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Nodulation and N_2 Fixation Effectiveness of *Bradyrhizobium* Strains in Symbiosis with Adzuki Bean, *Vigna angularis*

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ABSTRACT

In pot experiment, one isolate Knj from a Serbian soil, four strains of Bradyrhizobium japonicum *and three strains of* Bradyrhizobium *spp. were examined for the effect on adzuki bean nodulation and effectiveness in symbiotic N_2 fixation. All the tested strains produced root nodules in adzuki bean. Strains of* B. japonicum *showed high potential of N_2 fixation, particularly 525 and 542.* B. japonicum *strains resulted 65-71% shoot dry weight and 99-138% total N content of uninoculated control with full N content (100%). No significant difference was found between the plants inoculated with* Bradyrhizobium *spp. strains and uninoculated control plants without N (40-42 and 42% shoot dry weight, respectively), which indicated symbiotic N_2 fixation inactivity of the* Bradyrhizobium *spp. strains. Knj strain had the middle position (56% shoot dry weight). These data showed that* B. japonicum *525 and 542 strains could be used in further investigations in order to apply them as inoculants in microbiological N fertilizers.*

Key words: *Bradyrhizobium*, adzuki bean, nodulation, symbiotic N fixation

INTRODUCTION

Adzuki bean, *Vigna angularis* (Willd.) is an important legume and traditional pulse crop in East Asia, widely used as a source of protein for human nutrition, especially in developing countries. In Europe and America, it is valued for its high protein content (25%), low fat, natural sugar nutritional profile, high nutritious value and easy digestibility compared with *Phaseolus vulgaris*. It has following economic importance: environmental (soil improver as a green manure), animal food (forage), medicines (folklore-herb) and for human food (pulse, vegetables, beverage base) (Sato et al., 2005). The ability of adzuki bean to grow in different geographical regions brought the opportunity for introducing this legume into Serbian agricultural practice (Yee et al., 1999; Zong et al., 2003).

Adzuki bean has high physiological nitrogen (N) requirements. However, little is known about its assimilation of N from soil and atmosphere (Kimura et al., 2004). Robinson (1983) found that adzuki bean did not increase yield with inorganic N fertilization, indicating the priority of symbiotic nitrogen fixation (SNF). Adzuki bean fixed N_2 in symbiosis with some bacterial strains of the genera *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (Fujihara et al., 2006). The native populations of rhizobial strains are generally poor efficient in N fixation (Yadav et al., 1996; Vlassak and Vanderleyden, 1997) and inoculation with effective strains as active agent of microbiological N fertilizer is required as an important farming

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method. The formation of effective root nodules leads to N_2 fixation, which makes N mineral fertilizers superfluous. Hence, there is a need to conduct a screening of inoculant strains for better effectiveness.

In this study, some selected rhizobial strains (525, 526. 532, 542 of *B. japonicum*; Knj isolate and 2001, 2801 and 5388 of *Bradyrhizobium* spp) were examined for their ability to promote nodulation of adzuki bean and effectiveness in SNF in order to acquire highly effective strains as the basic agent of microbiological nitrogen fertilizers.

MATERIALS AND METHODS

Bacterial strains and host plant

Four strains of *Bradyrhizobium japonicum* (525, 526, 432 and 532), one isolate (Knj) of *Bradyrhizobium spp*, from the Collection of Institute of Soil Science, Belgrade and three *Bradyrhizobium* spp. strains (2001, 2801 and 5388), specific to adzuki bean from the Collection of Faculty of Agronomy, University of Buenos Aires were tested. Adzuki bean, *Vigna angularis* L., was used as the host-plant. The seeds (domestic population) were obtained from the "Selsem", company for breeding and seed production (Belgrade, Serbia).

