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Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa* L.)

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ABSTRACT Soil bacteria associated with plant roots that can exert beneficial effects on their hosts are designated as plant growth promoting rhizobacteria (PGPR). Some of these PGPR can enter the root interior and establish endophytic populations. The present study was performed to isolate non-rhizobial endophytes from the surface sterilized root nodules of alfalfa (*Medicago sativa* L.) and assess their effects on alfalfa growth. Out of 15 endophytic non-rhizobial strains isolated, 5 gram-positive strains were selected for further identification and characterisation. The strains LR1k, 4148pk and SNji formed one single cluster in rep-PCR analyses and partial sequences of 16S rRNA genes showed 100% similarity to *Bacillus megaterium*. Strains 251s and 236 displayed two separate rep-PCR patterns and according to 16S rRNA genes sequences they were closely related to *Brevibacillus chosinensis* and *Microbacterium trichothecenolyticum*, respectively. None of the non-rhizobial isolates was able to nodulate alfalfa when re-inoculated in gnotobiotic culture. Co-inoculation of all non-rhizobial strains with *S. meliloti* positively influenced nodule number of alfalfa, but was without significant effect on growth parameters with respect to inoculation with *S. meliloti* alone. However, single inoculation with non-rhizobial strains caused significant increase in shoot and root parameters compared to uninoculated plants, indicating that non-rhizobial strains possess some plant growth promoting potential. Further studies on the interactions among these endophytic bacteria and other legumes or non-leguminous plants are needed.

Key words: alfalfa, endophytic bacteria, plant growth promotion

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a very important forage crop in many countries. It is famous for its excellent nutritive value, high digestibility and a high biomass yield. This species forms N_2-fixing symbiotic association with *Sinorhizobium meliloti*, which may supply most of the N required for plant growth. In this way alfalfa contributes to the incorporation of nitrogen in agriculture systems, with a consequent economic benefit, helping to reduce the application of synthetic N fertilizers (Campillo *et al.* 2003; Jensen & Hauggaard-Nielsen 2003). Additionally, many studies have shown that simultaneous infection with rhizobia and some plant growth promoting bacteria (PGPB), increases nodulation and growth in a wide variety of legumes (Parmar & Dadarwal 1999; Bullied *et al.* 2002; Shaharoona *et al.* 2006; Tilak *et al.* 2006). Most of the tested PGPB strains are plant growth promoting rhizobacteria (PGPR), however endophytic bacteria have recently drawn particular attention as a

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group of potential PGPR (Sturz *et al.* 1997, 2000; Bai *et al.* 2002, 2003; Mrabet *et al.* 2006; Rajendran *et al.* 2008; Ibáñez *et al.* 2009). Endophytic bacteria have been isolated from legume plants such as alfalfa (Gagne *et al.* 1987), clover (Sturz *et al.* 1997), and soybean (Oehrle *et al.* 2000). More recent reports were published by Zakhia *et al.* (2006) who described the association of 14 bacterial genera with wild legume nodules in Tunisia, while Mureus *et al.* (2008) reported over 24 non-rhizobial taxa isolated from nodules of different wild legumes. Bacteria isolated from legume tissues include *Agrobacterium, Bacillus, Curtobacterium, Enterobacter, Erwinia, Mycobacterium, Paenibacillus, Pseudomonas, Phyllobacterium, Ochrobactrum, Sphingomonas* and others. Available reports indicate improved plant yield, plant health and nodulation when co-inoculated with nodule endophytes, compared to inoculation with rhizobia alone (Rajendran *et al.* 2008; Bai *et al.* 2002, 2003; Sturz *et al.* 1997). PGPR can contribute to plant growth by increasing nitrogen uptake, synthesis of phytohormones (auxin, cytokinin), solubilization of minerals, and iron chelation (Bowen & Rovira, 1999). They may suppress soil-born pathogens by producing siderophores, antimicrobial metabolites, or by competing for nutrients and/or niches (Nelson 2004).

However, in some cases inoculation with the nodule endophytic bacteria had a negative effect on growth and yield parameters. *Agrobacterium* strains specifically reduced the nodulation of *Rhizobium gallicum* in the common bean (Mrabet *et al.* 2006), but they did not affect nodulation of *Sinorhizobium meliloti* with alfalfa (Wang *et al.* 2006).

