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RESEARCH ARTICLE

SALT TOLERANCE AND BIOCHEMICAL CHARACTERIZATION OF RHIZOBIA ISOLATED FROM SOME WILD *CROTALARIA* SPP.

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Abstract

Thirty one isolates of Rhizobia isolated from root nodules of *Crotalaria pallida* Ait., *Crotalaria verrucosa* L. and *Crotalaria retusa* L. growing in coastal regions of Ratnagiri and Sindhudurg districts of Maharashtra showed 8% tolerance against NaCl (Sodium chloride). Physiological and biochemical characterization of these *Rhizobium* spp. were studied on non saline and modified saline YEMA medium. The aim of the experiments was to select salt tolerant Rhizobia which could be better perform in saline soil.

Introduction:-

Legume - Rhizobia symbiosis is well known to the world for its Nitrogen fixing mechanism. Rhizobia have considerable economic importance in agriculture as they play key role in Biological Nitrogen Fixation (BNF). Today agriculture faces a major problem of hypersalinity of soil. Some wild legumes and Rhizobia indigenous to coastal regions are likely to be adapted to salinity stress and prove themselves suitable for successful symbiosis in saline soils (Shishido and Pepper, 1990). Reclamation of this saline soils can achieved through the agencies of wild legume Rhizobial systems obtained from saline regions (Singleton *et al*., 1982). In the present investigation, 31 *Rhizobium* isolates were obtained from root nodules of different *Crotalaria* spp. *viz. C. pallida* Ait., *C. verrucosa* L. and *C. retusa* L. along the sea shore of Ratnagiri and Sindhudurga districts were screened for their salinity tolerance. Among them CPR-11, CVR-7 and CRR-4 showed maximum salt (NaCl) tolerance up to 8%. *Rhizobium* isolated from the nodules of *Crotalaria spp*. can be used to reclamate saline wastelands hence these salt tolerant Rhizobia further subjected to physiological and biochemical characterization.

Material and Methods:-

Collection of root nodules:-

Leguminous plants *viz. Crotalaria pallida* Ait., *C. verrucosa* L., *C. retusa* L. growing at sea coast of Ratnagiri (16° 59´ 0´´ North, 73° 18´ 0´´East) and Sindhudurg (16° 4´ 0´´ North, 73° 28´ 0´´East) districts of Maharashtra were collected for the studies. Collection of root nodules was done by the method given by Somasegaran and Hoben (1985). The nodule status was studied by the method of Vincent (1970).

Isolation of root nodulating bacteria

The plant roots were washed under running tap water. The nodules were separated from the roots and washed with sterilized distilled water (SDW) for several times. Surface sterilization of nodules was carried out with 70% ethyl

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alcohol for 10 seconds and then with 0.1% $HgCl_2$ solution for 1 to 2 minutes. Surface sterilized root nodules were crushed in sterilized distilled water. Following Serial dilution, bacterial suspension was streaked onto Cango Red – Yeast Extract Mannitol Agar (CRYEMA) plates and incubated at 28±2^{0}C for 2 to 3 days (Vincent, 1970). Rhizobial colonies were picked up and sub cultured on Yeast Extract Mannitol Agar (YEMA).

Authentication of Rhizobia:-
Authentication of Rhizobia was done by Cango Red absorption test (Vincent, 1970); Glucose peptone agar (GPA) test (Vincent, 1970); Hofer's alkaline medium test (Hofer, 1935) and Ketolactose test (Bernaerts and De Ley, 1963).

Cultural characters, staining and morphology:-
The cultural characters such as colony shape, colony size, elevation, pigmentation, margin, opacity, mucocity etc. were studied by using standard Microbiology protocols. Bacterial cultures were examined for Gram staining reaction by the method of Hucker and Conn, (1923) using 48 hrs. old cultures. Bacterium shape was observed under the microscope. Cell size was measured by using ocular and stage micrometer as well as photographic methods.

