Original scientific paper
DOI: /10.5513/JCEA01/20.4.2436
Journal of Central European Agriculture, 2019, 20(4), p.1116-1125

Phenotypic and genotypic diversity of *Sinorhizobium meliloti* strains isolated from soils in Zadar County

Fenotipska i genotipska raznolikost sojeva *Sinorhizobium meliloti* izoliranih iz tala Zadarske županije

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ABSTRACT

Soil bacteria, *Sinorhizobium melilo i* is of great agricultural importance because of its ability to fix atmospheric nitrogen in symbiosis with alfalfa, a very valuable forage crop. The main aim of this study was to evaluate tolerance of indigenous *S. melilo i* strains to stress environmental conditions. Twenty rhizobial strains, isolated from different regions in Croatia, were genotipically characterized to assess diversity amongst natural population. Stress tolerance assays were performed in order to select indigenous rhizobia with tolerance to unfavorable soil conditions. The growth of the strains was studied at different pH values, temperatures, NaCl and heavy metals concentrations. The results of 16S rDNA genotyping using PCR/RFLP analysis showed that 17 isolates could be assigned to *S. melilo i* while RAPD and ERIC-PCR fingerprints revealed significant genetic diversity among indigenous rhizobial strains. In comparison to the reference strain, higher resistance to adverse growth conditions of indigenous *S. melilo i* strains was determined. Most of the strains grew at temperatures higher than optimal and tolerated both acidic and alkaline environment. It was found that indigenous strains tolerate extremely high NaCl concentrations. Better understanding of rhizobial response to adverse environmental conditions is of potential value for improving rhizobial inoculants and efficiency of symbiotic nitrogen fixation.

Keywords: nitrogen fixation, indigenous strains, *Sinorhizobium meliloti*, genetic diversity, phenotypic characterization

SAŽETAK

Bakterija tla, *Sinorhizobium meliloti* važna je u poljoprivrednoj proizvodnji zbog sposobnosti fiksacije atmosferskog dušika u simbiozi s lucernom koja je vrlo vrijedna krmna kultura. Glavni cilj ovog istraživanja bio je procijeniti otpornost autohtonih sojeva *S. meliloti* na stresne uvjete okoliša. Najprije je genotipski karakterizirano dvadeset sojeva rizobija, izoliranih iz različitih regija u Hrvatskoj, kako bi se procijenila raznolikost unutar prirodne populacije. Ispitivanje otpornosti na stres provedeno je u cilju odabira autohtonih sojeva rizobija otpornih na nepovoljne uvjete u tlu. Ispitivan je rast sojeva pri različitim pH vrijednostima, temperaturama, koncentracijama NaCl-a i teškim metalima. Rezultati genotipizacije 16S rDNA PCR / RFLP analizom pokazali su da 17 izolata pripada vrsti *S. meliloti*, dok je primjenom RAPD i ERIC-PCR metode utvrđena značajna genetska raznolikost među autohtonim sojevima rizobija. Utvrđena je veća otpornost autohtonih sojeva *S. meliloti* na nepovoljne uvjete rasta u usporedbi s referentnim sojem. Većina sojeva rasla je na temperaturama višim od optimalnih i toleriralo je i kiselo i alkalno okruženje. Utvrđeno je da autohtoni sojevi podnose ekstremno visoke koncentracije NaCl. Bolje razumijevanje prilagodbe rizobija na nepovoljne uvjete okoliša važna je u svrhu poboljšanja inokuluma i učinkovitost simbiozne fiksacije dušika.

Ključne riječi: fiksacija dušika, autohtoni sojevi, *Sinorhizobium melilo i*, genetska raznolikost, fenotipska karakterizacija

INTRODUCTION

Nitrogen is the main limiting factor in production of agricultural crops. Majority of nitrogen stored in arable soils derives from biological fixation of molecular nitrogen from the atmosphere (Redžepović and Sikora, 2006). This natural process is of immense importance in sustainable agricultural production which is based on the reduction of agrochemicals use, primarily mineral fertilizers and plant protection products with emphasis on the exploitation of natural resources and renewable energy sources. Besides, it is well known that legume production has a unique role in sustainable agriculture (Strunjak and Redžepović, 1986; Komesarović et al., 2007).

