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Phylogenetic studies among different groundnut (*Arachis hypogaea* L.) root nodule bacteria (*Rhizobium)* isolated from Junagadh and Rajkot districts of Gujarat

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is one of the most important oilseed crops cultivated in different areas of Junagadh and Rajkot districts. The diversity of root nodule bacteria that can nodulate groundnut is poorly understood. Twenty one samples along with reference strains, TAL-1000, IGR-6 and NC-92 were analyzed using restriction patterns produced by amplified DNA coding for 16 s rDNA with two enzymes (*Hinf* I and *Alu* I) and were placed in four genotypes. Genetic diversity was also assessed by repetitive PCR using BOX primers and in all isolates were placed in five genotypes. Nitrogen-fixing ability of the isolates was confirmed by amplification of 781 bp *nif*H fragment. A considerable level of genetic diversity was determined among Rhizobial strains isolated from different areas of Junagadh and Rajkot districts.

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the most important oilseed crops, commercially popular due to its superior edible oil quality, protein in the meal and confectionery purposes. Groundnut has a wide range of adaptability in varying agro-climatic conditions and soils, which has made its cultivation possible in most of the tropical and sub-tropical countries in the world. The total area under groundnut cultivation in India during the year 2006-07 was 6.7 million hectares and total production was 6.6 million tons with the productivity of 985 Kg ha^{-1} (FAO Database, 2007). Among the major groundnut growing states of India, Gujarat ranks first, in groundnut production and second in area under production. In the districts of Junagadh and Rajkot of Gujarat state, groundnut is the major oilseed crop.

Besides its nutritional and agricultural importance, groundnut (as a legume) plays an important role in maintaining and improving soil fertility through the process of biological nitrogen fixation (BNF). Groundnut has been reported to form effective nodules with slow-growing rhizobia. Currently, Taurian *et al.* (2006) demonstrated that *Arachis hypogaea* L. is nodulated by *Bradyrhizobium* species and also by fast growing rhizobia closely related to *Rhizobium giardini* and *Rhizobium tropici* species.

Nodulation of groundnut by indigenous bacteria is usually assumed to be adequate, and inoculation is seldom practiced. However, survival and effective functioning of Rhizobium populations are reduced by high soil temperatures, salt and osmotic stress, soil acidity and alkalinity, pesticide and fungicide applications as well as nutrients deficiencies stress (Zahran, 1999).

The different areas of Junagadh and Rajkot districts contain *Rhizobium* strains, well adapted to varying soil and environmental conditions. The *Rhizobium* strain isolated from these areas will be very effective for evaluating as a groundnut inoculants in different areas of Saurashtra, because the inoculated *Rhizobium* should survive or persist in critical number for a long time in the rhizosphere of the groundnut to out-compete and/or complement the native microflora. To achieve the maximum biological nitrogen fixation in these areas,

there is need to identify and characterize root nodule bacteria before they are made commercially available for field application. All root nodule bacteria (RNB) isolates along with reference strain were studied genotypically using different molecular markers like PCR- RFLP, *Nif* H gene amplification and BOX PCR.

MATERIALS AND METHODS

Bacterial strains:

Rhizobia were isolated in the laboratory using groundnut as trapping host. A total of 21 RNB isolates were obtained from the nodules of groundnut from various regions of Junagadh and Rajkot district as described by Vincent, 1970. All strains were grown and maintained on yeast extract-mannitol (YM) with or without agar (Vincent, 1970). The reference strains NC-92, TAL-1000, IGR-6 were collected from Department of Microbiology, Directorate of Groundnut Research, Junagadh.

