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THE USE OF *STREPTOMYCES* ISOLATE WITH PLANT GROWTH PROMOTING TRAITS IN THE PRODUCTION OF ENGLISH RYEGRASS

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ABSTRACT

The aim of this study was the isolation and characterization of actinomycetes (*Streptomyces* sp.) with PGP traits in the rhizosphere of perennial ryegrass, and monitoring the impact of their use on the parameters of the English ryegrass (*Lolium perenne*) yield and microbiological activity in its rhizosphere. The isolates had different growth characteristics. All three isolates produced a greater amount of indole-acetic acid (IAA) in the medium with added L-tryptophan. Isolate A3 produced siderophors and hydrogen cyanide, while isolates A1 and A2 utilized organic phosphorus. All isolates were produced cellulase, urease and gelatinase but not amylase. Isolates A1 and A3 produced lipase while A2 produced a protease. Isolate A3 was chosen for the examination of the isolate impact on plant yield. When evaluated on English Ryegrass in the field, during the first year of experiment, the isolate A3 affected positively the yield of fresh and dry mass, root and stem length. During the second year, the isolate A3 statistically significant enhanced the measured parameters over the control. In relation to *Trichoderma asperellum,* application of isolate A3 had a better effect on the plant yield. The number of investigated group of microorganisms and dehydrogenase activity in the inoculated variant increased in comparison with the control.

Key words: actinomycetes, English ryegrass, plant-growth promoting microorganisms, yield.

INTRODUCTION

The use of excessive amounts of mineral fertilizers and pesticides leads to soil degradation, disturbance of natural balance and stability among organisms in soil. Due to the concern about the harmful effects of these chemical substances, there is a growing interest for deeper understanding of cooperation between rhizospheric microorganisms and plants. A large body of research has shown that PGPM (plant-growth promoting microorganisms) could be used as an alternative to chemical fertilizers which are used for plant growth promotion (Berg, 2009).

Actinomycetes belong to an extensive and diverse group of Gram-positive, aerobic, filamentous bacteria that play important ecological roles in soil nutrient cycling. Besides acting as organic matter decomposers, these microorganisms have a great potential as agents for control of root pathogenic fungi (Franco-Correa et al., 2010) and bacteria (Oskay et al., 2004) and/or for promotion of plant growth (Fermino-Soares et al., 2007). This is due to their capacity to produce antibiotics, phytohormones, extracellular enzymes, siderophores, enzymes that have antimicrobial activity, substances that promote plant growth, solubulization of phosphates and competition for nutrients and substratum with plant pathogens (Panhwar et al., 2012).

Most soil born actinomycetes belong to the genus *Streptomyces* (Suzuki et al., 2000). These bacteria are widely recognized as industrially important microorganisms because of their ability to produce many kinds of secondary metabolites, including antibiotics and extracellular enzymes (Chater et al., 2010). Plant growth promotion potential of *Streptomyces* was reported on pea (Tokala et al., 2002), bean (Nassar et al., 2003), tomato (El-Tarabily, 2008), wheat (Sadeghi et al., 2012) and rice (Gopalakrishnan et al., 2013).

Despite the well-documented history of *Streptomyces* in biocontrol and preliminary evidence of their capacity to enhance plant growth, *Streptomyces* species have been

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poorly investigated specifically for their potential as plant growth promotion microorganisms. On the other hand, the use of such preparations as an addition to, or instead of mineral fertilizers and pesticides has special significance for plant species such as forage grasses which are grown over large areas. It has been known that *T. asperellum* applications can improve plant growth and development of a broad range of species (De Souza et al., 2008). Because of that, in this study we did a comparative study of the effect of *Streptomyces* isolate and *T. asperellum* on the plant yield.

Therefore, the aim of this study was the isolation and characterization of actinomycetes (*Streptomyces* sp.) with PGP traits in the rhizosphere of perennial ryegrass and monitoring the impact of their use on the parameters of the English ryegrass (*Lolium perenne*) yield and microbiological activity in its rhizosphere.

