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PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *AZOSPIRILLUM* SPP. ISOLATED FROM MAIZE UNDER WATER STRESS

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Abstract

This study was designed to isolate and characterize *Azospirillum* strains from maize (*Zea mays* L.) grown under well-watered and water stressed conditions and to evaluate the ability of bacteria to produce plant growth promoting hormones like Indole Acetic Acid (IAA), Gibberellic Acid (GA), trans-Zeatin riboside (tzr) and abscisic acid (ABA). A total of eight strains of *Azospirillum* were isolated from rhizosphere and roots of maize plants grown in pots and it was observed that survival efficiency of *Azospirillum* from well watered plants was higher as compared to that of *Azospirillum* strains isolated from roots and rhizosphere samples of water stressed plants (having 8-12% soil moisture). Preliminary identification of isolates was made on the basis of morphological characteristics and carbon/nitrogen source utilization patterns using QTS-24 kits. The genetic diversity among the isolates was evaluated by Randomly Amplified Polymorphic DNA finger printing and similarity matrix. Inoculation of wheat with isolates from water-stressed plants induced tolerance to water stress in inoculated plants. Isolates from water-stressed conditions produced low concentration of indole acetic acid, gibberellic acid, and trans zeatin riboside but higher concentration of abscisic acid. The isolated bacterial strains have technological implications for inoculants formulation and improved growth of cereal crops.

Introduction

The use of beneficial soil microorganisms as agricultural inputs for improved crop production requires the selection of rhizosphere-competent microorganisms with plant growth-promoting attributes (Hynes *et al*., 2008). Water stress affects virtually all climatic regions of the world (Wilhite *et al*., 2000) and more than one-half of the earth is susceptible to drought every year (Hewitt, 1997; Kogan, 1997). Drought is one of the major environmental stresses that limit the growth of plants and the production of crops (Shinozaki *et al*., 2003).Desiccation affects microbial population structure (Ilyas *et al.,* 2008). Exposure to extreme environmental conditions as imposed during dry season alters soil microbial activity (Castro-Sowinski *et al*., 2007). The introduction of beneficial bacteria can normalize and, in some cases, improve plant performance in stressful environments and thereby preserve or enhance yield (Bensalim *et al*., 1998).The adapted micro-symbionts can be used to protect their hosts from these stresses (Vivas *et al*., 2003) so as to maintain adequate food production and be used as possible biosensor of the soil functioning (Köves-Péchy *et al*., 2003). Bacteria growing in sites where water is limited or where dry periods occur frequently have been shown to promote plant growth better than those growing in sites where water is abundant (Mayak *et al*., 2004). Many soil PGPRs, such as *Azospirillum* can compensate for the reduction in plant growth produced by drought stress (Zahir *et al*., 2007) and some other unfavorable environmental conditions.

Most species of the genus *Azospirillum* are known to act as plant growth-promoting rhizobacteria (PGPR) and stimulate plant growth directly either by synthesizing phytohormones or by promoting improved N nutrition through BNF(Jiang *et al*., 2007). PGPR also produce several other growth promoting substances including IAA, GA3, zeatin and ABA (Perrig *et al*., 2007). Members of genus *Azospirillum* are able to exert beneficial effects on plant growth and yield of many agronomic crops under a variety of environmental and soil conditions (Dobbelaere *et al*., 2001). *Azospirillum* inoculation alleviates low soil moisture on wheat and maize seedlings grown under water stress conditions (Pereyra *et al*., 2006). *Azospirillum* spp., isolated from moisture stressed conditions can improve tolerance to water stress, and thus they can be inoculated to promote plant growth on stressed sites, such as semiarid and arid regions (Ilyas & Bano, 2010).

In this paper we investigated the effect of water stress on occurrence, carbon/nitrogen utilization pattern, phytohormone production and genetic diversity of maize associated *Azospirillum* strains. The selected strain, which can be successfully used as inoculant under exposure to water stress, was identified on basis of 16S rRNA sequence analysis.

Materials and Methods

An experiment was conducted under natural condition in a net house of Quaid-i-Azam University, Islamabad, Pakistan. Seeds of two varieties (EV-4001 ad EV-1098) of maize *(Zea mayz* L.*)* were obtained from Crop Research Institute, National Agriculture Research Center (NARC) Islamabad and were sown in pots measuring 24 x 30 cm^2, filled with sand and soil (collected from nearby farmer's field) in 1:3 ratios, during first week of July for two consecutive years, 2006 and 2007. Soil pH and EC were determined with the help of pH and EC meter which were 8.2 and 126.67µs/cm, respectively. Surface sterilization of seeds was done with $HgCl_2$ (0.1%), followed by several washings with distilled water. Fertilizers were applied at the recommended rate of 140-90-60 kg ha^{-1} N, P and K, respectively in the form of urea, single super phosphate and potassium sulphate. Five pots were randomized at regular interval for each

treatment and irrigated at field capacity by daily addition of water. Five plants per pot were allowed to grow.

Water stress was induced at two stages (once at tillering stage of crop, i.e., 27 DAS in case of maize crop), (second at anthesis stage, 80 DAS for maize crop) by withholding the supply of water for 7 days. The control plants were sprayed with distilled water as required. During water stress treatment, plants were protected from rain by plastic sheet. After 7 days, plants were harvested and their root samples were collected and stored at 4°C till further analyses. For drought treatment, plants were covered with polypropylene until harvest. Soil samples for isolation of bacteria were also collected at end of each treatment.

