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Improvement of common bean growth by co-inoculation with *Rhizobium* and plant growth-promoting bacteria

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Abstract

In the greenhouse experiment, two Bacillus and two Pseudomonas strains were examined for their ability to promote common bean growth (Phaseolus vulgaris L.) when co-inoculated with Rhizobium phaseoli. Co-inoculation with Rhizobium and Pseudomonas sp. LG or Bacillus sp. Bx improved shoot dry weight, nitrogen and phosphorus contents in bean plants, compared to inoculation with Rhizobium alone. Pseudomonas sp. LG promoted bean growth and particularly P uptake more efficiently than Bacillus sp. Bx. In vitro screening for plant growth-promoting traits showed phosphate solubilization, IAA, ammonia and siderophore production by Pseudomonas sp. LG, as opposed to Bacillus sp. Bx, which showed only ammonia production. Multiple plant growth-promoting traits of Pseudomonas sp. LG may be associated with its ability to improve common bean growth and nutrient uptake efficiently.

Keywords: common bean, plant growth promotion, co-inoculation

Introduction

Leguminous plants include many important species that are used as food and fodder crop throughout the world. They can provide their own nitrogen requirements through nitrogen fixation in symbiosis with soil bacteria collectively known as rhizobia. These bacteria form root nodules on leguminous plants and convert atmospheric N_2 into a form usable by plants. Application of effective rhizobial strains as biofertilizers to improve legume production is an important approach in sustainable agriculture.

Many studies have recently shown that inoculation with some plant growth-promoting bacteria (PGPB), increases growth and yield in great number of plants including legumes [1, 2, 3]. PGPB can contribute to plant growth in different manners: by increasing nitrogen uptake, synthesis of phytohormones (auxin, cytokinin), minerals solubilization and iron chelation [4, 5, 6, 7]. They may suppress soil-borne pathogens by producing siderophores, antimicrobial metabolites, or competing for nutrients and/or niches [8]. PGPB also have beneficial effects on legume growth, and at least some strains enhance nodulation and nitrogen fixation by affecting interactions between plant and rhizobia. Available reports indicate improved legumes yield, health and nodulation when co-inoculated with PGPB, compared to inoculation with rhizobium alone [5, 9, 10, 11].

Beans are the most important grain legumes for direct human consumption in the world. In nutritional terms, beans are great protein source and they are rich in minerals (especially iron and zinc) and vitamins [12]. Symbiotic nitrogen fixing potential in common bean is considered to be low in comparison with other legumes. However, owing to genotypic variability for traits associated with N_2 fixation potential selection has produced breeding lines able to fix high levels of N_2. The selection of particular rhizobial strains with high nitrogen fixing potential is also important [13]. Phosphorus has a considerable influence on the legume-rhizobia symbiosis. Positive effects of P on nodulation and nitrogenase activity in common bean were observed. Nitrogen fixation in common bean is more affected by P deficiency than in other legume crops [14, 15]. Taking into account that some PGPB possess ability of phosphate solubilization, they could be useful in bean production improvement by increasing P content in the soil and enhancing nodulation and N fixation.

The aim of this research was to determine if the co-inoculation of common bean with *Rhizobium phaseoli* and some *Pseudomonas*, as well as *Bacillus* strains can improve plant growth and nutrient uptake. In addition, the bacterial strains were examined for their plant growth-promoting traits.

Material and methods

Bacterial strains and growth conditions

Strains luc2, LG and SNji were isolated from alfalfa root nodules, while Bx strain was isolated from soil rhizosphere. The identification of isolates to the genus level was performed by standard microbiological procedure [16]. *Rhizobium phaseoli* strain 123 (the Collection of Institute of Soil Science, Belgrade) was selected for its high capacity to fix nitrogen in symbiosis with common bean. For the inoculation study *R. phaseoli* strain was cultivated in yeast mannitol broth (YMB), *Bacillus* strains in nutrient broth medium and *Pseudomonas* strains in King B medium, in the 250 ml flasks shaken at 125 rpm at 28 ˚C. The 78 h old culture of *R. phaseoli* and 24 h old cultures of others strains were used as inoculants.