MATERIAL AND METHODS

Medium with glucose and nitrate was used for the isolation of actinomycetes (genus *Streptomyces*) (glucose 10 g/l, $NaNO_3$ 10 g/l, K_2HPO_4 10 g/l, KCl 10g/l, $MgSO_4$ x $7H_2O$ 10 g/l, agar 15 g/l). The culture characteristics (colony morphology) of the isolates were studied on the potato dextrose agar (PDA).

Physiological and biochemical characterization of *Streptomyces* isolates

Utilization of carbon sources was determined by using Hugh-Leifsson medium (pepton 2 g, K_2HPO_4 0,3 g, NaCl 5 g, 10 g of carbon sources (glucose, galactose, fructose, sacharose, lactose or xylose) brom-timol blue 0,03 g, agar 3 g) (Hugh and Leifsson, 1953) and observing the change of colony colour from greenish to yellow, in the case of a positive reaction.

For physiological growth characteristics, the isolates inoculated on PDA were incubated at different temperatures (5°C, 15°C, 28°C, 37°C, 45°C), pH levels (pH 4, 5, 6, 7), and salt concentrations (3%, 5%, 7%). For determination of pH and salt tolerance, the isolates were incubated at 28°C for seven days. After 7 days of incubation, the width of colony was measured and compared with the control.

Investigation of indol-3-acetic acid (IAA) production by bacterial isolates was performed according to the method of Gordon and Weber (1951). Actinomycete was grown on PDA 10 days after which the mycelia was scraped from the surface of the media and transferred into 100 ml of PDB (potato dextrose broth). Isolates were incubated for 7 and 14 days on PDB supplemented with 0, 200 and 500 µg/ml tryptophan. IAA was assayed by colorimetric method using Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$). Development of pink colour was assayed with spectrophotometer at 530 nm. Concentration of produced IAA was determined from a standard curve of IAA (1-50 µg/ml).

Bacterial ability to produce the siderophores was assayed on chrom-azurol S (CAS) medium by protocol of Milagres et al. (1999). Two halves of a Petri dish were filled with CAS medium and PDA, respectively. Bacterial inoculum was spread on PDA medium as near as possible to border line with CAS medium and incubated at 28ºC for 5 days. The medium discoloration from blue to orange indicated siderophores production and the diameter of discoloration was measured.

Bacterial isolates were inoculated on HCN induction medium (Tryptic Soy Broth 30 g/l, Glycine 4.4 g/l, agar 15 g/l (Frey-Klett et al., 2005). Inoculated plates with disk of Whatman paper, previously dipped in HCN revealing solution (0.5% picric acid and 2% Na_2CO_3) and placed on the media surface, were tightly sealed with parafilm and incubated at 28ºC for 5 days. Development of orange-brown colour of the paper indicated HCN synthesis ability.

The ability of the mineralization of phosphorus organic compounds was investigated on Menkina medium, modified by Rodina (Menkina, 1963). After 5 days of incubation at 28ºC the appearance of transparent zone around the colony was evidence of the ability to dissolve phosphate. Diameters of the zone were measured and

according to their values the relative efficacy of phosphate solubilization was evaluated.

Production of cellulase was tested on CMC agar (carboxy methyl cellulose agar) (Kasing, 1995). After incubation, the Petri dishes were overflowed with a solution of Congo-red (1 mg/cm^3 H_2O). After fifteen minutes, the Congo-red was decanted and the Petri dishes were overflowed with 1 M NaCl. Discoloured zone around the colony was proof of the cellulase activity.

Lipase activity was determined by growing the isolates on the medium with Tween 80 (pepton 10 g/l, NaCl 5 g/l, $CaCl_2 \cdot H_2O$ 0.1 g/l, agar 15 g/l) (Lanyi, 1987) and observing the presence and absence of a zone around the colony.

Urease activity was tested by using the urea agar (Cristensen, 1946). The appearance of the red color was proof of the urea decomposition.

