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Differences in symbiotic N_2 fixation of alfalfa, *Medicago sativa* L. cultivars and *Sinorhizobium* spp. strains in field conditions

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DUŠICA DELIĆ[1*], OLIVERA STAJKOVIĆ-SRBINOVIĆ[1], JASMINA RADOVIĆ[2], DJORDJE KUZMANOVIĆ[1], NATAŠA RASULIĆ[1], ALEKSANDAR SIMIĆ[3] AND JELENA KNEŽEVIĆ-VUKČEVIĆ[4]
[1]*Institute of Soil Science, Teodora Drajzera 7, Belgrade, Serbia*
[2]*Institute for Forage Crops, Krusevac, Serbia*
[3]*Faculty of Agriculture, University of Belgrade, Belgrade, Serbia*
[4]*Faculty of Biology, University of Belgrade, Belgrade, Serbia*
**Corresponding author: E-mail: vukmirdusica@yahoo.com Tel/fax: +381 11 2667 123*

Abstract

In a two-year field trial, four alfalfa cultivars grown in all combinations with five strains of Sinorhizobium spp. were assessed for N_2-fixing potential and compatibility of symbionts with the aim to co-select plant and rhizobia as alternative approach to symbiotic N_2 fixation improvement. It was found that rhizobial strains, host cultivars and interactions between them had a highly significant effects on shoot dry weight (SDW), total and fixed N content of alfalfa. A significant variation in N_2 fixation effectiveness of rhizobial strains, from ineffective to significantly effective depending on cultivars and year of utilization was detected. Effective strains increased SDW by 49-114% (2010) and 35-86% (2011). In the first year the highest effectiveness had strain L5 with cultivars K-28 and BL-88 and strain L3Si with BL-88 while in the following year all applied strains were effective with different extent. Only one strain (L5) was effective with most of alfalfa cultivars over a two-year period. Percentage of N_2 fixed in plant mass for strain L5 was 33% in 2010, and 38% in 2011. In addition, one cultivar (BL-88) was identified to have superior symbiotic performances, exhibiting its high capacity for N_2 fixation. Results indicate that simultaneous selection of both symbionts should be applied for improvement of N_2 fixation in alfalfa.

Keywords: alfalfa, rhizobia, symbiotic effectiveness, compatibility, co-selection

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important leguminous forage crops widely cultivated throughout the world. Its agronomical interest is based on high herbage yield and protein content, excellent nutritive value, high digestibility and the ability of symbiotic N_2 fixation (SNF) [1]. Deep and extensive root system of alfalfa reduces erosion, improves tilth and water infiltration. Alfalfa improves soil as a result of ability to fix atmospheric nitrogen and has a nitrogen replacement value in succeeding crops. SNF in legumes is a natural process of supplying legume host plant with N by reduction of atmospheric nitrogen (N_2) to NH_3, performed by N_2-fixing nodule bacteria collectively called rhizobia (fam. *Rhizobiaceae*). Before modern mineral fertilizers, legume SNF was the primary nitrogen input for agriculture. SNF is considered to be a significant boost to N fertilization, and additionally as a natural process, it is not hazardous to the environment [2, 3]. Thus it is a healthy, economical source of nitrogen for agriculture.

Symbiotic association between alfalfa, *Medicago sativa* L. and *Sinorhizobium meliloti* (*Ensifer meliloti*) is one of the most efficient interactions between legume plants and N_2-fixing bacteria with the average N_2 fixed amount of 140–210 kg ha^{-1} per year in the field and with the assessed potential of even 550 kg ha^{-1} per year [4].

Various associations of alfalfa cultivars and rhizobial strains differ in symbiotic N_2 fixation capacity which depends upon both symbionts, alfalfa host plant and rhizobium as

well as their interaction [5, 6]. Environmental factors affect the growth and activity of N_2-fixing symbiotic association so an important aim is also to select the symbiotic pairs in field conditions. Alfalfa is highly variable species due to its tetraploidy, cross-pollination and the synthetic status of the varieties. Genetic variation in alfalfa was also caused by allelic variations of primary symbiotic genes, so that symbiosis is a supplementary source of variability [7]. On the other hand, rhizobial variability, whether due to mutation, genome rearrangement or genetic exchange, is a fact of rhizobium life and several hundred of rhizobial "symbiotic" genes were identified controlling nodulation and symbiotic N_2 fixation [4, 8].