Nodule bacteria of the *Sinorhizobium meliloti* species establish a mutually beneficial relationship with one of the most important fodder legumes, alfalfa (*Medicago sativa* L.). The result of symbiosis is the formation of nodules on alfalfa root where an extremely important process for agricultural production called symbiotic nitrogen fixation takes place. Utilization of this process reduces the need for nitrogen fertilization which is of significant ecological, economical and energetic importance (Mitsch et al., 2017).

Among legume crops, a special place in agricultural production takes alfalfa which is considered as one of the oldest and most important fodder crops. Its economic value derives from production of voluminous feed, organic acids and minerals, and with a yield of 12 t / ha of dry matter represents the highest biologicaly valuable protein source (Langer et al., 2008).

Nowadays there is an increasing emphasis on the exploitation of biological nitrogen fixation and in the alfalfa cultivation, seed inoculation is widely applied. Inoculation of alfalfa seeds with selected, highly efficient strains of *S. meliloti* can fully substitute the plant needs for nitrogen. Selection of high-quality *S. meliloti* strains is of crucial importance for successful inoculation because strains differ significantly in their effectiveness, compatibility and competitiveness. Although commercial strains of symbiotic fixing bacteria are characterized by high symbiotic activity, they often have lower competitive ability for the nodulation site compared to indigenous strains present in the soil (Bakhoum et al., 2014). Therefore, in the production of high-quality inoculants it is of great importance to select highly efficient strains which are also highly adaptive to unfavourable soil conditions in order not to limit the success of this, very important agrotechnical measure (Sharma et al., 2004). Although the inoculation of alfalfa is not common practice in Zadar County, the production of this valuable crop is of great importance for this submediterranean region of Croatia.

The main assumption is that native *S. meliloti* strains are present in the soils of Zadar County and that their distribution is influenced by the agroecological conditions of this area. It is also assumed that the strains within the natural population differ genotypically and phenotypically and that some of them have potential for efficient nitrogen fixation under stress soil conditions. The main aim of this study is identification and characterization of indigenous alfalfa rhizobia isolated from soils of Zadar County.

MATERIALS AND METHODS

Isolation of rhizobia from nodules

Soil samples were collected from 13 different sites of two locations (Vrana and Vigens) in Zadar County (Table1). Trapping host method was performed to obtain 20 isolates of indigenous alfalfa symbionts. Rhizobia were isolated from fresh surface sterilized nodules by the standard method (Vincent, 1970). Hence, nodules were immersed in 95% ethanol (v/v) for 10 s and then surface sterilized in 0,1% $HgCl_2$ for 4 min, and washed three times in sterile distilled water. Sterilization was performed to eliminate the possibility of isolating surface-attached bacteria. Sterilized nodules were crushed with a sterile glass rod in a sterile test tube. One loop full of the nodule content suspension was streaked on yeast mannitol agar (YMA) plates containing 0.0025% (w/v) Congo red. After incubation for 3 to 7 days at 28°C, single colonies were selected and restreaked on YMA (Vincent, 1970). Pure cultures were preserved in 20% glycerol at -20°C until further use.

Table 1. Origin and designation of rhizobial isolates used in this study

Sampling site Location	Coordinates	Strain designation
Vigens	45°81'31.77"N 15°97'70.48"E	43°57'22"N 15°33'43"E
Vrana	43°57'22"N 15°33'43"E	43°57'22"N 15°33'43"E

Phenotypic characterization of rhizobial isolates

Colony morphology

The colony morphology of the isolates was examined on YMA plate. After an incubation of 3 to 7 days at 28°C, individual colonies were characterized based on their size, color, shape, mucosity, transparency, borders, elevation, and Gram stain reaction (Vincent, 1970).

Salt, temperature, and pH tolerance

Rhizobia were examined for their tolerance to salt on YMA supplemented with 0.5, 1, 2, and 3% (w/v) NaCl (Romdhane et al., 2009).

Temperature tolerance was tested by incubating the inoculated plates at 37 and 42°C (Niste et al., 2015).

The ability of the isolates to grow in acidic or alkaline medium was tested by streaking each isolate on separate Petri plates on YMA with pH adjusted to 4, 4.5, 5.0, 5.5, 8.0, 8.5, 9.0, 9.5, as a method indicated by Shamseldin and Werner (2005).