A composite soil sample from thoroughly mixed soil was taken to calculate soil moisture according to following formula:

$$\text{Soil moisture (\%)} = \frac{\text{Weight of wet soil (g) -Weight of dry soil (g)}}{\text{Weight of dry soil (g)}} \times 100$$

Isolation, morphology and population size of *Azospirillum* strains: Five plants each from irrigation and drought treatment were randomly selected for bacteria isolation. A series of decimal dilutions of rhizoshere soil were prepared by suspending 10 g rhizosphere soil in 90 ml of sterilized distilled water. An aliquot (100 µl) from three dilutions (10^{-1}, 10^{-5}, and 10^{-10}) was used to inoculate nitrogen-free (NF) medium in Mc Cartany's vials incubated at 30°C for 48 h. Vials with bacterial growth were used for inoculation of isolates on Luria Bertani (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. Roots (1 g) were surface-sterilized by shaking them in 0.1% $HgCl_2$ solutions for 1–2 min, followed by five to six washings with sterilized distilled water. One gram of surface-sterilized roots of plants was homogenized, and a series of decimal dilutions were prepared. Further purifications were made in same manner as that from rhizosphere. The vials with bacterial colonies were used to inoculate LB (Miller, 1972) agar plates to obtain pure colonies of *Azospirillum* species. Viable cell counts were calculated as suggested by James (1978):

Viable cell count (CFU/g) = (number of colonies × dilution factor/volume of inoculums)

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

Biochemical characterization: Gram staining of bacterial cultures was done as described previously by Vincent (1970). Oxidase test was performed according to Steel (1961) Kovac's reagent (1% N,N,N,N-tetramethyle-p-phenylene diamine dissolved in warm water and stored in dark bottle; Kovacs, 1956). A strip of filter paper was dipped in this reagent and air-dried. With the help of sterile wire loop, 1- day-old colonies of bacterial cultures 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 was performed according to the procedure of MacFaddin (1980). Bacterial 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. Physiological and biochemical tests 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. The obtained results were compared with already described standard species reported in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Genotypic characterization (RAPD-DNA finger printing and 16S-rRNA gene sequencing): DNA to identify bacterial isolates was extracted from *Azospirillum* isolates as described by Chen and Kuo (1993). Cultures of *Azospirillum* strains were streaked on LB plates and grown in incubators (incubation at 35°C for *Azospirillum*). From plates, single colonies of *Azospirillum* were inoculated into test tubes containing LB broth media and grown in shaker (Excella E24, New Brunswick Scientific USA) at 80 rpm overnight. DNA was extracted from culture as follows: 1.5 ml of a saturated culture was harvested with centrifugation for 3 min at (7,000×g). The cell pellet was resuspended and lysed in 200 µl of lysis buffer (40 mM Tris-acetate pH7.8, 20 mM sodium acetate, 0.5 mM EDTA, 1% SDS) by vigorous pipetting. To remove protein and cell debris, 65 µl of 5 M NaCl solution was added and mixed well, and the viscous mixture was centrifuged at (7,000×g) for 10 min at 4°C. After transferring the clear supernatant to a new vial, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was formed. Following centrifugation at (7,000×g) for 3 min, the extracted supernatant was transferred to another vial, and the DNA was precipitated with 100% EtOH, washed twice with 70% EtOH, and redissolved in 50 µl autoclaved cold water; DNA sample (5µl) was mixed with 2µl of 6× loading dye, loaded into 1% agarose gel (stained with 0.01 g/ml ethedium bromide), and electrophoresis was run at 100 V. After 30 min, bands were observed under UV transilluminator lamp. DNA ladder (1 kb) was used to mark the bands. Genetic biodiversity and polymorphism among the isolated strains were determined by the random amplification of polymorphic DNA–polymerase chain reaction (RAPD-PCR) technique adapted by Teaumroong and Boonkerd (1998). Randomly amplified

polymorphic DNA finger printing was done by using OP-01 (ACCTGGACAC) and OP-06 primer (AAGGC GGCAG). Genomic DNAs of *Azospirillum* isolates were amplified as described by Weisburg *et al*., (1991). The PCR was carried out by forward (fd1) primer with nucleotide sequence AGAGTTTGATCCTGGCTCAG and reverse rd1 primer (AAGGAGGTGATCCAGCC). The reaction was carried out in a thermocycler (Biometra, Germany). Each reaction volume (25µl) contained 1µl of template DNA, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 5µl of 10× Taq buffers, 1 unit of Taq DNA polymerase, and 10 pmol of each primer. The volume was raised to 25µl by autoclaved cold water. After denaturation at 95°C for 2 min, 30 cycles with temperature values of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min were followed by incubation at 72°C for 10 min. Then, 5µl of amplified PCR products were electrophoresed on 1.2% (w/v) agarose gel, in 1× TBE buffer at 80 V, and stained with ethidium bromide (0.01 g/ml). Gel was visualized under UV transilluminator lamp (S. N. 76 S/64069, Bio RAD, Italy) and photographed. As a marker, we used 1-kb DNA ladder (Fermentas, Germany). The DNA bands were excised from gel, and DNA was purified by gel purification kits (JET quick, Gel Extraction Spin Kit, GENOMED) and sequenced on automated sequencer (ABI PRISM 310 Genetic Analyzer). Amplification primers were used for sequencing both strands of PCR product. The sequence was compared with standard databases by BLAST (NCBI) software and deposited at NCBI databank and accession number GQ255950 was obtained.

