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MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF RHIZOBIA FROM HALOPHYTES OF KHEWRA SALT RANGE AND ATTOCK

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Abstract

Present investigation was aimed to isolate and characterize plant growth promoting rhizobacteria (Rhizobia) from rhizosphere (EC: 2300 µS/cm; pH: 8.6) of four halophytes: *Sonchus arvensis* L., (sow thistle), *Solanum surratense* Burm. F., (yellow berried night shade), *Lactuca dissecta* D. Don., (wild lettuce) and *Chrysopogon aucheri* (Boiss.) Stapf (golden beared grass) collected from Khewra Salt Range and compared with *Rhizobium* isolate from *Solanum surratense* Burm. F. of arid soil (EC: 210 µS/cm; pH: 7.9) of Attock (treated as control). The isolates were identified and characterized on the basis of colony morphology and biochemical traits viz gram staining, catalase and oxidase tests and carbon and nitrogen source utilization pattern. The survival efficiency of isolates was measured in culture (colony forming unit / g soil). The genetic diversity among the isolates assessed by RAPD-DNA finger printing and PCR was done for the presence of 16S-rRNA gene. On the basis of carbon / nitrogen source utilization patterns, *Rhizobium* isolates placed in five different groups and were designated as Rkh1, Rkh2, Rkh3, Rkh4 and Rak5 but 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.

Introduction

Salinity is one of the major environmental factors deleterious to plant growth and yield (Allakhverdiev *et al*., 2000). Khewra Salt Range is situated in the foothills of the Salt Range. It is located between longitudes 073 00, 26.9 E and latitudes 32 39, 03.4 N. The vegetation of region is exposed to severe habitat losses due to high EC and pH.

Increasing salt concentration may have detrimental effects on rhizobial population (Singleton *et al*., 1982). Salt tolerant rhizobia may have the potential to improve yield of legumes under salinity stress (El-Mokadem, 1991). Rhizobial inoculation increases nodule biomass thus encourages sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation (Adewusi *et al*., 2008). Various researches demonstrated the ability of *Rhizobium* to colonize roots of non-legumes (Matiru & Dakora, 2004) and act as phytohormone producer, phosphate solubilizer and to some extent as nitrogen fixer (Afzal & Bano, 2008).

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 (Mehmood *et al*.,

2008) used for phylogenetic relationships among and within closely related species (Williams *et al*., 1990). This paper reports the isolation of *Rhizobium* isolates from Khewra salt range and Attock and their characterization on the basis of morphological, biochemical and molecular characters.

Materials and Methods

Collection of samples: The rhizospheric soil of four halophytes: *Chrysopogonn aucheri* (Boiss.) Stapf. (Golden beared grass) → Poaceae; *Lactuca dissecta* D. Don. (Wild lettuce) → Asteraceae; *Solanum surattense* Burm. f. (Yellow berried night shade) → Solanaceae and *Sonchus arvensis* L. (sow thistle) → Asteraceae with five replicates were collected at vegetative stage from different localities of Khewra salt range (313-360 m.a.s.l; 32° 3860 North latitude and 73° 10 East longitude; pH: 8.6 and EC: 2300 µS/cm). *Solanum surrattense* Burm.f with five replicates was also collected from Attock (305-1067 m.a.s.l; 33° 46' 20 North latitude and 72° 22 East longitude; pH: 7.6 and EC: 210 µS/cm) and treated as control. The rhizospheric soil was sieved, and stored at 4 °C for further processing.

Isolation of *Rhizobium* and determination of survival efficiency: For isolation of *Rhizobium* species 10 g rhizosphere soil were suspended in 90 ml of sterile distilled water. Serial dilutions (10 X) were made with 9 ml distilled water and an aliquot (100 µl) from decimal dilutions was used to inoculate YEMA (Vincent, 1970) media for the growth of *Rhizobium*. The plates were incubated at 30°C for 24-72 h.

To measure the survival efficiency, colonies of *Rhizobium* from 10^8 dilutions were counted following the formula adapted by James (1978):

$$\text{Viable cell count (CFU/g soil)} = \frac{\text{Number of colonies}}{\text{Volume of inoculum}} \times \text{Dilution factor}$$

Colony and cell morphology: Bacterial strains from overnight grown cultures in YEMA (Vincent, 1970) broth were spread on YEMA agar plates and incubated at 30°C for 24 h. After 24 h the colour and shape of colonies was noted. Cell motility and shape of single colony was observed under light microscope (Nikon, Japan).

