

Full Length Research Paper

Effects of plant growth promoting rhizobacteria on maize in greenhouse and field trial

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The aim of the study was to investigate ability of *Pseudomonas* sp. Q4b, *Bacillus* sp. Q5a and *Azotobacter chroococcum* strain 8 to enrich a population of plant growth promoting rhizobacteria (PGPR) in rhizosphere and thus enhance maize growth (*Zea mays* L.). Experiments were carried out under greenhouse and field conditions, on a chernozem soil. *In vitro* screening for PGP properties showed significant difference between indole-3-acetic acid (IAA) and siderophores production and phosphosolubilization between *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a. *Pseudomonas* was estimated as a better producer of hydrogen cyanide (HCN) and siderophores than *Bacillus*. Bacterial treatments had a significant effect on the number of investigated microbial groups in rhizosphere, and maize growth and yield. Co-inoculation had an advantage compared to single inoculation, while in case of single strains, better effects were achieved in *Pseudomonas* and *Azotobacter* treatments. Inoculation increased the number of azotobacters (103.25%), pseudomonads (82.29%) and aerobic spore-forming bacteria (52.65%) as well as height (17.15%) and dry weight (35.48%) of maize plants. The highest plant height was recorded in treatment with *Pseudomonas* and *Bacillus*. The highest dry weight and yield were recorded in the treatment with mixture of these three strains.

Key words: Maize, inoculation, plant growth promoting rhizobacteria, yield.

INTRODUCTION

Maize (*Zea mays* L.) is an important crop in temperate and semi-arid regions and one of the three major food staple crops for the world's population. In Serbia, maize is grown on about 1 200 000 ha of soils per year and results of yield testing on numerous locations show its high genetic potential (Jocković et al., 2010). Beside the genetic potential, achieving higher yields also require appropriate application of agro-technical measures, especially fertilization. After nitrogen (N), phosphorus (P) is the most limiting nutrient for crop yields, and is essential for maize growth and development (Wu et al., 2005). In order to increase the natural fertility of soils and develop new approaches to reduce the need for chemical fertilizers and pesticides the rhizosphere microorganisms

especially some rhizobacteria called plant growth promoting rhizobacteria (PGPR) are recognized as an important factor in sustainable agricultural production (Mrkovački and Bjelić, 2011).

PGPR may be important for plant nutrition by increasing N and P uptake by plants, and playing a significant role in the biofertilization of crops (Cakmakci et al., 2005). Maize stimulates different N₂-fixers in its rhizosphere and the most abundant diazotrophs belonging to Enterobacteriaceae and Azotobacteraceae families. Numerous studies have shown that inoculation of maize plants with PGPR strains caused significant increase in plant height, plant dry weight, root length and weight, yield, leaf area, and plant nutrient uptake of N, P, K, Fe, Zn, Mn and Cu (Biari et al., 2008; Sachin, 2009; Yazdani et al., 2009).

Among the different bacterial genera that have been reported as PGPR (*Azospirillum*, *Agrobacterium*, *Rhizobium*, *Enterobacter*, *Beijerinckia*, *Klebsiella*, *Xanthomonas*, *Phyllobacterium*) (Lucy et al., 2004),

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Pseudomonas, *Bacillus* and *Azotobacter* are the most widely reported (Mrkovački and Milić, 2001; Joo et al., 2004; Poonguzhali et al., 2008; Đurić et al., 2011). Growth promotion and disease control by these bacteria are complex interrelated processes involving mechanisms that include synthesis of some phytohormones (auxins, cytokinins and gibberellins), production of siderophores, antibiotics, hydrogen cyanide (HCN), and volatile compounds. Indirect mechanisms include phosphosolubilization, competition and induced systemic resistance (Lugtenberg et al., 2002).

Effectiveness of PGPR inoculations on plant growth enhancement and crop yields depends upon its ability to survive and multiply in soils and is influenced by many abiotic and biotic factors including texture, pH, temperature, moisture content, soil type, soil amendment, nutritional status of the plant, plant species, plant age, microbial competition and predation (Van Veen et al., 1997; Marschner et al., 2004).

The aim of this study was to examine the effect of maize seed inoculation with *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains mixed with well known *Azotobacter* biofertilizer strain, applied in single or in co-inoculation, on microbial population in rhizosphere and maize growth and yield. In addition, *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains were tested for their plant growth-promoting properties.