DNA isolation:

The genomic DNA was obtained from overnight grown culture at 28^{0}C in Yeast extract mannitol broth (YEMB). Cells were pelleted by centrifugation at 10,000 rpm for 15 min. After that pellet was washed with TE buffer (10T/1E). Then it was dissolved in 400 µl TE. 40 µl of 10 per cent SDS and 5 µl proteinase K (20 mg/ml) was added. It was incubated at 56^{0}C for 45 minutes. 400 µl of Tris saturated phenol (pH 8.0) was added and then centrifuged. 200 µl of Tris saturated phenol and 200 µl of chloroform: isoamyl alcohol (24:1) was added in supernatant then it was centrifuged. Again 400 µl of chloroform: isoamyl alcohol (24:1) was added in supernatant and then it was centrifuged. 0.1 volume of 3M sodium acetate and 2 volume of chilled absolute ethanol were added to the supernatant. Then it was incubated at -20^0 C for 2 hrs. The pellet was collected after centrifugation and it was washed with 70 per cent ethanol twice. Air dried the pellet at room temperature and suspended the pellet in 100 µl of TE. The DNA samples were treated with 2 µl of RNase (18.9 mg/ml) for 2 hour at 37^{0}C. The enzyme was deactivated at 62^{0}C for 10 minutes.

PCR- RFLP of 16S rDNA:

The 1500 bp 16S rDNA was amplified using primers 16S rDNA F (GCT CAA GAT TGA ACG CTG GCG) and 16S rDNA R (CGG TTA CCT TGT TAC GAC TTC ACC). Amplification of 16S rDNA was conducted in 50 µl reaction volume using 50 ng DNA templates, 5 µl of 10X PCR buffer, 3 µl of 0.25 mM dNTP, 2 µl of 16S rDNA F and 2 µl of 16S rDNA R primers with 25 pmol, Taq DNA polymerase 2 U and makeup upto 50 µl with sterile distilled wáter. The PCR conditions for amplification were, Initial denaturation at 94^{0}C for 7 min followed by 35 cycles of denaturation at 94^{0}C for 40s, annealing at 55^{0}C for 1 min and extension at 72^{0}C for 2 min. Final extensions at 72^{0}C for 5 min. PCR products were digested with restriction endonuclease *Hinf* I and *Alu* I.

BOX PCR:

The genomic DNA fingerprinting was carried out by using random BOX AIR primer (GAT CGG CAA GGC GAC GCT GAC G). Amplification reactions were performed in 25 µl volume, containing 50 ng template DNA, 5 µl of 10X PCR buffer, 3 µl of 100 µM dNTP, 25 pmol BOX AIR primer, 1 U Taq DNA polymerase and make it up to 25 µl of sterile destille water. DNA amplification was performed with the following thermal profile: an initial denaturation at 94°C for 7 minutes followed by 35 cycles of denaturation at 94^{0}C for 1 minute, annealing at 53^{0}C for 1 minute, extension at 65^{0}C for 8 minutes and final extension at 65^{0}C for 16 minutes.

Nif H gene amplification:

Nif H gene encodes the identical subunits of component II of the enzyme nitrogenase. The *nif* H gene amplification was carried out by using primer *Nif* H1 (CGT TTT ACG GCA AGG GCG GTA TGC GCA) and *Nif* H2 (TCC TCC AGC TCC TCC ATG GTG ATC GG). The *nif* H primers used in this study were designed by aligning the *nif* H sequence of *B. japonicum* 110, *R. phaseoli* CFN 42, *R. trifolii* 329 and *S. melitoto* 41. All the RNB isolates and reference strains were grown on YEMA plates at 28^{0}C for 24 hours. A loopful colony was picked by sterile wire loop in aseptic conditions and suspended in 100µl of sterile DD water. Then it was incubated for 5 minutes in boiling water. This suspension was directly used as template DNA in PCR reaction. PCR reactions were performed in 50 µl volume, containing colony sample 3 µl, 10X PCR buffer 5 µl, 3 µl of 100 µM dNTP, *Nif* H 1 and *Nif* H 2 primers 6.25 pmol, 1 U Taq DNA Polymerase and make it up to 50 µl with sterile distilled water. DNA amplification was performed with the following thermal profile: an initial denaturation 94°C for 7 min followed by 30 cycles denaturation at 93^{0}C for 45 s, annealing at 65^{0}C for 30 s and extension at 72^{0}C for 30 s. A final extension was at 72^{0}C for 7 min.

Statistical analysis:

Data analyses were performed using the NTSYS-Pc (Numerical Taxonomy System, version 2.02). Presence of bands was scored as "1", absence of band as "0". The SIMQUAL program was used to calculate the Jaccard's coefficient. Dendrogram was constructed using unweighted pair group method for arithmetic mean (UPGMA) based on Jaccard's coefficient.

