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Studies on the screening of salt tolerance Rhizobia for the groundnut in Cuddalore District of Tamil Nadu

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ABSTRACT

Nitrogen is one of the major important nutrients essential for plants growth. The atmosphere contains about 10^{15} tonnes of N_2 gas and the nitrogen cycle involves the transformation of some $3x10^9$ tonnes of N_2 per year on the Global basis. However, transformations eg. N_2 fixation is not exclusively biological. Lightning probably accounts for about 10 per cent of the world's supply of fixed nitrogen. The fertilizer industry also provides very important quantities of chemically fixed nitrogen. World's production of fixed nitrogen from dinitrogen for chemical fertilizer accounts for about 25 per cent of earth's newly fixed nitrogen and the biological process accounts for about 60 per cent. The international emphasis on environmentally sustainable development with the use of renewable sources is likely to focus attention on the potential role of biological nitrogen fixation in supplying N for agriculture. Annually, biological nitrogen fixation is estimated to be around 175 million tonnes of which 79 per cent is accounted by terrestrial fixation. Legume-*Rhizobium* symbiotic association observed to fix 60-100 kg N ha^{-1} $crop^{-1}$. The phenolate type of siderophore production by the isolates ranged from 1.49 mg l^{-1} to 4.95 mg l^{-1}. The isolates GNBJ-9 produced maximum siderophore 4.95 mg l^{-1} which was followed by GNBJ-4, GNBJ-8 and GNBJ-28 respectively. The Intrinsic antibiotic resistances (IAR) of the 30 isolates were assessed against 5 antibiotics. The isolate GNBJ-9 possessed high level of antibiotic tolerance upto 200 ppm for Kanamycin, 300 ppm for Streptomycin, 250 ppm for Rifampicin and 200 ppm for Pencillin and sensitive to Gentamycin. The effect of salinity towards the growth of all thirty isolates was tested using different concentration of salt. All the isolates grew well in YEM liquid broth without salt, as the concentration increased, the growth get decreased.

Keywords: Rhizoium, Salt tolerance, Arachis hypogaea

INTRODUCTION

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Symbiotic nitrogen fixation (SNF) by legumes plays major role in sustaining crop productivity of marginal lands and in small holders systems. Farmers in the saline areas depend on legumes as an important crop in their cropping systems due to the capacity of these plants to fix nitrogen from air by the interaction with nitrogen fixing rhizobia. It is well known that nitrogen is abundant in the atmosphere, but plants cannot directly utilize the elemental nitrogen from the air. Symbiotic nitrogen fixation occurs mainly through symbiotic association of legumes with N_2 fixing rhizobia that convert elemental nitrogen fixation (BNF) in to ammonia. This type of biological nitrogen is therefore less costly and more sustainable as compared with nitrogen fertilizers for production of plant proteins. Scientific and technological progress has opened tremendous opportunities for the benefit of small farmers (Abdel Aal *et al.* 2004). BNF accounts for approximately two-thirds of the nitrogen fixed globally, while the rest of the nitrogen is industrially synthesized by the Haber–Bosch process (Rubio and Ludden, 2008). Biological nitrogen fixation occurs, generally at mild temperatures, by nitrogen fixing microorganisms, which are widely distributed in nature (Raymond *et al.,* 2004).

Nitrogen fixing organisms are generally categorized as (a) symbiotic N_2 fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (*e.g.* Rhizobia) (Ahemad and Khan, 2012b) and non-leguminous trees (*e.g. Frankia*) and (b) non-symbiotic (free living, associative and endophytes) nitrogen fixing forms such as cyanobacteria (*Anabaena, Nostoc*), *Azospirillum, Azotobacter, Gluconoacetobacter diazotrophicus* and *Azocarus etc.* (Bhattacharyya and Jha, 2012). However, non-symbiotic nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the bacterially-associated host plant requires (Glick, 2012). Salinity in the soil and irrigation water is an environmental problem and a major constraint for crop production. Currently, 20 % of the world's cultivated land is affected by salinity, which results in the loss of 50 % of agricultural yield. At present, there are nearly 954 million hectares of saline soils on the earth's surface. All these salt affected soils are distributed throughout the world. A large bulk of about 320 million hectares and of lands in South and South East Asia is under the grip of salinity (Aslam, 2006).

