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## **PHENOTYPIC AND MOLECULAR PROFILING OF INDIGENOUS CHICKPEA RHIZOBIA IN INDIA**

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### **ABSTRACT**

Development of rhizobial inoculants requires selection of isolates that are symbiotically efficient and well adapted to local edapho-climatic conditions. Our aim is to identify, characterize and improve indigenous chickpea rhizobia isolated from different agro-ecological regions of India. In this endeavor, 28 chickpea rhizobial strains were isolated and characterized in relation to two reference strains representing *Mesorhizobium ciceri*. Preliminary characterization of twenty eight chickpea rhizobial isolates done on the basis of phenotypic traits and growth kinetics revealed tremendous diversity. Molecular profiling by Randomly Amplified Polymorphic DNA (RAPD) further supported this heterogeneity. The results establish that India has a wide rhizobial diversity which can be used to develop efficient and suitable inoculants for chickpea.

**Key Words:** Chickpea, Rhizobia, Genotypic, RAPD.

### **INTRODUCTION**

Chickpea (*Cicer arietinum* L.) is the world's third most important food legume as it constitutes a valuable source of protein used both in human and livestock nutrition. Substantial research programmes are being carried out to improve its yield, disease resistance and nutritional value. More than 70% of the nitrogen economy of the crop is obtained through its symbiosis with nodulating bacteria, hence giving the chickpea rhizobia a great agricultural value. Inoculation of chickpea seeds with the selected rhizobial strains is being widely practised to ameliorate the plant yield by enhanced root nodulation and nitrogen uptake of the plant (Babic *et al.*, 2008). It has been recognized that an important requirement for the rhizobium-chickpea association is the ability of inoculant strains to compete with very diverse indigenous rhizobial strains. Therefore, in order to improve the beneficial effect of chickpea inoculation, it is important to determine the characteristics of rhizobial field populations (Sikora and Redzepovic, 2003). Recently, the assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention. Characterization of diversity among rhizobial strains is a prerequisite for exploitation of genetic resources for improvement of biological nitrogen fixation. The advent of various molecular genetic approaches has greatly expedited these investigations. The sensitivity of PCR based genotyping methods (Selenska Pobell *et al.*, 1995) has complemented the traditional microbiological and physiological methods, hence enabling the differentiation among closely related bacterial strains and the detection of higher rhizobial diversity than previously considered. India, has a large biodiversity of rhizobial resources, however, only few of them have been explored. The present investigation was therefore taken up with the following objectives:

- (i) To isolate and characterize indigenous rhizobia nodulating chickpea from soils of various chickpea growing regions of India.
- (ii) To evaluate genetic diversity among the chickpea rhizobial isolates by using both phenotypic and molecular approaches.

### **MATERIALS AND METHODS**

#### ***Isolation of Rhizobium strains***

Strains of rhizobia were obtained from soils collected from different chickpea field sites in chickpea

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growing belt of India. Rhizospheric soil samples were taken at a depth of 0-30cm. Under aseptically controlled conditions in the greenhouse, surface sterilized chickpea seeds (cv. Pusa C-235) were sown directly into soil samples collected. Plants were grown raised in growth chamber under 16/8h light/dark cycle and 28/20°C day/night temperature regimes. Nodules were excised 45 days after planting and rhizobial strains were isolated from surface sterilized nodules following standard protocol (Vincent, 1970). These presumptive rhizobial isolates were authenticated by nodulation test on their original host *C. arietinum* in monoxenic conditions. Tests were performed in triplicate. The yeast extract- mannitol (YEM) medium was routinely used for isolating, purifying and cultivating these rhizobia. All the positive strains were stored in 40% (v/v) glycerol at -80°C. The origin and designation of rhizobial isolates studied are presented in Table 1.

#### Colony morphology and growth characteristics

All the strains were grown to single colonies on YEMA medium and colony characteristics i.e. colony colour, shape and texture were studied. For bacterial growth study, bacteria were cultivated initially in flasks containing 50ml of YEM medium and incubated at 28°C on a rotary shaker (200 rev/ min). The cells, at the late growth phase, were used to inoculate new cultures to an initial optical density of 0.1 (600nm). Growth was followed by measurement of the optical density at 600nm every 2h. The generation time was calculated from growth in the logarithmic growth phase.

