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Original scientific paper

OSMOTIC STRESS TOLERANCE, PGP TRAITS AND RAPD ANALYSIS OF *Bradyrhizobium japonicum* STRAINS

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The osmotic stress tolerance of *B. japonicum* strains assessed according to their persistence in PEG solution. The lowest tolerance to osmotic stress was observed in strain 511 (43.3%), and the highest tolerance was observed for strain D216 (3.3% growth reduction in presence of PEG). PGP traits of *B. japonicum* strains were tested. None of five *B. japonicum* strains produced siderophore, strains 511 and 518 had the urease ability, and only *B. japonicum* 518 strain showed the ability to solubilize insoluble tricalcium phosphate. RAPD analysis, using AP10, BC318, AF14 and SPH1 primers, indicated genetic differences between *Bradyrhizobium* strains. The first group (strains 3, 6 and 518) showed more than 80% similarity. Strains 511 and D216 formed separate clusters. Difference between strains D216 and the other strains were more than 60%, with maximum value of 72% in comparison with strain 511. Plant-growth promoting (PGP) traits, osmotic stress tolerance and RAPD analysis highlighted strain D216 as useful for further investigation of *B. japonicum* impact on drought reduction in symbiosis with soybean.

Key words: *Bradyrhizobium japonicum*, osmotic stress tolerance, RAPD analysis, PGPR

INTRODUCTION

Rhizobia are soil bacteria that possess the unique ability to establish a symbiotic association with the roots of leguminous plants and form specialized structure – root nodule. Great environmental

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and agricultural significance of these bacteria is based on their ability to fix atmospheric nitrogen in symbiotic process, reduce it to ammonia and supply host plant with this essential nutrient. The most common soybean microsymbiont is *Bradyrhizobium japonicum*, rod-shaped, gram-negative, aerobic, motile, slow growing bacteria of the genus *Bradyrhizobium*. Besides biological nitrogen fixation, rhizobia can promote plant growth by synthesis of vitamins, phytohormones and enzymes, producing siderophores, dissolving phosphates and other nutrients and prevention deleterious effects of phytopathogenic microorganisms (BOIERO *et al*., 2007; HAYAT *et al*., 2010; AHEMAD and KHAN, 2011).

Survival and growth of rhizobia in soil are limited by severe environmental stress, mainly salinity and drought (MHADHBI *et al*., 2008). 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; CYTRYN *et al*., 2007). Capacity to overcome stressful situations varies within rhizobial strains and the strains with genetic potential for increased tolerance to this adverse environmental stress could enhance nitrogen fixation (REHMAN and NAUTIYAL, 2002).

Due to their importance and diversity within natural populations, identification and characterization of rhizobial strains imply a comprehensive and accurate approach. Traditional methods, based on morphological, physiological and biochemical assay, frequently failed in the identification of *Rhizobium* strains within a species and there are circumstances in which recognition of a particular strain (HAMEED *et al*., 2004) are very difficult. The development of numerous molecular methods has greatly contributed to these investigations (EL-FIKI, 2006). The availability of sensitive, easy, rapid, reliable and accurate PCR-based genotyping among closely related bacterial strains and the detection of higher rhizobial diversity have been greatly consider (VINUESA *et al*., 1998; DOIGON-BOURCIER *et al*., 2000; TAN *et al*., 2001; JOSIC *et al*., 2002; EL-FIKI, 2006; RAJASUNDARI *et al*., 2009). RAPD (randomly amplified polymorfic DNA) is frequently used technique for exploring genetic polymorphisms of *Bradyrhizobium* (SIKORA *et al*., 2002; HAMEED *et al*., 2004; EL-FIKI, 2006; RAJASUNDARI *et al*., 2009).

The aim of this study was to investigate osmotic stress tolerance ability of five *Bradyrhizobium japonicum* strains, combined with their plant-growth promoting activities (ability to solubize inorganic phosphate, produce siderophores and urea hydrolysis) and to compare it using RAPD analysis as useful molecular genetic tool.

MATERIALS AND METHODS

Five *Bradyrhizobium japonicum* strains used in this study were obtained from the collection of the Department of Microbiological Preparations, Institute of Field and Vegetable Crops, Novi Sad.

