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Effect of inoculation with *Bradyrhizobium* and phosphate solubilizing bacteria on soybean seed yield and composition

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Abstract

In the field experiment, the effect of co-inoculation with *Bradyrhizobium japonicum* and two *Pseudomonas* sp. strains on seed yield and macronutrient uptake in soybean (*Glycine max* L.) was evaluated. The results showed that inoculation and co-inoculation of soybean seeds with *B. japonicum* and *Pseudomonas* sp. strains increased seed yield (from 65 up to 134%), and uptake of N, P, K, Mg, and Ca (kg ha^{-1}) (from 65 to 167%), compared to the control plants (uninoculated, non-fertilized plants). Phosphorous concentration (mg kg^{-1}) was increased in inoculated and co-inoculated treatments (up to 15%), compared to the control. The N%, as well as the concentrations of K and Ca, did not differ significantly among treatments and control. Magnesium concentrations were increased in mineral fertilized and co-inoculated treatments. Uptake of all nutrients was in significant correlation with seed yield, while the concentration of P only correlated with seed yield. The results showed that co-inoculation with bradyrhizobial and some phosphate solubilizing bacteria can increase the seed yield and uptake of N and P in soybean.

Keywords: inoculation, bradyrhizobia, *Pseudomonas*, nitrogen, phosphorous

Introduction

Soybean (*Glycine max* L.) is a very important leguminous plant used for human nutrition as well as fodder crop. Soybean seeds contain a high amount of proteins (40-42%), oils (about 20%), and minerals (calcium, zinc, iron) (Argaw, 2012). Soybean can form a symbiotic association with soil bacterium rhizobium, *Bradyrhizobium japonicum*, and fix atmospheric nitrogen (N_2). By establishing a symbiotic relationship and performing symbiotic N_2 fixation, rhizobia improve the N content and soybean seed yield, thus reducing the need for nitrogen mineral fertilizer application in soybean cultivation. According to Unkovich and Pate (2000), the amounts of N_2-fixed (kg ha^{-1}) by soybean have been up to 450 kg N ha^{-1}. Besides N, phosphorous supply is very important for soybean since it is required for nodule development and functioning (Sa and Israel, 1991). However, the availability of P to plants in the soil is usually very low, mainly because P exists in the soil as insoluble inorganic and/or organic phosphorus (Walpola and Yoon, 2012). This depends on the pH of the soil where P can be immobilized in calcium phosphate ($Ca_3(PO_4)_2$) (in alkaline or neutral soils) or aluminum (AlPO) and ferrous phosphate (FePO) (in acidic soils) (Kalayu, 2019). The conversion of these insoluble P forms into

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soluble forms available for plant growth can be achieved using P solubilizing bacteria. In alkaline soil, these bacteria can dissolve the insoluble soil phosphates by producing organic acids that chelate the cations and compete with the phosphate for adsorption sites in the soil resulting in P solubilization. On the other hand, insoluble organic P can become bioavailable by mineralization using these P solubilizing bacteria. Several organic acids can be released by P solubilizing bacteria and their solubilization efficiency depends on the strength and nature of the acids (Kalayu, 2019; Alori et al., 2017). These soil bacteria could be useful in soybean production improvement by increasing P content in the soil and enhancing nodulation and N fixation. Many of them belong to the genera such as *Pseudomonas*, *Bacillus*, *Azotobacter*, *Paenibacillus*, *Serratia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Kushneria*, *Rhodococcus*, *Salmonella*, *Sinomonas*, *Thiobacillus* (Alori et al., 2017). The inoculation of soil or crop with P solubilizing/mineralizing bacteria (P biofertilizers) is an eco-friendly strategy for reduction application of chemical fertilizers that have a negative impact on the environment to improve P absorption by plants (Alori et al., 2012; Babalola and Glick, 2012). In addition, some studies indicated that rhizobia, besides N_2 fixation, may influence certain macro and micronutrient contents in leguminous plants and soil, such as various strains from *Rhizobium* and *Bradyrhizobium* (Alori et al., 2017; Ndakidemi et al., 2011; Bambara and Ndakidemi, 2010). Rhizobial strains enhanced the uptake of many macro and micronutrients in peanut nodules and seeds (Howell, 1987), soybean (Tairo and Ndakidemi, 2014 a,b), and common bean plants (Makoi et al., 2013; Ndakidemi et al., 2011).

