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Variation in *Rhizobium* and *Azospirillum* Strains Isolated from Maize Growing in Arid and Semiarid Areas

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ABSTRACT

Plant growth promoting rhizobacteria (*Rhizobium & Azospirillum)* were isolated from the roots and rhizosphere of maize plants grown at farmer's field of arid and semiarid areas and irrigated area. *Rhizobium* and *Azospirillum* isolated from irrigated areas showed higher colony count as compared to those from semi-arid areas particularly at reproductive stage. For *Rhizobium,* sodium citrate, acid from maltose, tryptophan deaminase, indole and acid from melibiose tests were negative for isolates from low soil moisture. The results for sodium citrate, acid from arabinose and lysine decarboxylase were negative for *Azospirillum* from low soil moisture. On the basis of carbon/nitrogen utilization pattern, three groups of *Rhizobium* isolates were identified that fell into two groups on the basis of banding pattern of RAPD. Similar grouping were recognized for *Azospirillum* on the basis of carbon/nitrogen utilization pattern and RAPD analysis.

Key Words: *Rhizobium; Azospirillum;* Maize

INTRODUCTION

Maize (*Zea mays* L.) is the third most important cereal after wheat and rice all over the world (Anonymous, 2000). Crops grown in soil may fail to respond to supplements of P or N, because of a complex of negative factors including inadequate moisture (Khan *et al*., 2001).

More than 70% of Pakistan (87.81 million ha) is arid or semi arid and rainfall usually support dry land or irrigated crops (Suleman *et al*., 1995). Much of the rainwater is lost by surface runoff or rapid evaporation due to high temperature. This probably affects the survival of indigenous rhizobia (Athar & Johnson, 1997) as well as host plant. *Rhizobium* spp. with the genetic potential for increased tolerance to these adverse environmental stresses could enhance production of food and forage legumes in semiarid and arid regions of the world (Brockwell *et al*., 1995).

Rhizobium with non-legumes could act as phosphate solubilizer, hormone producer and to some extent as N-fixer (Afzal & Bano, 2008). Inoculation with *Rhizobium* can consequently led to improved soil fertility and can reduce the production cost of next crop through reduced input in the form of nitrogen fertilizers, which in turn also minimize the health hazard effects (Ahmed *et al*., 2008).

Azospirillum–plant association is accompanied by biochemical changes in roots, which in turn; promote plant-growth and tolerance to low soil moisture (Pereyra *et al*., 2006). *Azospirillum*–plant interactions have been extensively studied since 1970s (Zahir*et al*., 2004). The bacteria stimulate plant-growth even in the presence of several stresses such as drought (Creus *et al*., 1996). *Azospirillum* inoculation alleviates low soil moisture effects on wheat plants grown under drought conditions (El-Komy *et al*., 2003).

Appropriate root colonization is known to result in growth promotion of wheat and maize seedlings, which are more evident under low soil moisture (Casanovas *et al*., 2000). Adapted micro-symbionts, therefore can be used efficiently to survive and protect their hosts from those stresses (Vivas *et al*., 2003). Present study aimed to investigate survival efficiency of *Rhizobium* and *Azospirillum* as well as the biochemical characterization of the isolates in the soil-maize systems, as a function of low soil moisture condition. Research was focused upon isolation of *Rhizobium* and *Azospirillum* from rhizosphere and roots of maize plants collected from semi arid areas (Attock & Kallar Sayedan) and irrigated areas of NARC (National Agriculture Research Centre, Islamabad). Characterization of *Rhizobium* and *Azospirillum* isolates on the basis of morphological characters, biochemical tests including microbial identification kits (QTS 24) and by DNA based technique: RAPD-PCR were made.

MATERIALS AND METHODS

Fresh maize rhizosphere soil (to a depth of 15 cm) and roots were collected from semi arid areas of Attock (266 m. altitude, latitude 33°46′N & longitude of 72°22′E, with 5-6% available soil moisture) and Kallar Sayedan (561 m

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altitude, latitude of 33°27′N & longitude of 73°16′E, with 7-8.5% soil moisture content). Soil samples collected from National Agricultural Research Centre, Islamabad (500 m altitude, at latitude of 33°42′N & longitude of 73°08′E) were taken as irrigated control and having 15-17% soil moisture content. Sampling was done during the vegetative (3-4 leaf stage, 27 days after sowing) and at anthesis stage of crop (55 days after sowing). During sampling intact plants were up-rooted along with its roots. Five plants were up-rooted from each site. The root system was gently removed from the soil and washed carefully to remove adhering soil particles.

