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Characterization of *Rhizobium* strains associated with *Cicer arietinum* grown in different agro climatic zones of West Bengal

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Altogether 63 isolates of Chickpea-*Rhizobium* were isolated from the fresh nodules of chickpea plants collected from 12 different localities of West Bengal. The morphological characteristics and biochemical properties of all the isolates are in agreement to the *Rhizobium* type as described in Bergey's Manual.

For this investigation, streptomycin resistant (500 µg/ml) marker isolates of the *Rhizobium* were developed following the method of Schwinghamer and Dudman (1973). The stability of the mutant isolates was checked periodically by growing them on YEMA containing 500 µg/ml streptomycin sulphate.

The morphological characteristics and biochemical properties of these natural isolates revealed the mutant isolates were comparable in all respects with the wild isolates except antibiotic resistance ability. Moreover, the mutant isolates were comparable to that of the natural isolates with respect to their ability of infecting the root hairs of host plants.

Key words : *Cicer arietinum*, *Rhizobium* sp. soils of agro climatic zones, West Bengal

INTRODUCTION

Legume seeds for their rich protein content are considered as one of the important sources of food of mankind since ancient times. Several food legume crops like chickpeas, beans, lentils, peas and others are being grown throughout the world. (Hardy and Silver, 1977).

Pulses are the major sources of proteins for all the sections of Indian population for several reasons and India enjoys the distinction of having the largest cultivated area under pulse crops in the World. In West Bengal pulses constitute one of the indispensable dietary items of the people. It has been estimated that annual requirement of pulses for the State of West Bengal is around 8 lakhs tonnes on the basis of per capita per day consumption of 30 g pulses which is considered to be far below the level of nutritionally balanced diet. The annual production of pulses in West Bengal is about 1.75 lakhs tonnes only (Anon., 1933).

Chickpea (*Cicer arietium* L.) is mostly cultivated in wide agro climatic regions of tropics and forms symbiosis with highly host specific root nodule bacteria, *Rhizobium* i.e. *Cicer*-Rhizobia (Gaur and Sen, 1979). According to them *Cicer*- Rhizobia form a bridge between the fast growing rhizobia of clover, pea and beans and the slow growing rhizobia of soybean, lupin and cowpea miscellany on the basis of cultural and physiological characteristics. In India very little work has so far been done on the *Cicer*-Rhizobia association except the reports of Gaur and Sen (1979, 1981, 1989). However, no extensive work has been done on the *Cicer*- Rhizobia association occurring in the soils of West Bengal except the works of Poi (1986) and Tallawi *et al.* (1986).

In the present investigation attempt has been made to study the morphology and physiological

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characteristics of the different isolates of rhizobia growing in association with *Cicer arietinum* in different agro climatic zones of West Bengal.

MATERIALS AND METHODS

Cicer arietinum L. (commonly known as Chickpea; Bengali vernacular *Chhola*) is an important pulse crop which is cultivated throughout West Bengal. The seeds of *C.arietinum* were collected from the Oil and Pulse Seed Research Station, Directorate of Agriculture, Government of West Bengal, Baharampore.

The following media were used during the present investigation : Yeast extract-mannitol-agar (YEM A), which consisted of K_2HPO_4, 0.5; $MgSO_4.7H_2O$,0.2; NaCl, 0.1; Yeast extract, 1 Mannitol, 10 (g/l); and Distilled water up to, 1000 ml.; Nutrient Agar which consisted of $CaSO_4.2H_2O$,1.003; $MgSO_4.7H_2O$, 0.493; K_2SO_4, 0.279 ; KH_2PO_4, 0.023 ; K_2HPO_4, 0.145; $CaCl_2$, 0.056 (g/l); Fe–EDTA, 16.67; H_3BO_3, 1.43; $MnSO_4.7H_2O$, 1.02; $ZnSO_4.7H_2O$, 0.22; $CuSO_4.5H_2O$, 0.08; $CoCl_2.6H_2O$, 0.10; $Na_2MoO_4.2H_2O$,0.05 (mg/l) and Agar, 20 (g/l) and Rose Bengal medium which consisted of Dextrose, 10.0; Yeast extract, 0.5; KH_2PO_4, 0.5; K_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; Peptone, 0.5; Agar, 25; Rose Bengal, 0.05; Streptomycin, 0.03; (gm/l), Distilled Water, 1000ml.