There are some researches regarding the co-inoculation of legume plants with a consortium of rhizobial and non-rhizobial bacteria (Marinković et al. 2018; Stajković et al., 2011; Argaw, 2012). Kumawat et al. (2019) used *Pseudomonas aeruginosa* (LSE-2) nodule endophyte with *Bradyrhizobium* sp. (LSBR-3) as biofertilizer of soybean. Argaw (2012) also studied the effect of co-inoculation of *Bradyrhizobium japonicum* (TAL-378 and TAL-379) and P-solubilizing bacteria *Pseudomonas* spp. on nodulation, yield, and yield components of soybean. Co-inoculaton with *Bradyrhizobium japonicum* and *Azotobacter chroococcum* had better effect on soybean yield compared to single inoculation with *Bradyrhizobium japonicum* (Marinković et al., 2018).

Therefore, the aim of this research was to determine if the co-inoculation of soybean with *Bradyrhizobium japonicum* and two *Pseudomonas* P-solubilizing strains can additionally improve seed yield and macro nutrient uptake, compared to rhizobial inoculation alone.

Materials and methods

Soil and experiment design

The experiment was set up in 2011 (N 44°41′38″, E 19°39′10″) in the field in the village of Boljevci (near Belgrade, Serbia), on Chernozem soil (FAO, 1998) with the following chemical characteristics (Table 1). In 2011., the average monthly temperatures during the vegetation period (from March to

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October) were 12.9°C with a maximum in June of 24.8°C, while the total amount of rainfall was 541.1 mm. In the past 10 years, legumes have not been grown in the experimental field.

Strains *Bradyrhizobium japonicum* 542, as well as *Pseudomonas* sp. L2Cr and LG from the Collection of the Institute of Soil Science were used for the inoculation or co-inoculation of soybean (variety Biser, maturity group 0). *Pseudomonas* strains were previously characterized and identified by Stajković et al. (2009) and Knežević et al. (2021). *Pseudomonas* strains were cultivated for 24 h in King B medium while *Bradyrhizobium* strains were grown in yeast mannitol broth (YMB) for 5 days. The culture of 40 mL of each strain was mixed with 100 g of sterile ground peat and after a 15-day incubation period, single inoculums consisting of approximately 10^9 bacteria per g peat were obtained (Somesegaran and Hoben, 1994). The trial was designed with 3 inoculated treatments *B. japonicum* 542, *B. japonicum* 542 + *Pseudomonas* LG (*Bradyrhizobium* + P1), *B. japonicum* 542 + *Pseudomonas* L2Cr (*Bradyrhizobium* + P1), treatment with mineral NPK fertilizer (N 60 kg ha^{-1}, P 100 kg ha^{-1} and K 100 kg ha^{-1}; NØ), and control without mineral N fertilizer and inoculation (Ø). In the co-inoculation treatment, the ratio of *Bradyrhizobium* and *Pseudomonas* inoculum was 1:1. The experiment was laid out in a completely randomized design in three replications. Each plot was planted in 6 rows of 2 m length with an inter-row spacing of 50 cm, with a final number of 40 plants per row. The seeds were sown during the first week of May 2011, and no mineral fertilizers or pesticides were used before sowing and during the vegetation period. Soybean seeds were hand-harvested at full maturity in the last week of August.

Soil and seed samples analyses

For mineral nutrient analysis, the seeds were dried at 65ºC for 72 h, ground in a mill to pass through a 1-mm mesh. The concentrations of N, C, and S were analyzed using an elemental CNS analyzer, Vario model EL III (ElementarAnalysensysteme GmbH, Hanua, Germany). To determine K, P, Mg, and Ca in the seed, the ground material was burned to ash at 550°C in a muffle furnace, and acid digestion with HNO_3 and HCl was performed (AOAC, 1990). Element concentrations were analyzed using a Thermo iCAP 6300 Duo (ICP-OES).

Soil pH was determined using a glass electrode pH meter in 1M KCl and in H_2O (soil: KCl or H_2O 1:2.5 ratio). Available P and K in soil were determined by the AL-method of Egner-Riehm (Egnér et al., 1960). Soil Ca and Mg were extracted with ammonium acetate and determined using a dual atomic adsorption spectrophotometer SensAA (Dandenong, Australia). Organic C and N in the soil were determined using an elementary CNS analyser, Vario model EL III (Hanau, Germany).

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Statistical analysis

The effect of the treatments was evaluated using analysis of variance (SPSS 16.0 program, 2007) and significant differences between means were tested by Duncan's multiple range test.