Therefore, the aim of this study was to isolate and identify non-rhizobial bacteria from alfalfa root nodules found in Serbian fields and to evaluate their influence on alfalfa alone and its symbiosis with *S. meliloti*.

MATHERIAL AND METHODS

Isolation of nodule endophytes. Root nodu-les were sampled from alfalfa plants grown in fields in the central part of Serbia. The nodules were selected randomly and the isolation of the nodule endophytic bacteria was performed by a standard procedure using yeast extract-mannitol agar medium (YMA) containing congo red (Vincent, 1970). To test the surface-sterilization process, aliquots of the sterile distilled water used in the final rinse were plated onto YMA medium and the plates were incubated at 28˚C for 4 days (Kuklinsky-Sobral *et al.* 2004). All strains were incubated at 28˚C. Gram staining was performed with exponentially growing cultures. The nodule formation was checked for each isolate by inoculating alfalfa seedlings as described by Vincent (1970). The non-symbiotic gram-positive bacteria were further characterized in this work.

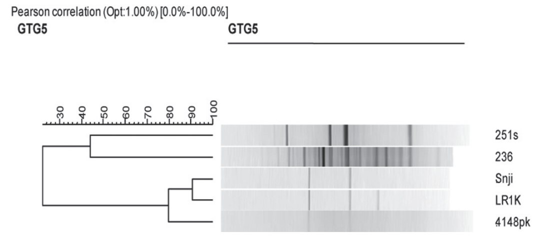

Fig. 1. Clustering and electrophoresis patterns generated by rep-PCR among non-rhizobial isolates from alfalfa.

Bacterial strains and growth conditions. Strains LR1K, 4148pk, SNji, 251s and 236 were isolated in this work. *Sinorhizobium meliloti* L3Si strain was previously identified and selected from field populations of alfalfa for its high capacity to fix nitrogen in symbiosis (Stajković *et al.* 2008). The strains were cultivated in yeast extract-mannitol broth in 250 ml flasks shaken at 125 rpm at 28˚C. The 78h old culture of *S. meliloti* and 24h old cultures of non-rhizobial strains were used as inocula.

Rep-PCR and 16S rDNA sequencing. The strains were characterised by rep-PCR analyses with GTG5-primer (Versalovic *et al.* 1994) and by partial 16S rDNA gene sequencing (Gaunt *et al.* 2001). Both data types were analyzed using BioNumerics 4.0 software (Applied Maths) and sequence data were submitted to a FASTA search (Pearson 1990).

Effects of nodule endophytic bacteria on alfalfa in sterile conditions. Seeds of alfalfa (*M. sativa* L. variety K28) were surface-sterilized with 0.1% $HgCl_2$ solution and seed germination, inoculation, and incubation of the plants were performed as described by Vincent (1970). The plants were grown in sterile conditions in 250 x 25 mm glass tubes containing 30 ml of Jensen medium (Vincent 1970). The isolates alone and the mixture of each isolate with *Sinorhizobium meliloti* L3Si (1:1) were used for inoculation of alfalfa; 0.5 ml of each bacterial culture was used for treatment. Blank controls without inoculation (without mineral nitrogen – Ø, and with full N content (0.5 g KNO_3 l^{-1}) - NØ) and nodulation control inoculated with *S. meliloti* L3Si were included for comparison. The plants were exposed to an 18h light regime and 26°C day/17˚C night in a growth chamber. The number of nodules and shoot and root length were recorded after 6 weeks of growth of the plants. Plant shoots, roots and nodules were dried in an oven at 70˚C to constant weight and the average dry weight per plant was calculated. The percentage of shoot and root nitrogen was determined from dried and grinded plant samples using the CNS analyser and it was used to calculate total N content in mg per plant.

