International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Mitigation of Soil Salinity Stress by Salt-Tolerant Rhizobacteria and *Saccharomycescerevisiae* in Wheat (*Triticum aestivum*)

Dalal H. Alkhalifah[1], Ahmed I. Elsayed[2], Serag A Farag[3]

[1]Microbiology of Biology Department, Faculty of Sciences Princess Nourah bint Abdulrahman

[2]Biology Department, Soil, Water and Environmental, Institute, Agriculture Res. Center, Giza, Egypt

[3]Univ.,Riyadh, Kingdom of Saudi Arabia, National Centre for Radiation Research & Technology, P.O.Box 8029.Nasr City ,Cairo ,Egypt.

Abstract: ***In the current study, A. chroococcum NRRL B-14346 and A. lipoferum showed grow well at 2.5% NaCl. Whereas, only A. lipoferum has growth at 3 % NaCl. A. chroococcum NRRL B-14346 isolates could tolerate 9.5 % NaCl. Our results proved that A. chroococcum NRRL B-14346 and A. lipoferum adapted to 1% increase in NaCl with a successful adaptation to higher concentration of NaCl. Only Azto4 and Azos9 were unstable after 60 days to a higher concentration of NaCl reached. All adapted isolates were further characterized for indole acetic acid production and nitrogenase production under 5 % NaCl concentration .Inoculation of adapted isolates with S. cerevisiae caused the significant increase in growth and yield parameters and chlorophyll concentration in both seasons. Inoculation of Azos6 with S. Cerevisiae recorded the maximum increase in 1,000-grain weight relative to the uninoculated control during two seasons. Co-inoculant A. lipoferum + S.cerevisiae recorded a significant increase in nitrogen concentration in leaves than uninoculated plants with 100% N fertilizers in both seasons under soil salinity stress. Co-inoculant A. lipoferum + S.cerevisiae and Azto4+ S. cerevisiae resulted in the highest increase of protein as leaves then the uninoculated control with 100% N fertilizers in both seasons at 60 days. Plants inoculated with Azos6 and co-inoculation Azos6+ S. cerevisiae had higher protein contents in seeds than uninoculated plants, uninoculated plants with 50% and 100% N in both seasons at 130 days.***

Keywords: Plant growth promoting rhizobacteria (PGPR); Adaptation; Abiotic stress; Wheat

Abbreviations

ARA Acetylene reduction assay
ARC Agriculture Research Center
OD Optical density
IAA Indole-3-acetic acid
CFU Colony-forming units
Fed Feddan
Chl. Chlorophyll

1. Introduction

Plants usually face several environmental stresses that can affect crop quality and productivity (Jones 2009). The need of the day is to decrease abiotic stress existing in soil for sustainable development for increasing international concern for food and environmental quality. Soil salinity is one of the main a biotic stress which affects the plant growth and productivity of agriculture. Nearly 40 % of world's surface has salinity problems (Jadhav *et al.* 2010).

Egypt is one of the countries that suffer from severe salinity problems. Salt stress negatively affects the establishment, growth and development of plantin several ways: water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Zhu, 2007; Moradi *et al.*2011).

Wheat is the most widely grown cereal crop in the world. It is cultivated on almost 215 million ha out of 670 million ha under cereals. Wheat is the most important grain crop in Egypt and grains.Wheat represents almost 10 percent of the total value of agricultural production and about 20 percent of all agricultural imports. Egypt remains is also the world's biggest wheat importer (FAO, 2017).Wheat is rated as moderately salt tolerant. Nevertheless, salinity reduces germination and delays emergence in this species and stands tend to be irregular with depressed crop yield. The significant decrease in wheat germination caused by salt stress (Ashraf and O'Leary1997) might be associated to elevated levels of salinity reducing seed germination and causing poor root growth (Zapata *et al*.2007).

To overcome this problem, suitable biotechnological approaches could be used to improve crop productivity in salt affected areas (Zahir *et al*.2004). One of these approaches is use of salt tolerant microbial strains associated with roots of different crops may improve plant resistance towards adverse salinity conditions (Yang *et al*. 2010). Moreover, salt-tolerant microorganisms may improve soil fertility through decomposition of organic matter and nutrient cycling, by fixation of atmospheric nitrogen or through production of growth hormones (Wu *et al*. 2009;).
The use of plant growth-promoting bacteria and symbiotic microorganisms has proved useful in developing strategies to facilitate plant growth in saline soils (Kohler *et al*., 2009). The mechanisms of alleviation of salt stress by PGPR include the production of phytohormones and competition for nutrient and niches. PGPR might also increase nutrient uptake from soils, thus reducing the need for fertilizers and preventing the accumulation of nitrates and phosphates in agricultural soils. A reduction in fertilizer use would lessen the effects of water contamination from fertilizer run-off and lead to savings for farmers (Yang *et al*. 2008).

