

Alfalfa yield and nutrient uptake as influenced by co-inoculation with rhizobium and rhizobacteria

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OLIVERA STAJKOVIĆ-SRBINOVIĆ^{1*}, DUŠICA DELIĆ¹, BORIS NERANDŽIĆ¹,
SRDJAN ANDJELOVIĆ², BILJANA SIKIRIĆ¹, DJORDJE KUZMANOVIĆ¹,
NATAŠA RASULIĆ¹

¹ Institute of Soil Science, Teodora Dradžera 7, Belgrade, Serbia

² Delta Agrar, Autoput za Zagreb 35, 11070 New Belgrade, Serbia

*Address for correspondence to: oliverastajkovic@yahoo.com

Abstract

A two-year field trial was conducted to evaluate the effects of single and co-inoculation of alfalfa with *Sinorhizobium meliloti* and different rhizobacteria on dry matter yield and nitrogen and phosphorus contents. Inoculation was performed with *S. meliloti* strain L3Si and strains of genera *Pseudomonas* (strains luc2 and LG), *Bacillus* (strains BB and SNji), *Azotobacter chroococcum* (strains AC and AZ) and *Enterobacter* (strain E1) at the time of sowing. There were 16 different treatments and the control without inoculation and fertilization. Improved dry matter yield and nitrogen content in inoculated alfalfa plants were noted in respect to control plants and/or inoculation with *Sinorhizobium* alone. In the first year, the increase in dry matter yield ranged between 17% (*Sinorhizobium*) and 67% (*Azotobacter* AC) in single inoculation and from 20% (*Sinorhizobium* + *Bacillus* BB) to 84% (*Sinorhizobium* + *Pseudomonas* LG) in co-inoculation over control. It is noticeable that single inoculation with some rhizobacterial strains, *Bacillus* sp. BB, *Enterobacter* E1, and *A. chroococcum* AC increased alfalfa yield even over rhizobium inoculation (22, 27 and 43% respectively over rhizobium inoculation). In most of the cases co-inoculation of rhizobacterial strains with rhizobium increased alfalfa yield over rhizobium inoculation, indicating positive interactions with rhizobium. In the second year, the increase in alfalfa yield was mainly reduced in the co-inoculated treatments compared to the first year, while in treatments with single rhizobacterial inoculation it retained the same % of increase over control and/or rhizobium inoculation.

Keywords: *Medicago sativa* L., plant growth promotion, co-inoculation, nitrogen, phosphorus

1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops in many countries due to a high biomass yield, excellent nutritive value and high digestibility. Alfalfa can establish a nitrogen fixing symbiosis with soil bacteria, rhizobia (*Sinorhizobium meliloti*) and fix atmospheric nitrogen of the benefit to the plant. Symbiotic association of alfalfa/*S. meliloti* is one of the most efficient interactions between rhizobia and legume plants, taking into account the amount of N usually fixed by alfalfa in the field of 140–210 kg ha⁻¹ per year with the assessed potential of even 550 kg ha⁻¹ per year (PROVOROV & TIKHONOVICH [1]). In this way, alfalfa contributes to the incorporation of N in the soil, with a consequent economic and ecologic benefit, helping to reduce the application of synthetic N fertilizers (CAMPILLO & al. [2], JENSEN & HAUGGAARD-NIELSEN [3]). Besides nitrogen-fixing symbiosis with rhizobia, alfalfa has the ability to tolerate heavy metals. Therefore, it could play an important role in the restoration of nitrogen-depleted soils and might be used as a

priority pioneer species for the rehabilitation of degraded and contaminated soils (GARDEA-TORRESDEY & al. [4], GUEYE & al. [5]).

