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Response of Halotolerant Rhizobia of *Glycine max* (L.) to Various Salts and pH Values

N.R. Shaikh and M.B. Gandhi

Department of Microbiology, Yashwantrao Chavan College of Science,
Karad Vidyanagar, Karad - 415 124, India.

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Over irrigation and poor water drain of agricultural land may create a problem of soil salinity. In Maharashtra the problem is increasing day by day. Salinity affects soil qualities adversely. Many microbial interactions in soil are victims of salinity. Symbiotic nitrogen fixation, fertility determining factor in the soil, can be one of them. The effect is not only due to decrease in the plant growth but also due to decrease in the useful microbial population in soil. However, new salt tolerant varieties of useful microbes may develop in such soils. If screened properly, they can be of use in saline soils. Halotolerant rhizobia can be of use in saline soils, if used along with their legume partners. This can add in to nitrogen content of the soil and help in reclaiming soil fertility. Four halotolerant rhizobial strains namely GMNW1, GMNW2, GMB2 and GMB1 of *Glycine* max (L.) have been isolated from few of such saline soils in Maharashtra in the Krishna river basin area at Satara and Sangli districts. The NaCl tolerance of the isolates was ranging from minimum up to 5 % in case of GmNW1 and GmNW2 to maximum up to 8% in GmB1 and GmB2. The isolate namely, GmNW1 tolerated 8% of KCl while remaining three isolates tolerated KCl up to 9%. Tolerance to carbonates and bicarbonates was variable for different isolates and was ranging from 0.1 % in case of GmNW1 to 0.5 % in case of GmB2. Presence of KCl in the medium lowered NaCl tolerance of the isolates. Although pH of the medium influenced NaCl tolerance of the isolates, it was variable at different pH. However, the tolerance was maximum at pH 7 for all the isolates.

Key words: Saline-environments, Salt-tolerance, Rhizobia, *Glycine max*(L.), Symbiotic nitrogen fixation.

Increasing salinity is causing loss of fertility of agricultural land. More than seven million hectares of land has been affected due to salinity in India. This accounts for 5% of the total land under cultivation. In Maharashtra, about 6 to 6.5 lakh hectares of land under cultivation has become infertile due to increased salinity. About 17,000 hectares of land in Krishna river basin has become saline and is increasing at the rate of 10 to 12 % every year.

Few wild leguminous plants can be cultivated in saline soils and then used as green manure. Some legumes like *Vicia faba, Phaseolus vulgaris* and *Glycine max*(L.) are comparatively more salt tolerant than others.[15] So if pulse legumes like *Glycine max*(L.) are grown in saline soils, then they will be of more benefit.

* To whom all correspondence should be addressed.
Tel.: +91-2164-224016; Off.: +91-2164-271356;
Mob.: +91-9422604116; E-mail: nrshaikh@ymail.com

Legumes, the macrosymbiont grow better if grown in association with rhizobia nodulating them. Salt tolerant rhizobia are much more effective than the non tolerant ones in saline condition.[7] This is necessary to screen salt tolerant rhizobia from saline soils which are capable of nodulating pulse legumes like *Glycin max.*(L.)

Four salt tolerant rhizobial strains viz, GMNW1, GMNW2, GmB1 and GMB2 which nodulated a cultivated pulse legume, soybean (*Glycine max.* L) were isolated from saline soils of Krishna river basin area at Narayanwadi and Bahe in south Satara and north Sangli districts of Maharashtra.

This paper reports the results of studies on tolerance of these isolates to various salts like NaCl, KCl, Na_2CO_3, $NaHCO_3$ and a mixture of NaCl and KCl, as well as effect of different pH values of the growth medium on salt tolerance of the isolates as it is reported that high salt tolerance adds in to tolerance to high pH.

Objectives

Saline soil can be one of the natural habitat for salt tolerant rhizobia. The project was under taken to isolate salt tolerant rhizobia of *Glycine max* (L.) from saline soils and to study their salt tolerance. Soil pH has great influence on the microbial population in soil, so the influence of pH on salt tolerance of the rhizobial isolates was also studied.

MATERIAL AND METHODS

Collection of Soil Samples

Samples were collected from Krishna river basin area at Narayanwadi and Bahe in southern part of Satara district and northern parts of Sangli district respectively in plastic bags which were then transferred to the laboratories on the same day. The samples were kept in refrigerator at 4° C, till use. They were then analyzed for pH and salinity and used for screening of salt tolerant rhizobia nodulating *Glycine max*(L.) plants.