Results

The soil of the experimental field had a neutral reaction and it was well supplied with N, P, and K (Table 1). The soil type was Chernozem, and according to the soil texture, it was clay loam (Table 2).

Table 1. Soil chemical properties

pH H_2O	pH KCl	P_2O_5	K_2O	N_{tot}	C_{org}
		mg 100 g^{-1}	mg 100 g^{-1}	%	%
7.00	6.65	21.81	33.59	0.27	3.7

Table 2. Soil texture

Coarse sand % >0.2 mm	Fine sand % 0.2-0.02 mm	Silt % 0.02-0.002 mm	Clay % <0.002 mm	Total send % >0.02 mm	Silt + clay % <0.02 mm	Textural classis
0.5	28.2	32.2	39.1	28.7	71.3	Clay loam

Soybean seed yield in all inoculated treatments was increased compared to the control, indicating symbiotic efficiency of the strains (Table 3). The highest yield was obtained in the treatment with NPK mineral fertilizer application and the treatment with co-inoculation with *B. japonicum* 542 and *Pseudomonas* LG. The co-inoculation with *B. japonicum* 542 and *Pseudomonas* L2Cr realised lower yield than single inoculation with *B. japonicum* 542, but without statistical significance. There were no significant differences in N percentages (N%) among treatments, which is probably due to very small yield in the control plants compared to inoculated treatments and dilution effect in higher yielded treatments.

Table 3. Seed yield and macronutrient concentrations of soybean plants inoculated with *Bradyrhizobium* and *Pseudomonas*.

Treatment	Grain yield	N	P	K	Ca	Mg
	t ha^{-1}	%	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
*Bradyrhizobium**	3.28b	6.46a	5.36a	15.70a	3.56a	2.54c
Bradyrhizobium + P1	4.19a	6.28a	5.36a	15.84a	3.76a	2.70b
Bradyrhizobium + P2	2.88b	6.51a	4.86b	15.95a	3.88a	2.64bc
Ø	1.79c	6.37a	4.70b	15.62a	3.49a	2.54c
NØ	4.35a	6.44ns	5.28a	15.92ns	3.32ns	2.91a

Ø- control (no inoculation and no fertilization); NØ- treatment with NPK fertilization and without inoculation; ns- statistically not significant; values followed by the same letter in a column are not significantly different (Duncan test, $P< 0.05$); *- *B. japonicum* 542; P1- *Pseudomonas* sp. LG; P2- *Pseudomonas* sp. L2Cr.

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However, the total N content (kg ha^{-1}) in all inoculated treatments was increased (Table 4). The concentration of P in soybean seeds was increased in all treatments compared to the control except in the co-inoculation with *B. japonicum* 542 and *Pseudomonas* L2Cr (Table 4). There were no differences between inoculation and co-inoculation with LG strain. The increase in P concentration was 14% for inoculation and co-inoculation with the LG strain, and 3% for co-inoculation with the L2Cr strain, compared to the control. Total P content (kg ha^{-1}) was increased in all treatments compared to control, with the highest values measured in co-inoculation with *B. japonicum* 542 and *Pseudomonas* LG and treatment with mineral fertilizer application (Table 4). The highest percentage increase in P uptake among treatments was in the treatment *Bradyrhizobium* + P1 compared to the control and was approximately 167% (Table 5).

Table 4. Macronutrients uptake in soybean seeds inoculated with *Bradyrhizobium* and *Pseudomonas* strains.

Treatment	N	P	K	Ca	Mg
	kg ha^{-1}				
*Bradyrhizobium**	211.89ab	17.58b	51.50b	11.68bc	8.33bc
Bradyrhizobium + P1	262.92a	22.44a	66.32a	15.74a	11.30ab
Bradyrhizobium + P2	187.49b	14.00b	45.94b	11.17c	7.60c
Ø	113.81c	8.40c	27.91c	6.23d	4.54d
NØ	279.93a	22.95a	69.20a	14.43ab	12.65a

Ø- control (no inoculation and no fertilization); NØ- treatment with NPK fertilization and without inoculation; ns- statistically not significant; values followed by the same letter in a column are not significantly different (Duncan test, P< 0.05); *- *B. japonicum* 542; P1-*Pseudomonas* sp. LG; P2- *Pseudomonas* sp. L2Cr.

Table 5. Percentage of increase in seed yield and macronutrient uptake of soybean inoculated with *Bradyrhizobium* and *Pseudomonas* strains compared to control.