MATERIALS AND METHODS

Bacterial strains

PGPR were isolated from maize rhizosphere growing on poor pseudogley soil in central Serbia (Progoreoci, Aranđelovac). Selection of isolates was performed on the basis of the PGPR traits. Identification of selected isolates (Q4b as *Pseudomonas* sp. and Q5a as *Bacillus* sp.) were done using biochemical analysis and 16S rDNA partial sequences.

Bacterial isolates - *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a were grown on yeast manitol agar (YMA) supplemented with 6% NaCl for salt tolerance test or nutrient agar (NA) medium for various pH (from 4.5 to 9.5) adjusted with 1 M HCl or 1 M NaOH for pH tolerance.

In vitro screening for plant growth-promoting properties

Phosphate solubilization

Spot inoculations on Pikovskaya agar with 0.5% tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$] were applied for solubilization assay (Pikovskaya, 1948). Appearing of clear zones were in positive correlation with phosphate solubilizing ability. Diameters of clear zones around the colonies after 5 days of incubation at 28°C were measured.

Siderophores production

Schwyn and Neilands (1987) chrome azurol S (CAS) assay was used for estimation of siderophore production. For CAS assay in solid medium, plates were spot inoculated with 10 μl of $\sim 5 \times 10^6$ CFU

mL^{-1} on the CAS medium supplemented with 1/10 Nutrient Agar. The intensity of siderophores production was measured by zone of color change (green-blue to orange) after 5 days of incubation at 28°C.

Hydrocyanic acid (HCN) production

HCN production was tested on King B medium supplemented with glycine (4.4 g L^{-1}) (Ayyadurai et al., 2007). Filter papers were impregnated with 0.5% picric acid and 2% sodium carbonate, placed on the lid of inverted plates and sealed with parafilm. Inverted plates were incubated during 4 days at 28°C and production of cyanide was detected by discoloration of yellow filter paper to orange or brown.

Indole acetic acid (IAA) production

For quantitative analysis of IAA production, a 10 μl of bacterial suspension (standardized to OD_{600} of 0.625) were inoculated in liquid NA medium supplemented without and with 5 mM of tryptophan (Glickman and Dessaux, 1995) and incubated 36h at standard temperature. Salkowski reagent (2% 0.5 M ferric chloride in 35% perchloric acid) was mixed with the supernatant (2:1 v/v) and intensity of developed color was measured at 530 nm for IAA production.

Greenhouse experiment

Experiment was carried out in pots under greenhouse conditions, using non-sterile chernozem soil. Soil chemical analyses were done before sowing. Ten seeds were sown at 4 to 5 cm depth of chernozem soil in each Mitscherlich pot, in three replications. The soil used had the following characteristics: pH (in H_2O) - 8.49; CaCO_3 - 5.04%; humus content 2.05%; available P and K contents - 12.8 and 17.3 mg 100 g^{-1} soil, respectively.

Field experiment

Experiment was conducted at the Rimski Šančevi Experiment Field of Institute of Field and Vegetable Crops in Novi Sad. The field is located on a calcareous chernozem on loess terrace. The chemical soil properties were: pH (in H_2O) - 7.43; CaCO_3 - 5.46%; humus content - 2.47%; available P and K contents - 62.22 and 32.82 mg 100 g^{-1} soil, respectively. The experimental design was a randomized, complete block with four replications. The length of the experimental unit was 5 m, the width was 3 m. The sowing was done in the spacing 75 x 22 cm.

Bacterial treatments

Seeds of maize hybrid NS 6010 developed at the Institute of Field and Vegetable Crops were inoculated with three bacterial strains: *Azotobacter chroococcum* strain 8 from the collection of the Institute of Field and Vegetable Crops in Novi Sad (NSCNFB, as registered in WDCM), *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a. *Azotobacter* was grown in Fiodor medium, *Bacillus* in the NA medium and *Pseudomonas* in King B medium, in liquid culture $\sim 6 \times 10^8$ CFU mL^{-1} . Different treatments were tested: 1. *Azotobacter*; 2. *Bacillus*; 3. *Pseudomonas*; 4. *Azotobacter* + *Bacillus*; 5. *Azotobacter* + *Pseudomonas*; 6. *Bacillus* + *Pseudomonas*; 7. *Azotobacter* + *Bacillus* + *Pseudomonas* (in a ratio 1:1:1). Maize seeds were soaked in 0.2% HgCl_2 and 70% alcohol for 3 to 5 min and washed with water several times. Seeds were treated with 1 ml

Table 1. Environmental adaptations and plant growth promoting properties of applied *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains.