Salt tolerance in Rhizobia:-
Sensitivity of Rhizobia to various salt (NaCl) concentrations was done on YEMA plates as well as in YEM broth. All the strains were inoculated in test tubes containing YEM broth with different concentrations of salts i.e. Sodium Chloride (1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 9.5% and 10%). Broth without NaCl served as 'Control'. The bacterial growth was recorded by using spectrophotometer (Shimadzu UV-1800) at 600 nm after 48 hr. incubation period at 28± 2^{0}C. YEMA plates supplemented with 1 to 10% NaCl were streaked out with isolates in triplicate. Plates were incubated at 28± 2^{0}C for 48 hours. The isolates which were highly tolerant to salinity were further subjected to physiological and biochemical characterization.

Biochemical and Physiological characterization of *Rhizobium:-*
Biochemical and Physiological tests such as reactions in YEMA Graham and Parker (1964), Reaction in litmus milk (Skerman, 1967), Amylase activity (Starch hydrolysis) (De Oliverira, 2007), Catalase Test (Graham and Parker, 1964), Gelatin Hydrolysis (Difco and BBL Manual, 2009), Nitrate reductase, Oxidase Test Kovasks, (1956), Lipid hydrolysis, Citrate utilization (Koser, 1923), Hydrogen sulfide (H_2S) production (Hunter and Cercelius, 1938), Methyl Red and Vegos Proskauer test (Voges and Proskauer, 1898), Crystal violet sensitivity were carried out with respective methods.

Carbohydrate nutrition:-
The test was carried out on 'non saline' and modified 'saline' Basal medium (Bishop *et. al* 1976). Different carbohydrates (galactose, glucose, sucrose, dextrose, fructose, maltose, lactose, mannitol and raffinose) were substituted for Mannitol. Every carbohydrate source was added at 1.0% (wt/vol) concentration. Bacterial growth on YEMA slants was removed with cotton swabs and suspended in sterile distilled water to a density 10^{-7} per ml. 10 fold diluted cell suspension was added to the wells of a inoculators plate and incubated on to the surface of carbohydrate contained plates. Bishop's agar plate without carbohydrate was served as 'control' the agar plates were incubated for a week at 28^{0}C.

Amino acid nutrition:-
To determine utilization of nitrogen, different amino acids such as Amino- N- butyric acid, alanine, aspergin, glycine, histidine, lysine, serine, tyrosine and valine were incorporated in non saline and saline YEM broth at 0.01% concentration. 0.1 µl bacterial cultures were added to each test tube. Broth without Yeast extract served as control. The bacterial growth was recorded by using (Shimadzu UV-1800) Spectrophotometer at 600nm after 48 hr. incubation period at 28± 2^{0}C.

Results :-

Authentication test:-
All strains inoculated on saline and non saline YEMA amended with Cango red were remained creamy. No strain absorbed Cango red. All strains were failed to grow on GPA medium. Rhizobial strains inoculated on Hofer's alkaline medium were unable to grow at high pH (11.00). Isolates were unable to produce 3- Ketolactase because no yellow ring was observed around the colony when flooded with Benedict's reagent on Lactose Agar medium.

Cultural characters, staining and morphology:-

Colony characters: All isolates were observed for cultural characteristics on saline and non saline YEMA after 48 hrs incubation at 28^0C. Colonies of all isolates on both the media were circular in shape and creamy in colour. All isolates on both media showed convex elevation, entire margins, mucoid consistency and opaque nature. Comparatively smaller colonies (1 to 3 mm diameter) were observed on saline YEMA while on non saline medium colonies ranged 2 to 6.5 mm in diameter. From above observations it was clear that these were fast growing Rhizobia.

Cell characteristics:-
All isolates were gram negative, rod shaped and non spore forming bacteria. The cell size ranged 0.5 to 1X 1.5 to 2.9 µm on non saline medium while 0.7 to 1.2X 1.7 to 2.9 µm on saline medium.

Salt tolerance:-
Sensitivity of these 31 isolates to NaCl ranged from 1 to 8%. Among the 31 isolates 3 isolates were resistant to 8% concentration of NaCl. From Table No. 1., CPR-11, CVR-7 and CRR-4 isolate having maximum (8%) tolerance to NaCl. From Fig. No. 1 to 3, It is clear that, Isolate CPR-11, CVR-7 and CRR-4 grew highest at concentration 2 to 3% C. growth was decreased slowly towards 8% NaCl. Beyond this they showed very poor or no growth at 9 % NaCl. All isolates showed significant growth at 8% NaCl.

