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Competitiveness of *Bradyrhizobium japonicum* inoculation strain for soybean nodule occupancy

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Abstract: The competitiveness of *Bradyrhizobium japonicum* inoculation strain against indigenous rhizobia was examined in a soil pot experiment. The effect of inoculation strain was evaluated under different soil conditions: with or without previously grown soybean and applied commercial inoculant. Molecular identification of inoculation strain and investigated rhizobial isolates, obtained from nodules representing inoculated treatments, was performed based on 16S rDNA and enterobacterial repetitive intergenic consensus (ERIC) sequencing. Inoculation strain showed a significant effect on the investigated parameters in both soils. Higher nodule occupancy (45% *vs*. 18%), nodule number (111% *vs*. 5%), nodule dry weight (49% *vs*. 9%), shoot length (15% *vs*. 7%), root length (31% *vs*. 13%), shoot dry weight (34% *vs*. 11%), shoot nitrogen content (27% *vs*. 2%), and nodule nitrogen content (9% *vs*. 5%) was detected in soil without previously grown soybean and applied commercial inoculant. Soil had a significant effect on the shoot, root and nodule nitrogen content, while interaction of experimental factors significantly altered dry weight and nitrogen content of shoots, roots and nodules, as well as number of nodules. Nodulation parameters were significantly related with shoot dry weight, shoot and nodule nitrogen content. Symbiotic performance of inoculation strains in the field could be improved through co-selection for their competitiveness and effectiveness.

Keywords: competitiveness for nodulation; *Glycine max*; nitrogen fixation; protein crop; symbiotic bacteria

Soybean (*Glycine max* (L.) Merrill) is one of the most important oil and protein crop in the world. Considering the high-quality chemical composition of soybean seeds, extensive efforts have been undertaken globally to enhance its production. In this context, symbiotic nitrogen fixation (SNF) contributes to yield improvement, input of mineral nitrogen, nodulation and nitrogen fixation ability. SNF is vital for reducing the nitrogen (N) fertilisation and achieving an economic N optimum for each legume. SNF returns about 20–400 kg N/ha to arable soil annually (Herridge et al. 2008). The amount of nitrogen fixed in SNF depends on the plant species, bacterial strain and numerous biotic and abiotic factors (Lindström and Mousavi 2020). This variation and contribution of SNF in total mineral nitrogen supplie require improving of symbiotic performance of the most effective bacterial strains.

A quality inoculant should contain rhizobial strains with high nitrogen fixation, high competitiveness for nodulation and high tolerance to specific environmental conditions. However, in the outer environment, numerous abiotic and biotic factors reduce the competitiveness and effectiveness of inoculation strains. Native rhizobia are quantitatively and qualitatively heterogeneous but poorly efficient, and often demonstrate a higher competitiveness compared to highly efficient inoculation strains (diCenzo et al. 2019). Inoculation strains should be able to survive and adapt to a new environment, and to outnumber and prevail the indigenous rhizobia present in soil (Yates et al. 2011).

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The most common microsymbionts of soybean are bacteria from the genus *Bradyrhizobium*. In our agricultural soils, highly efficient strains of *Bradyrhizobium japonicum* have been introduced for decades through the use of commercial inoculant. However, little is known about the competitiveness of inoculation strains. In order to investigate whether the bacteria from the inoculant are present in the root nodules of soybean grown under different soil conditions, the competitiveness and effectiveness of inoculation strain *vs*. native rhizobia were tested.

MATERIAL AND METHODS

This study was performed with *Bradyrhizobium japonicum* strain from the collection of the Laboratory for Microbiological Research, Institute of Field and Vegetable Crops Novi Sad (IFVCNS), Serbia. The strain enters the composition of commercial inoculant for soybean production. Inoculation strain was cultured in liquid yeast extract mannitol (YEM) medium at 28 °C, in shaker (SM-30 B, Edmund Bühler GmbH, Bodelshausen, Germany) for 72 h (Vincent 1970). Before experiment, the cell counts were adjusted to 10^9 CFU (colony forming unit)/mL of culture suspension.