Intrinsic resistance to heavy metals

Test resistance to heavy metals was conducted to assess the ability of the isolates to tolerate different heavy metals concentrations: cadmium (0.125, 0.25, 0.5, 1) mmol copper (0.5, 1, 1.5 I 2) mmol (zinc 0.25, 0.5, 1 and 2) mmol and manganese (1.5, 3, 6 and 9) mmol (Cevheri et al., 2011). The results were evaluated after one week of incubation at 28°C.

Genotypic characterization

The molecular study involved 20 selected rhizobial strains. Indigenous and reference strains *S. medicae* LMG 18864, *S. meliloti* 2011 and type strain *S. meliloti* 30135 were grown for three days in yeast mannitol broth liquid medium (YMB) at 29°C. Strains were then stored in glycerol at -20°C for further molecular analyzes. DNA isolation was performed using DNeasy® Blood & Tissue kite (QIAGEN, 2006, USA), according to manufacturer's instructions. The concentration of isolated DNA was determined using Lambda 12 spectrophotometer (Perkin Elmer, USA) on wavelength 260 nm.

PCR-RFLP analysis of 16S rDNA

The universal primers fD1 and rD1 were used for PCR amplification of 16S rDNA (Sikora et. al., 2003). 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 $MgCl_2$, 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 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72°C for 2 min; and final extension at 72 °C for 3 min. Amplified PCR products were digested with restriction endonucleases *RsaI* (Roche, Germany), as recommended by the manufacturer. The digests 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 restriction patterns were visualised under UV illumination after staining with ethidium bromide and photographed with Cannon Powershot A640 camera. A 1kb ladder (GenSura Laboratories, CA) was used as molecular size marker.

ERIC fingerprinting was performed with primers ERIC 1R and ERIC 2. Six arbitrarily chosen primers (P1-P6) used for RAPD fingerprinting were 10 nucleotides in length and had a GC content of 70 %. For both methods, primer sequences were previously described (Sikora et al., 2003)

and the PCR reaction mixture was the same as described above for PCR-RFLP analysis of 16S rDNA.

The cycling programs for RAPD and ERIC-PCR fingerprinting differed only in the annealing temperature and time. The reaction mixtures were incubated for 5 min at 95°C for initial denaturation, and then amplified for 35 cycles consisting of 30 s at 94°C, 30 s at 36°C (RAPD), and 30 s at 50°C and 1 min at 52 °C (ERIC-PCR), and 1 min at 72°C followed by a 7-min incubation at 72°C. The amplification products were separated by gel electrophoresis on precast 6% poly (NAT) gels and visualized as described above.

All restriction patterns as well as RAPD and ERIC-PCR fingerprints were coded in the binary form, and analyzed using NTSYS-pc package (Rohlf, 1990). A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

RESULTS AND DISCUSSION

Isolation and morphological characteristics of rhizobial isolates

A total of 20 rhizobial strains were isolated from root nodules of alfalfa. All isolates subjected to Gram staining and microscopic observation were all Gram negative. The majority of rhizobial isolates had the same colony morphology and growth rate on YMA medium. A high production of mucus was verified in all of the studied isolates. They formed transparent to creamy colonies with 1 to 3 mm in diameter after 3 to 5 days incubation on YMA plates.

Salt, temperature and pH tolerance of rhizobial isolates

Phenotypic characteristics of rhizobia such as growth on different pH values, temperatures, concentrations of salts or heavy metals and on various sources of carbohydrates, have been widely studied (Grossman et al., 2005; Moschetti et al., 2005; Mnasri et al. 2007; Shamseldin et al., 2009; Cevheri et al., 2011). It has been shown that low pH negatively affects the growth of *S. meliloti* strains isolated from alfalfa (Blažinkov et al., 2010).

In this study, growth of strains at pH values lower and higher than optimum was tested. The results showed a weak growth at lower pH and strain Z10A can be considered as the most tolerant to acid and alkaline conditions because it grew well at all tested values (Table 2).

