Resistance of indigenous *Bradyrhizobium japonicum* strains on moisture deficiency stress

Otpornost autohtonih sojeva *Bradyrhizobium japonicum* na stres izazvan nedostatkom vlage

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ABSTRACT

Drought is one of the most important factors limiting N fixation, growth and yield of soybean. In this study, fifteen indigenous *Bradyrhizobium japonicum* strains isolated from different regions of Croatia were subjected to *in vitro* investigations of different drought conditions (simulated by polyethilene glycol (PEG) 6000) and increased NaCl concentrations. ERIC-PCR method was employed in order to determine genetic variability of strains. At the concentration of 15% PEG 6000 indigenous strain *B. japonicum* IS1 isolated from east Slavonia region was most tolerant to the lack of water. At the concentraction of 30% PEG 6000, *B. japonicum* IS2, was distinctively resistant to osmotic pressure and the least tolerant was *B. japonicum* IS4, both isolated from eastern Slavonia. For all tested strains, good growth was observed at the concentration of 1% NaCl and moderate growth was recorded for the strains from Koprivnica region. Indigenous strans from Baranja, Istria and two from eastern Slavonia were very tolerant to high concentration of 4% NaCl. According to results of ERIC-PCR method genetic similarity was not related to location of the isolation. The greatest genetic similarity was observed for strains isolated from Baranja region, eastern Slavonia, Koprivnica and western Slavonia.

Keywords: *Glycine max* (L.) Merr, root colonizing bacteria, nitrogen fixation, *in vitro* drought, NaCl, ERIC-PCR

SAŽETAK

Suša je jedan od najvažnijih faktora koji ograničava fiksaciju dušika, rast i prinos soje. U ovom radu petnaest autohtonih sojeva *Bradyrhizobium japonicum* izoliranih iz različitih regija Hrvatske podvrgnuto je *in vitro* ispitivanjima otpornosti na sušu simuliranu polietilen glikolom (PEG) 6000, te na povišen sadržaj soli. ERIC-PCR metoda korištena je za utvrđivanje genetske varijabilnosti autohtonih bakterija. Pri koncentraciji 15% PEG 6000 autohtoni soj izoliran iz područja istočne Slavonije *B. japonicum* IS1 bio je najtolerantniji na nedostatak vode. Pri koncentraciji PEG 6000 od 30%, autohtoni soj *B. japonicum* IS2 bio je izrazito otporan na osmotski stres, dok je najmanje tolerantan bio soj *B. japonicum* IS4. Oba soja izolirana su iz područja istočne Slavonije. Kod svih ispitivanih sojeva uočen je dobar rast pri koncentraciji od 1% NaCl, a umjeren rast je utvrđen kod autohtonog soja iz područja Koprivnice. Autohtoni soj iz Baranje, Istre i dva iz istočne Slavonije bili su vrlo tolerantni na izrazito visoke koncentracije od 4% NaCl. Prema rezultatima ERIC-PCR metode utvrđeno je da genetska sličnost nije uvjetovana mjestom izolacije. Najveća genetska sličnost bila je utvrđena kod sojeva izoliranih iz područja Baranje, istočne Slavonije, Koprivnice i zapadne Slavonije.

Ključne riječi: *Glycine max* (L.) Merr, kvržične bakterije, fiksacija dušika, *in vitro* suša, NaCl, ERIC-PCR

INTRODUCTION

Soybean (*Glycine max* (L.) Merr) is one of the most important legumes in the world. It is mainly cultivated for oil and protein and is a major food and feed source (Abbasi and Sadeghipour, 2012). Legumes are agronomically and economically important in many cropping systems because of their ability to assimilate atmospheric N and this importance is anticipated to increase with the need to develop sustainable agricultural practices. Rhizobia are minor components of the soil microbiota and reach their maximum number and efficiency in association with legumes.

The world-wide soybean production in 2017/2018 reached 355.3 million metric tons (FAO, 2018). Sustainability of soybean yields is, however, threatened by predicted climatic changes with persistent droughts over many parts of the world (Foyer et al., 2016). Drought is one of the major causes of reduced growth, development and yield in field crops (Yucel et al., 2010). In leguminous plants, drought also reduces nitrogen fixation and its related traits (Abbasi and Sadeghipour, 2012). Selection of more drought-tolerant soybean cultivars is therefore required to address this imminent threat to food and protein security (Ku et al., 2013).