Many studies have shown that simultaneous infection with rhizobia and some plant growth promoting rhizobacteria (PGPR) can increase growth in respect to rhizobium inoculation alone in a wide variety of legumes including alfalfa (HUNGRIA & al. [6,7], STAJKOVIĆ & al. [8,9], ITZIGSOHN & al. [10]). Plant growth promoting bacteria represent a group of beneficial soil microorganisms capable of promoting plant growth by several biological processes, including the production of plant growth hormones, siderophore production, ability of phosphate solubilization, and also biological nitrogen fixation. The most studied PGPR and employed as inoculants worldwide belong to the genus *Bacillus*, *Enterobacter*, *Azotobacter* and *Pseudomonas*, *Azospirillum* etc. (HUNGRIA & al. [6]). Taking into account that phosphorus is a second major nutrient in plant nutrition and has a considerable influence on the legume-rhizobia symbiosis, PGPR which possess ability of phosphate solubilization could be of particular importance in legume production improvement by increasing the available P content in soil (COLLAVINO & al. [11]). This is also important due to the low available P soil supply in Serbia. In the rhizosphere, there are intensive interactions between the plant, soil, microorganisms and soil microfauna. Soil physical and chemical properties (pH, water availability, temperature, salinity, organic matter content, etc.), the presence or absence of pesticides and other substances can affect plant growth and their interaction with soil microflora and fauna (ANTOUN & PREVOST [12]). Taking into account the before-mentioned, the necessity for testing PGPR effects in the field conditions is indicated. In this study, we evaluated the effect of *S. meliloti* and rhizobacteria from the *Bacillus*, *Enterobacter*, *Azotobacter* and *Pseudomonas* genera on alfalfa dry matter yield and N and P contents in the two-year field experiment. All strains used were selected due to some plant growth promoting traits evaluated in the previous experiments.

2. Materials and Methods

The trial was set up in 2010 at Ratare village, Serbia (20°7'15.5"E and 44°39'0.3"N) on fluvisol (FAO, 2006) with clayed loam texture and the following granulometric content and chemical properties: sand 31.3%, silt 32.3%, clay 36.4%, pH 6.95 (in KCl), organic matter 4.23%, CaCO₃ 0.42%, N% 0.22, NH₄-N + NO₃-N 25 mg kg⁻¹, P 34.73 mg kg⁻¹, K 267.27 mg kg⁻¹. In 2010 and 2011 average monthly temperatures during the growing period (from March to October) were 17.3°C and 12.9°C with maximum in July 24.4°C and June 24.8°C, respectively while total amount of rainfall was 865.5 mm and 541.1 mm, respectively. In the past 10 years legumes have not been grown on the experimental field.

Strains *Sinorhizobium meliloti* L3Si, *Bacillus* sp. BB, *Bacillus megaterium* SNji, *Enterobacter* sp. E1, *A. chroococcum* strains AC and AZ, as well as *Pseudomonas* sp. strains luc2 and LG from the Collection of the Institute of Soil Science were used for the inoculation of alfalfa (variety K28, Institute for forage crops, Kruševac, Serbia). *Bacillus* and *Enterobacter* strains and *Pseudomonas* strains were cultivated for 24^h in nutrient broth medium and King B medium, respectively. *S. meliloti* strain was cultivated in yeast mannitol broth (YMB) for 48^h while *Azotobacter* strains were cultivated in N free mannitol broth for 72^h. The culture of 40 ml of each single strain was mixed with 100 g sterile ground peat and after a 15 day incubation period, single inoculums consisting of approximately 10⁹ bacteria g peat⁻¹ were obtained. Alfalfa seeds were inoculated either with *S. meliloti* or rhizobacterial strains alone, or by mixing rhizobium inoculums with *Bacillus*, *Pseudomonas*, *Enterobacter*, or *Azotobacter* inoculums in a ratio of 1:1. The trial was designed with 15 inoculated treatments, treatment with mineral fertilizer (N 60 kg ha⁻¹, P 100 kg ha⁻¹ and K 100 kg ha⁻¹),

