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PCR Studies on Genetic Diversity of Rhizobial Strains

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ABSTRACT

Genetic diversity in rhizobial strains was studied by using Random Amplified Polymorphic DNA (RAPD) markers. The strains isolated, using dilution plate method, from nodules of lentil plant grown at different sites of Punjab, Pakistan were used in the study. Slow and fast growing colonies of rhizobial isolates were selected, isolated and purified by streaking. About 10 mL of broth culture of each isolate was used for DNA extraction. Polymerase chain reaction (PCR) was performed using decamer oligonucleotide primers to study DNA polymorphism among strains. Cluster analysis divided the strains into two distinct groups A and B. The data showed that only the strains L-5, L-10, L-22 and S-26 were able to solublize soil phosphates. It is concluded that considerable genetic diversity in rhizobial strains exists with respect to their efficiency for solublizing soil phosphate and the strains can be identified using RAPD.

Key Words: PCR; Genetic diversity; Rhizobial strains

INTRODUCTION

Biological nitrogen fixation (BNF) is a cheap and environment friendly alternative source of nitrogen fertilizer in which atmospheric nitrogen (N_2) is converted into biologically active source of nitrogen and utilized by plants (Zahran, 1999). Biological nitrogen fixation takes place by the symbiotic relationship of nitrogen fixing rhizobia in leguminous plants. The benefits of BNF have led to various studies in which the genetic diversity and identity of the associated bacterial symbionts is investigated.

Rhizobia are soil bacteria that fix N_2 after becoming established inside root nodules of legumes *(Fabaceae*). Rhizobia are well known for their contribution to the soil nitrogen pool through N_2 symbiosis with leguminous plants. In those areas of soil where legumes are cultivated, indigenous populations of rhizobia are abundant. Studies of these populations have been carried out by ecological and agricultural considerations. In terms of agriculture, high abundance of rhizobia in the soil facilitates cultivation of legumes. However, soils containing indigenous rhizobia are also problematic as they create a barrier to the establishment of introduced efficient inoculants in nodules of target host plants. There is evidence of widespread sub-optimal efficiency of native strains with legumes. But it is generally argued that indigenous populations are highly adapted to their local soil environments and may form more effective symbioses than commercial inoculants isolated from a distant and un-related soil environment (Gandee *et al.*, 1999). Thus, selection of indigenous strains with high nitrogen-fixing capacity, adapted to a range of environmental conditions at a specific site, is an important strategy to maximize legume production.

Molecular techniques based on the polymerase chain reaction (PCR) are very convenient for characterization, because they are rapid, simple and discriminative. PCR has been found useful for rhizobial strain differentiation using GC-rich oligonucleotide primers. It has been possible to differentiate strains belonging to different colony morphology variants of the strain USDA110, which differ in symbiotic nitrogen fixation (Paffetti *et al.*, 1996). Random amplified polymorphic DNA (RAPD) was used in the present study to assess the genetic diversity of rhizobial strains from various districts of Punjab, Pakistan.

MATERIALS AND METHODS

The study was conducted in the Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad, Pakistan. Rhizobial cultures isolated from root nodules of lentil (*Lens culinaris* Medic) collected from various locations of Punjab i.e., Faisalabad (F1, F38), Gujranwala (G4, G9), Layyah (L3, L5, L10, L22 L29 & L45) and Sailkot (S7, S26) were used in the study.

Isolation of rhizobial strains. Isolation of rhizobial strains was carried out from the root nodules of lentil. The nodule

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surface was sterililized with 0.2% $HgCl_2$ for 2-3 min and crushed in sterilized distilled water in a test tube with the help of a glass rod. The nodule extract was streaked on yeast extract mannitol (YEM) agar (Vincent, 1970) supplemented with Congo red as an indicator (Nelson & Child, 1981) and allowed to grow at 29±1°C in an incubator. Single rhizobial colonies that appeared on YEM agar plates within 36 to 48 h of incubation were picked and sub-cultured repeatedly on fresh YEM media to obtain pure cultures.

Preparation of inoculum. Fresh inoculum was prepared for each experiment and diluted to get desired concentration (10^7 - 10^8 CFU mL^{-1}). Sterilized YMA medium was inoculated with each Rhizobial strain and incubated at 29±1°C with continuous shaking in mechanical shaker (100 rpm). Streaking of samples on YMA media was carried out on the sterilized petri plates, which were then shifted to an incubator at 29±1°C. After the completion of growth, strains were preserved at 4.0±1°C for further experimentation.