Gelatine hydrolysis was detected using a nutrient gelatine stub method. The inoculated tubes and an uninoculated control tube were incubated at 25°C for 7 days. Hydrolysed gelatine was detected in a liquid medium after exposed to cold temperature (ice bath), while the uninoculated control medium remained solid.

The ability of the hydrogen sulfide (H_2S) production was determined by the appearance of a change in colour (from orange to black) of deep-peptone-iron agar (pepton 15 g/l, proteose pepton 5 g/l, feri ammonium citrate 0,5 g/l, $Na_2S_2O_3$ 0,08 g/l, agar 15 g/l).

Hydrolysis of starch was performed by flooding iodine on one-week-old actinomycetes colonies grown on starch agar (starch 10 g/l, KH_2PO_4 0.5 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ x $7H_2O$ 0.2 g/l, agar 15 g/l) (Rodina, 1965), and by observing presence or absence of halo zone around the colony.

Evaluation of *Streptomyces* isolates for their PGP potential under semi-controlled conditions on English ryegrass

The experiment was conducted in Vojvodina (Serbia), in the soil having the following characteristics: 3.53% $CaCO_3$; 4.51% humus; 0.3% N; 20.89 mg P_2O_5 in 100 g of soil; 19.68 mg K_2O in 100 g of soil; pH in H_20 8.11; pH in KCl 7.59. According to FAO classification, the soil is classified as chernozem.

English ryegrass (*Lolium perenne* L. Calibra) was taken from the collection of Institute of Forage Crops, Kruševac, Serbia.

The experiment was set up following the randomised block system. The size of the experimental plot was 5 m^2. Each variant had four repetitions. The variants of the experiment were the following:

1. isolate *Streptomyces* sp. A3;
2. *T. asperellum* (microbiological preparation Trifender, Hungary);
3. control - no inoculation. Before sowing, 50 ml 10^8 CFU/ml of *Streptomyces* sp. A3 cells as well as 50 ml 10^8 CFU/ml of *T. asperellum* spores were introduced into 5 l of tap water each, and than evenly sprayed on the plot surface. The sowing was performed manually with 20 kg of English ryegrass per ha.

Three mowing (March, July and November) were performed during the two years. The following parameters were determined: yield of fresh and dry mass (t/ha), stem and root length (cm).

From each plot after the first and third mowing, one sample of rizospheric soil was taken for microbiological analysis, i.e. four samples for each variant. In the lab, each of the samples was analysed in three repetitions. The number of microorganisms was determined using the dilution method (Trolldenier, 1996). Appropriate nutrient media were used (Hi Media Laboratories Pvt. Limited, Mumbai, India): nutrient agar for the total number of bacteria and potato dextrose agar for the number of fungi. Dehydrogenase activity was determined by Lenhard's method (1956) modified by Thalmann (1968).

Statistical analysis

The data were statistically processed using the Statistics 10 software (Hamburg). The significance of the difference between the applied treatments was determined using Fisher's LSD test.

RESULTS AND DISCUSSION

Physiological and biochemical characterization of *Streptomyces* isolates

From the rhizosphere of perennial ryegrass 12 bacteria of the genus *Streptomyces* were isolated. Based on the morphological characteristics of the colony and cell, bacteria were classified into three groups. Representative isolates of each group (denoted as *Streptomyces* sp. A1, A2 and A3) were examined for different physiological and biochemical properties.

The isolates varied in terms of utilization of carbon sources such as glucose, galactose, fructose, sacharose, lactose and xylose (Table 1). The optimum pH for growth of all the isolates was 6 and 7, while only isolate A3 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 determined with the isolates A2 and A3, but it stimulated growth of isolate A1. All the isolates developed well grown colonies at 28 and 37ºC, however, one isolate A3 could grow at 45ºC.