MATERIALS AND METHODS

Siderophore production

Siderophore assay medium contained K_2HPO_4 0.05%, $MgSO_4$ 0.04 %, NaCl 0.01%, Mannitol 1%, glutamine 0.1%, NH_4NO_3 0.1% (Reeves *et al*., 1983).To 100 ml of the above media, two ml of Bradyrhizobial cultures having cell density of 10^9 CFU/ml

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were added and incubated at 30°C psychrotherm incubater for 72 hours. The cells were harvested at 4000 x g for 10 min. and the supernatant was collected. It was divided into three equal parts for estimation of catechol type, hydroxymate and salicylate type siderophore.

Catechol type and salicylate type siderophore

Salicylate type siderophore was measured as follows. To 20 ml of supernatant, 20 ml of ethyl acetate (pH 2) was added and extraction was done twice. Hathway reagent (1 ml of 0.1 M ferric chloride in 0.1 N HCl added to 100 ml of distilled water and to this 1 ml of 0.1 M Potassium ferric cyanide was added) was prepared (Reeves *et al*., 1983). 5 ml of the assay solution was added to 5 ml of Hathway reagent and absorbance was determined 560 nm with sodium salicylate as standard.

Hydroxymate type Siderophore

Hydroxymates were measured in the untreated culture supernatants of *Bradyrhizobium* isolate by the method of Gibson and Magrath (1969). To 0.5 ml of the culture supernatant, 0.5 ml 6M H_2SO_4 was added and the mixture was autoclaved in a glass tube. 1 ml of sulphanilic acid (1% m/v) in 30% acetic acid and 0.5 ml of 1.3% iodine in 30% acetic acid were added. The excess of iodine was destroyed by the addition of 1 ml of 2% (w/v) Na_3ASO_4 solution. A solution of 1 ml α-naphthylamine (0.3% in 30% acetic acid) was added and the total volume was mad up to 10 ml with distilled water. Hydroxylamine hydrochloride was used as a standard. Since the strains produced only phenolate type of siderophore therefore catechol type siderophore was estimated studies on the stress tolerance of *bradyrhizobium* isolates

Determination of Intrinsic Antibiotic Resistance (IAR) of isolates

Intrinsic antibiotic resistance (IAR) of *Bradyrhizobium* strains were determined by using Tryptone yeast extract agar medium by incorporating with five antibiotics *Viz.,* Streptomycin, Gentamycin, Rifampicin, Kanamycin and Penicillin. Stock solutions were prepared in deionised water, the Rifampicin was dissolved in methanol and were filter sterilized, refrigerated and used within 2 weeks. The appropriate amount of stock antibiotic solution was dispensed into autoclaved, cooled, (45-50°C) Tryptone yeast extract medium.

The amount of inoculum to each plate was about 10^6 cells for all strains. A inoculating needle (20 gauge nichrome wire) was used to transfer the inoculum to test plates. The screening was carried out until the maximum resistance for each strain was obtained. A maximum of 10 strains were inoculated per plate with the help of suitable reference spots marked on each plate

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using glass marker. The plates were incubated at 28°C for 7 days. The growth of the antibiotic plates was compared with that on Tryptone yeast extract agar control plates.

Determination of Salt tolerance level of the root nodule isolates

The Yeast extract manitol broth was prepared at different levels of sodium chloride concentrations *viz.*, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 per cent. pH adjusted to 7.2 and distributed in 100 ml quantities into 250 ml Erlenmeyer flasks and sterilized. Yeast extract mannitol broth without NaCl served as control. The flasks were inoculated with 1 ml of standardized inoculum ($1x10^7$ cells) of *Rhiobium* isolates. The flasks were incubated for 72 h at 30°C with intermittent shaking. The growth measured as absorbance at 520 nm in Spectronic-20 spectrophotometer.

