Selection of Stress Tolerant Indigenous Rhizobia Nodulating Alfalfa (*Medicago sativa* L.)

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Summary

Alfalfa (*Medicago sativa* L.) is a very important forage crop, which forms a symbiotic relationship with nodule bacterium *Sinorhizobum meliloti*. The main aim of this study was to evaluate the stress tolerance of indigenous *S. meliloti* strains to adverse environmental conditions. Twenty rhizobial strains, isolated from different regions in Croatia, were phenotypically characterized to assess diversity amongst natural field population. The growth of the strains was studied at different pH values, temperatures, carbohydrate sources and different concentrations of NaCl. The results showed that most of the strains can grow at temperatures higher than optimal and that strains tolerate both acidic and alkaline environment. It was found that indigenous strains can tolerate extremely high concentrations of NaCl. Most strains possess the enzyme urease while only a small number of them possess the enzyme catalase. The results showed that all indigenous strains belonged to the group of fast-growing rhizobia and that they were more tolerant to the antibiotics tested in comparison to the reference *S. meliloti* strain. Better understanding of rhizobial response to adverse environmental conditions is of important value for improving rhizobial inoculants and efficiency of symbiotic nitrogen fixation.

Key words

biological nitrogen fixation, phenotypic characterization, alfalfa (*Medicago sativa* L.), *Sinorhizobium meliloti*

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Introduction

Rhizobia are gram negative soil bacteria which are associated symbiotically with the roots of leguminous plants. The symbiosis is based on specific recognition of signal molecules produced by bacteria and its plant partners. Process of symbiosis results in biological nitrogen fixation in which atmospheric nitrogen (N_2) is converted into ammonia (NH_3) which is subsequently available for plants, and in turn plants provide nutrients to the bacteria. Presently, rhizobia are classified into genera *Rhizobium, Bradyrhizobium, Mesorhizobium, Azorhizobium, Allorhizobium* and *Ensifer* (formerly *Sinorhizobium*) and two new genera *Neorhizobium* and *Pararhizobium* (Velázquez et al., 2017).

Sinorhizobium meliloti is a gram-negative soil bacterium, and a member of the family *Rhizobiaceae* in the alpha subdivision of Proteobacteria. *S. meliloti* forms symbiotic associations with plant genera such as *Medicago*, including alfalfa (*Medicago sativa* L.), *Melilotus* (sweet clover *Melilotus alba*) and *Trigonella*. On the roots of these plants *S. meliloti* forms nodules within which the differentiated bacteroids reduce nitrogen gas to ammonia. In recent years, due to the need for reduced applications of nitrogen fertilizers, rhizobia have gained a great value and play an important role in improving soil fertility in farming systems (Zahran, 1999). Inoculation of alfalfa with efficient strains of rhizobia has significant economic and ecological benefits (Elboutahiri et al. 2010). Survival and effective functioning of indigenous and inoculated rhizobial populations are reduced by high soil temperatures, salt and osmotic stress, soil acidity and alkalinity. The presence of indigenous strains of rhizobia in the soils, highly competitive and well adapted to certain environment can reduce the inoculation benefits even with highly efficient strains. Inoculation with stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation under stress conditions. Rhizobial populations vary in their tolerance to major environmental factors (Biswas et al., 2008).

All soil microorganisms are frequently exposed to environmental stress, such as limitations in nutrient supply, sudden changes in osmolarity, and up or downshifts in temperature. Stress response in bacteria is essential for effective adaptation to changes in the environment, as well as to changes in the bacterial physiological state. This response is mediated by global regulatory mechanisms that operate in an effective method of transcriptional control, with the participation of specialized RNA polymerase subunits, the alternative sigma factors (de Lucena et al., 2010).

The main aim of this study was to select indigenous alfalfa rhizobial strains isolated from different regions of Croatia, tolerant to adverse environmental conditions.

Materials and methods

Isolation of rhizobia from soil

Alfalfa was grown in soil samples collected from the most important alfalfa production regions in Croatia (Istria, Zadar, Brod-Posavina and Koprivnica-Križevci County). Soil chemical characteristics of sampling sites are reported in Table 1.

Trapping host method was employed to obtain twenty indigenous rhizobial strains from alfalfa nodules using the standard procedure (Vincent, 1970). Rhizobia were isolated on Yeast-Mannitol-Agar medium (YMA-CR) supplemented with 0.0025 % (w/v) Congo red. The single colonies were selected and checked for purity by repeated streaking on YMA. Individual colonies morphology was characterized based on size, color, mucosity, borders, transparency, elevation and Gram stain reaction.

