Polish Journal of Microbiology
2017, Vol. 66, No 2, 269–272

SHORT COMMUNICATION

Influence of *Pseudomonas* and *Bacillus* Strains Isolated from *Lolium perenne* Rhizospheric Soil in Vojvodina (Serbia) on Planth Growth and Soil Microbial Communities

DRAGANA R. STAMENOV[1]*, SIMONIDA DJURIC[1], TIMEA HAJNAL-JAFARI[1] and SNEŽANA ANDJELKOVIC[2]

[1] Department of Field and Vegetable Crops, Faculty of Agriculture, University of Novi Sad, Novi Sad, Serbia
[2] Institute of Forage Crops, Kruševac, Serbia

Submitted 27 June 2016, revised 25 October 2016, accepted 16 January 2017

Abstract

The aim of this study was the isolation of *Pseudomonas* sp. and *Bacillus* sp. strains from rhizospheric soil and monitoring the impact of two isolates denoted as P12 (*Pseudomonas* sp.) and B1 (*Bacillus* sp.) on the parameters of English ryegrass (*Lolium perenne*) yield and activity of the soil microbial communities. During 2012–2014, a plot experiment was set up following the randomized block system. Better effect on the plant growth was recorded with the use of *Pseudomonas* sp. P12 isolate than with *Bacillus* sp. B1. Positive effect on the increase in the total number of microorganisms, aminoheterotrophs and azotobacter was also achieved. *Bacillus* sp. B1 increased only the number of actimycetes. Both isolates positively affected dehydrogenase activity (DHA).

Key words: *Bacillus* sp., *Lolium perenne*, *Pseudomonas* sp., DHA, plant growth, soil microbial community activity

The mechanisms by which plant-growth promoting rhizobacteria (PGPR) enhance plant growth are not fully understood, but it is believed that the PGPR promote plant growth and yield either by direct mechanisms such as: the ability to produce phytohormons like indolacetic acid, gibberellin, cytokinins and ethylene (Egamberdiyeva, 2007), asimbiotic N_2 fixation (Salantur *et al*., 2006), antagonism against phytopathogenic microorganisms by production of siderophores (Tian *et al*., 2009), and also solubilisation of mineral phosphates and other nutritients (Chen *et al*., 2006), or by indirect mechanisms: the extracellular production of antibiotics, synthesis of antifungal metabolites, production of fungal cell wall lysin enzymes, depletion of iron from the rhizosphere, competition for sites on roots and induced systemic resistance (Ahmad *et al*., 2006). To what extent this stimulative effect will affect the plant yield depends on the type of soil, effectivenes of the indigenous and introduced strains of microorganisms, plant species, agrotechnical measures *etc*. On the other hand, microbiological processes in the soil can additionally be stimulated by introducing PGPR. These microorganisms reproduce in soil and with their enzymatic activity raise and maintain the appropriate level of organic matter in soil (Dobbelaere *et al*., 2003). The effect of bacterial inoculation on the change of microbiological activity in soil depends on soil conditions, plant species, adaptability of introduced microorganisms *etc*. (Egamberdiyeva, 2007). Different bacteria have been reported as PGPR, but the most popular bacteria studied and exploited as biocontrol/promoter agents include the fluorescent species of *Pseudomonas* and *Bacillus* (Adesemoye *et al*., 2008).

The aim of this study was the isolation of *Pseudomonas* sp. and *Bacillus* sp. strains from rhizospheric soil and monitoring the impact of two isolates denoted as P12 (*Pseudomonas* sp.) and B1 (*Bacillus* sp.) on the parameters of the English ryegrass (*Lolium perenne*) yield and activity of the soil microbial communities.

Microorganisms were isolated from the roots and root-adhering soil of 30 days old plants (*L. perenne* L. Calibra) grown in carbonate chernozem type of soil (Stamenov *et al*., 2016). The 10 g samples of roots with tightly adhered soil were suspended in 90 ml of 0.1 M $MgSO_4 \cdot 7H_2O$ buffer and shaken for 10 min at 180 rpm on a rotary shaker. Hundred microliters of the suspension were spread onto King B agar (King *et al*., 1954) for *Pseudomonas* sp. isolates, and onto nutrient agar (Sambrook and Russell, 2001) for *Bacillus* sp. isolates, and incubated at 25°C for 48 h. Isolates

* Corresponding author: D.R. Stamenov, Faculty of Agruculture, University of Novi Sad, Novi Sad, Serbia;
e-mail: dragana.stamenov@polj.uns.ac.rs

which were established as gram-negative, rod-shaped cell and produce pigment fluorescein, were selected as *Pseudomonas* sp. isolates. Fluorescein production was tested on Pseudomonas Flo agar (Che *et al.*, 1999). Isolates which were established as catalase-positive, gram-positive, rod-shaped cells that form endospores, were classified as *Bacillus* sp. isolates. The presence of catalase was detected by the release of oxygen bubbles in contact with dilute hydrogen peroxide solution (Sneath, 1986).