#### Genomic DNA extraction

Total genomic DNAs from all strains were isolated using standard phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The concentration of DNA was estimated on nanodrop at 260nm.

#### RAPD fingerprinting

The PCR procedure was followed as described by Williams *et al.*, (1990). Out of thirty two random decamer primers (Operon Technologies, United States) used, nine were from OPK series, five from OPL series, two from OPA series and four from OPM, OPT, OPU and OPV series each. The reaction volume was 25µl containing 0.5µl (10mM) dNTP mix, 2µl (30ng) primer, 2.5µl 10X Taq buffer, 1µl (25ng) genomic DNA, 0.2µl (0.5U) Taq polymerase purchased from Bangalore Genei. The amplification reaction was carried out in a thermal cycler (Perkin Elmer model 9600) programmed for first denaturation step of template DNA at 92 °C for 5 min followed by 40 cycles of amplification (92 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min). Finally the reaction mixture was kept for extension step at 72 °C for 7 min. PCR products were separated on a 1.5% agarose gel containing ethidium bromide using 0.5X TBE buffer. Gel was electrophoresed for 2h at 90V. The sizes of the amplified fragments were determined by using size standards (100bp DNA plus ladder). DNA fragments were visualized under UV light and photographed using Gel Documentation System. All field isolates were compared with two reference strains TAL620 and H68 representing *Mesorhizobium ciceri* in this analysis.

#### Data analysis

The RAPD fingerprints obtained were scored qualitatively and coded in binary form and analysed using NTSYS-pc package (Rohlf, 1998). A simple matching coefficient was calculated to construct a similarity matrix. The UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

## RESULTS AND DISCUSSION

A total of 31 rhizobial isolates were obtained from diverse chickpea rhizospheric soils (Table 1) by trap host plants, all of which were authenticated to be chickpea rhizobia by reinoculation tests. Twenty eight out of thirty three strains formed effective, nodules on *C. arietinum* after 45 days. Therefore, these thirty strains were taken up for genetic diversity analysis.

#### Phenotypic characterization

The phenotypic traits of twenty eight chickpea rhizobial isolates were characterized on the basis of their colony shape, colour and texture (Table 2). Most of the *M. ciceri* rhizobia grew with dome shaped, milky or white coloured and gummy textured colonies with smooth margins which are typical features of *M.*

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**Table 1: Provenance of chickpea rhizobial isolates used in the study**

Location	Strain designation	Climate
1. Bellary	T1, T2, T3, T4, T5	Dry, arid
2. Nandyal	N1, N2, N3, N4, N5, N6	Semi arid
3. Nagpur	K1, K2, K3, K4, K5	Semi humid
4. Hissar	MC1, MC2, MC3, MC15, MC16, R1, R2, R3	Arid
5. Delhi	C1, C2, C3, C4	Semi arid

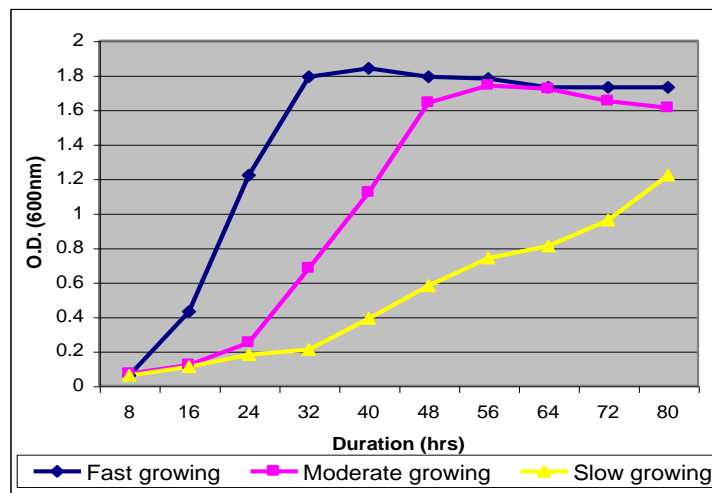
*ciceri* (Somasegaran and Hoben, 1994). The rhizobia secrete excessive exopolysaccharide which gives gummy texture to the colonies. Exceptionally two strains K2 and K3 grew with yellow coloured colonies which are a very uncommon feature in chickpea rhizobia.