The osmotic stress tolerance

The osmotic stress tolerance of *B. japonicum* strains was tested using polyethylene glycol (PEG) 6000 as a stress substance (RASANEN *et al*., 2004). The initial inoculums were grown in YEM medium (VINSENT, 1970) and 1ml of this culture was transferred to 50 ml of the same medium supplemented with 0% or 9% PEG. After 72 h incubation, at 28ºC with shaking at 150 rpm (Edmund Bühler SM-30 B), each sample was spotted on to YMA plates and incubated for five days. Strain growth was determined by the number of colonies formed (CFU ml^{-1}).

Plant-growth promoting activities

Bacterial ability to solubilize inorganic phosphate $Ca_3(PO_4)_2$ i $FePO_4$ x $2H_2O$ was assayed on Pikovskaya medium (yeast extract 0.5 g l^{-1}, glucose 10 g l^{-1}, $Ca_3(PO_4)_2$ ($FePO_4$ x $2H_2O$), 5 g l^{-1}, $(NH_4)_2SO_4$ 0.5 g l^{-1}, KCl 0.2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g l^{-1}, $MnSO_4 \cdot H_2O$ 0.0001 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 0.0001 g l^{-1} and agar 15 g l^{-1}). Plates were incubated at 28ºC for 5 days, and the presence of clear halo zone around bacterial colonies indicating the phosphate solubilization.

Siderophores production was tested on chrom-azurol S (CAS) medium described by MILAGRES *et al*. (1999). Plates were filled with CAS medium, and a half of this media was replaced with YMA medium. The bacterial inoculum was placed near the borderline between two media and incubated at 28ºC for 5 days. Siderophores production was determined by CAS medium discoloration from blue to orange.

The ability of bacterial strains to hydrolysis urea was determined by changing medium color from pale-pink to pink, using urea agar base (HIMEDIA, Spain) with the addition of urea (5%).

RAPD analysis

Genomic DNAs were extracted from 200 µl cultures according to SELENSKA-POBEL *et al*. (1996). Four primers for RAPD analysis were used. Primers AP10 (5'- CAG GCC CTT C-3') (SELENSKA-POBEL *et al*., 1996) and SPH1 (5'- GAC GAC GAC GAC GAC -3') (DOOLEY *et al*., 1993), already were used for rhizobial strain testing. Primers BC318 (5'- CGG AGA GCG A -3') and AF14 (5'- GGT GCG CAC T -3') (MLIKI *et al*., 2001), are used for plant materials, but not for investigation of rhizobia. PCR was carried out with DreamTaqGreen Master Mix (ThermoScientific Fermentas), 25 ng of total bacterial DNA as template and 100pM of appropriate primer. The amplification program: 5 min at 95°C for initial denaturation, (1 min at 95°C, 1 min at 37°C, 2 min at 72°C) for 35 cycles and 7 min at 72°C for final extension was used. Fragments were separated in 1.2% agarose gels, stained with ethidium bromide solution and visualized on UV/Vis transilluminator (Shimadzu, 160UV, Germany).

Strains similarities and cluster analyses were obtained using Statistica for Windows 10.

RESULTS

The osmotic stress tolerance

B. japonicum strains were tested based on their persistence in PEG solution and the results are shown in table 1. Osmotic stress was induced in flasks by supplementing the liquid nutrient YM medium with 9% concentration of polyehtylene glycol (PEG) 6000. *B. japonicum* cell survival was assessed by plate count analysis, following incubation period of 5 days, and expressed as $\log_{10}$ CFU ml^{-1}. Low water potential, induced by PEG, resulted in notably reduction in growth rates of all tested strains. PEG decreased the percentage of growth; however, all tested *B. japonicum* strains showed low growth in the presence of 9% PEG. The number of colonies formed by control culture was set to 100% of growth for each bacterial strain (represents control treatment) and the number of colonies formed by stressed cultures was normalized accordingly. In control treatment, $\log_{10}$ CFU ml^{-1} was 3.61 and 20.39 in strains 6 and 3, respectively. The growth of strain D216 was the least affected by PEG (compared with control, growth was reduced by 3.3). Strain 511 showed lowest tolerance to osmotic stress, and comparing to control, number of colonies in presence of PEG, was reduced to 43.3%.