One of common mechanism of salt tolerant microbial strains is adaptation to osmotic stress. Fortunately, bacteria have a

remarkable ability to adapt to environmental stress, and adaptive techniques are useful when beneficial mutations can be encouraged by environmental conditions. Various approaches of adaptive evolution have been employed including continuous cultivation with progressively increased feed concentrations (Liu *et al*. 2007) and shake flask cultures with prolonged exponential-phase growth (Charusanti *et al*. 2010). Recently, a device to facilitate adaptive evolution was developed (de Crécy *et al*. 2007), to make *E. coli* hermophilic (Blaby *et al*. 2012) and has been used to make *E. coli* sodium tolerant(Wu*et al*. 2014).Thus, selection of salt- tolerance PGPR strains, could be useful to design new inoculants to be used in salinity soil.

Plant growth promoting microorganisms with complementary functional traits and ecology can be combined and such microbial consortia have been shown to provide synergistic effects on growth promotion (Pii *et al*., 2015). Co-inoculation strategies might improve plant's performance. This approach is current with modern demands of agricultural, economic, social and environmental sustainability (Chaparro *et al*. 2012).

Usually, mixed inoculants are used for many crops grown under field condition are promote greater beneficial effects than single strain inocula. This was attributed in part to intensive population densities in mixed inocula and to the greater ability of the strains or species to cope with continually fluctuating conditions in the rhizosphere of inoculated plants. Also, strains in mixed inocula can have synergetic effect on the survival and persistence of other community members that are less competitor but desirable strains(Goddard *et al*. 2001). However, the knowledge about interactions between different plant growth promoting microorganisms is still limited (Larsen *et al*., 2015). Coinoculation of *Azotobacter chroococcum* and *Azospirillium* on wheat plants had positive effects on plant height, spike length, grain yield, biological yield and harvest index in various wheat genotypes Rai and Caur (1998).

Therefore, the present investigation aims at application of salinity tolerant *Azotobacter*and *Azospirillium* strains with *Saccharomyces cerevisiae* strains as biofertilizer for wheat cropunder saline soil conditions.

2. Materials and Methods

Strains and growth conditions

Bacterial strains and *Saccharomy cescerevisiae* used in this study and their sources are listed in Table1.*Azotobacter* strain was grown and maintained on Ashby's mannitol medium at 28±2 ◦C and 150 rpm according to Rao, (1984). However, *Azospirillum* was grown and maintained on Doberainer's Medium at30◦C under aerobic condition according to Döbereiner, (1976). *Saccharomy cescerevisiae* was maintained on standard yeast extract/peptone/dextrose (YPD) rich medium comprising in (w/v) %: Glucose, 2; Yeast extract, 1and Peptone, 2, otherwise stated agar, 2 %, Liu *et al*. (1997).

Table 1: Bacterial strains used in this study

Strains	Source of reference	Strains code
Azotobacter chroococcum NRRL B-14346	National center for Agriculture Utilization Research, USDA	A.*chroococcum* B-14346
Azospirillum lipoferum	Microbiology Dep., Soil , Water and Environmental Research Institute, Agriculture Research Center	*A. lipoferum*
*Saccharomy cescerevisiae*Y-389	National center for Agriculture Utilization Research, USDA	Y-389

Effect of NaCl on bacterial growth

Tolerance of *A.chroococcum* B-14346 and *A. lipoferum* to NaCl was evaluated on growth broth medium supplemented with increasing NaCl concentrations ranging between 0 and 4%. Flasks (125 mL) were incubated for seven days at standard conditions. The bacterial growth was monitored by measuring optical density at 600 nm (Pathak and Sardar 2012).