Isolation of *Rhizobium* and *Azospirillum*

Isolation from rhizosphere soil. Decimal dilutions were prepared by suspending 10 g rhizosphere soil in 90 mL of sterilized distilled water. For isolation of *Rhizobium* isolates aliquot (100 µL) from three dilutions (10^{-1}, 10^{-5} & 10^{-10}) was used to inoculate yeast mannitol agar medium in petri plates. The cultures were incubated at 30°C for 24 h. The colonies of *Rhizobium* thus obtained were counted to determine cfu mL^{-1} and recultured to obtain single pure colony.

For isolation of *Azospirillum* isolates 100 µL from three dilutions (10^{-1}, 10^{-5} & 10^{-10}) was used to inoculate vials containing nitrogen free medium (NFM) in Mc Cartany's vials. These vials were incubated at 30°C for 48 h. Vials showing *Azospirillum* growth were used for inoculation of *Azospirillum* on Lauria Beratni (LB) plates in order to obtain pure colonies. Single colonies appearing on these plates were transferred in liquid broth of LB and on agar slants for further study.

Isolation from roots. Roots (1 g) were surface sterilized by shaking them in 0.1% $HgCl_2$ solutions for 1-2 min, followed by 5-6 washings with sterilized distilled water. One gram of surface sterilized root of plants was crushed and decimal dilutions (10 X) were prepared. For isolation of *Rhizobium* isolates approximately 100 µL from three dilutions (10^{-1}, 10^{-5} & 10^{-10}) was used to inoculate in yeast mannitol agar medium and incubated at 30°C for 24 h. Similarly, for isolation of *Azospirillum* isolates 100 µL from three dilutions (10^{-1}, 10^{-5} & 10^{-10}) was used to inoculate 5 mL of nitrogen-free semi solid Combined Carbon Medium (CCM; Rennie, 1981) to which malic acid (5 g L^{-1}) was added as an additional source and Nitrogen Free Malate medium (NFM; Okon *et al.*, 1977). Inoculated vials were incubated at 30°C for 48 h, which was further diluted to10^{-10}.

The vials showing bacterial colonies were used to inoculate Luria Beratni (LB; Miller, 1972) agar plates to obtain pure colonies of *Azospirillum* species.

Viable cell count method. An original broth culture viable cell per mL was calculated as suggested by James (1987):

Viable cell count (CFU mL^{-1}) = (number of colonies×dilution factor/volume of inoculum).

Colony and cell morphology. Isolated strains of bacteria were identified on the basis of colony, cell morphology and biochemical tests (Holt *et al.,* 1994). Bacterial strains from overnight grown cultures LB (Miller, 1972) broth were spread on the agar plates of the medium. The morphology of the colonies (colour & shape) was noted after 24 h to study the cell motility and shape. Single colony from the agar plates was transferred on glass slide with a drop of sterile water and observed under light microscope (Nikon, Japan).

Oxidase test. Oxidase test was performed according to Steel (1961) for determination of the presence of oxidase enzyme in bacterial strains.

Catalase test. This test was performed according to the procedure of MacFaddin (1980) in order to study the presence of catalase enzyme in bacterial colonies.

Miniaturized identification system-QTS 24. Physiological and biochemical tests were performed using QTS 24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan) following the method of MacFaddin (1980).

DNA extraction. DNA was extracted from *Rhizobium* and *Azospirillum* isolates by using the method of Chen and Kuo (1993).

PCR analysis of genomic DNA by using random primers (RAPD-PCR). Genetic biodiversity and polymorphism among the isolated strains was determined by using RAPD-PCR technique adapted by Teaumroong and Boonkerd (1998). Randomly amplified polymorphic DNA finger printing was done by using OPI- 01 (ACCTGGACAC) and OPI-06 primer (AAGGCGGCAG).

Statistical analysis. The data were analyzed statistically by analysis of variance technique and comparison among means was made by Duncan's Multiple Range Test (DMRT) using MSTAT-C version 1.4.2.