The optimal temperatures for growth of species from *Sinorhizobium* genera are 26-30°C, while their generation time is 2-4 hours, and therefore they are classified into a group of fast-growing bacteria (Atlas, 1997). Strains from this study differentiaite in the ability to grow at temperatures higher than optimal. Similar results were published in paper Blažinkov et al. (2010) where good growth at 37°C was also determined. Strains Z2B, Z9E, Z11E and Z12A did not grow at 37°C, showing that temperatures above optimum inhibit their growth. Other strains showed good or at least weak growth at 37°C. Strains Z15D, Z3D, reference and type strain grew weak at temperature of 42°C, while the growth of all other strains was inhibited (Table 2).

This study investigated the ability of rhizobial strains to grow at elevated NaCl concentrations. The results showed higher tolerance of all tested strains to elevated NaCl concentrations than to changes of pH values. These findings were in contrast to the work of Blažinkov et al. (2010) but in agreement with previous studies (Glenn and Dilworth; 1994, Draghi et. al., 2010) which showed that *Sinorhizobium* strains were extremely sensitive to low pH.

Croatian soils, especially those in the coastal area, often contain more than 0.01% NaCl, which is the optimal for the growth of rhizobia. At 0.5% and 1% NaCl, all strains showed good growth with the exception of strain Z9 E and Z12A. Slow or good growth at 2% NaCl was determined for all strains (except Z9E and Z12A) while at 3% NaCl the growth was determined for 57% of strains.

Table 2. Effect of NaCl concentration, pH and temperature on rhizobial isolates

Strain	pH				NaCl (%)				Temperature (C°)	
	4	5	8	9.5	0.5	1	2	3	37°C	42°C
Z1A	--	--	++	++	++	++	+-	--	+-	--
Z2B	--	--	++	++	++	++	+-	--	--	--
Z3A	--	++	++	++	++	++	++	++	++	--
Z3D	--	++	++	++	++	++	++	++	++	+-
Z4D	--	--	+-	+-	++	++	+-	--	++	--
Z6D	--	++	++	++	++	++	++	++	++	--
Z7C	--	--	++	++	++	++	+-	--	+-	--
Z8C	--	--	++	++	++	++	+-	--	+-	--
Z8E	--	--	++	++	++	++	+-	--	+-	--
Z9E	--	--	+-	--	--	--	--	--	--	--
Z10A	+-	++	++	++	++	++	++	++	++	--
Z10B	++	++	++	++	++	++	++	++	++	--
Z11E	--	++	++	++	++	++	+-	--	--	--
Z12A	--	--	--	--	--	--	--	--	--	--
Z13A	--	++	++	++	++	++	++	++	+-	--
Z13B	--	++	++	++	++	++	++	++	++	--
Z13E	--	++	++	++	++	++	++	++	+-	--
Z14A	--	--	++	++	++	++	++	+-	++	--
Z14C	--	--	++	++	++	++	+-	+-	++	--
Z15D	--	--	++	++	++	++	+-	--	+-	+-
2011	+-	+-	++	+-	++	++	+-	+-	++	+-
30135	+-	+-	++	+-	++	++	+-	+-	++	+-
LMG 18864	+-	+-	++	+-	++	++	+-	+-	++	+-

Intrinsic resistance to heavy metals

Unlike Cevheri et al. (2011) who did not observe the growth of any strain at the highest investigated concentration of 1 mmol cadmium, in this study strains Z3A, Z6E, Z10A, Z10B, Z11E and Z13E (Table 3) showed good growth at all tested concentrations except at 1 mmol. No growth was observed for the strains Z1A, Z2B, Z9E and Z12/A at any concentration of cadmium while the other strains showed at least weak growth (Table 3).

Testing the resistance of strains to the presence of copper, only 22% of strains were able to grow at concentration of 0.5 mmol and no growth was observed at any other tested concentration in this research. This results are significantly different from those of Cevheri et al. (2011) in which 100% isolates grew up at a concentration of 0.5 mmol.

