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GROWTH PROMOTION OF ALFALFA, *MEDICAGO SATIVA* L. BY INOCULATION OF A PRECEDING CROP WITH RHIZOBACTERIA

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SUMMARY

In the greenhouse experiment, the possibility of alfalfa (*Medicago sativa* L.) growth promotion by inoculation of preceding barley (*Hordeum vulgare* L.), with plant growth promoting rhizobacteria (PGPR) was examined. The aim of experiment was to select the effective strains as biofertilizer applied in plant rotation. Effects of inoculation with two *Azotobacter* and two *Pseudomonas* strains as well as one *Sinorhizobium, Enterobacter* and *Bacillus* strain on shoot dry weight and total N content of alfalfa were determined. The results pointed out significant plant growth promotion abilities of strains A1, A2 and P1 which increased alfalfa shoot dry weight over untreated control Ø, by 41, 39 and 35 %, respectively. These three strains increased total N content of alfalfa plants by 34.92- 40.45% in respect to control Ø. The presented study showed a significant positive influence of preceding barley inoculation with rhizobacteria alone and their mixture on shoot yield and total N content of alfalfa. Results indicated that strains of *Azotobacter* sp., *Pseudomonas* sp. and *Enterobacter* sp. alone can be investigated in further researches as potential agents of biofertilizer for plant growth promotion of alfalfa.

Key words: alfalfa, plant growth promotion, inoculation, previous crop, barley

INTRODUCTION

Providing an adequate supply of nutrients is important for alfalfa (*Medicago sativa* L.) production and essential for maintaining high, quality and profitable yields. Application of mineral fertilizers significantly increases quality and quantity of yield because the content of plant nutrients in the soil is variable and often unavailable. However, in last decades with the increasing world population there have been economic and ecological problems associated with excessive use of mineral fertilizers and other synthetic chemicals. The principle goal of agriculture is the production of high quality, safe and affordable food for word population. Hence there has been an ever-increasing interest in the use of native and non-native beneficial microorganisms to improve plant health and productivity while ensuring safety for human consumption and protection of the environment (Avis et al., 2008; do Vale Barreto Figueredo et al., 2011). Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria which colonize the rhizosphere and plant roots and have ability to enhance plant growth by variety of mechanisms that involve increasing nitrogen (N) uptake (biological N fixation-BNF), solubilisation of mineral nutrients, stimulation of root growth (phytohor-

mone production) and suppression of root diseases (Martinez-Viveros et al., 2010; Bhattacharyya and Jha, 2012). Strains with PGPR activity belonging mainly to phylum Proteobacteria: genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas, Serratia* and rhizobial bacteria have been reported (Egamberdiyeva et al. 2004; Abbas-Zadeh et al. 2010; Antoun and Prévost 2000; Bhattacharyya and Jha, 2012). Due to ability of plant growth promotion, PGPR appeared as bacterial inoculants, a promising alternative for mineral and organic fertilization, pesticides and other supplements (Bhattacharyya and Jha, 2012).

Barley (*Hordeum vulgare* L.) is a major cereal grain followed by legumes in crop rotation. One of an important advantage of using barley as cover crops is its trait to suppress weeds through physical as well as chemical allelopatic effects (Nagabhushana et al., 2001; Saini, 2006). Good cover crop, inexpensive and easy to grow barley should be considered an integral part of any farming system that wants to efficiently utilize nutrients, improve soil quality, and increase farm profitability mostly by reducing herbicide costs. Based on these characteristics barley is suitable preceding crop to legumes particularly, alfalfa. Inoculation of preceding crops with PGPR could help establishment of population of these bacteria in soil and rhizosphere of the next crop (Goos et al., 2001).

In recent years there has been a growing interest in using bacterial inoculants as biofertilizers. Selection of effective strains with PGPR properties is one of the important steps in examination of potential bacterial inoculants. The experiment was designed to determine possibility of alfalfa growth promotion by inoculation of barley as preceding crop alfalfa with some rhizobacteria with the aim to select effective strains as biofertilizer applied in plant rotation.

MATERIAL AND METHODS

Sinorhizobium meliloti strain L3Si, *Bacillus megaterium* SNji, *Enterobacter* sp. strain E1 *Azotobacter* sp. strains A1 and A2, as well as *Pseudomonas* sp. strains P1 and P2 from the Collection of the Institute of Soil Science were used for the inoculation of barley. The inoculation effects of these PGPR on the yield of alfalfa cultivar K-28 as subsequent crop to barley were examined in pot experiment under greenhouse conditions.