Table 1. log_{10} CFU ml^{-1} Bradyrhizobium japonicum in nutrient medium under osmotic stress conditions

Strain	0% PEG		9% PEG	
	log CFU ml^{-1}	%	log CFUml^{-1}	%
D216	8,29	100	8,02	96,7
518	7,69	100	5,93	77,1
6	3,61	100	2,50	69,3
3	20,39	100	12,98	63,7
511	16,17	100	7,00	43,3

Plant-growth promoting activities

PGP trait of *B. japonicum* strains, such as P-solubilizing ability, siderophores production and urea hydrolysis were tested (table 2). None of the five tested *B. japonicum* strains produced siderophore. Phosphate solubilization test was conducted by plating bacteria in agar containing two different inorganic phosphate sources - tricalcium phosphate and iron phosphate. All *B. japonicum* strains tested grew on the agar with tricalcium phosphate as source of inorganic phosphate. However, when tricalcium phosphate was replaced by iron phosphate, only *B. japonicum* strains 511 and 518 had the ability to grow. The ability to solubilize precipitated tricalcium phosphate was positively exhibited only by strain *B. japonicum* 518. Although it grew on medium with iron phosphate, this strain had no ability to solubilize it. All five strains grew on urea agar base, but growth on this medium was weaker. Strains 511 and 518 had the ability to hydrolyse urea (table 2).

Table 2. Plant-growth promoting activities of Bradyrhizobium japonicum *strains*

Strain	Siderophore production	P-solubilization $Ca_3(PO_4)_2$		P-solubilization $FePO_4 \times 2H_2O$		Urease activity	
		growth	solubilization	growth	solubilization	growth	urea hydrolysis
3	—	+	—	—	—	+	—
6	—	+	—	—	—	+	—
511	—	+	—	+	—	+	+
518	—	+	+	+	—	+	+
D216	—	+	—	—	—	+	—

RAPD analysis

RAPD fingerprinting was used to determine genetic polymorphism among the five *B. japonicum* strains. Four different oligonucleotide arbitrary primers AP10, BC318, AF14 and SPH1 were used in amplification reaction. Multiple DNA fragments obtained with all primers ranging in size from 110 bp to more than 3000. Random priming amplification with AP10 primer produced the highest number of bands, ranged from 7 to 11 polymorphic bands per strain. The fragments of 3200, 750, 350 i 250 bp, present in four strain after AP10 amplification, are missing in strain D216 (figure 1). Amplification with BC318 (figure 2), AF14 (figure 3) and SPH1 (figure 4) primers resulted in higher uniformity of the obtained patterns. Size and position of bands obtained for strain D216

were very different compared with strains 3, 6 and 518 using BC318, AF14 and SPH1 primers. Amplification with AF14 and SPH1 primers resulted with few specific bands for strains 511 and D216, but number of bands characteristic for strain D216 was grater. Strains were divided into three groups based on dendrogram derived from RAPD analysis (primers AP10, BC318, AF14 and SPH1) (figure 5).

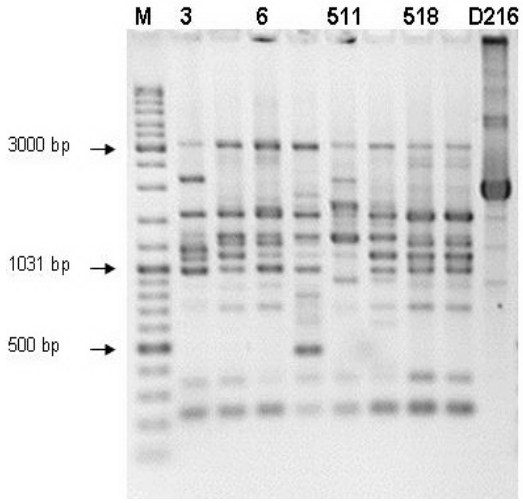

Figure 1. Genodiversity of *Bradyrhizobium japonicum* isolates based on PCR using AP10 primer