Extraction, purification and quantification of phytohormones from bacterial culture: Isolated bacterial strains were analyzed for abscisic acid, indole 3-acetic acid (IAA) and gibberellic acid (GA) production in pure culture. *Azospirillum* strains were grown in NF liquid medium. Ammonium chloride (1.0 g/l) and tryptophan (100 mg/l) were added in nitrogen-free medium. Tryptophan was added as precursor for IAA biosynthesis. Growth media (100 ml) were inoculated with 24-h-old bacterial cultures and placed on a shaker (EXCELLA E24, New Brunswick Scientific USA) at 100 rpm, for 7 days. Thereafter, cultures were centrifuged (6,000×g) for 15 min, and supernatant was used for extraction of growth hormones excreted in the growth medium. The pH of supernatant was adjusted to 2.8 with 1 N HCl. Phytohormones were extracted with an equal volumes of ethyl acetate, as described by Tien *et al*., (1979). This ethyl acetate extract was evaporated to dryness at 35°C, and the residue was dissolved in 1,500µl of pure methanol (Sigma Chemical Co). The samples were analyzed on HPLC (Agilent 1100) using UV detector and C18 column (39× 300 mm). For identification of hormones, a 100-µl sample was filtered through 0.45 Millipore filter and injected into column. Pure IAA, GA, and abscisic acid (ABA) (Sigma Chemical Co. USA), dissolved in HPLC grade methanol, were used as standards for identification and quantification of bacterial hormones. Growth hormones were identified on the basis of retention time and peak area of the standard. Methanol, acetic acid, and water (29:1:70) were used as mobile phase. Flow rate was adjusted at 1 ml/min, with an average run time of 20 min/sample. The wavelength used for detection of IAA was 280 nm (Sarwar *et al*., 1992), whereas for GA and ABA analysis, it was adjusted at 254 nm (Li *et al*., 1994).

Inoculation effects of *Azospirillum* strains: Growth-promoting effects of *Azospirillum* used as an inoculant were studied on wheat varieties. Two strains, MRA_7 (from roots of plants grown under well-watered conditions) and MRA_{10} (isolated from roots of plants growing under water stress at anthesis stage) were used. Seeds were surface-sterilized with $HgCl_2$ (0.1%) and successively washed several times with sterilized water. Seeds were soaked overnight in 7-day-old cultures of *Azospirillum* isolates with optical density of 0.99 at 600 nm (10^7cfu/ml). Then inoculated seeds were sown in plastic pots (14×14 cm^2) pre-sterilized with 10% chlorox and filled with sand and soil in 3:1 and autoclaved at 121°C and 15 psi. After 4 weeks, water stress was imposed by withholding water supply for a period of 7 days, while non-stressed plants were irrigated as required. After 1 week, plants were harvested, and growth parameters (shoot length, root length, and fresh and dry weight of shoot and root) were recorded.

Statistical analysis: Mean and standard deviation values of data were calculated from at least five replicates. The data were analyzed statistically by analysis of variance and comparison among means by Duncan's multiple range test using MSTAT-C version 1.4.2. In case of RAPD-PCR the presence of band was scored as 1 and absence as 0 and 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.

Results

Survival efficiency (measured as cfu/g soil and root) of isolates from irrigated pots (soil moisture 22%) revealed higher values than those of isolates from roots and rhizosphere soil of plants grown water stressed conditions (soil moisture 8%) . The effect of low soil moisture on colony forming unit was more pronounced at anthesis stage of plant growth than at compared to tillering stage (Fig. 1). At tillering stage, 20–28% decrease in colony counts was observed as compared to control, whereas at anthesis stage, the colony count decreased by 27–38% with corresponding decrease in soil moisture 8%.

On the basis of carbon/nitrogen utilization patterns, the isolates from rhizosphere soil and roots of maize grown in field at different moisture regimes were classified into three groups. Group I included isolates from rhizosphere soil and roots of plants growing under well watered conditions at both tillering and anthesis stages and were named as MRA_7, MSA_7, MRA_9, and MSA_9. Group II included isolates from rhizosphere soil and roots of plants growing under water-stressed conditions at tillering stage and were named MRA_8 and MSA_8. Group III included isolates from rhizosphere soil and roots of plants growing under water-stressed conditions at anthesis stage and were named as MRA_{10} and MSA_{10} (Table 1).