RESULTS AND DISCUSSION

Twelve each of *Rhizobium* and *Azospirillum* strains were isolated from rhizosphere soil and root samples (Table I). The colony morphology of *Rhizobium* strains on agar plates was 2-7 mm with copius mucoid slime after an incubation of 3 days at 28°C. Under the microscope all the rhizobial isolates were rod shaped. Under light microscope all *Azospirillum* were short or medium sized, motile rods.

The cfu of both *Rhizobium* and *Azospirillum* appeared to be the function of soil moisture. Data (Table II) revealed that *Rhizobium* and *Azospirillum* isolates obtained from roots and rhizosphere of plants growing under irrigated conditions (15-17% soil moisture) of NARC showed maximum value of colony count (as measured by log cfu mL^{-1}). Isolates from Attock region (soil moisture of 5-6%) had cfu less than isolates from Kallar Sayedan (available soil moisture 7-8.5%). Soil moisture had a more pronounced effect on log cfu at reproductive stage as compared to vegetative stage.

Maximum value of colony count was obtained for *Rhizobium* and *Azospirillum* isolates obtained from roots and rhizosphere of plants growing under irrigated conditions

Table I. *Rhizobium* and *Azospirillum* isolates collected from rhizospheric soil and roots of Maize plants collected from arid and semi arid areas (Attock & Kallar Sayedan) and well watered conditions NARC (National Agriculture Research Center, Islamabad)

Samples	*Root*			*Soil*		
	Irrigated area (NARC)	**Arid area (Attock)**	**Irrigated area (NARC)**	**Arid area (Attock)**	**Irrigated area (NARC)**	**Arid area (Attock)**
Rhizobium-vegetative stage	R_1RC	R_3RA	R_5RK	R_2SC	R_4SA	R_6SK
Rhizobium-reproductive stage	R_7RC	R_9RA	$R_{11}RK$	R_8SC	$R_{10}SA$	$R_{12}SK$
Azospirillum–vegetative stage	A_1RC	A_3RA	A_5RK	A_2SC	A_4SA	A_6SK
Azospirillum-reproductive stage	A_7RC	A_9RA	$A_{11}RK$	A_8SC	$A_{10}SA$	$A_{12}SK$

Table II. Log Colony forming unit(c.f.u/ml) of *Rhizobium* and *Azospirillum* isolates collected from rhizospheric soil and roots of Maize plants collected from arid and semi arid areas (Attock & Kallar Sayedan) and well watered conditions NARC(National Agriculture Research Center, Islamabad) at 5%level of significance

Samples	*Root*			*Soil*		
Tests	**Irrigated area (NARC)**	**Arid area (Attock)**	**Semi-arid Area (Kallar Sayedan)**	**Irrigated area (NARC)**	**Arid area (Attock)**	**Semi-arid area (Kallar Sayedan)**
CFU (*Rhizobium*-vegetative stage	3.28b	2.12f	2.61c	3.48a	2.56b	2.61c
CFU (*Rhizobium*-reproductive stage)	3.40a	2.26e	2.40d	3.35a	2.48e	2.51b
CFU(*Azospirillum*–vegetative stage	3.94b	3.07e	3.28d	4.12a	3.85b	4.09a
CFU (*Azospirillum*–reproductive stage	4.16a	3.67c	3.15e	3.83b	3.67c	3.71c

Table III. Morphological and biochemical characteristics of *Rhizobium* isolates(QTS) isolated from rhizospheric soil and roots of Maize plants collected from arid and semi arid areas (Attock & Kallar Sayedan) and well watered conditions NARC(National Agriculture Research Center, Islamabad)

Tests	*Reactions*	*Group I*	*Group II*	*Group III*
Isolates		**R1RC, R2SC, R7RC, R8SC**	**R5RK, R6SK, R11RK, R12SK**	**R3RA, R4SA, R9RA, R10SA**
CO	Catalse oxidase	+	+	+
OPNG	Ortho nitro phenyl β-D-galactopyranoside	+	+	+
CIT	Sodium citrate	-	-	+
MALO	Sodium malonate	+	-	-
LDC	Lysine decarboxylase	+	+	+
ADH	Arginine dihydrolase	+	+	-
ODC	Orthinine decarboxylase	+	-	-
H_2S	H_2S production	+	+	+
URE	Urea hydrolysis	+	-	-
TDA	Tryptophane deaminase	+	+	-
IND	Indole	+	-	-
VP	Voger Proskaur(acetion)	+	+	+
GEL	Gelatin hydrolysis	-	-	-
GLU	Acid from glucose	-	-	-
MAL	Acid from maltose	+	+	-
SUC	Acid from sucrose	-	-	-
MAN	Acid from mannitol	+	+	+
ARA	Acid from arabinose	+	+	+
RHA	Acid from rhammose	+	-	-
SOR	Acid from sorbitol	+	-	-
INO	Acid from inositol	-	-	-
ADON	Acid from adontol	+	+	-
MEL	Acid from melibiose	+	-	-
RAF	Acid from raffinose	-	-	-