Biochemical and physiological characterization:-
Reaction in YEMA: saline and non saline YEMA plates containing Bromothymol blue (BTB) were inoculated with *Rhizobium* impregnated paper disc showed yellow coloured zone around the disc. It indicated change in the pH of the medium was due to acid producers i. e. Fast growing *Rhizobium.* All isolates were acid producers and not affected by salinity. **Reaction in Litmus Milk:** Isolates CPR-11, CVR-7 and CRR-4 showed acidic reaction in saline and non saline litmus milk medium. **Amylase activity:** A clear halos in the midst of the dark plate was observed in isolate CRR-4 in both saline and non saline starch agar when iodine reagent is applied. **Catalase test:** All isolates showed positive Catalase activity. Air bubbles produced on the glass slide when H_2O_2 added to the smear of bacteria. **Gelatinase activity:** All isolates from saline and non saline nutrient gelatin medium showed liquefaction of gelatin. **Nitrate reductase activity:** All isolates from saline and non saline medium were reduced Nitrate. **Oxidase activity:** All isolates from saline and non saline medium showed positive results for oxidase test. **Lipid hydrolysis:** All isolates in both saline and non saline medium showed a clear halo surrounding the bacterial growth identifies the presence of lipase. Lipase production was not affected by salinity. **Citrate Utilization:** All isolates were grown on non saline Simmon's citrate agar medium and gave positive test by changing colour from green to blue. Isolates from saline medium remained green due to trace or no growth of *Rhizobium* isolate. **H_2S production:** No black precipitation was observed on Triple Sugar Iron (TSI) medium. Isolates produced gas only. Better results were found on non saline medium than saline medium. **Indole test:** Bacteria were unable to degrade tryptophan (amino acid). All isolates from modified saline and non saline Tryptophan broth showed negative Indole test. **Methyl Red and Vogas Proskaure test (MR-VP):** Both tests on saline and non saline MR-VP (Glucose Phosphate broth) were found negative. Physiological and Biochemical characteristics of Rhizobia are given in Table No. 2.

Crystal violet sensitivity:-
All isolates from saline and non saline media were sensitive to crystal violet having 1:1000; 1: 10000 and 1:50000 concentrations. CPR-11 and CVR-7 from both saline and non saline media grown on 1:100000 concentrations. While CRR-4 was unable to grow on both media at 1:100000 concentrations. All isolates from both the media tolerated 1:200000 concentrations. From, Table No. 3, it is proved that fast growing Rhizobia were less sensitive to high concentrations of Crystal violet .

Carbohydrate nutrition:-
Sugars like sucrose, galactose, fructose, ribose, raffinose, maltose, xylose and mannitol were utilized by all isolates from Bishop's media. CPR-11, CVR-7 and CRR-4 from non saline media utilized all carbohydrates except rhamnose. CPR-11 from saline medium was unable to utilize sorbitol. Arabinose from saline YEM broth was not utilized by CPR-11, CVR-7 and CRR-4. (Table No. 4).

Amino acid nutrition:-
Amino acids such as amino- N- butyric acid, alanine, aspergin, histidine, lysine, serine and tyrosine were utilized by all rhizobial isolates from both saline and non saline medium. Glycine and valine were not utilized by all rhizobial isolates. (Table No. 5).

Discussion:-

Results of authentication tests proved that all isolates were *Rhizobium* and different from *Agrobacterium* or other bacteria. Colonies obtained from non saline and modified saline media were circular, convex, opaque, mucoid with entire margin. The results were in agreement with Gauri *et al*., (2011). Comparatively smaller colonies were observed on saline medium. According to Steinborn and Roughly, (1975) and Singleton *et al*., (1982) salinity increases the generation time of Rhizobia, which results in formation of smaller colonies on saline YEMA. All isolates from non saline and modified saline media were gram negative, rod shaped and non spore forming. The results are similar with Sadowsky *et al*., (1983). There was a slight variation in size of cell under stressed condition. Rhizobia respond to stress by changing their size and morphology.