and control without mineral N fertilizer and inoculation (Ø). The experiment was laid out in completely randomized design in three replicates. Each plot was planted in 15 rows of 2 m length with 20 cm inter-row spacing according to seed rate of 20 kg ha⁻¹. Plants were harvested in full-bloom stage, in first and second cut, of both years (2010 and 2011). Plant shoots were dried in an oven at 70°C to constant weight and the average dry weight per plot was calculated. The percentage of shoot N was determined from dried and ground plant samples using the CNS analyzer (CNS analyzer, Vario model EL III, Elemental Analysis systems GmbH, Hanau, Germany). In order to determine P content, plant samples were burned to ash and acid digestion with HCl was performed according to Chapman & Pratt [13]. Phosphorus was measured by the colorimetric ammonium vanadate method (Egnér & al. [14]). The data were statistically processed by the LSD and Duncan test using the statistical program SPSS 10.0.

3. Results and Discussions

Two-year field experiment showed a significant effect of *Sinorhizobium meliloti* on alfalfa yield in respect to the control (Table 1). Some rhizobacterial strains individually or when co-inoculated with *Sinorhizobium* significantly increased yield and N content of alfalfa in respect to the control and/or inoculation with *Sinorhizobium* alone. In the first year, in all treatments, except *A. chroococcum* AZ, alfalfa yield increased significantly, compared to the control (Ø). The increase in dry matter yield ranged between 17% (*Sinorhizobium*) and 67% (*Azotobacter* AC) in single inoculation and from 20% (*Sinorhizobium* + *Bacillus* BB) and 84% (*Sinorhizobium* + *Pseudomonas* LG) in co-inoculation over non-inoculated control (Figure 1). Single inoculation with rhizobacterial strains, *Pseudomonas* sp. LG, *B. megaterium* SNji and *Pseudomonas* sp. luc2 increased alfalfa yield at the level of rhizobium inoculation, while strain *Bacillus* sp. BB, *Enterobacter* sp. E1 and *A. chroococcum* AC, increased alfalfa yield even over rhizobium inoculation (22, 27 and 43% respectively, over rhizobium inoculation). This indicates significant potential of these strains to improve legume growth alone. In most of the cases, co-inoculation of rhizobacterial strains and rhizobium increased alfalfa yield over rhizobium or their individual inoculation, indicating positive interactions with rhizobium. However, there were two exceptions, strain *A. chroococcum* AC and co-inoculation *Sinorhizobium* + *Azotobacter* AC had the same effect (46 and 43% respectively over rhizobium inoculation) while co-inoculation *Sinorhizobium* + *Bacillus* BB (3%) had even lower influence than BB (22%) inoculation alone, indicating possible negative interaction between these two strains. It is noticeable that *A. chroococcum* AC realized the same increase in dry matter of alfalfa as co-inoculation which suggests that strain AC alone has higher potential to improve yield alfalfa than *Sinorhizobium*. Plants of the fertilized treatment (NPK) showed middle position in dry matter production considering all treatments (57% increase compared to the control), possibly due to the smaller amount of fertilizer applied.

In the second year, the increase in alfalfa yield was mainly reduced in co-inoculated treatments and NPK fertilized treatment compared to the control (Table 1, Figure 2). Alfalfa dry matter yield in single inoculation retained the same % of increase over control in the second year. In the treatments *Sinorhizobium* + *Azotobacter* AC, *Sinorhizobium* + *Bacillus* SNji, and *Sinorhizobium* + *Pseudomonas* LG, the co-inoculation effect was diminished, while in the treatments *Sinorhizobium* + *Enterobacter* E1, *Sinorhizobium* + *Bacillus* BB, and *Sinorhizobium* + *Pseudomonas* luc2, yield increase corresponded to the single inoculation of rhizobacterial strains. This may indicate that the co-inoculation loses the effect in the second year. Treatment with *Sinorhizobium* + *Azotobacter* AZ retained the same level of increase above rhizobium inoculation alone.