Screening for plant growth-promoting traits

Detection of phosphate solubilizing ability. Bacterial strains were tested by plate assay using Pikovskaya medium (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP) [17, 18]. Strains were stabbed on plate in triplicate using sterile toothpicks. The halo appearing around the colony after 7 days of incubation at 28 ˚C indicates P solubilization ability of strains. Halo size was calculated by subtracting colony diameter from the total diameter.

Detection of ammonia. Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in tubes and incubated for 48–72 h at 28 ˚C. Nessler's reagent (0.5 ml) was added in each tube. The development of color from yellow to brown was a positive test for ammonia production.

Siderophore detection. Siderophore production was determined in appropriate medium according to the Milagres et al. procedure [19]. Color change of the medium from blue to orange is an indication of siderophore production by bacterial strains.

Test of IAA production ability. The strains for indole-3-acetic acid (IAA) detection were grown in nutrient broth medium or YMA (*Rhizobium*), supplemented with tryptophan (2 mg ml^{-1}), at 28 ˚C for 5 and 7 days respectively. Cultures were centrifuged and the two ml of supernatant was mixed with 2 ml of reagent which consisted of 4.5 g of $FeCl_3$ per litre in 10.8 M H_2SO_4. Development of a pink color indicates IAA production.

Evaluation of common bean growth when co-inoculated with Rhizobium and PGPB strains

The co-inoculation effects of PGPB and *Rhizobium* strains on bean growth were examined in pot experiment under greenhouse conditions, using non-sterile soil with no history of bean cultivation. The soil used had the following characteristics: pH (in H_2O) 7.4, 0.09 % N, 1.48 % C, 21 mg kg^{-1} available P. The experiment was designed with 5 co-inoculated treatments with 5 replications in a completely randomized system. Controls without inoculation (without mineral nitrogen (Ø) and with full N content (NØ)) and nodulation control inoculated with *Rhizobium phaseoli* strain 123 alone were included for comparison. The experiment was performed in 24 cm diameter plastic pots filled with soil. Common bean seeds (*Phaseolus vulgaris* L. cultivar Biser) were surface-sterilized with 0.1% $HgCl_2$ solution [20] and inoculated either with *R. phaseoli* strain alone, or with a *Bacillus* and *Pseudomonas* strains in a ratio of 1:1. Five seeds per pot were planted and thinning of the seedlings to 3 was done after 2 weeks. The pots were kept in a closed greenhouse in semi-controlled conditions for two months, and the plants were harvested in the flowering phase. Roots were carefully removed from the pots, washed free of soil and the root and shoot portions of bean were separated and measured. The number of nodules and shoot and root length were recorded. Plant shoots, roots and nodules were dried in an oven at 70 ˚C to constant weight and the average dry weight per plant was calculated. Symbiotic nitrogen fixing efficiency (SNF) was calculated as an increase in SDW in relation to SDW of NØ plants (100%). The percentage of shoot N was determined from dried and grinded plant samples using the CNS analyser (Vario model EL III (ELEMENTAR Analysensysteme GmbH, Hanua, Germani)) and it was used to calculate total N content in mg per plant. In plant material samples the P content was determined by dry ashing at 550 ˚C and acid digestion, after which, P was determined colorimetrically. The data were statistically processed by the LSD and Duncan test using SPSS 10.0 computer program.

Results and discussion

Plant growth-promoting traits

Bacterial strains used in this study, luc2, LG and SNji, originated from alfalfa root nodules and represent non-rhizobial nodule endophytes. Two of these strains, luc2 and LG, were found to belong to the genus *Pseudomonas*, while SNji strain was previously determined as *Bacillus megaterium* [21]. Strain Bx was isolated from soil rhizosphere and determined as *Bacillus* sp. Initial screening for plant growth-promoting traits demonstrated that all strains were positive for ammonia production (Table 1). Strains *Pseudomonas* spp. luc2 and LG produced siderophores intensively, and could strongly solubilize phosphate in both PVK and NBRIP media, together with *Rhizobium* strain which was however less efficient. *Rhizobium*, as well as LG and SNji strains produced IAA. *Bacillus* sp. Bx did not show any of the plant growth promotion traits tested, except ammonia production.