Adaptation to salinity: To adapt the strains for increased salt tolerance**,** each strain was tested for the final NaCl tolerated level**.**10 ml each strains were cultured in five independent 125-ml shake flasks overnight growing at 28°C in growth medium using rotary shaker at 120 revolutions per min (rpm) to giving finally 10^8 CFU/ml. Saline tolerant isolates were induced via subculture *A.chroococcum* B-14346and *A. lipoferum*strains in different NaCl concentrations using 1% intervals to enhancement the switch on of saline tolerance genes. Every 24 h, the optical density (OD) was measured, and 1 ml of the culture was transferred into 9 ml of a fresh medium. If the OD was much greater than that observed in the previous culture transfer, then the sea water concentrations was increased. This process was continued for 14 days, after which time asingle colony from each culture was isolated on solid (agar). Five single colonies from that appeared in every concentration were picked up and sub-cultured on Ashby's and Doberainer's slant agar medium. The resistant colonies obtained were retested and purified on the same medium containing the same concentration of sea water (Wu *et al*.2014).

Measurement stability of adaptive isolates

Stability of *A.chroococcum* B-14346 and *A. Lipoferum* isolates resulted from adaptation to high Nacl concentrations was confirmed by growing an aliquot of the frozen stock for each of the five isolates (and reference strains as a negative control) in growth medium without additional sea water, transferring once into the same medium, and then transferring into growth medium with the last concentration adapted. Salinitytolerance was quantified by growing *Azotobacter* and *Azospirillium* strains and each isolates in growth medium, then transferring the culture into a series of growth media containing different concentration of NaCl(Wu *et al*.2014)..

Quantification of IAA production

The production of IAA by the bacterial strains and their adaptive tolerance isolates was determined according to the method of Bano and Musarrat (2003). Production broth medium, 50 ml containing different concentration of NaCl supplemented with l-tryptophan(100 mg l^{-1}) at 30 ◦C for 72

were inoculated with the tested bacterium and incubated at 30°C with shaking at 120 rpm for 7 days. After centrifugation at 5000 rpm for 15 min, one milliliter of the supernatant was mixed with 2 ml of Salkowski reagent and the appearance of a pink color indicated IAA production. The absorbance was measured at 530 nm and the quantity of IAA produced was estimated against the IAA standard.

Plant growth promotion (PGP) traits

Acetylene reduction assay (ARA)

The Nitrogenase enzyme activity was estimated quantified by gas chromatography (GC) according to (Rao *et al*. 1983). Hewlett Packard chromatograph (Hp 6890 GC) fitted with a dual flame detector and a 150 cm × 0.4 cm diameter stainless steel column fitted with a Propa XR 100-120 mesh used for the determination. The bacterial strains and their adaptive tolerance isolates were incubated in 3 ml nitrogen-free mineral salt-yeast extract broth containing 1% NaCl for 72 h at 29°C in 7 ml tubes stoppered with cotton plugs. After visible growth, cotton plugs were aseptically exchanged with rubber stoppers and the headspace air was replaced with 10% (by volume) of high purity C_2H_2 gas by hypodermic syringe. The C_2H_4production was measured after 72 h incubation of the tubes in dark at 29°C. Tubes without C_2H_2 served as controls. The data are expressed as μmol/C_2H_4/h.

3. Field Experiments

Site description

The present study was conducted on the farmers' fields at Taq El-Ezz Research Station, Dakahlia Governorate, Egypt Nile delta during two seasons 2013 to 2014. Taq El-Ezz is locatedatlatitude+7 m altitude, 31^{O}36' latitude and 30^O 57' longitude. The total farm area used for experiment for the entire treatments was approximately 875 m^2 (5 Kirate). These field experiments tested the response of wheat to inoculation with different inoculants formulations, each containing individual or multiple bacterial strains, their adaptive tolerance isolates and *Saccharomy cescerevisiae*.

Figure 1: A Satellite image of the Nile Delta and the study region Taq El-Ezz- Dakahlia Governorate in the Egypt Nile delta where field inoculation trial were performed

Soil description

Soil samples were collected fromsurface soil to a depth of 30 cm just before tillage. Standard methods of analysis (Black *et al*. 1965; Jackson 1967) were used to assess soil textures, chemical, physical, and physicochemical properties that dominated the experimental fields during two seasons (Table 3).

Wheat seeds

Seeds of Misr 1variety was used, which obtained from field Crops Research Inst., Agriculture Research Center (ARC), Giza, Egypt.