The pot experiment was carried out in greenhouse. The individual pots were filled with 10 kg of soil and sand (3:1). The soil used for the experiment was collected from the experimental field of IFVCNS: soil 1, where only wheat was grown for the last ten years and commercial inoculant was not applied, and soil 2, where different plants including soybean were grown in the last ten years and commercial inoculant was applied. Prior to the experiment, soil chemical properties were determined at the Laboratory for Soil and Agroecology (IFVCNS). Soil pH was determined in 1 mol/L KCl, using a pH meter Mettler Toledo (Columbus, USA). Organic carbon content (%) was determined by oxidation of organic matter by the method of Tyurin. The contents of plant-available phosphorus (AP) and potassium (AK) were extracted according to the Egner-Riehm method. Total content of nitrogen was determined using the CHNS analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany). The number of native rhizobia in soil (CFU/g) was estimated by disk dilution method at the Laboratory for Microbiological Research. Both soils were classified as Haplic Chernozem (loamic). The host plant was soybean cv. Galina (IFVCNS).

Soybean seeds were surface disinfected in 2% NaClO and 70% ethanol and washed four times with sterile distilled water. Five seeds were sown into pots in April 2018. At sowing, except for the control, each seed of soybean was inoculated with a 1 mL culture suspension of the inoculation strain. Plant density was reduced to three plants per pot after seedling emerged. The experiment was set up as a two factorial design: soil × inoculation, in five biological replicates. The pots were placed in a greenhouse where the plants were subjected to environmental factors, but watered regularly to maintain soil humidity. Plants were grown under natural light, average temperature of 20.25 °C and mineral N-free conditions. At the flowering stage in July 2018, the plants were removed from the pots.

For each inoculated soil treatment a sample of 10 nodules was taken for isolation of rhizobia. After nodule surface sterilisation, each nodule was crushed and bacteria were cultivated on the YEMA plates. Followed by several successive recultivations of single colonies on the same medium, the isolates were selected on the basis of growth and purity, and further characterised according to their morphological and biochemical properties (Vincent 1970). Finally, the total number of 22 rhizobial isolates (11 isolates for each inoculated soil) was transferred into test tubes with a slant YEMA and subjected to polymerase chain reaction (PCR) analyses.

To obtain a DNA sequence, a total DNA was extracted from the 3 days old bacterial cultures, using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's instructions. Following the DNA extraction, the 16S rDNA gene fragments were amplified by PCR with the primer pair 27F and 1492R (Weisburg et al. 1991). The amplification was done in a Mastercycler PCR device (Eppendorf, Germany), using the program described by Laguerre et al. (1994). Amplicons were determined using electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 µg/mL). The presence of amplicon of expected size in all investigated samples was confirmed by comparing the amplified DNA fragments with the marker O'RangeRuler 100 bp DNA Ladder (SM0623), ready-to-use (Fermentas, Lithuania). Purification and sequencing of the PCR-amplified fragments were performed in the Macrogen Europe B.V., Amsterdam, the Netherlands.

Sequences of rhizobial isolates, inoculation strain and related *Bradyrhizobium* strains (GenBank at the National Center for Biotechnology Information), were subjected to phylogenetic analysis using the neighbor-joining (NJ) method within MEGA 7

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(Kumar et al. 2016). Additional PCR analysis was performed to estimate genetic differences between investigated rhizobial isolates and inoculation strain. The enterobacterial repetitive intergenic consensus (ERIC) sequences were amplified with the primer pair ERIC1R and ERIC2 (de Bruijn 1992). The amplifications were performed in Mastercycler PCR device (Eppendorf, Germany), using the program described by Versalovic et al. (1991). Amplified products were electrophoresed as described earlier. The expected size of the amplified fragments was estimated by comparison with TriDye™ 1 kb Plus DNA Ladder (N3270S) (New England Biolabs Inc., Tulsa, USA).

