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Screening of salt tolerant rhizobial isolates associated with *Vigna radiata* cultivated in saline soils of West Bengal

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Fifty one rhizobial strains were isolated from effective root nodules of 5 different cultivars of *Vigna radiata* growing in saline areas of South 24-Parganas, North 24-Parganas and Medinipur districts of West Bengal. Morphological, cultural and biochemical characteristics of these isolates revealed that they belong to *Bradyrhizobium* group of legume symbionts. Nearly some 10 % of the isolates tolerated 2 % (w/v) NaCl in the growth medium and four of them were resistant to 400μg/ml of streptomycin. Infectivity of these resistant isolates (74.2-75.2 %) was comparable to that of the wild type (74 %) while, the survival and recovery of cells were quite high (60 %) at an initial density of 50x 10^7 cells/g of sterile soil. Seed inoculation using these selected rhizobial isolates could be an effective tool in increasing the growth and yield of *Vigna radiata* under salt stressed condition.

Key words: *Bradyrhizobium, Vigna radiata*, saline soil, salt tolerant rhizobia

INTRODUCTION

Salinity is a serious threat to agricultural productivity in arid and semiarid tropical regions. According to Cordovilla *et al.* (1994) nearly 40 per cent of World's land surface is considered as having potential salinity problems and most of these areas are restricted to the tropics and Mediterranean regions. The increase in salinity levels of soil or in irrigation water often results in lowering productivity of several crops as a consequence of marked changes in the growth pattern of plants. Salinity decreases growth and yield of crop plants depending upon the species of crop, levels of salinity and ionic composition of the salts (Delgado *et al.*, 1994). Increasing salt concentrations have also been found to have a detrimental influence on microbial population of soil as a result of direct toxicity as well as through osmotic stress.

As such the legume-*Rhizobium* symbiosis and nodule formation in legumes have been reported to be more sensitive to salt or osmotic stress than the *Rhizobium* alone (El-shinnawi *et al.* 1989; Velagaleti *et al.* 1990). Moreover, the association was found to be successful depending completely on the (i) survival and persistence of "native" or introduced rhizobia in the rhizosphere soil of the legume; (ii) successful infection and initiation of development of nodule; and (iii) proper fixation of nitrogen by the developed nodules (Alexender, 1978).

In India, the productivity of coastal areas lags far behind that of inland areas. The entire area is almost mono-cropped with rain fed rice being the only crop during the monsoon period. The land remains fallow during the rest of the period of the year due to either lack of good quality irrigation water or a combination of factors like moderate to high level of salinity along with other soil and climatic constrains. Among all the states in India, West Bengal has the highest area of coastal saline land, which are spread over five districts, namely North 24 Parganas, South 24 Parganas, Hoara, East and West Medinipur.

Successful *Rhizobium*-legume symbiosis under salt stresed condition requires the selection of salt tolerant rhizobial isolates indigenous to saline soils.

The present studies was undertaken with a view to isolate and select some salt tolerant, antibiotic resistant marker strains of rhizobial isolate of *Vigna radiata* for effective and successful application in increasing productivity and yield of *Vigna radiata* cultivated in the saline belts of West Bengal.

MATERIALS AND METHODS

Isolation and maintenance of rhizobial isolates obtained

The rhizobial isolate used in this study were isolated from the fresh effective root nodules of different cultivars of *Vigna radiata* collected from farmers' field located in saline areas of North and South 24-Parganas and Medinipur districts of West Bengal. The root nodules were surface sterilized, crushed, serially diluted and plated on yeast extract mannitol agar medium (YEMA) according to the method of Aneja (1996). The strains were purified following dilution streaking and maintained on slopes of same agar medium at 30° C by regular subculturing at an interval of 30 days. The yeast-extract mannitol agar medium (YEMA) contained (g/l): mannitol, l0; K_2HPO_4, 0.5; $MgSO_4$ $7H_2O$, 0.2; NaCl, 0.1; agar, 20. (pH 7.0).

Salinity tolerance of rhizobial isolates

Tolerance to salinity of the rhizobial isolates were determined in yeast extract mannitol medium supplemented with 0.25-2.0 % (w/v) NaCl. The medium (20 ml/100 ml flask) was inoculated with freshly prepared inoculum and incubated at 30° C on a rotary shaker for 2-3 days. Growth of the isolates was determined by measuring the optical density at 625 nm in a Varian DMS 1005 Spectrophotometer.

Development of antibiotic-resistant mutants of rhizobia

Antibiotic resistant mutant of rhizobia was developed following the method of Mckovacki, (1990) using Bergersen's agar medium (1961) modified contained (g/l): mannitel, 3; sodium glutamate 0.5; yeast extract, 0.3; agar, 20. pH 7.0.