High effectiveness of symbiotic association was caused with high compatibility between symbionts and may supply most of the N required for plant growth [9]. Increased efficiency of symbiotic N_2 fixation will be achieved not only by separately selecting the best N_2-fixing host genotypes or rhizobial strains, but also selecting the best combinations of host genotypes and nodule bacteria [6, 10, 11]. Understanding and overcoming these biotic limitations to symbiotic N_2 fixation will be imperative to satisfy the demand for N in sustainable agricultural production system in the future [12].

Presented field experiment was carried out to assess N_2-fixing potential of alfalfa cultivars and specific rhizobial strains and their host-strain compatibility in the field as well as to emphasize the importance of their simultaneous co-selection.

Material and method

Strains and host plant.

Four effective *S. meliloti* strains (224, 218, L5 and L3Si) and one *S. medicae* strain (LR1KS) [13] from the Collection of Institute of Soil Science were applied for alfalfa inoculation. Four alfalfa cultivars from the Institute of forage crops (Serbia) were used as the host plants, K-28 (Serbia), BL-88 (Republic of Srpska), Soča (Slovenia) and Vanda (Slovakia).

Inoculum preparation.

Each of the single rizobial strains was grown in Yeast Mannitol Broth for 48 h at 28°C up to approximately 10^9 cell ml^{-1}. The culture of 40 ml of each single rhizobial inoculum was mixed with 100 g sterile ground peat. After a 15 day incubation period, single inoculums consisted of approximately 10^9 rhizobia g $peat^{-1}$ [14, 15].

Experimental site.

The trial was set up in 2010 and 2011 at Ratare village, Serbia (20˚7'15.5"E and 44˚39'0.3"N) on fluvisol with clayed loam texture and the following granulometric content and chemical properties in Amo horizon: sand 31.3%, silt 32.3%, clay 36.4%, pH 6.95, organic matter 4.23%, $CaCO_3$ 0.42%, N% 0.22, NH_4-N + NO_3-N 25 mg kg^{-1}, P 74.5 mg kg^{-1}, K 322.0 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 alfalfa has not been grown on the experimental field.

Experimental design of field trial.

The trial was designed as two-factorial (bacterial strain and plant cultivar) with 20 inoculated treatments and 8 uninoculated control treatments (4 controls without mineral N fertilizer-Ø and 4 controls with 100 kg of calcium-ammonium-nitrate fertilizer per ha (NH_4NO_3+$CaCO_3$, 27%N)-NØ). Seeds of alfalfa cultivars were inoculated with single strain inoculums. The experiment was laid out in completely randomized design in three replicates. Each plot was planted in five rows of 2 m length with 20 cm inter-row spacing according seed rate of 20 kg ha^{-1}.

Measurement of plant parameters.

Six weeks after sowing (in full-bloom stage) six plants from each plot were randomly collected to evaluate root nodulation. In the second cuts of both years (2010 and 2011), 10 plants (in the full-bloom stage) of each treatment were randomly selected and harvested to determine average values of symbiotic characters: plant shoot dry weight (SDW), as well as %N and total N content in shoot. Total N content in SDW was calculated by percentage of N (CNS analyzer, Vario model EL III, Elemental Analysis systems GmbH, Hanau, Germany). N_2 fixed in shoots was calculated by the N difference method according to equations [8]:

$$N_2 \text{ fixed in shoot} = (\%N \times SDW)_{\text{inoculated treatment}} - (\%N \times SDW)_{\text{Ø (uninoculated control treatment)}}$$

Additionally, percentage of N_2 fixed (%Ndfa) as percentage share of N_2 fixed in total N content was calculated. Data was analyzed using appropriate statistical analyses (ANOVA, correlation coefficient) and Statistica 8.0 software. The level of significance was determined by LSD testing. All references to significance in the text imply statistical significance at $P<0.05$, unless stated otherwise.