Table 1. Dry matter yield of alfalfa under the inoculation with *Sinorhizobium meliloti* and co-inoculation with plant growth promoting rhizobacteria (g plot⁻¹)

Treatment	2010			2011		
	1 st cut	2 nd cut	Σ	1 st cut	2 nd cut	Σ
<i>Sinorhizobium</i>	60 ^d	157 ^{gh}	218 ^g	550 ^{hi}	527 ^c	1077 ^{ij}
<i>Sinorhizobium</i> + <i>Azotobacter</i> AC	76 ^b	243 ^b	319 ^{bc}	625 ^g	510 ^{cd}	1135 ^{gh}
<i>Sinorhizobium</i> + <i>Enterobacter</i> E1	91 ^a	239 ^b	330 ^{abc}	788 ^c	547 ^c	1335 ^d
<i>Sinorhizobium</i> + <i>Bacillus</i> BB	39 ^f	186 ^{ef}	225 ^g	865 ^b	540 ^c	1406 ^{bc}
<i>Sinorhizobium</i> + <i>Pseudomonas</i> luc2	76 ^b	268 ^a	344 ^a	783 ^c	642 ^b	1425 ^b
<i>Sinorhizobium</i> + <i>Azotobacter</i> AZ	67 ^c	195 ^{cde}	262 ^e	721 ^d	539 ^c	1260 ^e
<i>Sinorhizobium</i> + <i>Bacillus</i> SNji	59 ^d	216 ^c	275 ^{de}	684 ^e	377 ^{fg}	1061 ^{ij}
<i>Sinorhizobium</i> + <i>Pseudomonas</i> LG	90 ^a	247 ^b	337 ^{ab}	645 ^{fg}	399 ^f	1044 ^j
<i>Azotobacter</i> AC	61 ^b	252 ^{ab}	312 ^c	952 ^a	641 ^b	1593 ^a
<i>Enterobacter</i> E1	77 ^b	201 ^{cde}	278 ^{de}	663 ^e	703 ^a	1366 ^{cd}
<i>Bacillus</i> BB	62 ^{cd}	204 ^{cde}	266 ^e	812 ^c	627 ^b	1439 ^b
<i>Pseudomonas</i> luc2	39 ^f	186 ^{ef}	225 ^g	636 ^{fg}	469 ^e	1106 ^{hi}
<i>Azotobacter</i> AZ	26 ^g	149 ^{hi}	175 ^h	535 ^{fg}	339 ^f	874 ^l
<i>Bacillus</i> SNji	49 ^e	172 ^{fg}	220 ^g	567 ^{gh}	518 ^{cd}	1085 ^{hij}
<i>Pseudomonas</i> LG	58 ^d	189 ^{def}	247 ^f	688 ^e	514 ^{cd}	1203 ^f
Ø	44 ^{ef}	138 ⁱ	182 ^h	496 ^j	448 ^e	943 ^k
NPK	85 ^a	207 ^{cd}	293 ^d	682 ^e	485 ^{de}	1168 ^{fg}
LSD	5.13	16.87	18.19	31.1	33.7	50.0

Ø non-inoculated control; NPK- treatment with NPK fertilizer; a-f: Means in a column followed by the same letter are not significantly different, according to Duncan's multiple range test at the 5% level ($p \leq 0.05$).