Gram staining: Gram staining was done by the method of Vincent (1970).

Oxidase test: Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates (Steel, 1961). Kovac's reagent (1% N, N, N.N-tetramethyle-p-phenylene diamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried. With the help of sterile wire loop, one-day-old rhizobial colonies from agar plates were transferred on this filter paper strip. The oxidase positive colonies turned lavender colored which became dark purple to black in color within 5 min.

Catalase test: This test was performed to study the presence of catalase enzyme in bacterial colonies. Rhizobial colonies (24 h old) were taken on glass slides and one drop of H_2O_2 (30 %) was added. Appearance of gas bubble indicated the presence of catalase enzyme. (MacFaddin, 1980).

Miniaturized identification system (QTS 24): Physiological and biochemical tests of *Rhizobium* isolates were performed using QTS-24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan) following the method of MacFaddin (1980). For these tests 24 h old bacterial cultures were used and results were noted after 18 h of incubation at 30°C.

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 4°C. The cell pellets were re-suspended and lysed in 200 µl lysis buffer (40 mM Tris-acetate pH 7.8; 20 mM Sodium-acetate; 1 mM EDTA and 1 % SDS). After vigorous pipetting 66 µl 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 4°C. 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 50 µl of pure water. The concentration and purity of DNA was estimated spectrophotometrically at 260-280 nm.

RAPD-PCR analysis of genomic DNA: Genetic diversity and polymorphism among strains was analyzed by RAPD-PCR technique adapted by Teaumroong & Boonkerd (1998). For RAPD-PCR 10 oligonucleotide OPI-06 primer (AAGGCGGCAG) was used. The PCR reaction mixture (25 µl) contained 1 µl of 50 µg genomic DNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase, 1 µM 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: Initial step of denaturation at 94°C for 30 sec, annealing at 35°C for 30 sec and elongation at 72°C for 1 min. An additional cycle for extension was conducted at 72°C 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). 1 Kb DNA ladder (Fermentas) was used as molecular marker.

PCR analysis of genomic DNA: The genomic DNA of *Rhizobium* isolates were amplified following the method of Weisburg (1991). 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 $MgCl_2$, 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.

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.

Results and Discussion

The isolates from rhizospheric soil of plants were designated as Rkh1 → from *Chrysopogonn aucheri* (Boiss.) Stapf, Rkh2 → from *Lactuca dissecta* D. Don, Rkh3 → from *Solanum surattense* Burm. f, Rkh4 → from *Sonchus arvensis* L. and RaK5 → from *Solanum surattense* Burm. f. The colonies of all isolates were almost circular, non-mucilaginous and transparent. Under the light microscope all the isolates were non-motile rods and were gram –ive. The microbes were positive for catalase and oxidase tests. The isolates from rhizospheric soil of plants collected from Khewra salt range showed higher reaction intensity in catalase tests as compared to the isolate from rhizospheric soil of plants collected from Attock.

Greater number of viable colonies (CFU/g) was recorded by RaK5 as compared to Rkh1-Rkh4 strains (Fig. 1). While among Rkh1-Rkh4 strains survival efficiency of Rkh2 and Rkh3 strains was greater. The difference in the survival efficiency (as measured by the colony count) among the isolates of Khewra Salt range may be attributed to the difference in the root exudates of different plants and their relative tolerance to saline condition. The amount of exudates produced by plants affects growth rate and metabolism of bacteria (Swedrzynska & Sawicka, 2001). The isolates ranked in the following order for colony counts: Rak5>Rkh2≥Rkh3>Rkh4≥Rkh1. Reinhold *et al*., (2005) isolated PGPR from salt tolerant Kallar grass grown in salt affected soil of Pakistan. Zahran (1999) reported that increasing salt concentration might have adverse affect on rhizobial population as a result of direct toxicity and indirectly by osmotic stress.