Collection of nodulated soybean plants from saline soils

The nodulated plant samples were collected to get entire root system intact. For this the plants were scooped carefully along with the soil in the circle of 12 to 15 cm radius around them. These plants were packed tightly with the clumps of soil in polythene bags and transported to the laboratory where they were used for the isolation of halotolerant rhizobia from the nodules at the root.

Screening of Halotolerant Rhizobia nodulating soybean plants: Screening of halo tolerant Rhizobia nodulating plants of *Glycine max*. L . was done by the method of Vincent[16]. Rhizobia were isolated on Congo red yeast extract mannitol agar (CRYEMA) containing 3 % of NaCl from the healthy intact nodules on the roots of the soybean plants collected from saline soils. Salt tolerant rhizobia nodulating *Glycine max*(L) were also isolated from the saline soil itself. For this the surface sterilized seeds of *Glycine max(*L) were sown in sterile garden soil in separate plastic pouches and saline soil was used as inoculum. The plants were grown for 40 days till they developed nodules on their roots which were then used for the isolation of rhizobia on CRYEMA with 3% of NaCl using method of Vincent[16].

Authentication of isolates as rhizobia

Isolates were confirmed as rhizobia by plant infection test, Congo red absorption test, growth on glucose peptone agar test[16], ketolactose test[1] and Nile blue reduction test[11].

Salt tolerance studies

Tolerance of all the obtained rhizobial isolates was tested for NaCl on CRYEMA as well as in yeast extract mannitol broth (YEMB) , and for KCl , Na_2CO_3, $NaHCO_3$ and a mixture of variable concentration of NaCl and KCl on CRYEMA. Concentrations of NaCl and KCl used were 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 gram /100 ml of water, however, of Na_2CO_3 and $NaHCO_3$ they were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 gram /100 ml of water. For the studies on the mixture of NaCl and KCl, a mixture of 3% of KCL and variable concentration of NaCl like 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 gram /100 ml of water were used. Each rhizobial isolate of cell density 10^8 per ml was inoculated in 0.01 ml aliquot in 5 ml of YEMB as well as streaked on CRYEMA and incubated at 28° C for 5 days. The isolates were also tested in the medium without any salt in it as a control i.e. 0.0% of salt. The results were recorded turbidometrically in the broth and as a presence or absence of growth on the solid culture medium.

Effect of pH of the growth medium on the salt tolerance of the rhizobial isolates

Effect of pH on salt tolerance of the rhizobial isolates was tested using YEMB with initial pH values viz, 4, 5, 6, 7, 8 and 9 in which 0.1 ml suspension of each of the rhizobial isolate of cell density 10^8 cells per ml was inoculated in 5 ml of YEMB and incubated at 28° C for 5 days. The results were recorded turbidometrically.

RESULTS AND DISCUSSION

It is seen from the table 1 that the soils samples collected from Krishna river basin areas for these studies were ranging in their pH values from 7.6 at B1 to 8.21 at B2. Their electrical conductivity was also ranging from 8.76 for NW2 to 10.13 dS/m for B1 respectively. The soil sample at B2 was saline alkaline.

It can be seen in Table 1 that in all four salt tolerant rhizobial isolates nodulating *Glycine max* plants were obtained. The isolate namely, GmB2 was obtained from soybean plant growing in saline soil and remaining three isolates namely GmNW1, GmNW2 and GmB1 were obtained from saline soil samples namely NW1, NW2 and B1 respectively. Finding of soybean plant in saline soil is in accordance with observation of Tilak[15] who reported *Glycine max* plants in saline soils.

All the four isolates namely GmNW1, GmNW2, GmB1 and GmB2 produced circular, mucoid colonies with entire margin of 3.9 to 5 mm in size within 5 days on CRYEMA with 3% of NaCl. Thus they were found to be fast growers. It is seen from table 2 that all the four root nodule bacterial isolates were authenticated as rhizobia.

Even though the natural root nodulating rhizobia of soybean plant are reported to be slow growing rhizobia (*Bradyrhizobium* spp.), many workers have also reported fast growing rhizobia of soybean plant[4,5,9,13]. Under stressful conditions of salinity, fast growing rhizobia survive better than slow growing ones[3].

