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Research article

Molecular characterization of rhizobium of Cajanus cajan plant from salt and herbicidal action

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ABSTRACT

The genetic diversity among the in vitro and in vivo Rhizobium species assessed by RAPD-DNA finger printing and PCR was done. Variations between the Rhizobium species of cajanus cajan plant; normal plant grown in normal conditions (N), normal plant in presence of 4% ai glyphosate (Ng), normal plant in presence of 300mM NaCl i.e Hallophillic (H) and normal plant in presence of 300mM NaCl and 6% ai glyphosate ie Hallophillic glyphosate (Hg) was concluded. Salinity-induced changes in the DNA profiles in Rhizobium sp. it exhibited alterations in the expression of Hg, which either showed an enhanced rate of synthesis as compared with their respective controls. RAPD tests categorized the isolates into two clusters. The RAPD results were further analyzed by MVSP software; similarity matrix was measured and converted into dendrogram using UPGMA clustering method.

Keywords: Cajanus Cajan, DNA, RAPD-PCR, Rhizobium, Nodules and stress.

INTRODUCTION

Excess amount of salt in the soil adversely affects plant growth and development, ultimately causing diminished economic yield and also quality of produce. Nearly 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity. In India saline and alkaline soil covers more than 9 million ha, and is rapidly expanding. Soil salinity adversely affects plant growth and development of crop plants. This is due to the cumulative effect over decades of adding water with some dissolved salts to the soil, along with excessive use of weedicides which affects the plant and soil micro flora, leading to the poor growth of crop and survival of these microorganisms in the soil [1].

Cajanus cajan (pigeon pea) leguminous plant is an important crop, offering high quality protein and increasing the input of combined N2 into the soil. However salt sensitivity can adversely affect yield in this crop and lead to marked changes in the growth pattern of plants. Although the effect of stress may have a detrimental effect on soil microbial population, and the introduction of plants capable of surviving under these conditions is worth investigating. There is currently a need to develop highly salt tolerant crops. Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes.

Various phenotypic and genotypic methodologies are being used to identify and characterize bacteria. Although phenotypic methods play a significant role in identification but the molecular methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Major molecular techniques include PCR (Polymerase chain reaction), RAPD (randomly amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeats) and 16S- rRNA gene sequencing. RAPD is the most reliable, rapid and practical method used for phylogenetic relationships among and within closely related species. This paper reports the isolation of in vitro and in vivo Rhizobium species grown in salt and

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herbicidal conditions and their characterization on the basis of morphological, biochemical and molecular characters [2].

MATERIAL AND METHOD

Collection of samples -The Rhizobium was isolated aseptically from the root nodules of Cajanus cajan (L.) Millsp. Plant of AKPH 2022 variety. The strains were identified and isolated according to Shende & Patil, 2005.

Rhizobuim inoculums: Fresh nodules were collected from Cajanus cajan plants. N plant grown in normal condition, NG grown in presence of 4% glyphosate, H grown in 300mM NaCl, HG grown in 300mM NaCl and 6% glyphosate. Four different types of nodules were collected, crushed and inoculated in YM medium aseptically.

DNA extraction: DNA was extracted from Rhizobium isolates following the method of Chen & Kuo (1993). Cultures of Rhizobium isolates were streaked on TY plates and incubated at 30°C. From these plates single colonies were incubated into test tubes containing TY broth respectively and grown in Shaker (Excella E 24, New Brunswick Scientific USA) at 80 rpm overnight. DNA was extracted as follows: TY Broth culture (1.5 ml) of Rhizobium isolates was centrifuged at 12,000 rpm for 10 min at 4ºC. The cell pellets were re- suspended and lysed in 200µl lysis buffer (40mM Tris-acetate pH 7.8; 20mM Sodium- acetate; 1mM EDTA and 1 % SDS). After vigorous pipetting 66µl of 5 M NaCl was added to remove cell debris and proteins. The viscous mixture was then centrifuged at 12,000 rpm for 10 min at 40oC. Supernatant was transferred into a new tube, an equal volume of chloroform was added and the tube was gently inverted at least 50 times until a milky solution was formed. Following centrifugation at 12,000 rpm for 3 min, the extracted supernatant was transferred into another microfuge tube and DNA was precipitated with 100 % ethanol, dried and re-dissolved in 50µl of pure water. The concentration and purity of DNA was estimated spectrophotometrically at 260-280 nm [3].