Rhizobium strains from well watered and water stressed conditions. R1RC: from roots of plants growing at NARC collected at vegetative stage; R2 SC: from Rhizosphere Soil of plants growing at NARC at vegetative stage; R3RA: from roots of plants growing at Attock at vegetative stage; R4SA: from Rhizosphere Soil of plants growing at Attock at vegetative stage; R5RK: from roots of plants collected from Kallar Sayedan at vegetative stage; R6SK: from rhizospheric soil of plants growing at Kallar Sayedan at vegetative stage; R7RC: from roots of plants growing at NARC collected at reproductive stage; R8SC: from Rhizosphere Soil of plants growing at NARC at reproductive stage; R9RA: from roots of plants growing at Attock at reproductive stage; R10SA: from Rhizosphere Soil of plants growing at Attock at reproductive stage; R11RK: from roots of plants collected from Kallar Sayedan at reproductive stage; R12SK: from rhizospheric soil of plants growing at Kallar Sayedan at reproductive stage.

at reproductive stage. Swêdrzyñska and Sawicka (2001) reported that developmental stage of a cultivated plant determines, to a sufficient degree, the number of bacteria occurring in a crop. A similar relationship with regard to the dependence of bacterium number on plant developmental stage was also recorded by Sawicka (1983). At reproductive stage there was more drastic difference in value of colony count of *Rhizobium* and *Azospirillum* isolates obtained from

Table IV. DNA number and Size in *Rhizobium strains* on the basis of RAPD-PCR analysis using random primer OP-01

Strains	*DNA number*	*Size of bands(bp)*								
		5000	4000	3000	2000	1500	1000	750	600	500
R_1RC	5	-	-		+	+	+	_	-	+
R_2SC	3	-	-	+	-	-	+	+	-	-
R_3RA	6	-	+	+	-	+	+	+		+
R_4SA	3	-	-	+	-	-	+	+	-	-
R_5RK	5	-	+	+	-	+	+	-	-	+
R_6SK	5	-	+	+	-	+	+	-	-	+
R_7RC	4	-	-	+	+	+	+	-	-	-
R_8SC	4	-	-	+	+	+	+	+	-	-
R_9RA	3	-	-	+	+	-	-	-	-	+
R_{10}SA	3	-	-	+	+	-	-	-	-	+
R11RK	4	-	-	+	+	+	-	-	--	+
R_{12}SK	6	+	-	+	+	+	-	-	+	+

Table V. DNA number and Size in *Rhizobium strains* on the basis of RAPD-PCR analysis using random primer OP-06

Strains	*DNA number*	*Size of bands(bp)*								
		5000	4000	3000	2000	1500	1000	750	600	500
R_1RC	6	-	-	-	+	+	+	-	+	+
R_2SC	3	-	-	+	-	-	+	-	-	-
R_3RA	6	-	+	+	+	-	+	+		+
R_4SA	3	-	-	+	-	-	+	+	-	-
R_5RK	7	-	+	+	-	+	+	-	+	+
R_6SK	6	-	+	+	-	+	+	-	+	+
R_7RC	4	-	-	+	+	+	+	-	-	-
R_8SC	5	-	-	+	+	+	+	-	+	-
R_9RA	3	-	-	+	+	-	-	-	-	+
R_{10}SA	3	-	-	+	+	-	-	-	-	+
R11RK	4	-	-	+	+	-	+	-	--	+
R_{12}SK	6	+	-	+	+	-	+	-	+	+

Table VI. Molecular characterization of *Rhizobium* strains based on RAPD (Randomly Amplified Polymorphic DNA) analysis

Band (bp)	*Group I R1RC, R2SC, R7RC, R5RK, R6SK, R8SC*	*Group II R3RA, R4SA, R9RA, R10SA,R11RK, R12SK*
5000	-	+
4000	+	-
3000	+	+
2000	+	+
1500	+	-
1000	+	+
750	-	+
600	+	-
500	+	+