It can be seen from Fig. 1 that on agar medium the isolates namely, GMNW2 and GMNW1 could tolerate NaCl concentration up to 5%, while GMB2 and GmB1 could tolerate up to 6%. The tolerance of all the four isolates to NaCl was comparatively more in YEM broth than on agar. The isolates namely GMNW2 and

Table 1. Soil characteristics along with the codes of the root nodule bacterial isolates of *Glycine max*

Sampling area	Soil Sample Code	pH	Electrical conductivity, EC (dS/m)	Codes of the root nodule bacterial isolate
Narayan wadi	NW1	8.08	9.82	GmNW1
	NW2	7.78	8.76	GmNW2
Bahe	B1	7.6	10.13	GmB1
	B2	8.21	9.78	GmB2*

*It is the isolate obtained from nodulated *Glycine max* plant from saline soil.

Table 2. Confirmatory tests of *Rhizobia* for the root nodule bacterial isolates of *Glycine max*

Rhizobial Strain	Congored adsorption test	Keotolactose test	Nile blue reduction test	Growth on Glucose Peptone agar	Test of Nodulation in soybean plant
GmNW1	-	-	-	-	+
GmNW2	-	-	-	-	+
GmB1	-	-	-	-	+
GmB2	-	-	-	-	+

- = negative results, + = positive results

GMNW1 showed NaCl tolerance up to 6% and 7% respectively, while remaining both the isolates namely GMB2 and GmB1 showed the tolerance up to 8%. The figure also shows that increasing NaCl concentrations reduced growth of all the four isolates. This observation is in accordance with the report that growth of the rhizobium decreases with increase in the salt concentration[8].

It cam be observed from Fig. 2 that pH of broth medium influenced the tolerance of the isolates to NaCl. Isolates namely, GMNW2, GMNW1 and GmB1 showed maximum NaCl tolerance at pH 7 which was 6%, 7% and 8% respectively. The tolerance declined with increase or decrease in pH values from 7. However the isolate GMB2 showed a different picture which could tolerate NaCl concentration maximum up to 8% at three different pH values as 6, 7 and 8. The tolerance was lowest at pH 4 for all the four isolates, which was 2% for GmNW2, 3% for GmNW1 and GmB1 and 4% for GmB2. These observations indicate that the most suitable pH for the growth of these rhizobial isolates was 7.

From Fig. 3 it can be seen that at pH 7 isolate namely GmB1, GmB2 and GmNW1 showed maximum growth at 0% NaCl, while GmNW2 showed maximum growth at 1% NaCl. At pH 8 the isolates namely, GmB1, GmB2 and GmNW2 showed maximum growth at 0% and 1 % NaCl, while GmNW1 showed maximum

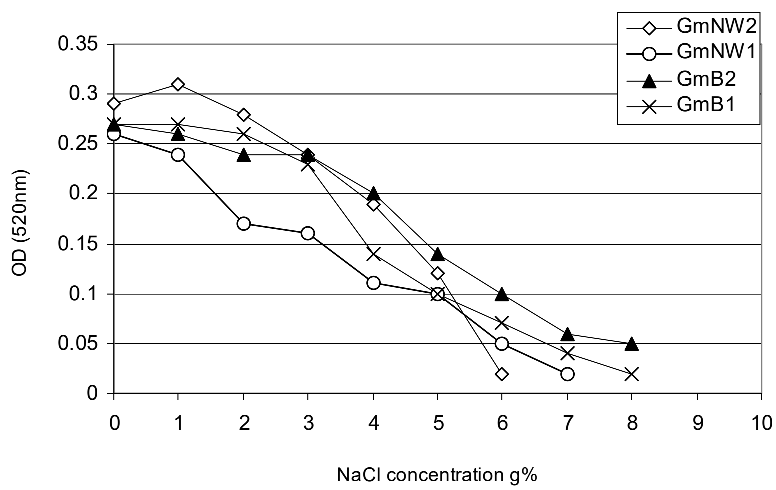

Fig. 1. Growth of rhizobial isolates as OD (520) after growth at different concentrations of NaCl after 5 days of incubation

