cultivated legumes in the world due to the chemical composition of its grain, which is about 40% protein and about 20% oil. This adds up to soybean having over 60% of different nutrients and therefore the plant can be used not only in the food sector, but also in various other industries. During a 10-year period from 2001 to 2010, world soybean production increased from 168 to 258 million metric tons (54% increase) (OHYAMA & al. [1]). The area of harvested soybean increased approximately from 79 million ha in 2001, to 102 million ha in 2010 (29% increase) (MASUDA & GOLDSMITH [2]). World trends to increase the area under soybean have reached our country, and today with the surface of over 100.000 ha, and the production of over 200.000 tons, soybean is an important factor of crop production in Serbia (HRUSTIĆ & MILADINOVIĆ [3]).
As a legume, soybean has the ability to fix atmospheric nitrogen and provide itself with sufficient amounts of available nitrogen, which makes this plant a very good fit for crop rotations. Symbiotic nitrogen fixation is a process by which the host plant (macrosymbiont) provides bacteria (microsymbiont) with energy, while bacteria provide the plant with reduced nitrogen from the atmosphere (DIXON & KAHN [4]). Bacteria that form nodules in symbiosis with soybean are *Bradyrhizobium japonicum*, *Bradyrhizobium elkani* and *Sinorhizobium fredii*. In this community, approximately 20-400 kg ha^{-1} per year of nitrogen is fixed and transferred to the plant, reducing the need for nitrogen fertilizer use, depending on the plant species, bacterial strain, and numerous biotic and abiotic factors (ZAHRAN [5]). A very small number of these bacteria are present in our agricultural soil hence the use of

microbiological fertilizers which contain selected, high effective strains of symbiotic nitrogen fixers was introduced as a regular measure in growing soybean (MRKOVAČKI [6]). Application of microbiological fertilizers allows successful nodulation of plants, better nitrogen fixation from the atmosphere, stimulation of plant growth and development, increase in biomass and nitrogen content in the plant, as well as yield and grain quality. Therefore it is considered as an alternative or supplement to the reduced use of chemicals in crop production.

Our previous research of rhizobia were performed in order to select the most effective strains of *Bradyrhizobium japonicum* which, besides the capacity for nitrogen fixation, must have the competitive ability in relation to the natural population which is most often inefficient in fixing nitrogen (MARINKOVIĆ [7]). Enhancement of legume nitrogen fixation by co-inoculation of rhizobia some plant growth-promoting bacteria (PGPR) is a good way to improve nitrogen availability in sustainable agriculture production (YUMING & al. [8]).

Diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*, *Azomonas*) rhizobacteria are used worldwide as bio-inoculants in order to promote plant growth and development by various mechanisms, including nitrogen fixation, production of siderophores, solubilization of minerals such as phosphorus, synthesis of phytohormones, etc. (COMPANT & al. [9]). Some rhizobacterial strains promote legume nodulation and nitrogen fixation by producing flavonoid-like compounds, and/or stimulating the host legume to produce more flavonoid signal molecules (PARMAR & DADARWAL [10]). Microbiological fertilizers that contain more than one species of microorganisms had a favorable effect on the growth and yield of different field and vegetable crops (MILOŠEVIĆ & GOVEDARICA [11]; MRKOVAČKI & al. [12]; JARAK & al. [13]). This effect was higher compared to their individual application, so it is assumed that co-inoculation may exert a beneficial effect on soybean production. The main objective of this research was to determine the effect of co-inoculation with *Bradyrhizobium japonicum* and PGPR strains on microbial activity in rhizosphere, nitrogen fixation parameters and yield of soybean.

2. Materials and Methods

The experimental site and soil chemical properties

A trial was set up in Rimski Šančevi, on the experimental field of Institute of Field and Vegetable Crops, Novi Sad, during the 2013 growing season on a chernozem soil using a randomized block design with four replicates. The size of experimental plots was 5 × 2 m. The chemical soil properties of experimental field are presented in Table 1.