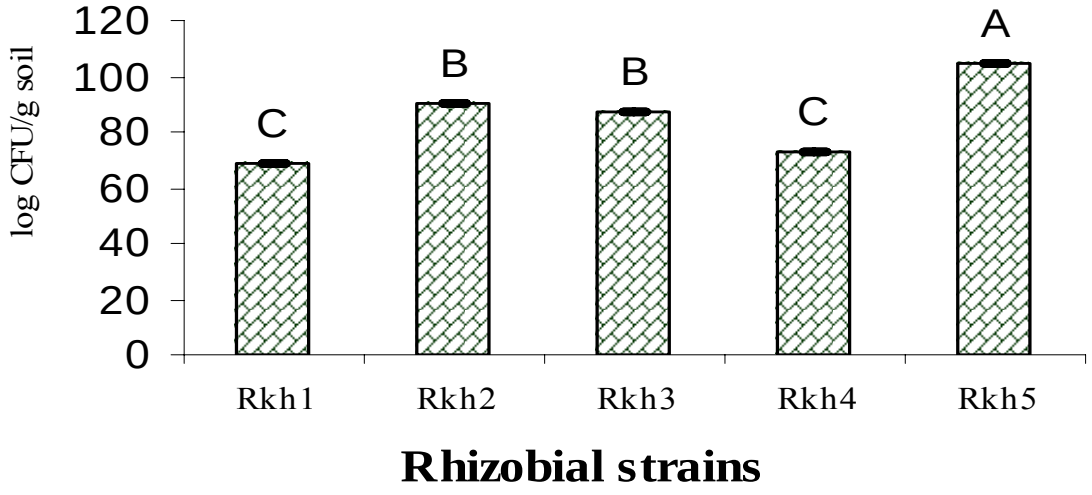

Fig. 1. Colony counts of *Rhizobium* strains. Rkh1-Rkh4 isolates isolated from rhizospheric soil of plants growing in Khewra salt range and Rak5 from rhizospheric soil of *Solanum surattense* growing in Attock.

All the rhizobial isolates were identified on the basis of biochemical tests included in microbial identification kits (QTS-24 miniaturized identification system). Mirza *et al*., (2007) and Erum & Bano (2008) characterized rhizobial strains on the basis of carbon / nitrogen source utilization patterns. On the basis of carbon / nitrogen source utilization patterns, the isolates were placed in five groups and were named as Rkh1, Rkh2, Rkh3, Rkh4 and Rak5. The isolates Rkh1-Rkh4 from rhizospheric soil of plants of Khewra Salt range showed better utilization of carbohydrates as compared to isolate Rak5 (Table 1). The greater utilization of carbohydrates by these isolates may an adaptive mechanism to cope with salt stress. Salt stress affects metabolic functions of microorganisms (Rivarola *et al*., 2004). Rhizobia use various mechanisms for osmotic adaptations under salt stress. These include accumulation of low molecular weight organic solutes: sugars, amino acids, polyamines and accumulation of ions (Miller & Wood, 1996; Zahran, 1999). The sodium citrate, sodium melonate, urea, indole and gelatin hydrolysis tests were negative for all the isolates. Arginine dihydrolase test was positive in Rkh3 being negative in other isolates. The isolate Rkh4 differ from other rhizobial isolates being negative to Tryptophan deaminase. The isolates Rkh1 and Rkh3 were positive to acid from glucose test. Rkh3 and Rkh4 were positive in acid from sucrose test while Rkh2 and Rkh3 differ from other strains in having acid from arabinose test positive. Acid from rhamnose test was negative in Rkh3 and Rak5. Rak5 also showed negative reaction to acid from sorbitol and acid from melibiose test while in other strains these tests were positive. The QTS results of all the isolates were compared with Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and were identified as *Rhizobium* on the basis of carbon / nitrogen source utilization pattern.

The genetic diversity among five isolates of *Rhizobium* was evaluated by RAPD-PCR analysis using OPI-06 primer. Genetic polymorphism of *Rhizobium* (Versalovic *et al*., 1994; Teaumroong & Boonkerd, 1998) strains had been studied by RAPD finger printing technique. PCR-RAPD is a useful tool to conduct persistence and competitiveness studies in rhizobia strains when inoculated in soils as inoculants (Pinto *et al*., 2004). Different DNA banding patterns, depending on the number and size of amplified products were observed for *Rhizobium* isolates (Table 2; Fig. 2). Total 28 bands were observed in *Rhizobium* isolates, their size ranged from 250-1225 bp. Out of 28 bands, 22 (78.5%) were polymorphic and were scored for analysis by Multivariate Statistical Package (version 3.1) software program, using Gower general similarity coefficient. Dendrogram fell in to two clusters (Fig. 3). The first cluster consisted only on Rak5 and the second cluster consisted on two groups. The first group consisted on Rkh1 and Rkh2 strains whereas Rkh3 and Rkh4 isolates fell in second group. The similarity matrix between *Rhizobium* isolates varied from 0.273-0.727 (Table 3). There was no 100 % similarity between any two strains. The highest similarity coefficient (0.727) was computed between strains Rkh1 and Rkh2, both of these isolates isolated from rhizospheric soil of plants of same area (Khewra salt range). The lowest similarity coefficient (0.273) was determined between isolates Rkh2 and Rkh3, both of these isolates were isolated from rhizospheric soil of plants of Khewra salt range and belonged to two different groups of cluster 2. The greater genetic difference among these isolates of same area (Khewra salt range) might be attributed to their adaptive factor which is possibly modulated by the differences in root exudates composition of different plants in the rhizosphere from where isolation was made. Young & Cheng (1998) determined genetic relationship among six strains of rhizobia. The greatest similarity was found between the strains isolated from same origin. Ilyas *et al*., (2008) characterized *Rhizobium* strains on the basis of RAPD-DNA finger printing and evaluated that environmental stress may favor adaptation of strains with genetic difference.