Table 3. Effect of heavy metals on rhizobial isolates

Strain	Cadmium (mmol)				Copper (mmol)				Zinc (mmol)				Manganese (mmol)			
	0.125	0.25	0.5	1	0.5	1	2	3	0.25	0.5	1	2	0.5	3	6	9
Z1A	--	--	--	--	--	--	--	--	++	+-	+-	+-	++	++	++	++
Z2B	--	--	--	--	--	--	--	--	++	+-	+-	+-	++	++	++	++
Z3A	++	++	++	--	--	--	--	--	++	++	++	++	++	++	++	++
Z3D	++	++	--	--	--	--	--	--	++	++	++	++	++	++	++	++
Z4D	++	+-	--	--	+-	+-	--	--	++	++	++	++	++	++	++	++
Z6D	++	++	++	--	--	--	--	--	++	++	++	++	++	+-	+-	+-
Z7C	++	--	--	--	--	--	--	--	--	--	--	--	++	++	++	++
Z8C	++	--	--	--	+-	--	--	--	++	++	++	++	++	++	++	++
Z8E	++	--	--	--	++	--	--	--	++	++	++	++	--	--	--	--
Z9E	--	--	--	--	--	--	--	--	--	--	--	--	++	+-	++	++
Z10A	++	++	++	--	--	--	--	--	++	++	++	++	++	+-	++	++
Z10B	++	++	++	--	--	--	--	--	++	++	++	++	++	++	++	++
Z11E	++	++	++	--	--	--	--	--	++	++	++	++	--	--	--	--
Z12A	--	--	--	--	--	--	--	--	--	--	--	--	++	++	++	++
Z13A	++	++	--	--	--	--	--	--	++	++	++	++	++	++	++	++
Z13B	++	++	--	--	--	--	--	--	++	++	++	++	++	++	++	++
Z13E	++	++	++	--	--	--	--	--	++	++	++	++	++	++	++	++
Z14A	++	--	--	--	++	--	--	--	++	++	++	++	++	++	++	++
Z14C	++	++	--	--	++	--	--	--	++	++	++	++	++	++	++	++
Z15D	++	+-	+-	--	--	--	--	--	++	++	+-	+-	++	++	++	++
2011	++	+-	--	--	--	--	--	--	++	+-	--	--	++	++	++	++
30135	++	+-	--	--	--	--	--	--	+-	+-	--	--	++	++	++	++
LMG 18864	+-	+-	--	--	--	--	--	--	+-	+-	--	--	++	++	++	++

The results for the growth of the strains on the medium with zinc are significantly different from those on the medium with cadmium and copper. Strains Z7C, Z9E and Z12A did not grow at any tested concentrations, while the vast majority of other strains showed very good growth at all concentrations of zinc (Table 3). The growth was observed for 87% of strains at 0.25 mmol and 0.5 mmol and 74% at 1 and 2 mmol Zn. Similar results were obtained in a study of Cevheri et al. (2011) where 100% isolates grew at 0.25 mmol, 55% at 0.5 mmol, 15% at 1 mmol and no growth was observed at 2 mmol. Rhizobial strains also showed a very good growth on the medium with manganese in all tested concentrations. The results of this study are different from that of Cevheri et al. (2011), in which tested strains did not grow at concentration of 6 mmol while only 20% grew at 3 mmol and 65% at 1.5 mmol Mn.

Genotypic characterization

The 16S rDNA PCR - RFLP method was used to identify strains from this study at species level. Reference strains *S. meliloti* 2011 and *S. medicae* LMG 18864, the type strain *S. meliloti* 30135 were included as well. Total genomic DNA was first amplified and the products were digested with *RsaI* enzyme. The results revealed that 85% of the isolates belong to *S. meliloti* species, while further analysis is needed to identify other 15% of the strains (data not shown).

Strains which were determined to belong to *S. meliloti* species as well as reference strain *S. meliloti* 2011 and the type strain *S. meliloti* 30135 were further analysed using RAPD method with three different oligonucleotide primers. Obtained fingerprints differed in the number and size of the fragment indicating the polymorphism within the investigated isolates. After the cluster analysis of the total number of amplified polymorphic fragments, a final dendrogram was constructed with the DendroUPGMA program used to differentiate strains and to study the genetic variability of *S. meliloti* natural population (Figure 1).

The dendrogram showed that the *S. meliloti* strains can be divided into two main groups based on their RAPD profiles at a relative similarity level of 0.72. Within the first main group, the greatest differences were determined between reference strain *S. meliloti* 2011 and all other strains (relative similarity of 0.73). The second main group comprises five rhizobial strains divided into two subgroups. The first subgroup contains isolates Z10A and Z10B isolated from the same soil sample with a similarity level of 0,82. These results revealed the moderate genetic diversity among indigenous alfalfa rhizobia. The obtained data showed that the significant genetic diversity is present even among the strains isolated from the same soil samples, as was confirmed also by other authors (Blažinkov et. al., 2010; Elboutahiri et. al., 2010).