Preparing seed

Wheat seeds (variety Misr 1) were surface sterilized by immersing in 70% ethanol for 5 min, followed by 0.1% mercuric chloride for 3 min and then rinsing several times with sterile distilled water. These seeds were air dried under laminar flow (Prasanna *et al.* 2009).

Preparation of inoculants for field experiments

For the inoculation treatments, the seeds were dressed using the inoculated peat mixed with10 mL of 10% sucrose solution as sticker. To prepare the inoculant carrier, peat was sterilized at 80 °C for4 h. Pure cultures of the test strains were grown in broth medium at 29 °C with rotary shaking(150 rpm) for 3 days to produce population densities of 10^8–10^9 colony-forming units (CFU)/ml. Similar culture volumes of either individual strain or balanced mixtures of strains (1/1 or 1/1/1 v/v) were mixed with the peat carrier to maintain a moisture content. The inoculum was kept at room temperature for 24 h and stored under refrigeration until used.

Field preparation

The 30 to 40 cm depth of the top soils of the experimental fields was plowed three consecutive times inperpendicular directions. Remains of the previous crop were removed after the second plowing. Ammonium nitrate (33%NH_4NO_3), Calcium super-phosphate (15 % P_2O_5) and potassium sulphate (48 % K_2O) were broadcasted at rates of 120, 100 and 120 kg/Feddan, respectively. Phosphorus was added as superphosphate (15.5% P_2O_5) at a rate of 200 kg fed-1 once during soil preparation. Potassium was added as potassium sulphate (48% K_2O) at a rate of 50 kg fed-1once before flowering stage (60 days from sowing). All treatments received 50 % of the recommended dose of nitrogen and phosphorus except the control, which received the full dose of chemical fertilizers.

Table 2: Mechanical and chemical properties in the experimental soil used in this study.

Physical properties Mechanical analysis (%)	Season 2013/2014	Season 2014/2015
Coarse sand	3.75	3.72
Fine sand	8.60	8.35
Silt	26.90	28.76
Clay	60.70	59.81
Textural class	Clayey	Clayey
Chemical properties		
Organic matter %	1.1	1.3
Total nitrogen mg kg^{-1}	22	23
CaCO3 %	2.2	2.3
Available K (ppm)	175.21	169
pH soil	8.25	7.9
E.C (ds.m-1)	10.9	9.0
Cations (meq/100 g soil)		
Ca^{2+}	7.9	9.46
Mg^{2+}	9.6	24.73
Na^{+}	32.9	49
K^{+}	1.13	1.76
Anions (meq/100 g soil)		
HCO_3^-	1.6	8.25
Cl^-	31	32
SO_4^{2-}	18.93	44.70

Field experiment

Field experiment were conducted at the experimental farm, of Taq El-Ezz Research Station, Dakahlia Governorate, Egypt Nile delta during two seasons of 2013/2014 and 2014/2015. This investigation aimed to evaluate the performance of bacterial strains, their adaptive tolerance isolates and *Saccharomy cescerevisia.* Treatments were arranged in randomized complete block design (RCBD) with three replicates. A total of 11 treatments with completely randomized design was replicated three times (Table 2). Control without anyinoculation with biofertilizers was also used. Application of 100% NPK chemical fertilizers (without inoculants), served as positive control, and 50% N, 100% Pand 100% K was used to compare the nitrogen-fixing potential of the inoculants.

Data recorded:

Wheat mature plants data:

Growth measurements, for the plants exposed to saline treatments, were taken at 60 days. The three replicates taken for each treatment, were used to calculate the mean of each measurement. The growth measurements taken at 60 days were the following: root dry weight (g), shoot dry weight (g), root length (cm) and plant height (cm). At physiological maturity, 3 months after seed germination10 plants per treatment were harvested and data regarding number of tillers/plant, Plant height (cm), Spike length (cm). number of spikes/plant, Spike dry weight (g), number of grains/spike, dry weight of 1000 grains (g), grain yield (Ton/fed),straw yield (Ton/fed), biological yield (Ton/fed) and harvest index were recorded.