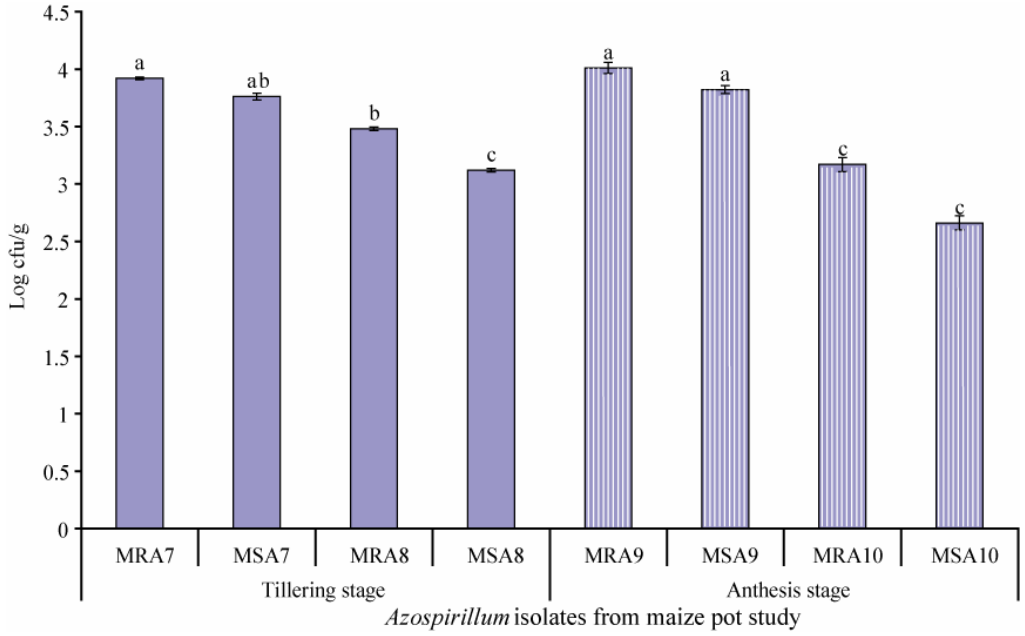

Fig. 1. Survival efficiency of strains of *Azospirillum lipoferum* isolated from roots and rhizosphere of irrigated and water stressed *Zea mays*. The bars bearing the same letter are not statistically different at 5 % level of significance.
MRA_7 Isolate from roots of well watered plants at tillering stage, MSA_7 Isolate from rhizosphere soil of well watered plants at tillering stage, MRA_8 Isolate from roots of water stressed plants at tillering stage, MSA_8 Isolate from rhizosphere soil of water stressed plants at tillering stage stage, MRA_9 Isolate from roots of well watered plants at anthesis stage, MSA_9 Isolate from rhizosphere soil of well watered plants at anthesis stage, MRA_{10} Isolate from roots of water stressed plants at anthesis stage, MSA_{10} *Azospirillum* Isolate from rhizosphere soil of water stressed plants at anthesis stage.

Table 1. Biochemical characterization of bacterial strains using QTS-24 miniaturized identification system.

Tests	**Group I** **MRA_7, MSA_7, MRA_9, MSA_9**	**Group II** **MRA_8, MSR_8**	**Group III** **MRA_{10}, MSA_{10}**
Colony morphology	Irregular, wrinkled, pink	Irregular, wrinkled, pink	Irregular, wrinkled, pink
Gram stain	-	-	-
Catalase and Oxidase	+	+	+
Ortho nitro phenyl β-D-galactopyranoside	-	-	-
Sodium citrate	+	+	+
Sodium malonate	+	+	+
Lysine decarboxylase	+	+	+
Arginine dihydrolase	+	+	+
Orthinine decarboxylase	+	+	+
H_2S production	-	-	-
Urea hydrolysis	+	+	+
Tryptophane deaminase	+	-	-
Indole	-	-	-
Voger Proskaur (Acetion)	-	-	-
Gelatin hydrolysis	+	+	-
Acid from glucose	+	+	+
Acid from maltose	-	-	-
Acid from sucrose	-	-	-
Acid from mannitol	+	+	+
Acid from arabinose	+	+	-
Acid from rhammose	-	-	-
Acid from sorbitol	-	-	-
Acid from inositol	-	-	-
Acid from adontol	+	-	-
Acid from melibiose	+	-	-
Acid from raffinose	+	+	-
Organism identified	*Azospirillum lipoferum*		

MRA_7 Isolate from roots of well watered plants at tillering stage, MSA_7 Isolate from rhizosphere soil of well watered plants at tillering stage, MRA_8 Isolate from roots of water stressed plants at tillering stage, MSA_8 MSA_7 Isolate from rhizosphere soil of water stressed plants at tillering stage stage, MRA_9 Isolate from roots of well watered plants at anthesis stage, MSA_9 Isolate from rhizosphere soil of well watered plants at anthesis stage, MRA_{10} Isolate from roots of water stressed plants at anthesis stage, MSA_{10} *Azospirillum* Isolate from rhizosphere soil of water stressed plants at anthesis stage.

The molecular weight of amplified products was determined by electophoresis on 1.2% (w/v) agarose gel. A band of 1.5 kb corresponding to 16SrRNA gene for each isolate was obtained. On the basis of colony morphology, gram staining, and carbon/nitrogen utilization pattern (QTS-24), the isolated strains were identified as members of genus *Azospirillum* (Bergey's Manual of Bacteriology). On the basis of unweighted pair group method with arithmetic means (UPGMA) cluster analysis of *Azospirillum* strains isolated from rhizosphere soil and roots of maize plants using OP -01 and OP-06 primer (Figs. 2a and 2b respectively), isolates were grouped into two clusters. UPGMA cluster analysis with OP-01 primer revealed that group I included isolates from well watered plants (at tillering and anthesis stage), whereas group II included isolates from water-stressed plants. UPGMA cluster analysis for OP-06 primer gave similar clusters as that of OP-01.

