



Evaluation of Salt-Tolerant *Azospirillum lipoferum* and Its Role in Improvement of Wheat Growth Parameters



Ibrahim El-Akhdar^{1*}, Mostafa El-Sheekh², Nanis G. Allam², Faiza Kamal¹, Reda Abou-Shanab³, Christian Staehelin⁴

¹Microbiology Department, Soils, Water and Environment Research Institute, ARC, Egypt.

²Botany Department, Faculty of Science, Tanta Univ. Egypt.

³Biotechnology Institute, College of Biological Sciences, University of Minnesota, Saint Paul, USA

⁴School of Life Sciences, State Key Laboratory of Biocontrol, Sun Yat-sen (Zhongshan) University, East Campus, Guangzhou 510006, China.

EIGHTEEN isolates of *Azospirillum* were collected from different sites at Kafrelsheikh Governorate. The isolates were cultivated on nitrogen-free malate (NFM) medium supplemented with different NaCl g l⁻¹ concentrations (0, 0.7 and 1.5%) and tryptophan to study the salt tolerant in vitro and indole acetic acid production. Pots were used to evaluate the most salt-tolerant and efficient species and soils of this experiment had artificially salinized with different salinity levels. The most salt-tolerant A₁₀ and A₁₁ species were genetically identified by 16S rRNA sequencing and the results revealed to be closest matched at 98% *A. lipoferum* strains and selected to inoculate wheat plants. The investigated *A. lipoferum* species had variable divergence growth at different salinity levels. Actually, increased salinity had deleterious effect on IAA production. In addition, salinity had deleterious effect on dry weights of plants, number and dry weight of branches, total chlorophyll, sodium and potassium% with increasing salinity levels. But, inoculation with associative nitrogen fixation *A. lipoferum* isolates enhanced the previous parameters. Thus, inoculation with the salt-tolerant *A. lipoferum* isolates decreased the deleterious effect of salt stress on wheat plants.

Keywords: Salinity, Wheat, 16srRNA, *Azospirillum lipoferum*, IAA.

Introduction

Forty percentage of the world's land surface categorized as having potential salinity problems, most of these areas had confined to the tropics and Mediterranean regions (Cordovilla et al., 1994). Salinity reduces the ability of plants to take up water and this quickly causes reductions in the growth rate, along with a suite of effects identical to those caused by water stress and reduce the photosynthetic capacity of the plant, affect growth via changed water relations, hormonal balance or carbon supply, the relative importance of each process depending on the time scale of the response (Munns, 2002) or

due to the effects of specific ions on metabolism, or adverse water relations. Different strategies had employed to maximize plant growth under saline conditions. One of them is to produce salt tolerant genotypes of different crops (Kaya et al., 2009).

Wheat (*Triticum aestivum*-L.) is one of the main winter cereal crops in Egypt for grain production and straw and grows on an area of 3.39 million feddan (feddan = 4200m²) with an annual production of about 9.28 million tones and with an average yield of 2.74 tons per feddan during 2014/2015 growing season (CLAC 2015; El-Temsah 2017).

*Corresponding author e-mail: dr.elakhdar@yahoo.com

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Use of microorganisms as environment friendly biofertilizer helps to reduce much expensive of fertilizers. Efficiency of biological nitrogen fixation increased the availability of Fe, Zn etc., through production of plant growth promoting substances (Kucey *et al.* 1989). The plant growth producing regulators (PGPR) influence the crop productivity, morphological characteristics and development by releasing plant growth regulators (Volpin and Philips 1998).

The most important microorganisms that play a significant role in soil fertility in tropical and temperate regions of the world and isolated from the rhizosphere of a diverse range of cereals, including corn, millet, sorghum, and wheat is *Azospirillum* sp. (Hamdia and El-Komy, 1998), play an important role in the promotion of plant growth (Steenhoudt and Vandeleyden 2000) and capable of affecting growth and yield of numerous plant species and its ability to produce various phytohormones that improve root growth adsorption of water, minerals that eventually yield larger and in many cases, more productive plants (Dobbelaerae *et al.* 2003) and can also mineralize nutrients from the soil, sequester Fe, survive under harsh environmental conditions, and support beneficial mycorrhizal-plant associations (Bashan *et al.* 2006). *Azospirillum* can help plants minimize the negative effects of abiotic stresses because, *Azospirillum* is the most studied PGPB and excluding rhizobia (Diaz-Zorita and Fernandez-Canigia, 2009 and Hartmann and Bashan, 2009), can survive in the absence of their host owing to the presence poly- β -hydroxybutyrate (PHB) (Okon and Itzigsohn 1992) and polysaccharide synthesis (Del Gallo and Idaegi, 1990)

Inoculated wheat plants with salt-tolerant *Azospirillum* enhanced non-symbiotic nitrogen fixation capability in salinity soil by produces growth-promoting substances such as IAA, gibberellins, pantothenic acid, thiamine and niacin which promotes root proliferation and improve the plant growth and yield. Inoculated wheat (*T. aestivum*) seedlings with *Azospirillum* subjected to osmotic stress developed significant increased coleoptiles, fresh weight, better water status, all yield components, grains number per spike, grains weight per spike, the length of spike, nitrogen content, grains protein content, straw protein content above un-inoculated wheat plants than non-inoculated seedlings. The effect of salinity on pigment concentrations

(chlorophyll a, b and total chlorophyll) appeared significant decrease of chlorophyll and pigment concentrations (chlorophyll a, b and total chlorophyll) had significantly positive affect by salt-tolerant *Azospirillum* inoculation (Zaied *et al.*, 2009; Sonia *et al.*, 2017).

Materials and Methods

Wheat grains (*Triticummae stivum-L* Sakha 93) were kindly supplied from Department of Cereals, Field Crop Research Institute, Agricultural Research Center, Sakha Agriculture Research Station, Kafrelsheikh, Egypt.

Soil samples

Soil samples were obtained from different sites at Kafrelsheikh Governorate (Balteim, Elhamoul, Kafrelsheikh). Soils were air dried, crushed and sieved through 2 mm sieve, and subjected to chemical analysis. The characteristics of the soil presented in Table 1. Using the spade, describe a circle with a radius of approximately 15 cm. This section had cut to a depth 20 cm at least. The cations analyzed in saturation extracts are Ca^{++} , Mg^{++} , K^{+} and Na^{+} while the anions are SO_4^{-} , CO_3^{-} , HCO_3^{-} and Cl^{-} (Meq/l) according to Richards (1954). Soil pH had measured in soil paste extract by using pH meter (model 3151/SET). Carbonate and bicarbonate (CO_3 and HCO_3) were estimated by titrating with KHSO_4 (N/50) using phenolphthalein indicator for the former and bromocrysol green for the latter (Richards, 1954). Chlorides Cl^{-} determined by titration (5 ml of samples) against standard solution of silver nitrate according to Moher's methods (Jackson, 1958). Electric conductivity (EC) dSm^{-1} : It was determined by measuring the electrical conductivity (E.C dS.m^{-1}) in soil paste extract according to Richards (1954).