Utilizing the ERIC-PCR method with specific primers, the total genomic DNA was amplified from 17 indigenous strains for which it was determined to belong to the *S. meliloti* species and from the reference strain *S. meliloti* 2011 and the type strain *S. meliloti* 30135. Sufficient number of polymorphic patterns was obtained to determine the variability of the strains. The distribution of strains in two main groups at the relative similarity level of 0.79 (Figure 2).

The first group contains two subgroups, of which the first comprises nine strains. Among them two identical strains (relative similarity 1,00) Z13A and Z13B were isolated from the same soil sample. In the second subgroup, strains Z14A and Z14C, were also identical while in RAPD analysis their similarity was 0.92. It is also shown (Figure 2) that the reference strain 2011 is different from the other strains in its group (relative similarity 0.81). The second main group consists of isolates of the same soil sample Z10A and Z10B with similarity 0.89. Obtained data indicate the existance of very similar or even identical strains within the natural population of *S. meliloti*. Although the variability between the strains was determinated with ERIC - PCR method, it was apparently lower in comparison to that obtained with RAPD - PCR method.

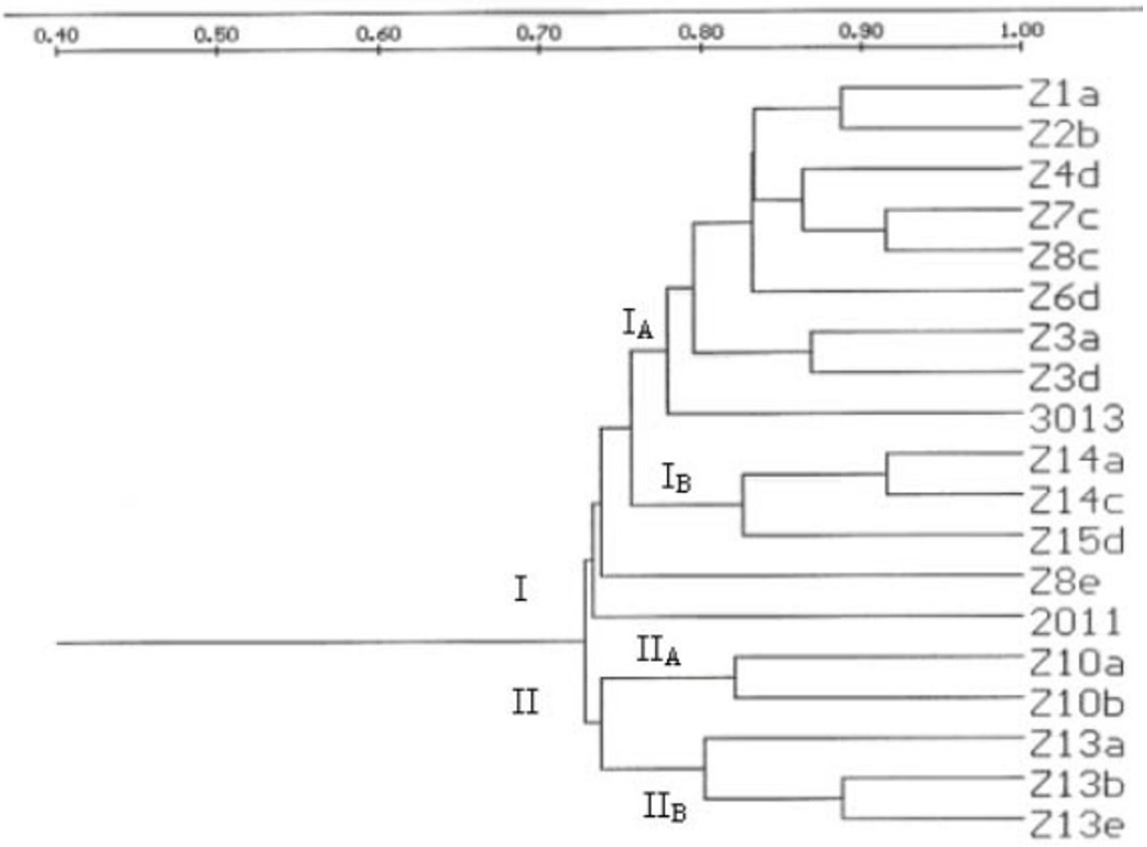

Figure 1. Dendrogram of *S. meliloti* strains derived from RAPD fingerprints generated by using three different primers