Rhizobial strains growth and culture maintenance. The isolated rhizobial strains were grown on YEM agar and cultured in YEM broth with constant shaking at 100 rpm on an orbital shaker. Mean generation time was recorded for each isolate and the isolates were gram stained after harvesting (Vincent, 1970). The morphological and growth characteristics of rhizobial isolates were studied by light microscopy.

Morphological and biochemical characterization of rhizobial strains. The purified Rhizobial strains were characterized for identification. For colony and cell morphology, the rhizobial strain isolates were streaked on YEM agar plates and incubated at 29±1°C for 36 to 48 h. Each colony was then identified on the basis of its growth rate, colour, shape and gum production. The rhizobial strains were observed under light microscope by taking a drop of culture suspension in saline medium and identified on the basis of cell shape, size and motility.

For Gram's strains reaction, the slides of isolated and purified rhizobial strains were prepared (Vincent, 1970). A drop of rhizobial culture was taken and a thin smear on glass slide was prepared. The smear was air dried heat fixed, stained with crystal violet stain for one minute and was washed with water. Then the smear was flooded with iodine solution. After 30 sec. the iodine solution was drained off and smear was de-colorized with 75% ethanol for 30 sec. The smear was washed with water and counter stained with safranin. It was then re-washed with water; air dried and observed under light microscope.

To determine the acid producing ability of the strains, each isolate was grown in duplicate on YEM agar plate containing 0.025% (w/v) bromothymol blue. After the incubation of 72 h at 28±1°C, the pH change was scored on the basis of the colour change of the medium (Chen & Lee, 2001).

For the measurement of phosphate solublization a single colony of rhizobial strain culture grown on yeast extract mannitol (YEM) medium was streaked on Pikovskaia's medium containing tricalcium phosphate (Pikovskaia *et al.*, 1948) and incubated at 28±1°C for days. The plates were observed for clear P-zone formation around colonies. Quantification of solubilized phosphate was carried out by phospho-molybdat method using spectrophotometer (Yoshida *et al.*, 1976).

Molecular characterization using RAPD technique. Genomic DNA was extracted by the alkaline lyses method for different rhizobial strains (Sambrook *et al.*, 1989) with slight modifications. The pure rhizobial strains were grown in YEM broth for 2-3 days (or depending upon growth condition) at 28±2°C with constant shaking in mechanical shaker. The pure rhizobial strain cultures (1.5 mL, each) with sufficient growth were harvested and centrifuged at 13000 rpm for 10 min and pellet was isolated. The pellet was suspended in 500 µL Tris Acetate buffer and 100 µL of lysozyme (15 mg mL^{-1}) was added. RNase of 10 µL (10 mg mL^{-1}) was added and mixed gently by inverting the tube for several times and incubated at 37°C for 30 min. A volume of 30 µL SDS (10%) was added, and mixed thoroughly and incubated at 70°C for 20 min. ProtienaseK (20 mg mL^{-1}) of 10 µL was added and incubated at 45°C for 2 h. The mixture was centrifuged at 13000 rpm for 10-12 min. and supernatant was collected. The same volume of chloroform: isoamylalcohol (24:1) was added, mixed gently by inverting the tube to form an emulsion, centrifuged at 13000 rpm for 15 min. Aqueous layer was taken in a new tube and discarded the remaining chloroform phase. The same volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added, centrifuged at 13000 rpm for 10-12 min. Aqueous layer was taken, added 1/10 volume of sodium acetate (3 M) and 0.6 volume of chilled isopropanol stored at -20°C for 1 h, spun at 13000 rpm for 10-12 min. The pellet was washed with 85% ethanol to remove the bases and then added absolute ethanol to dehydrate the pellet. The pellet was air dried and resuspended in 50-100 µL TE buffer or d_3H_2O. The concentration of DNA was measured with spectrophotometer at 260 nm wavelength. The quality of DNA was observed by running on agarose gel (1.2%). The DNA was stored at 4.0°C.