Isolates	<i>Pseudomonas</i> sp. Q4b	<i>Bacillus</i> sp. Q5a
Halo tolerance ability (6% NaCl in YMA)	+ ^a	±
Alkali tolerance ability (pH 9.5 of NA)	+	±
Acid tolerance ability (pH 4.5 of NA)	+	±
Phosphate solubilization - zones (mm/5 day)	10.67 ± 1.34 ^b	4.6 ± 0.87
Siderophores production - zones (mm/5 day)	7.67 ± 0.44	4.8 ± 0.12
HCN production	+	±
IAA production (µg mL ⁻¹)	NB without triptophane supplemented	7.92 ± 0.17
	NB supplemented with 5 mM triptophane	20.97 ± 0.25
		0.88 ± 0.14
		7.75 ± 0.36

^a, (+) bacterial growth; (±) poor bacterial growth; (-) no growth; ^b, means and standard deviations values.

of inoculum. No treated seeds were designed as control.

Effect on microbial population in maize rhizosphere

The rhizosphere soil for microbiological analysis was sampled at the three- and six-leaf stage of maize. The total microbial count was done in soil agar (dilution 10⁷), the fungi in potato dextrose agar (dilution 10⁴), the pseudomonads in King B medium (dilution 10⁶), the aerobic spore-forming bacteria and non-spore forming bacteria in NA medium (dilution 10⁶), and the number of azotobacters in Fiodorov medium (dilution 10²) (Jarak and Đurić, 2004). All microbiological analyses were performed in three replications and the average number of microorganisms was calculated at 1.0 g absolutely dry soil.

Effect on maize growth

Maize plants were harvested at the three- and six-leaf stage and carefully separated from soil. Plant height (cm plant⁻¹) and dry weight of plant (g plant⁻¹) of each plant were recorded and the average per plant was calculated. Dry weight was recorded after drying in an oven at 50°C to constant weight.

Data analysis

Data were analyzed by analysis of variance (ANOVA); LSD and Duncan test were used to separate treatment means when there was a significant difference at the P < 0.05 level. Time of sampling was considered a random effect and treatments was a fixed effect. All analyses were conducted using the statistical software package STATISTICA 10.0 (StatSoft Inc. USA) (Mead et al., 1996).

RESULTS AND DISCUSSION

Plant growth promoting properties of *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains

Soil properties have a strong impact on a range of processes influencing crop yield, including microbial diversity. According to Rengel and Marschner (2005), chemical changes in the rhizosphere result in altered

abundance and composition of microbial communities. Adaptations of bacteria to different soil properties and a broad range of pH and salinity may lead to better competition and survivals. Investigated bacteria in our work expressed good potential for adaptation. Adaptations to high salt concentration and pH values ranged from 4.5 to 9.5, and plant growth promoting properties of *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains are shown in Table 1.

Investigated bacteria in our work expressed good potential for adaptation. *Pseudomonas* sp. strain Q4b showed better tolerance abilities to high (9.5) and low (4.5) pH value and to high salt concentration than *Bacillus* sp. Q5a strain.

PGPR use one or more mechanisms to improve the growth and health of plants and can be active simultaneously or independently at different stages of plant growth. Among these, phosphosolubilization, improvement of other plant nutrients uptake, and phytohormone production (indole-3-acetic acid) are some of the regulators that influence plant growth (Zaidi et al., 2009). Production of IAA in liquid nutrient medium without triptophane added were observed for *Pseudomonas* sp. Q4b in the same range as for *Bacillus* sp. Q5b in the same medium supplemented with 5 mM of triptophane. Substantial amount of indole-3-acetic acid were produced by Q4b strains in the presence of triptophane – 20 µg mL⁻¹.

Large proportion of phosphorus in soil is insoluble and therefore unavailable to plants (Singh and Kapoor, 1994). Plant rhizospheric bacteria *Bacillus* and *Pseudomonas* are able to solubilize phosphates *in vitro* and most of them act as PGPR (Joo et al., 2004; Poonguzhali et al., 2008). Investigated strains Q4b and Q5a showed good phosphosolubilization ability -4.6 to 10.67 mm after 5 days of cultivation on Pikovskaya agar plates supplemented with calcium phosphate.

Rhizospheric bacteria act as biological agents through the production of antibiotics, lytic enzymes, hydrogen cyanide, and siderophores or through competition for

Table 2. Effect of PGPR on total microbial number, number of azotobacters and pseudomonads in maize rhizosphere (g soil⁻¹).