Bradyrhizobium japonicum is slow growing root nodule symbiont, which is widely used as an inoculant in soybean fields throughout the world. Generally, soybean inoculated with *B. japonicum* forms highly effective nodules and frequently increased soybean yields, especially in fields where soybeans are cultivated for the first time (Uma et al., 2013). Survival and growth of rhizobia in soil are limited by severe environmental stress, mainly salinity and drought (Mhadhbi et al., 2011). Success of symbiotic nitrogen fixation is often strongly inhibited in arid and semiarid soils due to poor survival of rhizobia under desiccation stress (Rehman and Nautiyal, 2002).

Capacity to overcome stressful situations varies within rhizobial strains and therefore the strains with genetic potential for increased tolerance to this adverse environmental stress could enhance nitrogen fixation (Marinkovic et al., 2013).

Soybean has an allorhizic root system consisting of a primary root (taproot) and lateral (basal) roots (Fenta et al., 2014). Decreased root lengths and dry biomass accumulation have been reported in many soybean accessions under drought conditions (Kunert et al., 2016). Drought not only changes root architecture but also partitioning of root to shoot biomass with an increase in root mass (Franco et al., 2011).

Despite symbiotic nitrogen fixation is adequate to meet the nitrogen needs of the soybean crop, high-yielding soybeans benefit from supplemental N applications, since nitrogen fixation capacities are not always sufficient to produce high yields (Kunert et al., 2016). Nodule numbers are only decreased when soybean plants are subjected to severe drought conditions (Márquez-García et al., 2015).

Nodule drought tolerance has been linked to the ability of plant to sustain a supply of photosynthate to the nodules during drought and to greater nodule biomass (King and Purcell, 2001). More robust rhizobia with better osmo-tolerance to longer persistence in drought soils might also be a contributor (Mhadhbi et al., 2013).

Recent research has also provided evidence that plant growth-promoting rhizobacteria (PGPR) improve plant adaptation to drought by stimulating lateral root formation and increasing shoot growth (Rolli et al., 2015) with stimulation partly caused by bacterium-produced volatile organic compounds (Wintermans et al., 2016).

The aim of this study was to access rhizobial tolerance to sodium chloride (NaCl) and polyethylene glycol (PEG) in culture media and to study genetic diversity of isolates using ERIC-PCR method.

MATERIALS AND METHODS

Genotypic diversity and phenotypic characterization performed in this study was conducted on fifteen isolates previously identified as *B. japonicum* and reference strain *B. japonicum* 344.

Determination of drought tolerance level of the isolates

The effect of drought on the growth of *Bradyrhizobium* isolates was studied using polyethylence glycol (PEG) 6000 at different concentration ranging from 0 to 30% in Yeast Extract Mannitol (YEM) broth. The initial inoculums were grown in YEM medium (Vincent, 1970). After that, 100 µl of culture was transferred to 20 ml of the same medium supplemented with 15% or 30% PEG. After 7 days incubation, at 28 °C with shaking at 150 rpm (Orbital Shaker-Incubator ES-20), the bacterial growth was measured spectrophotometrically (Spectrometer Lambda EZ 210) at OD 600 nm (Uma et al., 2013).

Determination of salt tolerance level of the isolates

As for salinity tolerance, the experiment was carried out by cultivating *Bradyrhizobium* isolates on different NaCl concentrations (1.0, 2.0, 3.0 and 4.0%) in YMA medium plates plus the control treatment (0.01% NaCl). Isolates were incubated at 28 °C for 7 days.

Genetic diversity of the isolates by ERIC-PCR

Total DNA was extracted using DNeasy® Blood & Tissue kite (QIAGEN, 2006, USA), according to manufacturer's instructions. ERIC fingerprinting was performed with primers ERIC 1R and ERIC 2. Amplification reactions were performed in a 25 µL volume, containing: 20 mmol/L Tris-HCl (pH=8.4), 50 mmol/L KCl, 2.0 mmol/L MgCl2, 200 µmol/L of dNTPs, 1 µmol/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase (TaKaRa Bio, USA). The temperature profile was as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, and extension at 72 °C for 1 min; and final extension at 72 °C for 7 min. Fragments were separated by submerged gel electrophoresis on precast 6 % poly (NAT) gels run in SEA 2000 apparatus (Elchrom Scientific AG, Switzerland) for 2.5 h at 7 V/10mm and 20 °C. The amplification patterns were visualised under UV illumination after staining with ethidium bromide (Sigma Chemical Co. Ltd) and photographed with Cannon Powershot A640 camera. Strains similarities and cluster analyses were obtained using BioNumerics Seven, Applied Maths (Gent, Belgija).