RAPD-PCR analysis of genomic DNA: Genetic diversity and polymorphism among strains was analyzed by RAPD-PCR technique adapted by Teaumroong & Boonkerd (1998) (8). For RAPD-PCR 20 commercially available decamer primers (OPA-1 to OPA-20) from Operon technologies (Alameda, CA, USA) were used in the present study for RAPD-PCR amplification. The PCR reaction mixture (25µl) contained 1µl of 50µg genomic DNA, 0.2mM dNTPs, 2.5mM MgCl2, 1.5 U of Taq DNA polymerase, 1µM primer and rest of the volume was adjusted with autoclaved pure water. The amplification was performed in programmable thermocycler (Biometra, Germany). The amplification reaction composed of 35 cycles with each cycle having the following steps [4]. Initial step of denaturation at 94oC for 30 sec, annealing at 35oC for 30 sec and elongation at 72oC for 2 min. An additional cycle for extension was conducted at 72oC for 10 min. Amplified PCR products were separated by gel electrophoresis on 1.5% (w/v) agarose gel and visualized under UV trans-illuminator lamp (CUV 30A, Diamate Bio Technology UK) after staining with ethedium bromide (0.01g/ml). A known DNA size marker was run with every gel (1Kb DNA ladder from Bangalore Genei, India) [5].

PCR analysis of genomic DNA: The genomic DNA of Rhizobium isolates were amplified following the method of Weisburg (1991) (9). The polymerase chain reaction (PCR) was performed by using two primers rd1 (AAGGAGGTGATCCAGCC) and fd1 (AGAGTTTGATCCTGGCTCAG). Each 25 µl reaction volume contained 1 µl of 50 µg of genomic DNA, 0.2 mM dNTP mix, 1.5 mM MgCl2, 5 µl of 10 X Taq buffer, 1 U Taq DNA polymerase, 10 pmoles of each primer and rest of the volume was adjusted by autoclaved pure water. The reactions were carried out in thermocycler (Biometra, Germany). After denaturation at 95°C for 2 min, samples were cycled for 30 cycles through the following temperature profile: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2 min plus one additional cycle for chain elongation at 72°C for 10 min. Amplified PCR products were separated by gel electrophoresis on 1.2% (w/v) agarose gel and visualized under UV trans-illuminator lamp after staining with ethedium bromide (0.01g / ml). 1 Kb DNA ladder was used as molecular marker [6].