Table 1. Biochemical characterisation of the strains. *diameter of halo in mm; Neo (Neomycin 120 μg/plate), Nov (Novobiocin 5 μg/plate), Trim (Trimetoprim 5 μg/plate), Bac (Bacitracin 40 U/plate), Cef (Cefalexin 30 μg/plate), Cli (Clindamicine 2 μg/plate)

Strain	Gram reaction	P solubilization		NH_3	Organic acid	Antibiotics resistance					
		PVK*	NBRIP*			Neo	Nov	Trim	Bac	Cef	Cli
S. meliloti L3Si	-	9	9	-	-	-	-	+	-	+	+
Bacillus sp. LR1k	+	1	-	+	-	-	-	+	-	-	+
Bacillus sp. 4148pk	+	1	-	-	-	-	-	+	-	-	+
Bacillus sp. SNji	+	2	-	+	-	-	-	+	-	-	-
Microbacterium sp. 236	+	1	-	-	-	+	+	+	-	+	+
Brevibacillus sp. 251s	+	1	-	+	-	-	-	+	-	-	-

Effects of *Bacillus* spp. strains on alfalfa in non-sterile soil. For detecting the co-inoculation response under non-sterile soil conditions, unsterilized soil poor in *S. meliloti* population was used. The experiment was designed with 3 inoculated and 2 uninoculated treatments (Ø and NØ) with three replications in a randomized complete block system. For this experiment, 12 cm diameter plastic pots were filled with soil. The soil was saturated with water before sowing. The surface-sterilized seeds were inoculated either with *S. meliloti* L3Si strain alone, or with a mixed culture of *Bacillus* spp. strains in a ratio of 1:1:1; 1ml of each bacterial culture or mix was used for treatment. Ten seeds per pot were planted. Thinning of seedlings to 5 was done after 2 weeks. The pots were kept in a closed greenhouse in semicontrolled conditions for two months. Roots were carefully removed from the pots, washed free of soil and the root and shoot portions of alfalfa were separated and measured. Shoot and root dry weight, as well as the percentage of nitrogen, were determined as in the experiment in sterile conditions. The data were statistically processed by the LSD and Duncan test using SPSS 10.0 computer program.

Phosphate solubilization. Bacterial strains were tested by plate assay using Pilovskaya medium (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP) supplemented with 1.5% Bacto-agar (Pikovskaya 1948; Nautyal 1999). Strains were stabbed on plate in triplicate using sterile toothpicks. The halo and colony diameters were measured after 3 days of incubation at 28°C. Halo size was calculated by subtracting colony diameter from the total diameter.

Organic acid production. Bacterial cultures were grown in MM9 agar medium (Sambrook & Russell 2001) and observed for drop in pH using methyl red as an indicator dye which changed from yellow to pink below pH 5.0. Isolates having the ability to produce organic acid gave a pink zone around the colony.

Ammonia production. Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72h at 28°C. Nessler's reagent (0.5 ml) was added in each tube. The development of colour from yellow to brown was a positive test for ammonia production (Cappuccino & Sherman 1992).

Intrinsic antibiotic resistance. Intrinsic tole-rance to different antibiotics (Neomycin 120 μg/plate, Novobiocin 5 μg/plate, Trimetoprim 5 μg/plate, Bacitracin 40 U/plate, Cefalexin 30 μg/plate, Clindamicine 2 μg/plate) was tested by streaking the strains on YMA supplemented with antibiotics and incubating at 28°C for 24h. The appearance of visible growth within 24h was taken as an indicator of resistance to the applied concentration of the antibiotic.

RESULTS AND DISCUSSION

Isolation and characterization of endo-phytic bacteria. Out of 15 endophytic non-rhizobial strains isolated, 5 strains showing gram positive reaction (Table 1) were selected for further characterisation considering that some nodule endophytes identified as *Bacillus* spp. improved nodulation and plant yield when co-inoculated with rhizobium (Bai *et al.* 2002, 2003; Rajendran *et al.* 2008). All isolated strains coexisted with symbiotic *Rhizobium* strains, since both were isolated from the same nodules. Not even one bacterial colony was observed from the aliquots of the sterile distilled water used in the final rinse of nodule surface sterilization. None of these non-rhizobial isolates could nodulate alfalfa when re-inoculated in gnotobiotic culture.

Strains 4148pk and SNji showed large (5 mm), opaque, light red colonies with circles, while LR1k formed large intensely red colonies, also with circles. Orange, medium-sized, opaque colonies were observed for strain 236, while small (1.5 mm), transparent colonies were obtained for strain 251s.