Media used:

Medium 1: nitrogen-free malate (NFM) used for isolating and growing *Azospirillum* spp. (Dobereiner and Day, 1976; Bergey's Manuals Systematic of bacteriology, 2005). Malic acid: 5.0 g, K_2HPO_4 : 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.2 g, NaCl: 0.1 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.002, Fe-EDTA (1.64% w/v aqueous): 4.0 ml, Trace element solution 2.0 ml (Bromothymol blue (0.5% alcoholic solution), 2.0 ml: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 1.4 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$: 1.6 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 5.0 mg, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mg) and Vitamin solution 1.0 ml: (Biotin: 10.0 mg, Pyridoxin: 20.0 mg), KOH: 4.0 g, Agar (Semi solid): 1.75 g and Distilled water 10000 ml.

Medium 2: Nutrient Agar supplemented with different carbon sources (Starch, Manitol, Sucrose, Succinate and Fructose) for growing *Azospirillum* spp. (Difco, 1985).

Carbon sources: 5 gl⁻¹, Beef Extract: 3 gl⁻¹, Peptone: 5 gl⁻¹, Dist water: 1000, pH: 7.0 and autoclaved at 121°C for 15 min.

Medium 3: Requirement of biotin for growing *Azospirillum* spp. (Tarrand et al., 1978)

K₂HPO₄: 0.5, Biotin: 5.0, FeSO₄·7H₂O: 0.01, Na₂MoO₄: 0.002, MgSO₄: 0.2, NaCl: 0.1, CaCl₂·2H₂O: 0.02, (NH₄)₂SO₄: 1.0, pH: 7.0. The component dissolved/L and autoclaved at 121°C for 15 min. Utilization of sugars media 1 used with replace Malic acid with each studied sugar separately.

Medium 4: Nutrient solution for wheat growing (Shrdleta et al., 1984)

K₂SO₄: 0.486 gl⁻¹, K₂HPO₄: 0.200 gl⁻¹, MgSO₄·7H₂O: 0.200 gl⁻¹, FeCl₂: 0.010 gl⁻¹, CaCl₂: 0.376 gl⁻¹, H₃PO₄: 1.855mg/l, ZnSO₄·7H₂O: 0.280 mg/l, MnSO₄·H₂O: 2.231 mg/l⁻¹, CuSO₄·5H₂O: 0.25mg/l⁻¹, NaMoO₄: 0.412mg/l⁻¹. All those contents were dissolved in 1 liter water and the pH of solution was adjusted to pH 6.9 using KOH.

24 Isolates of *Azospirillum* spp. were isolated from collected different soils at Kafrelsheikh Governorate only 18 isolates were chosen. Isolation of free N-fixing organisms from soils were carried out by the enrichment culture technique using semisolid malate medium supplemented with 50 mg·l⁻¹ yeast extract. The bacterial isolates showing sub-surface pellicle formation were selected and further purified by streaking on Congo red agar medium. The purified isolates were stored at 4°C and sup-cultured every 1-2 months (Dobereiner and Day, 1976).

Characterization of *Azospirillum* isolates

The following physiological characterizations were conducted to identify the isolated *Azospirillum*. Cell morphology and mobility had examined under a light microscope. Gram staining and substrate utilization using malate medium were carried out for species identification. In addition, glucose assimilation in nitrogen free broth (NFB) medium and biotin requirement for growth were determined (Tarrand et al., 1978). Identification of obtained isolates had carried out according to keys of **Bergey's Manual Systematic of bacteriology (2005)**. Cell shape, the purified cultures after 72 h grown had microscopically examined for the cell morphological characters.

TABLE 1. Some chemical characteristics of soil samples collected from different sites at Kafr Elsheitkh Governorate used for *Azospirillum* isolation

Soil samples	Soluble anions (meq l ⁻¹)				Soluble cations (meq l ⁻¹)				EC* dSm ⁻¹	pH	
	SO ₄ ⁻	Cl ⁻	HCO ₃ ⁻	CO ₃ ⁻	Mg ⁺⁺	K ⁺	Na ⁺	Ca ⁺⁺			
Balteim	A1	156.43	642.0	6.00	0.0	187.2	15.43	546	56.8	51.6	8.45
	A2	155.43	640.0	6.00	0.0	187.2	13.43	544	56.8	50.6	8.45
	A3	330.6	1520	6.00	0.0	374.4	17.10	135.2	113.6	64.32	8.34
	A11	53.0	210.0	4.00	0.0	65.5	24.5	128	35.5	23.04	8.68
	A13	120.5	460.0	4.00	0.0	77.2	9.98	390.0	56.8	36.48	8.44
	A20	53.0	210.0	3.5	0.0	65.5	24.5	128	35.5	23.04	8.3
	A5	128.85	300.0	6.50	0.0	194.2	4.85	1060	92.3	16.32	8.3
	A7	33.0	200.0	4.00	0.0	37.7	4.85	167	17.75	13.44	8.3
Elhamoul	A14	31.88	340.0	4.00	0.0	81.4	1.58	65.0	28.4	13.44	8.34
	A15	27.00	240.0	5.00	0.0	47.9	1.48	4.0	113.6	10.56	8.9
	A16	31.60	170.0	0.5	0.0	54.0	0.68	101.0	49.7	12.48	8.7
	A18	31.88	340.0	4.00	0.0	81.4	1.58	65.0	28.4	13.44	8.34
	A19	33.0	300.0	2.0	0.0	37.7	4.85	167	17.75	13.44	8.4
	A21	27.00	240.0	4.5	0.0	47.9	1.48	170.0	113.6	10.56	8.3
	A24	31.60	170.0	3.75	0.0	54.0	0.68	101.0	49.7	12.48	8.55
	A10	4.9	480.0	5.50	0.0	5.05	0.84	26.25	45.60	4.42	8.6
Kafr Elsheitkh	A12	38.5	400.0	12.50	0.0	4.08	0.35	4.00	1.42	1.44	8.5
	A22	38.5	400.0	2.25	0.0	4.08	0.35	4.00	1.42	1.44	8.3

A: *Azospirillum* isolates

EC: Electric conductivity

Motility, using cavity slide for bacterial motility (Hanging drop technique) (**Facklam and Elliott, 1995**), Gram reaction; Gram staining was carried out as mentioned by **Rangaswami and Bagyaraj (1993)**, Utilization of glucose as a sole carbon source; as a sole carbon source for growth in yeast malate modified (malate replaced by 1% glucose) medium was carried out **Tarrand et al. (1978)**, Utilization of other sugar by the same methods malate replaced with the studied sugar. Requirement of biotin; the producer recommended by **Tarrand et al. (1978)** used for determination of biotin requirement by *Azospirillum*. Media with or without biotin (0.1 mg/L) had prepared and dispensed in test tube inoculated with 24 old culture and incubated for 48 hrs at 37 °C. Positive tubes were recognized by microbial growth.