R1RC: from roots of plants growing at NARC collected at vegetative stage; R2 SC: from Rhizosphere Soil of plants growing at NARC at vegetative stage; R3RA: from roots of plants growing at Attock at vegetative stage; R4SA: from Rhizosphere Soil of plants growing at Attock at vegetative stage; R5RK: from roots of plants collected from Kallar Sayedan at vegetative stage; R6SK: from rhizospheric soil of plants growing at Kallar Sayedan at vegetative stage; R7RC: from roots of plants growing at NARC collected at reproductive stage; R8SC: from Rhizosphere Soil of plants growing at NARC at reproductive stage; R9RA: from roots of plants growing at Attock at reproductive stage; R10SA: from Rhizosphere Soil of plants growing at Attock at reproductive stage; R11RK: from roots of plants collected from Kallar Sayedan at reproductive stage; R12SK: from rhizospheric soil of plants growing at Kallar Sayedan at reproductive stage

Fig. 1. PCR amplification of DNA isolated from *Rhizobium* strains using random primer OP-01

In this and subsequent figures: Lane1=1kb DNA ladder Lane 2(R1RC): from roots of plants growing at NARC collected at vegetative stage; Lane 3(R2SC): from Rhizosphere Soil of plants growing at NARC at vegetative stage; Lane 4 (R3RA): from roots of plants growing at Attock at vegetative stage; Lane 5 (R4SA): from Rhizosphere Soil of plants growing at Attock at vegetative stage; Lane 6 (R5RK): from roots of plants collected from Kallar Sayedan at vegetative stage; Lane 7(R6SK): from rhizospheric soil of plants growing at Kallar Sayedan at vegetative stage; Lane 8 (R7RC): from roots of plants growing at NARC collected at reproductive stage; Lane 9 (R8SC): from Rhizosphere Soil of plants growing at NARC at reproductive stage; Lane 10 (R9RA): from roots of plants growing at Attock at reproductive stage; Lane 11 (R10SA): from Rhizosphere Soil of plants growing at Attock at reproductive stage; Lane 12(R11RK): from roots of plants collected from Kallar Sayedan at reproductive stage; Lane 13 (R12SK): from rhizospheric soil of plants growing at Kallar Sayedan at reproductive stage.

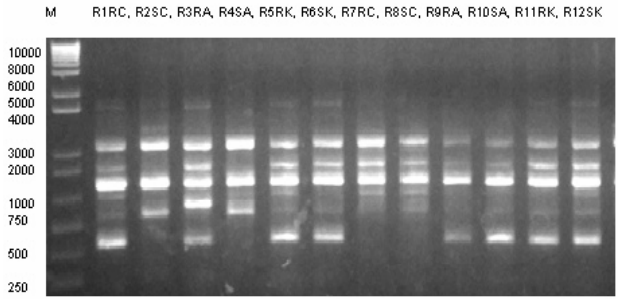
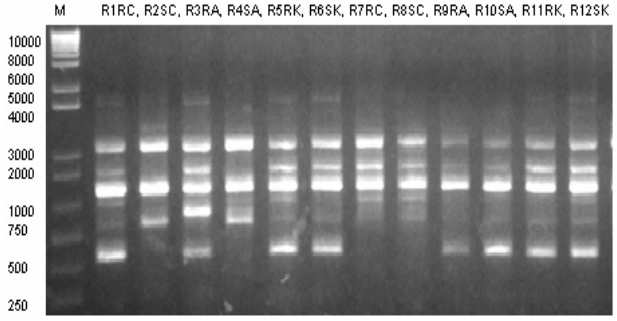


Fig. 2. PCR amplification of DNA isolated from *Rhizobium* strains using random primer OP-06



roots and rhizosphere of plants growing under low moisture conditions as compared to those of irrigated conditions. There was little chance for recovery from low soil moisture in the reproductive stage (Franson *et al.*, 1991).