The pots were filled with 1.9 kg of non-sterile soil with following characteristics: pH (in H_2O) 7.4, 0.09% N, 1.48% C, 21 mg kg^{-1} available P (P_2O_5), 400 mg kg^{-1} K_2O. The experiment was designed with 7 inoculated treatments which represented alfalfa plants as subsequent crop grown in pots after cutting inoculated barley. Treatments were compared with two control treatments without inoculation: Ø (alfalfa plants after uninoculated preceding crop) and Øo (alfalfa without preceding crop). The experiment was carried out with 3 replications in completely randomised system and the pots were kept in greenhouse conditions. *Bacillus* and *Enterobacter* sp. strains and *Pseudomonas* sp. strains were cultivated for 24h in nutrient broth medium and King B medium, respectively (King et al., 1954; Vincent, 1970; Bergey, 1984-1989; Sarić, 1989). *S. meliloti* strain was cultivated in yeast mannitol broth (YMB) for 48h while *Azotobacter* sp. strains were cultivated in N free mannitol broth with Vinogradsky solution for 72h .

Barley (*Hordeum vulgare* L.) seeds were surfaced-sterilized with 0.1% $HgCl_2$ solution (Vincent, 1970) and inoculated (2 ml $plant^{-1}$ of liquid culture) with single strains or with their mixture in a ratio 1:1. Liquid culture of single strain contained $>10^9$ cells ml^{-1}. Ten seeds per pot were planted and after 2 weeks seedlings were thinned to 5 plants pot^{-1}. Barley plants were removed in filleting stage and alfalfa (*Medicago sativa* L.) as subsequent crop was sown in the same pots. Alfalfa seeds were surfaced-sterilized, planted and thinned as the same way like barley. The plants were kept for six weeks. Plant shoots were separated from roots and dried in an oven at 70 °C to constant weight and the average dry weight per plant was calculated. The percentage of shoot N was determined from dried and ground plant samples using the CNS analyser and it was used to calculate total N content in mg per pot. The data were statistically processed by the LSD and Duncan test using the statistical program SPSS 10.0. Correlation coefficients were calculated to study the associative relations among the measured traits. All references to significance in the text imply statistical significance at $P<0.05$, unless otherwise stated.

RESULTS

In our study barley as crop preceding alfalfa was inoculated with seven single effective rhizobacterial strains belonging to *S. meliloti, B. megaterium*, *Enterobacter* sp., *Azotobacter* sp. and *Pseudo-*

monas sp. as well as their mixture. Effects of barley inoculation with PGPR strains on alfalfa yield were examined. The height of the alfalfa plants was 29.95–35.89 cm without significant differences among treatments. The greatest average values of alfalfa shoot dry weight (SDW) were obtained in the inoculated treatments with *Azotobacter* sp. strains A1 and A2 followed by *Pseudomonas* sp. strain P1, (698.59, 690.62 and 672.65 mg plant^{-1}, respectively) (Table 1). These results indicated that alfalfa shoot dry weight (SDW) was significantly influenced by inoculation the preceding barley with A1, A2 and P1 strains in respect to the other inoculated treatments and control Ø. The results pointed out significant plant growth promotion abilities of strains A1, A2 and P1 which increased alfalfa SDW over control Ø, by 41, 39 and 35 %, respectively. Shoot dry weight values were in highly significant positive correlation with shoot total N content (r=0.98). The highest average values of total N content was detected also, in A1, A2 and P1 treatments and there are no significant differences between them. Among the strains applied, the treatment with a *Pseudomonas* sp. strain P1 resulted in the greatest value of total N content (21.162 mg plant^{-1}) followed by A1 and A2 strains. These three strains increased total N content of alfalfa plant by 34.92- 40.45% in respect to control Ø.

However, it should not neglect influence of strain mixture as well as *Enterobacter* sp. E1 and *Pseudomonas* sp. strain P2 on plant parameters bearing in mind that these strains significantly increased (by about 27%) SDW and total N content of alfalfa over control Ø.

B. megaterium strain SNj and *S. meliloti* strain L3Si did not improve alfalfa properties investigated in this study in comparison with control Ø. Small and white nodules scattered on alfalfa roots were indicators of ineffective symbiotic association between host plant and rhizobial strain L3Si.

Table 1. Effect of barley inoculation with PGPR on parameters of alfalfa as subsequent crop.
Tabela 1. Efekat inokulacije ječma PGPR rizobakterijama na neke osobine lucerke kao narednog useva.