Effect of temperature, pH and NaCl concentrations on Azospirillum growth

A fixed number (5×10^8 cfu) of pure isolated *Azospirillum* was added in 250 ml conical flask have liquid free nitrogen malate medium (**Dobereiner and Day, 1976**) at different degrees of temperature (27, 29, 32, 35 and 37°C), pH (5, 5.5, 7, 7.5, 8) and different NaCl concentrations (Zero, 7 and 12 g l⁻¹). The cultures were incubated at 30°C for 3 days until *Azospirillum* growth were appeared and the growth was measured at 600 nm using UV/Visible spectrophotometer (Model 6705) against the reagent blank.

In vitro evaluation of Azospirillum isolates abilities to nitrogen fixation

N-free malate medium was prepared and 100 ml added to a 250 ml conical flask and autoclaved. From 24 h old culture one ml of inoculum added to each flask which incubated at 37°C for seven days. Homogenized cultures were digested with 5 ml of concentrated H₂SO₄ along with 0.2 g digestion catalyst mixture K₂SO₄: CuSO₄: selenium (100:10:1). Volume made up to 10 ml with distilled water and transferred to microKjeldhal distillation unit. 20 ml of 40 percent NaOH mixed with sample and the distilled ammonia evolved was trapped in four percent boric acid mixed indicator (Bromocresol green 0.066 g and methyl red 0.033 g in 100 ml methanol) till the solution turned from pink to green. The N% was determined according to this equation (**Chapman and Parkar, 1963**) $N\% = (\text{Titration value} \times 0.014 \times \text{Normality of H}_2\text{SO}_4 \times \text{Vol. / Volume of sample used}) \times 100$.

Assessment of of Indole-3-Acetic Acid (IAA) production

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For rapid quantitative determination of Indole-3-Acetic Acid (IAA), *Azospirillum* was grown and then 1 ml of the inoculums was transferred to each Erlenmeyer flask containing 50 ml of specific medium (NFB), supplemented with 0.3 g/l tryptophan and incubated at 28° C with agitation at 100 rpm in the dark. The cultures were centrifuged at 4000 rpm for 15 min and two milliliters of the supernatant were mixed with one milliliter Pilet-Chollet reagent which consists of 12 g FeCl₃·l⁻¹ of 7.9 M H₂SO₄ and the mixture was lifted in the dark for 30 minutes at room temperature observing. Development of pink color indicated that IAA was produced. The absorption spectra of the mixtures were determined at 530 nm (**Pilet and Chollet, 1971**). The level of produced IAA had estimated from stander IAA curve.

DNA Extraction, 16S Ribosomal RNA (rRNA) amplification and sequencing

Genomic DNA of selected bacteria was extracted from 10-ml bacterial according to the method described by (**Dobereiner and Day 1976**). Bacterial pellets suspended in mixture of TE buffer, SDS (10%) and proteinase K were inoculate for 1hr at 37 C. NaCl (5 M) and CTAB/ NaCl solution (4.1 g NaCl and 10 g CTAB [N-cetyl-N,N,N,-Trimethyl ammonium bromide] in pre-warmed 100 ml distilled water). The mixture was inoculated for 10 min at 65 C. the solution was extracted with 780 µl of chloroform- isoamyl alcohol (24:1), centrifuged for 5 min and the aqueous phase was further extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The DNA present in the aqueous phase precipitated with 0.6 vol. isopropanol and the precipitate was washed with 70% ethanol. TE buffer used to re-suspended DNA pellet (Abou-Shanab et al. 2006). 16S Ribosomal RNA (rRNA) amplification and sequencing by using oligonucleotide primers primers 16Sa (GGCGAGGCTTAACA) with specificity for eubacterial 16S rRNA, and 16Sb (CCAGCCGCAGGTTCCCCT) **Van Berkum and Fuhrmann (2000)**, were used to amplify the 16S rRNA gene fragments with template DNA originating from Gram-negative bacteria, PCR mixtures contained 300 mM Tris-HCL pH 9, 7.5 mM MgCl₂, 75 mM (NH₄)₂SO₄, 10 mM each dDTPs, 10 pmol of each primer, 10-50 ng of the extracted DNA 3U of Taq DNA polymerase were used with the reaction volume broth up to 120µl using sterile distilled water. (**Van Berkum and Fuhrmann, 2000**). A search for GenBank with BLAST was used to identify bacterial species (Altschul et al., 1997).

Preparation of pots

Plastic pots used to an *in vivo* evaluate the most salt-tolerant and efficient and N₂-fixation *Azospirillum* isolates prepared as followed: Sand soil had washed several times with 0.1 % HCl solution followed by washing with distilled water in order to remove nitrogen as well as other minerals. Each pot had filed with 250g of sand soil. Pots were autoclaved twice at 1.5 par, 121°C for 4 hours (El Nady and Belal 2005). The soil had salinized with NaCl and CaCl₂ with the concentrations of normal, 5, and 10 dS m⁻¹ according to **Manual of salinity research methods (1992)**.

Soil salinization

The salinity levels were expressed as electrical conductivity (EC). Artificial salinization with different levels of EC had prepared using NaCl and CaCl₂ salts. Time domain reflectometer (TDR) device used with moisture meter type HH2 using wet sensor (Type WET1) who measured soil salinity, moisture content and soil temperature at the same time. To obtained a fixed pressure and avoid moisture stress, water losses by evaporation was compensated. The plants had irrigated to field capacity (FC) to avoid the losses of salts by drainage. Artificial salinization of soil was prepared at constant sodium adsorption ratio (SAR). Suppose one wants to develop salinity in a soil to obtain the characteristics according to Manual of salinity research methods (1992).

Sterilized pots were arranged as complete randomize design with 5 replicates. Wheat grains were surface sterilized in order to eliminate possible contamination by native bacteria by rinsing in ethanol (75%) for 3-5 minutes and soaking for 4 minutes in hydrogen peroxide (3% v/v) followed by washing in sterile distilled water several times. 10 grains/pot were sown. Seedlings were thinned to five/pot then inoculated with liquid cultures of different *Azospirillum* isolates 5 ml (1x 10⁸ cfu ml⁻¹) for pot prepared in addition to *Azospirillum* free liquid medium as control. Irrigation was carried out twice weekly by free of nitrogen nutrient solution according to prevailing climatic conditions (Shrdleta et al., 1984). After 45 days of sowing, plants were collected and subjected to the following analyses; fresh and dry weight of plant, N, K, P % and N, K, and P content. The most salt-tolerant and efficient N-fixer isolates of *Azospirillum lipoferum* have been selected and used in a pot experiment to study effect of

inoculation with and/or *Anabena oryza* on wheat plant growth and tolerance to different saline concentrations.