The ability of *Rhizobium* sp. to survive at a low water potential in soil has been established by many studies in which viability was assessed by determining colony forming ability on agar plates (Matt *et al*., 1989). Drought is an important environmental factor that can affect rhizobial competition and survival (Athar & Johnson, 1997; Serraj *et al.*, 1999). The *Rhizobium* isolates obtained from well watered conditions showed better utilization of carbohydrates as compared to isolates of drought conditions. The low utilization of carbohydrates may be due to low metabolic activity of these *Rhizobium* isolates under low

Table VII. Morphological and biochemical characteristics of *Azospirillum* strains(QTS) isolated from rhizospheric soil and roots of Maize plants collected from arid and semi arid areas (Attock & Kallar Sayedan) and well watered conditions NARC(National Agriculture Research Centre, Islamabad)

Tests	***Reactions***	***Group I***	***Group II***	***Group III***
Isolates		**A1RC, A2SC, A7RC, A8SC**	**A5RK, A6SK, A11RK, A12SK**	**A3RA, A4SA, A9RA, A10SA**
CO	Catalse oxidase	+	+	+
OPNG	Ortho nitro phenyl β-D-galactopyranoside	-	-	-
CIT	Sodium citrate	+	+	+
MALO	Sodium malonate	+	-	-
LDC	Lysine decarboxylase	+	+	-
ADH	Arginine dihydrolase	+	+	+
ODC	Orthinine decarboxylase	+	+	+
H_2S	H_2S production	-	-	-
URE	Urea hydrolysis	+	+	+
TDA	Tryptophane deaminase	+	-	-
IND	Indole	-	-	-
VP	Voger Proskaur(acetion)	-	-	-
GEL	Gelatin hydrolysis	+	+	-
GLU	Acid from glucose	+	+	+
MAL	Acid from maltose	-	-	-
SUC	Acid from sucrose	-	-	-
MAN	Acid from mannitol	+	+	+
ARA	Acid from arabinose	+	-	-
RHA	Acid from rhammose	-	-	-
SOR	Acid from sorbitol	-	-	-
INO	Acid from inositol	-	-	-
ADON	Acid from adontol	+	-	-
MEL	Acid from melibiose	+	-	-
RAF	Acid from raffinose	+	+	-

Azospirillum strains from well watered and water stressed conditions.

A1RC: from roots of plants growing at NARC collected at vegetative stage; A2 SC: from Rhizosphere Soil of plants growing at NARC at vegetative stage; A3RA: from roots of plants growing at Attock at vegetative stage; A4SA: from Rhizosphere Soil of plants growing at Attock at vegetative stage; A5RK: from roots of plants collected from Kallar Sayedan at vegetative stage; A6SK: from rhizospheric soil of plants growing at Kallar Sayedan at vegetative stage; A7RC: from roots of plants growing at NARC collected at reproductive stage; A8SC: from Rhizosphere Soil of plants growing at NARC at reproductive stage; A9RA: from roots of plants growing at Attock at reproductive stage; A10SA: from Rhizosphere Soil of plants growing at Attock at reproductive stage; A11RK: from roots of plants collected from Kallar Sayedan at reproductive stage; A12SK: from rhizospheric soil of plants growing at Kallar Sayedan at reproductive stage

Table VIII. DNA number and Size in *Azospirillum strains* on the basis of RAPDPCR analysis using random primer OP-01

Strains	***DNA number***	***Size of bands(bp)***							
		4000	**3000**	**2000**	**1500**	**1000**	**750**	**500**	**300**
A_1RC	6	-	-	+	+	+	+	+	+
A_2SC	4	-	+	+	-	+	+	-	-
A_3RA	5	+	+	+	-	+	+	-	-
A_4SA	6	-	+	+	-	+	+	+	+
A_5RK	6	+	+	+	+	+	+	-	-
A_6SK	7	+	+	+	+	+	+	+	-
A_7RC	5	-	+	+	+	+	+	-	-
A_8SC	5	-	+	+	+	+	+	-	-
A_9RA	5	-	+	+	-	+	+	+	-
A_{10}SA	5	-	+	+	-	+	+	+	-
A11RK	4	-	+	+	-	+	+	-	-
A_{12}SK	5	-	+	+	-	+	+	+	-

soil moisture. Swaine (2006) demonstrated that the growth of rhizobia at low osmotic potentials requires the production of many polyols or amino compounds, which demand considerable amounts of energy, leading to increased specific respiration rates and enhanced heat production. Both of these responses are associated with reduced growth efficiency of rhizobia at low matric potentials.