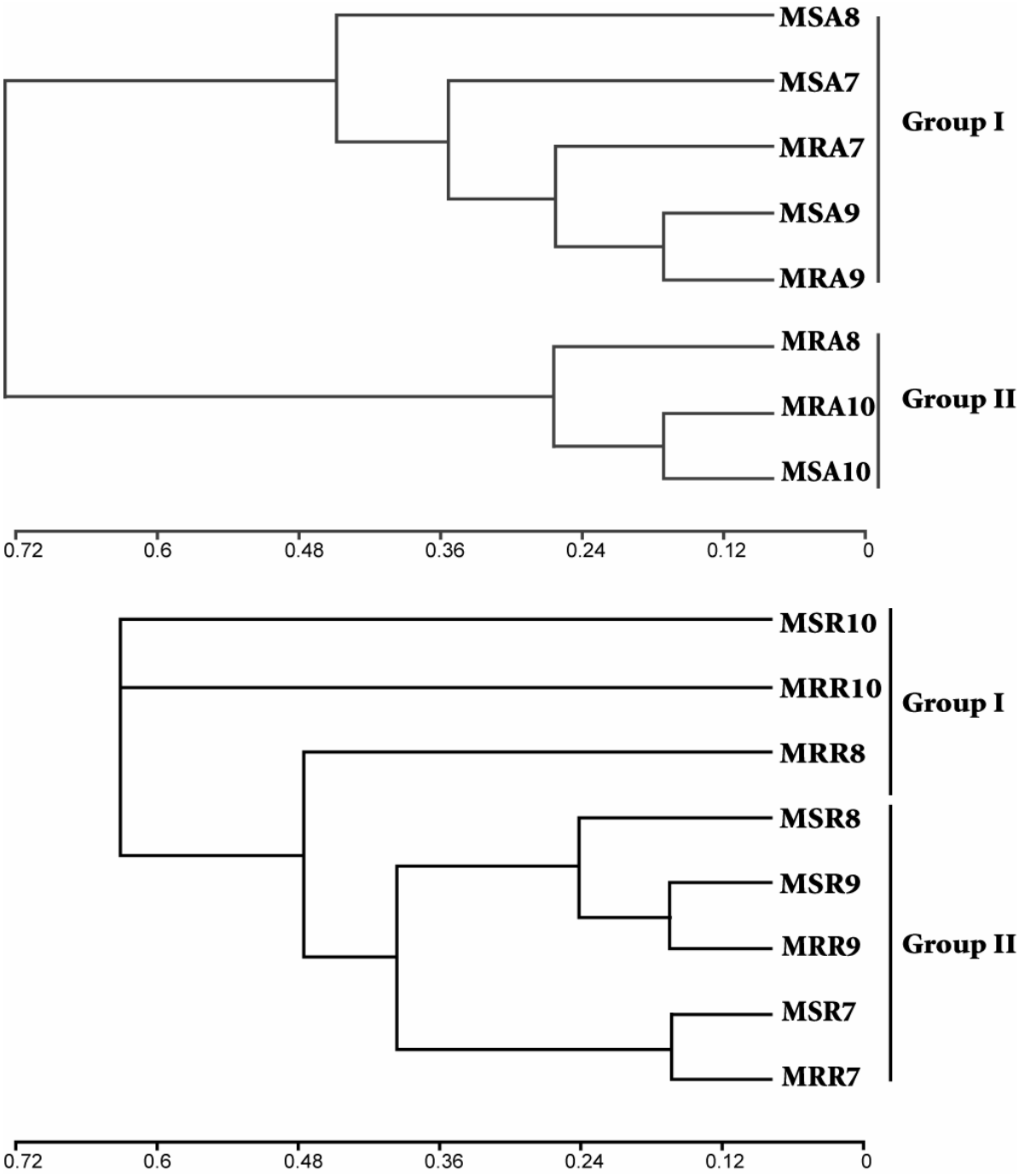

Fig. 2(a, b). The UPGMA Analysis of RAPD banding scores of *Azospirillum lipoferum* strains with OP-01 and OP-06 primers respectively.
The details of samples are similar as previously described in Fig 1.

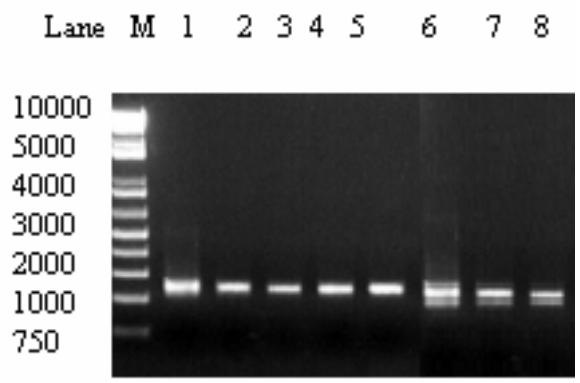

Fig. 3. PCR product of 16S rRNA gene of *Azospirillum* strains, from rhizospheric soil and roots of maize plants collected from water stressed conditions (soil moisture 12 and 8%) and well watered conditions (soil moisture 22%).
Lane M=1kb DNA ladder, Lane 1 *Azospirillum* isolated from roots of plants growing under well watered conditions at tillering stage, Lane 2 *Azospirillum* isolated from rhizosphere soil of plants growing under well watered conditions at tillering stage, Lane 3 *Azospirillum* isolated from roots of plants growing under water stressed conditions at tillering stage, Lane 4 *Azospirillum* isolated from rhizosphere soil of plants growing under water stressed conditions at tillering stage, Lane 5 *Azospirillum* isolated from roots of plants collected from pot study growing under well watered conditions at anthesis stage, Lane 6 *Azospirillum* isolated from rhizosphere soil of plants growing under well watered conditions at anthesis stage, Lane 7 *Azospirillum* isolated from roots of plants growing under water stressed conditions at anthesis stage, Lane 8 *Azospirillum* isolated from rhizosphere soil of plants collected from pot study growing under water stressed conditions at anthesis stage.