The effectiveness of inoculation strain or indigenous rhizobia was determined based on the growth, nodulation and nitrogen fixation parameters of soybean. For each pot, all the nodules formed on the three plants were counted, and nodule occupation by inoculation strain was examined by comparing the number of nodules formed in inoculated and control soil treatments. Also, length, dry weight and nitrogen content of shoots, roots and nodules were determined. For dry weight determination, plant material was oven dried at 50 °C to a constant weight. Total N was determined using CHNS analyser and expressed in % of the analysed plant material dry weight (DW).

The data were statistically processed by the Statistica 10 programme (StatSoft Inc., Ipswich, USA). The variables were analysed using the analysis of variance (ANOVA) statistical method, followed by mean separation according to the Tukey's test ($P < 0.05$). The relationship between nodulation and growth or nitrogen fixation parameters was determined by correlation analysis.

RESULTS AND DISCUSSION

Molecular identification of inoculation strain and 22 rhizobial isolates based on 16S rDNA homology revealed the presence of amplified fragments of the expected size in inoculation strain and all rhizobial isolates. Comparison of the sequences with the *Bradyrhizobium* ID-database showed that inoculation strain and investigated isolates belong to *B. japonicum*. Phylogenetic analysis involved 36 nucleotide sequences of the isolated and related *Bradyrhizobium* species (Figure 1). A neighbor-joining tree shows that the most isolates could be divided into two major groups. Inoculation strain *B. japonicum* 1/13 and rhizobial isolates 2, 3, 4, 7, 9, 16, 18, 19, 20, 21 were grouped together with *B. japonicum* USDA138 (MK782151.1) and NAZ 505 (MK480229.1) strains from database. Rhizobial isolates 12, 14, 22, 23, 24 were closely related to *B. japonicum* MN-139 strain (KF995106.1). Also, high homology was detected between isolates 6, 8 and *B. japonicum* NA110 (JN392462.1), isolates 10, 11 and strains USDA110 (AF363150.1) and NARS-B10 (MF817963.1), isolates 5, 17 and strain USDA123 (MK782148.1), isolate 15 and strain N-86 (KF995101.1) (Figure 1).

Additionally, ERIC-PCR patterns were recorded to determine genetic differences between inoculation strain and rhizobial isolates (Figure 2). If the analysed patterns of investigated isolates were identical with the inoculation strain, it was apparent that *B. japonicum* inoculation strain was able to occupy nodules and exhibited its competitiveness and N_2 fixation ability. Otherwise, it was considered that indigenous rhizobia from soil outnumbered the inoculation strain. In this study, inoculation strain showed 45% of nodule occupancy in soil 1, and 18% of nodule occupancy in soil 2 (Figure 2). Similarly, Šimon and Salava (2006) reported 0% and 60% of nodule occupancy for two inoculation strains at same inoculation level. Both molecular methods resulted in almost identical grouping of the *Bradyrhizobium* isolates. Nevertheless, rhizobial isolates 18, 19 and 21 which were closely related to the inoculation strain on the basis of 16S rDNA, clearly differed on the basis of ERIC-PCR. Likewise, *B. japonicum* isolate 22 differed from the group of isolates 12, 14, 23, 24.

Investigated soils differed in chemical and microbial properties (Table 1). Soil 1 (without previously grown legumes and applied commercial inoculant) had a slightly acidic pH reaction, medium nutrient supply and low population of native rhizobia. Soil 2 (with previously grown legumes and applied commercial inoculant) had a slightly alkaline pH reaction, high nutrient supply and high population of native rhizobia.

Table 2 showed a significant influence of inoculation on the tested plant parameters. Investigated parameters were affected by soil, which had a significant effect on the shoot, root and nodule nitrogen content. Interaction of experimental factors significantly altered dry weight and nitrogen content of shoots, roots and nodules, as well as number of nodules.