Results and discussion

In the field trial four alfalfa cultivars were estimated for their symbiotic characteristics (SDW, total and fixed N content in shoot) with five rhizobial strains (one *S. medicae* strain and four *S. meliloti* strains). Screening of inoculated alfalfa cultivars showed that there were nodules on the roots of tested cultivars while there were no nodules on control plants (data not shown). Analysis of variance in this two-factor experiment indicated that rhizobial strains, host cultivars and interactions between them (strain x cultivar) had highly significant ($P<0.001$) effects on symbiotic characteristics in both experimental years (Table 1) which is in correlation with results of some authors [8, 10, 16, 17].

Effectiveness of N_2 fixation varied in different rhizobia-host combinations in both subsequent years (Table 2 and 3). SDW of all inoculated cultivars ranged 4.74-11.15 g plant^{-1} and 27.05-61.65 g plant^{-1} in 2010 and 2011, respectively. Their shoot total N content was 215.34-473.59 mg plant^{-1} and 952.42-1843.44 mg plant^{-1} in 2010 and 2011, respectively. There was highly positive correlation between SDW and total N content in the both years (2010 and 2011, r=0.881*** and 0.940***, respectively). N_2 fixed varied widely 7.50-248.36 mg plant^{-1} and 230.36-825.35 mg plant^{-1}, in 2010 and 2011, respectively. Only in the year of alfalfa establishment there were highly positive correlations between N_2 fixed and tested symbiotic parameters, SDW (r=0.786***) and total N content (r=0.910***). However, in the second year of alfalfa cultivation, there was low correlation between N_2 fixed and SDW (r=0.280*) as well as total N content (r=0.255*).

Table 1. ANOVA-two factorial analysis of variance for plant characteristic of four alfalfa cultivars inoculated with five *Sinorhizobium* spp. strains

Source of variance	F-value [a]					
	Shoot dry weight (g plant^{-1})		Total N content (mg plant^{-1})		Fixed N content (mg plant^{-1})	
	2010	2011	2010	2011	2010	2011
Cultivars	16.180***	49.609***	40.380***	167.648***	219.591***	27.165***
Strains	8.299***	51.852***	25.587***	247.568***	55.355***	21.591***
Interaction	4.307***	23.066***	14.890***	103.246***	120.997***	20.846***

[a] *, **, *** Significant at $P<0.05$, 0.01 and 0.001, respectively

[a] F-value- calculated for 10 plants

SDW is the best parameter to evaluate symbiotic activity of legume-rhizobium associations [18, 19]. Rhizobial strain is considered effective if SDW of inoculated plants is significantly higher than SDW of control untreated plants-Ø. Our results showed that in 2010 strain activities were ranged from ineffective to effective while in 2011 strains were characterised by different extent of effectiveness. In the first year (Figure 1), SDW of plants inoculated with effective strains was increased by 49-68% and even up to 114% in cultivar K28 (comparing to uninoculated control Ø) while in the second year the increase was 35-86% (Figure 2). These results indicate significant ability of particular *Sinorhizobium* strains to improve yield of alfalfa cultivar in the field conditions.

