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Isolation, selection and characterization of salt tolerant *Rhizobium*

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Abstract
The present study was focused on isolation, selection and characterization salt tolerant *Rhizobium* from root nodules of soybean. Out of fourty samples total thirty three rhizobial isolates were obtained and maintained on Yeast Extract Manitol Agar (YEMA) medium. For assessment of salt tolerancy the medium was supplemented with different NaCl concentrations, $MgCl_2$ concentration and different pH level. All the isolates showed promising salt tolerance. Out of thirty three isolates twenty isolates were extremely salt tolerant (more than 5.4 % salt tolerance limit). Four isolate were tolerant to NaCl concentration (2.10- 3.6 % salt). Nine isolates were moderately tolerant to NaCl concentration (0.09-1.50 % salt). Classification of salt tolerant *Rhizobium* was done on the basis of salt tolerance limit. Out of thirty three isolates, twenty isolates showed growth at $MgCl_2$ concentration of 0.75 %, four isolates showed growth at 0.50 % $MgCl_2$ concentration and nine isolates showed growth at 0.40 % $MgCl_2$ concentration. Among thirty three isolates, twenty isolates showed growth at 8.50, four isolates showed growth at pH 8.00 and nine isolates showed growth at pH 7.50. Out of thirty three isolates twenty isolates were classified under extremely salt tolerant were characterized for morphological, cultural, physiological and biochemical characterization. The work was carried out for the isolation, selection and characterization of salt tolerant strains of *Rhizobium* which would be highly important inoculum to improve the growth and development of the leguminous plant under saline environment. There is currently need to develop highly salt-tolerant *Rhizobium* strains.

Keywords: Isolation, selection, characterization, salt tolerant, *Rhizobium* sp

Introduction
Salinity is a serious threat to agriculture in arid and semiarid regions (Rao and Sharma, 1995) [26]. Nearly 40 % of total world land is affected due to salinity. Most of the leguminous plants are more sensitive to salinity and they require slightly acidic soil for N_2 fixation (Waroporn, 2006) [33]. Soil salinity is a potential problem in irrigated soils which is responsible for reducing soil and water quality, limiting crop growth, and leading to the abandonment of agricultural land (Egamberdiyeva *et al.,* 2007) [9].
Nitrogen is one of the most essential nutrients required for growth and development of plants. *Rhizobium*acts as primary symbiotic fixer of nitrogen by infecting root of leguminous plants. Kiers *et al.,* (2003) [15] state that, *Rhizobium*bacteria stimulates the growth of leguminous plants by fixing atmospheric nitrogen into soil by symbiotically interaction. Several environmental stresses may affect the nitrogen fixation in plants. It includes salinity, water stress, soil pH, temperature and heavy metals (Kucuk and Kivanc 2008) [16]. Increasing salt concentration might have detrimental effects on rhizobial population (Singleton *et al.*, 1982) [30]. Soil salinity particularly disturbed the symbiotic interaction between legumes and rhizobia (Marcar *et al.*, 1991) [19]. According to Zahran, (1999) [34] salt stress directly affects symbiosis than free living *Rhizobia*. The ability of legume hosts to grow and survive in saline conditions was improved when they were inoculated with salt tolerant strains of rhizobia (Zou *et al.*, 1995; Hashem *et al.,* 1998; Shamseldin and Werner, 2005) [36, 12, 29]. An efficient *Rhizobium*-legume symbiosis under salt stress required also the selection of salt-tolerant rhizobia (Zahran, 1999) [34]. Bhardwaj (1972) [4] had reported that Rhizobia isolated from legumes of saline alkaline soil gave red, orange or yellow pigmented colonies. The colonies were usually mucoid appearing opaque with little firm gum, or translucent appearing glistening or dull with gummy, soft slime. They might or might not be with opaque centers (Jordan, 1984) [14]. Zahran *et al.* (1995) [35] reported that the majority of *Rhizobium*strains isolated from saline soils were salt tolerant, being able to grow in media containing more than 510 mM (2.9835 %) NaCl. Salt tolerant rhizobia might have the potential to improve yield of legumes under salinity stress (EI-Mokadem *et al.*, 1991) [10].

Thus, the isolation salt tolerance *Rhizobia* would be the highly important inoculums to improve the growth and development of the leguminous plants under saline environment.