Treatment	Investigated microbial groups		
	Total microbial number ($\times 10^7$)	Azotobacters ($\times 10^2$)	Pseudomonads ($\times 10^6$)
<i>Azotobacter</i>	255.43	191.69*	79.43
<i>Bacillus</i>	358.81	183.63*	70.42
<i>Pseudomonas</i>	304.02	68.76	130.82*
<i>Azotobacter</i> + <i>Bacillus</i>	458.06	258.05*	110.23
<i>Azotobacter</i> + <i>Pseudomonas</i>	773.61*	244.11*	363.63*
<i>Bacillus</i> + <i>Pseudomonas</i>	455.71	254.41*	131.03*
<i>Azotobacter</i> + <i>Bacillus</i> + <i>Pseudomonas</i>	567.92	280.89*	105.04
Average	453.37	211.65*	141.51*
Non inoculated control	500.52	104.13	77.63
Increase (%)	-9.42	+103.25	+82.29
LSD 5%	170.3	40.7	34.3

*p < 0.05 (significant).

Table 3. Effect of PGPR on number of fungi, spore-forming and non-spore forming bacteria in maize rhizosphere (g soil⁻¹).

Treatment	Investigated microbial groups		
	Fungi ($\times 10^4$)	Spore-forming bacteria ($\times 10^6$)	Non-spore forming bacteria ($\times 10^6$)
<i>Azotobacter</i>	27.12	192.93	324.75
<i>Bacillus</i>	56.78*	657.63*	899.47
<i>Pseudomonas</i>	52.97*	642.97*	502.42
<i>Azotobacter</i> + <i>Bacillus</i>	47.60	200.58	952.77
<i>Azotobacter</i> + <i>Pseudomonas</i>	45.45	384.69	1314.45*
<i>Bacillus</i> + <i>Pseudomonas</i>	31.37	289.29	374.35
<i>Azotobacter</i> + <i>Bacillus</i> + <i>Pseudomonas</i>	35.73	761.35*	826.86
Average	42.43	447.06	742.15
Non-inoculated control	41.31	292.87	875.92
Increase (%)	+2.71	+52.65	-15.27
LSD 5%	9.85	277.0	183.1

*p < 0.05 (significant).

nutrients and space. Biological control of plant pathogens and deleterious microbes can improve significantly plant health, as evidenced by increases in seedling emergence, vigor and yield (Antoun and Kloepper, 2001). *Pseudomonas* sp. Q4b was estimated as better producer of HCN and siderophores than *Bacillus* sp. Q5a strain and assumed to be better in promoting maize growth.

Microbial population in maize rhizosphere

Rhizosphere is a dynamic system controlled by the combined effects of soil properties, plants characteristics and the interactions of plant roots with microorganisms and the surrounding soil (Bowen and Rovira, 1999). High concentration of easily degradable substrates in root exudates leads to a proliferation of microorganisms in

rhizosphere (Bais et al., 2006) and thus plant create its specific microflora that may have neutral, deleterious or beneficial effects on the plant. Greenhouse experiment was established on a chernozem soil that provides optimum conditions for microbiological activity (Đurić et al., 2008). Quantitative difference between the total number of microorganisms and their enzymatic activity in soil is a reliable indicator of its potential and effective fertility (Govedarica et al., 1992). Pot experiment showed a significant effect on the number of investigated microbial groups that varied depending on the applied strains (Tables 2 and 3).

Inoculation increased the number of azotobacters (103.25%), pseudomonads (82.29%) and aerobic spore-forming bacteria (52.65%), while the total microbial number and the number of aerobic non-spore forming bacteria was decreased by 9.42 and 15.27%, in relation

Table 4. Effect of PGPR on maize growth.

Treatment	Tested growth parameters	
	Plant height (cm plant ⁻¹)	Dry weight (g plant ⁻¹)
<i>Azotobacter</i>	27.85	0.42*
<i>Bacillus</i>	27.91	0.37*
<i>Pseudomonas</i>	28.74*	0.38*
<i>Azotobacter</i> + <i>Bacillus</i>	28.30*	0.41*
<i>Azotobacter</i> + <i>Pseudomonas</i>	28.48*	0.43*
<i>Bacillus</i> + <i>Pseudomonas</i>	29.49*	0.45*
<i>Azotobacter</i> + <i>Bacillus</i> + <i>Pseudomonas</i>	27.68	0.50*
Average	28.35	0.42
Non-inoculated control	24.20	0.31
Increase (%)	+17.15	+35.48
LSD 5%	3.71	0.02

