SALINITY EFFECT ON THE GROWTH OF RHIZOBIA *IN VITRO*

Monica NISTE[1], Roxana VIDICAN[1], Ioan ROTAR[1], Raluca MICLEA[1], Rodica POP[1]

e-mail: monikniste@yahoo.com

Abstract

This study aimed to isolate and collect different strains from soil and to characterize their performance against salinity. Two strains *Rhizobium leguminosarum* bv. *trifolii* and two *Sinorhizobium meliloti* strains were used in this study to characterize the growth performance against salinity. Nodules collected from this plants were surface sterilized and presumptive test for rhizobia was conducted before authentication according to Somasegran and Hoben (1994). Yeast extract-mannitol medium (YEM), was used for routine cultivation of rhizobia, and when necessary the medium was solidified with 1.2 % agar (YEMA). To test the rhizobial growth at different salt concentration we supplemented the medium by adding 0, 50, 100, 150 and 300 mM sodium chloride (NaCl). Rhizobial growth after incubation at 28°C with shaking for three days was measured spectrofotometrically at OD_{600} nm. Rhizobial strains were classified by their growth response at 300 mM regarding their optical density (OD). Result showed the two *Rhizobium leguminosarum* bv. *trifolii* strains tested , about half were able to tolerate up to 300 mM of salt, and the best performance strains were RtR 2. Tolerance of high levels of NaCl varies for *Sinorhizobium meliloti* and all strains showed good salt tolerance efficiency compared with *Rhizobium trifolii*. The best *Sinorhizobium meliloti* strain was SmM 2. The two rhizobia species examined best development has been observed on the *Sinorhizobium meliloti*. At the other species studied in the presence of salt was observed that the number of colony development was adversely affected. These salt tolerant strains are excellent models to study the mechanisms of the resistance and the impact of NaCl on rhizobia tolerance.

Key words: *Rhizobium trifolii*, *Sinorhizobium meliloti*, sodium chloride, tolerance.

Leguminous plants have been used in agriculture for their importance for human food and for animal feed. Alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) are grown over extensive areas as forage crops for grazing or as dry hay, and they furnish not only high quality protein but also a variety of biologically active molecules such as vitamins, minerals and other nutrients (Gauri *et al*., 2011). Rhizobia are soil bacteria of great economic importance. They have the ability to invade the roots and form nodules on leguminous plants.

The legume-*Rhizobium* symbiosis is one of the most important sources of biologically fixed nitrogen in agricultural systems, and since it is a biological process, it does not depend on external sources of energy, except for free and renewable sunlight, and has few detrimental ecological effects (Abd-Alla *et al*., 2014).

In the *Rhizobium*–legume symbiosis, the process of nitrogen fixation is strongly related to the physiological state of the host plant (Keneni *et al*., 2010). Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing) impose limitations on the vigor of the host legume (Zahran Hamdi Hussein, 1999).

Salinity stress is one of the most serious factors which is limiting the productivity of agriculture and affects approximately 7% of the world's total land area and nearly 40% of the world land surface can be categorized as suffering from potential salinity problem (Fahmi *et al*., 2011). Salinity affects growth and survival of these genera, there have been reports of the detrimental effects of salt stress on plant growth, multiplication of rhizobia, nodulation and nitrogen fixation (Elsheikh, 1998). Salinity stress is one of the most serious factors limiting the productivity of agriculture.

The detrimental effects of salt on plants are a consequence of both a water deficit, resulting in osmotic stress, and effects of excess sodium ions on critical biochemical processes (Abolhasani *et al*. 2010). Saline soils may be characterized by the presence of high levels of neutral salts (Abdelmoumen *et al*., 1999).

[1]University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca

Salt may affect symbiosis by its effects on the growth and survival of rhizobia in soil, restriction on root colonization, inhibition, of processes of infection and nodule development, or impairment of active nodule functioning (Abolhasani *et al*. 2010).

This study aimed to isolate different strains of *Rhizobium leguminosarum* biovar *trifolii* and *Sinorhizobium meliloti* and to characterize their performance against salinity.