Harvesting of Bacterial Cells

Since a bacterial cells of *Rhizobium* secretes gummy extra cellular material, so it was not so easy to harvest the rhizobial cells. The broth was collected after the incubation period and centrifugation was done at 8,000 rpm for 30 minutes (Hitachi). Repeated (3 times) washing with sterilized distilled water was done following the same centrifugation steps. Then inoculum was prepared by adding sterilized distilled water or by adding required solution.

TAL 640, TAL 1145 and TAL 1148, the bacteria were surface plated (10^7 cells/plate) on agar containing streptomycin sulphate at 500 µg/ml. Selected resistant colonies were sub cultured and maintained on antibiotic agar. Then the cells from slants (antibiotic containing) were enriched in broth containing respective concentration of antibiotic. Then the additional selection pressure was applied i.e. gradual increase of strength of antibiotic µ/ml was done and corresponding growth pattern was studied. In case of streptomycin sulphate strength reached up to 500 µg/ml. additional selection pressure was given to eliminate the cells resistant to immediate lesser strength of the respective antibiotic. After getting the stable mutants of the respective parent strains, strains were subcultured on respective antibiotic containing Bergersen's agar medium. After subculturing all clones were grown on non-antibiotic agar. Tests for level of resistance of clones and for cross resistance were made by spotting a loopful of cells suspension 5×10^7 ml on agar containing the antibiotic at a series of concentrations and noting the concentration at which growth was not inhibited relative to growth on non antibiotic agar. Clones reisolated from surface sterilized nodules were similarly tested for the presence of resistance markers.

Isolation of Mutants

Before mutant isolation the level of natural resistance of the parent strains to the antibiotics was determined by spotting a loopful of bacterial cell suspension (about 10^7 or 10^8 cells/ml in 0.1M phosphate buffer, pH 7.0) onto Bergersen's agar containing the antibiotic at a series of concentrations, and noting the concentration at which growth was not inhibited relative to growth on non-antibiotic agar. Tests for level of resistance of mutant clones were carried out in the same way.

Cultures were grown in Bergersen's broth at 28°C on a rotary shaker (100 rpm) until the mid logarithmic phase of growth was reached. The bacteria were harvested by centrifugation and resuspended in sterile phosphate buffer to give an OD 600 nm of 0.4 (about 10^9 cells ml). A sample (0.1ml) of this suspension was then spread onto the surface of Bergersen's agar containing 2 or 4 times the concentration of antibiotic able to prevent growth of the parent strain. After incubation resistant colonies were counted and 5 to 10 of them were subcultured on to Bergersen's agar containing the same level of antibiotic before transferring to Bergersen's agar slants.

Identification of mutants showing changed ability for symbiosis

Rhizobium strains were reisolated from nodules for the purpose of identification, selected nodules were immersed in ethanol/H_2O_2 (1:1) for 2 minutes, washed several times in sterile water and crushed in drop of YEMA broth. This homogenate was then streaked onto YEMA (antibiotic containing 500 µg/ml of streptomycin and the plate showed vigorous growth on YEMA of the mutant strain.

RESULTS AND DISCUSSION

During the survey in legume cultivation fields of different districts of West Bengal, several plants of *Cicer arietinum* along with well developed nodules were collected from 12 localities and brought to laboratory immediately. From these fresh nodules isolations were made on YEMA medium and cultural and biochemical characterizations were studied. The data obtained are presented in the Tables 1-3.