Two series (A & C) of 30 RAPD primers were used in the analysis to amplify the DNA of the strains. The PCR was performed in a volume of 25 µL containing 2.5 µL 10X [(750 mM Tris-HCL (pH 8.8), 200 mM $(NH_4)_2SO_4$], 3 µL $MgCl_2$ (25 mM), 2.5 µL 0.001% Gelatin, 1 µL each of dATP, dCTP, dTTP and dGTP (2.5 mM), 2 µL primer (15 ng μL^{-1}), 2.5 µL of genomic DNA (15 ng μL^{-1}), 0.2 µL (1 unit) Taq polymerase and 8.3 µL dH_2O. *Taq* polymerase together with buffer, $MgCl_2$ and dNTPs were purchased from MBI, Fermentas. Amplification was performed in Eppendorf DNA thermal cycler 9600 programmed for a first denaturation step of 5 min. at 94°C followed by 40 cycles of 1 min at 94°C, 1 min. at 36°C and 2 min. at 72°C. The reactions mixtures were kept at 72°C for 10 min for final extension step. To confirm that the observed bands were amplified from genomic DNA and not primer artifact,

genomic DNA was omitted from control reaction.

Amplification products were analyzed by electrophoresis in 1.2% agarose gel in 0.5 X Tris Borate EDTA buffer and detected by staining with ethidium bromide. Before loading PCR products in the gel, 5 µL of bromophenol blue dye mixed with 10% glycerol, 0.1 M EDTA and 2% SDS was added. Only 10 µL of the reaction mixture was loaded on gel. DNA ladder (Mass Ruler TM DNA ladder mix) from MBI Fermentas, USA with known molecular weight bands was loaded on both or either side of the gel to calculate the size/molecular weight of the polymorphic DNA fragments. Samples were electrophoresed for approximately 2 h at 50 volts. After electrophoresis, the amplified products were viewed under ultraviolet transilluminator and photographed using the Syngene Gel Documentation System. Good quality photographs were used to read the amplification profiles. All visible and un-ambiguously scorable fragments amplified by primers were scored under the heading of total scorable fragments. Bands of less than 200 bp were in some cases difficult to score and were not considered.

Statistical analysis. Multivariate analysis was conducted to generate a similarity matrix using Popgene 32 software, version 1.44 (Yeh *et al*., 2000) based on Nei's un-weighted paired group of arithmetic means averages (UPGMA) to estimate genetic distance and relatedness of rhizobial strains.

RESULTS AND DISCUSSION

Morphological and physiological characterizations. Reliable identification of specific rhizobial strains is necessary for the study of their symbiotic association with plants. The characterization of new prospective isolates for application requires the development of easy, rapid and reliable methods for their identification (Selenska-Pobell *et al.*, 1996). From the root nodules of lentil (*Lens culinaris* Medic) 12 rhizobial isolates were obtained. Fast growing rhizobial strains showed sufficient growth and more gum production having a mean generation time of 24-36 h., while slow growing rhizobial strains showed sufficient growth but less gum production, having a mean generation time of 36-48 h.

The colonies obtained were gummy, translucent and circular with entire or smooth margins. All the strains were gram negative and rod shaped as revealed by Gram's staining technique. The growth patterns, morphological and physiological characteristics of the strains such as source, gum production, acid/alkali production and phosphates solubilization were studied for the rhizobial isolates. Ten rhizobial strains i.e., F-1, L-3, G-4, L-5, S-7, G-9, S-26, L-29, F-38 and L-45, turned the yeast extract mannitol agar medium from green to yellow bromothymol blue (BTB) showing that these were acid producers. Other two strains, L-10 and L-22 changed the colour from green to medium blue showing that were alkali producers with a mean generation time of 48 to 72 h.

Fig. 1. RAPD product of oligonucleotide primer GLA-18 of 12 rhizobial strains (F-1, L- 3, G-4, L-5, S-7, G-9, L-10, L-22, S-26, L-29, F-38 and L-45)
M is DNA Ladder.

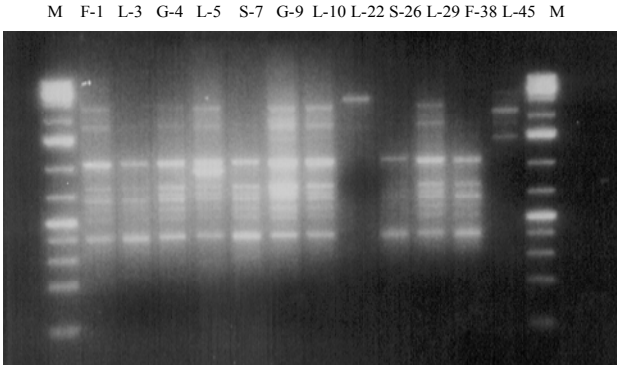


Fig. 2. RAPD product of oligonucleotide primer GLC-19 of 12 rhizobial strains (F-1, L-3, G-4, L-5, S-7, G-9, L-10, L-22, S-26, L-29, F-38 and L-45)
M is DNA Ladder.