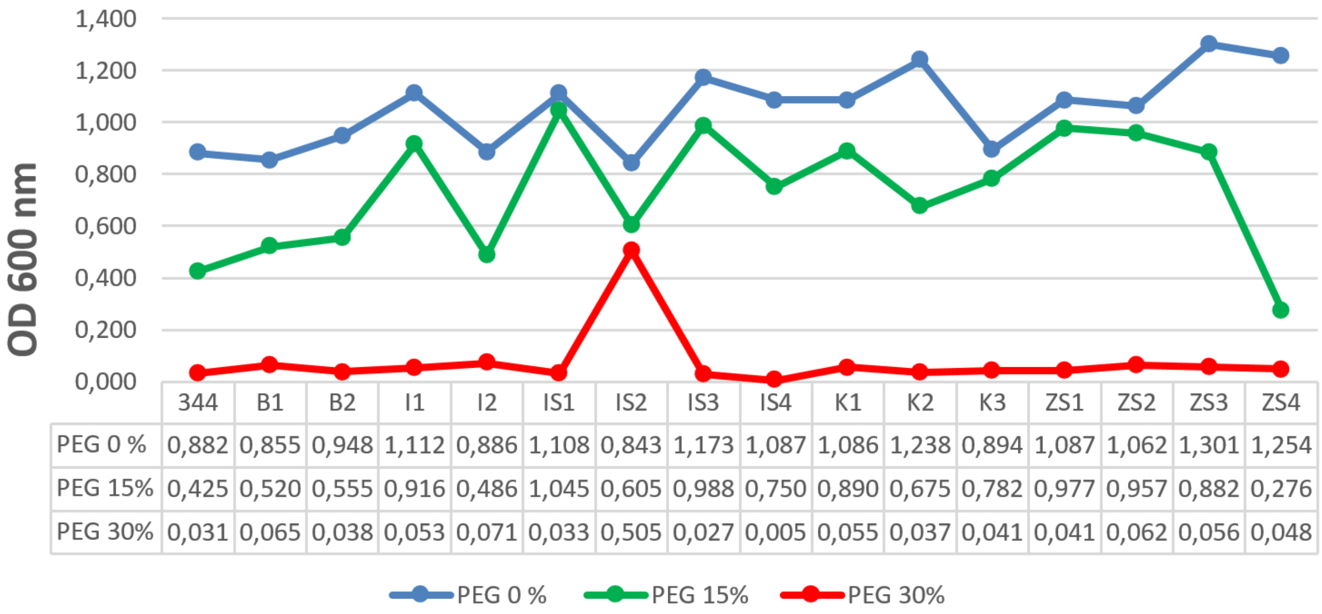
RESULTS AND DISCUSSION

Studies of the resistance of symbiotic nitrogen fixators to abiotic environmental factors can largely overcome the obstacles in soybean production. It is very important to select and identify not only symbiotic nitrogen fixators but also PGPR bacteria to reduce the pesticides and mineral fertilizer usage in order to mitigate the negative influence on environment which can directly affect the plant yield.

To induce osmotic stress, a solution of PEG 6000 was used. The higher percentage of PEG 6000 is considered to present the higher osmotic stress, i.e. higher drought induced in the YMB nutrient solution. The PEG 6000 adsorbes water, and this molecule is big enough not to enter the bacterial cell.

The bacterial growth turbidity under controlled conditions (0% PEG 6000) ranged between the highest measured absorption value of 1,301 and the lowest measured value of 0,843. The average bacterial growth at 0% PEG was 1.051. The highest growth in control conditions was that observed for the indigenous strain ZS3, while the lowest measured value was measured for indigenous strain IS2 (Figure 1). By increasing PEG 6000 concentration to 15%, bacterial growth was reduced. Even seven out of sixteen strains, showed very good growth at increased osmotic pressure. The best growth was obtained for indigenous strain IS1 with an absorption value of 1,045, followed by strains IS3 (0,988), ZS1 (0,977), ZS2 (0,957), I1 (0,916), K1 (0,890), ZS3 (0,882). At this concentration of PEG 6000, the lowest absorption value of 0.276 was measured for the indigenous strain ZS4, followed by the reference strain 344 (0.425).

Drastic decrease in bacterial growth was observed when the PEG 6000 concentration was increased to 30%. Only one strain, IS2 had a good growth at this concentration where the absorption was 0.505. All other strains had absorption values lower than 0.100 and ranged between 0.071 and 0.027.



