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A COMPARATIVE ANALYSIS OF PLANT GROWTH-PROMOTING TRAITS OF *Pseudomonas* AND *Bacillus* STRAINS ISOLATED FROM *Lolium perenne* RHIZOSPHERIC SOIL IN VOJVODINA (SERBIA) AND THEIR EFFECT ON THE PLANT YIELD

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ABSTRACT

The objective of this work was to do a comparative study of *Pseudomonas* and *Bacillus* isolates for their plant growth-promoting (PGP) potential, monitoring the impact of selected isolates on the yield of English ryegrass (*Lolium perenne*). Isolation, physiological and biochemical characterization, in vitro assay of enzymatic and plant-growth promoting activities of isolates were done. *Pseudomonas* isolates have been shown to have the ability to use different sources of carbon, to live in the condition of low pH as well as temperature and to produce siderophore. On the other hand, *Bacillus* isolates have the ability to solubilize phosphate, to produce a greater amount of indol-3-acetic acid (IAA) than *Pseudomonas* isolates and have an inhibitory effect on the growth of phytopathogenic fungi. In other investigated traits, isolates were similar. The use of *Pseudomonas* sp. P12 and *Bacillus* sp. B1 isolates had a positive effect on the plant mass and total yield, which indicate that the use of these isolates can result in a better yield of forage crops.

Key words: antifungal activity, auxin, enzymatic activities, siderophores, grass

INTRODUCTION

During the last couple of decades, the use of plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture has increased in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported by many authors [Stamenov and Jarak 2012]. The PGPR activity of *Pseudomonas* and *Bacillus* strains has been known for many years [Spiers et al. 2000, Alina et al. 2015].

Among Gram-negative soil bacteria, *Pseudomonas* is the most abundant genus in the rhizosphere of many plants. Their plant growth-promoting activities include production of hydrogen cyanide (HCN), siderophores, protease, antimicrobial secondary metabolites, auxin and phosphate-solubilizing activity [Suresh et al. 2010]. All these metabolites strongly affect the environment, both because they inhibit the growth of other deleterious microorganisms and because they increase nutrient availability to the plant. The use of *Pseudomonas* sp. has a positive effect on the yield and growth of the chickpeas, on the fresh and dry mass of the sugar cane, the yield of legumes,

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wheat plants, potatoes and radish [Rokhzadi et al. 2008, Mehnaz et al. 2009].

Bacillus species demonstrated to have a wide spectrum of plant protection and growth promoting abilities [Alina et al. 2015]. There are a number of metabolites, such as auxins, cytokinins, gibberellins and different polyamines released by these strains, which strongly affect the parameters of plants growth. *Bacillus* spp. strains also could act as biological control agents (BCAs) to provide plant protection against microbial and insect pathogens by various mechanisms such as production of extracellular lytic enzymes, antagonism, nutrients and niche competition, quorum quenching and induced defense responses in host plants [Balasubramanian and Simões 2014]. *Bacillus* sp. has a positive effect on the yield of tomato and pepper, raspberry plant under organic growing conditions, cucumber, suggesting its potential use as fertilizer [Supanjani et al. 2006].

Growth promotion and disease control by *Pseudomonas* and *Bacillus* are complex interrelated processes. In these processes, there are some similarities and differences between *Pseudomonas* and *Bacillus* strains. Due to the concern about the harmful effects of mineral fertilizers and pesticides, there is a growing interest for a deeper understanding of cooperation between rhizospheric microorganisms and plants, especially plant species such as forage grasses which are grown over large areas. Therefore, the objective of this work was to do a comparative study of *Pseudomonas* and *Bacillus* isolates for their plant growth-promoting (PGP) potential monitoring the impact of selected isolates on the yield of English ryegrass (*Lolium perenne*).

MATERIALS AND METHODS

Investigations were carried out in 2015–2016 at Faculty of Agriculture in Novi Sad, Serbia.

Isolation of *Pseudomonas* and *Bacillus* strains. Microorganisms were isolated from rhizospheric soil of English ryegrass, from chernozem in Vojvodina (Latitude 45.246650, Longitude 20.586940), Serbia.