The data in Table-1 revealed that altogether 63 isolates were obtained, of which 4 from Kalyani and Krishnagar, 5 were from Baharampore, Arambag, Memari, Chinsurah, Purulia, Bankura, Birbhum and Maldah, 6 from Baruipur and 9 from Midnapore only. Cells of all the isolates were found to be motile, gram negative, and rod shaped in nature. The colonies formed on YEMA type were found to be circular, non spreading, translucent to opaque, convex, smooth, entire, non-chromogenic, glistering, butyrous, form emulsion in water and odourless. Similar observations were also reported by Gaur and Sen (1981) on root nodule bacteria of chickpea of North-Western India. All these characters are also in agreement to the *Rhizobium* type as described in Bergey's Manual (Jordan and Allen, 1952).

The data in Tables 2 and 3 indicated clearly that all the isolates did not absorb congo-red, failed to grow on YEMA in 24 h, ability to form serum zone coupled with acid production in litmus milk, ability of production of hydrogen sulphide, utilizing power of citrate, product of mannitol, and reduction of nitrate. During growth in mannitol medium 62 isolates produced acid and 1 produced alkali. The data in Table 3 showed that when compared with cultural and biochemical characteristics of the isolates of *Rhizobium* of other hosts the present isolates showed similarities in several aspects. Similar reports were also published by Gaur and Sen (1981) and Kleczkowska *et al.*(1968). Isolates even of the same origin group exhibited varied responses among themselves.

The streptomycin sensitivity of chickpea-*Rhizobium* isolates were studied and compared with the sensitivity of rhizosphere soil microflora of chickpea. The test organisms on YEMA, NA or Martin's rose Bengal agar medium contaning different doses of streptomycin sulphate and the data obtained are given in the Table 4.

The data revealed that all the wild type isolates of

Table 1 : Isolates of *Cicer*-rhizobia

Locality	Soil Type	Soil pH	Host cv	Isolate No.	Total No. of Isolates
Baharampore (I)	Alluvium loamy	6.5	BG 240	I 1,2,3,4,45	5
Kalyani (II)	Alluvium loamy	6.6	BG 240	II 12,15,16,57	4
Krishnagar (III)	Alluvium loamy	6.6	BG 240	III 19,29,30,56	4
Baruipur (IV)	Deltaic alluvium	7.2	BG 256	IV 5,6,7,8,9,10	6
Arambag (V)	Alluvium loamy	5.0	BG 256	V 11,13,14,31,55	5
Memari (VI)	Alluvium loamy	6.5	BKG 60	VI 17,18,32,54,58	5
Chinsurah (VII)	Alluvium loamy	6.0	ICCV90254	VII 28,33,34,35,36	5
Purulia (VIII)	Red and Gravelly	6.3	Phule G-81-1-1	VIII 25,26,27,53,59	5
Bankura (IX)	Laterite	7.2	GL 86066	IX 24,46,47,48,49	5
Birbhum (X)	Laterite	7.0	BKG 51	X 21,22,50,51,52,	5
Maldaha (XI)	Red soil	7.0	BGM 478	XI 23,41,42,43,44	5
Midnapore (XII)	Laterite	6.0	JG 62	XII 20.37,38,39,40,60,61,62,63	9

Table 2 : Cultural and biochemical characteristics of all the isolates of *Cicer-Rhizobia*