Chemical analyses

For determination of N, P, K % plant samples or grains were dried and 0.2 g were digested in 5 ml H₂SO₄ and 1 ml Perchloric acid in a conical flask as described by **Chapman and Parker (1963)**. The digested materials were completed to 50 ml and then distilled by micro-Kjeldahl methods and the nitrogen % of distillate was determined by titration against 0.02 normal H₂SO₄ according to Black et al. (1965), Phosphorus % of samples was determined calorimetrically according to the methods described by Snell and Snell (1967). And Sodium and Potassium were determined in the digested solution by flam photometer (No, 712700 REG. DES No, 866150) as described by (Jackson, 1967). N, P, K and Na contents were determined according to Black et al. (1965). Element content = element% x dry weight/ 100. Total chlorophyll was determined by Minolta chlorophyll meter SPAD-502 in the felid after 60 days of sowing.

Statistical analysis

The obtained collected data were subjected to the statistical analysis, using the analysis of variance (ANOVA). The LSD range tests were used to compare between the means (**Steel and Torrie, 1980**).

Results and Discussion

Eighteen isolates had been obtained from different sites at Kafrelshiekh, Balteim and Elhamuol. Soil analysis showed a variation in EC, pH, CO₃⁻, HCO₃⁻, Cl₁⁻, SO₄⁻, Mg⁺⁺, K⁺, Na⁺ and Ca⁺⁺ (Table 1). Morphological characters of isolated *Azospirillum* had studied. The isolates were rod and vibroid in shape. Only three isolates were rod and another 15 isolates were vibroid (Table 2). This result was confirmed by **Dobereiner and Day (1976)** and **Usha and Kanimozhi (2011)**. Also, **Krieg and Dobereiner (1984)** reported that microscopic examination revealed polymorphism, but the dominant forms on a solid malate medium are characteristic curved rods of various sizes with predominant refractive fat droplets. Eighteen isolates showed white dense and undulating fine pellicle as showed in Fig. 1. The pellicle formation is characteristic of *Azospirillum* spp. In this concern **Dobereiner and Day (1976)** reported that microaerophilic growth in semisolid agar stagnant conditions were helpful for the inoculation of the organism

since *Azospirillum lipoferum* grown in a typical pellicle 1 to 4 mm below the surface; this method was particularly useful for studying the substrates and growth conditions for nitrogen fixation. All the obtained isolates were gram-negative bacteria, motile and had character by white colour on NFM medium as reported in Table 2. As reported by Usha and Kanimozhi (2011) and Nadeem *et al.*, (2016) for differentiated *Azospirillum* species. Biochemical characters of all isolates were carried out and revealed that 15 *Azospirillum* isolates

grown well in biotin medium but only 3 gave poor growth with medium supplemented with biotin. Usha and Kanimozhi, (2011) reported that *A. lipoferum* and saline tolerant species *A. halopraeferens* require biotin for its growth and N_2 -fixation.

In the present study, all the investigated 18 *Azospirillum* isolates utilized manitol, sucrose, succinate and fructose. Only two isolates can't utilize sucrose (A_5 and A_{20}) and 2 isolates cannot utilize succinate (A_{12} and A_{22}). In this context,

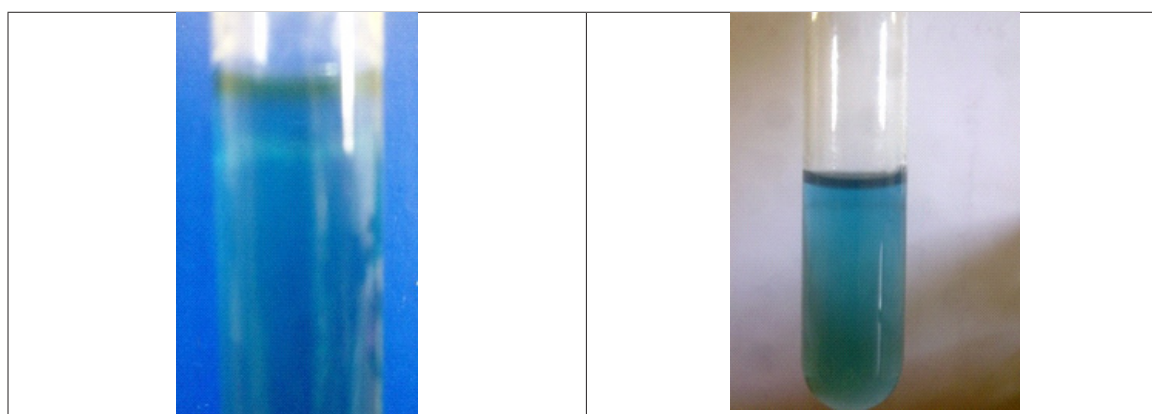


Fig. 1. Undulating fine pellicle characteristic of *Azospirillum* isolates

TABLE 2. Morphological characters and carbon utilization of *Azospirillum* isolate.

Isolate Number	Morphological characterisation					Carbon utilize reaction					
	Cell Shape	Colony Character	Gram Reaction	Motility	Malic acid	Biotin	Starch	Manitol	Sucrose	Succinate	Fructose
A ₁	Rod	White	Negative	Motile	+	-	+	+	+	+	+
A ₂	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₃	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₅	Vibroid	White	Negative	Motile	+	+	+	+	-	+	+
A ₇	Rod	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₀	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₁	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₂	Vibroid	White	Negative	Motile	+	+	-	+	+	-	+
A ₁₃	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₄	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₅	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₆	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₈	Rod	White	Negative	Motile	+	+	+	+	+	+	+
A ₁₉	Vibroid	White	Negative	Motile	+	-	+	+	+	+	+
A ₂₀	Vibroid	White	Negative	Motile	+	+	+	+	-	+	+
A ₂₁	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+
A ₂₂	Vibroid	White	Negative	Motile	+	-	+	+	+	-	+
A ₂₄	Vibroid	White	Negative	Motile	+	+	+	+	+	+	+

A: *Azospirillum* isolate (+) Presence of growth (-) Absence of growth

Krieg and Dobereiner (1984) found that the organic acids support the vigorous growth and nitrogen fixation of all *Azospirillum* spp. but the organisms have very different capabilities for the utilization of sugars. *A. lipoferum* effectively utilizes glucose and *A. amazonense* utilizes sucrose. It has been suggested that chemotaxis to organic acid, sugars and amino acids is important for the establishment of root-associated growth of Azospirilla in their ecological niche. Eckert et al. (2001) reported that all these features were very similar to other *Azospirillum* sp. The trait that differentiates, the species from other based on its ability to use several sugars and some minute genetic detail. It could conduct that the optimum growth occurs at 30°C and at pH values between 6.0 and 7.0.

Effect of different degrees of temperature (27-37 °C) and pH of media were (5-8) and had reported in Table 3. All *Azospirillum* isolates gave good growth at different temperatures (27, 29, 32, 35 and 37°C) and gave good optimum growth at 5.5 to 7.0 and 7.5 pH while no growth at 5.0 and 8.0 pH except isolate *Azospirillum* (A₁). This results had been conducted to **Usha and Kanimozhi, (2011)** showed that *Azospirillum* species had the ability to grow at different degree of temperatures and different pH values.