In present study all the PGPR strains showed differential capacities to produce phytohormones Indole-3-acetic acid (IAA), Gibberellic acid (GA3), trans-zeatin riboside (t-zr) and Abscisic acid (ABA) in culture media supplemented with and without tryptophan (Fig. 4a–b). Isolates from roots and rhizosphere soil at anthesis stage produced markedly greater concentration of IAA, GA, and t-zr than those of tillering stage in each level of soil moisture content. However, significantly ($p \leq 0.05$) less IAA, GA, and t-zr were produced in isolates from roots of plants grown in water stressed conditions as compared to that of irrigated control. Maximum amounts of IAA, GA, and t-zr were produced by MRA_9 and MSA_9 from roots of plants (at anthesis stage) grown in pots under well-watered conditions, while the least amounts of IAA, GA, and t-zr were produced by isolate MRA_8 and MSA_8 (from roots of plants grown under water-stressed conditions at 8% soil moisture).

In contrast to other phytohormones, ABA production of all isolates from rhizosphere soil and roots of plants growing under water-stressed conditions was significantly ($p \leq 0.05$) higher than that of the isolates from rhizosphere soil and roots of well-watered plants. At anthesis stage, maximum ABA was produced by MRA_{10} (isolated from roots of plants growing under water stress at anthesis stage), while the least amounts of ABA was produced by MRA_7 (from roots of plants grown under well-watered conditions). Tryptophan addition to culture media markedly increased the production of all phytohormones. The magnitude of increase was the highest for IAA production. All *Azospirillum* isolates from roots and rhizosphere of maize plants grown under well-watered conditions showed approximately eight times increase in IAA and five times increase in GA and t-zr production in culture medium supplemented with tryptophan as compared to the culture without tryptophan (Fig. 4a–b).

The results (Fig. 5) revealed that treatment T_2, i.e., inoculation with isolates MRA_7 (from roots of plants grown under well-watered conditions), significantly ($p \leq 0.05$) increased shoot length (37%), root length (45%), shoot dry weight (50%), root dry weight (61%), and root to shoot ratio as compared to uninoculated (To) wheat plants. The inoculation of wheat (T_4) with isolate MRA_{10} (isolated from roots of plants growing under water stress at anthesis stage) also stimulated wheat growth but with lower magnitude. Under induced water stress of 7 days, the inoculum with *Azospirillum* isolates from water-stressed potted plants increased plant biomass more than with *Azospirillum* isolates from well-watered potted plants.

PCR amplification for 16S rRNA gene was carried out and the product was run on gel and visualized (Fig. 3) On the basis of phytohormone production and tolerance to water stress, MRA_{10} *Azospirillum* (isolated from roots of maize plants growing under induced water-stressed conditions at anthesis stage) were characterized by 16S rRNA gene sequence analysis. The comparison of the sequence show highest similarity (98%) to *Azospirillum lipoferum.*

Discussion

Low soil moisture adversely affected the survival of *Azospirillum* isolates from roots and rhizosphere samples of plants exposed to water stress, with greater effects at anthesis stage of plant. The difference in the response to soil moisture for different phases of plant growth may be due to differences in composition and yield of root exudates, which markedly affect bacterial activity of rhizosphere soil. Association of microbes depends on developmental stage and root architecture of plants (Ilyas & Bano, 2010). Adaptation to water stress in microbes is a very complex phenomenon involving many physiological and biochemical processes and physiological/morphological changes such as cyst formation (Bashan, 1991; Sadasivan and Neyra, 1987), floc formation (Neyra *et al*., 1995), melanin production (Givaudan *et al*., 1993), poly-B-hydroxybutyrate synthesis (Okon and Itzigsohn 1992), and polysaccharide synthesis (Del Gallo & Haegi, 1990). The observed decreased use of carbohydrates by group II may be attributed to stress induced changes in morphology/physiology of *Azospirillum* under water stress. Persistence of stress usually results in a cell that is smaller and much harder than a cell not subjected to stress. Stress also affects the metabolic functioning of microorganisms, reducing the use of nutrients (Maot *et al*., 2002). The isolates were classified in three groups, on the basis of carbon/nitrogen utilization pattern, and in two groups on the basis of RAPD analysis. The chemical and physical differences between soils may be at least partially responsible for the genetic differences among the strains (Paffetti *et al*., 1996). Genomic DNA fingerprinting using RAPD has been found useful in differentiating very closely related bacteria (Oliveira *et al*., 2000).