Overall, inoculation had a positive effect on the investigated parameters compared to control, in both soils (Table 3). The increase of the most studied parameters after inoculation was higher in soil 1

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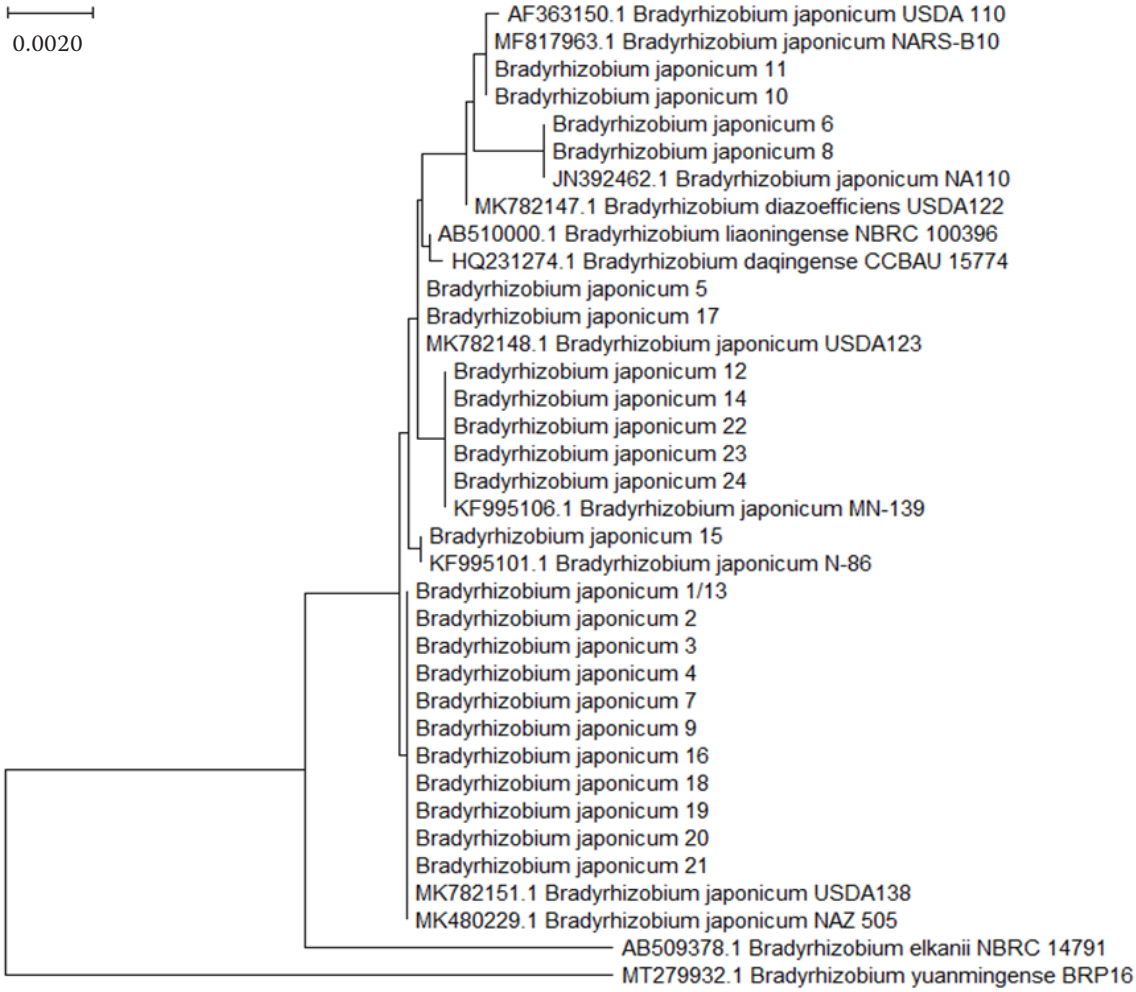