Phenotypic characterization of rhizobial isolates

In order to determine generation time for isolates, each one was streaked on YMA plates and a single colony from the respective isolates was transferred into test tubes containing 10 ml YMB and incubated, on a rotary shaker (125 rpm /min) at room temperature for 48 h. One ml of cell suspension from each culture broth was transferred into 250 ml Erlenmeyer flasks containing 100 ml of YEM broth and incubated on a rotary shaker (125 rev/min) at 28 °C. Turbidity was measured every 2 h for three days at 540 nm using UV/VIS spectrophotometer Lambda 12 (Perkin Elmer, USA). Mean generation time or doubling time was calculated from the logarithmic phase. A loop full of test isolates from broth culture of 72h was streaked on YMA medium and incubated at 28 °C. After 3-5 days, colony diameter and morphology of the isolates was recorded.

Acid-base production test

The ability of isolates to produce acid or base in the medium was evaluated by inoculating into YMB containing the Bromothymol blue (BTB) (0.125 %) as a pH indicator. A color change of the colonies was observed after 3-5 days of incubation.

Salt, temperature and pH tolerance

As for salinity tolerance, this experiment was carried out by cultivating rhizobial isolates on YMA medium with different NaCl concentrations (1.0, 2.0, 3.0 and 4.0%) plus the control treatment (0.01% NaCl). Isolates were incubated at 28 °C for 3-5 days.

The ability of isolates to grow at high and low temperatures was also determined using YMA medium incubated at temperatures ranging from 4-42 °C (Hameed et al., 2014).

Concerning the ability of the isolates to grow in acid and alkaline media, the isolates were inoculated on YMA media in which pH was adjusted to 4.5, 5.5, 8, and 10 by using sterile HCl or NaOH (Bernal and Graham, 2001) and the isolates were kept at 28 °C for 3-5 days.

Antibiotic resistance (AR)

Resistance of isolates to different antibiotics was performed using ampicilin (10 µg/ml), streptomycin (10 µg/ml), erythromycin (15 µg/ml) and kanamycin (30 µg/ml).

Intrinsic resistance and minimum inhibitory concentration for four different antibiotics was tested on YMA by using disc method (Lebrazi et al., 2018). Strains were considered resistant when growth occurred and sensitive when zone of inhibition was formed.

Table 1. Strain designation and chemical properties of soils used for their isolation

Rhizobial isolates	pH (in water)	pH (in MKCl)	*w* (humus)/%	*w* (nitrogen)/%	*w* ($P_2 0_5$)/mg/100g	*w* ($K_2 0$)/mg/100g
I1	7.44	7.13	3.60	0.10	0.70	20.00
I2	7.32	7.21	1.30	0.08	3.70	11.80
I3	6.75	6.07	2.50	0.15	1.00	12.20
I4	7.18	6.93	3.70	0.20	13.50	40.00
I5	7.45	7.17	2.70	0.15	2.10	19.50
Z1	8.11	7.86	4.70	0.24	6.00	5.00
Z2	8.05	7.99	5.00	0.28	15.00	4.00
Z3	8.02	7.60	3.60	0.20	15.50	44.50
Z4	8.08	8.04	2.70	0.15	6.60	5.00
Z5	8.06	7.74	6.00	0.34	9.50	24.00
NG1	5.50	4.10	2.00	0.13	27.00	12.60
NG2	7.93	6.78	3.40	0.21	9.10	14.00
NG3	7.95	7.00	1.90	1.10	16.60	12.80
NG4	7.28	6.43	2.50	0.150	29.40	19.50
NG5	6.65	5.56	3.60	0.20	45.9	33.5
K1	5.01	4.21	1.50	0.10	11.20	12.20
K2	7.42	7.27	2.10	0.14	9.90	6.80
K3	7.45	7.10	1.40	0.08	24.30	5.00
K4	7.45	7.10	1.40	0.08	24.30	5.00
K5	7.45	7.10	1.40	0.08	24.30	5.00

Enzymes Test

Catalase and urease tests were performed according to Hameed et al. (2014) and Ronald et al. (1995) respectively.

Results and discussion

Twenty isolates were obtained from nodules of alfalfa plants after 3-5 days of growth on YMA medium. Colonies formed were white translucent, glistening with diameters of 1-3 mm (Table 2). All bacteria were gram negative rod-shaped. Colonies of the isolates appeared to be sticky indicating the production of mucous substances which is one of the characteristics of rhizobia (Singh et al., 2013). Eighteen isolates changed the green color of yeast extract mannitol broth bromothymol blue (YMB-BTB) to yellow (Table 2.), indicating the production of acid which is one of the characteristics of fast-growing rhizobial strains (Talukder et al., 2008). However, isolate NG5, NG4, I2 and I4 changed the color into blue which is typical for base production by slow growing rhizobia (Talukder et al., 2008).