Rhizobial cells grown in Bergersen's broth were harvested by centrifugation from mid logarithmic phase of growth and suspended in sterile phosphate buffer (pH 7.0) to attain a density of 10^9 cells ml^{-1}. An aliquot (0.1ml) of this suspension was spread onto the surface of Bergersen's agar supplemented with different concentration of streptomycin. After an incubation of 48-96 h, resistant colonies were selected and maintained on Bergersen's agar medium.

Infectivity of rhizobial isolates

To find out the infectivity of wild and mutant rhizobial isolates, the seedling roots (5 day old) of *V. radiata* cv. B1 were inoculated with a cell suspension of $7x10^7$ cells/ml. Sterile water was used as the control and incubated for 24 h. The percentage of root infection was recorded.

Survival of rhizobial isolates in soil

In order to find out the survival capability, the selected rhizobial strains were inoculated in sterile and non sterile soils and incubated under field capacity moisture, (85%-90% humidity) at 28° C in Petri plates for 10 days. After the desired period of incubation, rhizobial cells were recovered from soil by standard soil dilution plate technique on YEMA containing 400 μg/ml streptomycin and 2% NaCl.

RESULTS AND DISCUSSION

Isolation and identification of rhizobial isolates

The rhizobial isolates were isolated from the freshly collected effective root nodules of *Vigna radiata* growing in different experimental areas of saline zones of South 24-Parganas, North 24-Parganas and Medinipur districts of West Bengal. The method as described by Aneja (1996) was principally followed for the purpose of isolation. Altogether 51 isolates were obtained from *Vigna radiata* cultivated in saline zones of Basanti, Canning and Kakdwip of South 24-Parganas, Deuli, Kharampur and Saimalpur of North 24-Parganas and Contai and Tamluk of Medinipur districts of West Bengal (Table 1).

All the fifty one (51) rhizobial isolates isolated from the nodules of *Vigna radiata* were rod-shaped, Gram-negative and motile in nature. The cultural and biochemical characteristics of these isolates, viz. growth pattern in congo-red test, and on YEMA medium, pH of the medium, serum zone formation, acid production in litmus milk, reduction of nitrate, production of hydrogen sulphide, production of acid or alkali on mannitol were also studied. On the basis of these features, the rhizobial isolates were tentatively placed in the *Bradyrhizobium* group which also includes the cowpea-miscellany rhizobia. Yang *et al.*(2008) in a recent study has clustered. mung bean rhizobial isolates from different geographical areas of China into four groups. In addition to *Bradyrhizobium* spp. these groups included isolates related to the genera *Sinorhizobium, Rhizobium* and *Mesorhizobium.*

Salinity tolerance and antibiotic resistance

The sensitivity of rhizobial isolates of *Vigna radiata* to NaCl was tested in yeast-extract mannitol medium supplemented with wide range (0.75-2.0 %, w/v) of NaCl. Results as presented in Table 2 indicate that the number of tolerant isolates gradually decreases with increase in NaCl concentration in the medium. A total of 5 isolates showed moderate to good growth in 2 % (w/v) NaCl indicating significant salt tolerance. These strains were CAN 11, CON 30, KAK 08, TAM 49 and KHA 16

These findings support the views of earlier workers El-Sheikh *et al.,* 1989; El-Shinnawi *et al.,* 1989; Kumar *et al.,* 1990, Chien-Ching-Te *et al.,* 1992 and Craig *et al.,* 1991) and indicate that tolerance of rhizobial isolates to salinity varied widely even from a small geographical regions and that there may be

Table 1. Isolation of rhizobial isolates from root nodule of *Vigna radiata* cultivated in saline zones of West Bengal

Locality	Soil type	Soil pH	Soil salinity (dSm^{-1})	*Vigna* cultivar	Rhizobial isolate No	Total no. of isolates
South 24 Parganas						
Canning	Silt Clay	7.85	7.50	B 48	CAN 03, 09, 11, 15, 19, 35	6
Kakdwip	Silt Clay	8.25	7.20	B 152	KAK 01, 06, 08, 10, 48	5
Basanti	Silt Clay	8.10	5.80	B 295	BAS 02, 04, 05, 07, 41, 46	6
North 24 Parganas						
Deuli	Loamy	4.70	6.00	B 1	DEU 12, 14, 18, 20, 24, 36, 45	7
Saimalpur	Silt Clay	7.00	2.60	B 48	SAI 17, 24, 25, 26, 29, 40, 44	7
Kharampur	Silt Clay	5.00	7.20	PDM 89-139	KHA 13, 16, 21, 22, 23, 27, 37	7
Medinipur						
Contai	Lateritic	5.50	2.40	B 48	CON 28, 30, 31, 32, 33, 34	6
Tamlak	Lateritic	6.50	6.00	B 152	TAM 38, 39, 42, 43, 47, 49, 50	7

Table 2. Sodium chloride tolerance of rhizobial isolates obtained from *Vigna radiata* cultivars