Table 1. Chemical soil properties of experimental field

pH		**$CaCO_3$ %**	**Humus %**	**Total N %**	**AL-P_2O_5 mg/100g**	**AL-K_2O mg/100g**
in KCl	in H_2O					
7.33	8.39	2.21	2.24	0.166	22.83	26.3

Plant and microbial germplasm

Soybean seeds used in this study were obtained from the soybean cultivar “Galina“ (Institute of Field and Vegetable Crops, Novi Sad, Serbia), a cultivar with a short vegetative cycle (00). Mixture of five *Bradyrhizobium japonicum* strains, commercially used as microbiological fertilizer (NS-Nitragin), for soybean production in Serbia was used for inoculation.

Bradyrhizobium japonicum strains used in this study were obtained from the collection of the Department of Microbiological Preparations, Institute of Field and Vegetable Crops, Novi Sad. *Bacillus subtilis*, *Bacillus megaterium* and *Azotobacter chroococcum* strains used in this study were also obtained from the collection of the Department of Microbiological Preparations, Institute of Field and Vegetable Crops, Novi Sad, Serbia.

Experimental design

Five inoculation treatments were tested: 1. *Bradyrhizobium japonicum* (*B. j.*), 2. *Bradyrhizobium japonicum* + *Azotobacter chroococcum* + humic acid (*B. j.* + *A .c.* + H. A.), 3. *Bradyrhizobium japonicum* + *Azotobacter chroococcum* (*B. j.* + *A. c.*), 4. *Bradyrhizobium japonicum* + *Bacillus subtilis* (*B. j.* + *B. s.*), and 5. *Bradyrhizobium japonicum* + *Bacillus megaterium* (*B. j.* + *B. m.*), 6. Non-inoculated seeds – control. Seeds were inoculated 30 minutes before sowing, and sterilized peat was used as carrier (sterilization with γ rays – Vinča Institute of Nuclear Sciences, Serbia). Non-inoculated seeds were designed as control.

Preparation of bacterial inoculum

Bradyrhizobium japonicum strains, for the preparation of inoculum, were cultured for 72 h in yeast-mannitol medium (YEM) (SOMASEGERAN & HOBEN [14]). *Azotobacter chroococcum* strain was cultured for 72 h in Burk's N-free broth (WILSON & KNIGHT [15]), and *Bacillus subtilis* and *Bacillus megaterium* strains were cultured for 48 h in nutrient broth. Bacteria for the inoculum were grown at optimal temperature of 28°C, at the shaking rate of 150 rpm (on shaker SM-30 B, Edmund Bühler GmbH, Germany). Cell suspensions contained about 5×10^{11} CFU ml^{-1} for each isolate.

Soil microbiological analysis

Microbial activity in soybean rhizosphere was determined using the number of microorganisms and enzyme dehydrogenase activity - DHA (EC 1.1.1.). Rhizosphere soil samples were taken at the full bloom stage (R2). Indirect dilution plate method on appropriate solid nutritive media was used to determine the number of microorganisms. Total number of microorganisms was determined on soil agar (soil dilution 10^{-7}) (POCHON & TARDIEUX [16]); number of azotobacter (soil dilution 10^{-2}) and N_2-fixers (soil dilution 10^{-6}) were done on nitrogen-free medium (Fyodorov's medium) (ANDERSON & DOMASCH [17]). Number of ammonifiers were determined on mesopeptone agar (soil dilution 10^{-6}), actinomycetes on Krasilnikov's agar (Scharlau Microbiology, Barcelona, Spain) and fungi on Czapek-Dox agar (Scharlau Microbiology, Barcelona, Spain) (soil dilution 10^{-4}). Incubation temperature was 28°C, while incubation time depended on the tested group of microorganisms. The abundance of examined microbial groups was assessed over number of colony forming units (CFU), and the average number for all samplings was calculated per 1.0 g of absolutely dry soil. DHA (μg TPF g^{-1} soil) was done according to the method using triphenyltetrazolium chloride (TTC) (EN ISO 23753-1:2005). All microbiological analyses were performed in three replications.

