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Nitrogen fixing activity, plasmid profiles and protein patterns of *Bradyrhizobium japonicum* strains

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Abstract

Nitrogen fixation activity of four strains of Bradyrhizobium japonicum strains 507, 518, 524 and 542 in two soybean cultivars (Hodgson and Aura) growing in greenhouse pots on two different soil types (alluvium and chernozem) free of a indigenous population of B. japonicum were investigated. Nitrogen fixation activity of the investigated strains was determined by the methods of ^{15}N isotope dilution and total N difference. Soybean plants were fertilized with low amounts of enriched $(^{15}NH_4)_2SO_4$ (30 mg · pot^{-1}). The degree of enrichment constituted 10% ^{15}N. Oat (Avena sativa) and uninoculated soybean were the referent plants in the experiment.

Grain yield was measured at the end of vegetation, at which time the percentage and amount of N in the grain and the $^{15}N/^{14}N$ ratio were also determined with a mass spectrometer. The percentage of fixed N in the soybean grain in relation to the total N content in the grain as determined by the difference method was 85.3-90.6% in alluvium and 65.7-73.3% in chernozem, with a slightly higher percentage of fixed N recorded in the Hodgson cultivar than in Aura. Strains 542 and 518 of B. japonicum showed higher nitrogen fixing activity than strains 507 and 524. The amounts and percentages of fixed N obtained by the ^{15}N isotope dilution method were somewhat higher than those obtained by the difference method.

MW of the plasmids from investigated strains was estimated. Strains 507 and 518 contain the plasmids with the size of 250 kb. Strains 532 and 542 contain plasmid band of 300 and 180 kb respectively. All strains have different protein pattern. The protein patterns between strains 518 and 524 were very similar, while strains 507 and 518 were very different.

Keywords: *B. japonicum* strains; nitrogen fixation; soybean cultivars; inoculation; ^{15}N isotope dilution; difference method, plasmids, protein pattern.

Introduction

Growing demand for soybean (*Glycine max* L.) in this country is the result of increasing needs for protein-rich foodstuffs and the fact that soybean is exceptionally abundant in proteins (35-45%). Furthermore, soybean proteins are rich in essential amino acids, especially lysine and methionine, and their biological value is closest to animal proteins. Besides proteins, the soybean grain also contains 18-24% oil, which makes it an oleiferous crop.

Similar to other legumes, soybean can use atmospheric N in symbiosis with highly specific root nodule bacteria (*B. japonicum*) in its process of protein synthesis. The process is known as symbiotic biological nitrogen fixation.

The characteristic ability of legumes to use atmospheric N through symbiosis with appropriate nodule bacteria (*Rhizobium spp*) has an enormous economic impact in agricultural production in terms of considerable savings on mineral nitrogen fertilizers, and especially production energy (over $25 \cdot 10^9 J \cdot t^{-1}$). Besides, soybean and other legumes leave considerable amounts of pure N in soil for succeeding crops to use.

Hitherto international research has shown that soybean seed inoculation increases yield by 30-50%, and contents of oil and proteins in grain by 5-10%. Furthermore, it speeds up growth and maturation by 7-10 days [13, 14, 7, 12, 24, 3, 4, 5].

Successful soybean cultivation under domestic conditions would require artificial inoculation, i.e. introduction of specific nodule bacteria (*B. japonicum*) that are not part of the autochthonous micro flora of domestic soils by way of inoculated seeds during sowing. One of the most important factors regarding the effect of inoculation on yield and grain quality of soybean is the activity of *B. japonicum* strains used in the inoculum in terms of N fixation. Successful inoculation would depend on a series of other factors as well: inoculum density and method of application, type of soil and general conditions existing for the development of host plants, strain ability to adapt to such conditions, presence of indigenous strains in soil, as well as an affinity (compatibility) between the symbionts.