	344	B1	B2	I1	I2	IS1	IS2	IS3	IS4	K1	K2	K3	ZS1	ZS2	ZS3	ZS4
PEG 0 %	0,882	0,855	0,948	1,112	0,886	1,108	0,843	1,173	1,087	1,086	1,238	0,894	1,087	1,062	1,301	1,254
PEG 15%	0,425	0,520	0,555	0,916	0,486	1,045	0,605	0,988	0,750	0,890	0,675	0,782	0,977	0,957	0,882	0,276
PEG 30%	0,031	0,065	0,038	0,053	0,071	0,033	0,505	0,027	0,005	0,055	0,037	0,041	0,041	0,062	0,056	0,048

Figure 1. Growth *B. japonicum* at various levels of polyethilene glycol (PEG 6000) for selecting drought tolerant strains. OD value was used to group the bacteria for growth estimation

The lowest value was 0.005 obtained for the indigenous strain IS4 (Figure 1). The obtained results show that three strains from Western Slavonia and two strains from Eastern Slavonia have very good adaptation to the *in vitro* lack of moisture. In the presence of the highest percentage of PEG 6000 the best resistance to osmotic stress was observed for indigenous strain isolated from Eastern Slavonia (IS2).

According to Marinković et al. (2013) *B. japonicum* strains in nutrient medium supplemented with 9% PEG 6000 showed a slight growth decrease in comparison to the medium without the addition of PEG 6000. In their study, *B. japonicum* strain 511 had the least tolerance to osmotic stress, for which the growth between 0 and 9% PEG 6000 decreased for 43.3%, while strain D216 was tolerant to osmotic stress, as growth between control and % PEG 6000 was reduced only for 3.3%. In the study Kukreja (2013) who tested 91 soybean rhizobia, the growth at an extremely high PEG 6000 content of 30% was observed for only four strains. The obtained absorption values ranged between 0.485 and 0.606.

According to the results of this study it is apparent that all analyzed indigenous *B. japonicum* strains and the reference strain 344 have the ability to grow at 1% NaCl concentrations (Table 1).

Table 1. Tolerance degree of rhizobial strains on different NaCl concentrations

Strains	1% NaCl	2% NaCl	3% NaCl	4% NaCl
344	++	+	-	-
B1	±	±	±	±
B2	+	±	±	-
I1	++	±	±	±
I2	++	±	±	-
IS1	±	±	±	±
IS2	++	±	±	-
IS3	+	±	±	±
IS4	++	±	-	-
K1	+	±	±	-
K2	++	+	-	-
K3	±	±	-	-
ZS1	±	-	-	-
ZS2	±	-	-	-
ZS3	±	±	-	-
ZS4	±	±	-	-

+ (growth), ± (moderate growth), - (no growth)

Six strains (344, I1, I2, IS2, IS4 and K2) showed a very good growth, while moderate growth was obtained for three strains (B2, IS3, K1) and the rest of the strains showed weaker growth at same concentration. By increasing the NaCl concentration to 2%, two strains (ZS1 and ZS2) did not grow, while only the reference strain 344 and the indigenous strain K2 showed moderate growth. Other analyzed strains showed weak growth. At the 3% NaCl concentration, eight tested strains (B1, B2, I1, I2, IS1, IS2, IS3 and K1) showed weak growth, while the other strains did not grow at this concentration of NaCl. At the highest tested concentration (4% NaCl), only four indigenous strains (B1, I1, IS1, IS3) formed visible colonies on the YMA nutrient medium but their growth was weak. Other strains did not grow on a nutrient medium (Table 1). In the research of Mandala (2014), growth of all 27 *Rhizobium trifolli* investigated strains was determined at concentration of 0.25% NaCl. Increasing the salt concentration in nutrient medium to 0.5% NaCl, five strains were sensitive, i.e. no growth was observed while at the highest tested NaCl concentration (3% NaCl), five strain formed visible colonies so they were considered tolerant to high salinity. In contrast, Dong et al (2017), tested four strains of *B. japonicum* isolated from roots of *Stylosanthes* spp. for susceptibility to 0.1 M, 0.2 M, 0.3 M, 0.35 M, 0, 4 M and 0.5 M NaCl and the growth was recorded for only one strain with 0.5 M NaCl (about 3%). Sadowsky and Graham (2013) recorded that different *Bradyrhizobium* species did not grow at concentrations higher than 2%. It is considered that the slow growing rhizobia are less tolerant to increased salt concentration in the soil than the fast growing (Zahran, 1999, Elsheikh, 1998).

According to the aforementioned research, it can be seen that very small number of rizobia can grow at high salt percentages which can be up to four times higher than optimal for rhizobia. The results obtained in this paper indicate that four indigenous strains, isolated from Baranja, Istria and Eastern Slavonia tolerate high salt content, and are resistant to extremely unfavorable NaCl concentrations.