On the basis of carbon/nitrogen source utilization patterns the *Rhizobium* isolates were classified into three groups (Table III). In case of *Rhizobium*, sodium malonate, gelatin hydrolysis and tryptophan deaminase tests were negative for isolates from low soil moisture. The *Rhizobium* isolates R_1RC, R_2SC, R_7RC; R_8SC (from well watered conditions) showed better utilization of carbohydrates as compared to isolates R_3RA, R_4SA, R_9RA and R_{10}SA obtained from arid (Attock) area. The isolates R_5RK, R_6SK, R_{11}RK, R_{12}SK (from Kallar Sayedan) showed different carbon/nitrogen utilization pattern and these were placed in separate group.

Azospirillum strains were also placed into three groups on the basis of their carbon/nitrogen utilization pattern (Table VII). The tests result of acid from arabinose, acid from adontol, acid from melibiose, acid from raffinose and tryptophan deaminase were negative for *Azospirillum* isolates from low soil moisture. The grouping was similar to that of *Rhizobium*. Isolates of group I A_1RC, A_2SC, A_7RC, A_8SC (from well watered conditions) showed better utilization of carbohydrates as compared to A_3RA, A_4SA, A_9RA, A_{10}SA (group II) which were obtained from arid region of Attock. The strains A_5RK, A_6SK, A_{11}RK, A_{12}SK (from Kallar Sayedan) showed different carbon/nitrogen utilization pattern and these were placed in separate group

Table IX. DNA number and Size in *Azospirillum strains* on the basis of RAPD-PCR analysis using random primer OP-06

Strains	*DNA*	*Size of bands(bp)*								
	number	**5000**	**4000**	**3000**	**2000**	**1500**	**1000**	**750**	**600**	**500**
A_1RC	5	-	-	+	-	+	+	-	-	-
A_2SC	3	-	-	+	-	+	+	-	+	-
A_3R*A*	5	-	+	+	+	-	+	+	-	-
A_4SA	5	-	+	+	+	-	+	+	-	-
A_5RK	5	-	+	+	-	-	+	-	-	-
A_6SK	4	-	+	+	-	+	+	-	-	-
A_7RC	4	-	+	+	-	+	+	-	-	-
A_8SC	4	-	+	+	-	+	+	-	-	-
A_9RA	4	-	+	+	+	-	+	+	-	-
A_{10}SA	5	-	+	+	+	-	+	+	+	-
A11RK	4	-	+	+	+	-	+	+	-	-
A_{12}SK	7	+	-	+	+	-	+	+	+	+

Table X. Molecular characterization of *Azospirillum* strains based on RAPD-PCR (Randomly Amplified Polymorphic DNA) analysis

Band (bp)	*Group I* *A1RC, A2SC, A5RK, A6SK , A7RC, A8SC*	*Group II* *A3RA, A4SA, A9RA, A10SA, A11RK, A12SK*
5000	-	+
4000	+	+
3000	+	+
2000	-	+
1500	+	-
1000	+	+
750	-	+
600	+	-
500	-	+

A1RC: from roots of plants growing at NARC collected at vegetative stage; A2 SC: from Rhizosphere Soil of plants growing at NARC at vegetative stage; A3R*A*: from roots of plants growing at Attock at vegetative stage; A4SA: from Rhizosphere Soil of plants growing at Attock at vegetative stage; A5RK: from roots of plants collected from Kallar Sayedan at vegetative stage; A6SK: from rhizospheric soil of plants growing at Kallar Sayedan at vegetative stage; A7RC: from roots of plants growing at NARC collected at reproductive stage; A8SC: from Rhizosphere Soil of plants growing at NARC at reproductive stage; A9RA: from roots of plants growing at Attock at reproductive stage; A10SA: from Rhizosphere Soil of plants growing at Attock at reproductive stage; A11RK: from roots of plants collected from Kallar Sayedan at reproductive stage; A12SK: from rhizospheric soil of plants growing at Kallar Sayedan at reproductive stage

III. Swêdrzyñska and Sawicka (2001) found that increased growth rate and metabolism of bacteria is probably related with the development of root system, photosynthetic activity and the amount of exudates associated with this and produced by plants.

The genetic diversity among the isolates was assessed by RAPD analysis. All the isolates showed reproducible DNA banding pattern. Diversity among the isolates was assessed on the basis of variation of size number and intensity of bands (Saleena *et al*., 2001). DNA was amplified by using RAPD-PCR revealed banding patterns depending on the number and size of amplified products were observed for *Rhizobium* and *Azospirillum* isolates (Table IV,V VIII & IX; Fig 1 − 2). All the *Rhizobium* and *Azospirillum* isolates shared different DNA banding pattern. The strains with identical DNA fingerprints were placed in one group.