RESULTS AND DISCUSSION

Groundnut soil samples were collected from thirty different locations in Cuddalore district of Tsunami affected saline areas of Tamil Nadu and their physico-chemical properties were analysed. The groundnut root nodules isolates GNBJ-1 to GNBJ-30 were screened for their efficiency based on IAA and EPS production, nodulation, nodule ARA activity, leghemoglobin content and nodule N content.

Screening of Bradyrhizobial isolates for siderophore production

The development of wine colour and absence of orange colour by the addition of 1 mm $FeCl_3$ to spent medium of *Bradyrhizobium* suggested that hydroxymate type of siderophore was absent. The nil absorbance at 560 nm indicated the absence of salicylate type of siderphore. None of the strains could produce hydroxymate type of siderophore produce by the strains was estimated by measuring the OD value at 700 nm using 2.3-D HBA standard. The siderophore production ranged from 1.49 to 4.95 mg l^{-1} of the culture medium. Among the 30 isolates, the isolate GNBJ-9 produced maximum siderophore of 4.95 mg l^{-1} which was followed by GNBJ-8, GNBJ-28 and GNBJ-4 respectively. The results were presented in Table-1. It has been postulated that indole acetic acid (IAA) as the most probable initiator substance to cause the curling effect (Subba Rao, 1977). The ability to produce curling of root hair is necessary for a rhizobial strain to be able to cause nodulation (Sahlaman and Fahraeus, 1962). In the present study, all the 30 isolates had the ability to produce IAA under *in vitro* condition. The production of IAA ranged from 0.70 to 7.80 μg ml^{-1}.

Table-1: Screening of Groundnut Bradyrhizobial isolates for Siderophore production

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S.No.	Isolates	Hydroxymate type in mg l^{-1}
1	GNBJ-1	2.69
2	GNBJ -2	3.75
3	GNBJ -3	3.45
4	GNBJ -4	4.70
5	GNBJ-5	1.80
6	GNBJ-6	2.57
7	GNBJ-7	3.98
8	GNBJ-8	4.80
9	GNBJ-9	4.95
10	GNBJ-10	3.75
11	GNBJ-11	1.76
12	GNBJ-12	3.00
13	GNBJ-13	3.65
14	GNBJ-14	2.95
15	GNBJ-15	1.72
16	GNBJ-16	3.28
17	GNBJ-17	1.90
18	GNBJ-18	1.60
19	GNBJ-19	3.86
20	GNBJ-20	2.64
21	GNBJ-21	3.10
22	GNBJ-22	2.75
23	GNBJ-23	2.00
24	GNBJ-24	3.37
25	GNBJ-25	2.58
26	GNBJ-26	4.00
27	GNBJ-27	1.49
28	GNBJ-28	4.78
29	GNBJ-29	3.15
30	GNBJ-30	2.38
SED		0.14
CD (P=0.05)		0.26

The isolate GNBJ-9 produced the maximum IAA of 7.80 μg ml^{-1}. To initiate the groundnut cultivation in certain areas, where the soil has low nutrients, it is essential to obtain some effective *B. japonicum* strains having better adaptability to survive in such soil conditions. Keeping the above view in the mind, the present investigation was made and the symbiotic effectiveness of some salt tolerant *B. japonicum* strains has been evaluated in low nutrients soil. These results were conformity with the result of Sayyed *et al.,* (2011); Marczak *et al.,* (2013); Castellane *et al.* (2014).

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Screening of groundnut root nodule isolates for Intrinsic Antibiotic Resistance (IAR)

The intrinsic antibiotic resistance of the thirty isolates were determined against five antibiotics such as Kanamycin, Streptomycin, Rifampicin, Gentamycin and Penicillin. The maximum tolerance level of the isolates for each antibiotic was calculated and the results were observed in Table-2. All the thirty isolates had a variation in their antibiotic resistance considerably. The isolate GNBJ-28 found to be sensitive to all the antibiotics at different concentrations tested. The isolate GNBJ-9 had highest level of antibiotic tolerance up to 200 ppm for Kanamycin, 300 ppm for Streptomycin, 250 ppm for Rifampicin, 200 ppm for Penicillin and sensitive to Gentamycin. The bacterial strains possessing intrinsic antibiotic resistance (IAR) are reported to possess better competitive survival. This character of the nodulating *Rhizobium* could be exploited for monitoring their population and to determine the percentage of nodules formed by inoculated culture.