It was observed that, sensitivity of these 31 isolates to NaCl ranged from 1 to 10% NaCl. Among the 31 isolates six isolates were resistant to 8% concentration of NaCl. There are many workers who have also recorded sensitivity of Rhizobia to various concentrations of NaCl. According to Kucuk *et al*., (2006) *Rhizobium* isolated from root nodules of *Phaseolus vulgaris* tolerated 5% NaCl. Sharma *et al*., (2013) reported that Rhizobia isolated from root nodules of *Sesbania sesban, Lablab purpureus* and *Cajanus cajan* shown growth on medium containing 40 dsm^{-1} of NaCl. According to Mandal, (2014) out of 27 strains of *Rhizobium trifolli* isolated from root nodules of *Trifolium alexandrinum* tolerated 3% concentration of NaCl. Ali *et al*., (2009) reported that Rhizobia isolated from *Leucaena leucocephala, Tephrosia purpurea* and *Crotalaria medicagina* tolerated 4.5% concentration of NaCl. Bajekal (1996) found that, out of 16 strains of halotolerant rhizobia, two tolerated 3.5% salt, one strain 4.5, two strains, 5.0, four strains, 5.5, two strains, 6.0, three strains, 6.5 and two strains tolerated up to 7.0% NaCl. *Rhizobium* is more tolerant to salts than their host legume; hence survive in saline condition (Subha Rao *et al*., 1972 and 1974). El- Mokadem *et al*., (1991) concluded that, salt tolerant Rhizobia can improve yield of legumes under salt condition. Hashem *et al*., (1998); Shamseldin and Werner, (2005) found that, legume plants grew and survived well in saline condition when they were inoculated with salt tolerant Rhizobia. According to Mandal, (2014) salt tolerant strains of *Rhizobium trifolli* can be useful under stress condition.

On the basis of biochemical tests; isolate showed acidic to strongly acidic reaction in litmus milk like the previous results of Sadowsky *et al*., (1983). A yellow ring or colouration around the culture disc on YEMA amended with Bromothymol blue indicated isolates were acid producing or fast growing root nodulating bacteria Rhizobia. The results were in close agreement with Norris (1965). Positive tests were found for Catalase, gelatinase, oxidase and nitrate reductase from non saline and saline condition. A lipid hydrolysis test was also found positive. These results were similar with Sadowsky *et al*., (1983) and Deshwal and Chaubey, (2014). According to De oliveria *et al*., (2007) *Rhizobium* utilized starch from different sources. Most of the Rhizobia were unable to produce enzyme gelatinase. Hunter *et al*., (2007) observed negative gelatinase activity. According to Sadowsky *et al*., (1983) fast growing Rhizobia isolated from soybean root nodules could produce enzyme gelatinase but slow growers were unable to produce gelatinase. These tests were not affected by salinity. All isolates utilized citrate from non saline medium but were unable to utilize from modified Simmon's Citrate Agar. According to Gauri *et al*., (2011) all isolates of *R. trifolii* were unable to utilize citrate. Isolates found negative for Methyl Red (MR), Voges Proskauer (VP) and Indole tests. These findings were closely in agreement with Shahazad *et al*., (2012) characterized *Rhizobium* strain from root nodules of Alfalfa. From the above observations these Rhizobia were fast growing Rhizobia which are more salt tolerant.

Conclusion:-

Efforts were carried out to develop salt tolerant Rhizobia. The fast growing strains of *Rhizobium* are alkali tolerant. Rhizobia are more tolerant to alkalinity than their legume host. Hence these Rhizobia improve the alkali tolerance of legumes and become helpful in the amelioration of alkaline soil.

Acknowledgement:-

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Table No. 1:- Sensitivity of *Rhizobium* isolates to NaCl (%) *in vitro*.