Plant material analysis

The effectiveness of nitrogen fixation was determined based on the number and mass of nodules and nitrogen content in aboveground plant parts, roots, nodules and seeds. The number and dry matter mass of nodules, and nitrogen content in aboveground plant parts, roots and nodules were tested at the full flowering stage (R2) of soybean, while the nitrogen content in seeds was determined at full maturity of soybean (R8). Plant material was dried at 50°C during 24 h, and nodule dry weight was measured afterwards. Total nitrogen content was determined using the method AOAC 972.43:2000 of Elemental Analysis (Elementar VARIO EL III). The effect of inoculation on yield, pod number, seed number and seed mass was determined at full maturity of soybean. All plant analyses were performed in ten replications and the average number for all samplings per plant was calculated. Soybean yield was presented as kg ha^{-1} (based on the 14% moisture content).

The variables were analyzed using analysis of variance (ANOVA) statistical method, followed by mean separation according to Fisher's LSD test at the $p < 0.05$ level of probability (STANKOVIĆ & al. [18]).

3. Results and discussion

Presence of a large number of different microbial groups indicates good soil quality and proper functioning of processes that result in the activation of nutrients for crop production (CARDOSO & al. [19]). Microorganisms with their enzymatic systems are involved in 60-90% of total soil metabolic activity. One of the best indicators of microbial activity in soil is dehydrogenase activity, closely related to the process of respiration (DAS & VARMA [20]).

Additional activation of microbial processes can be achieved through inoculation with highly effective strains of PGPR which favored proliferation of rhizobia in crop rhizosphere due to better plant growth. Establishment of a significant number of PGPR in rhizosphere and plant tissues by inoculation led to an increase in biomass (even in grain production) in later stages of development (BASHAN & al. [21]). The effects of single and co-inoculation of soybean are presented in tables 2, 3 and 4.

Table 2. Effect of inoculation on microbial activity in soybean rhizosphere

Treatments	***Azoto-bacter*** **×10^2**	**Total microbial number ×10^7**	**Ammoni-fiers ×10^6**	**N - fixers ×10^6**	**Fungi ×10^4**	**Actino-mycetes ×10^4**	**Dehydro-genase activity**
	CFU g^{-1} absolutely dry soil						μg TPF g^{-1} soil
B.j.	142.4	297.6*	111.8	216.0*	20.6	59.5	575.0
B.j.+A.c.+ H.A.	132.7	248.0	99.0	140.1	24.3	60.1	488.0
B.j. + A.c.	177.3*	269.3*	115.2	204.3*	20.6	67.9	604.0*
B.j. + B.s.	152.1*	242.0	157.3*	187.4	18.2	58.2	591.0*
B.j. + B.m.	123.5	238.7	130.5	181.0	38.5	68.9*	523.0
Average	**145.6***	**259.1***	**122.8**	**185.8**	**24.4**	**62.9**	**556.2**
Control	113.0	209.0	96.8	143.0	33.4	58.4	486.0
Increase (%)	**28.8**	**24.0**	**26.8**	**29.9**	**-26.9**	**7.7**	**14.4**
$LSD_{0.05}$	30.8	46.1	34.4	52.2	5.8	10.4	97.9

The data represent mean values of three replicates; values marked with * are significantly different for $p < 0.05$

The analysis of variance of microbial activity based on the LSD test showed that inoculation had a significant effect on several analyzed microbial parameters. The applied inoculant positively influenced microbial activity in soybean rhizosphere (Table 2). On average, inoculation increased the number of azotobacters (28.8%), total microbial number (24%), ammonifiers (26.8%), N-fixing bacteria (29.9%), actinomycetes (7.7%), and dehydrogenase activity (14.4%). Inoculation with *Bradyrhiyobim japonicum* had a significant positive effect on the total microbial number and the number of N-fixers. Co-inoculation prevailed over single inoculation, so a significantly higher number of azotobacter, total microbial number and the number of N-fixers were obtained through co-inoculation with *A. chroococcum,* while a higher number of azotobacter and ammonifiers were recorded through co-inoculation with *B. subtilis*. The highest number of actinomycetes (68.9×10^4 g^{-1}) was obtained by co-inoculation with *B. megaterium* (Table 2).