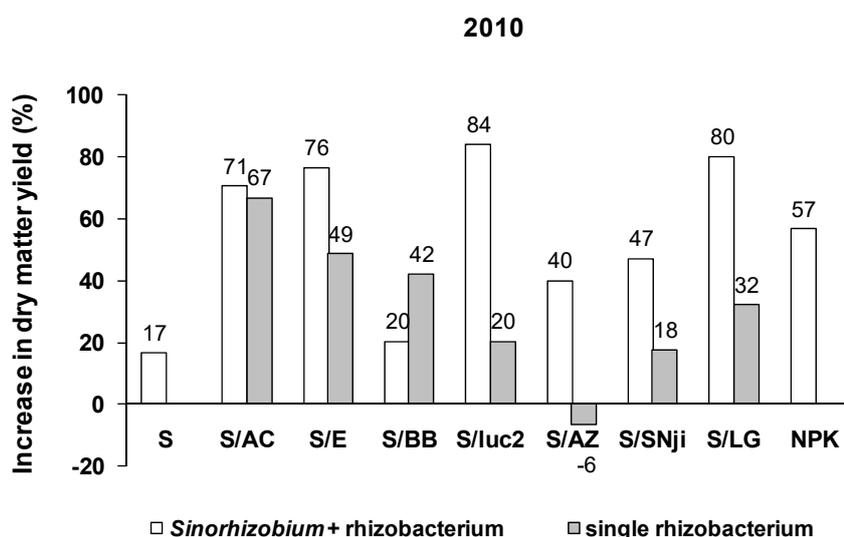


Figure 1. Increase in alfalfa dry matter yield (%) in respect to the control in 2010

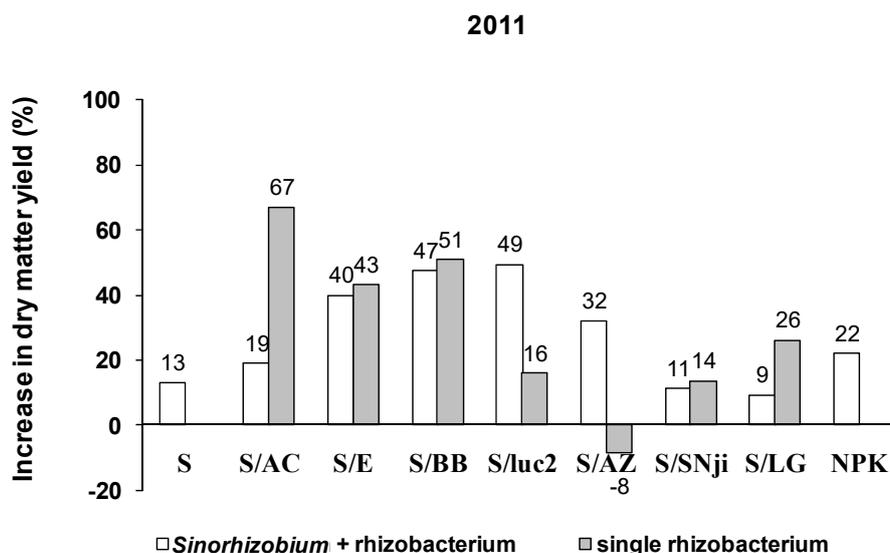


Figure 2. Increase in alfalfa dry matter yield (%) in respect to the control in 2011

Table 2. Nitrogen and phosphorus contents of alfalfa dry matter under the inoculation with *Sinorhizobium meliloti* and co-inoculation with plant growth promoting rhizobacteria (% of dry weight)