Sr. No	Rhizobium isolates	Concentration of NaCl in %																		
		1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10
1.	CPR-1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
2.	CPR-2	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
3.	CPR-3	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
4.	CPR-4	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
5.	CPR-5	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
6.	CPR-6	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
7.	CPR-7	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8.	CPR-8	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
9.	CPR-9	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	CPR-10	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
11	CPR-11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
12	CPR-12	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
13	CPR-13	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
14	CPR-14	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
15	CVR-1	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
16	CVR-2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
17	CVR-3	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
18	CVR-4	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
19	CVR-5	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
20	CVR-6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
21	CVR-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
22	CVR-8	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
23	CVR-9	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
24	CVR-10	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
25	CRR-1	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
26	CRR-2	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
27	CRR-3	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
28	CRR-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
29	CRR-5	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
30	CRR-6	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
31	CRR-7	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

+ = Bacterial growth - = No Bacterial growth

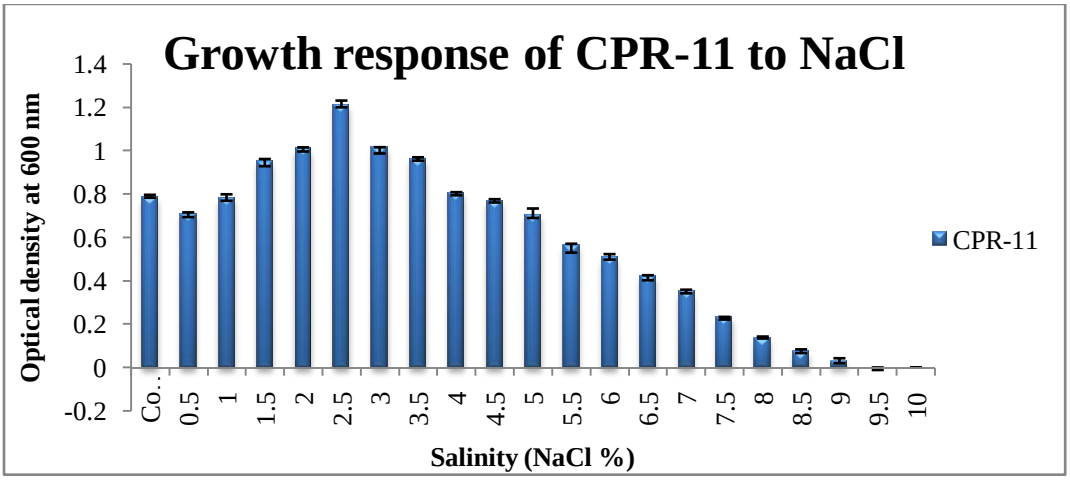

Fig.1:- Dose response curve of CPR-11 to various concentrations of NaCl on YEM broth

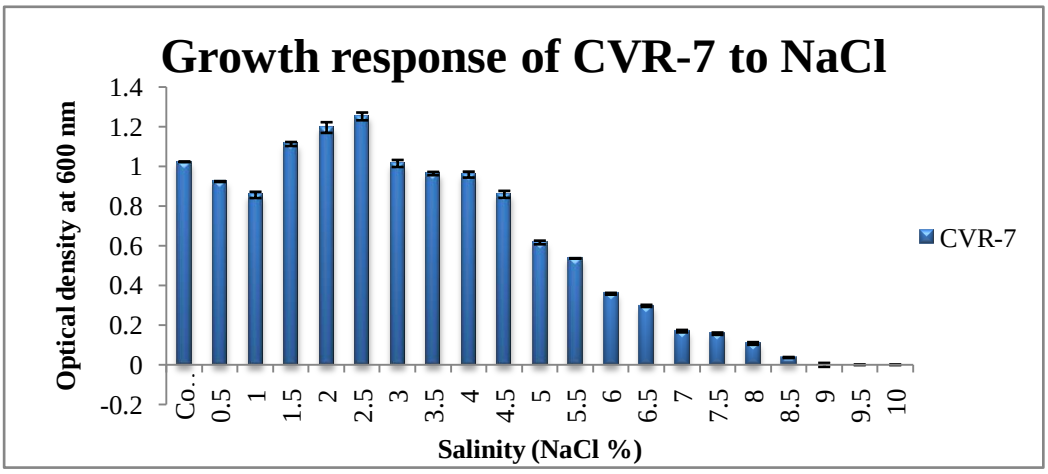

Fig. 2:- Dose response curve of CVR-7 to various concentrations of NaCl on YEM broth.