Isolates which were established as Gram-negative, rod-shaped cell and produce pigment fluorescein, were selected as *Pseudomonas* isolates. The fluorescein production was tested on Pseudomonas Flo agar (protease peptone 20.0 g l^{-1}, maltose 10.0 g l^{-1}, K_2HPO_4 1.5 g l^{-1}, $MgSO_4$ 0.73 g l^{-1}, glycerol 10 ml l^{-1}, 15 g l^{-1}). Isolates which were established as catalase-positive, Gram-positive, rod-shaped cells that form endospores, were chosen as *Bacillus* isolates. The presence of catalase was detected by releasing the oxygen bubbles in contact with diluted hydrogen peroxide solution.

Physiological and biochemical characterization of *Pseudomonas* and *Bacillus* isolaties. Utilization of carbon sources was determined by using Hugh-Leifson medium (peptone 2 g l^{-1}, K_2HPO_4 0.3 g l^{-1}, NaCl 5.0 g l^{-1}, 10 g l^{-1} of carbon sources (glucose, galactose, fructose, saccharose, lactose or xylose) Bromtimol blue 0.03 g l^{-1}, Agar 3.0 g l^{-1}) and by observing the change of colony colour from greenish to yellow, in the case of a positive reaction.

The *Pseudomonas* isolates inoculated on King-B and *Bacillus* isolates on nutrient agar were incubated at different temperatures (3, 13, 28, 37 and 45°C), pH levels (4, 5, 6, 7), and salt concentrations (3, 5 and 7%), for 4 days. The width of the colony was measured and compared with the control.

***In vitro* assay of enzymatic activities.** Ability to produce cellulase was tested on CMC agar (carboxymethyl cellulose agar). After incubation, bacterial cultures were overflowed with a solution of Congo-red (1 mg cm^{-3} H_2O). After 15 min, the Congo-red was decanted and the bacterial cultures were overflowed with 1 M NaCl. A discolored zone around the colony was proof of the cellulase activity.

Lipase activity was determined by growing the isolates on the medium with Tween 80 (peptone 10 g l^{-1}, NaCl 5 g l^{-1}, $CaCl_2 \cdot H_2O$ 0.1 g l^{-1}, Agar 15 g l^{-1}) and observing the presence and absence of a zone around the colony.

Urease activity was tested by using the Christensen's urea agar (urea 20 g l^{-1}, peptone 1.0 g l^{-1}, KH_2PO_4 2.0 g l^{-1}, glucose 1.0 g l^{-1}, NaCl 5.0 g l^{-1}, Agar 15.0 g l^{-1}, phenol red indicator 0.012 g l^{-1}). The pH of the media was adjusted to 6.8 ±0.2. Since urea is unstable, it was filtered through 0.2 µm filter and added separately. The appearance of the red color was proof of the urea decomposition.

Gelatin hydrolysis was detected using a nutrient gelatine stub method (meat extract peptone broth (MPB), 15% gelatin). The inoculated tubes and an uninoculated control tube were incubated at 25°C for 7 days. Degradation of gelatin indicates the presence of gelatinase enzyme [Aneja 2003].

Production of hydrogen sulfide was determined by the appearance of a change in color (from orange to black) of deep-peptone-iron agar (peptone 15.0 g l^{-1}, protease pepton 5.0 g l^{-1}, ammonium ferric citrate 0.5 g l^{-1}, sodium glycerophosphate 1.0 g l^{-1}, sodium thiosulfate 0,08 g l^{-1}, Agar 15.0 g l^{-1}).

Hydrolysis of starch was performed by flooding iodine on colonies grown on starch agar (starch 10.0 g l^{-1}, KH_2PO_4 0.5 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $MgSO_4 \times 7\ H_20$ 0.2 g l^{-1}, Agar 15.0 g l^{-1}), and by observing the presence or absence of a halo zone around the colony.

Plant-growth-promoting activities. Bacterial ability to produce the siderophores was assayed on chrome-azurol S (CAS) medium [Schwyn and Neilands 1987]. The medium discoloration from blue to orange indicated siderophores production.

Bacterial ability to solubilize sparingly soluble $Ca_3(PO_4)_2$ was assayed on Pikovskaya [1948] medium. Phosphate solubilization was verified by clear halo appearance around colonies.

To estimate HCN production, bacterial cultures were streaked on tryptic soy agar amended with 4.4 g l^{-1} glycine. A filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of each plate [Frey-Klett et al. 2005]. Plates were sealed with parafilm and incubated at 28°C. Development of color from yellow to light brown, moderate brown or strong brown indicated HCN production.