Table 1- Some plant growth-promoting traits of bacterial strains.

Strain	P solubilization*		NH_3	IAA	Siderophore
	PVK	NBRIP		production	
Rhizobium phaseoli	0	3	-	+	-
Bacillus megaterium SNji	1.5	0	+	+	-
Bacillus sp. Bx	0	0	+	-	-
Pseudomonas sp. luc2	10	13	+	-	+
Pseudomonas sp. LG	10	15	+	+	+

*diameter of halo in mm; +, positive reaction; -, negative reaction.

Plant growth and inoculation in non-sterile soil

Pot experiment with non-sterile soil showed a significant effect of some PGPB strains when co-inoculated with *Rhizobium* on nodulation and growth parameters of bean in respect to inoculation with *Rhizobium* alone.

Co-inoculation of bean with *Bacillus* strains SNji and Bx was found to positively influence nodule number (106.67 and 76.67 nodule number plant^{-1}, respectively) (Table 2), and in the case of SNji nodule dry weight also (90.33 mg plant^{-1}), compared to inoculation with *Rhizobium* alone (54 nodule number plant^{-1} and 53.12 mg plant^{-1}). Maximum nodule number recorded in the *Rhizobium* and SNji co-inoculation doubled compared to the *Rhizobium* control. High positive correlation between nodule number and nodule dry weight was noted (Table 4).

Table 2. Average nodule number, nodule dry weight, shoot and root length and root dry weight of inoculated common bean plants.

Treatment	Nodule N^{o} plant^{-1}	Nodule dry weight (mg plant^{-1})	Shoot height (cm)	Root length (cm)	Root dry weight (g plant^{-1})
Rhizobium	54.33^{c}	53.12^{b}	127.57^{b}	31.83^{a}	0.920^{a}
Rhizobium + SNji	106.67^{a}	90.33^{a}	127.14^{bc}	31.05^{a}	0.829^{a}
Rhizobium + Bx	76.67^{b}	63.67^{b}	115.97^{d}	32.00^{a}	0.870^{a}
Rhizobium + luc2	59.02^{c}	55.93^{b}	119.77^{bcd}	38.52^{a}	0.973^{a}
Rhizobium + LG	63.25^{c}	56.67^{b}	113.70^{d}	36.09^{a}	0.900^{a}
MIX	57.00^{c}	54.57^{b}	127.27^{bc}	33.70^{a}	0.822^{a}
Ø	34.51^{d}	30.33^{c}	119.35^{cd}	33.73^{a}	0.504^{b}
NØ	27.22^{d}	31.61^{c}	140.49^{a}	37.07^{a}	0.841^{a}
LSD 0.05	8.44	9.39	7.52	7.51	0.131

Non-inoculated controls: NØ-with N and Ø-without N; MIX- mixture of all strains; a-d: Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test at the 5% level ($p \leq 0.05$).

The maximum bean shoot height was recorded in the NØ control (140 cm) and it was significantly superior to all other treatments tested. On the other hand, the maximum root length was detected with luc2 strain co-inoculation, but without significant differences. There was no correlation between shoot height and root length.

Table 3. Average shoot dry weight, total and fixed N content, P content of inoculated common bean plants indicating plant growth-promoting potential of strains.

Treatment	Shoot dry weight		Shoot N (%)	Shoot total N content (mg plant^{-1})	Shoot fixed N content (mg plant^{-1})	Shoot P (%)	Shoot P content (mg plant^{-1})
	(g plant^{-1})	Symbiotic efficiency (%)					
Rhizobium	2.40^{c}	101.27	2.34	56.11^{c}	14.31^{c}	0.59	14.16^{de}
Rhizobium + SNji	2.41^{c}	101.69	2.17	52.30^{c}	10.45^{d}	0.65	15.67^{cd}
Rhizobium + Bx	2.75^{b}	116.03	2.65	72.87^{a}	31.03^{a}	0.70	19.25^{bc}
Rhizobium + luc2	2.37^{c}	100.00	2.18	51.67^{b}	9.82^{d}	0.74	17.54^{bcd}
Rhizobium + LG	3.07^{a}	129.54	2.36	72.45^{a}	30.60^{a}	0.90	27.63^{a}
MIX	2.57^{bc}	108.44	2.53	65.02^{b}	23.17^{b}	0.79	20.30^{b}
Ø	1.86^{d}	74.26	2.35	41.85^{d}	/	0.60	11.16^{e}
NØ	2.37^{c}	100.00	2.14	50.72^{c}	/	0.83	19.67^{bc}
LSD 0.05	0.21			7.40	3.44		3.81

Non-inoculated controls: NØ-with N and Ø-without N; MIX- mixture of all strains; a-e: Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test at the 5% level ($p \leq 0.05$).