MATERIAL AND METHOD

Red clover (*Trifolium pratense* L.) with isolates RtS1 and RtR2 and alfalfa (*Medicago sativa*) also with two isolates SmM1 and SmM2 were used for isolation and to test the salinity effect.

Roots were washed thoroughly to remove soil. Undamaged nodules were picked off the roots and surface sterilized in 20 ml of sodium hypochlorite solution (commercial product Domestos) for five minutes. Sterile water was used to remove the desinfectant action. The nodules were crushed with a sterile forceps directly into the surface of Yeast Extract Mannitol Agar (YEMA) plate and incubated at 28°C for three days. Rhizobial colonies were subjected to different cultural test for example growth behavior on YEMA medium and Congo red test was also perform.

Liquid Yeast Mannitol (YEMB) and Yeast Mannitol Agar media (Somasegaran and Hoben, 1985), were used for routine cultivation of rhizobia.

All Rhizobium strains were tested against different NaCl concentrations, in YEMB and YEMA medium. The salinity of the medium used was adjusted with 0, 50, 100, 150, 300 mM NaCl. One mililitre of the *Rhizobium* suspension (10^{-5} cells mL^{-1}) was added to each test tubes and Petri plates according to Somasegaran and Hoben (1985). After inoculation, test tubes and Petri plates were incubated at 28°C for five days with continuous shaking at 150 rpm.

The growth rate of the strains in tubes were assessd by the change of OD. Readings of OD were performed using a spectophotometer at a wavelength of 600 nm immediately after inoculation and then daily until the growth had reached a stationary phase between 3 and 5 days. Sterile media of similar salinities served as the standards (Mihammad *et al.*, 19991). The growth rate on Petri plates were performed by counting the CFU and each treatment was done in triplicates.

RESULTS AND DISCUSSION

Four *rhizobial* strains were isolated from each vegetation plot. The isolates were fast growing acid producing, similar to the type strain *Rhizobium leguminosarum* biovar *trifolii* and *Sinorhizobium meliloti*. They were differentiated from one another on the basis of morphological, cultural and some important biochemical characters

Strains of *Rhizobium* usually cannot tolerate or function under high levels of osmotic stress caused by salinity or drought (Abdel-Salam, 2010).

This study aimed to isolate and characterize different indigenous *Rhizobium leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti* strains. It was also aimed the study of the different concentration of salt effect and to select the best strain for application in certain ecological areas**.**

In *figure 1* is presented the growth of the two *Rhizobium trifolii* isolates and they differ in their absorbance at 0 hours and at three days after inoculation on the YEMB. The best growth at a 50mM concentration of NaCl RtS1 have OD_{600} = 1.34 and RtR2 have OD_{600}=1.47 after 72 hours. The growth of RtR2, SmM1 and SmM2 was unaffected at 50 mM, and was only slightly reduced for the RtS1 strain for the same concentration.

For isolate RtS1 the differences at 50mM and 100mM NaCl are not that significant. For RtR2 isolate the differences are higher between the concentration 50 mM and 100 mM. The best grown at 72 hours after inoculation at 300mM NaCl was observed in the isolate RtR2 (OD_{600} = 0.92) and the weakest growth was observed in the isolate RtS1 (OD_{600} = 0.79).

Strains isolated from *Medicago sativa* L. were more tolerant to salinity than isolates from *Trifolium pratense* L. The results shown in Figure 2 demonstrate that among the two strains of *S. meliloti* analyzed we found a high degree of diversity. One strain was highly tolerant to a salt concentration up to 300 mM NaCl (SmM2). The differences between control, 50 mM, 100 mM and 150 mM NaCl are not considerable fot the strain SmM1. But a significant decrease is shown at 300 mM NaCl (OD_{600} = 0.72) after 72 hours. However, the reduction of SmM2 growth was much less pronounced than in SmM2.

All *Sinorhizobium meliloti* strains showed good salt tolerant efficiencies compared with *Rhizobium trifolii*, where both of them could tolerate up to 300 mM NaCl. The best *R. meliloti* was SmM2 strain, which could grow up to OD_{600} = 0.95.