This research aimed to investigate nitrogen fixing abilities of four *B. japonicum* strains and their mixtures applied to two soybean cultivars using the methods of ^{15}N isotope dilution and total-N-difference in order to identify the most effective strains to be used in practice as inoculum for soybean production. Also, MW of the plasmids and protein patterns of applied strains were determined.

Materials and methods

Two simultaneous experiments were carried out in greenhouse pots on two types of soil containing no indigenous population of *B. japonicum*. Two soybean cultivars, Hodgson and Aura, were used in both trials, as well as 4 strains of *B. japonicum*: 507, 518, 524 and 542, all from the Institute's collection of strains.

Each pot was filled with 4.5 kg fresh top soil over a layer of gravel for drainage. Alluvial soil from the surroundings of Velika Moštanica near Belgrade was used in the first trial, and chernozem collected from the Zemun surroundings in the other. The basic chemical properties of the two soils were: (1) Alluvium – pH (nKCl) 6.5; humus 2.6%; P_2O_5 39.5 and K_2O 43.3 mg $\cdot$ 100^{-g}; Chernozem – pH (nKCl) 7.2; humus 4.3%; P_2O_5 28.8 and K_2O 33.4 mg $\cdot$ 100^{-g}.

Soybean was inoculated with single strain and multiple strain inoculum (mixture of four strains). Each variant in both trials had four replicates with 4 plants per pot. As the objective was to test nitrogen fixing activity of *B. japonicum* strains on two cultivars using the ^{15}N isotope dilution method, soybean was fertilized with low amounts of ^{15}N (30 mg $\cdot$ pot^{-1}), which is equivalent to 20 kg of pure N $\cdot$ ha^{-1} in the form of enriched $(^{15}NH_4)_2SO_4$. Enrichment was 10% ^{15}N. In both trials, oat (*Avena sativa*) and uninoculated soybean were used as referent plants.

The plants were grown in a greenhouse to prevent contamination, each pot was watered by plastic tube with a stopper, and the surface of each pot was covered with granulated sterile gravel after soybean emergence.

In the trial on alluvial soil, soybean was watered during vegetation with distilled water and occasionally with weak nitrogen-free Jensen solution, while soybean on chernozem was

watered only with distilled water. Soil moisture was maintained at 75% of maximum water capacity.

Grain yield was recorded after vegetation, and total N analysis in grain was done by Kjeldahl procedure and $^{15}N/^{14}N$ ratio measurement using mass spectrometer as previously described Rennie et al. [24, 15]. Based on the data acquired, we determined the percentage and amount of fixed N in grain for each cultivar/strain variant using the ^{15}N isotope dilution method and a formula employed in earlier research [21, 24, 25, 19, 28].
The percentage of fixed N in total N was determined according to formula:

$$\% \, Hdfa = \left(1 - \frac{atom\%\ ^{15}N \text{ excess in (fc)}}{atom\%\ ^{15}N \text{ excess in (nfc)}}\right) x100$$

where

Ndfa = N derived from the atmosphere
fc = test crop (fixing crop)
nfc = control crop (non fixing crop)
Atom % ^{15}N excess was calculated with reference to the natural ^{15}N abundance of the atmosphere, which is 0.3663 atom % ^{15}N [1]

The amount of fixed nitrogen, expressed as $g \cdot pot^{-1}$, was determined according formula:

$$N_2 \text{ fixed } (g \cdot pot^{-1}) = \% \text{ Ndfa} / 100 \text{ x N yield } (g \cdot pot^{-1})$$

or

$$N_2 \text{ fixed} = \% \text{ Ndfa} \cdot \text{N yield} / 100\text{:(fc)}$$

where N yield is the total N content in grain.

The amount of fixed nitrogen expressed as $g \cdot pot^{-1}$ employing the total N difference method was determined using the formula:

$$N_2 \text{ fixed} = \text{N yield (fc)} - \text{N yield (nfc)}.$$

Plasmid profiles of the strains were obtained by AGE [23]. MW of the plasmids from investigated strains was determined by comparison with the *Agrobacterium rhizogenes* A4M70 Gus strain that has one plasmid of 254 kb. Protein analysis by SDS-PAGE was performed according Laemmli [29].