The plot experiment was set up following randomized block system, during 2012–2014. The size of the experimental plot was 5 m^2. Each variant was conducted in four repetitions. From each repetition (from each plot) it was taken one sample for analysis, *i.e.* four samples for each variant. In the lab, each of the samples was analyzed in three repetitions. The variants of the experiment were the following: 1. plots inoculated with *Pseudomonas* sp. P12, 2. plots inoculated with *Bacillus* sp. B1, 3. control – plots without inoculation. *Pseudomonas* sp. isolate was cultivated on King- B medium whereas *Bacillus* sp. isolate was cultivated on nutrient agar. Before sowing, 50 ml (10^8 CFU/ml) of *Bacillus* sp. as well as 50 ml (10^8 CFU/ml) of *Pseudomonas* sp. isolate cells was introduced into 5 l of tap water, respectively and then evenly sprayed on the plot surface. The sowing was performed manually with 20 kg of English ryegrass per hectare. The experiment was conducted in chernozem soil (FAO classification).

Three mowings (March, July and November) of the grass were performed yearly. The following parameters were determined: yield of fresh and dry mass (t/ha), stem and root length (cm). After the third mowing, the number of microorganisms was determined using the dillution method (Trolldenier, 1996) and dehydrogenase activity (DHA) was measured in accordance with Thalman (1968). Appropriate nutrient media were used (Hi Media Laboratories Pvt. Limited, Mumbai, India): nutrient agar for the total number of bacteria, synthetic agar for the number of actinomycetes, potato dextrose agar for the number of fungi, meat peptone agar for the number of aminoheterotrophs, and nitrogen free medium with manitol for the number of *Azotobacter* sp.

The data were statistically processed by Statistics12.0 programme. The significance of the difference between the applied treatments was determined using Fisher's LSD test.

Seventeen *Pseudomonas* sp. and 23 *Bacillus* sp. strains were isolated. This research was focused on examining the effect of isolate *Pseudomonas* sp. P12 which produces indole-3-acetic acid (IAA), siderophores, cellulase, lipase, urease and gelatinase and isolate *Bacillus* sp. B1, IAA producer and phosphorus solubilizing bacteria, on the growth of English ryegrass (Stamenov, 2014).

Table I
Plant yield (t/ha) and the length of stem and root (cm) of English reygrass