Table 1. Growth of streptomyces isolates in culture medium with different sources of carbon, at different pH, NaCl levels and temperature

Isolates	Sugars						pH				NaCl (%)			T(ºC)				
	G	Ga	F	S	L	K	4	5	6	7	3	5	7	3	13	28	37	45
A1	+	+	+	+	-	+	-	-	++	++	++	++	+++	-	++	++	++	-
A2	+	+	-	-	+	-	-	++	++	++	++	++	+	-	+	++	++	-
A3	+	+	+	+	-	+	+	++	++	++	++	+	+	-	+	++	++	+

G – glucose; Ga – galactose; F – fructose; S – sacharose; L – lactose; K – xylose;
- absence of growth; + minimal growth; ++ optimal growth; +++ intense growth.

The ability of *Streptomyces* sp. to grow in harsh pH and temperature and higher concentration of salinity was reported by Malviya et al. (2013). According to the Sadeghi et al. (2012), metabolic diversity allows actinomycetes to survive in saline, acid and high temperature environments, indicating their good ability to adapt to adverse environmental conditions. The results of this work indicate the adaptability of these isolates, specially isolate A3, showing that they have a good ability to survive under different environmental conditions.

All three selected isolates had the ability to produce IAA without tryptophan supplementation (Table 2). Isolates produced a greater amount of IAA in the medium with added L-tryptophan. The amount of IAA increased with time, thus higher values of IAA were measured after 14 days in comparison to the values on the 7 days of the experiment.

IAA and siderophore producing microbes are widely recognized as industrially important microorganisms, because of their ability to improve plant growth by increasing seed germination, root elongation and root dry weight (El-Tarabily, 2008). Similarly, Hamdali et al. (2008) reported that all tested isolates produced greater amount of IAA in medium with added L-tryptophan.

Table 2. Production of indole acetic acid (µg/ml) by streptomycete isolates

Isolates	L tryptophan (µg/ml)					
	0	200	500	0	200	500
	After 7 days			After 14 days		
A1	0,71	0,96	1,07	1,36	5,64	1,21
A2	1,86	1,82	2,82	2,32	2,68	3,61
A3	0,71	0,75	2,78	1,75	2,28	6,03

One isolate (A3) was found to be positive for production of siderophores and hydrogen cyanide, while two isolates (A1 and A2) utilized organic phosphorus (Table 3). All the isolates showed ability to produce cellulase, urease and gelatinase, but not amylase. Two isolates (A1 and A3) produced lipase, while only isolate A2 produced a protease (Table 3). These results indicate that these isolates having these traits can be exploited for biological control of plant pathogens, which indirectly promotes plant growth.

Table 3. Production of siderophores, hydrogen cyanide, phosphate utilization and enzymatic activity

Isolates	Siderophore[a]	P[b]	Cellulase[c]	Lipase	Urease	Gel	Prot	Amyl
A1	-	++	+	+	+	+	-	-
A2	-	+	+	-	+	+	+	-
A3	+	-	+	+	+	+	-	-

[a] width of orange zone : + 0-10 mm; ++ ≥ 10 mm; - no zone;
[b] efficacy of phosphate solubilization evaluated according to zone diameter: + represents 4mm/day; ++ represents 5-8 mm/day; +++ ≥8 mm/day;
[c] Cellulase, lipase, urease, gelatinase (Gel), protease (Prot), amylase (Amyl) activities: + hydrolysis; - no hydrolysis.

Evaluation of *Streptomyces* isolates for their PGP potential under semi controlled conditions on English ryegrass

Previous studies indicated the positive effect of different microbial inoculants on the yield of some grass species (Ratti et al., 2001; Dragomir and Moisuc, 2007). In our study, isolate A3 affected positively the yield of fresh and dry mass of English Ryegrass (Table 4). During the first year of experiment, statistical analyses showed that inoculation did not have a significant impact on plant growth parameters in relation to the control treatment,but in the second year, a statistically significant yield increase was obtained in both variants. During the first year, the yield of fresh mass of the plants inoculated with isolate A3 was by 12,1% higher than in the control, whereas the yield of dry mass was by 14,2% higher (Table 4). During the second year, the yield of fresh mass of plants was by 63.2% higher than in the control whereas the yield of dry mass was by 85.9% higher in variant with *Streptomyces* isolate. Similar to this study, Stamenov et al. (2012) identified the positive effect of three *Streptomyces* sp. strains on the height and dry weight of two cultivars of English ryegrass (Eminent and Leia).