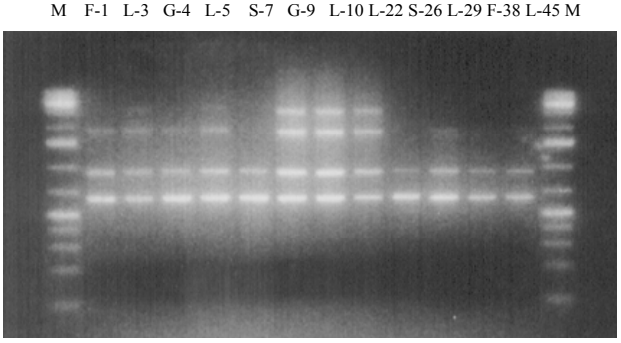
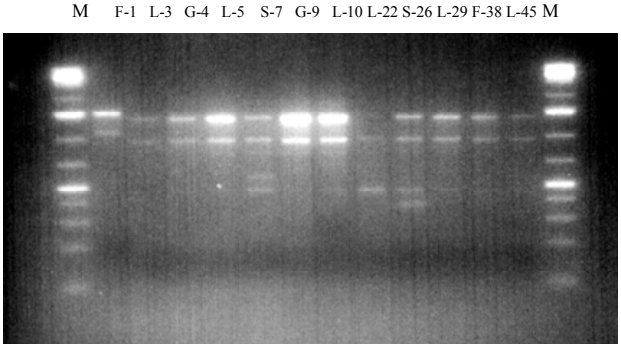


Fig. 3. RAPD product of oligonucleotide primer GLC-11 of 12 rhizobial strains (F-1, L-3, G-4, L-5, S-7, G-9, L-10, L-22, S-26, L-29, F-38 and L-45)
M is DNA Ladder.



Phosphate solubilization. Phosphate solublization data showed that only L-5, L-10, L-22 and S-26 rhizobial isolates were able to solubilize soil phosphates, among these, L-22 showed maximum phosphate solublization, L-5 and L-10 showed medium phosphate solublization, while S-26 showed low phosphate solublization. The other strains,

F-1, L-3, G-4, S-7, G-9, L-29, F-38 and L-45 showed no phosphate solubilizing ability.

In earlier studies a number of phosphates solubilizing rhizobial strains showed an effective role in phosphorus uptake and growth enhancement of plants by dissolution of inorganic insoluble phosphate (Belimov *et al*., 1995). It is known that mineralizing organic phosphorus compounds and by converting inorganic phosphorus into available form, these soil phosphate solubilizing strains can increase the availability of phosphorus to plants (Marschner, 1995; Baryosef *et al*., 1999). Phosphate solubilizing strains when grown in liquid cultures show a fall in pH by production of organic acids (Asea *et al.*, 1988; Rodriguez & Fraga, 1999). The phosphate solubilization results of the present study showed that most of the rhizobial isolates did not have the ability to solubilize phosphates. It was observed that the isolates with phosphate solubilizing ability were fast growing and could form colonies with clear zones around them indicating their ability to solubilize phosphates.

Molecular characterization. The previous methods used for distinguishing rhizobial strains were morphological, physiological and biochemical (Echevemgaray *et al.*, 2000). However, these traditional methods of *Rhizobium* characterization frequently fail in the identification of strains within a species. So, it is necessary to obtain a better understanding of microbial diversity, molecular methods should be adopted for strain identification. In this study, analsis of twelve rhizobial strains by RAPD using 30 oligonucleotide primers was made (Table I). PCR amplifications with some primers indicated that each primer-template yielded district, easily detectable bands of variable intensities (Fig. 1–3). The bands used for fingerprinting were those reproducible over repeated runs with sufficient intensity to detect presence or absence with confidence.

Considering all the primers and rhizobial strains, a total of 1480 bands were amplified in the PCRs of 12 strains, out of which 663 were polymorphic, showing 44.80% of overall polymorphism. The polymorphism percentage was lower than that obtained by Hameed *et al.* (2004) in *Rhizobium, Bradyrhizobium* and *Agrobacterium* strains (99.8%). Number of bands produced per genotype ranged from 93 to 147 with an average of 123 bands per genotype (Table II). Among the strains, maximum number of bands were produced by the G-9 that was 147 (70 polymorphic), while minimum number of bands were produced by strain L-22 that was 93 (45 polymorphic). The polymorphic bands percentage per genotype ranged from 35% (in strain L-3) to 50.9% (in strain S-26). The low degree of similarity (monomorphic bands) indicated high divergence between the strains. The number of amplification products produced per primer varied from 4 to 9 with an average of 6 bands per primer. Maximum numbers of bands were produced by the primer GLA-11 (94) in which 50 were polymorphic, while a minimum number of bands were produced by primer GLC-6 (19) with 10 polymorphic bands. The polymorphism percentage per primer ranged from 33.92% (GLA-5) to 59.52% (GLC-15).