Increased efficiency of symbiotic N_2 fixation can be achieved by selecting the best rhizobial strains as well as best combinations of plant genotypes and nodule bacteria [4, 6]. A coordinated plant-bacteria breeding is required to create the optimal combinations of partners' genotypes. Therefore, considerable efforts over the years have been detected towards selecting the optimal combination of rhizobial inoculums and the legume genotype for high yield and plant mass quality [4, 6, 17]. In this research, based on SDW in the year of alfalfa establishment there were six highly effective symbiotic associations (6/20) without significant differences between them: L3Si/BL-88, L5/K-28, L5/BL-88, L3Si/Soča, 224/BL-88 and 218/BL-88. Among them symbiotic pairs L3Si/BL-88 and L5/K-28 were the most effective in N_2 fixation because fixed significantly the greatest amount of N_2 (248.36 and 230.82 g plant^{-1}, respectively) followed by L5/BL-88. It can be assumed that these two the most effective pairs showing the highest %Ndfa (49% for L3Si/BL-88 and 54% for L5/K-28) possess the greatest N_2-fixing effectiveness as a result of high compatibility. Results are in agreement with the fact that a strain showing superior N_2-fixing characteristics in one cultivar may produce a suboptimal response in another [1].

In the second year the other symbiotic pairs (LR1KS/BL-88, 218/Vanda, L5/Vanda) expressed the highest SDW, significantly different from 50% of all the pairs. They were followed by the next five highly effective pairs: LR1KS/K-28, L5/BL-88, L3Si/Vanda and the best symbiotic pairs from the first year, L3Si/BL-88 and L5/K-28. Values of %Ndfa of this two pairs, L3Si/BL-88 (35%) and L5/K-28 (37%) together with %Ndfa of L5/BL-88 (36%) belonged to the high values of the most of symbiotic pairs. Results are in agreement with a very low coefficient of correlation between N_2 fixed and other two the symbiotic characteristics measured in the second year.

These results showed that rhizobial N_2- fixing activity depended on the host cultivars and the year of alfalfa cultivation. All these effective combinations could replace mineral N fertilizer with 27 kg N ha^{-1} applied in NØ control. This variation in symbiotic efficiency of different symbiotic pairs during the both year of utilization can be affected by the inputs of uncontrolled factors such as individual plant heterogeneity for symbiotic traits, random phenotypic modifications of these traits in each strain-cultivar combination, a plasticity of bacterial genome as well as environmental factors [4, 20].

Table 2. Symbiotic characters of alfalfa cultivars inoculated with different *Sinorhizobium* spp. strains in the first year (2010)

Alfalfa cultivar	Strain 224	L5	LR1KS	L3Si	218	Ø	NØ	Mean
Shoot dry weight (g plant^{-1})								
Vanda	7.25	6.01	7.83	7.03	6.65	4.65	6.49	6.56
Soca	6.87	8.63	6.67	10.16	6.76	6.82	9.86	7.97
BL-88	10.10	10.43	9.02	11.15	9.99	6.87	8.09	9.38

K-28		8.52	11.03	4.74	5.55	6.24	5.15	10.79	7.43
Mean		8.19	9.02	7.06	8.48	7.41	5.87	8.81	
LSD 0.05	Cultivars	0.83							
	Strains	1.10							
	Interaction	2.24							
Total N content (mg plant^{-1})									
Vanda		303.87	231.15	309.99	260.12	289.17	188.89	264.96	264.00
Soca		264.62	330.08	273.46	418.92	287.02	256.83	410.15	319.91
BL-88		336.56	400.68	397.69	473.59	413.28	244.12	317.73	369.37
K-28		353.90	457.84	215.34	236.39	257.45	209.48	421.53	307.32
Mean		314.67	354.93	299.16	347.61	311.96	224.78	353.00	
LSD 0.05	Cultivars	19.32							
	Strains	25.56							
	Interaction	51.11							
Fixed N content (mg plant^{-1})									
Vanda		114.97	42.26	121.01	71.21	100.27			89.97
Soca		7.50	73.26	16.97	162.09	30.20			58.05
BL-88		92.44	156.55	153.57	230.82	170.74			160.82
K-28		144.43	248.36	5.86	26.90	47.11			94.54
Mean		89.88	130.12	74.38	122.75	87.08			
LSD 0.05	Cultivars	8.32							
	Strains	9.31							
	Interaction	18.60							

Uninoculated controls: NØ-with N and Ø-without N; LSD at 0.05 level of probability