Results and discussion

There have been a growing number of studies over the last 10 years in which the method of ^{15}N isotope dilution was used to determine nitrogen fixation by different legumes. The method is based on a concept developed by Fried and Middleboe [21] proposing that legumes dilute their uptake of ^{15}N from soil with atmospheric nitrogen.

In contrast to the acetylene reduction method, which allows instantaneous measurements of nitrogen activity, i.e. the amount of N fixed from the atmosphere, this method clearly has the advantage of providing a more accurate estimate of the integral amount of N fixed from the atmosphere during a plant's vegetation period [9]. However, the method's accuracy greatly depends on the referent plant used [20, 18, 17]. A basic precondition for applying this method is to ensure that the test and control (referent) plants grow under identical conditions in a soil enriched with equal amounts of ^{15}N and uptake identical $^{15}N/^{14}N$ ratios from soil even when the amounts of N from soil and fertilizer are different [22, 9, 28, 11]. Another important precondition for its application is to ensure that

the referent plants do not fix N from the atmosphere. A referent plant could be the same as test plant, but only provided that its soil is free of an indigenous population of rhizobia. If, however, rhizobia are present in soil, their isolines could be used that produce no nodules.

Barley, oat, wheat, ryegrass and rice are some of the most frequent referent plants used in experiments. In this study, oat was used along with uninoculated soybean as another referent plant.

The method of total N difference is based on estimating the difference between total N accumulated by a N fixing test plant and a control plant that does not fix N on condition that both grow in the same soil and under identical ecological conditions (N_2 fixed = Nfc – Nnfc).

The results of our investigation are presented in Tables 1 and 2. In the alluvial soil trial, the percentage of fixed N in total N in grain determined by the difference method was found to be very high, i.e. 85.3% in the cultivar Aura inoculated with strain 507, and 90.6% in the cultivar Hodgson inoculated with strain 542 (Table 1).

Table 1. N_2 fixing activity of *B. japonicum* strains and their mixture with two soybeans cultivars estimated by the "total-N-difference" and "N^{15}-isotope-dilution" methods. (Test in pots on alluvial soil)

B.japonicum strain	Grain yield g pot^{-1}	N content in grain		Fixed N in grain				
		%	g pot^{-1}	Total N-diff. method		N^{15}-isotope dilution method		
				g pot^{-1}	%	% N^{15} at excess	N dfa %	N dfa g pot^{-1}
Cultivar Hodgson								
507	24.04 d	5.69 c	1.42 d	1.25 d	88.0 d	0.037	90.08	1.29
518	26.80 bc	6.29 a	1.68 b	1.51 b	89.9 b	0.021	94.80	1.59
524	26.60 c	5.99 b	1.59 c	1.42 c	89.3 c	0.029	92.80	1.47
542	29.62 a	6.15 a	1.82 a	1.65 a	90.6 a	0.015	96.30	1.75
Mix	27.96 b	6.29 a	1.76 a	1.59 a	90.3 ab	0.023	94.30	1.66
X	27.20	6.08	1.65	1.48	89.7	0.025	93.80	1.55
Ø	4.47 e	3.78 d	0.17 e			nfc=0.403		
Cultivar Aura								
507	26.50 c	5.67 c	1.50 b	1.28 c	85.3 b	0.049	87.80	1.32
518	27.47 b	6.02 ab	1.65 a	1.43 a	86.7 a	0.027	93.30	1.54
524	27.08 abc	5.66 c	1.53 b	1.31 bc	85.6 b	0.037	90.80	1.39
542	26.68 bc	6.20 a	1.65 a	1.43 a	86.7 a	0.018	95.50	1.58
Mix	27.82 a	5.38 bc	1.62 a	1.40 ab	86.4 a	0.031	92.30	1.49
X	27.11	5.88	1.59	1.37	86.2	0.032	92.10	1.46
Ø	5.50 d	4.03 d	0.22 c			nfc=0.403		