Figure 1. Phylogenetic tree based on the neighbor-joining (NJ) analysis of 16S rDNA gene sequences for inoculation strain (*Bradyrhizobium japonicum* 1/13) and rhizobial isolates (*B. japonicum* 2–12 isolates from soil 1 and *B. japonicum* 14–24 isolates from soil 2), and other *Bradyrhizobium* spp. strains from National Center for Biotechnology Information (NCBI) database

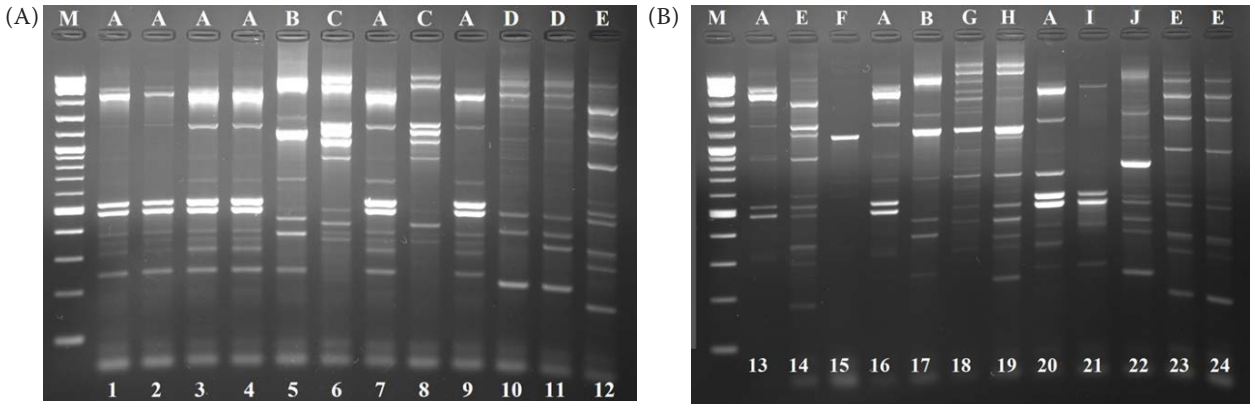

Figure 2. The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) patterns of rhizobial isolates from (A) soil 1; the number at the bottom (2–12) and (B) soil 2; the number at the bottom (14–24) represents each rhizobial isolate; a letter at the top corresponds to a particular pattern. lane M – molecular-weight marker; lane 1 – inoculation strain

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Table 1. Chemical and microbial properties of soil 1 and soil 2

Parameter	Soil 1	Soil 2
pH_{KCl}	6.63	7.47
Organic carbon (%)	1.19	1.71
Total nitrogen (%)	0.154	0.222
Available phosphorus (mg P/100 g)	12.9	38.4
Available potassium (mg K/100 g)	17.5	42.25
Number of native rhizobia (CFU/g)	10^3	10^5

CFU – colony forming unit

compared to soil 2, suggesting the better response of inoculation with *B. japonicum* in slightly acidic soil with lower fertility and smaller population of indigenous rhizobia (Tables 1 and 3). Inoculation effect was the most pronounced in nodulation parameters i.e. nodule number (111% and 5% increase in soil 1 and soil 2, respectively), and nodule dry weight (49% and 9% increase in soil 1 and soil 2, respectively), indicating higher effectiveness and better performance of inoculation strain in nodule occupation under investigated soil conditions. Additionally, a higher effectiveness of inoculation in soil 1 *versus* soil 2 was observed for shoot length (15% and 7%), root length (31% and 13%), shoot dry weight (34% and 11%), shoot nitrogen content (27% and 2%), and nodule nitrogen content (9% and 5%). Conversely, only root dry weight (26% and 31%) and root nitrogen content (10% and 19%) had lower increase by inoculation in soil 1 compared to soil 2.