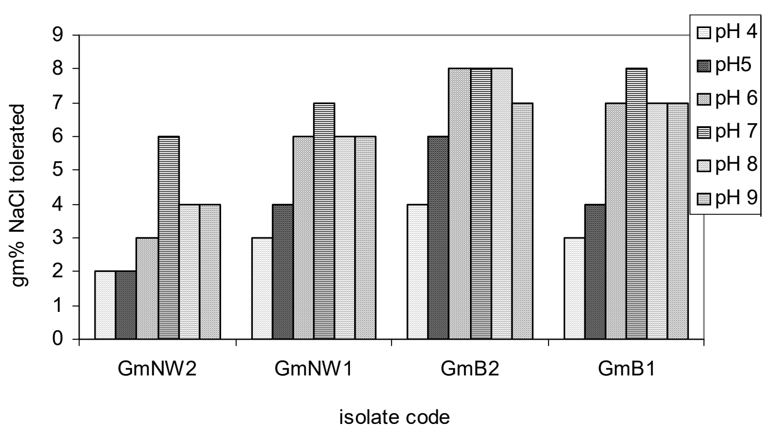

Fig. 2. NaCl tolerance of the rhizobia of *Glycine max* at variable pH values

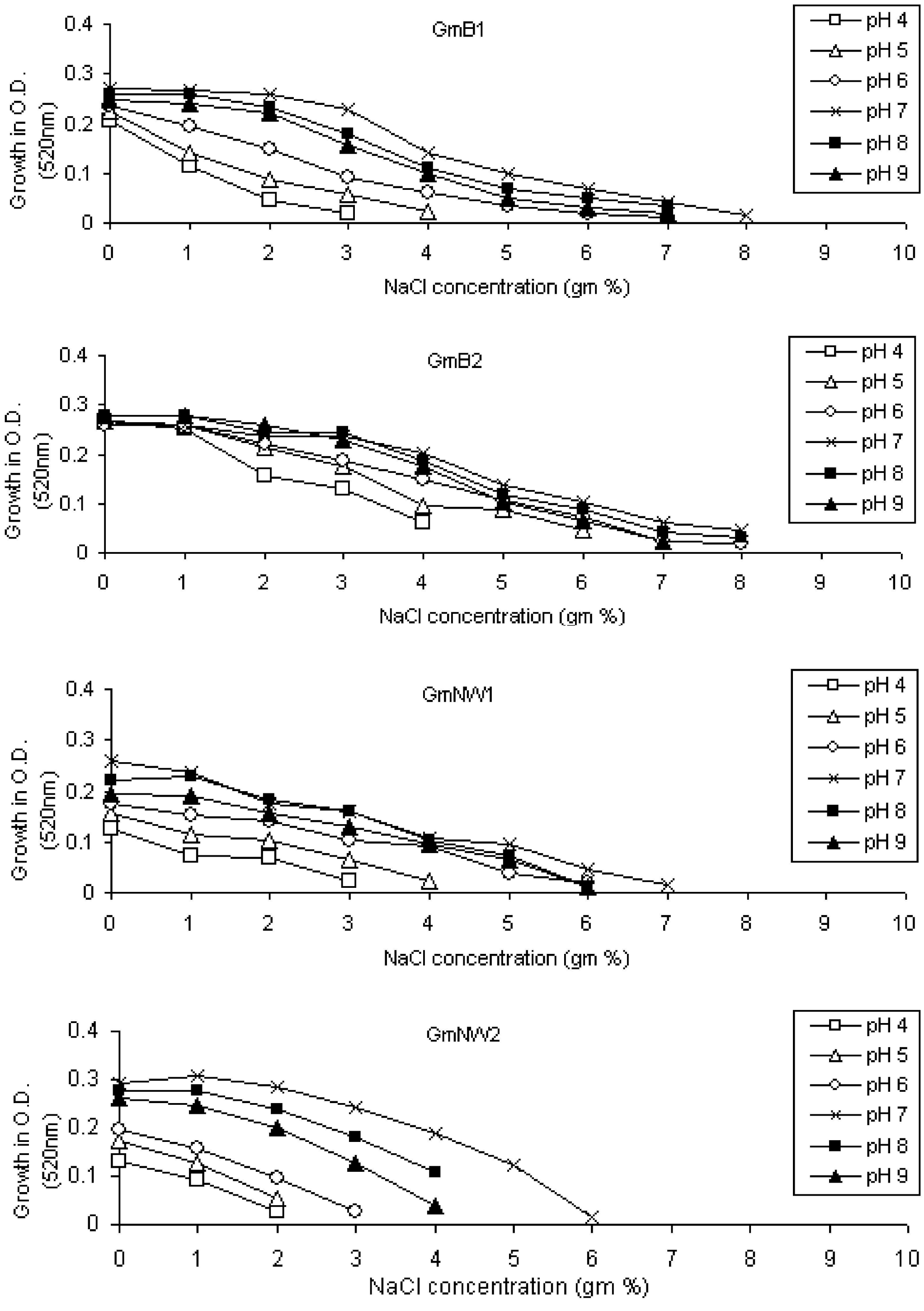

Fig. 3. Growth response in terms of O.D. (520nm) of the rhizobial isolates of *Glycine max* at different pH values