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 ($p \leq 0.05$). Mix-mixture of strains; X-average grain yield; Ø-uninoculated plants (control)

Such a high percentage of fixed N was obtained because the soybean plants growing under experimental conditions in pots had little mineral N in soil, so that the inoculated plants supplied most of the required N by fixation, which was not the case with uninoculated plants. Each pot containing 4.5 kg soil had four plants, which created a deficiency in nutrients, including mineral N. This resulted in a high difference between the inoculated and uninoculated plants, with grain yield of the inoculated plants being 4.9-fold higher on the average in the cultivar Aura, and 6.0 fold in Hodgson, compared to control.

As we mentioned before, soybean in this experiment was occasionally watered with weak non-nitrogen Jensen solution, which fully provided the plants with all macro- and micro-elements except N. Unlike uninoculated plants, which had limited amounts of mineral N on their disposal, inoculated plants made up for the deficient N from the atmosphere by nitrogen fixation involving rhizobia of *B. japonicum*. The yield difference between inoculated and uninoculated plants therefore results exclusively from their unequal supply of N.

Compared to the alluvial soil trial, the percentage of fixed N in grain in the trial on chernozem was considerably lower, 65.7% in the cultivar Hodgson inoculated with strain 507, and 73.3% in both cultivars inoculated with strains 542 and 518 (Table 2).

Table 2. N_2-fixing activity of *B. japonicum* strains and their mixture in symbiosis with two soybean cultivars estimated by total N difference and ^{15}N isotope dilution methods. (Test in greenhouse pots on chernozem soil)

B.japonicum strain	Grain yield g pot^{-1}	N content in grain		Fixed N in grain				
		%	g pot^{-1}	Total method	N-diff.	N^{15}-isotope dilution method		
				g pot^{-1}	%	% N^{15} at. excess	N dfa	
							%	g pot^{-1}
Cultivar Hodgson								
507	6.69 b	5.00 c	0.35 b	0.23 b	65.7 b	0.100	74.70	0.26
518	7.74 a	5.57 a	0.43 a	0.31 a	72.1 a	0.078	80.30	0.34
524	7.00 b	5.18 b	0.36 b	0.24 b	66.7 b	0.121	69.40	0.25
542	8.13 a	5.54 a	0.45 a	0.33 a	73.3 a	0.101	74.40	0.33
Mix	8.14 a	5.41 a	0.44 a	0.32 a	72.7 a	0.086	78.20	0.34
X	7.59	5.34	0.41	0.29	70.7	0.097	75.40	0.31
Ø	3.12 c	3.82 d	0.12 c			nfc=0.395		
Cultivar Aura								
507	7.39 c	5.01 c	0.37 c	0.25 c	67.6 b	0.131	66.80	0.25
518	8.53 a	5.32 b	0.45 a	0.33 a	73.3 a	0.100	74.70	0.34
524	6.50 d	5.07 c	0.33 d	0.21 d	63.6 c	0.140	64.60	0.21
542	7.59 b	5.37 b	0.43 b	0.31 b	71.1 a	0.118	70.10	0.30
Mix	6.89 c	5.58 a	0.39 c	0.27 c	69.2 b	0.116	70.60	0.27
X	7.47	5.27	0.39	0.27	69.2	0.121	69.40	0.27
Ø	3.09 e	3.96 d	0.12 e			nfc=0.395		

a-d: Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level ($p \leq 0.05$). Mix-mixture of strains; X-average grain yield; Ø-uninoculated plants (control)

Soybean was not watered with Jensen mineral dilution in this trial so that the limiting factor for its development was an insufficient content of other macro- and micro-elements, which explains the fact that even inoculated plants had lower yield here than in the trial on alluvial soil. It also caused a significantly smaller difference in grain yield between the inoculated and uninoculated plants, i.e. 2.4-fold for both cultivars compared to alluvial soil. Grain yield, total N content in grain and the percentage of fixed N were significantly higher in soybean on alluvial soil than on chernozem. Besides, an increase in the amount and percentage of fixed N (alluvium trial) caused a decreased difference in the activity of strains and vice versa, the difference in activity became more evident between strains (chernozem trial) with decreasing amounts and percentages of fixed N.