Similarly, Raposeiras et al. (2006) described that the inoculation effect is more evident in soils with poor rhizobial population, especially due to the absence of host legume and lower levels of fertility. Other studies showed that soil properties such as pH and nutrient supply did not show a significant influence on the nodule mass or number, but have an influence on the abundance of *Bradyrhizobium* sp. (Griebsch et al. 2020).

The correlations between competitiveness for nodulation and growth or nitrogen fixation were found (Table 4). Nodule number and nodule dry weight were positively related with other parameters and had a significant relationship with shoot dry weight, shoot nitrogen content and nodule nitrogen content.

Table 2. Analysis of variance (*P*-value) for shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), nodule number (NNo), nodule dry weight (NDW), shoot nitrogen content (SN), root nitrogen content (RN) and nodule nitrogen content (NN) under different soil and inoculation treatments

	SL	RL	SDW	RDW	NNo	NDW	SN	RN	NN
Soil (S)	0.869	0.373	0.146	0.317	0.943	0.569	0.000	0.000	0.000
Inoculation (I)	0.004	0.023	0.000	0.000	0.000	0.002	0.000	0.000	0.000
S × I	0.204	0.262	0.001	0.001	0.000	0.025	0.000	0.000	0.000

Table 3. Effect of inoculation on soybean parameters in soil 1 and soil 2

Parameter	Soil 1		Soil 2	
	control	inoculation	control	inoculation
Shoot length (cm/plant)	53.23^{b}	61.26^{a}	55.69^{ab}	59.35^{ab}
Root length (cm/plant)	36.42^{a}	47.78^{a}	43.18^{a}	48.59^{a}
Shoot dry weight (g/plant)	2.783^{c}	3.719^{a}	3.195^{b}	3.531^{a}
Root dry weight (g/plant)	0.585^{c}	0.740^{b}	0.651^{c}	0.850^{a}
Nodule number (No/plant)	46.44^{c}	97.81^{a}	69.88^{b}	73.75^{b}
Nodule dry weight (g/plant)	0.160^{b}	0.238^{a}	0.197^{ab}	0.215^{a}
Shoot nitrogen content (%)	2.233^{d}	2.834^{a}	2.316^{c}	2.368^{b}
Root nitrogen content (%)	2.027^{d}	2.223^{b}	2.170^{c}	2.578^{a}
Nodule nitrogen content (%)	4.701^{d}	5.120^{b}	4.908^{c}	5.148^{a}

Differences between treatments were analysed using the Tukey's test. The different letter indicates a significant difference at $P < 0.05$

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Table 4. Pearson correlations between nodule number (NNo) and nodule dry weight (NDW) with shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), shoot nitrogen content (SN), root nitrogen content (RN), and nodule nitrogen content (NN)

Trait	SL	RL	SDW	RDW	SN	RN	NN
NNo	0.61	0.44	0.88**	0.54	0.85**	0.36	0.79**
NDW	0.52	0.16	0.71**	0.43	0.69**	0.45	0.76**

**$P < 0.01$

In conclusion, the results showed an influence of different soil conditions on the competitiveness of inoculation strain, indicating that the strain persistence in the soil depends on the environment, in particular native rhizobial population and soil characteristics. Higher effect of inoculation on nodule occupation, growth and nitrogen fixation ability was recorded in soil with smaller native population of rhizobia and lower levels of fertility, i.e. in the absence of host legume and applied inoculant. The competitiveness of inoculation strains along with their effectiveness should be first considered in developing rhizobial inoculants. Mono-inoculation experiments are advantageous for evaluating the ability of strain to form efficient symbiosis, and the inoculation response is more noticeable. This is the first study on competitiveness of inoculation strain which is included in commercial inoculant for soybean production in Serbia. Since competitiveness for nodulation is controlled by genetic factors from both soybean and rhizobia, future research should focus on testing the individual competitiveness of each inoculation strain, together with its interaction with other inoculation strains and native rhizobia, in different cultivars and soil conditions.

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