Previous studies have reported that inoculation of soybean with rhizobacteria produced a wide range of effects in plant development (LEE & al. [22]). BAI & al. [23] showed high ability of three *Bacillus* strains co-inoculated with *Bradyrhizobium japonicum* to improve soybean nodulation and growth under low temperature stress in greenhouse and field experiments. Similarly, STAJKOVIĆ & al. [24] showed that co-inoculation with *Rhizobium* and *Pseudomonas* or *Bacillus* strains improved shoot dry weight, nitrogen content and phosphorus content in bean plants, compared to inoculation with *Rhizobium* alone, whereby *Pseudomonas* stimulated bean growth and particularly phosphorus uptake more efficiently than *Bacillus*. CHEBOTAR & al. [25] observed that co-inoculation with *B. japonicum* and PGPR strains increased *B. japonicum* colonization on soybean roots, nodule number and acetylene reduction activity, due to production of growth-promoting substances, and that co-inoculation effects were strain dependent. MASCIARELLI & al. [26] indicated that co-inoculation of soybean plants with *Bacillus* strain, able to produce high levels of auxin, gibberellins and salicylic acid, and natural symbiont *B. japonicum* altered plant growth parameters and significantly improved nodulation.

Table 3. Effect of inoculation on nitrogen fixation parameters of soybean

Treatments	**Nitrogen fixation parameters**					
	Nodule		**Nitrogen content (mg plant^{-1})**			
	Dry matter mass (g plant^{-1})	**Number (plant^{-1})**	**Nodules**	**Roots**	**Above ground parts**	**Seeds**
B.j.	0.314*	38.7*	8.1*	13.5	266	589*
B.j. + *A.c.* + H.A.	0.188	20.5	6.0	10.6	219	367
B.j. + *A.c.*	0.264*	32.3*	6.5	12.1	232	481*
B.j. + *B.s.*	0.204	24.2	4.8	13.1	304*	348
B.j. + *B.m.*	0.211	25.3	5.2	9.7	205	445
Average	**0.236***	**28.2***	**6.12**	**11.8**	**245**	**446**
Control	0.134	18.6	4.6	10.0	185	328
Increase (%)	**76.1**	**51.6**	**33.0**	**18.0**	**32.4**	**36.0**
$LSD_{0.05}$	0.092	8.6	3.1	5.1	94.6	147.0

The data represent mean values of ten replicates; values marked with * are significantly different for $p < 0.05$

On average, inoculation increased dry matter mass (76.1%) and the number of nodules (51.6%), as well as nitrogen content in nodules (33%), roots (18%), aboveground parts (32.4%), and seeds (36%) of soybean (Table 3). The highest effect on the studied parameters of nitrogen fixation was obtained by single inoculation with *Bradyrhizobium japonicum* which caused a significant increase of dry matter mass, number of nodules, nitrogen content in nodules and nitrogen content in seeds, compared to the control. Co-inoculation with *Azotobacter chroococcum* significantly increased dry matter mass, the number of nodules, and nitrogen content in seeds. Co-inoculation with *Bacillus subtillis* showed positive effect on nitrogen content in the aboveground parts (Table 3).

Positive impact of inoculation with nitrogen-fixing bacteria *Bradyrhizobium japonicum* which increased the number of studied microorganisms in rhizosphere, nitrogen fixation parameters, as well as soybean yield, was also reported in study of BALEŠEVIĆ-TUBIĆ & al. [27].