Table-2: Screening of Groundnut Bradyrhizobial isolates for Intrinsic Antibiotic Resistance (IAR)

S.No.	Isolates	Maximum tolerance level of different antibiotics (μg ml^{-1})				
		Kanamycin	Streptomycin	Rifampicin	Gentamycin	Penicillin
1	GNBJ-1	85	100	-	20	-
2	GNBJ -2	140	100	130	-	100
3	GNBJ -3	100	50	75	-	75
4	GNBJ -4	150	100	25	-	80
5	GNBJ-5	100	85	75	25	-
6	GNBJ-6	100	25	-	-	25
7	GNBJ-7	120	135	120	-	140
8	GNBJ-8	185	275	210	-	165
9	GNBJ-9	200	300	250	-	200
10	GNBJ-10	100	70	100	25	75
11	GNBJ-11	75	45	30	-	-
12	GNBJ-12	100	75	25	-	50
13	GNBJ-13	50	25	-	-	25
14	GNBJ-14	100	100	150	-	-
15	GNBJ-15	-	-	75	-	25
16	GNBJ-16	100	175	125	-	75

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17	GNBJ-17	75	50	25	-	75
18	GNBJ-18	150	-	-	-	75
19	GNBJ-19	50	75	25	-	25
20	GNBJ-20	-	175	50	-	75
21	GNBJ-21	-	125	50	-	50
22	GNBJ-22	100	160	150	-	125
23	GNBJ-23	175	50	-	-	50
24	GNBJ-24	25	25	-	-	50
25	GNBJ-25	25	50	25	-	75
26	GNBJ-26	100	25	150	50	-
27	GNBJ-27	-	-	-	-	-
28	GNBJ-28	155	185	160	-	170
29	GNBJ-29	-	25	25	-	25
30	GNBJ-30	125	-	25	25	75

In the present study, the strain GNBJ-9 was found to tolerate up to 200 ppm for Kanamycin, 300 ppm for Streptomycin, 250 ppm for Rifampicin and 200 ppm for Penicillin but it is sensitive to Gentamycin. The similar, Elbouthari *et al.* (2010) reported that *Sinorhizobium. meliloti* and *S. medicae* were resistant to streptomycin, chloromphenicol, tetracycline and streptomycin. Therefore, isolation of rhizobia strains that are capable to tolerate these stresses is essential for efficient nitrogen fixation (Abd El-Halim *et al*., 2001, Abdel - Salam *et al*., 2002, Diouf 2007, Essendoubi *et al*., 2007).

Screening of *Bradyrhizobium* isolates for salt tolerance

The effect of salt towards on the growth of all the 30 isolates was studied. The salt tolerance potential of the thirty isolates were tested by growing them in Yeast extract-Mannitol (YEM) liquid medium prepared with salt 6000 at different concentrations (from 0%, 1.0%, 2.0%, 3.0%). The results were presented in Table-3.

All the isolates grow well in YEM liquid broth without NaCl as the concentration increased, the growth get decreased. Among the isolates tested, the isolates GNBJ-4, GNBJ-8, GNBJ-9 and GNBJ-28 were found to grow at 3.0% salt concentration. The isolate GNBJ-9 was able to grow up to OD 520=1.53 at 3.0% salt concentration.

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The four isolates of *B. japonicum* namely GNBJ-4, GNBJ-8, GNBJ-9 and GNBJ-28 were selected for further study based on their efficiency and salt tolerance level.