The strains were grouped into three different rep-PCR types, taking into account the coefficient value 0.85 as a cut-off. Strains LR1k, 4148pk and SNji formed one single cluster, while strains 236 and 251s each displayed a unique rep-PCR profile (Fig. 1). Partial sequences of 16S rRNA genes of LR1k, 4148pk and SNji strains showed 99.6%, 100% and 100% similarity to *Bacillus megaterium*, respectively. Strains 251s and 236 according to 16S rRNA genes sequences were closely related to *Brevibacillus chosinensis* and *Microbacterium trichothecenolyticum* respectively (99.5% and 99.1% similarity).

None of the non-rhizobial strains could strongly solubilize phosphate in PVK and NBRIP media, as opposed to *S. meliloti* L3Si. None of the tested strains could produce organic acids, but strains SNji, 251s and LR1k were positive for ammonia production. All strains were sensitive to 40 U/plate of bacitracin and resistant to 5 μg/plate of trimetoprim; 4148pk was additionally resistant to clindamicine, while strain 236 was resistant to all tested antibiotics (Table 1).

Inoculation in gnotobiotic conditions. Plants inoculated with *S. meliloti* L3Si strain showed significant increase in all investigated growth parameters in comparison to control uninoculated plants without N (Ø) (Tab. 2).

Additionally, the values of shoot length and shoot dry weight as well as of shoot nitrogen content achieved by these plants were similar to those obtained for control uninoculated plants with full N content (NØ) indicating highly effective symbiosis of alfalfa and *S. meliloti* L3Si strain. Single inoculation with all non-rhizobial strains caused significant increase in shoot and root parameters compared to Ø control, but obtained values were lower than in NØ and L3Si (Table 2). The strains of *Bacillus* sp. were more efficient than the remaining two strains.

Co-inoculation of all non-rhizobial strains with *S. meliloti* L3Si was found to positively influence nodule number, while in the case of LR1k and SNji strains nodule dry weight was also increased, with respect to inoculation with *S. meliloti* L3Si alone. Additionally, an increase in shoot and root dry weight in co-inoculated treatments was detected, however without significance (Table 2).

Inoculation in soil. Experiments with non-sterile soil in pots showed extra-ordinary capacity of *S. meliloti* L3Si strain to fix N in the symbiosis with alfalfa; the significant increase in all measured parameters was observed in respect to NØ control. Inoculation with the *Bacillus* strains mix (LR1k, 4148pk, SNji) increased the root dry weight and root nitrogen to the level detected in the NØ control while all the other parameters were at the level of uninoculated control Ø (Table 3). Co-inoculation of *S. meliloti* and *Bacillus* strains caused decrease in all parameters with respect to inoculation with *S. meliloti* alone.

Root nodules of leguminous plants were found to host large population of endophytic bacteria of diverse genera and species which are unrelated to rhizobial symbiotic nitrogen fixing bacteria (de Lajudie *et al.* 1999; Gao *et al.* 2001; Zakhia *et al.* 2006, Kan *et al.* 2007; Mureus *et al.* 2008; Li *et al.* 2008). These non-rhizobial nodule endophytes improved plant growth and nodulation when co-inoculated with rhizobium, compared to inoculation with rhizobium alone (Mishra *et al.* 2009; Rajendran *et al.* 2008; Bai *et al.* 2002, 2003).

In this study we identified the alfalfa nodule enodophytes that belonged to three different genera: *Bacillus*, *Microbacterium* and *Brevibacillus*. *Bacillus* species comprise one of the most common soil bacteria and they are frequently isolated from the rhisospheres of plants, as well as from different plant tissues. The occurrence of *Bacillus* species as nodule endophytes has been reported for soybean (Bai *et al.* 2002), red clover (Sturz *et al.* 1997), pigeon pea (*Cajanus cajan*) (Rajendran *et al.* 2008), Kudzu (*Pueraria thunbergiana*) (Selavkumar *et al.* 2008), *Calycotome villosa* (Zakhia *et al.* 2006) and different wild legumes (Mureus *et al.* 2008). *Microbacterium* and *Brevibacillus* species were also isolated from different tissues and plant nodules (Zakhia *et al.* 2006; Sturz *et al.* 1997).