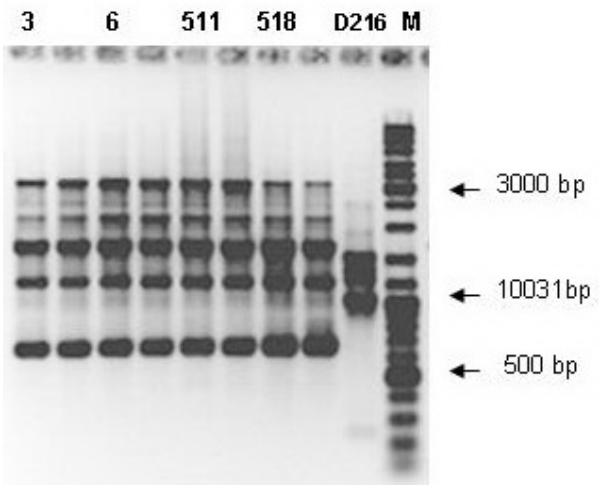

Figure 2. Genodiversity of *Bradyrhizobium japonicum* isolates based on PCR using BC318 primer

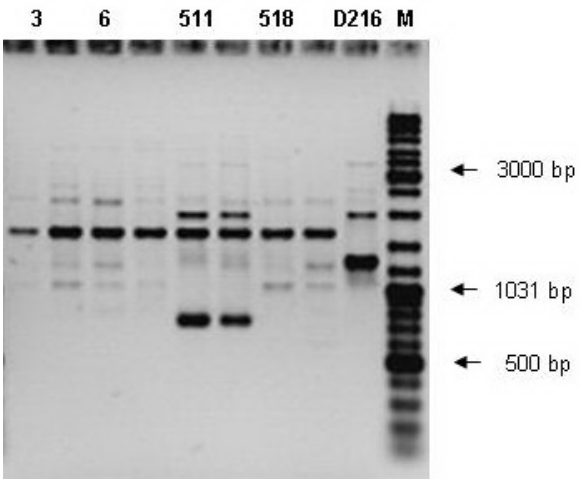

Figure 3. Genodiversity of *Bradyrhizobium japonicum* isolates based on PCR using AF14 primer

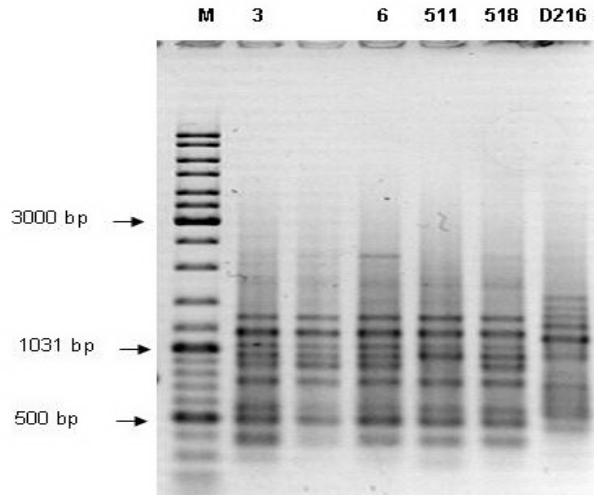

Figure 4. Genodiversity of *Bradyrhizobium japonicum* isolates based on PCR using SPH1 primer

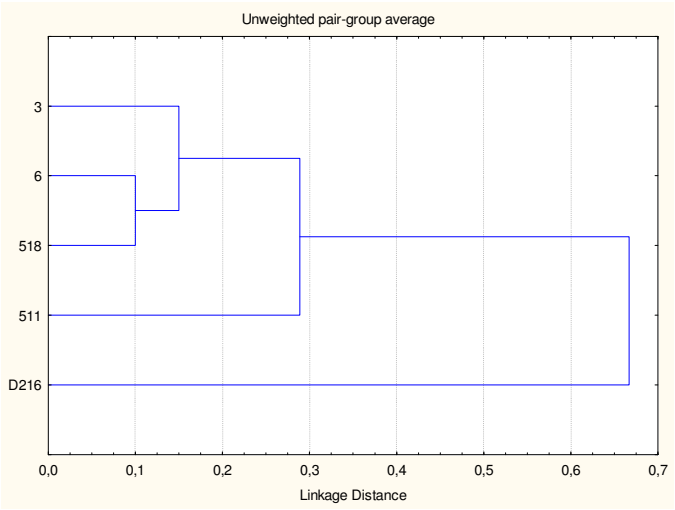

Figure 5. Cluster analysis of *Bradyrhizobium japonicum* strains obtained by RAPD method using four primers (AP10, BC318, AF14 and SPH1)