Table 1. Morphological, physiological and biochemical characteristics of *Rhizobium* strains obtained from rhizospheric soil of plants collected from Khewra salt range and Attock.

Reactions	Tests	Rkh1	Rkh2	Rkh3	Rkh4	Rak5
CS	Colony shape	Round, transparent, non-mucilaginous				
CES	Cell shape	Rods				
CM	Cell motility	Non-motile				
GS	Gram staining	-ive				
Oxid	Oxidase	+	+	+	+	+
Cat	Catalase	+	+	+	+	+
ONPG	Ortho nitro phenyl β-D-galactopyranoside	+	+	+	+	+
CIT	Sodium citrate	-	-	-	-	-
MALO	Sodium melonate	-	-	-	-	-
LDC	Lysine decarboxylase	+	+	+	+	+
ADH	Arginine dihydrolase	-	-	+	-	-
ODC	Ornithine decarboxylase	-	-	-	-	-
H2S	H_2S production	+	+	+	+	+
URE	Urea hydrolysis	-	-	-	-	-
TDA	Tryptophan deaminase	+	+	+	-	+
IND	Indole	-	-	-	-	-
VP	Voges proskauer	+	+	+	+	+
GEL	Gelatin hydrolysis	-	-	-	-	-
GLU	Acid from glucose	+	-	+	-	-
MAL	Acid from maltose	+	+	+	+	+
SUC	Acid from Sucrose	-	-	+	+	-
MAN	Acid from mannose	+	+	+	+	+
ARA	Acid arabinose	-	+	+	-	-
RHA	Acid from rhamnose	+	+	-	+	-
SOR	Acid from sorbitol	+	+	+	+	-
INO	Acid from inositol	+	-	+	-	+
ADON	Acid from adonitol	-	+	+	+	+
MEL	Acid from melibiose	+	+	+	+	-
RAF	Acid from raffinose	+	-	-	+	+
Organisms identified		***Rhizobium* sp.**				

Table 2. DNA number and size in *Rhizobium* isolates on the basis of RAPD-PCR analysis using random primer OPI-06.

Strains	DNA bands #	Size of bands (bp)										
		1225	875	750	685	625	580	500	415	375	330	250
Rkh1	6	+	+	+	+	-	-	+	-	+	-	-
Rkh2	5	+	+	-	+	-	+	-	-	+	-	-
Rkh3	7	+	+	+	-	+	-	+	+	-	+	-
Rkh4	7	+	+	+	+	+	-	+	-	-	-	+
Rak5	3	-	-	+	-	+	-	-	-	+	-	-

Table 3. Similarity matrix between *Rhizobium* isolates constructed from RAPD-PCR banding pattern.

	Rkh1	Rkh2	Rkh3	Rkh4	Rak5
Rkh1	1.000				
Rkh2	0.727	1.000			
Rkh3	0.545	0.273	1.000		
Rkh4	0.427	0.455	0.636	1.000	
Rak5	0.545	0.450	0.455	0.455	1.000