Material and methods

Total fourty root nodules samples along with rhizosphere soil samples with intact root nodules of soybean plants were collected from saline tract of five districts of Western Maharashtra to isolate salt tolerant *Rhizobium*. From each site, soybean plants were randomly collected. Ten active nodules were randomly chosen from each samples of the plants. All the nodules were placed on cotton in screw cap plastic vials containing calcium carbonate as dessicant below cotton at the bottom and stored in refrigerator until isolation. Besides root nodules, the sub samples of rhizosphere soil were collected for the analysis of chemical and nutrient characteristics. Isolation of *Rhizobium* from root nodule was done by the method of Samosegaran and Hoben (1985) [27], from each sample, two-three nodules were picked up and washed thoroughly with sterile distilled water. After washing, nodules were surface sterilized in 95 per cent alcohol for 30-40s to remove wax coating if any and subsequently immerged in 4 per cent sodium hypochlorite for 3-4 min. Then nodules were immediately washed 5-6 times with sterile distilled water to remove traces of sodium hypochlorite. The surface-sterilized nodules were transferred to sterile tubes containing 100 µl sterile distilled water. Nodules were crushed with the help of sterile glass rod and then were streaked one loopful of milky suspension on Congo red yeast extract mannitol agar (CRYEMA) and incubated at 28^0C. Single unique colonies were picked up and were streaked on YEMA medium until pure culture was obtained.

Assessment of salt tolerant *Rhizobium* at different NaCl level

To confirm the salt tolerance of *Rhizobium* isolates, they were tested against different concentrations of NaCl salt and classified on the basis of salt tolerancy method given by Cardoso *et al*, (2014). For this, YEMA medium supplemented with 0.075, 0.15, 0.3, 0.6, 1.2, 1.8, 2.1, 2.4, 3.0, 3.6, 4.2, 4.8, 5.4, 6.0, 7.2, 8.4, 9.6 and 10.8 per cent NaCl. The plates of YEMA with above NaCl concentrations were prepared on which each *Rhizobium* isolate were inoculated by streaking and incubated at 28^0C for 2-7 days. After completion of incubation period, growth of salt tolerant *Rhizobium* isolates was observed for growth and rated as +++: full growth, ++: scanty growth +: and -: no growth. The full growth rhizobial isolates were selected for further studies.

Assessment of salt tolerant *Rhizobium* on different $MgCl_2$ concentration

To confirm the salt tolerance of *Rhizobium* isolates, they were tested against different concentrations of $MgCl_2$ salt. For this, YEMA medium supplemented with 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90, 1.00 % $MgCl_2$. The plates of YEMA with above $MgCl_2$ concentrations were prepared on which each salt tolerant *Rhizobium* isolate was inoculated by streaking and incubated at 28^0C for 2-7 days. After completion of incubation period, growth of salt tolerant *Rhizobium* isolates was observed for growth and rated as +++: full growth, ++: scanty growth +: and -: no growth. The full growth rhizobial isolates were selected for further studies.

Assessment of salt tolerant *Rhizobium* on different pH level

To confirm the salt tolerant *Rhizobium* isolates, they were tested against different pH levels in the medium. For this, YEMA medium, pH was adjusted by using HCl and NaOH to 7.5, 8.00, 8.50 and 9.00. The plates of YEMA with above pH levels were prepared on which each salt tolerant *Rhizobium* isolate was inoculated by streaking and incubated at 28^0C for 2-7 days. After completion of incubation period, growth of salt tolerant *Rhizobium* isolates was observed and rated as +++: full growth, ++: scanty growth +: and -: no growth. The full growth rhizobial isolates were selected for further studies.

Characterization ofselectedsalt tolerant *Rhizobium*

Morphological and cultural characterization

The salt tolerant *Rhizobium* isolates were examined for cell shape, size, arrangement, motility test as per the standard procedures. Overnight grown culture of all the selected isolates were streaked onto Yeast extract mannitol agar medium and observed for colony morphology *viz*., growth, form, margin and elevation of colonies after 48 hrs incubation at 28 ± 2^0C. Gram reaction were recorded as per the standard procedures given by Barthalomew and Mittewer (1950) [3] and Anon (1957) [1].