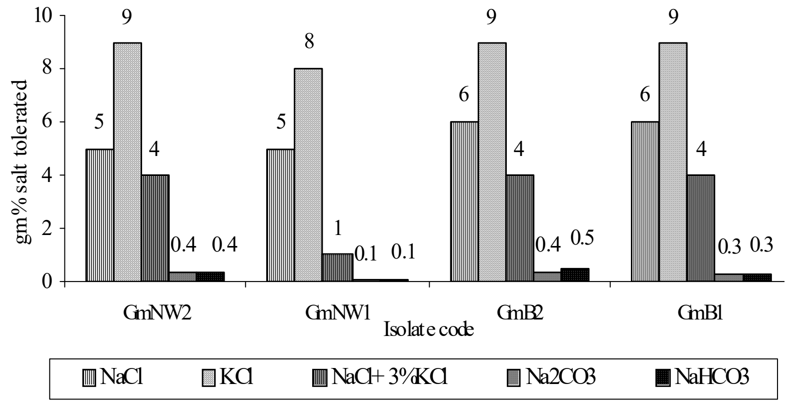

Fig. 4. Salt tolerance of the isolates to NaCl, KCl, mixture of variable concentration of NaCl with 3% of KCl, Na_2CO_3 and $NaHCO_3$ on CRYEMA

growth at 1% NaCl. However for remaining pH values namely 4, 5, 6, and 9 all these four isolates namely GmNW1, GmNW2, GmB1 and GmB2 showed maximum growth at 0% NaCl. At pH 6, 8 and 9, isolates namely GmB2 showed comparatively more growth at 0 and 1% NaCl, while for remaining pH values namely 4, 5 and 7 the maximum growth was shown at 0% NaCl concentration. These observations indicate that different isolates showed variable response to NaCl at different pH values.

All the rhizobial isolates of *Glycine max* showed better growth at pH 7. Alkaline pH values were found to be better than acidic values. These observations are in accordance with the studies on effect of salt and pH stress on temperature tolerant rhizobium by Kulkarni & Nautiyal[6] who have reported that high salt tolerance adds in tolerance to high pH.

It can be observed from Fig. 4 that the isolates namely, GMNW2, GMB2 and GmB1 tolerated KCl up to 9% while GMNW1 tolerated up to 8%. Tolerance of all the four isolates was comparatively more to KCl than NaCl on solid medium. However, it was observed that the tolerance to NaCl was decreased when tested in presence of 3% of KCl in the medium. The NaCl tolerance of GMNW1 was found to be lowered up to 1 % in presence of KC1, however for the isolates GMNW2, GMB2 and GmB1 it was lowered up to 4%.

Fig. 4 shows that all the four rhizobial isolates of *Glycine max* showed comparatively very low tolerance to carbonates and bicarbonates. GMNW1 tolerated both Na_2CO_3 as well as $NaHCO_3$ up to 0.1 % , while tolerance to both the salts was found to be 0.4 and 0.3 % respectively for GMNW2 and GmB1. GMB2 could tolerate Na_2CO_3 and $NaHCO_3$ up to 0.4 % and 0.5 % respectively. Tolerance to carbonates and bicarbonates ranged from 0.1 to 0.5 %. There are reports about rhizobia tolerating up to 0.5 % of carbonate and bicarbonate.[14] Reports of Singh *et al.*[10] regarding rhizobia of lucerne (*Medicago sativa* L.) mentioning inhibition of nodulation at 0.1 to 0.2 % of carbonates and bicarbonates also indicate that rhizobia have very high sensitivity to carbonates and bicarbonates.[2,12]

CONCLUSIONS

- Salt tolerant rhizobia nodulating *Glycine max* were screened from saline soil as well as from the nodulated soybean plants from the saline soil.
- In YEM broth the tolerance of all the four rhizobial isolates to NaCl was comparatively more than on agar.
- In broth maximum tolerated NaCl concentration was 8% and was by GmB1 and GmB2.
- On agar the maximum tolerance to NaCl was up to 6% and was by GMB1 & GmB2.
- GMB2 and GmB1 were the isolates of *Glycine max* having maximum tolerance to NaCl.

- pH of the medium was influencing tolerance of the isolates to NaCl.
- The isolates showed comparatively more tolerance to KCl than to NaCl.
- Tolerance of the isolates to NaCl was reduced by the presence of KCl in medium.
- The isolates were comparatively more sensitive to carbonates and bicarbonates.

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