*p < 0.05 (significant).

to control. The best effects on the number of investigated microbial groups were achieved in the treatment with strains applied in mixture which caused a proliferation of total microbial number (567.92×10^7), number of azotobacters (280.89×10^2), pseudomonads (105.04×10^6) and aerobic spore-forming bacteria (105.04×10^6). Some previous researches suggested that the presence of microorganisms in the maize rhizosphere varies and that total number of microorganisms and number of azotobacters increases in the course of growing season (Govedarica et al., 1999; Bjelić et al., 2010). Balašević-Tubić et al. (2011) reported that abundance of microorganisms in the rhizosphere, as well as soybean yield using microbiological fertilizer was stimulated by plowing under crop residues. Picard et al. (2008) observed that PGPR were earlier stimulated in the rhizosphere of a hybrid maize than in those of its two parental lines and that each maize genotype stimulated the AMF (Arbuscular Mycorrhizal Fungi) population differently.

Azotobacter and *Pseudomonas* co-inoculation led to a significant increase in total microbial number (773.61×10^7), number of azotobacters (244.11×10^2), pseudomonads (363.63×10^6) and aerobic non-spore forming bacteria (1314.45×10^6) compared to control. Jarak et al. (2005) showed that number of azotobacters in the first ten days decreases from 10^8 to 10^2 cells, but after twenty days increases to more than 10^3 which suggest that this soil provides favorable conditions for bacteria that require a well-controlled water-air regime, enough easily degradable substances and neutral reaction.

The maximum number of investigated microorganisms was recorded when *Azotobacter* co-inoculated with *Pseudomonas*, subsequently with *Bacillus*, while the least effect was detected when *Bacillus* co-inoculated with *Pseudomonas*, regarding the effect of two strains

together. Observed by individual strains best effects were obtained in the *Pseudomonas* treatment, superior in relation to *Bacillus* or *Azotobacter* treatment. Inoculation with *Pseudomonas* led to an increase in the number of pseudomonads (130.82×10^6), spore-forming bacteria (642.97×10^6) and fungi (52.97×10^4). *Bacillus* caused a decrease in the number of azotobacters, pseudomonads and total microbial number compared to control, while good effect was recorded on the number of spore-forming, non-spore forming bacteria and fungi. Treatment with *Azotobacter* significantly increased the number of azotobacters (191.69×10^2), while the effect on the other microbial groups was better when *Azotobacter* is combined with *Bacillus* or *Pseudomonas* and in treatment with all three strains. Plant growth promotion is crucial in early developmental stages such as germination and seedling growth (Gholami et al., 2009). Establishment of a significant number of PGPRs in rhizosphere and plant tissues leads to an increase in biomass (even grain production) in later developmental stages (Bashan et al., 2004).

Maize growth

The applied PGPR strains significantly affected the height and dry weight of maize plants (Table 4). Responses of greenhouse-grown maize to PGPR inoculation also depended on applied strains that had similar effects as on microbial population in maize rhizosphere.

Results reveal that mentioned growth parameters increased in PGPR treated plants over non-inoculated control and that increase was significant for most of strains. The highest plant height (29.49 cm plant⁻¹) was recorded in treatment with *Pseudomonas* and *Bacillus*. The highest dry weight (0.50 g plant⁻¹) was recorded in the treatment with mixture of all three strains. In