Treatment	Grain yield	N	P	K	Ca	Mg
*Bradyrhizobium**	84	87	109	85	87	84
Bradyrhizobium + P1	134	131	167	138	152	149
Bradyrhizobium + P2	61	65	67	65	79	68
Ø	0	0	0	0	0	0
NØ	143	146	173	148	131	179

Ø - control (no inoculation and no fertilization); NØ- treatment with NPK fertilization and without inoculation; *- *B. japonicum* 542; P1-*Pseudomonas* sp. LG; P2-*Pseudomonas* sp. L2Cr.

Concentrations of K and Ca did not differ among treatments, while Mg concentrations were increased only in mineral fertilizer and *Bradyrhizobium* + P1 co-inoculation treatments, compared to the control (Table 3). Total uptake of K, Ca, and Mg was increased in all treatments and there were no differences between mineral fertilizer and *Bradyrhizobium* + P1 co-inoculation treatment (Table 4).

There were no negative correlations between any of the soybean seed parameters (yield, macronutrient concentrations) and total macronutrient content (Table 6). Table 6 shows only significant

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correlation values. Positive and very significant correlations were observed between grain yield and all macronutrients uptake, as well as significant correlations between P concentration and P uptake.

Table 6. Significant correlations among soybean seed parameters.

	Yield	N	P	K	Ca	Mg	N#	P#	K#	Ca#	Mg#
N#	.999**										
P#	.997**		.900*				.994**				
K#	1.000**						.999**	.995**			
Ca#	.976**						.972**	.971**	.977**		
Mg#	.991**						.991**	.982**	.992**	.949*	

**p*<0.05 significant correlation; ***p*<0.01 very significant correlation; #-uptake.

Discussion

The inoculation of soybean with rhizobacteria produced a wide range of effects in plant development (Marinković et al., 2018). Simultaneous inoculation with rhizobia and some plant-growth promoting rhizobacteria (PGPR) can increase growth and yield, compared to rhizobium inoculation alone in leguminous plants including soybean (Hungria et al., 2015; Stajković et al., 2009; Itzigsohn et al., 1993). However, published results mainly showed that co-inoculation of soybean with *Bradyrhizobium* and other PGPR could substantially increase the number of nodules, nodule biomass, root biomass, and shoot biomass in soybean, but no significant differences in shoot N content and grain were observed (Zeffa et al., 2020). In addition, the co-inoculation effects were more evident in pot experiments than in the field (Zeffa et al., 2020). Due to this, the effects of soybean co-inoculation with bradyrhizobia and Pseudomonads on soybean seed yield and nutrient content in field conditions were tested in this study.

The seed yield increased in the co-inoculation treatment *Bradyrhizobium* + P1 by 27% compared to a single inoculation with *Bradyrhizobium*. Compared to the control, this increase was as much as 2.3 and 1.64 times higher for the *Bradyrhizobium* + P1 and *Bradyrhizobium* + P2 treatments, respectively. An increase in seed yield with co-inoculation with *B. japonicum* and P solubilizing *Pseudomonas* sp. was previously reported by Argaw, 2012, together with an increase in N% and P uptake (kg ha^{-1}). *B. japonicum* TAL 378 and *Pseudomonas* spp. treatment achieved significantly higher seed yield per hectare than the negative control among the tested co-inoculation treatments and amounted to 36.3% (Argaw, 2012), which was less than co-inoculation treatments in this study. Marinković et al. (2018) also observed an increase in seed yield with co-inoculation treatment with *B. japonicum* and *Azotobacter chroococcum* about 46% compared to the control. These authors reported that inoculation/co-inoculation with highly effective PGPR could activate the microbial process in the crop rhizosphere and potentiate better plant growth by favoring rhizobia proliferation. The change in the

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number of PGPR in the crop rhizosphere was not monitored in this study, but the establishment of a significant number of these bacteria may lead to an increase in biomass and grain production at a later stage of soybean development (Bashan et al., 2004; Marinković et al., 2018). In addition, an increase in the grain yield might be the result of the microorganisms involved in P solubilization. These bacteria can enhance plant growth by increasing the efficiency of biological fixation, enhancing the bioavailability of trace elements, and by the production of plant growth- promoting substances (Argaw, 2012).