Physiological characterization (Seeley *et al.*, 1991)[28]

Test tubes of yeast-extract-tryptone agar were melted and held at 100^0C for 10 minutes to expel dissolved oxygen, cooled to a temperature of 42-45^0C and inoculated heavily with test bacterial cultures. Tubes were solidified by placing the tubes in cool water (below 40^0C) and incubated at 37^0C for 2 days and observed the location and appearance of growth *viz.,* surface growth (Aerobic), growth only in depth of agar (Anaerobic) and growth throughout agar shake (Facultative).

Biochemical characterization

The selected salt tolerant isolates were subjected to biochemical characterization employing the standard procedures given by Seeley *et al.* (1991) [28] and Cappuccino and Sherman (1987) [6]. Different biochemical tests were performed.

Result

Total thirty three *Rhizobium* isolates were obtained. These *Rhizobium* isolates were further assessed for tolerance ability on different NaCl cocentration, $MgCl_2$ concentrations and pH level by adjusting NaCl cocentration, $MgCl_2$ concentrations and pH level of culture media. Among thirty three isolates twenty isolates were extremely salt tolerant (more than 5.4 % salt tolerance limit. Four isolates were tolerant to NaCl concentration (2.10- 3.6 %). Nine isolates and reference strain were moderately tolerant to NaCl concentration (0.09-1.50 %) (Table 1). Classification of salt tolerant *Rhizobium* was done on the basis of salt tolerance limit (Table 2).Among thirty three isolates (Table 3) twenty isolates were salt tolerant *Rhizobium* which showed growth upto 0.75 % $MgCl_2$ concentration. Four isolates showed growth upto 0.50% $MgCl_2$ concentration. Reference strain along with ten isolates showed growth upto 0.40% $MgCl_2$ concentration. Out of thirty three isolates (Table 4), twenty isolates showed growth at pH 8.50, four isolates showed growth at pH 8.00 and ten isolates including reference strain showed growth at pH 7.50.

Morphological and cultural characterization of salt tolerant *Rhizobium*

Thirty three isolates of Rhizobia were assessed for salt tolerance ability among all the isolates, nine isolates were characterized which were tolerant to salt upto 5.4 per cent under *invitro* condition.

Morphological and cultural characterization of salt tolerant *Rhizobium*

The selected nine (Table 5) isolates categorized under extremely salt tolerant Rhizobia were examined for morphological characters. All the isolates of salt tolerant Rhizobia (STR) were Gram negative rods. The cells of all salt tolerant *Rhizobium* isolates were rod shaped, motile. All salt tolerant *Rhizobium* isolates showed negative result for endospore formation; STR 2, STR 17 and reference strain were dull white; STR 25 was milky white in colour and remaining isolates were whitish pink in colour and glistering colony. All the isolated colonies were 2-3 (mm) in size, isolate STR 4 was 3-4 (mm) in size.

Cultural characterization of salt tolerant *Rhizobium*

The selected nine isolates (Table 5) categorized under extremely salt tolerant *Rhizobium* were examined for cultural characters. All the isolates of salt tolerant *Rhizobium* (STR) formed circular colonies with entire margin with convex elevation.

Physiological characterization of salt tolerant *Rhizobium*

The selected nine isolates (Table 5) categorized under extremely salt tolerant *Rhizobium* isolates were aerobic in nature.

Biochemical characterization of salt tolerant *Rhizobium*

The selected nine isolates (Table 6) categorized under extremely salt tolerant *Rhizobium* were examined for Biochemical characters viz., catalase, oxidase, hydrolysis of gelatine, starch, Voges-Proskauer (VP) test, methyl red test, H_2S production and urease activity etc. The test of catalase, oxidase, urease activity and citrate utilization were positive for all the nine isolates. The test of hydrolysis of gelatine, starch, Voges-Proskauer (VP) test, methyl red test and H_2S production were negative for all nine isolates.

Table 1: Assessment of Salt tolerant *Rhizobium* from root nodule of soybean on different NaCl concentrations