PGPR*	Alfalfa parameters Parmetri lucerke				
	Height cm	**SDW mg plant^{-1}**	**Total N content mg plant^{-1}**	**SDW index**	**Total N content index**
	Visina cm	**SDW mg biljka^{-1}**	**Sadržaj ukupnog N mg biljka^{-1}**	**SDW indeks**	**Indeks ukupnog sadržaja N**
Ø**	34.1 ab	495 de	15.1 d	100	100
Øo***	33.2 ab	543 d	15.9 d	110	106
A1	35.0 a	699 a	20.3 ab	141	135
A2	34.8 a	691 a	20.5 ab	140	138
P1	34.6 a	673 ab	21.2 a	136	140
P2	30.5 ab	607 c	19.1 bc	123	127
L3Si	30.0 ab	411 f	13.2 e	83	88
SNj	28.5 b	488 e	15.1 d	99	100
Mix***	31.9 ab	630 bc	19.3 bc	127	128
E1	35.9 a	640 bc	18.6 c	129	124
LSD 0.01 LSD 0.05	6.98; 5.27	57.41; 43.34	1.73; 1.31	-	-

PGPR* - plant grow promoting rhizobacteria; Ø**- control treatment-alfalfa plants after uninoculated preceding crop; Øo*** control treatment- alfalfa without preceding crop; Mix***- mixture of strains applied; Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test (P≤0.05).

PGPR*- rizobakterije koje poboljšavaju rast biljke; Ø**- kontrolni tretman-biljke lucerke posle neinokulisanog prethodnog useva; Øo***- kontrolni tretman-biljke lucerke bez prethodnog useva; Mix***- mešavina sojeva; Na osnovu Duncan's multiple range test srednje vrednosti u kolonama označene istim slovom nisu statistički značajno različite (P≤0.05).

DISCUSSION

There has been a growing interest in using bacterial inoculants (biofertilizers) in agricultural production including legumes and cereals (Figueredo et al., 2008; do Vale Barreto Figueredo et al., 2011). Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been extensively reported (Kloepper et al. 1978; Zhang et al. 1996; Gupta et al. 2002; Vessey 2003; Gray and Smith 2005; Figueiredo et al. 2008). In addition, there is interest in inoculation of preceding crops with PGPR with intention to establish PGPR population in soil and rhizosphere of the next crop (Domit et al., 1990; Goos et al., 2001).

In our study possibility of alfalfa growth promotion by inoculation of preceding barley with PGPR was determined in order to evaluate the plant growth promoting potential of some strains as inoculums as well as their influence on subsequent crop. Presented results indicated that among 5 strains with significant plant growth promoting activity (A1, A2, P1, P2 and E1), strains of *Azotobacter* sp. and one strain (P1) of *Pseudomonas* sp. own the greatest plant growth promoting potential.

The both applied strains of *Azotobacter* sp. significantly increased alfalfa SDW (about 40% over control) probably due to its N fixing or the other plant growth promoting abilities which is in agreement with results of some authors (Kennedy et al. 2004; Milošević et al., 2012). It was reported that particular species of *Azotobacter* (*A. paspali*) with some cereal can fixed 15-90 kg N ha^{-1}, which indicates *Azotobacter* genera as a good diastrophic useful for both cereals and legumes (Franche et al., 2009).

Presented results showed different plant growth promotion abilities of *Pseudomonas* sp. strains P1 and P2 indicating that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivar and genotype (Lucy et al. 2004). Direct plant grow promoting effect of boiocontrol agent such as *Pseudomonas* sp. in pathogen-free environment is often associated with following mechanism solubilization insoluble P source and regulation of plant growth regulators (Avis et al., 2008). In our results, middle PGPR effectiveness of *Pseudomonas* sp. P2 and *Enterobacter* sp. strain E1 should not be neglected because these strains significantly increased SDW over Ø by 22% and 29 %, respectively. Particular *Pseudomonas* species could increase yield of some plant species by 25% as well as 60% (Adjanohoun, 2011). *Enterobacter and Pseudomonas* genera have been identified as diazotrophic rhizobacteria with nitrogen-fixing and PGPR ability in rhizosphera of various plants that increase the height and plant yield (Minorsky, 2008; Franche et al., 2009; Zabihi et al., 2010).

Species of *Bacillus* are the most extensively studied (Minorsky, 2008; Hayat et al., 2010). Diversified populations of aerobic endospore forming bacteria of *Bacillus* species occur in agricultural fields and contribute to crop productivity. It is very likely that plant growth promotion by rhizosphere bacilli may be a result of combined action of two or more of these mechanisms (Richardson et al. 2009; Kumar et al., 2011). These bacteria competitively colonize the roots of plant and can act as biofertilizers and/or antagonists (biopesticides) or simultaneously both. However, in presented study *B. megaterium* SNji and *S. meliloti* strain L3Si did not show promoting abilities. The results of some authors concerning *Bacillus* species also pointed to its negative effect on maize (Adjanohoun, 2011).