**Table 2: Phenotypic characteristics of *M. ciceri* isolates**

Strain	Shape	Colour	Texture
MC3, R2, K4	Flat	Milky white	Dry
K2, K3	Flat	Yellow	Gummy
N1, N3, K1, K5, C2, N6, R2, T1-T5	Domed	Milky white	Gummy
MC1, 2, 16, C1, C3, C4, N2, N4, N5, R1, R3	Domed	Watery	Gummy

The results for growth kinetics shows that the strains varied for generation time on the basis of which they were categorized into fast ( $GT < 1.5h$ ), moderate ( $3h < GT < 5h$ ) and slow ( $6h < GT < 9h$ ) growers. The growth curve of one of the representative from each group is shown as line graph in fig 1. More than 70% of the strains had moderate growth rate. Therefore, phenotypically majority isolates had traits of *Mesorhizobium* genus (Jarvis *et al.* 1997). Moderate growers formed the major group with 21 strains in it, originating from various locations followed by fast growers, which included four isolates. Three rhizobial isolates (T2, T5 and N6) had very slow growth rate taking 8 days to reach log phase with  $10^8$  cfu/ml. Correlating this data with colony morphology, all the isolates with gummy texture had slow or moderate growing phenotype. Three out of four isolates with fast growth rate formed dry colonies. Chickpea rhizobia have been shown previously to have both fast and slow growing strains (Chakrabarti *et al.*, 1986; Nour *et al.* 1994a, 1994b).

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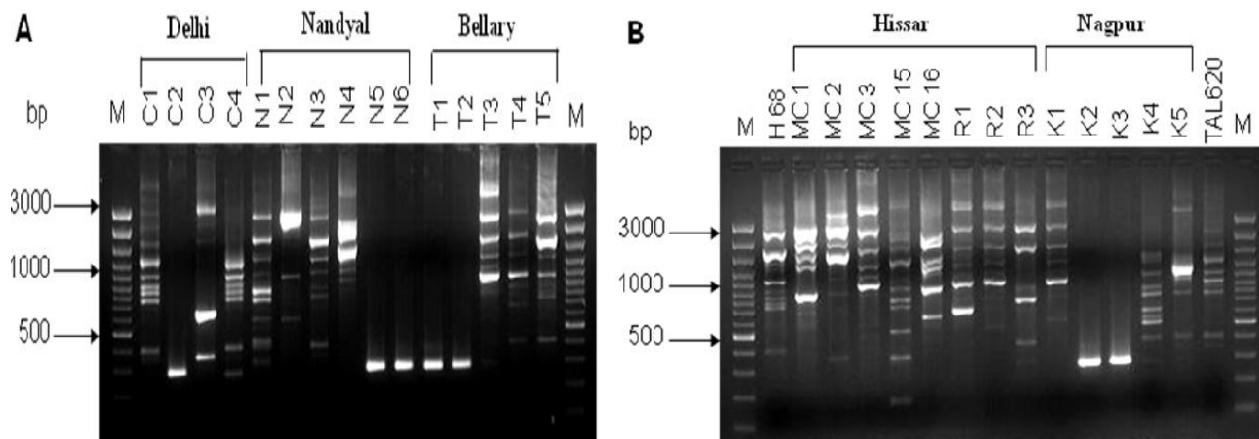


**Figure1: Growth kinetics of *M ciceri* isolates**

- i) Fast growing GT < 50min - MC3, R2, K4, K3
- ii) Moderate growing 3h < GT < 5h - Twenty one strains
- iii) Slow growing 6h < GT < 8h - T2, T5 and N6

## RAPD fingerprinting

All chickpea rhizobial isolates were genotypically characterized by RAPD fingerprinting. Total genomic DNAs were amplified with thirty two oligonucleotide primers. All primers produced multiple DNA products ranging in size from 0.1kb to 4kb, with an average of five bands per strain. The amplification pattern revealed a high level of polymorphism among all the strains. An example of RAPD fingerprint generated with primer K4 is shown in Figure 2 (A, B).