Investigation of indol-3-acetic acid (IAA) production by bacterial isolates was performed according to the method of Etesami et al. [2015]. Development of pink color was assayed with a spectrophotometer at 530 nm. The concentration of produced IAA was determined from a standard curve of IAA (1–50 µg ml^{-1}).

Relationship between the studied bacterial isolates and certain fungi (*Aspergillus niger*, *Aspergillus flavus*, *Sclerotinium sclerotiorum*) was investigated using the method by Toure et al. [2004]. The isolates inhibitory effect on the fungus can be expressed as a percentage according to the formula: I (%) = (C − T) / C × 100, where I is inhibition; C – length of mycelia in the control Petri dishes; T – length of mycelia in the experimental Petri dishes.

Evaluation of isolates for their PGP potential under semicontrolled conditions on English ryegrass. The experiment was set up following randomized block system. The size of the experimental plot was 5 m^2. Each variant was conducted in four repetitions. According to FAO classification, the experiment was conducted in chernozem soil. English ryegrass (*Lolium perenne*) was taken from the collection of Institute of Forage Crops, Kruševac, Serbia.

The variants of the experiment were the following: 1. isolate *Pseudomonas* sp. P12, 2. Isolate *Bacillus* sp. B1, and 3. control – no inoculation. Before sowing, 50 ml 10^8 CFU ml^{-1} of *Pseudomonas* sp. P12 as well as Bacillus sp. B1 was introduced into 5 l of tap water each, and then evenly sprayed on the plot surface. The sowing was performed manually with 20 kg of English ryegrass per ha. The following parameters were measured: a mass of 50 plants (fresh and dry), during the spring, autumn and winter; total yield of fresh and dry mass (t ha^{-1}).

Statistical analysis

The data were statistically processed using the Statistics 13 software (Hamburg). The significance of the difference between the applied treatments was determined using Fisher's LSD test.

RESULTS AND DISCUSSION

Isolation and characterization of bacterial isolates. From the rhizosphere of perennial ryegrass different bacteria strains of the genus *Pseudomonas* and *Bacillus* were isolated. Representative isolates of each group (denoted as *Pseudomonas* sp. P1, P9, P12, and for *Bacillus* sp. B1, B2 and B3) were examined for different physiological and biochemical properties.

The isolates varied in terms of utilization of carbon sources such as glucose, galactose, fructose, saccharose, lactose and xylose (Tab. 1). All the isolates developed well-grown colonies at 13, 28 and 37°C, with minimal growth at 45°C; however, only *Pseudomonas* isolates could grow at 3°C. The optimum pH for the growth of all isolates was 6 and 7, while only *Pseudomonas* isolates could grow at pH 4. All isolates had optimal growth on medium containing 3% and 5% NaCl. On medium containing 7% NaCl, minimum growth was observed for the isolate B6 and optimal growth for isolate P9 and B1.

The ability of *Pseudomonas* and *Bacillus* strains to grow at extreme pH values and temperatures and at high concentrations of NaCl is reported by Karagoz

Table 1. Growth of isolates in culture medium with a different source of carbon, at different temperatures, pH and NaCl levels

Isolates	Sugars						T (°C)					pH				NaCl (%)		
	G	Ga	F	S	L	K	3	13	28	37	45	4	5	6	7	3	5	7
P1	+	+	+	+	–	+	+	++	++	++	+	–	++	++	++	++	++	–
P12	+	+	+	+	–	+	+	++	++	++	+	–	++	++	++	++	++	–
P9	+	+	+	+	–	+	+	++	++	++	+	–	++	++	++	++	++	++
B1	+	–	+	–	–	+	–	++	++	++	+	–	–	++	++	++	++	++
B3	+	–	+	–	–	+	–	++	++	++	+	–	–	++	++	++	++	–
B6	+	–	+	–	–	–	–	++	++	++	+	–	–	++	++	++	++	+

G – glucose, Ga – galactose, F – fructose, S – saccharose, L – lactose, K – xylose; + minimal growth, ++ optimal growth, +++ intense growth

Table 2. Production of siderophores, phosphate utilization and enzymatic activity

Isolates	Cellulase[1]	Lipase[1]	Urease[1]	Gelatinase[1]	Protease[1]	Amylase[1]	Siderophore[2]	P[3]	HCN[4]
P1	–	+	+	–	–	–	+++	+	–
P12	+	+	+	+	–	–	++	–	–
P9	+	–	+	–	–	–	+++	–	–
B1	–	+	+	+	–	–	–	+	+
B3	–	+	+	+	–	+	–	++	+
B6	+	+	+	+	–	+	–	+	+