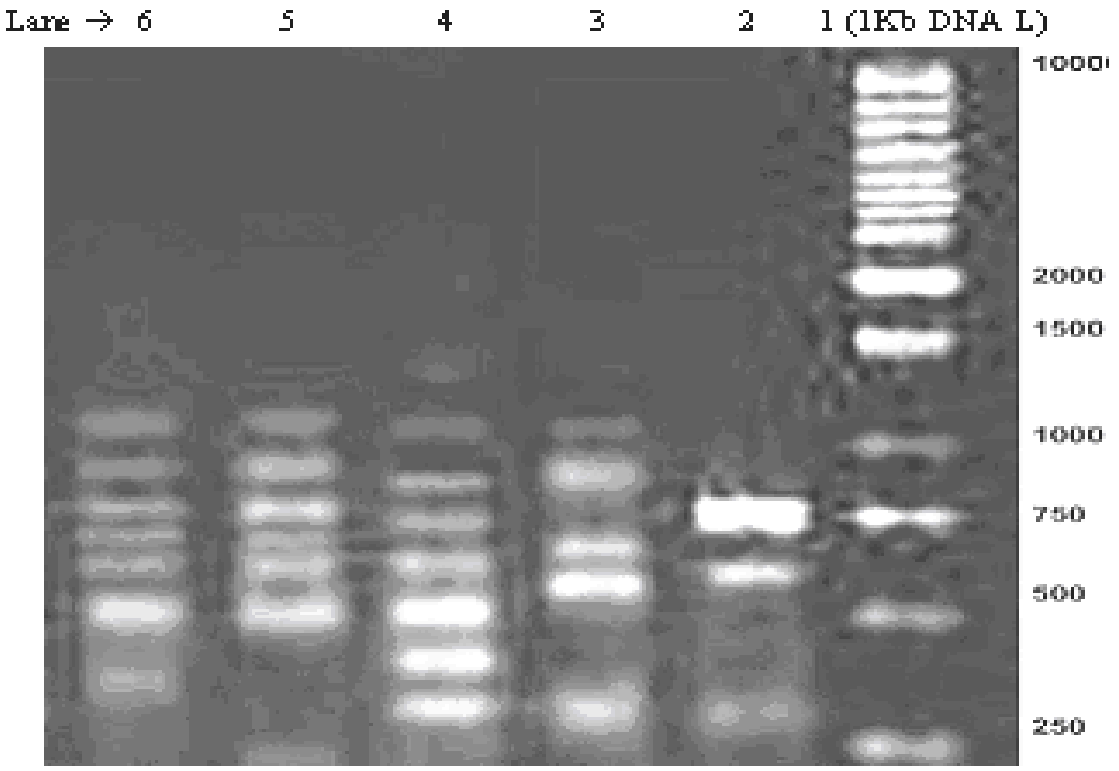

Fig. 2. RAPD-PCR amplification of DNA isolated from *Rhizobium* isolates using primer OPI-06.

In figure, Lane 1=1 Kb DNA Ladder; Lane 2-5=DNA banding pattern of Rkh1-Rkh4 isolates from rhizospheric soil of plants of Khewra salt range; Lane6=DNA banding pattern of Rak5 isolates from rhizospheric soil of plants of Attock.