Table 3: Details of experimental treatments

Treatment	Details	Strains used	Carrier based application with
T1	No chemical fertilizers	Uninoculated control	No microbialinoculant
T2	100% N 100% P 100% K	Uninoculated control	No microbialinoculant
T3	50% N 100% P 100% K	Uninoculated control	No microbialinoculant
T4	*A.chroococcum* B-14346	*Azotobacter chroococcum* NRRL B-14346	Single cultureinoculant
T5	$Azto_4$	*AdaptedAzotobacter chroococcum* NRRL B-14346 isolate	Single cultureinoculant
T6	*A. lipoferum*	*Azospirillum lipoferum*	Single cultureinoculant
T7	$Azos_6$	Adaptive *Azospirillum lipoferum isolate*	Single cultureinoculant
T8	*A.chroococcum* B-14346+ *S. cerevisiae*	*Azotobacter chroococcum* NRRL B-14346+*S. cerevisiae*	Co –culture inoculant
T9	*A. lipoferum + S.cerevisiae*	*Azospirillum lipoferum + S. cerevisiae*	Co -culture inoculant
T10	$Azto_4$+ *S. cerevisiae*	Adaptive *Azospirillum lipoferum isolate + S. cerevisiae*	Co -culture inoculant
T11	$Azos_6$+ *S. cerevisiae*	Adapted *Azospirillum lipoferum isolate + S. cerevisiae*	Co -culture inoculant

Photosynthetic pigments

One gram of fresh tissue, taken from the third and fourth leaf, was extracted by grinding in a mortar using 20 ml 80% methanol. Chlorophyll (chl. a, b and total) were determined spectrophotometrically after stored the extracted solution for twenty four hours in a refrigerator and calculated according to the Lichtenthaler and Wellburn (1983) formulae. Three replicates were used for each treatment, and the amount of pigment present in each sample

Plant analysis

For dry weight analysis, different parts of plants (shoots and roots) at 60-days-plant-old were oven dried at 70°C until reached to a constant mass and then turned immediately to weight. The wheat plants dried and ground for nitrogen and phosphorus analysis. N content was determined by a micro Kjeldahl method **(**Yuen and Pollard, 1953**)**and P content was estimated by vanado-molybdate method (Jackson, 1967).Crude protein was calculated by multiplying total nitrogen N-content by 5.75 AOAC, (2005).

Total protein assay

Protein was extracted from seeds at harvesting time by dilute alkaline hydrolysis. Then proteins in the supernatants were quantified by the Coomassie Brilliant Blue procedure for protein determination (Bradford, 1976) was used to determine protein concentration. However, Bovine serum albumin ranging in concentrations from 0 to 100µg/ml was used as the standard from the standard curve.

Statistical analyses

Analysis of variance (ANOVA) between the biochemical parameters of the inoculants was carried out by completely randomized design (CRD) while the same between the crop biometric experimental field measurements was carried out by randomized block design (RBD). The least significant (LSD) difference test was applied to evaluate the significance of difference between individual treatments at 5% and 1 % probability level according to Snedecor and Cochran (1955).Mean and standard error of three replicates were calculated using Microsoft Excel 2013 software.

4. Results and Discussion

Effect of different NaCl concentrations on growth of bacterial strains

The two diazotrophic strains *A. chroococcum* NRRL B-14346and *A. Lipoferum* were screened for salt tolerance with various concentrations of NaCl. The results showed that high levels of NaCl repressed bacterial growth, where strains *A. chroococcum* NRRL B-14346and *A. lipoferum* were tolerate NaCl until 2.5%, however *A. Lipoferum* only reached to 3 % NaCl. The results obtained here are in agreement with those reported by Paul *et al*.(2014)found that the growth and plant growth promoting activities of *Azotobacter chroococcum* was significantly reduced in high salt concentration (1.5M NaCl) and low salt concentration (0.3 M). Nevertheless, Chaudhary *et al*. (2013)found that *Azotobacter* strains ST3, ST6 and ST24 tolerance up to 8 % sodium chloride, whereas other strains ST9 and ST17 showed tolerance only up to 6%NaCl concentration.Viscardi*et al*. (2016) found that two *Azotobacter chroococcum* strains exhibited high tolerance to salt and could alleviate the negative effects exerted by abiotic stress on tomato plants. Similar results were found by Rojas-Tapias *et al*. (2012**)** who reported that salt did not exert negative effects on microbial growth of two selected strains of A. *chroococcum*, which reached an O.D.600 of about 0.2 at 5.85 g NaCl l^{-1}.Whereas, Holguin and Bashan (1996**)** found that *A. brasilense* Cd (the wild-type strain) could tolerated 2% NaCl when co-cultured with *Staphylococcus* sp.