[1] – no hydrolysis, + hydrolysis; [2] width of orange zone: + 0–10 mm, ++ 5–20 mm, +++ ≥20 mm, – no zone; [3] phosphate solubilization: + represents 4 mm per day; ++ represents ≥5 mm day^{-1}; [4] + HCN production, – not detected

Table 3. Production of indole-3-acetic acid (IAA) by *Pseudomonas* and *Bacillus* isolates in the presence and absence of tryptophan

Isolates	Concentration of IAA (µg ml^{-1})					
	24 h			48 h		
	L tryptophan (µg ml^{-1})			L tryptophan (µg ml^{-1})		
	0	200	500	0	200	500
P1	1.93	3.18	3.25	7.06	7.68	11.00
P12	2.42	5.39	7.07	5.32	12.82	12.93
P9	2.61	4.96	6.61	4.89	5.11	8.07
B1	3.25	4.60	6.43	3.78	9.71	10.03
B3	12.07	19.75	20.53	16.93	26.89	31.71
B6	3.72	5.96	7.50	6.93	13.43	15.00

et al. [2012]. According to the same author, metabolic diversity allows these bacteria to adapt to adverse environmental conditions. The results of this work indicate the adaptability of these isolates, showing that they have a good ability to survive under different environmental conditions.

Enzymatic and plant-growth promoting activities. Enzymes play an important role in the promotion of plant growth and biological control of plant diseases. Microorganisms can act as biocontrol agents on cellulose cell wall bearing microorganisms such as *Phytophthora* and *Pythium*. Besides that, the cell walls of plant pathogens also contain lipids. Lysis of this by lipase- producing bacteria leads to leakage of cell content and the death of the pathogenic fungi [Beneduzi et al. 2012]. *Pseudomonas* isolates were found to be positive for production of urease, though not amylase and protease (Tab. 2). Isolates P12 and P9 showed the ability to produce cellulase, isolates P1 and P12 lipase, while only isolate P12 produced gelatinase. All *Bacillus* isolates showed ability to produce lipase, urease, gelatinase but not protease. Isolate B3 and B6 produced amylase, while isolate B6 produced cellulase. The results of this work indicate that these isolates, especially isolates P12 and B6, having these traits can be exploited for biological control of plant pathogens, which indirectly promotes plant growth.

An important trait of PGPR, that may influence the plant growth indirectly, is the production of siderophores [Shameer and Prasad, 2018]. The amount of soluble Fe in the soil is much less than the total Fe contents. Thus, iron remains unavailable for crops even in iron-rich soils and contributes little in crop production. A large number of soil bacteria produce siderophores that bind Fe^{3+} and help in iron uptake because crops can absorb bacterial Fe^{3+} siderophore complexes. Furthermore, through this process, iron is made unavailable in the natural habitat of phytopathogens [Ramesh et al. 2009]. Pseudomonads are leading siderophore producers among PGPR [Suresh et al. 2010]. In our study, *Pseudomonas* isolates had the ability to produce siderophores, while *Bacillus* isolates did not have. Two of *Pseudomonas* isolates, P1 and P9, showed strong activity in producing siderophores.

Phosphorus solubilizing bacteria transform compounds of phosphorus to forms that can easily be taken up by crops. Bacteria assimilate soluble phosphorus and make it available by preventing it from adsorption [Khan and Joergensen, 2009]. Bacteria also enhance phosphorus availability to crops by solubilizing precipitated forms of phosphorus. Various bacterial species in the genera *Bacillus* and *Pseudomonas* have proven to be the most powerful phosphate solubilizing bacteria [Suresh et al. 2010, Alina et al. 2015]. In this study, all *Bacillus* isolates solubilized phosphate, especially isolate B3, while only one *Pseudomonas* isolate (P1) solubilized phosphate.

Datta et al. [2011] reported that hydrogen cyanide production has a beneficial effect on plants. Three strains of *Bacillus* produced HCN as evidenced by the change in the color of filter paper from yellow to moderate and reddish brown. Production of HCN by *Pseudomonas* isolates was not detected. Our results are in agreement with the results of Alina et al. [2015].