Generation time could be defined as time needed for a strain to reach logarithmic growth phase. This important parameter is prerequisite for any study of rhizobia. The doubling time of most isolates was found to be relatively fast (1.60- 1.73 h). Isolates I5, NG3, Z1, Z2 and K4 exhibited the fastest generation time to double its population (Table 1). According to the classification of the family *Rhizobiaceae* (Somasegaran and Hoben, 1994), isolates in the current study might show typical properties of fast-growing rhizobia.

Symbiotic nitrogen fixation by rhizobia in root nodules of grain and forage legumes provides substantial economic and environmental benefits. Symbiotic nitrogen fixation is dependent on the host plant genotype, the rhizobia strain, and the interaction of these symbionts with the pedoclimatic factors and the environmental conditions. *Sinorhizobium* strains could be used on stressed sites as inoculum to promote the growth of leguminous plants. Tolerance of rhizobial isolates to elevated NaCl concentration showed variation among strains. All isolates were able to grow on YMA containing 0.1 % NaCl.

Table 2. Morphology and growth characteristics of alfalfa rhizobial isolates grown on YMA and incubated at 28 °C

Rhizobial isolates	IS	CD	MGT	Growth on YMA-CR	Growth on YMA-BTB
I1	IC	2.0	1.68	Colorless	yellow
I2	IC	1.5	1.68	Colorless	blue
I3	IC	1.5	1.70	Colorless	yellow
I4	IC	3.0	1.68	Colorless	blue
I5	IC	3.0	1.65	Colorless	yellow
Z1	ZC	2.0	1.65	Colorless	yellow
Z2	ZC	2.5	1.65	Colorless	yellow
Z3	ZC	2.0	1.73	Colorless	yellow
Z4	ZC	2.0	1.70	Colorless	yellow
Z5	ZC	3.0	1.73	Colorless	yellow
NG1	BSC	2.0	1.68	Colorless	yellow
NG2	BSC	1.0	1.68	Colorless	yellow
NG3	BSC	1.0	1.65	Colorless	yellow
NG4	BSC	2.5	1.68	Colorless	blue
NG5	BSC	2.0	1.70	Colorless	blue
K1	KKC	2.5	1.67	Colorless	yellow
K2	KKC	2.5	1.70	Colorless	yellow
K3	KKC	2.0	1.70	Colorless	yellow
K4	KKC	3.0	1.60	Colorless	yellow
K5	KKC	2.5	1.68	Colorless	yellow
18864^T	LMG	3.0	1.68	Colorless	yellow
2011	IGER	2.0	1.68	Colorless	yellow

Keys: IS = isolation site; CD = Colony diameter; MGT = Mean generation time; IC = Istria County; ZD = Zadar County; BSC = Brod - Posavina County; KKC = Koprivnica - Križevci County; LMG = BCCM/LNG Bacteria Collection, Ghent, Belgium; IGER = Institute of Grassland and Environmental Research, UK

The results shown in Table 3 demonstrate a high diversity among isolates, 75 % of isolates were highly tolerant to salt concentration from 3 % to 4 % NaCl. Table 3 also shows that 20 % of isolates were less tolerant to salt stress in comparison to other strains since their growth was recorded on plates with 1 % and 2 % NaCl but not on higher concentrations. Strains K3, K5 and the reference strain 2011 were the most sensitive to elevated salt concentration. These strains grew only at 0.01 % NaCl (Table 3).

Rhizobia are mesophiles and grow poorly at temperatures below 10 °C or above 37 °C (Graham, 1992). For most rhizobia, the optimum temperature range for growth is from 28 to 30 °C. (Harwani, 2006). All the isolates including reference and type strain were able to grow at 28 °C while 80 % continued to grow at 37 °C. As indicated in Table 3., 40 % of isolates were tolerant to high temperature as they could grow at 42 °C. Ihsan (2000) reported that it is rare for rhizobia to grow at 4 °C. Our results confirm that, since none of the isolates could grow at that temperature level. Ahamad et al. (1981) pointed out that geographical origin plays an important role in the temperature tolerance of certain strains. Furthermore, rhizobial strains isolated from very dry and hot areas have the ability to survive better at higher temperatures than those isolated from colder geographical regions. In this study correlation between temperature tolerance and geographical origin was also observed; strains isolated from Mediterranean Zadar County could grow better at higher temperature than isolates obtained from continental regions of Croatia (Table 3).