Rhizobial growth at different NaCl concentrations is present in Table 1. Among the *Rhizobium leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti* strains, all were able to tolerate up to 300 mM NaCl. The best performance strains were SmM2, (63.00) follow by SmM1 (47.00). Strain SmM1 compared with RtR2 (42.00) the differences are not significant. Table 1 also showed that *Rhizobium trifolii* strain (RtS1) was very sensitive to salt stress and at 300 mM NaCl the number of colonies was low (16.00).

Study of different salt concentrations has a negative effect on the number of CFU. It can be seen that the numbers of CFU are distinct significant negative to control. But very small amount of salt doesn't effect that much the rhizobial growth in most observation data.

The *figures 3* and *4* show a correlation of +0.90 for all concentrations and the relationship betwen control and salt concentration is a strong positive uphill linear.

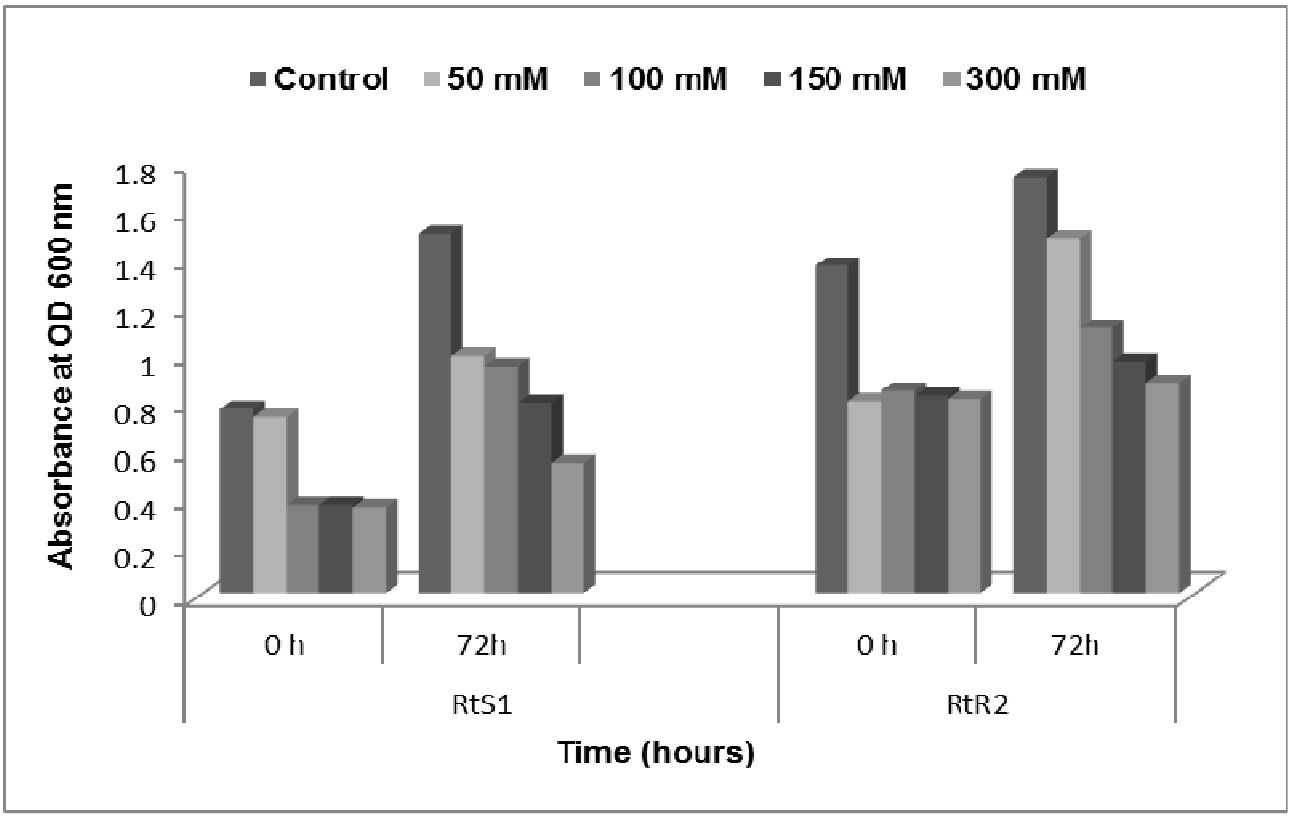

Figure 1 ***Rhizobium*** **growth on YEMB medium amended with 50 mM, 100 mM, 150 mM and 300 mM NaCl**