Another PGP feature that may play an important role in biocontrol is production of phytohormones. Among PGPRs, auxins, gibberellins, cytokinins, abscisic acid and ethylene are well documented. The most common and active auxin is indole-3-acetic acid (IAA), which directly influence plant growth and development. This phytohormone plays a very important role in the stimulation of root elongation and proliferation of root hairs and lateral roots [Davies, 2010] and it has been shown to have a stabilizing effect under unfavorable environmental conditions [Bianco et al. 2009]. In this study, all selected isolates had the ability to produce IAA without tryptophan supplementation (Tab. 3). Also, all isolates were able to produce IAA from tryptophan added to the culture medium, which is in agreement with numerous studies that demonstrated that IAA is the common product of tryptophan metabolism for several rhizobacteria [Ahmad et al. 2012]. Higher amounts of IAA were measured for all isolates in media with tryptophan amendment after 24 and 48 h of incubation than without tryptophan. The amount of IAA was grown with time, thus higher values of IAA were measured after 48 h in comparison to the values after 24 h of the experiment. The isolate B3 produced the greatest amount of IAA in comparison with the other isolates.

PGPR indirectly help in plant growth by suppression of deleterious microorganisms that inhibit plant growth or root pathogens through antibiosis, parasit-

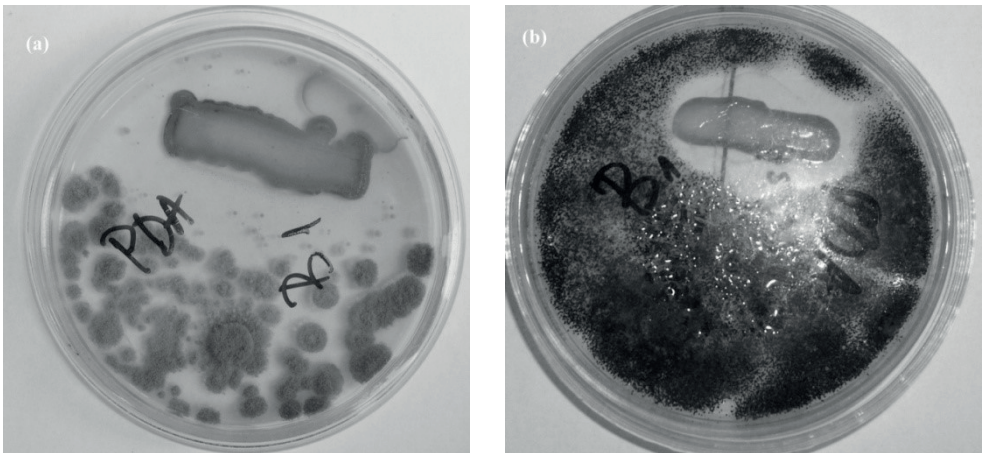

Fig. 1. Antagonistic activity of *Bacillus* isolate B1 against *Aspergillus flavus* (a) and *Aspergillus niger* (b). In both figures: bottom of Petri-dish, agar plug of *A. flavus* (a) and *A. niger* (b) with radial growth; top of Petri-dish, *Bacillus* isolate B1; clear zones around *Bacillus* isolate indicate inhibitory effect of bacterial isolate against mycelial growth of *A. flavus* (a) and *A. niger* (b)

ism, competition for nutrients and space within the vicinity of plant roots, and/or activation of host defense responses. The strains of *Bacillus subtilis* are the most widely used PGPR due to their disease reducing and antibiotic-producing capabilities [Alina et al. 2015]. Fluorescent pseudomonads are also known to suppress soil born fungal pathogens by producing antifungal metabolites and by sequestering iron in rhizosphere through the release of iron-chelating siderophores, rendering it unavailable to other organisms [Suresh et al. 2010]. In this study, it was found that the isolates did not have an inhibitory effect on the growth of fungi, in addition to a *Bacillus* isolate. Isolate B1 had an inhibiting effect on *Aspergillus flavus* (Fig. 1a) and *Aspergillus niger* (Fig. 1b). The growth of *A. flavus* was 52.8%, and the growth of *A. niger* was 60% lower in the experimental Petri dishes in relation to the growth of fungi in the control.

Several studies have demonstrated that production of siderophores, other secondary metabolites and lytic enzymes by microorganisms was the most effective in controlling the plant root pathogens including *A. niger*, *A. flavus* and *Rhizoctonia solani* [Hassanein et al. 2009, Kim et al. 2009, Mishra and Arora 2018]. However, the present of siderophores was not expected at this isolate (B1) because it was negative for siderophore production. The antifungal activity of the isolate B1 might be due to its ability to produce HCN. Further studies on the B1 isolate will uncover the right mechanism of fungal growth suppression.