Characteristics : Cultural and Biochemical		Isolate No. and No. of Isolates used in tests[a]												Total
		I (5)	II (4)	III (4)	IV (6)	V (5)	VI (5)	VII (5)	VIII(5)	IX (5)	X (5)	XI (5)	XII (9)	63
Size of colonies on YEMA (mm)[b]	3 mm	0	0	0	0	1	2	0	0	0	1	1	2	7
	4 mm	3	3	2	2	2	1	2	2	2	2	2	2	25
	8 mm	2	1	2	4	2	2	3	3	3	2	2	5	31
Congored Staining														
Colonies colourless		0	0	1	0	0	1	0	0	0	0	1	1	4
Colonies coloured at centre		4	4	3	6	5	4	5	5	5	5	4	8	58
Growth on YEMA in (h)	48 h	0	0	2	1	2	0	0	2	2	2	2	4	17
	72 h	3	3	2	5	3	4	4	2	2	2	2	3	35
	96 h	5	4	4	6	5	5	5	5	5	5	5	9	63
Production of hydrogen sulphide[c]		0	0	2	3	2	0	0	2	1	2	1	5	18
Utilization of citrate[c]		0	0	0	1	0	1	0	0	1	1	0	3	7
Mannitol production of[c]														
acid		5	4	4	6	5	5	5	5	5	5	5	8	62
alkali		0	0	0	0	0	0	0	0	0	0	0	1	1
Litmus milk test[d]														
Serum zone; alkali		0	1	0	1	2	0	0	1	2	1	1	3	12
No serum zone; alkali		5	3	4	5	3	5	5	4	3	4	4	6	51
Reduction of nitrate[c]		2	1	1	2	1	1	0	0	0	1	1	0	10

a Isolate numbers were from Table 1
b Data were taken on 7th days of growth
c Data were taken on 14th days of growth
d Data were taken on 40th days of growth

Table 3 : Cultural and biochemical characteristics of *Cicer-Rhizobium* isolates with other group of *Rhizobium*

Cultural and Biochemical characteristics[a]	Isolates of *Rhizobium* from							
	Medic[b]	Clover[b]	Pea[b]	Bean[b]	Lupin[b]	Cowpea[b]	Soybean[b]	Chickpea[b,c]
Serum zone with acid reaction	3+	-	-	-	-	-	-	-
Serum zone with alkaline reaction	+	3+	3+	3+	-	-	-	+
Fast growth on YEMA	4+	4+	4+	4+	-	+	-	3+
Acid production on mannitol	3+	3+	3+	3+	+	+	+	3+
Nitrate reduction	4+	3+	3+	3+	+	+	+	2+
Alkali production on mannitol	+	+	+	+	4+	3+	3+	+
No serum zone with alkaline reaction	-	+	+	+	4+	4+	4+	3+
Butyrens growth on YEMA	-	-	-	-	4+	3+	4+	4+
Non spreading growth on YEMA	-	-	-	-	4+	3+	4+	4+

a All these cultural and morphological tests were performed following the standard methods which were described in Materials and Methods sections; 4+ = < 75 - 100%; 3+ = < 50 - 75%; 2+ = < 25 - 50%; + = 0- 25%; - = nil.
b From the data of Raju (1938), Johnson and Allen (1952), Graham and Parker (1964), Norris (1965), Brockwell *et al.* (1966), Kleczkowska *et al.* (1968), Jordan and Allen (1974), Gaur and Sen (1981).
c Data of 64 isolates examined in present study.

Table 4 : Sensitivity of *Rhizobium* isolates and rhizosphere microorganisms of chickpea to different doses of streptomycin

Microorganisms	Isolates	Growth[a] on medium containing streptomycin (μg/ml)				
		100	200	300	400	500
Chickpea-*Rhizobium*	I 1	-	-	-	-	-
	I 4	-	-	-	-	-
	TAL 640	-	-	-	-	-
	TAL 1145	-	-	-	-	-
	TAL 1148	-	-	-	-	-
	I 1 Mutant	+	+	+	+	+
	I 4 (M)	+	+	+	+	+
	TAL 640 (M)	+	+	+	+	+
	TAL 1145 (M)	+	+	+	+	+
	TAL 1148 (M)	+	+	+	+	+
Rhizosphere micro organism	Bacteria	+	+	-	-	-
	fungi	-	-	-	-	-

a Isolates of *Rhizobium*, soil bacteria and soil fungi were grown on YEMA, NA and Rose Benal Agar media supplemented with different concentrations of streptomycin sulphate respectively. Observation were average of five replications.