The importance of host/strain interaction indicates that symbionts must be matched carefully for optimum N_2 fixation [1]. Cultivar BL-88 followed by K-28 expressed stable high symbiotic efficiency in both years while Soča and Vanda had high effectiveness only in the one year of investigations: Soča in the first year and Vanda in the second one (Tables 1 and 2). Our results indicated that the highest effectiveness had strain L5 with two cultivars (K-28 and BL-88) and L3Si strain with one (BL-88) cultivar in 2010 while in 2011 all applied strains were effective in symbiosis with different extent depend on cultivars (with exception the strain 218 with K-28). Beside SDW and %Ndfa, one of the criteria for selection of the best N_2-fixing strain in two years period is the year of alfalfa establishment bearing in the mind that this year is important for root establishment as well as long life and optimal yield of alfalfa. Thus we could conclude that only one strain (L5) was broadly effective inoculant with most of alfalfa cultivars, including K-28 and BL-88, over a two-year period, spatially in the year of alfalfa utilisation with mean %Ndfa values of 33% in 2010 and 38% in 2011 achieved in fluvisol with optimal content of available N. Some results indicate that highest intensity of N_2 fixation is usually achieved under low or moderate levels of nitrogen fertilizers covering no more than 20–30% of the plant demands [4].

Table 3. Symbiotic characters of alfalfa cultivars inoculated with different *Sinorhizobium* spp. strains in the second year (2011)

Alfalfa cultivar	Strain 224	L5	LR1KS	L3Si	218	Ø	NØ	Mean
Shoot dry weight (g plant^{-1})								
Vanda	38.77	56.52	50.52	55.40	55.78	37.73	49.78	49.35
Soca	28.33	38.54	31.85	27.05	38.83	20.83	78.67	37.73
BL-88	46.14	55.78	61.65	52.71	51.50	34.37	47.92	50.01
K-28	52.27	55.05	56.00	49.02	38.20	36.30	49.57	48.06

Mean		41.37	51.47	50.00	46.04	46.31	32.31	56.49	
LSD 0.05	Cultivars	2.32							
	Strains	3.07							
	Interaction	6.13							
Total N content (mg plant^{-1})									
Vanda		1422.74	1537.42	1682.21	1783.88	1729.86	1192.37	1603.02	1564.46
Soca		1008.31	1152.35	952.42	984.62	1308.68	483.68	2753.57	1234.71
BL-88		1458.02	1751.49	1843.44	1786.76	1700.00	1127.23	1648.45	1616.29
K-28		1594.13	1635.89	1674.40	1549.03	1379.02	1027.29	1457.36	1473.43
Mean		1370.67	1519.08	1538.17	1517.92	1536.92	957.42	1865.67	
LSD 0.05	Cultivars	36.98							
	Strains	48.92							
	Interaction	95.73							
Fixed N content (mg plant^{-1})									
Vanda		230.36	345.04	489.83	591.51	537.49			438.85
Soca		524.98	669.01	469.08	501.29	825.35			597.94
BL-88		330.80	624.27	716.21	626.20	603.18			580.13
K-28		566.84	607.60	647.11	521.74	351.73			539.00
Mean		413.25	561.48	580.56	560.18	579.44			
LSD 0.05	Cultivars	39.03							
	Strains	43.64							
	Interaction	87.45							

Uninoculated controls: NØ-with N and Ø-without N; LSD at 0.05 level of probability

Cultivar BL-88 in symbiosis with all investigated strains expressed stable high symbiotic efficiency in the both years; N_2 fixed of 92.44-230.82 mg plant^{-1} was in 2010 and of 330.80-716.21 mg plant^{-1} in 2011. Average %Ndfa of BL-88 was 44 and 36% in the 2010 and 2011, respectively which is similar with results of Viands et al. [21]. As suggested by Drew and Ballard [8] alfalfa cultivars with a high level of symbiotic characteristics (cultivar BL-88) combined with broadly effective strains as inoculants (strain L5) will give growers the best opportunity of maximising N_2 fixation in the field.