Treatment	2010			2011		
	N %	Crude protein %	P %	N %	Crude protein %	P %
<i>Sinorhizobium</i>	4.45 ^b	27.79	0.21 ^{cde}	4.02 ^{ab}	25.13	0.22
<i>Sinorhizobium</i> + <i>Azotobacter</i> AC	4.93 ^a	30.81	0.20 ^{de}	3.67 ^c	22.92	0.22
<i>Sinorhizobium</i> + <i>Enterobacter</i> E1	3.95 ^{def}	24.70	0.20 ^{de}	4.09 ^a	25.54	0.22
<i>Sinorhizobium</i> + <i>Bacillus</i> BB	3.77 ^d	23.57	0.24 ^b	4.25 ^a	26.59	0.20
<i>Sinorhizobium</i> + <i>Pseudomonas</i> luc2	4.39 ^b	27.46	0.21 ^{cde}	3.75 ^c	23.43	0.21
<i>Sinorhizobium</i> + <i>Azotobacter</i> AZ	3.90 ^{def}	22.40	0.20 ^{de}	4.06 ^{ab}	25.19	0.22
<i>Sinorhizobium</i> + <i>Bacillus</i> SNji	3.98 ^{def}	24.16	0.21 ^{cde}	3.66 ^c	25.80	0.21
<i>Sinorhizobium</i> + <i>Pseudomonas</i> LG	3.84 ^{ef}	27.60	0.24 ^b	3.96 ^{bc}	25.36	0.23
<i>Azotobacter</i> AC	3.58 ^g	24.89	0.22 ^c	4.03 ^{ab}	23.56	0.20
<i>Enterobacter</i> E1	3.87 ^{ef}	25.74	0.23 ^{bc}	4.13 ^a	25.65	0.23
<i>Bacillus</i> BB	4.42 ^b	26.64	0.21 ^{cde}	4.06 ^{ab}	23.92	0.21
<i>Pseudomonas</i> luc2	3.98 ^{def}	25.33	0.27 ^a	3.77 ^{bc}	26.63	0.23
<i>Azotobacter</i> AZ	4.12 ^{cd}	24.38	0.19 ^c	4.10 ^a	25.35	0.20
<i>Bacillus</i> SNji	4.26 ^{bc}	24.88	0.21 ^{cde}	3.83 ^{bc}	22.87	0.21
<i>Pseudomonas</i> LG	4.05 ^{cde}	23.99	0.22 ^{bcd}	4.26 ^a	24.77	0.21
Ø	4.00 ^{def}	24.98	0.22 ^{bcd}	4.19 ^a	26.20	0.22
NPK	3.75 ^{fg}	23.45	0.21 ^{cde}	4.13 ^a	25.81	0.24
LSD	0.22		0.024	0.20		0.030ns

Ø non-inoculated control; NPK- treatment with NPK fertilizer; a-f: Means in a column followed by the same letter are not significantly different, according to Duncan's multiple range test at the 5% level ($p \leq 0.05$).