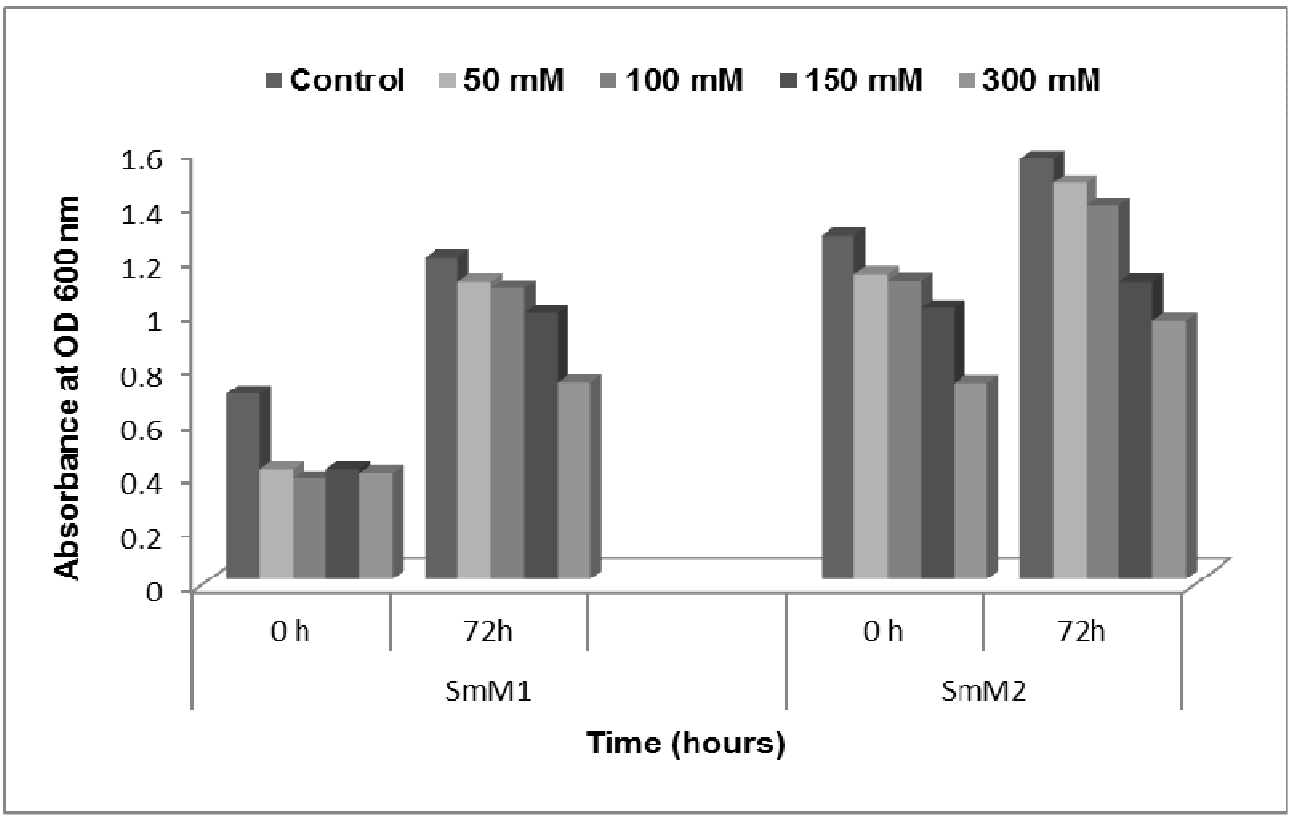

Figure 2 ***Sinorhizobium*** **growth on YEMB amended with 50 mM, 100 mM, 150 mM and 300 mM NaCl**