Azospirillum isolates had been evaluated for their tolerance against salt stress (*in vitro*), by growing on LNFM supplement with different salt concentrations (Table 4). There was a variation among the different isolates when grown on media amended with different salt levels. However, bacterial number decreased with increasing salinity concentrations. This result was similar to those obtained by Suhal et al. (2011) who indicated that increasing salinity levels (0 to 6) dSm⁻¹ caused a decreasing in number of bacteria cells. Among studied isolates A₂ and A₁₁ were the most salt-tolerant *Azospirillum* species. Viviana (1998) showed that growth of bacteria in the presence of different NaCl concentrations could tolerate up to 300 mM NaCl. Obtained results were similar to those observed by Soumitra et al, (2007) who reported a reduction in the growth of *A. brasilense* bacteria by addition of NaCl, by accumulation of organic osmolytes as flexible modes of adaptation and osmoadaptation strategies, which become the crucial factor for the stability of enzymes and other cellular components under salt stress. Bacteria adapt to salinity by cell envelope (Lopez et al, 2000). Also, potassium ions, glycine betaine, proline, proline betaine, trehalose and ectoines could accumulating as osmolytes. These compatible solutes maintain the appropriate cell volume and protect intracellular macromolecules

TABLE 3. Physiological characterization of *Azospirillum* isolates.

Isolate Number	Temperatures / C °					pH				
	27	30	33	35	37	5.0	5.5	7.0	7.5	8.0
A ₁	+	+	+	+	+	-	+	+	+	+
A ₂	+	+	+	+	+	-	+	+	+	-
A ₃	+	+	+	+	+	-	+	+	+	-
A ₅	+	+	+	+	+	-	+	+	+	-
A ₇	+	+	+	+	+	-	+	+	+	-
A ₁₀	+	+	+	+	+	-	+	+	+	-
A ₁₁	+	+	+	+	+	-	+	+	+	-
A ₁₂	+	+	+	+	+	-	+	+	+	-
A ₁₃	+	+	+	+	+	-	+	+	+	-
A ₁₄	+	+	+	+	+	-	+	+	+	-
A ₁₅	+	+	+	+	+	-	+	+	+	-
A ₁₆	+	+	+	+	+	-	+	+	+	-
A ₁₈	+	+	+	+	+	-	-	+	+	-
A ₁₉	+	+	+	+	+	-	-	+	+	-
A ₂₀	+	+	+	+	+	-	+	+	+	-
A ₂₁	+	+	+	+	+	-	+	+	+	-
A ₂₂	+	+	+	+	+	-	+	+	+	-
A ₂₄	+	+	+	+	+	-	+	+	+	-

A: Azospirillum isolate (+) Presence of growth (-) Absence of growth

TABLE 4. Evaluation of salt tolerant *Azospirillum* species to different salinity levels (in vitro), estimation of nitrogen fixation ability (mg g⁻¹ of malate) and production of Indole-3-Acetic Acid (IAA) under different salinity levels (µgml⁻¹).

Isolates	Total <i>Azospirillum</i> count (O.D)			IAA (µg ml ⁻¹)			Fixed nitrogen (mg g ⁻¹ malate)		
	Salinity levels of NaCl								
	Normal	0.7 %	1.5 %	Normal	0.7 %	1.5 %	Normal	0.7 %	1.5 %
A ₁	0.520	0.433	0.337	1.01	0.7	0.3	0.13	0.08	0.03
A ₂	1.200	1.013	0.740	12.1	0.8	0.4	0.17	0.12	0.04
A ₃	1.053	0.617	0.603	8.4	2.1	0.7	0.21	0.12	0.10
A ₅	0.733	0.653	0.510	12.9	3.1	1.1	0.31	0.18	0.09
A ₇	1.093	0.793	0.513	6.1	4.1	0.9	0.21	0.14	0.11
A ₁₀	0.923	0.610	0.377	85	20	12.5	0.7	0.37	0.26
A ₁₁	1.163	0.937	0.683	85	21.1	12.1	1.75	0.8	0.34
A ₁₂	1.173	0.903	0.670	84	5.9	1.21	0.66	0.35	0.22
A ₁₃	1.053	0.650	0.660	12.0	4.2	0.9	0.58	0.21	0.11
A ₁₄	0.910	0.653	0.417	13.1	7.1	1.2	0.41	0.22	0.12
A ₁₅	1.133	0.647	0.420	73	21	3.3	0.33	0.23	0.09
A ₁₆	0.860	0.670	0.500	30	6.3	1.2	0.55	0.12	0.09
A ₁₈	0.843	0.690	0.630	0.9	0.43	0.38	0.48	0.21	0.089
A ₁₉	0.913	0.767	0.623	0.8	0.43	0.29	0.61	0.31	0.11
A ₂₀	0.900	0.770	0.630	40	12.1	2.1	0.51	0.22	0.12
A ₂₁	0.897	0.700	0.587	23	9.1	1.9	0.46	0.11	0.098
A ₂₂	0.810	0.763	0.560	29	7.1	1.2	0.53	0.21	0.07
A ₂₄	0.813	0.600	0.413	15.1	3.6	1.09	0.12	0.12	0.087

A : *Azospirillum* isolate.

(Csonka and Epstein, 1996). Accumulation of these osmoprotectants is achieved by either endogenous de novo synthesis or uptake mediated by specific transporters (Kemp and Bremer, 1998). Zaied et al. (2009) showed that the studied *Azospirillum* isolates and their mutants were tolerated to NaCl up to 50%.

In the present study, *in vitro* estimation of *Azospirillum* ability species to fix nitrogen under normal and salt stress were assayed by growing bacteria in liquid malate medium supplement with different NaCl levels (Table 4). A variation in N-fixation ability of different species to grow was lowered with rising salinity levels. *Azospirillum* species A₁₀, A₁₁ and A₁₂ were the most N-fixer among *Azospirillum* isolates. This observation was similar to Miller and Woods (1996) and Nadeem et al. (2016), who found that salinity

significant inhibits nitrogen fixation. Hamdia et al. (2004) and Nadeem et al., (2016) who observed *Azospirillum brasilense* adapted to salinity stress due to their Acc (deaminase aminocyclopropane-1-carboxylate) or expolysaccharides (Eps) activity and could produce expolysaccharides (Eps) to bind cations including sodium (Geddie and Sutherland, 1993).