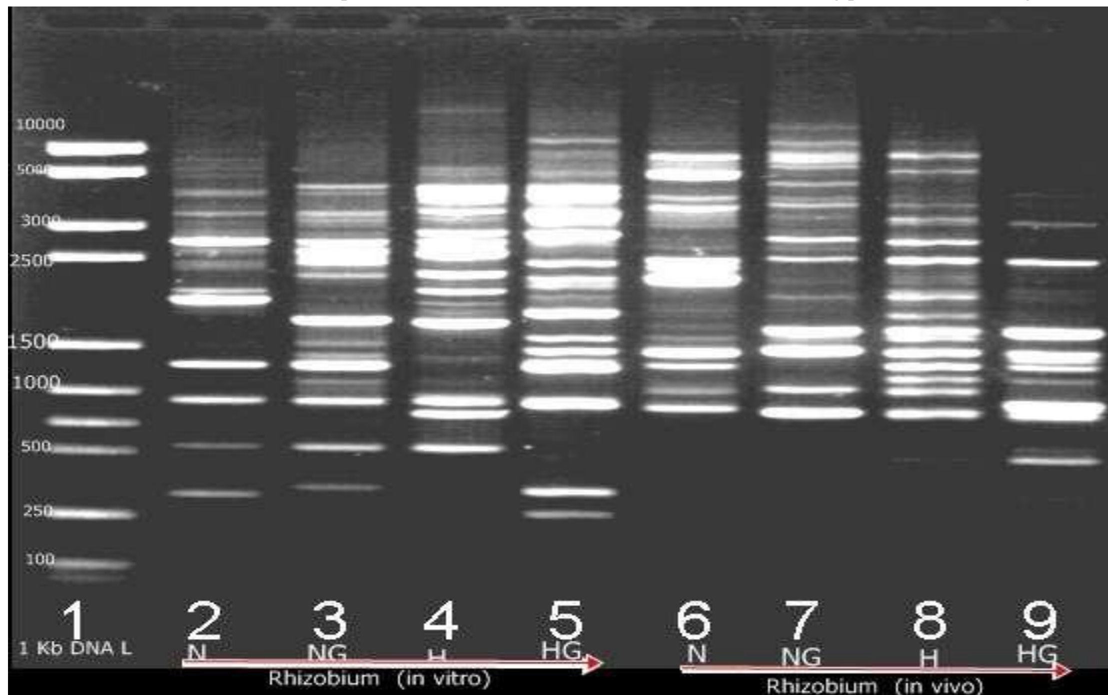
Statistical analysis of DATA: The colony forming unit (CFU/g soil) data of Rhizobium isolates were analyzed statistically by factorial analysis of variance and completely randomized design test (CRD) with least significant difference (LSD) using MSTAT- C version 4.00. In case of RAPD-PCR the presence of band was scored as 1 and absence as 0. The bands with same mobility were treated as identical bands. The RAPD-PCR data were analyzed by software package MVSP (Multivariate Statistical Package) version 3.1. The similarity matrix was measured by Gower General similarity coefficient. The similarity matrix values were converted into Dendrogram using UPGMA (unweight pair group method with the arithmetic average) clustering method [7].

RESULT AND DISCUSSION

The symbiont Rhizobium N, Ng, H & Hg strains were isolated from the healthy root nodules of Cajanus cajan plant. The identification of the bacteria was done according to Manual of Microbiological methods following Bergey's Manual. The plants of different strains (N) was grown in normal conditions, (NG) in normal conditions containing 4% glyphosate,(H) in halophillic conditions with 300mM NaCl and (HG) with 300mM NaCl 6% containing glyphosate. The Cajanus cajan plants were grown in above aseptical conditions and the nodulation was rechecked.

The genetic diversity among in vitro and in vivo isolates of Rhizobium was evaluated by RAPD-PCR analysis using OPA-20 primer. Genetic polymorphism of Rhizobium strains had been studied by RAPD finger printing technique

Figure 1: RAPD-PCR amplification of DNA isolated from Rhizobium isolates using primer OPA-20 In figure