RESULTS AND DISCUSSION

All the 21 RNB isolates and three reference strains produced a band of 1500 bp in PCR of 16S rDNA (Fig. 1). The

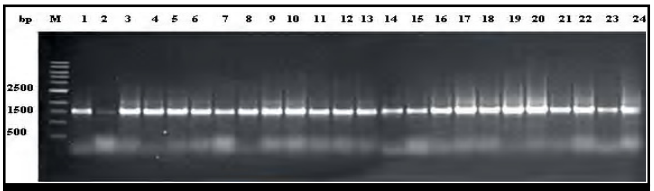

Fig. 1 : PCR amplified 16S rDNA region of RNB isolates and referencestrains (M- 500 bp ladder)

Fig. 5 : BOX-PCR amplification of RNB isolates and referencestrains (M-500 bp ladder)

PCR products were digested with endonuclease namely, *Alu* I *and Hinf* I. Thus, the size of PCR products estimated by summing the size of restricted fragments ranged from 1,200 to 1,500 bp (Fig. 2 and 3). It was smaller than or equal to the size of undigested PCR products depending on the endonuclease employed and the strain used. According to UPGMA dendogram, all RNB isolates and reference strains were divided into four groups (Fig. 6). Group I included nine RNB isolates like JND-1, JND-4, JND-5, JND-8, JND-14, RJ-3, RJ-4, RJ-7 and one reference sample NC-92. There was 81 per cent similarity between group I and group II. Group II included eight RNB isolates viz, JND-2, JND-3, JND-6, JND-7, JND-9, JND-12, JND-13 and one reference sample IGR-6. Group III contained four RNB isolates, namely RJ-1, RJ-2, RJ-5, RJ-6 and one reference sample TAL-100. Group IV included two RNB isolates JND-10 and JND-11. They both showed 27 per cent similarity with all other RNB isolates and reference strains The pattern of molecular genetic diversity for PCR-RFLP of 16s rDNA revealed that RNB isolates from different Talukas of Junagadh district were classified in group II. Group III included RNB isolates collected from various talukas of Rajkot district. Group I included mixture of isolates collected from both districts. This findings revealed that there was presence of geographical diversity among RNB isolates for PCR-RFLP of 16s rDNA. Similar findings were also reported by Wang *et al.* (1999). They observed nineteen different RFLP ribotypes of 16S rDNA gene among *Amorpha fructicosa* isolates and reference strains of *Rhizobium.* However, in the present study, 28 different RFLP ribotypes were observed for RNB isolates and reference strains of *Rhizobium.*

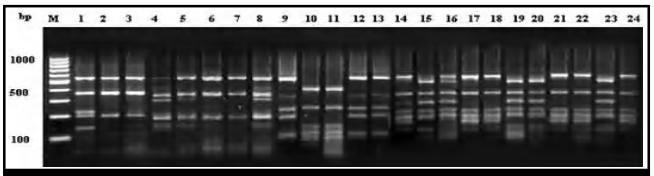

Fig. 2 : 16S rDNA PCR-RFLP of RNB isolates and referencestrains using restriction enzyme *Alu I* (M-100 bp ladder)

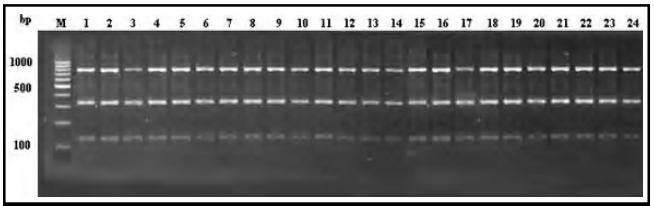

Fig. 3 : 16S rDNA PCR-RFLP of RNB isolates and referencestrains using restriction enzyme *Hinf* I (M-100 bp ladder)

The dendrogram constructed based on the data of BOX PCR, which clustered all the RNB isolates and reference strains into five main clusters (Fig. 6). The first group comprised of six isolates *viz.,* JND-1, JND-2, JND-11, JND-13, RJ-4 and RJ-7 and two reference strains (IGR-6 and TAL-1000). RJ-4 showed 81 per cent similarity with other Rhizobia of same group. Group II contained four RNB isolates JND-6, JND-8, JND-10, RJ-60