Production of IAA by *Azospirillum* species under salt stress was measured by growing the isolates on liquid malate medium supplement with tryptophan. Ability of *Azospirillum* species to produced IAA was variable when media amended with different salinity levels (Table 4). However, production of IAA decreases with increasing salinity levels. Among the *Azospirillum* species A₁₀, A₁₁ and A₁₂ were the most IAA producers. Conducted to obtain results Omay et al. (1993) who reported

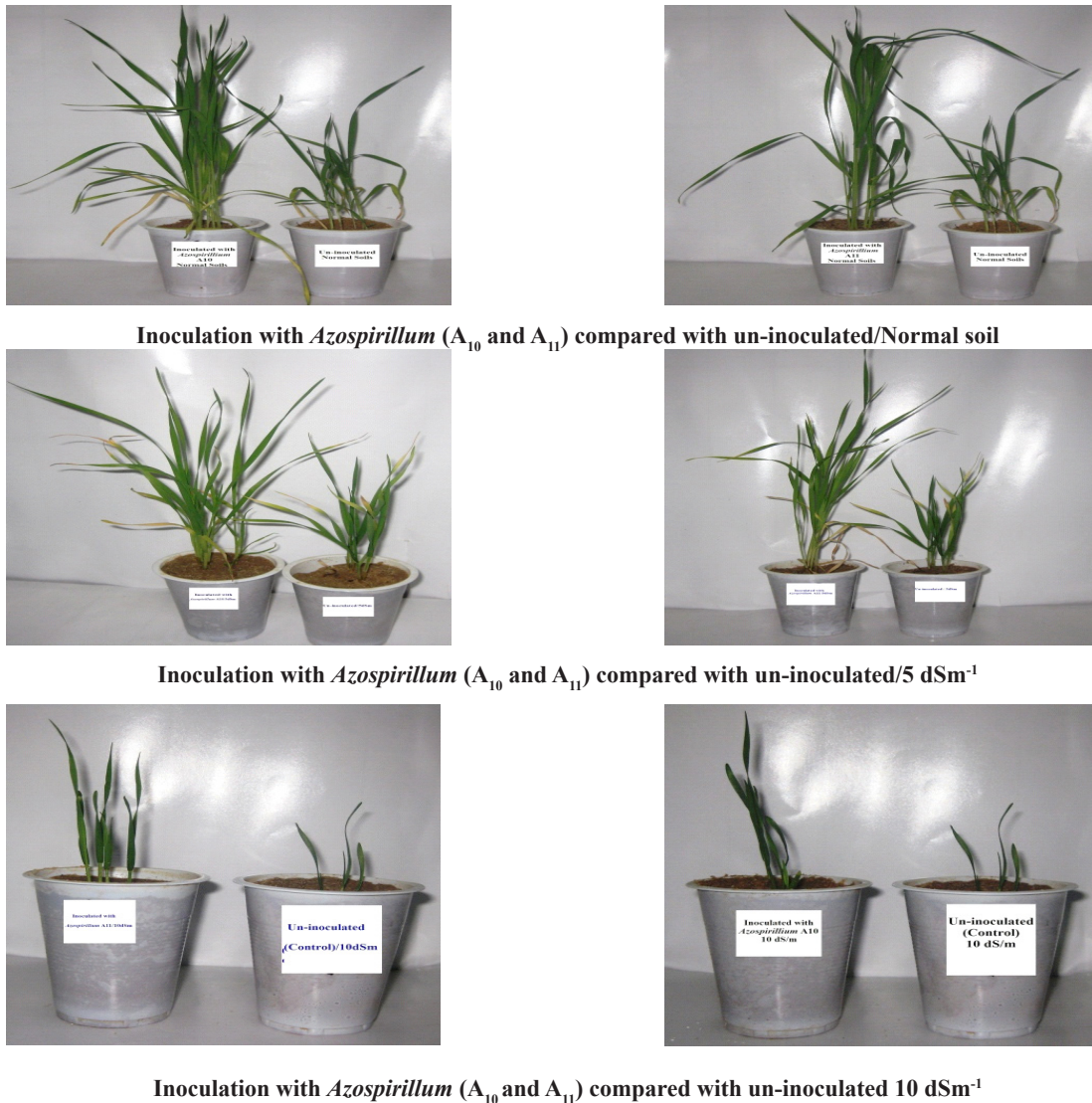


Fig. 2. Effect of inoculation with *Azospirillum* (A10 and A11) isolates on wheat growth parameters after 45 days of sowing under different salinity levels compared with un-inoculated control.

that in liquid culture *Azospirillum* spp. produced several plant hormones, e.g. isobutyric acid, IAA, and cytokinins which involved in the regulation of salinity stress (Walker and Dumbroff, 1981). While increasing salinity levels lead to diminution number of bacteria cells because of osmotic pressure out of the cells killed it, toxic effects of ions, particularly an ionic sodium and chloride (Soumitra et al. 2007).

In the pots experiment (*in vivo*), *Azospirillum* isolates had evaluated for their tolerance against salt stress and N fixing, wheat seedlings subjected to different concentrations of salt affected soils (Normal control (2), 5 and 10 dSm⁻¹) inoculated with the studied *Azospirillum* species. The

obtained results in Table 5 after 45 days of sowing showed a decrease in root wheat plant length with increasing salinity concentrations. Figure 3 showed a variation of wheat plant height in the plastic pots. This is in agreement with Munns (2002) reported that salinity reduces ability of plants to take up water and this quickly causes reductions in the growth rate. Also, Boghdady and Ali (2013) who found that salt-tolerant *Azospirillum* bacteria enhancing the root proliferation, increase in the lateral roots and root hair formation. Verma et al. (2001) illustrated that inoculation with salt-tolerant *Azospirillum* increased wheat vegetative growth and Somayeh et al. (2012) showed that inoculation of wheat

TABLE 5. Effect of inoculation with *Azospirillum* isolates on plant high and shoot length (cm/plant) of wheat plants under salinity stress after 45 days of sowing.

Treatment	Plant high			Shoot length			Dry weight g/plant		
	Salinity Levels								
	Normal	5 dSm ⁻¹	10 dSm ⁻¹	Normal	5 dSm ⁻¹	10 dSm ⁻¹	Normal	5 dSm ⁻¹	10 dSm ⁻¹
Un-inoculated	21.05	15.67	7.10	10.85	8.83	5.42	1.26	0.48	0.215
A ₁	21.90	17.20	8.30	10.77	9.40	6.22	1.58	0.755	0.238
A ₂	26.87	25.05	8.87	12.75	10.92	5.77	1.54	0.722	0.335
A ₃	27.40	23.82	7.75	12.95	11.52	5.90	2.02	0.760	0.512
A ₅	26.30	23.12	7.40	12.7	11.25	6.27	1.81	0.698	0.395
A ₇	27.82	24.45	7.55	14.00	11.40	6.47	1.82	0.688	0.425
A ₁₀	28.85	25.27	9.07	15.57	12.37	7.50	2.17	0.930	0.628
A ₁₁	27.45	23.60	7.60	16.35	12.15	7.15	2.22	0.945	0.632
A ₁₂	26.10	24.05	7.15	14.00	11.75	5.85	1.91	0.733	0.440
A ₁₃	26.35	23.42	7.12	12.92	10.30	6.02	1.67	0.725	0.412
A ₁₄	26.52	23.32	6.92	12.52	11.37	6.93	1.72	0.730	0.447
A ₁₅	26.82	23.55	6.87	12.45	11.15	6.42	2.05	0.913	0.700
A ₁₆	26.02	23.60	6.95	13.42	11.78	6.12	1.99	0.810	0.613
A ₁₈	22.87	23.37	6.35	11.07	10.85	5.67	1.85	0.830	0.628
A ₁₉	26.15	23.65	6.17	12.72	11.42	6.20	1.73	0.825	0.508
A ₂₀	25.32	23.40	6.85	13.22	11.17	6.20	1.70	0.752	0.508
A ₂₁	25.65	23.67	6.47	12.47	11.52	6.32	1.70	0.735	0.538
A ₂₂	25.95	23.12	6.30	13.37	11.42	6.05	1.48	0.655	0.432
A ₂₄	26.65	24.47	7.05	12.67	11.05	6.60	1.55	0.672	0.447
Means	25.89	23.04	7.25	12.99	11.13	6.27	1.78	0.75	0.47
LSD 5 %	S**		0.4013	S**		0.360	S**		0.0332
	I**		0.0867	I**		0.654	I**		0.108
	SI**		1.503	SI**		1.134	SI**		0.187