Effect of selected *Pseudomonas* and *Bacillus* isolate on the growth and yield of English ryegrass. This research focused on examining the impact of two isolates *Pseudomonas* sp. P12 (which produce cellulase, lipase, urease, gelatinase, indole-3-acetic acid and siderophores) and *Bacillus* sp. B1. (which produce lipase, urease, gelatinase, HCN, indole-3-acetic acid and siderophores and decompose tricalcium phosphate) on the yield of English ryegrass.

During the first year, in comparison with the control, a statistically significant increase in the mass of 50 plants and total yield of fresh and dry plants was recorded in the variants with *Pseudomonas* and *Bacillus* isolates (Tab. 4). In average, the use of *Pseudomonas* sp. P12 had a better effect on the plant's growth and total yield than isolate *Bacillus* sp. B1. The highest total yield was recorded in a variant with *Pseudomonas* sp. P12 isolate and it was 24 t ha^{-1} of fresh and 11 t ha^{-1} of dry plant.

During the second year, a statistically significant increase in the mass and total yield of the plant was recorded in both variants. In average, a better effect

Table 4. The impact of isolates use on the mass of 50 plants (g) during the three seasons, and total yield of English ryegrass (t ha^{-1})

Period	I year			II year		
	control	P12	B1	control	P12	B1
	fresh plants			fresh plants		
Spring	12_b	15.3_a	13_b	22_b	22.5_b	24.7_a
Autumn	13.5_a	19.6_b	18.7_b	10c	18.5_b	29a
Winter	6.4_c	14_a	9.5_b	7_c	14_b	20_a
Total yield	$17,5_b$	24_a	23_a	16_b	25_a	23_a
	dry plants			dry plants		
Spring	4_a	7.5_b	6_b	7.1_a	10.1_b	10.5_b
Autumn	7.2_a	9.2_b	9.7_b	5_c	11_b	22_a
Winter	1.9_a	2_a	3_a	2.8_c	6_b	9.5_a
Total yield	8_c	11_a	10_{ab}	5_b	9_a	7_a

Mean values with the same subscript(-s) are not significantly different according to Fisher LSD test ($p < 0.05$); P12, B1 – isolates *Pseudomonas* sp. P12 and *Bacillus* sp. B1, respectively

on the measured parameters was achieved with the *Bacillus* sp. B1 isolate than with *Pseudomonas* isolate. However, the highest total yield was denoted in variant where *Pseudomonas* sp. P12 was introduced, but there was no significant difference in comparison with variant with *Bacillus* isolate. Plant growth promoting activities include production of siderophores, auxin, and phosphate solubilizing activity because they increase nutrient availability to the plant [Suresh 2010]. In this study, the ability of isolates to produce enzymes and materia that promote plant growth (such as auxin and siderophores) could be the explanation of the isolates positive effect on the parameters of plant growth. Comparing these two isolates, isolate P12 produce more IAA than B1, and being siderophore producing bacteria, while isolate B1 is not. Probably these two properties have made isolate P12 had a better effect on the growth and yield of the plant. Similarly to our results, numerous investigations into the influence of inoculation on growth of forage grasses, have proved positive effects of inoculation with microorganisms [Garcia et al. 2004, Rokhzadi et al. 2008, Stamenov et al. 2012]. The use of microbiological fertilizers as an addition to, or instead of chemical substances has special significance for plant species which are grown over large areas.

CONCLUSION

The isolates of the genus *Pseudomonas* P1, P9, P12 and *Bacillus* isolates B1, B3 and B6 have different mechanisms for plant growth promotion.

Pseudomonas isolates have been shown to have the ability to use different sources of carbon, to live in the condition of low pH as well as temperature and to produce siderophore. *Bacillus* isolates have the ability to solubilize phosphate, to produce a greater amount of indol-3-acetic acid (IAA) than *Pseudomonas* isolates, to produce HCN and have an inhibitory effect on the growth of phytopathogenic fungi. In other investigated traits, isolates were similar.

The use of *Pseudomonas* sp. P12 and *Bacillus* sp. B1 isolates had a positive effect on the plant mass and total yield, which indicate that the usage of these isolates can result in a better yield of forage crops.

Results of this study suggest that isolates having these properties can be tested for usage as biofertilizers in agriculture for promoting plant growth, as well

as for the biological control of plant pathogens, which indirectly promotes plant growth.

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