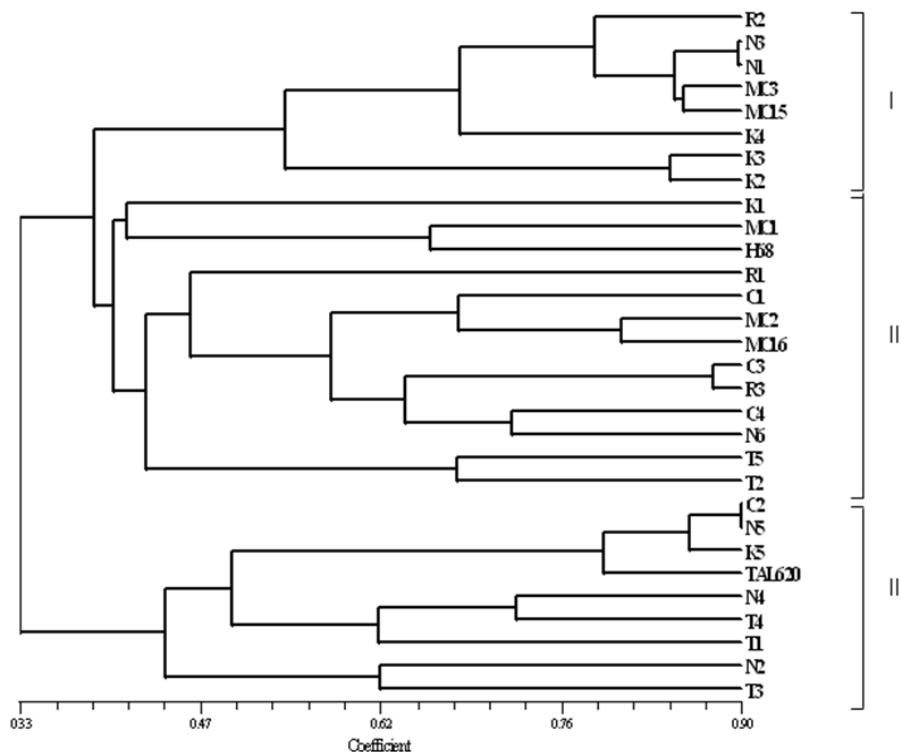


**Figure 2: Representative RAPD profile (A, B) of chickpea rhizobial isolates from each location and *M ciceri* reference strain; M: 100bp DNA plus ladder.**

These RAPD profiles were used for cluster analysis to present genetic relationships in the form of dendrogram. The dendrogram (Fig 3) shows that all isolates could be divided into two major clusters. Within the first major cluster, two very distinct subgroups of strains could be distinguished. Eight isolates

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that formed the first subgroup diverged from the other subgroup represented by H68 at a similarity level of 0.53. The second major cluster comprised the reference strain TAL 620 and eight field isolates that were related at a similarity level of 0.45.



**Figure 3: Dendrogram of chickpea rhizobial isolates from RAPD fingerprints using 32 different primers.**

The dendrogram grouped the slow growing isolates T2, T5 and N6 and most of moderate growers, gummy textured strains together with reference strain H68 in Cluster II and with other reference strain TAL620 in Cluster III. However, the grouping was partially congruent with the phenotypic characters studied as few of moderate growers were clustered along with three fast growers in Cluster I. The clustering of isolates did not correlate with their geographical origin since isolates from same origin were included in different clusters and isolates from diverse origins were found in the same group. The latter was strikingly exemplified by two of our isolates, C2 and N5 which had diverse origins (Delhi and Nandyal respectively) but clustered together in the dendrogram. Both phenotypic and genotypic approaches proved to be similarly sensitive in demonstrating the large diversity amongst these bacteria. In congruence with previous reports that showed chickpea rhizobia are more closely related to *Mesorhizobium* species (Nour *et al.*, 1995; Jarvis *et al.*, 1998), majority of our isolates clustered with *M. ciceri* reference strains in the RAPD profiles. The large number of different genotypes obtained from single species *M. ciceri* suggests that Indian soil holds a vast pool of genetic material which can be effectively used to yield superior inoculant strains.

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