A: *Azospirillum* isolate S: Salinity I: Inoculation SxI: Interaction between Salinity and Inoculation *: significant
 **: Highly significant NS: Not significant

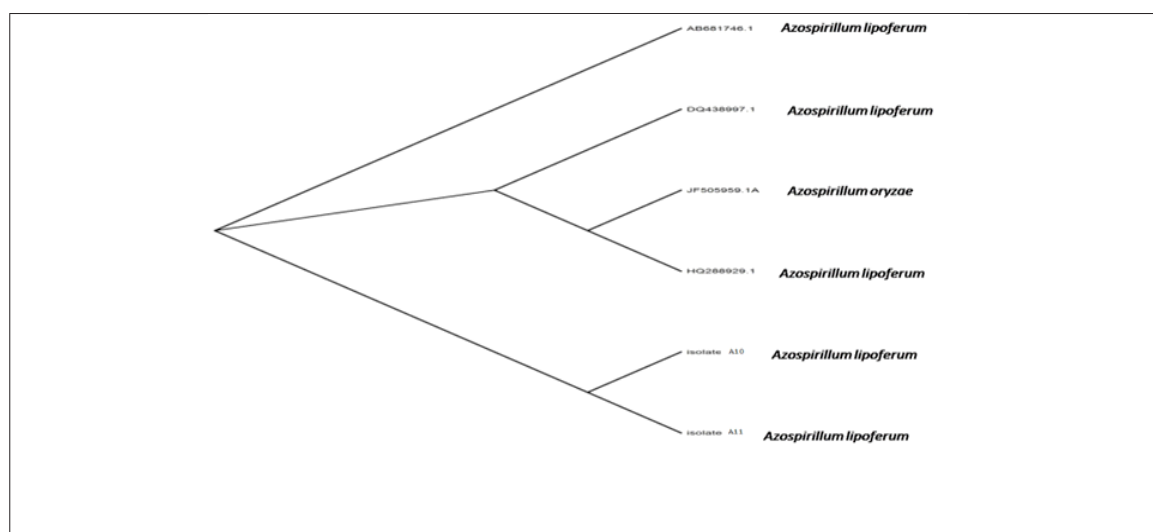


Fig. 3. Phylogenetic dendrogram showing the taxonomic positions of *Azospirillum* sp. type strains based on the 16S rDNA partial sequences.

plants grown under salt stress with *Azospirillum* isolates significantly increased growth parameter.

A decrease in dry weight of plants with rising salinity levels had been occurred as reported in Table 5. This is in agreement with **Munns (2002)** who reported that salinity reduces the ability of plants to take up water, and this quickly causes reductions in the dry weight of wheat. In addition, **Zaied et al. (2009)** showed that salinity caused reduction in dry weight of root and shoot at 60 days plant-old. Salts in the soil can reduce evapotranspiration by making soil water less available for plant root extraction and plant growth usually reduced by reducing the rate of leaf elongation, enlargement and cells division in the leaves (**Allen et al., 1998**). Otherwise, indicated that inoculation with N_2 -fixing *Azospirillum* that had a highly significant increase in dry weight of

the plant compared with control (un-inoculated). A_{10} and A_{11} isolates inoculation gave the best dry weight compared to other treatments under all salinity levels. In context, **Boghdady and Ali (2013)** indicated that, wheat (*T. aestivum*) seedlings inoculated with *Azospirillum* subjected to osmotic stress developed significant higher coleoptiles, higher fresh weight and better water status than un-inoculated seedlings. Also, inoculation with *Azospirillum* increased shoot and root dry matter of wheat (**Somayeh et al., 2012; Boghdady and Ali, 2013**). *Azospirillum* can also promote plant growth by mechanisms of tolerance of abiotic stresses, named as induced systemic tolerance, mediated by antioxidants, osmotic adjustment, production of phytohormones, and defense strategies such as the expression of pathogenesis-related genes (**Josiane 2018**).

TABLE 6. Effect of inoculation with Azospirillum isolates on N% and content (mg/plant) of wheat plants under salinity stress after 45 days.

Treatment	N %			N-content (mg/plant)		
	Salinity Levels					
	Normal	5 dSm ⁻¹	10 dSm ⁻¹	Normal	5 dSm ⁻¹	10 dSm ⁻¹
Un-inoculated	2.025	1.45	1.20	25.5	7.2	2.5
A₁	2.37	1.74	1.32	38.3	13.9	3.1
A₂	2.42	1.92	1.44	37.3	13.8	4.8
A₃	2.42	1.96	1.47	48.6	14.9	7.4
A₅	2.32	1.96	1.59	41.8	13.6	6.2
A₇	2.06	1.88	1.50	37.6	14.3	6.5
A₁₀	2.52	2.04	1.81	55.8	18.8	11.3
A₁₁	2.60	2.07	1.88	57.8	19.5	13.6
A₁₂	2.52	1.99	1.68	42.8	13.6	6.6
A₁₃	2.12	1.80	1.68	35.3	13.0	6.4
A₁₄	2.06	1.70	1.63	35.0	12.4	7.2
A₁₅	2.10	1.95	1.74	42.0	17.5	11.1
A₁₆	2.22	1.87	1.63	44.4	15.0	9.9
A₁₈	1.97	1.80	1.66	36.0	15.1	10.2
A₁₉	2.02	1.78	1.51	35.1	17.3	7.6
A₂₀	1.96	1.70	1.46	33.3	12.0	7.7
A₂₁	2.10	1.79	1.42	35.6	13.1	7.7
A₂₂	1.94	1.78	1.46	28.6	16.7	6.7
A₂₄	1.98	1.80	1.40	30.4	12.0	6.4
Mean	2.181	1.844	1.556	39.05	14.13	7.53
LSD 5 %	S**	I**	SI**	0.0561	S**	I**
				0.267	I**	2.51
				0.146	SI**	4.34

A: Azospirillum isolate S: Salinity I: Inoculation SxI: Interaction between Salinity and Inoculation *: significant **: Highly significant NS: Not significant

TABLE 7. Effect of inoculation with *Azospirillum* isolates on P% and P-content (mg/plant) of wheat plants under salinity stress after 45 days.