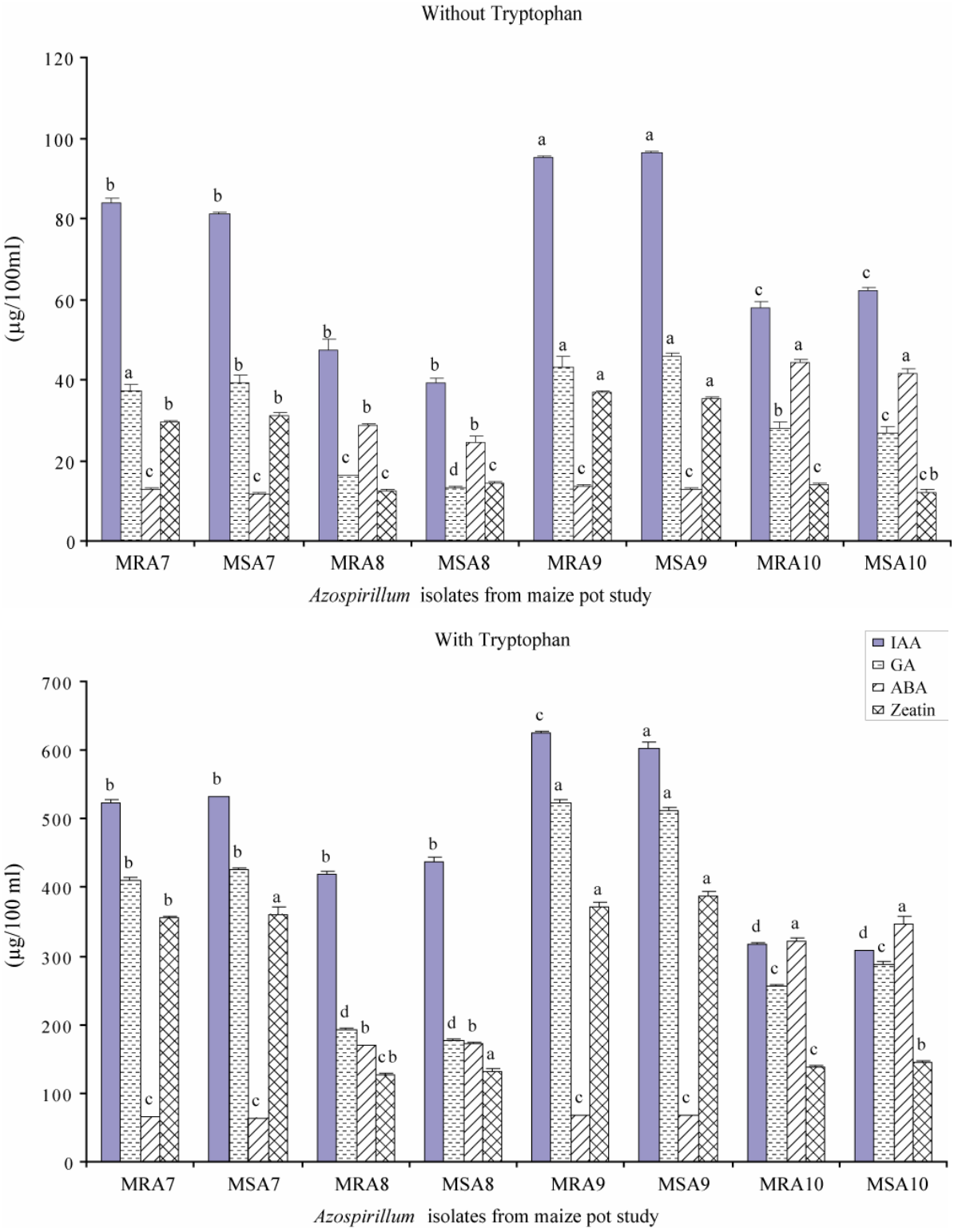

Fig. 4. Estimation of phytohormones produced (µg/100ml) by *Azospirillum lipoferum* strains in culture medium without and with tryptophan respectively. The bars bearing the same letter are not statistically different at 5 % level of significance.
MRA_7 Isolate from roots of well watered plants at tillering stage, MSA_7 Isolate from rhizosphere soil of well watered plants at tillering stage, MRA_8 Isolate from roots of water stressed plants at tillering stage, MSA_8 MSA_7 Isolate from rhizosphere soil of water stressed plants at tillering stage stage, MRA_9 Isolate from roots of well watered plants at anthesis stage, MSA_9 Isolate from rhizosphere soil of well watered plants at anthesis stage, MRA_{10} Isolate from roots of water stressed plants at anthesis stage, MSA_{10} *Azospirillum* Isolate from rhizosphere soil of water stressed plants at anthesis stage.

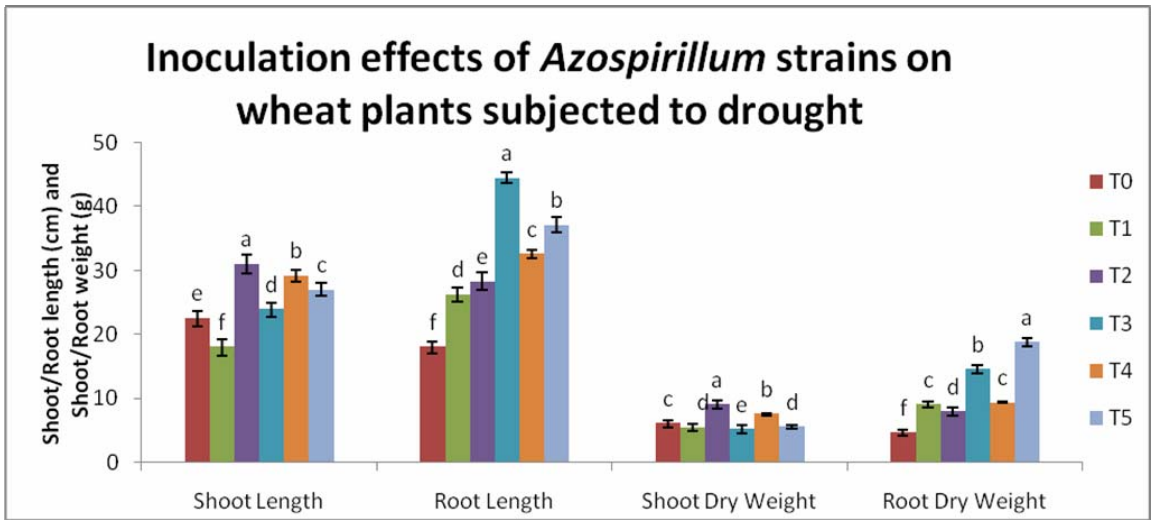

Fig. 5. Inoculation effects of *Azospirillum* strains on shoot and root dry weight and length of 45 days old *Triticum aestivum* L., plants growing under induced water stress and normal conditions. The values followed by same letter are not statistically different at 5% level of significance.