Nodulation test and symbiotic properties

Strain nodulation ability was tested on adzuki bean plants in a pot experiment conducted in a semi-controlled greenhouse environment (Vincent, 1970). The clay pots were filled with fine grade (2-3 mm) sand substrate, covered with plastic wrap and sterilized by autoclaving for two hours twice. The seeds for sowing were surface sterilized by 96% ethanol (30s) and 2‰ $HgCl_2$ (3 min), respectively and washed 6-8 times with sterile water. The seedling was performed by sterile pincers, five seeds per pot. The experiment was designed with eight inoculated and two controlled uninoculated treatments with three replications in a randomized complete block system. The seeds of each inoculated treatment were inoculated with 1 ml of suspension of single strain which were grown in yeast extract mannitol broth for 72 h at 28^0C. All the operations (inoculation and planting) were carried out aseptically in a laminar flow chamber. Inoculated plants were grown on sand with N-free Jensen nutrient solution. Uninoculated control plants were grown on sand with N-free (Ɵ) and full N rate as KNO_3 (0.05%, i.e. 500 mg l^{-1}) (NƟ) Jensen nutrient solution (Vincent, 1970). The plants were watered according to need and with sterile Jensen nutrient solution (with or without N) at 15-days intervals. They were harvested in phase of the third trefoil of NƟ (six weeks). The number of nodules was recorded after uprooting the plants, followed by dipping the roots in water to remove sand without losing the nodules. Nodules were collected from the roots and counted. Their average number was expressed as number of nodules per plant. The size of nodule clusters was measured by ruler while the size of single nodules was determined by sieving successively trough screens with different openings (Fujihara et al., 2006). The cross-sections of nodules and differences in the colour of nodular tissue were used for preliminary fast screening effective and ineffective nodules. The ineffective nodules were white to light green inside, while the effective nodules were characteristically pinkish brown (Gwata et al., 2003).

The root and shoot portions of adzuki bean were separated and nodules' fresh weight, shoot and root were measured. Plants shoots, roots and nodules were dried in an oven at 70^0C to constant weight and the dry weight per plant was determined.

The percentage of shoot and root nitrogen was determinated by micro-Kjeldahl method and it was used to calculate total and fixed N (only for shoot) contents in mg per plant. All the data were statistically processed by the LSD and Duncan test using SPSS 10.0 computer program. Correlation analysis between parameters of nitrogen fixation was also conducted.

RESULTS

All the tested strains were able to nodulate adzuki bean. However, there were major differences in nodule number, position and the looks of nodules induced with different strains. Also, there were differences in habitus of plants. Plants inoculated with the strains of *B. japonicum* had dark green leaves and dark-beige, circular shape nodules (1-2 mm) in the crown of the root system, forming a cluster (0.5-1.0 cm), or being arranged in rows on the root hair. These were visual indicators of active nodules. The plants inoculated with the Knj isolate had light green leaves and dark-beige, circular shape nodules (1-2 mm) placed on the root hair, usually arranged in rows and in sporadic clusters. The interior nodules colour of *B. japonicum* strains and Knj was pinkish-brown, indicating effective nodules. The plants inoculated with strains of *Bradyrhizobium* spp. had light green leaves, single light-beige nodules (1 mm) placed on the root hair all over the initial third part of the root. White interior colour of these nodules indicated either poor effective, or ineffective nodules. The average number of nodules ranged from 21.1 to 31.4 for Knj and *B. japonicum* strains and from 6 to 7 per plant in *Bradyrhizobium* spp. strains. Similar to the average nodule number, Knj and *B. japonicum* strains stood out compared to *Bradyrhizobium* spp. strains in respect to the average nodule dry weight. The average nodule dry weight per plant ranged from 43.2 to 68.8 in strains *B. japonicum* and Knj and from 2.8 to 21.45 mg plant^{-1} in *Bradyrhizobium* spp. Maximum number of nodules (31.4 nodule plant^{-1}) was induced by 526 strain, closely followed by Knj (28.8 nodule plant^{-1}), both of them being significantly superior over all other treatments (Table 1). The highest nodule dry weight per plant was recorded with strain 532 (68.88 mg plant^{-1}), which was significantly higher than the next highest nodule dry weight reached with 526 strain (62.17 mg plant^{-1}). The strains of *Bradyrhizobium* spp. had very low nodule number as well as nodule dry weight per plant comparing to the strains of *B. japonicum* and Knj.