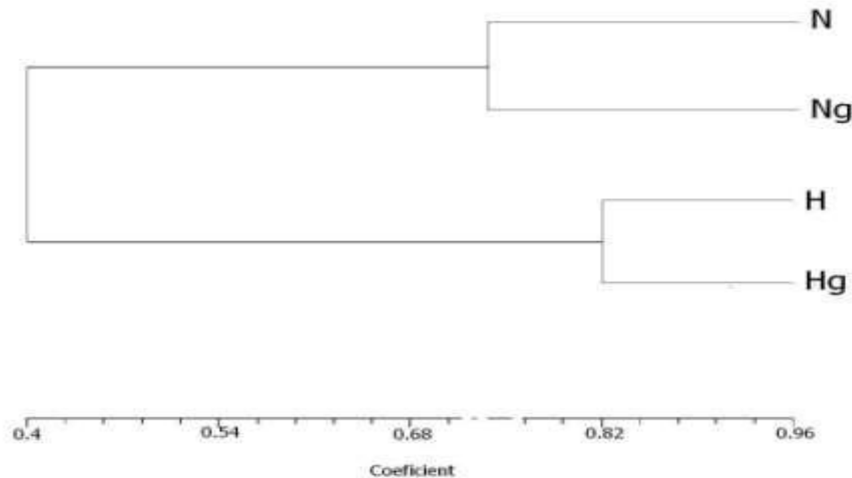


Lane 1=1 Kb DNA Ladder; Lane 2-5=DNA banding pattern of N, Ng, H & Hg Rhizobium grown in NaCl and glyphosatic condition in YM medium; Lane6-9=DNA banding pattern of isolates formed from nodules of Cajanus cajan plants grown in presence of NaCl and glyphosate. (N strain isolated from nodules of normal plants, Ng strain isolated from nodules of plants grown in presence of glyphosate 4% ai., H strain isolated from nodules grown in 300 mM NaCl & Hg strain isolated from nodules grown in presence of 6% ai glyphosate and 300 mM NaCl of Cajanus cajan plant)

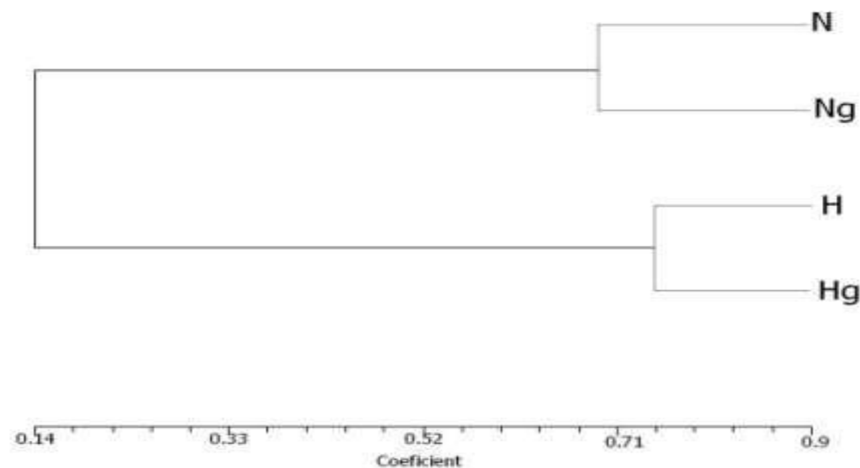
Table 1: DNA number and size in Rhizobium isolates on the basis of RAPD-PCR analysis using random primer OPA-20.

Size of bands (bp)	(In Vitro)				(In Vivo)			
	N(2)	Ng (3)	H (4)	Hg (5)	N (6)	Ng (7)	H (8)	Hg (9)
7200	-	-	-	-	+	+	+	-
5000	-	-	-	-	+	+	+	-
4250	-	-	+	+	+	-	-	-
3750	-	-	+	+	+	+	-	-
3000	+	+	+	+	-	-	+	+
2750	+	+	+	+	-	+	+	+
2500	-	+	+	+	-	+	+	-
2000	+	+	+	+	-	-	+	+
1750	-	+	+	+	-	-	+	+
1500	-	-	+	+	+	+	+	+
1250	+	+	-	+	+	-	+	+
1000	-	-	-	-	+	+	+	+
900	+	+	+	+	+	+	-	-
750	-	-	+	-	+	+	+	+
500	+	+	+	-	-	-	+	+
300	+	+	-	+	-	-	+	-
250	-	-	-	+	-	-	-	-
DNA bands	7	9	11	12	9	9	13	9 79 total

Graph-1 UPGMA Dendrogram showing the similarities values among four *Rhizobium* isolates of N, Ng, H & Hg (in Vitro) on the basis of RAPD-DNA finger printing



Graph-2 UPGMA Dendrogram showing the similarities values among four *Rhizobium* isolates of N, Ng, H & Hg (in Vivo) on the basis of RAPD-DNA finger printing.