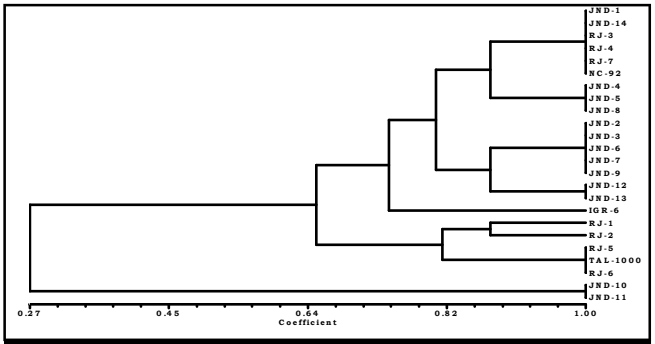

Fig. 4 : UPGMA dendrogram generated from 16S rDNA PCR-RFLP analysis of RNB isolates and referencestrains

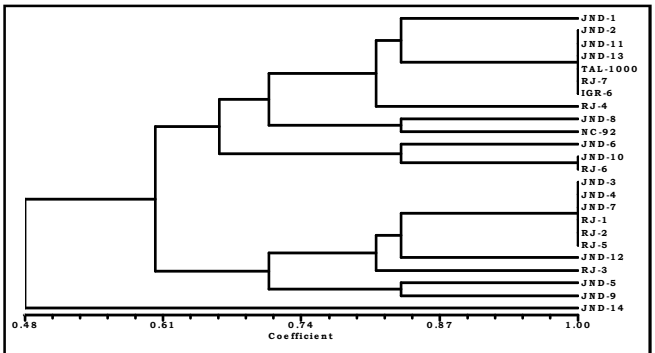

Fig. 6 : UPGMA dendrogram generated from the BOX PCR analysis of RNB isolates and referencestrains

and one reference strain NC-92. RJ-6 and JND-10 as well as JND-8 and NC-92 of this group showed 100 per cent similarity. JND-6 showed 83 per cent similarity with rest of the four isolates of same group. Group III included eight RNB isolates namely, JND-3, JND-4, JND-7, JND-12, RJ-1, RJ-2, RJ-3 and RJ-5. RJ-3 showed 84 per cent similarity with other isolates of same group. Group IV included two RNB isolates (JND-5 and JND-9). There was 71 per cent similarity between group III and group IV. Group V included only one RNB isolates (JND-14). JND-14 had 48 per cent similarity with all other RNB isolates.

In the present investigation, BOX PCR technique was unable to group the RNB isolates according to their host specificity or the geographical region, as pattern observed in PCR-RFLP of 16s rDNA (Fig. 4 and 5). Similar results were reported by Prakash and Annapurna (2006) for *Bradyrhizobium* isolates of soyabean. They also reported that there was no correlation between the group formed by phenotypic and molecular methods.

In *Nif* H gene amplification only one unique band was generated in all RNB isolates and reference strains with approximately 781 bp (Fig. 7). This finding confirmed nitrogenase activity of all RNB isolates and reference strains. Similar findings were reported by Pandey *et al.* (2004). They reported one unique band of 781 bp of *Nif H* gene in Rhizobia isolated from five medicinal legumes *viz.*, *Trigonella foenumgraecum, Abrus precatorius, Mucuna pruriens, Melilotus officinalis and Vicia angustifolia.*

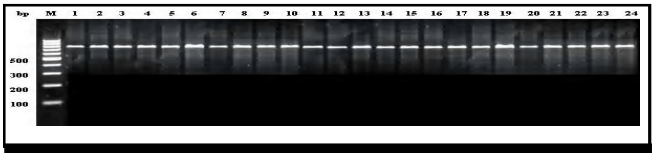

Fig. 7 : ***Nif*** **H gene amplification of RNB isolates and referencestrains (M-100 bp ladder)**

So, it is concluded that all RNB isolates showed variability in PCR-RFLP. All of the RNB isolates having nitrogenase activity. BOX profiles represent assessment of genetic variability in the entire genome using rep primer which has a base length of 22.

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