	plant	I year			II year			III year		
		Ø+	*P12*	*B1*	Ø	*P12*	*B1*	Ø	*P12*	*B1*
I	Fresh mass	2.3^{a}*	7.3^{b}	5.0^{b}	3.0^{a}	8.0^{b}	6.3^{bc}	22.0^{b}	40.9^{a}	27.2^{c}
	Dry mass	1.0^{a}	2.6^{b}	2.3^{b}	0.9^{c}	1.8^{a}	1.0^{c}	6.0^{b}	9.0^{a}	6.0^{b}
II	Fresh mass	3.0^{a}	3.7^{a}	3.3^{a}	5.3^{c}	8.0^{b}	8.0^{b}	15.7^{d}	19.9^{a}	14.3^{d}
	Dry mass	1.0^{b}	1.1^{b}	1.1^{b}	2.7^{a}	5.0^{cb}	4.3^{c}	4.2^{c}	7.0^{b}	5.5^{bc}
III	Fresh mass	12.0^{a}	13.0^{a}	15.0^{a}	8.0^{c}	8.6^{cb}	9.0^{cb}	22.5^{b}	36^{d}	44.0^{d}
	Dry mass	6.0^{a}	7.0^{a}	6.5^{a}	1.6^{b}	1.6^{b}	1.6^{b}	3.1^{d}	8.9^{b}	14.4^{a}
Total yield	Fresh mass	17.3^{b}	24.0^{a}	23.3^{a}	16^{b}	24.6^{a}	23.3^{a}	60.2^{d}	97.1^{a}	85.5^{b}
	Dry mass	8.03^{c}	10.7^{a}	9.9^{ab}	5.1^{b}	8.7^{a}	6.9^{a}	13.3^{c}	25^{b}	25.9^{b}
Total %	Fresh mass	–	38.5	34.6	–	51	63	–	61.3	42
	Dry mass	–	33.9	23.8	–	64	85.5	–	87.2	94.7
I	Stem	10.5^{b}	19.0^{a}	18.5^{a}	12.0^{a}	23.0^{c}	21.0^{cb}	24.0^{c}	34.5^{a}	28.5^{d}
	Root	3.0^{b}	5.0^{a}	4.5^{ab}	3.75^{a}	5.0^{a}	4.5^{a}	3.85^{b}	6.34^{a}	3.5^{b}
II	Stem	10.7^{a}	12.0^{a}	11.75^{a}	17.0^{a}	23.5^{b}	22.0^{cb}	26.7^{cb}	34^{b}	25.9^{c}
	Root	2.5^{a}	4.0^{a}	4.0^{a}	4.0^{a}	4.75^{a}	4.75^{a}	1.9^{ab}	2.7^{ab}	3.16^{b}
III	Stem	18.7^{b}	25.0^{a}	20.5^{b}	19.5^{a}	24.5^{cb}	26.0^{b}	32.0^{d}	39.2^{a}	28.7^{d}
	Root	4.7^{a}	5.5^{a}	5.0^{a}	4.75^{a}	5.0^{a}	5.5^{a}	4.0^{a}	4.5^{a}	3.75^{a}
Average	Stem	13.3	18.6	16.9	16.2	23.7	23.0	27.5	35.9	27.7
	Root	3.4	4.83	4.5	4.2	4.9	4.9	3.25	4.5	3.45

* Mean values with the same superscript(s) are not significantly different according to Fisher LSD test ($p < 0.05$);
+Ø – control; isolates *Pseudomonas* P12 and *Bacillus* B1.

In comparison with the control, a statistically significant increase in the yield of fresh and dry mass was recorded in plots inoculated with the microorganisms (Table I). During the first year, the total yield of fresh mass of the plants inoculated with isolate P12 was 38% higher than in the control, whereas the yield of dry mass was 33% higher. The yield of fresh and dry mass were also increased by the inoculation with B1 isolate for 34 and 23%, resectively. During the second year, the total yield of fresh mass of plants was 51 and 64% higher than in the control whereas the yield of dry mass was 64 and 85% higher in variant with *Pseudomonas* sp. and *Bacillus* sp. isolate, respectively. Results of the third year of experiment showed very similar results. In average, there was no significant statistical difference in the effect on the yield between the *Bacillus* sp. B1 and *Pseudomonas* sp. P12 isolates, except in the third year of experiment when the yield of fresh mass was statistically higher in the variant with *Pseudomonas* sp. P12 than in the variant with *Bacillus* sp. B1.

In comparison with the control, it was observed that isolates *Pseudomonas* sp. P12 *Bacillus* sp. B1 had a positive effect on the length of stem and root of English ryegrass. Better effect on the plant growth was recorded with the use of *Pseudomonas* sp. P12 isolate. Similarly to this study, Stamenov *et al.* (2012a) identified the positive effect of *Pseudomonas fluorescens* and *Bacillus subtilis* strains on the height and dry weight of English ryegrass. Garcia *et al.* (2004) noticed a positive effect of inoculation with *Bacillus lichenoformis* on plant yield. Stamenov *et al.* (2012b) investigated the effect of *P. fluorescens*, *B. subtilis*, *Streptomyces* sp. and *Trichoderma asperellum* on the yield of English ryegrass Esquire variety. On average, the best effect was achieved with *B. subtilis*, whereas the weakest effect was recorded in the variant with *T. asperellum*. Ratti *et al.* (2001) found that the combination of the arbuscular mycorrhizal fungus *Glomus aggregatum*, and the PGPR *Bacillus polymyxa* and *Azospirillum brasilense* maximized biomass and P content of the aromatic grass palmarosa (*Cymbopogon martinii*) when grown with an insoluble source of inorganic phosphate. Plant growth promoting activities include production of siderophores, phosphate solubilizing enzymes and auxin, strongly affect the environment, both because they inhibit growth of other deleterious microorganisms and because they increase nutrient availability for the plant (Suresh, 2010). In this study, isolates ability 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 plants growth.