Average shoot dry weight (SDW), total and fixed N contents in plants were used for quantitative and qualitative symbiotic nitrogen fixing efficiency (SNF) assessment as well as plant growth-promoting effect (Table 3). Plants inoculated with *Rhizobium* strain alone achieved similar SDW to control plants with N (NΘ), indicating its significant nitrogen fixing potential (Table 3). Co-inoculation of common bean with *Rhizobium* and Bx strain, as well as with *Rhizobium* and LG strain significantly increased SDW of plants (2.75 and 3.07 mg plant^{-1}, respectively), in respect to *Rhizobium* inoculation alone, showing the potential of these strains to improve dry matter accumulation over *Rhizobium* alone. Elevated N content was also detected in these two treatments and both strains showed the same N content per plant, regardless of the higher SDW in LG treatment. Shoot dry weight values were in a highly significant positive correlation with shoot total N (*r*=0.929).

The highest root dry weight (RDW) was recorded by co-inoculation with *Rhizobium* and luc2 (0.973 mg plant^{-1}) however, there was no significant improvement in RDW by co-inoculation in respect to the inoculation with *Rhizobium* alone.

The shoot P content was highly affected by co-inoculation with LG strain which showed phosphate solubilizing activity *in vitro*. Inoculation with other efficient phosphate solubilizing strain luc2 also increased the percentage of shoot P but not as much as LG strain and without positive effect on SDW increase. The P content in shoot was also somewhat elevated by Bx strain although its P solubilizing activity was not detected in this study. Significant correlation between P and total N content in the shoot was noted.

Table 4. Correlation (r) between parameters of nitrogen fixation.

	Shoot DW	Root DW	Nodule number	Nodule DW	Shoot total N	Shoot P	Shoot length	Root length
Shoot DW	1.000							
Root DW	0.662*	1.000						
Nodule number	0.383	0.337	1.000					
Nodule DW	0.407	0.437	0.989***	1.000				
Shoot total N	0.929***	0.512	0.353	0.350	1.000			
Shoot P	0.902***	0.519	0.095	0.124	0.771**	1.000		
Shoot length	-0.331	0.040	-0.316	-0.229	-0.431	-0.207	1.000	
Root length	0.063	0.241	-0.532	-0.487	-0.124	0.396	0.054	1.000

*P < 0.05 (significant), ** P < 0.01 (highly significant), *** P < 0.001 (extremely significant).

The use of plant growth-promoting bacteria to increase the soil fertility and improve growth and yield of agronomical important crops is a significant alternative to chemical fertilizers in sustainable agriculture. Strains of the *Pseudomonas* and *Bacillus* genera are the most well-known PGPB, which when co-inoculated with *Rhizobium* can improve growth of different legumes, including bean [22, 23, 24, 25].

In the present work, the results showed improved common bean growth, nitrogen fixation and phosphorous uptake when co-inoculated with *Pseudomonas* sp. LG, as well as *Bacillus* sp. Bx in respect to the inoculation with *Rhizobium* alone. The highest shoot dry weight as the main indicator of nitrogen fixing ability and growth promotion [26, 27] was detected in plants co-inoculated with *Rhizobium* and LG strain. The SDW increase of 29.54% over *Rhizobium* control was statistically significant, compared to *Rhizobium* inoculation alone, indicating considerable plant growth-promoting effect of LG strain.

Most of the tested PGPB strains are isolated from rhizosphere and represent PGPR. However, some endophytic bacteria also have the potential of plant growth promotion [28, 29, 30]. Moreover, *Pseudomonas* strains were often isolated from root nodules of leguminous plants and some of them showed plant growth potential [29, 31, 32], which was the case with LG strain in our research.