In our experiments, the positive effect of alfalfa inoculation obtained with all non-rhizobial strains alone with respect to inoculation with *S. meliloti* strain L3Si was minor. Moreover, co-inoculation of the plants with the non-rhizobial strains and *S. meliloti* L3Si in laboratory conditions was without significant effect on growth parameters, compared to inoculation with *S. meliloti* L3Si alone, although it positively influenced nodule number. Rosas *et al.* (2006) assessed that co-inoculation of alfalfa with *S. meliloti* and *Pseudomonas* strains did not differ in respect to inoculation with *S. meliloti* alone, but the same *Pseudomonas* strains when co-inoculated with *B. japonicum* promoted both nodulation and growth of soybean. The results of this and our study imply that the inoculation with effective *S. meliloti* strains alone could be sufficient for successful alfalfa growth. On the other hand, considering that the effects of PGPR depend on the host plant (Rosas *et al.* 2006; Mrabet *et al.* 2006; Wang *et al.* 2006) the non-rhizobial strains isolated in our study could be tested for plant growth promotion in other legumes.

In the soil experiment when alfalfa was co-inoculated with the mixed culture of *Bacillus* spp. isolates and *S. meliloti* L3Si, a decrease in all growth parameters was detected compared to inoculation with L3Si alone. It is known that the performance of PGPR may differ due to environmental factors, including the presence of other microorganisms that may affect growth of PGPR and exert their own effect on the plant (Chanway & Holl, 1993;

Table 2. The effect of inoculation with non-rhizobial strains and co-inoculation with *S. meliloti* on nodulation and growth parameters of alfalfa in gnotobiotic conditions. The means marked with the same letter do not differ significantly (p<0.05)

Strain	Nodule number per plant	Nodule dry weight (mg plant^{-1})	Shoot length (cm)	Shoot dry weight (mg plant^{-1})	Root dry weight (mg plant^{-1})	Shoot nitrogen (mg plant^{-1})
L3Si	5.40 c	1.27 c	26.62 ab	65.81 b	17.67 b	2.476 ab
LR1k	0	0	13.17 c	25.43 c	5.10 e	0.381 c
4148pk	0	0	15.08 c	28.74 c	10.37 d	0.373 c
SNji	0	0	11.55 c	26.61 c	9.67 d	0.372 c
236	0	0	12.00 c	13.11 d	5.37 e	0.157 cd
251s	0	0	9.47 c	12.51 d	5.95 e	0.187 cd
LR1k + L3Si	7.33 b	2.13 a	27.40 a	68.30 ab	18.37 b	2.664 ab
4148pk + L3Si	5.75 bc	1.70 b	26.12 ab	66.93 ab	18.01 b	2.610 ab
Snji + L3Si	7.50 b	2.45 a	28.33 a	70.15 a	16.30 bc	2.666 ab
236 + L3Si	11.60 a	1.12 c	25.30 b	64.78 b	17.50 b	2.397 b
251s + L3Si	10.70 a	1.05 c	24.10 b	63.45 b	16.09 c	2.411 b
Ø	0	0	6.35 d	6.50 d	4.98 e	0.070 d
NØ	0	0	28.00 a	74.44 a	23.27 a	2.749 a
LSD 0.05	1.43	0.40	1.32	3.02	1.04	0.170

Table 3. The effect of inoculation with non-rhizobial strains and co-inoculation with *S. meliloti* on alfalfa growth in the non-sterile soil. *Bacillus* spp. mix contained LR1k, 4148pk and Snji strains; the means marked with the same letter do not differ significantly (p<0.05)

Strain	Shoot length (cm)	Root length (cm)	Shoot dry weight (mg plant^{-1})	Root dry weight (mg plant^{-1})	Shoot nitrogen (mg plant^{-1})	Root nitrogen (mg plant^{-1})
L3Si	31.68 a	17.13 a	230.73 a	90.43 a	8.58 a	2.40 a
Bacillus mix	21.00 c	17.80 a	183.53 b	63.17 b	6.51 c	1.89 b
Bacillus mix + L3Si	26.50 b	17.85 a	115.00 c	48.17 c	6.08 c	1.36 bc
Ø	21.37 c	16.84 a	71.50 d	29.67 d	3.80 d	0.97 c
NØ	27.37 ab	16.60 a	183.78 b	71.57 b	7.21 b	1.77 b
LSD 0.05	2.07	0.94	12.43	7.14	0.72	0.67