Isolate No.	Salt tolerance limit (NaCl %)									Class
	0.15	0.60	0.90	1.50	2.10	3.60	5.40	6.00	7.20	
STR 1	+++	+++	+++	+++	+++	++	+	--	--	Extremely tolerant
STR 2	+++	+++	+++	+++	+++	++	+	--	--	Extremely tolerant
STR 3	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 4	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 5	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 6	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 7	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 8	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 9	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 10	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 11	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 12	+++	+++	++	+++	++	+	--	--	--	Tolerant
STR 13	+++	+++	+++	+++	++	+	--	--	--	Tolerant
STR 14	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 15	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 16	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 17	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 18	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 19	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 20	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR 21	+++	+++	+++	+	--	--	--	--	--	Moderately tolerant
STR 22	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR 23	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR 24	+++	+++	+++	+++	++	+	--	--	--	Tolerant
STR25	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR26	+++	+++	+++	+++	+++	+++	++	+	--	Extremely tolerant
STR27	+++	+++	++	+++	++	+	--	--	--	Tolerant
STR28	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR29	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR30	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR31	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR32	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
STR 33	+++	+++	++	+	--	--	--	--	--	Moderately tolerant
Reference strain	+++	+++	+++	++	+	--	--	--	--	Moderately tolerant

++ +: thick colony, + +: flattened, +: growth, -: No growth

Extremely sensitive :tolerance limit less than 0.15 % salt
Moderately sensitive :tolerance limit 0.15-0.60 % salt
Moderately tolerant :tolerance limit 0.90-1.50 % salt
Tolerant :tolerance limit 2.10-3.60 % salt
Extremely tolerant :tolerance limit more than 5.40 % salt
(Cardoso *et al.*, 2014).

Table 2: Classification of salt tolerant *Rhizobium* based on salt tolerance limit

Salt tolerance limit	Isolate No.	No. of isolates	Source of prevalence
less than 0.15 % salt (Extremely sensitive)	–	0	–
0.15-0.60 % salt (Moderately sensitive)	–	0	–
0.09-1.5 % salt (Moderately tolerant)	STR-21, STR-22, STR-23, STR-28, STR-29, STR-30, STR-31, STR-32, STR-33,reference strain	10	Malshiras Tehsil of Solapur Ditrict, Rahuri and Sangamner Tehsil of Ahmednagar District
2.1-3.6 % salt (Tolerant)	STR-12, STR-13, STR-24 STR-27.	04	Walva Tehsil of Sangli District and Madhaa Tehsil of Solapur Distict.
more than 5.4 % salt (Extremely tolerant)	STR-1, STR-2, STR-3,STR-4, STR-5, STR-6,STR-7, STR-8, STR-9,STR-10, STR-11, STR-14, STR-15, STR-16, STR-17, STR-18, STR-19, STR-20, STR-25, STR-26.	20	Shirol Tehsil of Kolhapur district, Miraj and Valva Tehsil of Sangli district, Karad Tehsil of Satara District and Madhaa Tehsil of Solapur District.

Table 3: Assessment of *Rhizobium* for salt tolerancy on different $MgCl_2$ concentration on medium

Isolate No.	$MgCl_2$ concentration (%)						
	0.00	0.20	0.30	0.40	0.50	0.75	1.00
STR 1	+++	+++	+++	+++	++	+	--
STR 2	+++	+++	+++	+++	++	+	--
STR 3	+++	+++	+++	+++	++	+	--
STR 4	+++	+++	+++	+++	++	+	--
STR 5	+++	+++	+++	+++	++	+	--
STR 6	+++	+++	+++	+++	++	+	--
STR 7	+++	+++	+++	+++	++	+	--
STR 8	+++	+++	+++	+++	++	+	--
STR 9	+++	+++	+++	+++	++	+	--
STR 10	+++	+++	+++	+++	++	+	--
STR 11	+++	+++	+++	+++	++	+	--
STR 12	+++	+++	+++	++	+	--	--
STR 13	+++	+++	+++	++	+	--	--
STR 14	+++	+++	+++	+++	++	+	--
STR 15	+++	+++	+++	+++	++	+	--
STR 16	+++	+++	+++	+++	++	+	--
STR 17	+++	+++	+++	+++	++	+	--
STR 18	+++	+++	+++	+++	++	+	--
STR 19	+++	+++	+++	+++	++	+	--
STR 20	+++	+++	+++	+++	++	+	--
STR 21	+++	+++	++	+	--	--	--
STR 22	+++	+++	++	+	--	--	--
STR 23	+++	+++	++	+	--	--	--
STR 24	+++	+++	+++	++	+	--	--
STR25	+++	+++	+++	+++	++	+	--
STR26	+++	+++	+++	+++	++	+	--
STR27	+++	+++	+++	++	+	--	--
STR28	+++	+++	+++	+	--	--	--
STR29	+++	+++	++	+	--	--	--
STR30	+++	+++	++	+	--	--	--
STR31	+++	+++	++	+	--	--	--
STR32	+++	+++	++	+	--	--	--
STR 33	+++	+++	++	+	--	--	--
Ref. strain	+++	+++	++	+	--	--	--