Plants were harvested 45 days after sowing T_0 Not inoculated and kept well watered, T_1 Not inoculated and exposed to induced water stress for 7 d, T_2 Inoculated with MRA_7 (from roots of plants grown under well-watered conditions) and kept well watered, T_3 Inoculated with MRA_7 (from roots of plants grown under well-watered conditions) and exposed to water stress for 7 d, T_4 Inoculated with MRA_{10} (isolated from roots of plants growing under water stress at anthesis stage)and kept well watered, T_5 Inoculated with MRA_{10} (isolated from roots of plants growing under water stress at anthesis stage) and exposed to water stress for 7 d.

The *Azospirillum* isolates obtained from well-watered conditions showed higher production of IAA, GA, and t-zr and less production of ABA as compared to isolates from water-stressed plants. Kolb and Martin (1985) and Cohen *et al*., (2007) detected ABA in the culture medium of *A. brasilense*. IAA is one of the most physiologically active auxins. According to Spaepen *et al*., (2007), environmental stress, such as acidic pH, water stress, and carbon limitation, can affect IAA synthesis. The application of exogenous tryptophan increased markedly the IAA production, which is in accordance with the earlier studies for *Azospirillum* (Patten and Glick 2002; Theunis *et al*., 2004). Mutual interaction between Cytokinins, ABA, and IAA has been reported (Polanska *et al*., 2006; Woodward & Bartel 2005), and alteration in cytokinin level can affect IAA and ABA contents (Polanska *et al*., 2006). The ABA inhibition of auxin biosynthesis has been reported (Milborrow, 1966; Anker, 1975).

Inoculation of wheat plants with *Azospirillum* isolates has increased the plant biomass, which appears to be correlated with the production of growth-promoting hormones. In addition to nitrogen fixation, the production of phytohormones by plant growth-promoting rhizobacteria is considered to be an important mechanism by which these bacteria can promote plant growth (Tien *et al*., 1979; Bashan *et al*. 2004; Spaepen *et al*., 2008). When plants were exposed to water stress for 7 days, stimulatory effects of *Azospirillum* isolates from plants exposed to water stress on root shoot length and fresh and dry weight of root and shoot were higher than those of *Azospirillum* isolates from well-watered plants in pots. One of the possible reasons for better performance of these isolates is higher ABA production and maintenance of higher ABA/t-zr ratio, indicating a decrease in cytokinin content. This higher amount of ABA produced by *Azospirillum* isolates may be taken up by inoculated plants and subsequently results in improved tolerance of plants to water stress. ABA has been suggested to be a key component in water deficit-induced responses. Taylor *et al*., (2000) and Fulai *et al*., (2005) reported that drought decreased water potential and increased ABA content. In another study *Azospirillum* strain from this experiment was used to assess the effects of inoculation of wheat seedlings on amelioration of adverse effects of drought stress on wheat plants. Two wheat varieties cvv. GA-2002 and Faisalabad 2008 were grown under normal and drought stressed conditions. *Azospiriilum* treatment under drought significantly increased stomatal conduction, osmotic potential, protein, proloine, soluble sugars, glycine betaine, phytohotmones like abscisic acid, fresh and dry weight of root and shoot, biological and grain yield (Sarwar, 2008).

Mayak *et al*., (2004) have hypothesized that those bacteria of sites where water is limited and repeated dry periods occur frequently are likely to stimulate better plant growth than bacteria isolated from sites where water sources are abundant. El-Komy *et al*., (2003) reported that *Azospirillum* mitigated adverse effects of low soil moisture on wheat plants grown under water-stressed conditions. *Azospirillum* strains isolated from moisture-stressed conditions imparted tolerance to host plant, which received bacterial inoculation. This was shown by the better growth of plants and drier biomass of shoot and root of inoculated than those of uninoculated seedlings. This hypothesis is further verified by the observation that root to shoot ratio, which is an index of stress tolerance in plants, was significantly ($p \leq 0.05$) greater in plants inoculated with T_4 group II (isolates from water stressed) than in plants inoculated with T_2 (isolates of group I,

well-watered conditions). Possibly the higher ABA production by these isolates may be responsible for the tolerance to water stress (Fig. 4). Plants inoculated with *Azospirillum* exhibit enhanced root system with more lateral roots and enlarged root hairs, therefore occupying an enhanced soil volume (Bertrand *et al*., 2000). The number and length of wheat roots were significantly increased by inoculation with IAA producing A. brasilense strains, and enhanced root growth of maize was found after inoculation of GA producing *Azospirillum* lipoferum (Barbieri *et al*., 1986; Fulchieri *et al*., 1993). Evaluation of strains for production of various bioactive metabolites ACC deaminase, osmoregulants and antioxidants is required for selection of the strains as bio-inoculant under stress environment.

Conclusion

The results indicated that the isolated *Azospirillum* strains are affected by water stress and are adopted consequently. The selected bacterial strain, *Azospirilum lipoferum* (MRA_{10}), can be used to promote cereal plant growth under normal and water stress conditions and enhance production of stress-hormones (ABA) along with higher ABA/trans zeatin riboside ratio, through inoculation of these strains in plants under stress condition.

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