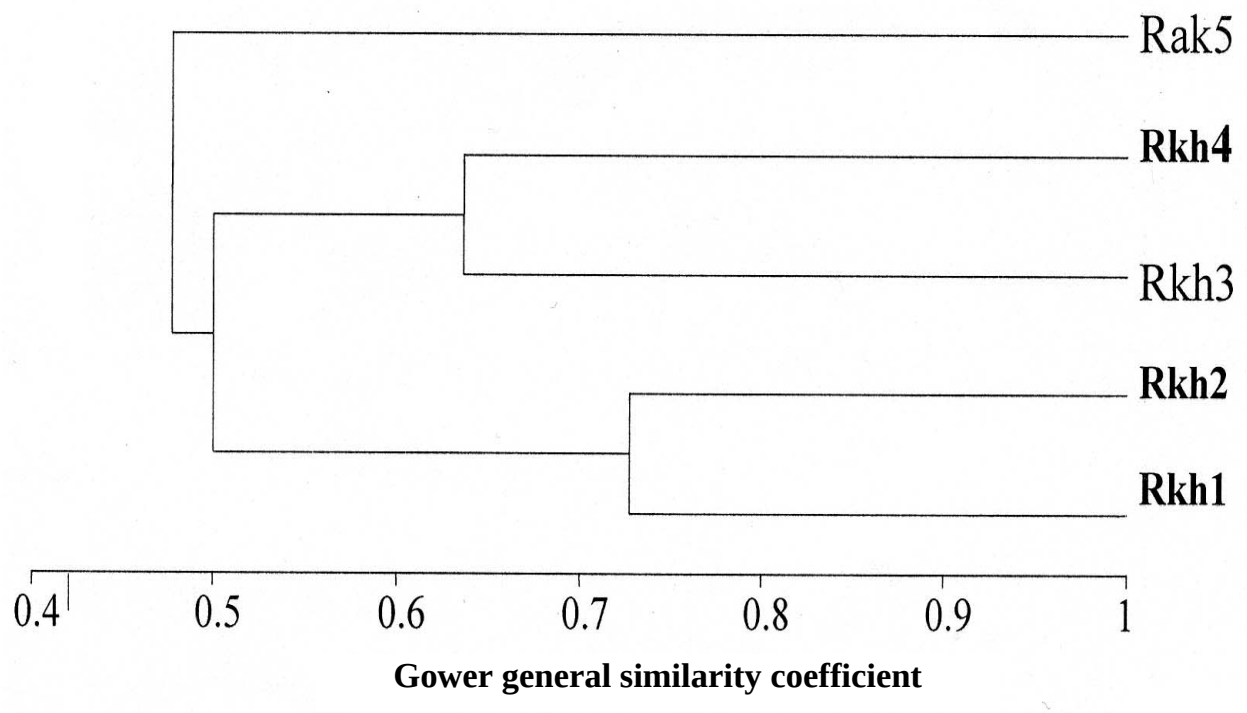

Fig. 3. UPGMA Dendrogram showing the similarities values among five *Rhizobium* isolates on the basis of RAPD-DNA finger printing.

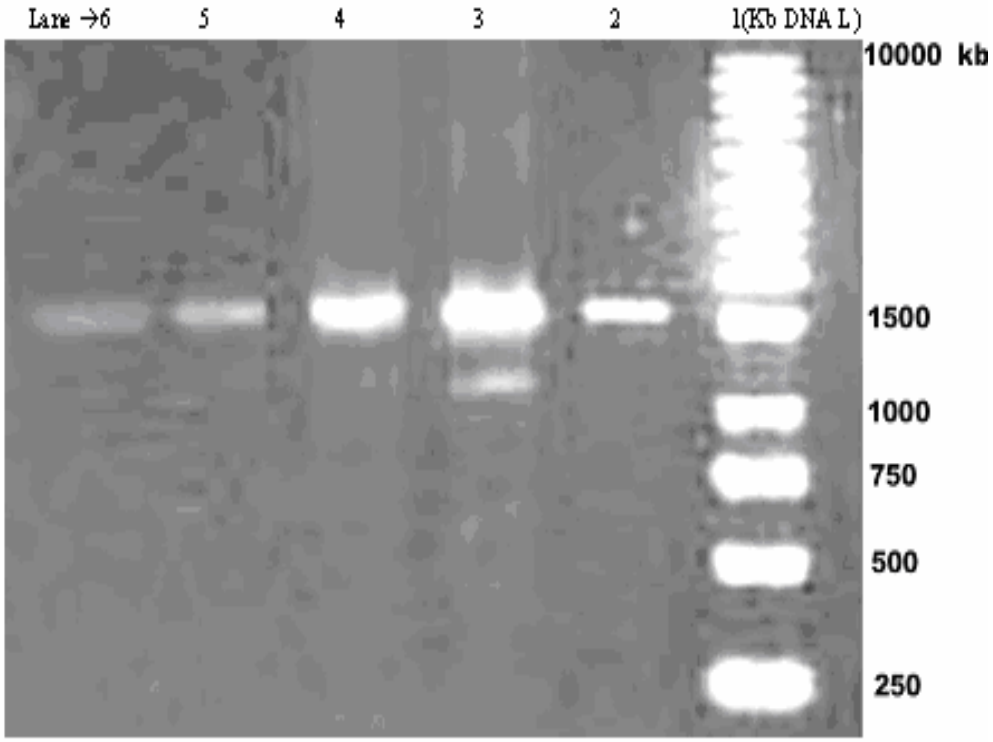

Fig. 4. PCR amplification of DNA isolated from *Rhizobium* isolates using primers: fd1 and rd1.
In figure, Lane 1 = 1 Kb DNA Ladder; Lane 2-5 = band of 1500 bp of Rkh1-Rkh4 isolates from rhizospheric soil of plants of Khewra salt range; Lane6 = band of 1500 bp of Rak5 isolate from rhizospheric soil of plants of Attock.

The PCR results indicated the presence of band of 1500 bp in all isolates (Fig. 4). This band is used for sequencing of 16S-rRNA gene as preferred phylogenetic marker used in bacteria ecology (Normand, 1996). 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 (Ludwig & Schleifer, 1999; Normand, 1999).

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* as observed between the isolates of Khewra salt range and that of arid area of Attock. The difference may also exist among the isolates of same ecological conditions on the basis of association with different plants.

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