Table 1

The influence of different salt concentration on the *Rhizobium trifolii* and *Sinorhizobium meliloti* growth

Izolatul	Concentratia	Media	% to control	Difference to control	Significance of the difference	Duncan test
RtS1	Control	104.00	100.0	0.00	Mt.	HIJ
	50 mM	88.67	85.3	-15.33	0	FGH
	100 mM	66.67	64.1	-37.33	000	DE
	150 mM	46.33	44.6	-57.67	000	BC
	300 mM	**16.00**	15.4	-88.00	000	A
RtR2	Control	119.33	100.0	0.00	Mt.	JK
	50 mM	113.33	95.0	-6.00	-	IJK
	100 mM	85.00	71.2	-34.33	000	FG
	150 mM	58.67	49.2	-60.67	000	CD
	300 mM	**42.00**	35.2	-77.33	000	B
SmM1	Control	127.33	100.0	0.00	Mt.	K
	50 mM	114.67	90.1	-12.67	-	IJK
	100 mM	92.33	72.5	-35.00	000	GH
	150 mM	75.67	59.4	-51.67	000	EF
	300 mM	**47.00**	36.9	-80.33	000	BC
SmM2	Control	154.67	100.0	0.00	Mt.	L
	50 mM	128.33	83.0	-26.33	000	K
	100 mM	100.33	64.9	-54.33	000	GHI
	150 mM	89.00	57.5	-65.67	000	FGH
	300 mM	**63.00**	40.7	-91.67	000	DE
DL (p 5%)				14.18		
DL (p 1%)				19.07		DS 14.21
DL (p 0.1%)				25.26		

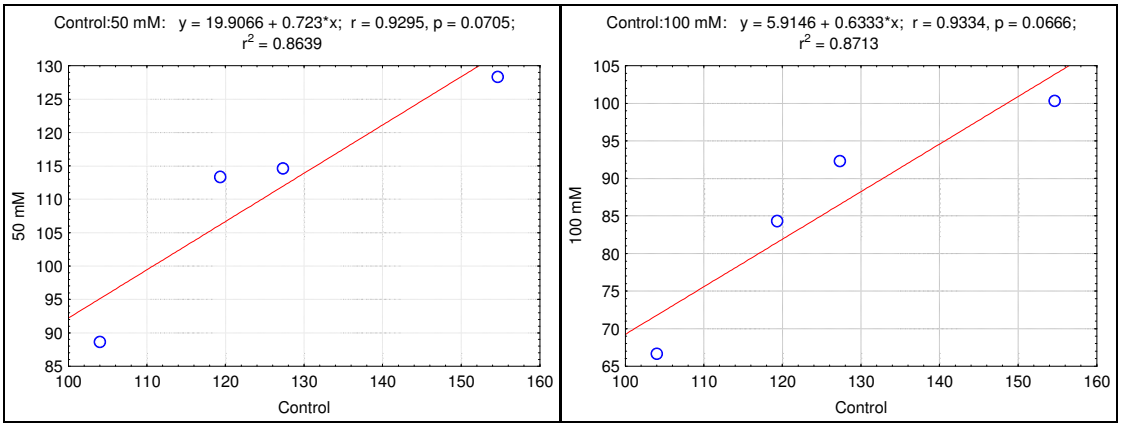

Figure 3 **Correlation between the number of CFU on medium with 50 mM and 100 mM salt and control**