This investigation also included observing the quantitative changes in the microbial population in the rhizosphere of English ryegrass. Results of this research showed that the number of the investigated groups of microorganisms, apart from the number of fungi during the second year, increased in both variants in comparison with the control, (Table II).

In average, the use of *Pseudomonas* sp. isolate P12 had a better effect on the increase in the total number of microorganisms, aminoheterotrophs and *Azotobacter* sp. whereas the use of *Bacillus* sp. isolate B1 had a better effect on the increase in the number of Actinomycetes. According to the results of Schrader and Blevins (2001), biomass production of Actynomycetes was generally higher at the higher phosphorus concentrations than at the lower. These results suggest that the phosphor-solubilizing activities of isolate B1 directly influence the number of Actynomycetes. Dehydrogenase activity reflects the total range of oxidative activity of soil microorganisms (Liang *et al.*, 2014). In comparison with the control, introduction of both isolates positively affected the DHA. The highest DHA was recorded in the variant where isolate P12 was used. Our results are in agreement with the results of Nannipieri *et al.* (2003), who pointed out that the use of bacteria in plant production increases the number and enzymatic activity of microorganisms which enhaces the productive capability of soil. Our results are supported also by the research of Han *et al.* (2006).

Table II
The effect of inoculation on the number of soil bacteria (CFU/g dry soil) and DHA (TPF g^{-1} soil)

The number of microorganisms in	I year			II year			III year		
	Ø+	*P12*	*B1*	Ø	*P12*	*B1*	Ø	*P12*	*B1*
Total number, 10^6	25.74^{b}	76.71^{a}	34.68^{b}	15.77^{c}	90.61^{a}	30.51^{b}	$5,07^{c}$	$214,06^{a}$	$151,0^{b}$
Fungi, 10^4	5.3^{b}	5.68^{b}	14.90^{a}	20.1^{ab}	14.1^{bc}	10.7^{bc}	$10,1^{bc}$	$42,46^{a}$	$20,24^{b}$
Actinomycetes,10^5	7.8^{a}	11.37^{a}	15.9^{a}	7.87^{a}	22.44^{a}	8.08^{a}	$10,99^{a}$	$21,22^{a}$	$28,03^{a}$
Aminoheterotrophs,10^6	4.54^{b}	67.47^{a}	6.38^{b}	30.59^{b}	32.42^{b}	54.75^{b}	$86,2^{bc}$	$213,17^{a}$	151^{ab}
Azotobacter, 10^2	162.8^{b}	177.57^{b}	223.45^{a}	54.6^{bc}	151.7^{a}	91.9^{ab}	$86,6^{ab}$	$163,6^{ac}$	$73,96^{b}$
DHA	1118	1713.3	1219.4	768.2	896.7	871.3	680.9	974.3	951.9

* Mean values with the same superscript(s) are not significantly different according to Fisher LSD test ($p < 0.05$);
+Ø – control; isolates *Pseudomonas* P12 and *Bacillus* B1.

They improved the biological properties of soil by introducing *Bacillus* sp.

In this study, the increase in the number of microorganisms in soil and the positive effect of inoculation on the plant suggest that the use of *Pseudomonas* sp. P12 and *Bacillus* sp. B1 can result in a better yield of forage crops, especially in organic production, where mineral fertilizers are not used. Introducing of isolate P12 had a better effect on the stem and root length, total number of microorganisms, aminoheterotrophs, *Azotobacter* sp. and DHA than isolate B1, which might be due to ability of isolate P12 to produce siderophores and enzymes. On the other hand, isolate B1 more influenced the number of Actinomycetes, which can be explained by the fact that *Bacillus* sp. isolate B1 has ability to solubilize phosphate. Further studies on the isolates will uncover the exactly mechanisms by which they promote plant growth. Isolates P12 and B1, with the ability to promote plant growth, may represent a biological alternative for chemical fertilizers application in agriculture. In order to achieve the best results, it is necessary to isolate as many microorganisms as possible from the rhizosphere of grasses, determine their effect on the plant growth and find the most adequate way of applying the inoculants.

Acknowledgement

This research was supported by the Ministry of education and science, Republic of Serbia, project III 43002.

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