DISCUSSION

Several studies showed the influence of drought conditions on the survival of rhizobia in culture, using non-permeating substances polyethylene glycol (PEG) to lower external water potential, because PEG reduces water availability by binding water molecules without penetrating the cell wall (REHMAN and NAUTIYAL, 2002; RASANEN *et al*., 2004; CYTRYN *et al*., 2007; ABDEL-SALAM *et al*., 2010). Decrease of water potential, by adding PEG, resulted in a growth reduction of all strains tested in this investigation. Among the strains, significant reaction differences to osmotic stress were obtained. Strain D216 showed the highest tolerance with growth reduction by 3.3%, while strain 511 was the most sensitive with the reduction of growth by 56.7%. CYTRYN *et al*. (2007) also demonstrated that PEG in liquid medium reduced viability *B. japonicum*, and direct microscopic observation showed a 49% survival ratio. In contrast, plate count analysis showed only 17% survival of desiccated cells, suggesting the presence of a large number of viable but nonculturable cells and significant physiological changes caused by osmotic stress. Differences in morphological and physiological responses to osmotic stress between rhizobial strains is confirmed in studies of REHMAN and NAUTIYAL (2002), RASANEN *et al*. (2004) and ABDEL-SALAM *et al*. (2010). *B. japonicum* directly responds to water deficit conditions by the induction genes and proteins involved in the synthesis of trehalose, oxidative stress responses, proteins involved in protection of the cell membrane and repair of DNA damage (CYTRYN *et al*., 2007). Specific differences in structure and expression of these genes may explain strain specific rhizobial tolerance to water stress conditions.

Although many rhizobial species may produce siderophore, this property is considered a strain-specific and previous investigations have proven a great diversity among species and strains (HAYAT *et al*., 2010). In this study, none of five strains produced siderophore, which is consistent

with findings reported by BOIERO *et al*. (2007) and JOSIC *et al*. (2010) for other *B. japonicum* strains. However, MAHMOUD and ABD-ALLA (2001) tested several rhizobium and bradyrhizobium strains and all strains showed a positive reaction on CAS medium. Experiments of ANTOUN *et al*. (1998) demonstrated siderophore production in several strains of different rhizobial species, but less percentage of *B. japonicum* strains, showed positive reaction. Bradyrhizobia may not possess highly developed iron acquisition systems, having evolved in the acid soils of the tropics, where iron is more generally available than in neutral or high pH soils (MAHMOUD and ABD-ALLA, 2001). Iron is essential in symbiotic nitrogen fixation, but the ability of strains to produce siderophore does not always limit binding of nitrogen (AHEMAD and KHAN, 2011). This can be explained by the fact that some strains may only use siderophores produced by other microorganisms, although they themselves do not synthesize it (BENSON *et al*., 2005).

Among tested strains, only strain 518 had the ability to solubilize calcium phosphate. Phosphate solubilization activity of rhizobia is associated with the production of 2-ketogluconic acid, due to its ability to reduce pH of the medium (HAYAT *et al*., 2010). During phosphate solubilization, not all strains of rhizobia produced the same organic acid and it has been suggested that the nature of organic acid produced is more important than the quantity (HAYAT *et al*., 2010). Results of several authors emphasize certain strains of *R. leguminosarum* (bv. *viciae*, bv. *phaseoli*, bv. *trifolii*), *R. leguminosarum* sp. and *S. meliloti* as good P-solubilizers (ANTOUN *et al*., 1998; PEIX *et al*., 2001; ALIKHANI *et al*., 2006; DAIMON *et al*., 2006), as well as strains of *Mesorhizobium ciceri* i *Mesorhizobium mediterraneum* (PEIX *et al*., 2001). However, ANTOUN *et al*. (1998), PEIX *et al*. (2001), ALIKHANI *et al*. (2006) and BOIERO *et al*. (2007), reported that fewer strains of *B. japonicum* had the ability to solubilize inorganic phosphate, as demonstrated in this study.