Table 5 : Morphological and biochemical properties of wild and mutant isolates of *Rhizobium* isolated from chickpea

Properties	Isolates of *Rhizobium*	
	Wild	Mutant
Gram staining	-[a]	-
Congo-red staining	+	+
Spore formation	-	-
Presence of flagella	+	+
Presence of capsule	+	+
Catalase activity	+	+
Strach hydrolysis	-	-
Gelatin hydrolysis	-	-
Voges-Proskauer test	+	+
Nitrate reduction	++	++
Starch iodine test	+	+
Litmus milk test		
Formation of Serum zone	+	+
Time required	After 4 days	After 4 days
Reaction type	Alkaline	Alkaline
pH value (initial 7.0)	7.5	7.5
Utilization of carbohydrate		
Mannitol	G[b]-0; R[c]-A	G-0; R-A
Sorbitol	G-0; R-A	G-0; R-A
Glucose	G-0; R-N	G-0; R-N
Sucrose	G-0; R-A	G-0; R-A
Lactose	G-0; R-N	G-0; R-N
Starch	G-0; R-N	G-0; R-N

[a]Presence or absence of a property is indicated by plus (+) or minus (-) sign : '+' = slight reaction; '++' = good reation

[b]G = Gas production;

[c]R = Reaction type;

All observations are average of five replications.

Table 6 : Infectivity of different isolates of Chickpea - *Rhizobium* to roots of chickpea cv. BG 240

Root[a] treated with	Percentage of root hair[b] of chickpea infection
Control (Water)	0
Cell suspension of chickpea - *Rhizobium*	
Wild type[c]	73.6
Mutant[d]	75.0

a Chickpea seedling (3 to 4 day old) roots were inoculated by flooding with water or 10^7 cells/ ml suspension of *Rhizobium* isolate;

b Percentage of root hair infection was scored after 12 h of inoculation with respect to control;

c Wild type of *Rhizobium* isolate was obtained from nodule of chickpea;

d Streptomycin resistant mutant of chickpea isolate were developed following the method of Schwinghamer and Dudman (1973).

Rhizobium obtained from the nodules of chickpea were highly sensitive to streptomycin and completely failed to grow on media containing 100 µg/ml of streptomycin. A few colonies of rhizosphere bacteria could grow on NA with 200 µg/ml of streptomycin and completely failed to grow on media containing more than 200 µg/ml of streptomycin. On the other all the rhizosphere fungi completely failed to grow on medium containing 100 µg/ml streptomycin.

The mutant isolates of Chickpea–*Rhizobium* could grow on YEMA contaning 500 µg/ml streptomycin (Table 4).

The morphological and biochemical characteristics of wild and mutant isolates or Chickpea–*Rhizobium* was compared and the results (Table 5) clearly indicated that all the properties of the mutant isolates were comparable to that of the wild type isolates except the streptomycin resistant character.

The roots hair infectivity ability of the mutant isolate was compared with that of wild type isolate by flooding the root hairs of 3-4 day-old-seedlings of chickpea with cell suspension of both the types in petridishes. The percentage of root hair infected was determined and the data are given in Table 6. The data revealed that mutant isolate was comparable to that of wild type isolate with respect to its ability of infecting the root hairs of host plants.

It was also found that acid or alkali production on mannitol was independent of the pH of the soil from where they were isolated. The present finding supports the reports of Brockwel *et al.* (1966); Raju (1938); Johnson and Allen (1952), but did not agree with the hypothesis of Norris (1965) that tropical rhizobia produce alkali.

On the basis of some cultural and biochemical characteristics, the isolates were found to be mainly of fast growing type and a few were found to be of slow growing type (De Ley and Rassel, 1965; T'Mamnetjc, 1967; Jordon and Allen, 1974; Graham, 1963). The observation also supports the report of Gaur and Sen (1981) and it is assumed that this group might be a link between fast growing and slow growing rhizobial groups. But, however, this needs further elaborate studies on DNA hybridization and character of flagella for confirmation. The isolates exhibited variability to antibiotic tested. Similar findings were also reported on other rhizobia by several scientists

earlier (Karanja and Wood, 1988; Young *et.al.*, 1989; Mr. Kovacki, 1990.

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