Table 4: Assessment of salt tolerant *Rhizobium* atdifferent pH level

S. No.	Isolate No.	Ph				
		7.00	7.50	8.00	8.50	9.00
1	STR 1	+++	+++	++	+	--
2	STR 2	+++	+++	++	+	--
3	STR 3	+++	+++	++	+	--
4	STR 4	+++	+++	++	+	--
5	STR 5	+++	+++	++	+	--
6	STR 6	+++	+++	++	+	--
7	STR 7	+++	+++	++	+	--
8	STR 8	+++	+++	++	+	--
9	STR 9	+++	+++	++	+	--
10	STR 10	+++	+++	++	+	--
11	STR 11	+++	+++	++	+	--
12	STR 12	+++	++	+	--	--

13	STR 13	+++	++	+	--	--
14	STR 14	+++	+++	++	+	--
15	STR 15	+++	+++	++	+	--
16	STR 16	+++	+++	++	+	--
17	STR 17	+++	+++	++	+	--
18	STR 18	+++	+++	++	+	--
19	STR 19	+++	+++	++	+	--
20	STR 20	+++	+++	++	+	--
21	STR 21	++	+	--	--	--
22	STR 22	++	+	--	--	--
23	STR 23	++	+	--	--	--
24	STR 24	+++	++	+	--	--
25	STR 25	+++	+++	++	+	--
26	STR 26	+++	+++	++	+	--
27	STR 27	+++	++	+	--	--
28	STR 28	++	+	--	--	--
29	STR 29	++	+	--	--	--
30	STR 30	++	+	--	--	--
31	STR 31	++	+	--	--	--
32	STR 32	++	+	--	--	--
33	STR 33	++	+	--	--	
34	Reference strain	++	+	--	--	--

++ + : thick colony, + +: flattened,
+ : growth, -: No growth

Table 5: Morphological, cultural and physiological characteristics of salt tolerant *Rhizobium*

S. No.	Isolates	Colony morphology					Gram reaction	Colony characters			Physiological Characters
		Colour	Shape	Size (mm)	Endo-spore	Motility		Form	Margin	Elevation	aerobic/ anaerobic
1.	STR2	Dull white	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
2.	STR4	Whitish pink and glistering	Rod	3-4	-	+	-ve	Circular	Convex	Entire	Aerobic
3.	STR5	Whitish pink and glistering	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
4.	STR10	Whitish pink and glistering	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
5.	STR14	Whitish pink and glistering	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
6.	STR17	Dull white	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
7.	STR18	Whitish pink and glistering	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
8.	STR19	Whitish pink and glistering	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
9.	STR25	Milky white	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic
10.	ReferenceStrain	Dull White	Rod	2-3	-	+	-ve	Circular	Convex	Entire	Aerobic

Table 6: Biochemical characteristics of salt tolerant *Rhizobium*

S. No.	Characteristics	STR 2	STR 4	STR 5	STR 10	STR 14	STR 17	STR 18	STR 19	STR 25	References train
1	Catalase	+	+	+	+	+	+	+	+	+	+
2	Oxidase	+	+	+	+	+	+	+	+	+	+
3	Hydrolysis ofStarch	-	-	-	-	-	-	-	-	-	-
4	GelatinLiquefication	-	-	-	-	-	-	-	-	-	-
5	Methyl redTest	-	-	-	-	-	-	-	-	-	-
6	Voges-proskaurTest	-	-	-	-	-	-	-	-	-	-
7	Production ofHydrogen sulphide	-	-	-	-	-	-	-	-	-	-
8	Urease activity	+	+	+	+	+	+	+	+	+	+
9	Citrate Utilization	+	+	+	+	+	+	+	+	+	+
10.	Probable genus	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*	*Brady-Rhizobium spp.*