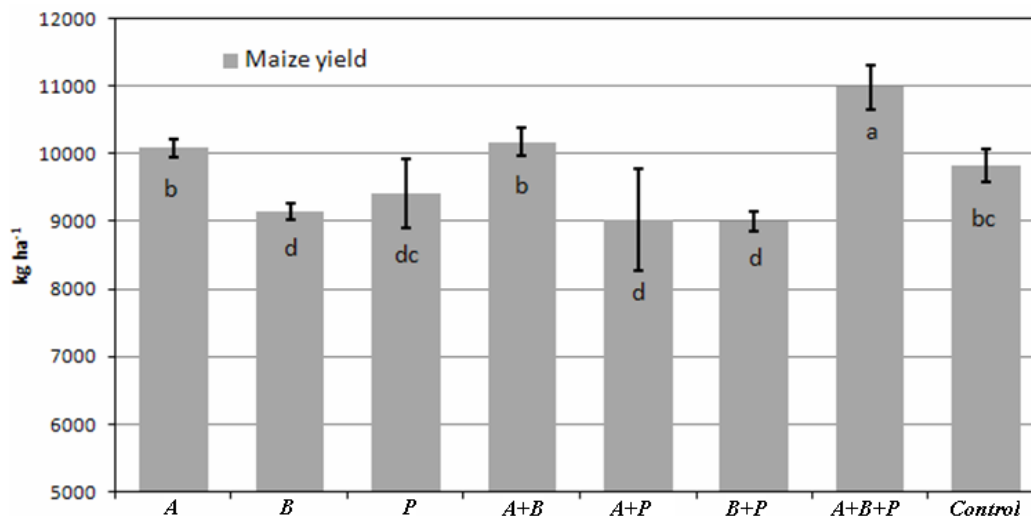


Figure 1. Effect of PGPR on maize yield (A: *Azotobacter*; B: *Bacillus*; P: *Pseudomonas*; A+B: *Azotobacter* + *Bacillus*; A+P: *Azotobacter* + *Pseudomonas*; B+P: *Bacillus* + *Pseudomonas*; A+B+P: *Azotobacter* + *Bacillus* + *Pseudomonas*).

comparison to single strains, combination of two or all three strains showed better results. In case of single strains, the best effect on plant height (28.74 cm plant⁻¹) was achieved in treatment with *Pseudomonas* and in treatment with *Azotobacter* for dry weight of plant (0.42 g plant⁻¹). Similarly, Stajković et al. (2011) showed that *Rhizobium* co-inoculation with *Pseudomonas* or *Bacillus* strains improved shoot dry weight, nitrogen and phosphorus contents in bean plants, compared to inoculation with *Rhizobium* alone, whereby *Pseudomonas* promoted bean growth and particularly P uptake more efficiently than *Bacillus*. Cassan et al. (2009) showed that *Azospirillum brasilense* and *Bradyrhizobium japonicum*, inoculated singly or in combination, promote seed germination and early seedling growth in soybean and maize, while root and shoot dry weight for co-inoculation was higher than for control or single-inoculation treatments. These results are in agreement with our assumptions that *Pseudomonas* will be better in maize growth promotion. Considering all PGP parameters tested in this study, there were significant difference between IAA and siderophores production and phospho-solubilization between *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains. Greenhouse experiment confirmed growth promotion by representative *Azotobacter* strain, as well as the mixture of all three strains. However, greenhouse investigation showed little variations in bacterial effectiveness among *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains applied as single inoculum, as well as among all treatments, although the difference compared to non-inoculated control was significant. Based on the results, strains used in this study can be recommended as maize growth promoting rhizobacteria.

Maize yield

In the combined analysis of variance over treatments the effects of microbes showed significant F-test ($P < 0.05$) among selected treatments (Figure 1).

The higher maize yield was observed after application of three strains indicating that favorable soil condition was achieved. Though, control plot had higher yield possibly as a result of imbalance in microbial population in those treatments with single application that resulted with immobilization of some nutrients or suppression to other microorganisms. Also, our research indicates that application of *Azotobacter* could significantly increase maize yield compared with other two strains. The lowest yield was found at plots where both *Pseudomonas* sp. Q4b + *Bacillus* sp. Q5a were applied. Standard deviation represented with error bars showed higher variation in *Azotobacter* + *Bacillus* treatment that could be explained with uneven performance of applied strains across replication. Govedarica et al. (2001) found enrichment in investigated microbial population and yield increase for both tested hybrids and strains applied (single and co-inoculation with *A. chroococcum* and *Bacillus megatherium*). Similar results - an increase of microbial count and maize yield in response to *A. chroococcum*, *Azotobacter vinelandii*, *Azospirillum lipoferum*, *B. megatherium*, *Bacillus subtilis* and *Klebsiella planticola* application - were obtained by Cvijanović et al. (2007).

Conclusion

This study confirmed plant growth ability of *Pseudomonas* sp. Q4b and *Bacillus* sp. Q5a strains.

Investigated strains mixed with well known *Azotobacter* biofertilizer strain had significant impact on microbial population in rhizosphere and maize growth and yield, suggesting that can be applied as biofertilizers for improved maize production. The best results were achieved in the treatments with strains applied in mixture indicating that co-inoculation had an advantage over single inoculation. Further greenhouse and field studies should provide more definitive information about the movement and uptake of macroelements (N and P) to plants with the impacts of PGPR-based inoculants.

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