Zhender *et al.* 1999; Bent *et al.* 2001). The negative effect of co-inoculation of *Bacillus* spp. strains and *S. meliloti* in the soil experiment could be due to the lower density of rhizobia than in the laboratory experiment (Mrabet *et al.* 2006), or the consequence of competition of *Bacillus* spp. strains with each other or with other soil bacteria for environment (nodules) and nutrients. To clarify the differences observed in laboratory and soil experiment further investigations are required.

It has been reported that the bacteria from genera *Bacillus*, *Microbacterium* and *Brevibacillus* promoted growth and yield of different non-leguminous plants (Karlidag *et al.* 2007; Orhan *et al.* 2007; Silva *et al.* 2008). In our study, inoculation with non-rhizobial strains alone significantly increased the shoot and root parameters in alfalfa with respect to uninoculated plants (Ø), both in laboratory and soil experiment, indicating that the non-rhizobial strains possess some plant growth promoting potential and could be useful for non-leguminous plants. PGPR can affect plant growth through various mechanisms and most PGPR may have multiple mechanisms of action (Bowen & Rovira, 1999; Nelson 2004). In our experiments, the non-rhizobial strains were poor phosphate solubilizers, but were positive for ammonia production; however, additional studies are needed to elucidate mechanisms by which the non-rhizobial strains elicit plant growth promotion.

CONCLUSION

The present study showed the presence of endophytic non-rhizobial bacteria in alfalfa nodules. They belonged to some of the most common genera connected with PGPR (*Bacillus*, *Brevibacillus*, *Microbacterium*). Although the results indicate that co-inoculation of *S. meliloti* and these strains do not affect alfalfa growth, the fact that non-rhizobial strains showed some plant growth promoting potential when inoculated alone indicates that they should be used in further research with other legumes or non-leguminous plants.

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REZIME

Izolacija i karakterizacija endofitnih nerizobijalnih bakterija iz nodula lucerke (*Medicago sativa* L.)

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Zemljišne bakterije koje u asocijaciji sa korenima biljaka pozitivno utiču na rast svojih domaćina označene su kao PGPR (plant growth promoting rhizobacteria). Neke od PGPR mogu da prodru u unutrašnjost korena i formiraju endofitne populacije. U ovom radu je izvršena izolacija endofitnih nerizobijalnih bakterija iz nodula lucerke (*Medicago sativa* L.) i ispitan njihov efekat na rast ove leguminoze. Od 15 nerizobijalnih izolata, 5 gram-pozitivnih sojeva je izabrano za dalju identifikaciju i karakterizaciju. Na osnovu rep-PCR profila sojevi LR1k, 4148pk i SNji formirali su jedan klaster, a parcijalno sekvenciranje 16S rRNK gena pokazalo je 100% sličnosti sa *Bacillus megaterium*. Sojevi 251s i 236 pokazali su dva odvojena rep-PCR profila i po sekvenci 16S rRNK gena bili su veoma slični *Brevibacillus chosinensis*, odnosno *Microbacterium trichothecenolyticum*. Nijedan od nerizobijalnih izolata nije nodulisao lucerku kada je izvršena reinokulacija u kontrolisanim uslovim. Koinokulacija lucerke sa nerizobijalnim sojevima i *S. meliloti* pozitivno je uticala na broj nodula, ali nije imala značajan efekat na parametre rasta u odnosu na inokulaciju sa *S. meliloti*. Međutim, inokulacija nerizobijalnim sojevima uzrokovala je značajno povećanje parametara rasta u odnosu na neinokulisane biljke, ukazujući da nerizobijalni sojevi poseduju PGP potencijal. Potrebna su dalja ispitivanja interakcije ovih endofitnih bakterija sa drugim leguminozama ili neleguminoznim biljkama.

Ključne reči: lucerka, endofitne bakterije, poboljšanje biljnog rasta

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