Dark green leaves and chlorotic plants with green-yellow leaves were observed in NΘ and Θ controls, respectively.

The maximum shoot length of adzuki bean was recorded in the NΘ control, which was significantly superior over all other treatments. The strains 525 and 532 had the highest shoot length among inoculated plants, but without significant statistic differences. The adzuki plants inoculated with 542 strain recorded the maximum root length (31.06 cm); however, without significant differences compared to almost all other treatments. Strain 5388 showed the lowest value of both shoot and root length.

Average shoot dry weight (SDW), total and fixed N contents in plants were used for quantitative and qualitative SNF efficiency assessment (Table 2). Based on the average SDW, all the strains were classified into three significantly different declining active groups. Shoot dry weight values were in high significant positive correlation with shoot total N and fixed N content (Table 3). *B. japonicum* strains 525, 532, 542 and 526 of the first group were the most effective. Shoot dry weight, total and fixed N content ranged from 290-320, 4.64-6.25 and 3.81-5.71 mg plant^{-1}, respectively.

Table 1 - Average nodule number, nodule dry weight, shoot and root length, root dry weight and total root N content of nodulated adzuki bean, *Vigna angularis*.

Treatments inoculated and [1]uninoculated	**Nodule N^o plant^{-1}**	**Nodule dry weight mg plant^{-1}**	**Shoot length (cm)**	**Root length (cm)**	**Root dry weight mg plant^{-1}**	**Root total N content mg plant^{-1}**
525	23.0 b	55.20 c	23.63 b	31.06 a	179 a	2.72 ab
532	24.6 b	68.88 a	23.64 b	27.02 abc	180 a	2.59 b
542	21.1 c	44.31 d	22.74 bc	28.72 ab	180 a	3.24 a
526	31.4 a	62.17 b	22.80 bc	30.93 a	160 abc	1.58 c
Knj	28.8 a	43.20 d	22.40 bc	22.75 bcd	170 ab	1.70 c
2001	6.5 d	21.45 e	22.12 bc	24.85 abc	140 bcd	0.81 d
2801	6.0 d	5.40 f	22.10 bc	26.58 abc	120 d	0.62 d
5388	7.0 d	2.80 f	20.97 c	18.32 d	130 cd	0.74 d
NΘ	/	/	27.16 a	23.79 bcd	170 ab	1.70 c
Θ	/	/	21.13 c	20.96 cd	120 d	0.54 d
LSD 0.05	3.5	6.08	1.55	4.57	26	0.67
0.01	6.0	7.98	2.05	6.03	34	0.82

[1] uninoculated controls: NΘ-with N and Θ-without N; a-f: Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level ($p \leq 0.05$).

Table 2 - Average shoot dry weight, total and fixed N content of nodulated adzuki bean, *Vigna angularis* indicating to potential nitrogen fixation activity of *Bradyrhizobium* strains.

Treatments inoculated and [1]uninoculated	Shoot dry weight		%N	Shoot total N content		Shoot fixed N content mg plant^{-1}	[2]Active groups of strains
	mg plant^{-1}	Symbiotic effectivity (%)		mg plant^{-1}	Symbiotic effectivity (%)		
525	320 b	71	1.77	5.73 ab	122	4.90 ab	
532	310 bc	69	1.68	5.37 bc	114	4.54 bc	
542	300 bc	67	2.16	6.52 a	138	5.71 a	I
526	290 bc	65	1.27	4.64 bc	99	3.81 c	
Knj	250 cd	56	1.27	3.17 d	67	2.34 d	II
2001	210 de	47	0.67	1.40 e	30	0.63 e	
2801	190 e	42	0.56	1.08 e	23	0.32 e	III
5388	180 e	40	0.71	1.26 e	27	0.46 e	
NΘ	450 a	100	1.04	4.71 bc	100	/	Controls
Θ	190 e	42	0.45	0.83 e	18	/	
LSD 0.05	47.9			0.870		0.867	
0.01	63.0			1.150		1.147	

[1] uninoculated controls: NΘ-with N and Θ-without N; [2] I-*B. japonicum*, II- Knj *and* III- *B. spp*. a-e: Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level (p≤0.05).