Vigna radiata cultivars	No. of rhizobial isolates	No. of isolates growing in YEM medium supplemented with NaCl, % (w/v)						Isolates tolerating 2% (w/v) NaCl
		0.75	1.00	1.25	1.50	1.75	2.00	
B 1	7	7	4	2	-	-	-	-
B 48	19	16	13	12	7	4	2	CAN 11, CON 30
B 152	12	12	12	11	8	6	2	KAK 08, TAM 49
B 295	6	6	6	3	1	-	-	-
PDM 89 - 139	7	7	7	6	4	4	1	KHA 16
Total	51	48	42	34	20	14	5	5

Isolates of rhizobia were grown on YEM medium supplemented with different concentrations (%) of sodium chloride. '-' means no growth.
Values represent average of five replications.

broad genetic base in the salt tolerance character of *Rhizobium* gene pool (Nair *et al.*, 1993).

Antibiotic resistance in *Rhizobium* sp. from different crops has been tested (Karanja and Wood 1989; Young *et al.*, 1989 and Mckovacki, 1990). During the present study all 5 salt tolerant rhizobial isolates were evaluated for their growth performance in medium containing increasing concentration of streptomycin. It revealed that out of 5 *Rhizobium* isolates, CAN 11, KHA 16, and TAM 49 of *Vigna* radiata were able to tolerate 400 μg/ml, of streptomycin (Table 3.)

Table 3. Sensitivity of selected rhizobial isolates of *Vigna radiata* to streptomycin

Vigna radiata cultivar	Rhizobial isolate	Growth on Bergersen's medium supplemented with streptomycin, μg/ml.			
		100	200	300	400
B 1	DEU 20	-	-	-	-
B 48	CAN 11	+	+	+	+
	CON 30	+	+	+	+
B 152	KAK 08	-	-	-	-
	TAM 49	+	+	+	+
PDM 89 - 139	KHA 16	+	+	+	+

Isolates of *Rhizobium* were grown on medium supplemented with different concentrations μg/ml of streptomycin sulphate, '-' means no growth. '+' means growth.
Result represent average of five replications.

Infectivity of rhizobial isolates

The root hair infectivity of wild type and salt tolerant streptomycin resistant mutant rhizobia were tested against *V. radiata* cv. B1. and the results are presented in Table 4. It was evident from the data that infectivity of the mutant rhizobial isolates was comparable to that of the wild type isolate and there was no significant variation in their power of infectivity when inoculated in the host. These observations are in agreement with those of Zelazna-Kowalska (1971) and Bhaumick (1995).

Table 4. Infectivity of rhizobial isolates to roots of *Vigna radiata* c.v. B - 1

Treatment [a]	Percentage of root hair Infection [b]
Control (Sterile water)	0
Rhizobial cell suspension	
Wild type isolate	74.0
Streptomycin resistant isolate	
CAN 11	75.2
KHA 16	75.0
CON 30	74.8
TAM 49	74.2

[a] *Vigna radiata* (5 day old) root were inoculated by flooding with sterile water or 7 X 10^7 cell / ml suspensions of wild and mutant rhizobial isolates.

[b] Percentage of roots hair infection was scored after 12 h of incubation

Survival of rhizobial isolates in soil

The successful application of rhizobial isolates in any soil depends mainly on its capability to survive in that soil and multiply. In the present study streptomycin resistant mutants rhizobial isolates of *Vigna radiata* was used to study its survival in soil. The experimental data (Table 5) on the survival and recovery of the streptomycin resistant cells of rhizobia of *Vigna radiata* revealed that the survival as well as recovery of cells were dependent on the initial density of cells added to the soil. The survival and the recovery of the cells were high at an initial cell density of 10^7 cells / g. of soil or just around it and were low at very low or high initial cell density which might be a factor in the survival of the rhizobial cells in soil. These observations correlate the findings of Bower and Kennedy (1959) and Bhaumick (1995) and appear to be a generalized feature for the survival of rhizobial isolates in nature.

Table 5. Survivality of streptomycin resistant rhizobial isolate (CAN 11) in soil

Initial cell concentration X10^7/g	Non sterile soil [c]		Sterile soil [b]	
	Cells recovered X X10^7/g	Per cent recovery[d]	Cells recovered X10^7/g	Per cent recovery
1000	400	40	410	41
500	210	42	225	45
100	50	50	58	58
50	27	54	30	60
10	3	30	4	40
5	1	20	2	20

[a] Cells of rhizobial isolates resistant to streptomycin and tolerant to NaCl were recovered from soil by standard soil dilution plate technique on YEMA containing 400 μg/ml streptomycin and 2 % NaCl.

[b] Soil samples were sterilized at 121° C for 1 h on three successive days.

[c] Non sterile soils were collected from the experimental fields.

[d] Percentage change was calculated based on cells initially added to soil.

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