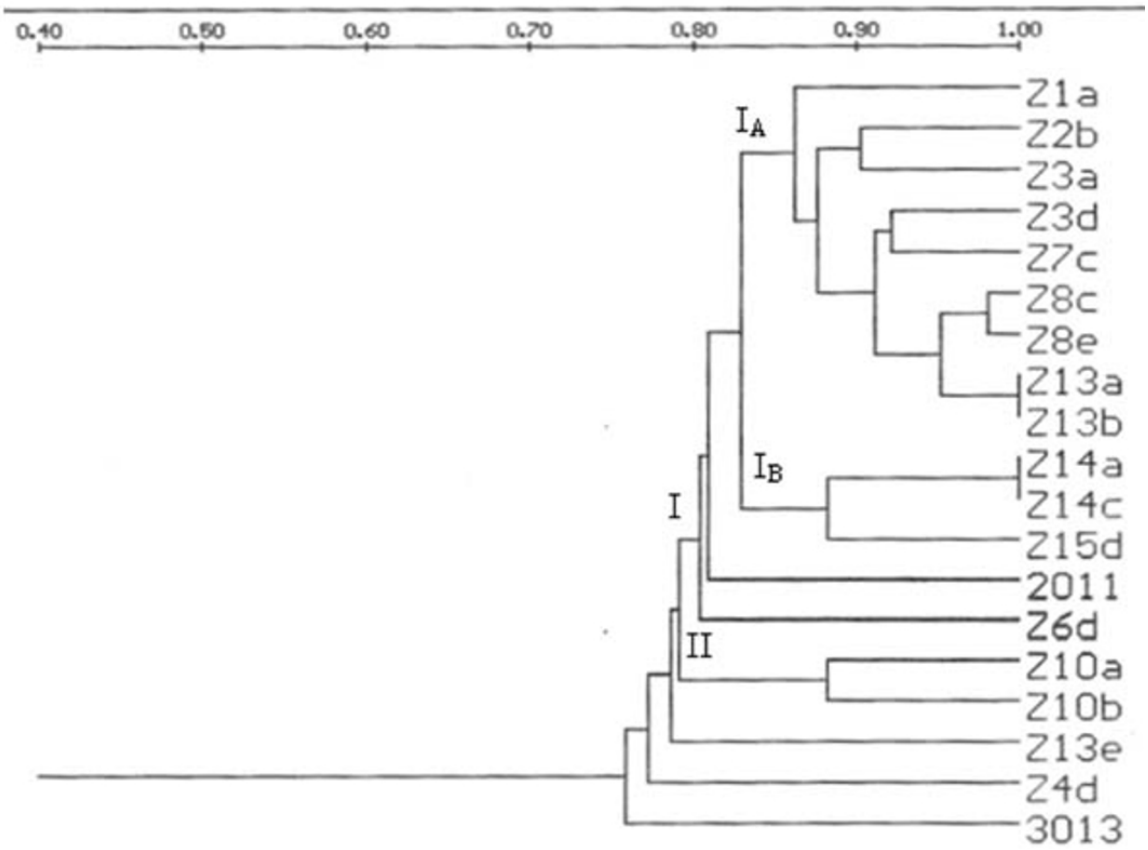

Figure 2. Dendrogram of *S. meliloti* strains derived from ERIC fingerprints generated by using ERIC 1R and ERIC 2 primers

CONCLUSION

The results of the present study revealed moderate diversity among investigated rhizobial strains isolated from Zadar County. However, the difference between strains from the same soil sample was obtained. The studied strains were tolerant to high concentrations of NaCl. Hence, these isolates may be the candidates for use in the saline soil such as those in the Croatian coastal area. The selected isolates were also tolerant to high temperatures and to extreme pH from 4.5 to 9.5. The highest resistance to heavy metals was recorded for zinc and manganese, while resistance to cadmium and copper was moderate.

All indigenous strains used in this study will be further characterized for their symbiotic properties in order to select the high quality and the most suitable strains for alfalfa inoculation under specific agroecological conditions.

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