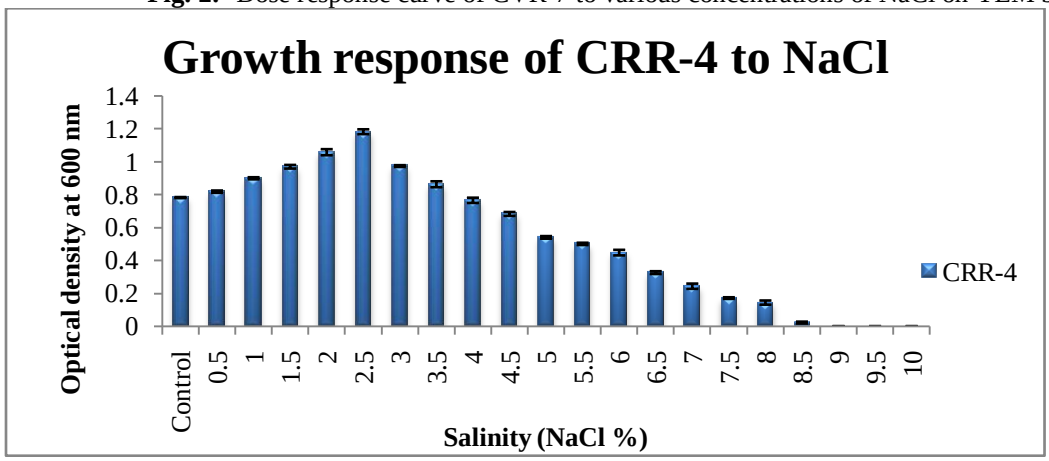

Fig.3:- Dose response curve of CRR-4 to various concentrations of NaCl on YEM broth.

Table No. 2:- Physiological and Biochemical characteristics of Rhizobia.

Biochemical and Physiological characteristics	***Rhizobium* Isolates**					
	CPR-11		**CVR-7**		**CRR-4**	
	NS	S	NS	S	NS	S
Reaction in YEMA	Ac	Ac	Ac	Ac	Ac	Ac
Reaction in Litmus Milk	Ac	Ac	Ac	Ac	Ac	Ac
Amylase	-	-	-	-	+	+
Catalase	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Lipid hydrolysis	+	+	+	+	+	+
Citrate Utilization	+	-	+	-	+	-
H_2S production	-	-	-	-	-	-
Methyl Red Test	-	-	-	-	-	-
Vogas Proskaure test	-	-	-	-	-	-
Indol	-	-	-	-	-	-

NS =Non saline medium **S** =Saline medium + =Positive test - =Negative test **Ac** =Acidic reaction

Table No. 3:- Sensitivity of Rhizobia to the Crystal violet.

Rhizobial strain	Crystal violet concentration									
	1:1000		1:10,000		1:50,000		1:1,00,000		1:2,00,000	
	NS	S	NS	S	NS	S	NS	S	NS	S
CPR-11	-	-	-	-	-	-	+	+	+	+
CVR-7	-	-	-	-	-	-	+	+	+	+
CRR-4	-	-	-	-	-	-	-	-	+	+

NS =Non saline medium, S =Saline medium, + =Positive test, - =Negative test

Table No. 4:- Effect of various Carbohydrate sources on the growth of Rhizobial isolate on Basal medium.

Sugar 1.0%	Rhizobial isolate					
	CPR-11		CVR-7		CRR-4	
	NS	S	NS	S	NS	S
Sucrose	+	+	+	+	+	+
Sorbitol	+	-	+	+	+	+
Glucose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Ribose	+	+	+	+	+	9+
Raffinose	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Xylose	+	+	+	+	+	+
Arabinose	+	-	+	-	+	-
Mannitol	+	+	+	+	+	+

NS =Non saline medium, **S** =Saline medium, + =Bacterial growth, - =No bacterial growth

Table No. 5:- Effect of various amino acids on the growth of Rhizobial isolate in YEM broth.

Amino acid 0.01%	Rhizobial isolates					
	CPR-11		CVR-7		CRR-4	
	NS	S	NS	S	NS	S
Amino- N- butyric acid	+	+	+	+	+	+
Alanine	+	+	+	+	+	+
Aspergin	+	+	+	+	+	+
Glycine	-	-	-	-	-	-
Histidine	+	+	+	+	+	+
Serine	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+
Valine	-	-	-	-	-	-

NS =Non saline medium, **S** =Saline medium, + =Bacterial growth, - = No bacterial growth

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