Table-3: Screening of Bradyrhizobial isolates for Salt tolerance OD at 520nm for 72hr broth culture

S. No.	Isolates	Sodium chloride concentration (%)						
		0%	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%
1	GNBJ-1	0.265	0.097	0.034	0.011	-	-	-
2	GNBJ -2	0.274	0.094	0.038	0.017	0.007	-	-
3	GNBJ -3	0.315	0.250	0.119	0.049	0.019	-	-
4	GNBJ -4	0.276	0.130	0.048	0.036	0.019	0.015	0.08
5	GNBJ-5	0.285	0.100	0.045	0.008	-	-	-
6	GNBJ-6	0.296	0.119	0.068	0.030	0.09	-	-
7	GNBJ-7	0.295	0.115	0.078	0.010	-	-	-
8	GNBJ-8	0.295	0.130	0.056	0.040	0.024	0.018	0.010
9	GNBJ-9	0.328	0.135	0.064	0.038	0.028	0.020	0.013
10	GNBJ-10	0.228	0.159	0.037	0.059	-	-	-
11	GNBJ-11	0.252	0.077	0.027	-	-	-	-
12	GNBJ-12	0.310	0.259	0.030	-	-	-	-
13	GNBJ-13	0.291	0.117	0.055	0.014	-	-	-
14	GNBJ-14	0.271	0.084	0.024	0.004	-	-	-
15	GNBJ-15	0.285	0.104	0.044	-	-	-	-
16	GNBJ-16	0.275	0.108	0.037	0.025	-	-	-
17	GNBJ-17	0.260	0.074	0.040	0.007	-	-	-
18	GNBJ-18	0.280	0.109	0.040	0.006	-	-	-
19	GNBJ-19	0.273	0.064	-	0.004	-	-	-
20	GNBJ-20	0.283	0.104	0.109	0.017	-	-	-
21	GNBJ-21	0.287	0.119	0.009	0.014	-	-	-
22	GNBJ-22	0.279	0.088	0.019	-	-	-	-
23	GNBJ-23	0.286	0.108	0.024	0.004	-	-	-
24	GNBJ-24	0.269	0.049	0.034	-	-	-	-
25	GNBJ-25	0.309	0.244	0.019	0.044	-	0.010	-
26	GNBJ-26	0.280	0.069	0.024	-	-	-	-
27	GNBJ-27	0.215	0.044			-	-	-
28	GNBJ-28	0.289	0.128	0.050	0.039	0.020	0.016	0.09
29	GNBJ-29	0.312	0.247		-	-	-	-
30	GNBJ-30	0.250	0.049		-	-	-	-

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Uyanoz and Karaca (2011) used salt supplemented YEM broth to screen salt tolerant rhizobia. In the present study we included salt at different levels (0, 1.0, 2.0, and 3.0%) to screen salt tolerant isolates. All the isolates grew well in YEM liquid broth without salt as the concentration increased the growth get decreased. Four isolates (GNBJ-4, GNBJ-8, GNBJ-9 and GNBJ-28) were found to grow at 3.0% NaCl saline 6000. The isolates GNBJ-9 was able to grow upto 1.53 at 30% salt 6000. These results were conformity with the result of Abdel - Salam *et al*. (2010).

Siderophore production

Siderophore assay medium contained K_2HPO_4 0.05%, $MgSO_4$ 0.04 %, NaCl 0.01%, Mannitol 1%, glutamine 0.1%, NH_4NO_3 0.1% (Reeves *et al*., 1983).

To 100 ml of the above media, two ml of Bradyrhizobial cultures having cell density of 10^9 CFU/ml were added and incubated at 30°C psychrotherm incubater for 72 hours. The cells were harvested at 4000 x g for 10 min. and the supernatant was collected. It was divided into three equal parts for estimation of catechol type, hydroxymate and salicylate type siderophore.

Catechol type and salicylate type siderophore

Salicylate type siderophore was measured as follows. To 20 ml of supernatant, 20 ml of ethyl acetate (pH 2) was added and extraction was done twice. Hathway reagent (1 ml of 0.1 M ferric chloride in 0.1 N HCl added to 100 ml of distilled water and to this 1 ml of 0.1 M Potassium ferric cyanide was added) was prepared (Reeves *et al*., 1983). 5 ml of the assay solution was added to 5 ml of Hathway reagent and absorbance was determined 560 nm with sodium salicylate as standard.

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