Thirty primers allowed discrimination of all the possible pair-wise comparisons between genotypes. Although some bands were monomorphic, most rhizobial strains produced unique amplification profiles sufficient to distinguish them from the other tested genotypes. These

Table I. Oligonucleotide decamer primers along with their sequences used in the study

Primer Names	Sequences
GL DecamerA-02	TGCCGAGCTG
GL DecamerA-04	AATCGGGCTG
GL DecamerA-05	AGGGGTCTTG
GL DecamerA-07	GAAACGGGTG
GL DecamerA-08	GTGACGTAGG
GL DecamerA-09	GGGTAACGCC
GL DecamerA-10	GTGATCGCAG
GL DecamerA-11	CAATCGCCGT
GL DecamerA-12	TCGGCGATAG
GL DecamerA-13	CAGCACCCAC
GL DecamerA-14	TCTGTGCTGG
GL DecamerA-15	TTCCGAACCC
GL DecamerA-16	AGCCAGCGAA
GL DecamerA-18	AGGTGACCGT
GL DecamerA-19	CAAACGTCGG
GL DecamerA-20	GTTGCGATCC
GL DecamerC-01	TTCGAGCCAG
GL DecamerC-02	GTGAGGCGTC
GL DecamerC-05	GATGACCGCC
GL DecamerC-06	GAACGGACTC
GL DecamerC-08	TGGACCGGTG
GL DecamerC-10	TGTCTGGGTG
GL DecamerC-11	AAAGCTGCGG
GL DecamerC-12	TGTCATCCCC
GL DecamerC-13	AAGCCTCGTC
GL DecamerC-14	TGCGTGCTTG
GL DecamerC-15	GACGGATCAG
GL DecamerC-16	CACACTCCAG
GL DecamerC-18	TGAGTGGGTG
GL DecamerC-19	GTTGCCAGCC

Fig. 4. Dendrogram of 12 rhizobial strains obtained from similarity matrix based on Nie's UPGMA

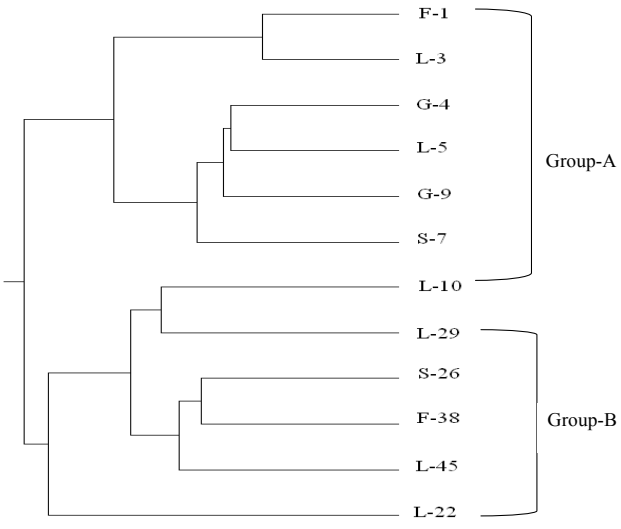


Table II. Polymorphism shown by the 12-rhizobial strains in PCR using 30 primers

Genotype	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	Polymorphism percentage
F-1	100	40	60	40.0
L-3	100	35	65	35.0
G-4	136	57	79	41.9
L-5	141	68	73	48.2
S-7	129	64	65	49.6
G-9	147	70	77	47.6
L-10	146	67	79	45.9
L-22	93	45	48	48.4
S-26	114	58	56	50.9
L-29	128	55	73	43.0
F-38	122	51	71	41.8
L-45	124	53	71	42.7