Treatment	P %			P-content (mg/plant)		
	Salinity Levels			Salinity Levels		
	Normal	5 dSm ⁻¹	10 dSm ⁻¹	Normal	5 dSm ⁻¹	10 dSm ⁻¹
Un-inoculated	0.074	0.070	0.068	0.92	0.34	0.14
A ₁	0.084	0.080	0.072	1.35	0.59	0.18
A ₂	0.082	0.080	0.074	1.25	0.58	0.25
A ₃	0.082	0.079	0.075	1.65	0.60	0.38
A ₅	0.080	0.079	0.074	1.43	0.55	0.29
A ₇	0.081	0.079	0.075	1.47	0.63	0.33
A ₁₀	0.083	0.079	0.075	1.79	0.73	0.47
A ₁₁	0.082	0.079	0.075	1.83	0.64	0.44
A ₁₂	0.081	0.076	0.073	1.67	0.58	0.32
A ₁₃	0.080	0.074	0.073	1.33	0.54	0.30
A ₁₄	0.079	0.075	0.072	1.35	0.55	0.32
A ₁₅	0.079	0.077	0.074	1.63	0.68	0.48
A ₁₆	0.079	0.078	0.074	1.58	0.63	0.46
A ₁₈	0.078	0.077	0.074	1.45	0.65	0.47
A ₁₉	0.078	0.077	0.074	1.37	0.63	0.37
A ₂₀	0.079	0.079	0.074	1.35	0.56	0.42
A ₂₁	0.078	0.077	0.073	1.34	0.57	0.40
A ₂₂	0.078	0.075	0.071	1.16	0.49	0.31
A ₂₄	0.077	0.077	0.072	1.20	0.51	0.31
Mean	0.080	0.077	0.073	1.43	0.58	0.35
LSD 5 %	S**		0.000125	S**		0.0012
	I**		0.00025	I**		0.0025
	SI*		0.00044	SI**		0.0153

A: *Azospirillum* isolate S: Salinity I: Inoculation SxI: Interaction between Salinity and Inoculation *: significant
 **: Highly significant NS: Not significant

Actually, results obtained showed a decrease in N, P, K concentrations and content of wheat by increasing the salinity concentrations (Tables 6,7 and 8). In this context, **Boghdady and Ali (2013)** showed that salinity decreased N, P, K concentrations and content of wheat plants by rising salinity concentrations. Furthermore, inoculation with *Azospirillum* isolates compared with un-inoculated control gave highly significant increase of N, P, K % and contents above the un-inoculated plants. This agreed with, **Somayeh et al. (2012)** and **Boghdady and Ali (2013)** reported that grains inoculation with salt-tolerant *Azospirillum brasilense* resulted in greater N, P, and K% and content of wheat compared to un-inoculated (N-fertilized). Plant

inoculation with *A. brasilense* promoted greater uptake of NO³⁻, K⁺ and H₂PO₄ in wheat. **Sarig et al. (1984)** and **Mertens and Hess (1984)** This well documented enzymatic activity is of sufficient magnitude to account for the increase in total N-yield of inoculated plants if the entire fix N is incorporated into the plants. Thus, the increased phosphate uptake observed in roots inoculated with *Azospirillum* could result from an increase in acid phosphatase activity (**Brahmaprakash and Sahu, 2012**). From the results obtained the best salt-tolerant, IAA production and efficient N-fixer *Azospirillum* species A₁₀ and A₁₁. The chosen isolates were subjected to 16S Ribosomal RNA (rRNA) amplification and sequencing.

TABLE 8. Effect of inoculation with *Azospirillum* isolates on K%, and K-content (mg/plant) of wheat plants under salinity levels after 45 days.

Treatment	K%			K-content (mg/plant)		
	Salinity Levels					
	Normal	5 dSm ⁻¹	10 dSm ⁻¹	Normal	5 dSm ⁻¹	10 dSm ⁻¹
Un-inoculated	3.63	3.53	3.43	46.0	17.6	7.4
A ₁	3.91	3.59	3.60	62.5	26.5	8.6
A ₂	4.01	3.66	3.62	61.7	26.8	12.2
A ₃	3.67	3.51	3.65	66.1	25.1	14.4
A ₅	3.83	3.44	3.58	71.6	25.9	14.1
A ₇	3.80	3.56	3.44	68.6	26.2	15.3
A ₁₀	4.07	3.75	3.63	88.6	34.9	23.0
A ₁₁	4.02	3.71	3.76	89.5	34.3	27.3
A ₁₂	3.86	3.65	3.53	67.5	25.1	15.5
A ₁₃	3.93	3.65	3.67	67.3	29.1	15.3
A ₁₄	3.90	3.72	3.46	81.1	27.3	15.4
A ₁₅	3.94	3.66	3.55	73.7	34.7	22.7
A ₁₆	3.70	3.67	3.60	72.8	31.0	22.0
A ₁₈	3.91	3.70	3.56	67.7	30.6	22.3
A ₁₉	3.89	3.76	3.60	64.9	31.2	18.2
A ₂₀	3.78	3.58	3.63	65.3	26.1	18.4
A ₂₁	3.83	3.71	3.68	55.3	27.6	20.1
A ₂₂	3.89	3.62	3.58	57.9	23.7	15.7
A ₂₄	3.91	3.66	3.57	60.6	24.5	15.9
Mean	3.871	3.643	3.590	68.6	27.8	17.0
	S**		0.0887	S**		1.39
LSD 5 %	I**		0.1196	I**		4.60
	SI**		0.27	SI**		7.97

A: *Azospirillum* isolate S: Salinity I: Inoculation SxI: Interaction between Salinity and Inoculation *: significant **: Highly significant NS: Not significant.

The obtained results clearly demonstrated that the study of 16S rDNA partial sequence spacer polymorphisms is a valuable tool to infer the nucleotide polymorphism position of the unknown isolates of *Azospirillum* sp. Moreover, even they were very closely related *A. lipoferum* isolate when we cannot distinguished with other available methods. Furthermore, the 16S rDNA partial sequences of the investigated *A. lipoferum* appear to be highly similar with few nucleotide differences compared with other sequences from Gene Bank. Our idolaters were highly conserved with other *Azospirillum* 16SrDNA sequences with high similarities with *A. lipoferum* than *A. oryzae* as shown in Fig. 3 and Appendix. This could help to be support the suggestion that rDNA were

evaluated based on concerted evolution (Austen and Kobayashi, 2007). Torbaghan et al. (2017) showed that inoculation with halotolerant bacteria improved the reduction of salinity effects on dry weight, plant height and production of wheat plants compared with un-inoculated treatments.

Conclusion

In conclusion, salt-tolerant *Azospirillum lipoferum* strains isolated from salt-affected soils are more efficient than other isolates and it increases wheat production under salinity condition. *A. lipoferum* have the ability to produce IAA and fixed Nitrogen so, it should be used to increase wheat productivity.

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