Table 3 - Correlation (r) between parameters of nitrogen fixation.

	Shoot DW	Root DW	N^{o} nodule plant^{-1}	Nodule DW	Shoot total N	Root total N	Shoot length	Root length
Shoot DW	1							
Root DW	0.757*	1						
No nodule plant^{-1}	0.809*	0.667*	1					
Nodule DW	0.914***	0.741*	0.893**	1				
Shoot total N	0.768**	0.939***	0.621	0.717*	1			
Root total N	0.619	0.924***	0.630	0.729*	0.958***	1		
Shoot length	0.959***	0.604	-0.025	0.090	0.551	0.434	1	
Root length	0.399	0.557	0.607	0.721	0.693	0.692	0.299	1

*P < 0.05 (significant),
** P < 0.01 (highly significant)
*** P < 0.001 (extremely significant).

Strain 525 showed the greatest SDW, while strain 542 had the largest amount of fixed and total N content. Symbiosis between adzuki bean plants and the Knj strain held the middle position (SDW, total and fixed N were 250, 3.17, 2.34 mg plant^{-1}, respectively). The plants with low values of the investigated parameters were inoculated with *Bradyrhizobium* spp. strains; 2001, 2801 and 5388 belonging to the third group. In this group, SDW was between 180 and 210 mg plant^{-1}, total N content 1.08-1.40 and fixed N content 0.32-0.63 mg plant^{-1}.

No significant difference was found between the plants inoculated with the strains of the third group and Θ control plants, which indicated SNF inactivity of the *Bradyrhizobium* spp. strains. A significant difference was found between the inoculated plants of the first and third groups. Compared to these two groups, the Knj isolate did not differ significantly for SDW, except strains 525, 2801 and 5388, but differed for the total and fixed N content.

The highest values of SDW were obtained in NΘ control with significant difference existing compared to all inoculated treatments. Symbiotic efficiency (%) is the ability of inoculated plant to fix N_2 in relation to use full mineral N content (100%) of uninoculated control (NΘ). In symbiotic associations, strains of the first groups showed 65-71% SDW and 99-138% total N content of the NΘ control (Table 2). High symbiotic efficiency of adzuki bean in symbiosis with strains 525 and 542 were achieved because these strains showed 71% SDW and 138% total N content, respectively. It indicated that *B. japonicum* strains with high symbiotic efficiency were able to provide the plant with necessary N in process of N_2 fixation.

Root dry weight and total N root content had similar trend like the same parameters of shoot, except in the case of NΘ control (Table 1). The maximum root dry weight was observed in adzuki bean inoculated with strains 542, 532 and 525 (180 mg plant^{-1}), (I group in relation to SDW), which was significantly better than the values reached by *Bradyrhizobium* spp. strains (III group in relation to SDW). In contrast to shoot dry weight, where NΘ control showed significantly superior value over all other treatments, root dry weight of NΘ did not significantly differ from almost all other treatments. The highest total N content in root, as in the case of total N content in shoot was achieved by strain 542 (3.24 mg plant^{-1}), followed by strains 525 and 532 (2.72, 2.59 mg plant^{-1}, respectively).

DISCUSSION

There is a constant search for superior rhizobial strains, characterized by high rates of N_2 fixation, high nodule efficiency and competitiveness, which would allow an increase in legume yields and N content (Hungria et al., 1996). The data reported by some authors indicated a need to measure the rhizobial population characteristics directly with the host plants of interest in order to obtain an accurate assessment of the need to inoculate (Thies et al., 1991). *Bradyrhizobium spp*. strains are known to nodulate specifically plants of the cross-inoculation *Vigna* group (cowpea group), while *B. japonicum* specifically nodulates *Glycine max,* belonging to another cross-inoculation group (Dénarié et al., 1992). In the present study, an attempt was made to test the nodulation and efficacy of *B. japonicum, Bradyrhizobium* spp. strains and Knj isolate specific to cowpea group on adzuki bean. All the tested strains were capable of nodulating adzuki bean, but there were different responses to the strains inoculation (Tables 1-3).