Until now, it was determined that Trichoderma species can improve plant growth and development in a broad range of species, such as carnation, corn, cotton, millet, ornamental grasses and English ryegrass (De Souza et al., 2008; Stamenov et al., 2011). However, in this study the application of *Streptomyces* sp. A3 had a better effect on the values of all measured plant yield parametersthan *T. asperellum*, especially in the second mowing of the second year (Table 4).

The use of bacteria in plant production increases the number and enzymatic activity of microorganisms, which enhances the productive capability of soil (Nannipieri et al., 2003). These microorganisms reproduce in soil and, with their enzymatic activity, increas and maintain the appropriate level of organic matter in soil (Jarak et al., 2009). 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). This research showed that the number of the investigated groups of microorganisms and dehydrogenase activity increased in both variants in comparison with the control. On average, the use of *Streptomyces* isolate A3 had a better effect on the increase of dehydrogenase activity, whereas the use of *T. asperellum* had a better effect on the increase in the total number of bacteria and fungi.

Table 4. Plant yield (t ha^{-1}) and the length of stem and root (cm) of English ryegrass

	Plant	I year			II year		
		Control	*T. asperellum*	A3**	Control	*T. asperellum*	A3
I	Fresh mass	2,33[a*]	2,5[a]	2,43[a]	3,0[a]	6,6[b]	4,35[b]
	Dry mass	1,03[a]	1,4[a]	1,22[a]	0,86[a]	1,16[a]	1,53[b]
II	Fresh mass	3,0[a]	3,67[a]	4,0[a]	5,33[a]	8,0[a]	9,0[b]
	Dry mass	1,0[b]	1,1[b]	1,67[a]	2,66[a]	4,0[b]	6,0[c]
III	Fresh mass	12,0[a]	12,3[a]	13,0[a]	8,00[a]	11,7[b]	11,0[b]
	Dry mass	6,0[a]	6,2[a]	6,35[a]	1,6[a]	2,2[a]	2,0[a]
Total yield	Fresh mass	17,33[a]	18,47[a]	19,43[a]	16,3[a]	26,3[b]	26,61[b]
	Dry mass	8,03[a]	8,7[a]	9,17[a]	5,12[a]	7,36[b]	9,52[b]
Total yield (%)	Fresh mass	100	106,6	112,1	100	161,3	163,2
	Dry mass	100	108,3	114,2	100	143,7	185,9
I	Stem	10,5[a]	11,0[a]	11,5[a]	12,0[a]	19,5[b]	21,5[c]
	Root	3.0[a]	3,75[a]	3,75[a]	3,75[a]	3,45[a]	4,0[a]
II	Stem	10,75[a]	11,0[a]	11,5[a]	17,0[a]	21,5[b]	22,0[b]
	Root	2,5[a]	3,0[a]	3,5[a]	4,0[a]	4,5[a]	4,0[a]
III	Stem	18,75[a]	20,0a	19,25[a]	19,5[a]	22,75[b]	23,5[b]
	Root	4,75[a]	4,75[a]	4,0[a]	4,75[a]	4, 75[a]	4,17[a]
Average	Stem	13,3	14,0	14,1	16,2	21,25	22,3
Average	Root	3,42	3,83	3,75	4,17	4,23	4,06

Mean values with the same superscript(s) are not significantly different according to Fisher LSD test ($p<0.05$).
**A3-isolate *Streptomyces* sp. A3.

Similarly, Stamenov et al. (2012) concluded that all three strains of *Streptomyces* sp. (5, 7, 9 k) had a positive effect on the yield of English ryegrass and on the number of microorganisms in the rhizosphere of the plant.

The increase in the number of microorganisms and dehydrogenase activity in soil and the positive effect of inoculation on the plant growth indicated that isolate *Streptomyces* sp. A3 had a great potential as agent to promote growth of English ryegrass, especially in organic production, where mineral fertilizers are not used.

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