Various direct and indirect mechanisms of plant growth promotion have been associated with *Pseudomonas* and *Bacillus* strains capable of enhancing plant growth. In the present study, *Pseudomonas* sp. LG showed multiple plant growth-promoting traits including

IAA production, phosphate solubilization, ammonia production and siderophores production, as opposed to strain Bx, which only showed ammonia production.

The production of IAA by PGPB was often directly associated with their potential to stimulate plant growth. The endogenous IAA level in plant regulates growth of shoots and roots, and in the case of legumes formation of nodules. Combined inoculations with *Rhizobium* and root colonizing *Pseudomonas* spp. producing IAA improved growth and symbiotic performance of fodder galega [5]. On the other hand, bacterial IAA biosynthesis alone cannot explain improved plant growth [33]. In our research, SNji strain expressing IAA production did not show plant growth-promoting potential in common bean.

Phosphate solubilizing bacteria improve the P supply in plants as a consequence of their capacity for P solubilization, and in that way contribute to plant nutrition fundamentally, and improve plant growth [34]. *Pseudomonas* and *Bacillus* strains have been described as effective phosphate solubilizers [34]. Significant potential of LG strain to solubilize phosphates *in vitro* could play a role in improved plant nutrient supply and increase SDW. Besides the highest SDW, plants co-inoculated with LG strains and *Rhizobium* showed the highest shoot P content as well; considerably higher than in *Rhizobium* control. In addition, strain luc2 capable of phosphate solubilization under *in vitro* conditions, slightly increased P content in plants, however did not increase shoot weight. In this way previous findings have been confirmed: the abilities of bacteria to solubilize P *in vitro* and promote plant growth are not necessarily associated with each other and such plant growth-promoting potential should be confirmed by plant inoculation [6].

Siderophore production by PGPB plays a vital role in Fe nutrition of plants and therefore in plant growth promotion. Inoculation of chickpea (*Cicer arietinum* L.) and soybean seed with a siderophore-producing fluorescent *Pseudomonas* resulted in increased seed germination growth, and plant yield [35]. In our study, both *Pseudomonas* strains showed siderophore production, while only LG strain promoted plant growth, as in the case of phosphate solubilization.

On the other hand, the absence of any of the plant growth-promoting traits *in vitro* does not guarantee that a particular isolate is not PGPB [33]. This is the case with Bx strain which despite the lack of plant growth-promoting traits *in vitro* testing (except ammonia production) showed potential of improving bean growth. It is possible that improved bean growth induced by Bx strain is the results of enhanced N fixation, since somewhat enhanced nodulation and high N% in plant shoot were detected [36, 37]. There may be some other PGPB trait(s) this strain was not tested for.

Increased nodulation is also connected with plant growth promotion [36, 37]. In our study, co-inoculation with *B. megaterium* SNji promoted bean nodulation very efficiently, but without effect on SDW increase or significant nitrogen accumulation. Similar effect was also noted when alfalfa was inoculated with SNji strain [21], as well as in red clover inoculated with some *Bacillus* strains [38]. Some previous researches suggested that the nodule number was not an appropriate trait for selection of the most effective N fixing rhizobium-legume associations and the selection of such symbiotic associations should be done on the basis of shoot and root dry weights [26].

In this study we used non-sterile soil due to the fact that in many researches PGPR showed plant growth promotion only under gnotobiotic conditions [33, 39, 40], where these bacteria do not compete with the normal array of soil microorganisms. However, besides the presence of other microorganisms, additional different environmental factors may affect performances of PGPB and their interaction with the plant as well. Therefore, it is important to examine the way PGPB exert their effects on plants under field conditions. Consequently, LG and Bx strains expressing plant growth-promoting ability in this study should be tested further with the aim of confirming the good results under field condition.

Conclusion

The present study demonstrated a significant positive effect of co-inoculation with *Pseudomonas* sp. LG and *R. phaseoli* on growth, N and P contents of common bean plants compared to inoculation with *Rhizobium* alone. These data suggest that *Pseudomonas* sp. LG strain can be used in further investigations as a potential agent of new biofertilizer for improved bean production.

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