Strains 542 and 518 thus evidently had higher activity than strains 507 and 524. The parameters measured show that the strain mixture was mostly between the active and less active strains with a strong inclination to the active.

Regarding the total N content of grain, the difference between strains and cultivars was more prominent than regarding yield, so that total N was a better indicator of symbiotic activity than grain yield.

The data also show higher amounts and percentages of fixed N in the cultivar Hodgson than Aura, although the difference in grain yield was insignificant on the average for all strains in both cultivars in both trials.

Slightly higher percentages and amounts of fixed N and greater difference in that respect between strains and cultivars were obtained using the method of ^{15}N isotope dilution than the difference method. The amounts of fixed N in the soybean determined by either method were very close and consistent regarding the relationships between strains and cultivars. Our results in this respect agree with earlier reports [10, 18, 16, 25, 19, 28].

Even though the method of ^{15}N isotope dilution ensured greater precision in measuring nitrogen fixation than the difference method, we are reluctant to assume that the former gives a more accurate estimate of nitrogen fixation than the latter because reliable evidence is lacking that the test and referent plants uptake equal amounts of N from soil and fertilizer (Chalk, 1985).

Low values of fixed N restrict the application and reliability of this method, especially when the percentage of fixed N is below 10-15% of total N [22, 6, 26, 27].

Plasmids obtained from investigated strains were visualized by 0.65% AGE. Dominant strains of *B. japonicum* showed the presence of plasmids in the size range of 180-300kb. MW of the plasmids was determined by comparison with the *Agrobacterium rhizogenes* A4 Gus strain that has one plasmid of 254 kb. Strains 507 and 518 contain the plasmids with the size of 250 kb. Strains 532 and 542 contain plasmid band of 300 and 180 kb respectively. All strains have different protein pattern (marked from A to D). The protein patterns between strains 518 and 524 were very similar, since strains 507 and 518 were very different.

Plasmid profile analysis and protein pattern showed differences between investigated strains. The strains 507 and 518 showed the same plasmid size, but significant differences in protein patterns. Plasmid profiles in the analysis may be significant, but limited values, because plasmids are easily transferable from one cell to another and are not related to chromosomal variation [2, 8].

Conclusion

The percentage of fixed N in soybean grain, compared to total content in grain, determined by the method of difference is very high, 85.3-90.6% in alluvial soil and 65.7-73.3% in chernozem. The high percentage of N results from low amounts of mineral N in soil, so that soybean plants provided most of the required N by nitrogen fixation; of the four *B. japonicum* strains tested, strains 542 and 518 showed greater nitrogen fixing activity than strains 507 and 524. Differences between strains were higher under unfavorable conditions for soybean development; total N content in grain is a better indicator of their symbiotic activity than grain yield; the cultivar Hodgson showed a better nitrogen fixing ability than Aura; the method of ^{15}N isotope dilution detected slightly higher percentages and amounts of fixed N and greater differences in that respect between strains and cultivars than the difference method; the amounts of fixed N determined by either method are very close and consistent; the method of difference, being simpler and more inexpensive, can provide sufficiently reliable data for measuring nitrogen fixation.

Strains 507 and 518 contain the plasmids with the size of 250 kb. Strains 532 and 542 contain plasmid band of 300 and 180 kb respectively. All strains have different protein pattern (marked from A to D). The protein patterns between strains 518 and 524 were very similar, while strains 507 and 518 were very different.

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