Table III. Polymorphism of primers in PCR of 12 rhizobial strains

Primer Name	Number of bands	Polymorphic Bands	Polymorphism percentage
GL DecamerA-02	32	15	46.87 %
GL DecamerA-04	46	21	45.65 %
GL DecamerA-05	56	19	33.92 %
GL DecamerA-07	38	17	44.73 %
GL DecamerA-08	24	7	54.16 %
GL DecamerA-09	67	31	46.26 %
GL DecamerA-10	37	15	40.54 %
GL DecamerA-11	94	50	53.19 %
GL DecamerA-12	47	24	51.06 %
GL DecamerA-13	46	20	43.47 %
GL DecamerA-14	35	14	40.00 %
GL DecamerA-15	61	23	37.70 %
GL DecamerA-16	22	4	40.90 %
GL DecamerA-18	57	24	42.10 %
GL DecamerA-19	64	28	43.75 %
GL DecamerA-20	72	30	41.66 %
GL DecamerC-01	54	26	38.88 %
GL DecamerC-02	72	33	45.83 %
GL DecamerC-05	49	23	46.93 %
GL DecamerC-06	19	13	52.63 %
GL DecamerC-08	29	13	44.82 %
GL DecamerC-10	59	24	35.59 %
GL DecamerC-11	47	24	51.06 %
GL DecamerC-12	57	27	47.36 %
GL DecamerC-13	59	25	42.37 %
GL DecamerC-14	43	20	46.51 %
GL DecamerC-15	42	25	59.52 %
GL DecamerC-16	48	19	39.58 %
GL DecamerC-18	59	22	37.28 %
GL DecamerC-19	45	27	60.00 %

Table IV. Similarity matrix developed from 12 rhizobial strains using RAPD analysis

Genotypes	L-3	G-4	L-5	S-7	G-9	L-10	L-22	S-26	L-29	F-38	L-45
F-1	0.8162	0.6649	0.6703	0.6703	0.6324	0.6324	0.5027	0.5568	0.4486	0.5189	0.4973
L-3		0.6541	0.6595	0.6811	0.7730	0.4541	0.5784	0.5243	0.4054	0.5405	0.5514
G-4			0.7784	0.7351	0.7730	0.6378	0.6000	0.6324	0.5784	0.5730	0.6162
L-5				0.7405	0.7676	0.6757	0.5514	0.6270	0.5622	0.6000	0.5568
S-7					0.7459	0.6432	0.6054	0.6486	0.5297	0.6324	0.6649
G-9						0.7351	0.5568	0.6324	0.6216	0.6595	0.6270
L-10							0.5568	0.6324	0.6216	0.6595	0.6270
L-22								0.6432	0.5459	0.5838	0.6595
S-26									0.6973	0.7459	0.7027
L-29										0.6703	0.6378
F-38											0.7405

results confirm the efficiency of RAPD markers for the identification of rhizobial strains. The combined analysis of the amplification products generated by primers was enough to asses the genetic diversity among the strains (Hameed *et al.*, 2004).

Multivariate analysis was conducted to generate a similarity matrix using Polgene 32 software, version 1.44 (Yeh *et al*., 2000) based on Nei's un-weighted paired group

of arithmetic means averages (UPGMA) to estimate genetic distance and relatedness of rhizobial strains (Table IV). Similarity value ranged from 0.4054 to 0.8162 with an average of 0.6262. The mean similarity in this study was lower than that obtained by Gracia *et al.* (2001) for *Bacillus* (0.63), while studying genetic variability of rhizobacteria from wild populations of four lupins species based on RAPD analysis. However, the mean similarity value of the present study was higher than that obtained by Hameed *et al.* (2004) in a study of *Rhizobium, Bradyrhizobium* and *Agrobacterium* strains isolated from cultivated legumes. In the present study, the highest similarity (0.8162) was found between strains F-1 and L-3, suggests that these strains are very similar in their genetic make up. The lowest similarity (0.5946) was present between L-3 and L-29.

Dendrogram drawn for the genetic distances (Fig. 4). Rhizobial strains cluster analysis defined two distinct groups. Group A consisted of 6 rhizobial strains, F-1, L-3, G-4, L-5, G-9 and S-7. The group A was further subdivided into two subgroups; first subgroup consisted of 2 strains including, F-1 and L-3, while the second subgroup consisted of 4 strains including G-4, L-5, G-9 and S-7. Group B consisted of 6 rhizobial strains, L-10, L-29, S-26, F-38, L-45 and L-22. The group B was further subdivided into three subgroups. First subgroup consisted of two strains including L-10 and L-29. Second subgroup consisted of three strains including S-26, F-38 and L-45.

CONCLUSION

A considerable genetic diversity in rhizobial strains exists with respect to their efficiency for solublizing soil phosphate and the strains can be identified using RAPD.

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