Rhizobia are a vast group of soilborne rhizobacteria with representatives that have proven plant growth promoting activities through N fixation. Rhizobal strains are well known N fixers which in symbiosis with legumes form N fixing nodules but they can associate with roots of non-legumes without forming true nodules resulting in the growth promotion of legumes and non-legumes by different mechanisms (Avis et al., 2008; Mehboob et al., 2009). These bacteria can equally produce plant growth regulations-phytohormones and solubilize organic and inorganic phosphates that would have a role in their plant grow promoting activities. Also presence of rhizobial strains indirectly stimulate the plant to active its defence mechanisms when challenged with pathogen through the production plant defence compounds (Avis et al., 2008). Rhizobia have a diverse range of activity. In contrast, some studies have revealed that rhizobial inoculation may also have deleterious effect on growth and yield of non- legumes and legumes and only specific rhizobial strains had potential to be used as PGPR (Antoun and Prevost, 2000). In this study *S. meliloti* strain L3Si applied as inoculant of preceding barley was ineffective in spite of its high effectiveness in BNF with alfalfa in our previous experiments (Delić et al., 2012). Significantly lower values of alfalfa parameters in comparison the control-Ø indicated bad relationship between the symbionts. Reasons for these results can be founded in natural variations in environment, cultivar, soil and indigenous microflora of a specific area (Mehboob et al.,

2008) and represent the major challenges in the use of bio-inoculants. Characterization of the degree to which symbiotic microbes vary in the provision of mutualistic benefits in relation to environmental quality, host species and plant community structure is critical to developing an understanding of their role as agents of productivity and selection in natural populations (Martines-Viveros et al., 2010; Thrall, 2011).

The mixture of strains applied in presented study promoted alfalfa SDW similar like middle effective strains E1 and P1 (by 27% over Ø). Except strain competition for place on rhizoplan and internal of the root tissue, the important role is played by plants in selecting and enriching the types of bacteria by the constituents of their root exudates. Therefore, the bacterial community in the rhizosphere develops depending on the nature and concentrations of organic constituents of exudates, and the corresponding ability of the bacteria to utilize these as sources of energy (Saharan and Nehra, 2011). It is not certain if plants actively select beneficial soil microbial communities in their rhizosphere. Composition of root exudates was shown to vary with plant species and stage of plant growth (Jaeger et al. 1999).

The presented study showed a significant positive influence of preceding barley inoculation with rhizobacteria alone and their mixture on shoot yield and total N contents of alfalfa. Results indicated that strains of *Azotobacter* sp., *Pseudomonas* sp. and *Enterobacter* sp. alone can be investigated in further researches as potential agent of biofertilizer for plant growth promotion of alfalfa.

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POBOLJŠANJE RASTA LUCERKE, *MEDICAGO SATIVA* L. POMOĆU INOKULACIJE PREDUSEVA RIZOSFERNIM BAKTERIJAMA

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REZIME

U eksprimentu u sudovima ispitana je mogućnost poboljšanja rasta lucerke (*Medicago sativa* L.) pomoću inokulacije ječma (*Hordeum vulgare* L.) kao preduseva bakterijama koje poboljšavaju rast biljaka (PGPR). Cilj eksperimenta je bio odabiranje efikasnih sojeva koji bi se primenili u plodoredu u formi biološkog đubriva. U radu je korišćeno sedam sojeva koji pripadaju sledećim bakterijskim vrstama: *Sinorhizobium meliloti*, *Bacillus megaterium*, *Enterobacter* sp, *Azotobacter* sp, kao i vrsti *Pseudomonas* sp. Efekat inokulacije ječma primenjenim sojevima je određen na osnovu suve nadzemne mase (SDW) i sadržaja ukupnog azota (N) u biljnoj masi lucerke. Rezultati su ukazali na sposobnost nekih sojeva da poboljšaju rast biljaka. Značajno je povećan prinos lucerke u odnosu na kontrolu Ø (lucerka gajena posle neinokulisanog ječma kao preduseva) inokulacijom sojevima *Azotobacter*-a A1 (41%) i A2 (39%) i *Pseudomonas*-a P1 (35%). Sadržaj ukupnog N je bio u korelaciji sa vrednostima SDW. Rezultati su ukazali na uticaj inokulacije ječma kao preduseva na prinos lucerke i njen kvalitet kao i da sojevi *Azotobacter* sp., *Pseudomonas* sp. i *Enterobacter* sp. imaju PGPR potencijal što daje osnovu za dalja ispitivanja i mogućnost primene kao biođubriva.

Ključne reči: lucerka, poboljšanje rasta biljaka, inokulacija, predusev, ječam

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