The strains of *B. japonicum* showed ability to establish high effective nodulation in adzuki bean according to parameters of effective symbiosis, both SDW and N content (total and fixed), which have also been applied elsewhere (Yadav et al., 1996; Mayz et al., 2003). As observed in previous studies (Delić et al., 1997), the shoot dry weight and total N content per plant among the *B. japonicum* strains mostly depended on the strain activity and these parameters were in high correlation (Table 3). The most active strains were 525 and 542 for these parameters. The strain 542 had significantly better quality of yield than the NΘ control, while strains 525, 532 and 526 with a quality similar to the NΘ clearly indicated the SNF efficiency of these strains. There were symbiotic associations with the potential of achieving higher shoot quality by N fixation than with using N from mineral fertilizers (Phillips and Teuber, 1985). Symbiosis between adzuki bean plants and the Knj strain held the middle position; therefore, Knj should be investigated in symbiosis with slightly more compatible adzuki bean genotypes.

Present results showed that the *Bradyrhizobium* spp. strains were ineffective in symbiosis with the investigated adzuki bean species, due to very low SDW values and total N content achieved by these plants, which was within the level of un-inoculated control without nitrogen indicating ineffective symbiosis. The rhizobia classified as *Bradyrhizobium* spp. comprise a highly heterogeneous group of bacteria that exhibit differential symbiotic characteristics on hosts in the cowpea miscellany cross-inoculation group (Thies et al., 1991).

According to criteria of Gwata et al., (2003), additional confirmation that the plants inoculated with *B. japonicum* formed effective symbiosis in the sixth week of developement was the dark green colour of their leaves contrary to the light leaves colour of uninoculated plants without N as well as *Bradyrhizobium* spp. inoculated plants.

The interior pinkish brown colour and size of 1-2 mm found in the effective nodules of *B. japonicum* and Knj showed that their stage of development lasted for six weeks, similar to the data reported by other authors (Gwata et al., 2003; Fujihara et al., 2006). The inoculation with effective *B. japonicum* strains and Knj had a dramatic effect on the nodule number formed by these strains compared to ineffective *Bradyrhizobium* spp. *B. japonicum* strains induced at least three times higher and more number of nodules compared to *Bradyrhizobium* spp. strains. Consequently, the nodule dry weight per plant was significantly higher in *B. japonicum* inoculated plants. There was significant correlation between the nodule number per plant, nodule dry weight and shoot dry weight (Table 3).

However, despite such significant correlation between the nodulation and symbiotic effectiveness expressed by SDW, considering only effective strains of *B. japonicum* (due to the extremely poor nodulation of *Bradyrhizobium* spp. strains), there was no correlation between SDW and nodule number as well as SDW and nodule dry weight per plant. This was in accordance with previous results, which found that nodule number was not an appropriate trait for selection of the most effective N_2 fixing rhizobium-legume association (Delić et al., 1997; Hefny et al., 2001).

As presented in Table 3, the values of root dry weight significantly correlated with total root N content, as well as with shoot dry weight of investigated strains (Hefny et al., 2001; Kuang et al., 2005). Root dry weight of plant inoculated with *B. japonicum* strains and Knj was similar to the NΘ control, but total N content in root of all *B. japonicum* strains, except strain 526, was significantly higher than those of NΘ control (Table 1), which was in agreement with previous findings (Hefny et al., 2001).

The present work on adzuki bean demonstrated a positive effect of *B. japonicum* strains on the nodulation and N_2 fixation for this plant. The strains of *B. japonicum* showing high potential of N_2 fixation in pot experiment could be used in further investigation for their efficiency under field condition for inoculation as microbiological N fertilizers. These results indicated that it would be possible to reduce the use of nitrogen mineral fertilization, or completely avoid it in adzuki bean production through inoculation with highly effective strains.

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