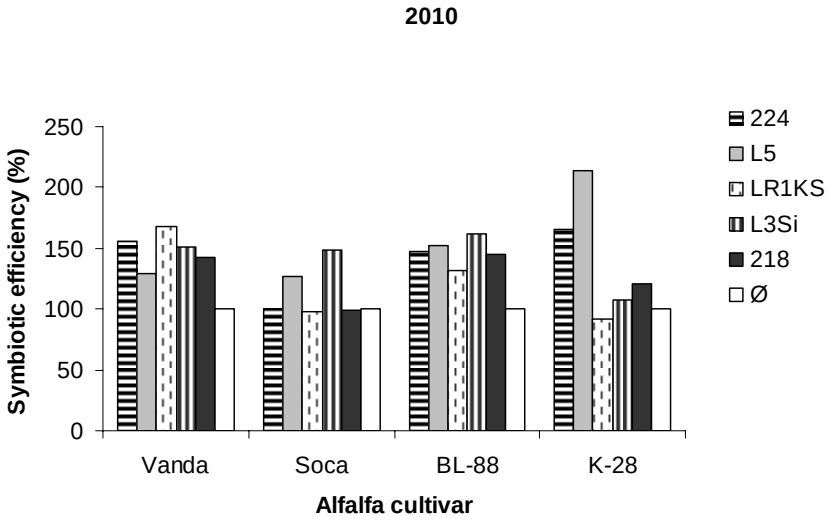

Figure 1. Symbiotic efficiency of different alfalfa cultivar/*Sinorhizobium* pairs in 2010. *S. meliloti* strains: 224, L5, L3Si, 218 and *S. medicae* LR1KS. Ø-uninoculated control without N mineral fertilizer. Symbiotic efficiency- increase of shood dry weight compared to Ø

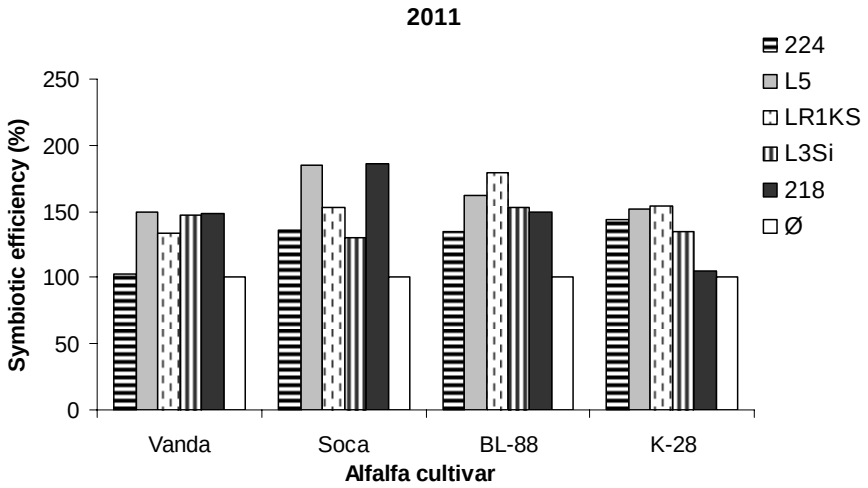

Figure 2. Symbiotic efficiency of different alfalfa cultivar/*Sinorhizobium* pairs in 2011. *S. meliloti* strains: 224, L5, L3Si, 218 and *S. medicae* LR1KS. Ø-uninoculated control without N mineral fertilizer. Symbiotic efficiency- increase of shood dry weight compared to Ø

Conclusions

The results of the present study suggest that SNF in alfalfa depends on plant and bacterial genotypes and simultaneous selection of both symbionts should be applied for improvement of N_2 fixation in alfalfa. Cultivar BL-88 had promiscuous relationship to all strains and could be a good genotype in breeding programs for improved N_2-fixing capacity. The only one strain (L5) was broadly effective inoculant with most of alfalfa cultivars (including BL-88) over a two-year period with ability to improve yield of alfalfa cultivar in the field conditions.

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