The strains of *B. japonicum* were genetically very different. According to the dendrogram (Figure 2), the strains are divided into two main groups (1 and 2). Among them, the relative similarity was 20%. The main group 2 containing the majority of strains was divided into two subgroups (2a and 2b). The subgroup 2a contains indigenous strain IS2 which shows the highest similarity (75%) with reference strain (344). The third strain is B1 with relative similarity of 41% compared to other strains of this subgroup. Subgroup 2b contains the largest number of isolated strains. The highest relative similarity (99%) of all analyzed strains was determined among the indigenous strains I1, IS4, K1, K3, ZS1 and ZS2. Strain B2 showed the lowest relative similarity (50%) with the rest of the subgroup. Group 1 contains two subgroups 1a and 1b. Group 1a includes strains IS1 and K2 with relative similarity 80%. Indigenous strain IS3 has a relative similarity 65% in a relation to IS1 and K2, while ZS3 has a relative similarity of 57% to mentioned subgroup 1a. The only strain in subgroup 1b is I2 with a relative similarity of 30%.

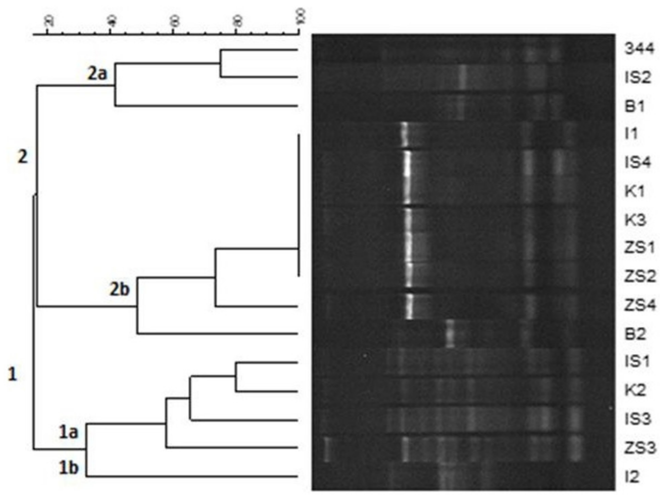


Figure 2. Dendrogram of *B. japonicum* strains derived from ERIC fingerprints generated by using ERIC 1R and ERIC 2 primers

Sequencing two strains of *B. japonicum* USDA6^T and *B. japonicum* USDA110 Kaneko et al. (2011) found that they possess similar genomic regions, but also the existence of major differences in their genomes. Since they have a different genetical structure although isolated from the same site, they have different resistance to environmental stresses.

Jida and Assef (2012) and Jebara et al. (2001) also confirmed that bacterial tolerance to adverse external conditions may differ between strains.

From the dendrogram (Figure 2) it can be clearly seen that unrelated to the location of the isolation, most of the strains were genetically very different. According to the results of this study, reference strain 344 is most similar to IS2, tolerant to high NaCl concentration (3%) and resistant to in vitro moisture deficiency. Indigenous strains I1, IS4, K1, K3, ZS1, ZS2, isolated from different regions of the Republic of Croatia showed a very high relative similarity.

CONCLUSION

Based on the results of research of 16 indigenous strains of *B. japonicum* isolated from different regions of Croatia it was found that all 15 isolates and reference strain 344 could grow at a 1% concentration of NaCl. Increasing the salt content reduced the growth of bacteria on nutrient substrates as half strains did not grow on 3% of NaCl.

In the absence of in vitro moisture, seven strains isolated from different regions of Croatia were tolerant to the higher concentration of PEG 6000. The highest resistance to 30% PEG 6000, or lack of water, showed an indigenous strain isolated from Eastern Slavonia.

Applying the ERIC-PCR method, genetic diversity has been found among *B. japonicum* strains. The highest genetic similarity was found within indigenous strains isolated from Istria (one strain), Eastern Slavonia (one strain), Koprivnica (two strains) and Western Slavonia (two strains) and their relative similarity was higher than 99%. Reference strain 344 showed the highest relative similarity to the indigenous strain from Eastern Slavonia, which had high tolerance to water and salt stress (growth was observed at 30% of PEG 6000 and 3% NaCl).

Although this preliminary study covered a small number of isolates, the obtained in vitro results suggest that there is a difference between strains that are capable of growing in unfavorable environmental conditions. In further research, it would be useful to include a greater number of isolates with the aim of characterizing their phenotypic and genotypic characteristics in order to select potential rhizobial strains for application in under adverse soil conditions.

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