Fig. 3. PCR amplification of DNA isolated from *Azospirillum* strains using random primer OP-01

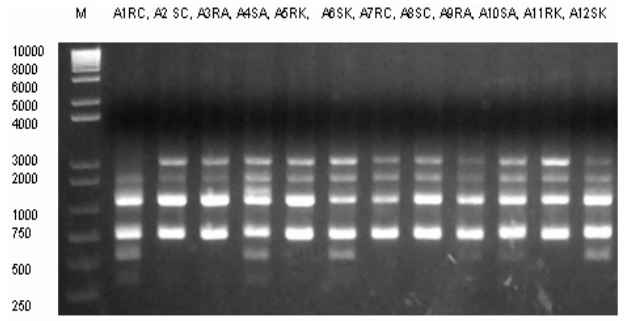
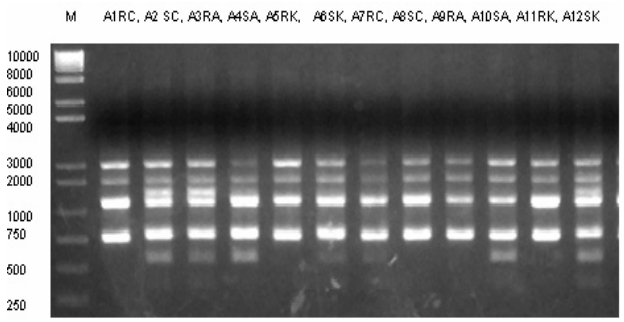


Fig. 4. PCR amplification of DNA isolated from *Azospirillum* strains using random primer OP-06



The *Rhizobium* isolates fell into three groups on the basis of carbon/nitrogen utilization pattern but classified into two groups on the basis of banding pattern of RAPD. The results showed that though R_5RK, R_6SK, R_{11}RK, R_{12}SK belonged to different groups on the basis of carbon/nitrogen utilization, genetically they differed from each other. R_5RK, R_6SK were more similar to R_1RC, R_2SC, R_7RC, R_8SC and R_{11}RK, R_{12}SK to R_3RA, R_4SA, R_9RA and R_{10}SA, respectively (Table VI). Similarly, the *Azospirillum* isolates were placed into three groups on the basis of carbon/nitrogen utilization pattern but fall into two groups on the basis of banding pattern of RAPD (Table VII). The results showed that though A_5RK, A_6SK, A_{11}RK, A_{12}SK showed to be a different group on the basis of carbon/nitrogen utilization source, genetically they were different. A_5RK and A_6SK were more similar to A_1RC, A_2SC, A_7RC, A_8SC and A_{11}RK, A_{12}SK to A_3R*A*, A_4SA, A_9RA and A_{10}SA, respectively (Table X). RAPD technique has been frequently used for identification and differentiation of bacterial strains such as *Rhizobium* (Hebb *et al*., 1998) and *Azospirillum* (Fani *et al*., 1993). According to Young and Cheng (1998), RAPD is a potential tool for the identification of the genetics and systematics of different populations. Possible reason for genetic diversity might be that low soil moisture may have resulted in genetic adaptations of the strains. However, variation among different strains of diverse origin suggested that there is genetic potential to improve tolerance to environmental

stress factor such as low soil moisture.

The results showed the diversity of *Rhizobium* and *Azospirillum* field populations and different types of strains isolated from different moisture regimes. It is well documented that Rhizobia are capable of surviving under low water potential (Mahler & Wollum, 1981; Fuhrmann *et al*., 1986). It is possible to identify *Rhizobium* and *Azospirillum* strains from nature that have a distinct tolerance for waters stress, high temperature, pH and salt concentration (Sauvage *et al*., 1983). *Rhizobium* and *Azospirillum* strains growing under moisture regimes showed differences in biochemical and genetic characterization. Gene sequencing is essential for final grouping of isolates.

CONCLUSION

These results assume a significant role of *Rhizobium* and *Azospirillum* in the improved fertility of soil in arid and semi arid habitats. On the basis of colony morphology and biochemical tests the rhizobial isolates were tentatively identified belonging to *Rhizobium leguminosarum*, whereas *Azospirillum* isolates were tentatively identified as *Azospirillum lipoferum*.

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