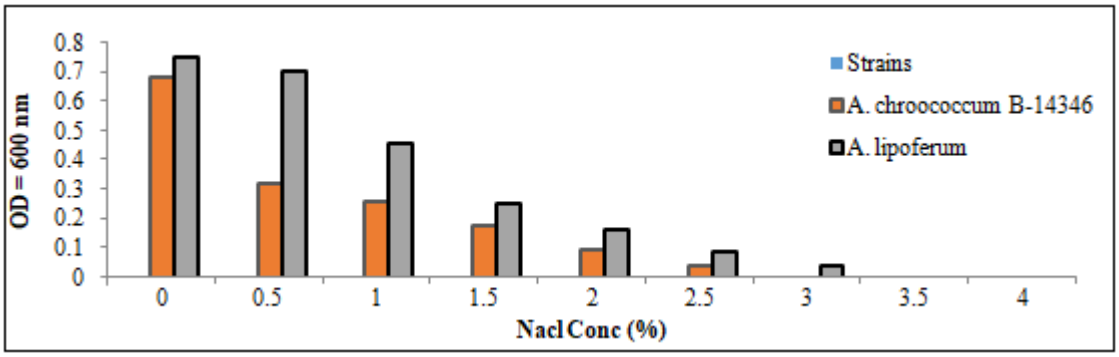
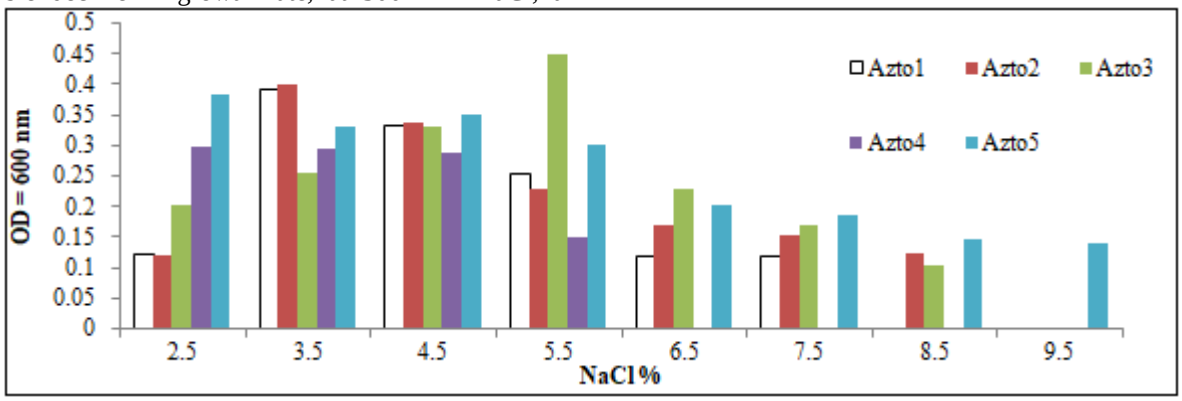


Figure 2: Growth of bacterial strains in response to various salt stresses The figure shows the maximum growth of each strain in their respective tolerance levels of NaCl. Each value is the mean of three replicates