In this research, N% was not increased compared to the control in any inoculated treatments, probably due to very low yield in the control treatment and nitrogen dilution in high yield obtained by inoculation (Jarrell and Beverly, 1981; Timmer, 1991). However, the total N uptake was increased significantly. Compared to the control, P concentration increased up to the same level in inoculation with *Bradyrhizobium* and in co-inoculation with *Pseudomonas* LG, while strain L2Cr had no influence on P concentration. Total P uptake was increased in all treatments compared to the control. The treatment of *Bradyrhizobium* + P1 in comparison with the treatment with mineral NPK fertilizer achieved similar concentrations of N and P in soybean seed and whose differences were not significant. This could be due to the fact that this co-inoculation provided similar N and P nutrients as the chemical fertilizer (Argaw, 2012). Phosphorus uptake increased in co-inoculation mainly as a result of P solubilizing microorganisms, which in addition to solubilizing P, produce a necessary phytohormone, indole-3-acetic acid, thereby enhancing root growth and increasing nutrient uptake (Argaw, 2012). In addition, *Pseudomonas* LG strain showed good results for the total content of N and P in the biomass of common bean in co-inoculation with the *Rhizobium phaseoli* 123 in comparison with single inoculation with rhizobium and control in a previous study by Stajković et al. (2011). Increasing the availability of other macroelements (K, Ca Mg), as well as N and P could be associated with the production of siderophores, IAA, and/or ammonia production. These abilities have been previously confirmed for the strain LG (Stajković et al., 2011).

Previously, the co-inoculation of soybean seeds with *B. japonicum* and *P. fluorescens* in conjunction with either 75% or 100% of the recommended dose of nitrogenous and phosphatic fertilizers significantly increased different plant parameters, including grain yield, N and P uptake in soybean (Pawar et al., 2018). Co-inoculation of *Bradyrhizobium* and *P. Pseudomonas* strain 54RB with the P_2O_5 treatment resulted in an increased grain yield of 38% in pot experiments and 12% in the field experiment, compared to the P_2O_5 treatment alone (Azfal et al., 2010). In the same research, maximum seed P%, N%, and protein% were recorded by *Bradyrhizobium*-*Pseudomonas*-P_2O_5 treatment. Production of plant growth regulators, P solubilization activity, and increased colonization in the rhizosphere suggest the mechanism of action of co-inoculated N-fixing and P-solubilizing microorganisms (Azfal et al., 2010).

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Conclusions

All treatments (inoculation and co-inoculations) had a positive effect on soybean seed yield, while the highest increase was obtained by co-inoculation with *B. japonicum* 542 and *Pseudomonas* L2Cr. The best effect on macronutrients uptake was also achieved by co-inoculation treatment with *Pseudomonas* L2Cr. The absence of significant differences between co-inoculation treatment with *Pseudomonas* L2Cr and treatment with mineral NPK fertilizer on the uptake of N, P, Ca Mg, and K give the possibility of applying microbial instead of mineral fertilizer. Replacement of mineral nitrogen fertilizers with microbial fertilizers represents a well-justified ecologically acceptable perspective in the production of soybeans and high-quality organic food.

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Efekat inokulacije bradirizobijalnim i fosfosolubilizirajućim bakterijama na prinos i sastav semena soje

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Izvod

U poljskom eksperimentu je ispitan efekat koinokulacije sojevima *Bradyrhizobium japonicum* i *Pseudomonas* sp. na prinos semena i usvajanje nutrijenata kod soje (*Glycine max* L.). Rezultati su pokazali da inokulacija i koinokulacija semena soje sa *B. japonicum* i sojevima *Pseudomonas* sp. povećava prinos semena (od 63 do 134%) i usvajanje N, P, K, Mg i Ca (kg ha^{-1}) (od 65 do 167%), u semenu u poređenju sa kontrolnim biljkama (neinokulisanim, neđuberenim biljkama). Koncentracija fosfora (mg kg^{-1}) je bila povećana u inokulisanim i koinokulisanim tretmanima u poređenju sa kontrolom (do 15%). Procenat N, kao i koncentracije K i Ca nisu se značajno razlikovale između tretmana i kontrole. Koncentracija Mg je bila povećana u koinokulisanim tretmanima i tretmanu sa mineralnim đubrivom. Usvajanje svih nutrijenata je bilo u pozitivnoj korelaciji sa prinosom semena, dok je koncentracija P jedina bila u pozitivnoj korelaciji sa prinosom semena. Studija je pokazala da koinokulacija bradirizobijalnim i fosfosolubilizirajućim bakterijama može da poveća prinos i usvajanje N i P kod semena soje.

Ključne reči: inokulacija, bradirizobije, *Pseudomonas*, azot, fosfor

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