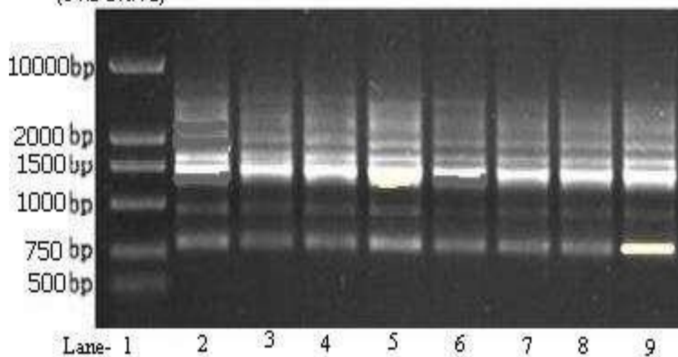


PCR-RAPD is a useful tool to conduct persistence and competitiveness studies in rhizobial strains when grown and inoculated in saline and herbicidal conditions. Different DNA banding patterns, depending on the number and size of amplified products were observed for Rhizobium isolates (Table 1; Fig. 2). Total 79 bands were observed in Rhizobium isolates 39 bands In vitro and 40 In vivo, their size ranged from 250-7200 bp. Out of 79 bands,
67 (84.8 %) were polymorphic and were scored for analysis by Multivariate Statistical Package (version 3.1) software program, using Gower general similarity coefficient. Two Dendrogram of In vitro and In vivo were formed (Graph 1&2). In Vitro the similarity coefficient was 0.82 in H and Hg and 0.73 in between N and Ng. The similarity coefficient computed between strains H and HG and N and Ng resembled as both of these isolates were isolated from YM medium grown in glyphosate and NaCl condition respectively. In vivo H and Hg showed 0.748 and N and Ng 0.682 and the cluster was formed at 0.14 for N and H strains. There was no 100 % similarity between any two strains, though some resemblance could be seen in N and NG strains grown in Nacl and H and Hg strains grown in glyphosatic conditions. The strains taken from same species of cajanus cajan plant were grown in different conditions of salt and weedicide though showed less resemblance, it might be attributed to their adaptive factor in such conditions which changed their genetic adaptibility. Determined genetic relationship among six strains of rhizobia. The greatest similarity was found between the strains isolated from same origin. (14) Characterized Rhizobium strains on the basis of RAPD-DNA finger printing and evaluated that environmental stress may favor adaptation of strains with genetic difference.

The PCR results indicated the presence of band of 1500 bp in all isolates (Fig. 2). This band is used for sequencing of 16S-rRNA gene as preferred phylogenetic marker used in bacteria ecology the availability and use of PCR based amplification methods and sequencing of the PCR products has rapidly extended RNA data bases during the past few years. QTS-24 and RAPD-PCR results both in combination can help in

the identification and characterization of genetically diverse Rhizobium isolates. Although RAPD is an authenticated technique to study genetic diversity of rhizobial strains but nevertheless, investigations need to be made using AFLP (amplified fragment length polymorphism) and SSR (single sequence repeat) and 16S-rRNA gene sequencing for further characterization of the microbes at species level. More physiological characterization of the microbes is imperative for their implication as bioinoculant in agriculture. It is inferred from the present finding that adaptive factors like soil pH, salt concentration and soil moisture induces biochemical and genetic differences in the Rhizobium and thus a potent and adaptive strain is recommended to overcome such stress.

Figure 2: PCR amplification of DNA isolated from Rhizobium isolates



Using primers: fd1 and rd1. In figure, Lane 1 = 1 Kb DNA Ladder; Lane 2-5 = band of 1500 bp of N, Ng, H & Hg (In vitro) isolates of Rhizobium grown in NaCl and glyphosatic condition in YM medium ; Lane 6-9 = band of 1500bp & 750bp of isolates from nodules of Cajanus cajan plants grown in presence of NaCl and glyphosate (In Vivo). (N strain isolated from nodules of normal plants, Ng strain isolated from nodules of plants grown in presence of glyphosate 4% ai., H strain isolated from nodules grown in 300 mM NaCl & Hg strain isolated from nodules grown in presence of 6% ai glyphosate and 300 mM NaCl of Cajanus cajan plant)

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