Table 3. Tolerance degree of rhizobial strains to different NaCl concentrations, pH and temperature

Rhizobial isolates	NaCl % tolerated	pH tolerated	T(C°) tolerated
I1	0.01-4	4.5-10	28-37
I2	0.01-3	4.5-10	28-37
I3	0.01-3	5.5-10	28-37
I4	0.01-1	4.5-10	28-37
I5	0.01-4	4.5-10	28-37
NG1	0.01-4	4.5-10	28-37
NG2	0.01-3	4.5-10	28-37
NG3	0.01-4	5.5-10	28-37
NG4	0.01-4	5.5-10	28-37
NG5	0.01-3	5.5-10	28-37
Z1	0.01-3	5.5-10	28-42
Z2	0.01-3	5.5-10	28-42
Z3	0.01-4	5.5-10	28-42
Z4	0.01-2	4.5-10	28-42
Z5	0.01-3	5.5-10	28-42
K1	0.01-4	5.5-10	28-42
K2	0.01-2	4.5-10	28-42
K3	0.01	5.5-10	28-37
K4	0.01-1	5.5-10	28-42
K5	0.01	5.5-10	28-37
18864^T	0.01-3	5.5-10	28-37
2011	0.01	5.5-10	28-37

In general, the optimum pH for rhizobial growth was reported to be between six and seven (Mensah et al., 2006). The results of this study showed that all isolates grew at pH 10 while 40 % of them grew at pH 4.5. The results agreed with those presented by Hasani et al., 2010. They stated that all strains tolerate salt concentration ranging from 3 % to 4 % NaCl and were highly resistant to alkaline conditions (pH 8 and 10). Our data (Table 3) agrees with those of Ali et al., (2010) who reported that isolates collected from dry geographical regions tolerate better the lower pH levels. Rhizobial strains isolated from Istria County, a dry region in Croatia, were able to grow at pH 4.5. Although some authors (Thqmi-Alami et al., 2010) found that rhizobia nodulating alfalfa plant were acid sensitive.

All 20 isolates were tested for resistance to four antibiotics at different concentrations (10, 15 and 30 $\mu g \cdot mL^{-1}$). The results revealed that all the isolates had intermediate resistance to kanamycin, erythromycin and streptomycin in all indicated concentration levels and 31 % of isolates were resistant to ampicillin at concentration 10 $\mu g \cdot mL^{-1}$, 20% at concentration of 15 $\mu g \cdot mL^{-1}$ and 14% at concentration of 30 $\mu g \cdot mL^{-1}$ (Table 4).

Table 4. Antibiotic resistance of rhizobial isolates to different concentration of antibiotics

Antibiotic	Resistance of isolates, %		
	10 $\mu g\ mL^{-1}$	15 $\mu g\ mL^{-1}$	30 $\mu g\ mL^{-1}$
Ampicillin	31	20	14
Streptomycin	5	3	0
Erythromycin	19	14	9
Kanamycin	0	0	0

The results revealed that isolates I1, I2, I3, I4, I5, NG1, Z4 and Z5 were positive to catalase. Other isolates were negative to the catalase test. As clearly shown in Table 3, 75 % of salt tolerant isolate gave a strong positive reaction to this enzyme. This result goes in agreement with that obtained by Haythem et al. (2011) indicating that NaCl and polyethylene glycol (PEG) tolerant strains showed increased catalase production under water deficient conditions. As for urease test, the results showed (Table 5) that 30 % of the isolates were negative.

Table 5. Enzymes reaction pattern of rhizobial isolates

Rhizobial isolates	Catalase	Urease
I1	+	+
I2	+	+
I3	+	+
I4	+	+
I5	+	+
NG1	+	+
NG2	–	–
NG3	–	+
NG4	–	+
NG5	–	+
Z1	–	+
Z2	–	+
Z3	–	–
Z4	+	–
Z5	+	+
K1	–	–
K2	–	–
K3	–	+
K4	–	–
K5	–	+
18864^T	–	+
2011	–	–

Conclusion

The present study reveals phenotypic diversity of *indigenous* rhizobia nodulating alfalfa (*Medicago sativa* L.) in different regions of Croatia. Some of the strains showed remarkable physiological characteristics such as high salt tolerance, resistance to antibiotics and survival at low or high pH values. According to these results, approximately 35-40% of isolates could be selected based on their resistance to adverse conditions. Results demonstrate that *in vitro* selection of stress tolerant strains may be a useful tool in the search for rhizobial strains better suited for soils in which NaCl and pH constitute a limitation for symbiotic nitrogen fixation. Therefore, due to their tolerance to environmental stress, these rhizobia could be included in the production of hight quality inoculant. However, these results present the first part of the selection program which is to be followed by detailed identification and characterization of strains with the emphasis on testing their symbiotic performance in greenhouse and field trials.

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