Based on the observed effects of co-inoculation, better results were achieved with *A. chroococcum*, whereby higher increase of nitrogen content in aboveground parts was obtained in treatment with *Bacillus subtillis*. Similarly, positive effects of wheat seed inoculation with this strain of *Azotobacter chroococcum* were obtained in the study of MILOŠEVIĆ & al. [28]. The possibility of using non-symbiotic *Azotobacter* as microbial inoculant through nitrogen fixation and production of growth substances and their effects on the plant has markedly enhanced crop production. *Azotobacter* fixes about 10 mg nitrogen g^{-1} of carbon source under *in vitro* conditions, while in field conditions the amount of fixed nitrogen is about 20 – 60 kg ha^{-1} (MRKOVAČKI & MILIĆ [29]). KOZIEL & al. [30] observed significantly higher number of azotobacter in soybean rhizosphere, and significantly improved nodulation of soybean plants and seed yield through co-inoculation with *Bradyrhizobium japonicum* and *Azotobacter chroococcum* compared to control and single inoculation. SUBRAMANIAN & al. [31] indicated that treatment of *Bradyrhizobium japonicum* along with *Bacillus megaterium* and *Methylobacterium oryzae* exhibited an increase in nodule number and nodule activity, measured in terms of nodule leghemoglobin content, nodulated root acetylene reducing activity (ARA), and total plant nitrogen content, compared to single inoculation of *B. japonicum*.

Table 4. Effect of inoculation on soybean yield parameters

Treatments	**Yield parameters**			
	Number of pods ($plant^{-1}$)	**Number of seeds ($plant^{-1}$)**	**Dry seed mass (g $plant^{-1}$)**	**Yield (kg ha^{-1})**
B.j.	30.3*	119.5*	13.7*	4472*
B.j. + *A.c.* + HA	24.1	89.8	9.38	4215
B.j. + *A.c.*	29.5*	97.5	11.52	4588*
B.j. + *B.s.*	27.6	94.3	10.62	4328
B.j. + *B.m.*	22.2	83.6	8.68	4263
Average	**26.7**	**96.9**	**10.78**	**4373**
Control	22.4	78.1	8.37	4208
Increase (%)	**19.2**	**24.1**	**28.8**	**3.92**
$LSD_{0.05}$	6.5	22.1	4.2	219.1

The data represent mean values of ten replicates; values marked with * are significantly different for $p < 0.05$

Application of *Bradyrhizobium japonicum* significantly increased the number of pods, number and mass of seeds, and yield, while the co-inoculation with *A. chroococcum* led to an

increase in the number of pods and yield, in comparison to the control treatment. On average, yield increase obtained by inoculation amounted to 3.92% compared to the control. Co-inoculation had a better effect on yield than single inoculation, and a significant increase in yield was obtained in the treatment with *A. chroococcum* (4588 kg ha^{-1} – 9%) (Table 4).
Similarly, MILIĆ & al. [32] showed that co-inoculation with *B. japonicum* and *A. chroococcum* resulted in plant growth promotion, as well as increase in microbial number, number of nodules and yield of soybean. MARINKOVIĆ & al. [33] concluded that inoculation with *B. japonicum* statistically increased pod and seed number, seed mass, and soybean yield. Results of AFZAL & al. [34] showed that co-inoculation of soybean with *Bradyrhizobium* and PGPR strains combined with P_2O_5 treatment increased grain yield of 38% in pot experiments, and 12% in field experiments, compared with the P_2O_5 treatment alone, as well as higher survival efficiency of applied strains due to co-inoculation as compared with its single inoculation. PRAKAMHANG & al. [35] observed that PGPR exhibited a significant capability of promoting N_2-fixation, nodule number, nodule and plant dry weight, with both commercial bradyrhizobial strains, and achieved 9.7 to 43.6% increase in seed yield in field conditions, which was higher compared to those uninoculated or with a single inoculation.

4. Conclusion

The best effect on nitrogen fixation and yield parameters was achieved by single inoculation, while co-inoculation had a better effect on the number of microorganisms, dehydrogenase activity, and yield of soybean. All treatments had a positive effect on soybean yield, while the largest increase was obtained by co-inoculation with *A. chroococcum*. Increase in yield of cultivated plants, along with decrease in the use of mineral fertilizers, has caused a more intense application of biological nitrogen as a microbial fertilizer. Replacement of mineral nitrogen fertilizers with microbial fertilizers is well-justified from the perspective of energy, economy and ecology, and their application enables the production of high-quality organic food.

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