Discussion

Simililary, Annapurna *et al.* (2007) [2] collected single pink nodule from each plant was removed and isolations of root nodule bacteria was carried out as described by Vincent (1970) [32]. Similarly, Patil *et al.* (2014) [21] isolated three *Rhizobium* strains from Soybean, *Trigonella* and Groundnut and maintained on Yeast Extract Mannitol Agar medium (YEMA). YEM broths were supplemented with different concentration of NaCl (0.2, 0.4, 0.6, 0.8, 1.2 %).The same fact was reported by results have been reported by Date (1982) [7] during isolation of rhizobia from soil with some extreme conditions such as salinity or acidity, rhizobia could grew better in a medium modified to resemble closely the soil conditions. Similarly Rai and Prasad (1986) [23] have reported the NaCl tolerant rhizobial isolates up to 400 mM (2.32 %) concentrations of NaCl. Paknikar (1987) [22] have reported tolerance to 2.5 per cent of NaCl in rhizobia of wild legumes of *Vigna* species, while Lal and Khanna (1994) [18] have reported salt tolerance up to 600 mM of NaCl in the rhizobia of *Vigna* species. Zahran *et al.* (1995) [35] reported *Rhizobium*strains isolated from saline soils were salt tolerant, being able to grow in media containing more than 510 mM (2.9835 %) NaCl. Kulkarni and Nautiyal (1999) [17] have reported about the Rhizobia of *P. juliflora* who could be found in alkaline soil, and tolerate up to 32 % NaCl for up to 8 h, 55^{0}C up to 3 h, and 45^{0}C with salt at pH 12.

Morphological characteristics of salt tolerant *Rhizobium*

In present study the isolates of salt tolerant *Rhizobium* (STR) showed Gram negative rods. The cells of all salt tolerant Rhizobial isolates were rod shaped, motile. All salt tolerant *Rhizobium* isolates showed negative result for endospore formation; STR 2, STR 17 and reference strain were dull white; STR 25 was milky white in colour and remaining were whitish pink and glistering colony in colour. All the isolates colonies were 2-3 (mm) in size isolate STR4 was 3-4 (mm) in size.

As given in Bergey's Manual of Systematic Bacteriology (1994), cells of *Rhizobium*can grow on yeast mannitol-mineral salts agar giving circular, convex, semitransparent (translucent), raised and mucoid colonies of 2-4 mm in size within 3 to 5 days. The results are in line with Gachande and Khansole (2011) [11] revealed that the colonies of *Rhizobium japonicum* syn. and *BradyRhizobium japonicum* were circular, light pink, convex, entire and opac.Similarly Bhardwaj (1972) [4] had reported that Rhizobial isolated from legumes of saline alkaline soil give red, orange or yellow pigmented colonies. The colonies were usually mucoid appearing opaque with little firm gum, or translucent appearing glistening or dull with gummy, soft slime. Trinick and Parker (1982) [31] have described rhizobial colonies to be white, milky or creamy, convex, glistening or dull with a regular entire margin.

Cultural characterization of salt tolerant *Rhizobium*

All the isolates of salt tolerant *Rhizobium* (STR) formed circular colonies with entire margin with convex elevation. Similarly Jadhav (1969) [13] and, Rangaswami and Oblisami (1962) [25] also have studied some cultural and physiological characteristics for rhizobia of some wild and cultivated legumes. Nimbalkar (1986) [20] studied these characters in 14 slow growing and 5 fast growing rhizobia.

Physiological characterization of salt tolerant *Rhizobium*

In present study the selected isolates categorized under extremely salt tolerant *Rhizobium* are aerobic in nature. Similarly the results were obtained by Gachande and Khansole (2011) [11] who studied physiological characters of *BradyRhizobium japonicum* and revealed they were aerobic in nature. Variation in the physiological characters of root nodule bacteria of cowpea, gram and dhaincha were observed by Raju (1938) [24].

Biochemical characterization of salt tolerant *Rhizobium*

In present study the selected nine isolates showed positive for catalase, oxidase, urease activity and citrate utilization test. The test of hydrolysis of gelatine, starch, Voges-Proskauer (VP) test, methyl red test and H_2S production were negative for all nine isolates.

The results are similar with Gachande and Khansole (2011) [11] revealed *Rhizobium japonicum* syn. and *BradyRhizobium japonicum* showed negative chemical reaction for indole, methyl red, Voges-Proskaur, hydrogen sulphide production, utilization of carbohydrate and gelatin hydrolysis. It showed positive reaction for citrate utilization, catalase and ammonia production from peptone and urea.

Dye, (1979) [8] have given some cultural, biochemical and physiological characteristics with the help of which one can differentiate *Rhizobium*and *Agrobacterium* from each other.

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