Only strains 511 and 518 had the ability to hydrolysis urea. Urease allows many soil bacteria to use urea as nitrogen source (HASAN, 2000; TOFFANIN *et al*., 2002). The ability of *R. leguminosarum* bv. *viciae* strains to use urea as nitrogen source was documented by TOFFANIN *et al*. (2002). Similar results were obtained for fast-growing rhizobia such as *Rhizobium* (*Vigna unguiculata*) (de OLIVEIRA *et al*., 2006), whereas a larger variability in this trait seems to occur in slow-growing *B. japonicum* strains (de OLIVEIRA *et al*., 2006; DELIĆ *et al.,* 2010).

Identification and characterization of the rhizobia population plays an important role both in studying of soil biodiversity and biological nitrogen fixation. Findings of SIKORA and REDŽEPOVIĆ, (2000) and SIKORA *et al*. (2002), indicate that RAPD fingerprinting method reliably distinguish different *B. japonicum* strains, which by conventional methods could not be observed. RAPD method has been found to be useful in differentiating between closely related strains in genetic analysis of *Bradyrhizobium* sp. (KHBAYA *et al*., 1998; GONZALEZ-ANDRES and ORTIZ, 1998) and *Rhizobium* sp. strains (RAJASUNDARI *et al*., 2009). With genomic patterns generated by three AP short arbitrary primers SELENSKA-POBELL *et al*. (1996) distinguished several species of the genus *Rhizobium* at strain level. Using AP10 and SPH1 primers, JOŠIĆ (2004), JOŠIĆ *et al*. (2002; 2008) and JOSIC and RADIN (2012) estimated biodiversity of *R. leguminosarum* bv. *trifolii* populations within several types of soil in Serbia.

The results of molecular strain characterization indicated a large genetic distance between strain D216 and other strains. Amplification with RAPD primers showed the greatest genetic diversity between strains D216 and 511, and the greatest differences in tolerance to osmotic stress in liquid culture. These results will be used in further investigation of osmotic stress tolerance of *B. japonicum* and impact on draught reduction in symbiosis with soybean.

CONCLUSIONS

The decrease of water potential resulted in growth reduction of all tested strains and significant reaction differences to osmotic stress were obtained among the strains. Strain D216 showed the highest tolerance with growth reduction by 3.3%, while the most sensitive was the strain 511 with the reduction by 56.7%. None of five *B. japonicum* strains produced siderophore, only strain 518 showed the ability to solubilize insoluble phosphate and strains 511 and 518 had the urease ability. RAPD analysis indicated that genetic difference between strains D216 and the other strains was greater than 60%. Amplification with RAPD primers showed the greatest genetic diversity between strains D216 and 511, and the greatest differences in tolerance to osmotic stress in liquid culture.

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TOLERANTNOST PREMA OSMOTSKOM STRESU, PGP OSOBINE I RAPD ANALIZA SOJEVA *Bradyrhizobium japonicum*

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Izvod

Tolerantnost sojeva *B. japonicum* prema osmotskom stresu ispitana je na osnovu preživljavanja sojeva u rastvoru PEG-a. Najmanju tolerantnost prema osmotskom stresu pokazao je soj 511 (43.3%), dok je najtolerantniji bio soj D216 (sa 3.3% smanjenja rasta u prisustvu PEG-a). Ispitane su PGP osobine sojeva *B. japonicum*. Nijedan od pet sojeva *B. japonicum* nije produkovao siderofore, ureaznu aktivnost pokazali su sojevi 511 i 518, a sposobnost solubilizacije neorganskog tri-kalcijum fosfata imao je samo soj *B. japonicum* 518. RAPD analiza, prajmerima AP10, BC318, AF14 i SPH1, ukazala je na genetske razlike između sojeva *Bradyrhizobium*. Prva grupa (sojevi 3, 6 i 518) pokazala je više od 80% sličnosti. Sojevi 511 i D216 formiraju zasebne klastere. Razlika između soja D216 i ostalih sojeva bila je veća od 60%, a maksimalna divergentnost od 72% zabeležena je u poređenju sa sojem 511. PGP osobine, tolerantnost prema osmotskom stresu i RAPD analiza izdvojili su soj D216 kao veoma značajan za dalja istraživanja *B. japonicum* u smanjenju negativnih posledica suše na simbioznu zajednicu sa sojom.

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