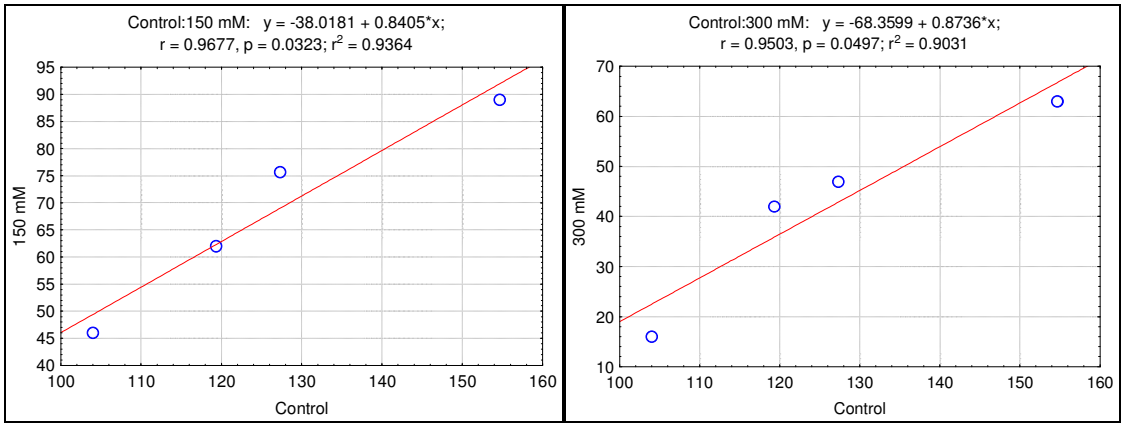

Figure 4 **Correlation between the number of CFU on medium with 150 mM and 300 mM salt and control**

CONCLUSIONS

In conclusion, our results showed that rhizobial growth was affected by salt. *Sinorhizobium* strains are more salt-tolerant than the strains of *Rhizobium*. Further studies are required in order to test these resistant strains as inoculums for alfalfa plants in order to evaluate their symbiotic interaction.

ACKNOWLEGMENTS

This paper was *published under the frame of* European Social Fund, *Human Resources Development Operational Programme 2007-2013, project no.* POSDRU/159/1.5/S/132765

REFERENCES

Abd-Alla Mohamed Hemida, Ahmed A. Issa, Takuji Ohyama, 2014, *Impact of Harsh Environmental Conditions on Nodule Formation and Dinitrogen Fixation of Legumes*, Advances in Biology and Ecology of Nitrogen Fixation, Prof. Takuji Ohyama (Ed.), ISBN: 978-953-51-1216-7, InTech, DOI: 10.5772/56997.

Abdelmoumen, H., Filali-Maltouf, A., Neyra, M., Belabed, A., El Idrissi, M.M., 1999, *Effect of high salts concentrations on the growth of rhizobia and responses to added osmotica*, Journal of Applied Microbiology, vol. 86, p. 889-898.

Abolhasani, M., Lakzian, A., Tajabadipour, A., Haghnia G., 2010, *The study salt and drought tolerance of Sinorhizobium bacteria to the adaptation to alkaline condition,* Australian Journal of Basic and Applied Sciences, vol. 4(5), p. 882-886.

Elsheikh, E. A. E., 1998, *Effects of salt on rhizobia and bradyrhizobia: a review*, Ann. appl. Biol, vol. 132, p. 507-524.

Fahmi, A. I., Nagaty, H. H., Eissa, R. A., Hassan, M. M., 2011, *Effects of salt stress on some nitrogen fixation parameters in faba bean***,** Pakistan Journal of Biological Sciences, vol 14(6), p. 385-391.

Gauri, Singh, A. K., Bhatt, R. P., Pant, S., Bedi, M. K., Nsglot, A., 2011, *Characterization of Rhizobium isolated from root nodules of Trifolium alexandrinum,* Journal of Agricultural Technology, vol. 7(6), p 1705-1723.

Keneni, A., Assefa, F., Prabu, P.C., 2010, *Characterization of acid and salt tolerant Rhizobial strains isolated from faba bean fields of Wollo, Northern Ethiopia*, J. Agr. Sci. Tech., vol 12, p. 365-376.

Mohammad, R. M., Akhavan-Kharazian, M., Campbell, W. F., Rumbaugh, M. D., 1991, *Identification of salt- and drought-tolerant Rhizobium Meliloti L. strains*, Plant and Soil, vol. 134, p. 271-276.

Somasegaran, P. and Hoben, H. J., 1985, Methods in legume-Rhizobium technology, Hawai:Niftal Project.

Zahran Hamdi Hyssein, 1999, *Rhizobium – Legume symbiosis and nitrogen fixation under severe conditions and in an arid climate*, Microbiology and Molecular Biology Reviews, vol. 63(4), p. 968-989.