In all treatments, N content in alfalfa dry matter was adequate or high (3.58-4.93%) (Table 2). According to BERGMAN [15], optimal N content is 3-5%, while some authors consider values above 4% as excess. The highest N content, in the first year, was detected in the treatment *Sinorhizobium* + *Azotobacter* AC 4.93% which can be contributed to the mixture of two N-fixing bacteria, but the lowest N content, 3.58% was also in AC inoculation. This decrease can be the result of rapid plant growth and high dry matter production which cause the dilution effect of N and its decrease in plants (TIMMER [16]). Similarly, in the control treatment with low dry matter, N content was the same as in NPK treatment with higher dry matter. The following treatments had the N content among the highest values: *Sinorhizobium* 4.45%, *Bacillus* sp. BB 4.42%, *B. megaterium* SNji 4.26 and *A. chroococcum* AZ 4.12%. However, N content in this treatment is considerably lower in the second year of cultivation and was among the lowest N content values of all treatments. In the second year N content was mainly lower compared to the first, which can be contributed to the overall higher dry matter production and N dilution effect in plants and decreased efficiency of N-fixation. According to BERGMAN [15], optimal P content in alfalfa is 0.3-0.6%, while some authors consider values of 0.2-0.7% sufficient. In this study P content was at the lower limit level or fluctuated about this value, which is possibly the consequence of low P soil supply as common characteristic of soils in Serbia. Significant differences among treatments were determined only in the first year. Noticeably higher P values were in the *Pseudomonas* sp. luc2 0.27% followed by *Bacillus* sp. BB 0.24% and *Pseudomonas* sp. LG 0.24% of P. Some of these strains showed phosphate solubilizing ability in *in vitro* conditions (STAJKOVIĆ & al. [9]). Similar to our study, significant positive effect of alfalfa co-inoculation with rhizobium and rhizobacterial isolates (*Bacillus* and *Azospirillum* sp.) on different growth parameters were previously reported: an increase in plant biomass, accumulation of protein, and increase in the leaf area (STAJKOVIĆ & al. [8], ITZIGSOHN & al. [10]). On the other hand, there are also the studies showing the lack of positive effects of co-inoculation in alfalfa in respect to single rhizobium inoculation, which is also the case with some rhizobacterial strains in our study. ROSAS & al. [17] assessed that co-inoculation of alfalfa with *S. meliloti* and *Pseudomonas* strains did not differ in respect to inoculation with *S. meliloti* alone, while GUIÑAZU & al. [18], reported increased root dry weight, root length, root surface, root surface area and partly shoot N content, but not the shoot dry weight of alfalfa co-inoculated with *Bacillus* or *Pseudomonas* compared to *Sinorhizobium* inoculation alone. Most of the studies evaluated the effects of PGPR on the early aspects of the alfalfa symbiotic relationship, mainly in laboratory or greenhouse conditions, with a few field experiments particularly in multi-year trial. For some other legumes, such as soybean and common bean, there is more evidence about the co-inoculation effect of rhizobia and *Azospirillum* on yield increase in the field condition in multi-year trial (HUNGRIA & al. [6,7]). BAREA & al. [19] reported the interactive effects of *Rhizobium*, mycorrhizal fungi and *Enterobacter* (the phosphate solubilizing rhizobacterium) inoculants to improve shoot dry weight, content of N and P, and the agronomic efficiency of rock phosphate, only if the organic matter amendment was applied. In addition, co-inoculation with rhizobium and *Enterobacter* did not have any effects without mycorrhizal fungi on alfalfa growth. In our research there was no increase in P content in plants inoculated or co-inoculated with phosphate solubilizing strains (LG, luc2, SNji) which is in agreement with BAREA & al. [19] taking into account that neither organic P, nor rock phosphate was added. The lack of the co-inoculation effect of *Bacillus* sp. BB with *S. meliloti* (promoted growth individually) in the first year, could be the result of its lower density in co-inoculation or the consequence of strains competition with each other or with other soil bacteria for environment (nodules) and nutrients (MRABET & al. [20]). The decrease of co-inoculation effect in the second year, besides many other factors, could be the result of the rhizobacteria influence, primarily in the early stage of fast plant development or

the decreased number of rhizobacteria in the soil over time. The later is supported by the fact that annual legumes should be inoculated every time, even if planted on the same location. Data from the field experiment, allow us to validate the interactive effects of rhizobium and rhizobacteria inocula but the mechanisms of such an effect was not easy to resolve. All the strains investigated showed two or more mechanisms which might be involved in plant growth promotion (STAJKOVIĆ & al. [8, 9]). Most of the applied rhizobacteria belong to nitrogen-fixing and phosphate solubilizing bacteria that may be important for plant nutrition by increasing N and P plant uptake (CAKMAKCI & al. [21]. In addition, all the strains in this study produced IAA, phytohormone, which is often directly connected with rhizobacterial potential to stimulate plant growth (SPAEPEN & al., [22]). Phosphate solubilization cannot be considered as the main mechanism, because in numerous studies there was no consistency between the abilities of bacteria to solubilize P and their ability to enhance plant growth and P uptake in crop plants (COLLAVINO & al. [11]) which is also the case in this study. Plant growth promotion could be the result of simultaneous activity of different mechanisms.

4. Conclusion

The results presented showed a significant positive effect of inoculation with rhizobium and different rhizobacteria on growth and N contents of alfalfa plants compared to control and/or the inoculation with rhizobium alone. Co-inoculation of alfalfa with rhizobium and some rhizobacterial strains increased yield over rhizobium inoculation, indicating positive interactions with rhizobium. In the second year, the increase in alfalfa yield was mainly reduced compared to the first year. Our results demonstrate that seed co-inoculation is agronomically efficient and could be of practical benefit in sustainable agricultural practices.

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