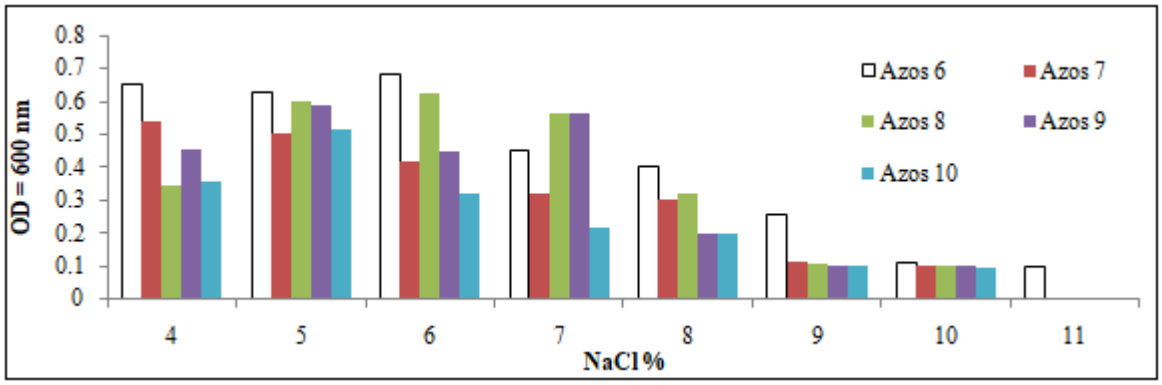
Adaptive evolution of PGPR bacteria

The adaptation to osmotic stress is crucial for microbial growth and survival due to exposure to salinity stress environments triggers rapid fluxes of cell water along the osmotic gradient out of the cell, thus causing a reduction in turgor and dehydration of the cytoplasm. So, the adaptation of diazotrophs strains to osmotic stress is great significance, because soil salinity inhibits many of the vital bacterial plant growth-promoting activities, such as nitrogen fixation and phytohormone production (Miller and Wood, 1996). In order to select adaptive bacterial strains to salinity stress, five independent cultures for every strain were exposed to in parallel to increasing concentration of NaCl as described previously and five isolates for each strain at final tolerance to NaCl concentration were selected. The adaptation of isolates resulted from *Azotobacter chroococcum* NRRL B-14346is shown in Fig.3, all strains tested started from the last level of NaCl, which reached in the previous test (Fig. 2). The results obtained clearly appeared that $Azto_2$, $Azto_3$, $Azto_5$ adapted from 3.5% up to 7.5 % NaCl, whereas isolate $Azto_5$ adapted to 9.5 % NaCl. In addition, $Azto_4$ reach to 6.5 % NaCl. This indicated that 9.5 % NaCl was a top level at which *Azotobacter chroococcum* NRRL B-14346 isolates could tolerated. On the other hand, all *Azospirillum lipoferum* adaptive isolates adapted from 4% up to 10 % NaCl whereas isolate $Azos_6$ adapted to 11% NaCl. In this study, all the isolates at higher NaCl concentrations grew. This could be due to the synthesis of protective factors and adaptation of current environmental conditions (Finkeland Kolter 1999).How *et al*. (2013) similarly reported that *E. coli* able to persist in high NaCl concentrations after multiple generations and that suggests that *E. coli* may be able to become increasingly halophilic over time. Whereas, Wu *et al*.(2014) reported that the adaptation of *E. coli* MG1655 enhanced Na tolerance compared to the wild type and that the adaptation significantly improved lactate production. Chowdhury *et al*. (2007) found that the growth of seven *Azospirillum brasilense* strains under300mMNaCl stress indicated a variability in salt tolerance from the most salt-sensitive strain Cd to the most salt tolerant strain MTCC4036.Tanaka *et al* (2004), who found that high-pressure adaptation was examined using a moderately halophilicbacterium (*Micrococcus roseus*) isolated from open seawater and capable of growing in 15 % w/v NaCl

(optimum NaCl concentration 3 % w/v). It was cultured in 1, 3, 5, 10 and 15% NaCl, the survival ratio proportionally increased at increased NaCl concentration. On the other hands, Suhail and Mahdi,(2011) demonstrated that increasing levels of salinity (0 to 6) ds.m-1caused to a decreasing number of bacteria the plant growth promoting rhizobacterium *Azotobacter chroococcum*.Rivarola *et al.* (1998), who found that growth of *Azospirillum brasilense* Cd in the presence of different NaCl concentrations showed that it tolerated up to 200 mM NaCl in the medium, without appreciable decline in growth rate, at 300 mM NaCl, a decrease of 66% in growth was observed at 24 h of culture and at 48 h of culture, bacteria in the presence of 300 mM NaCl reached the maximum optical density value that was attained at 12 h by control cultures. However, Jofré *et al.* (2009) generated an *A. brasilense* Cd Tn5 mutant that showed exopolysaccharides overproduction, decreased tolerance to saline conditions, altered cell morphology, and increased sensitivity to detergents.



A



B

Figure 3: Growth of *Azotobacter chroococcum* NRRL B-14346and *Azospirillum lipoferum*adaptive isolates during different grades of NaCl concentrations.
A: Growth of *Azotobacter chroococcum* NRRL B-14346 adaptive isolates
B**:** Growth of *Azospirillum lipoferum* adaptive isolates

Stability Testing of Adaptive Isolates: AdaptiveIsolates resulted from adaptation to high NaCl concentrations were tested after different time (20, 30, 40, 50and 60)days of frozen preservation to the last concentration adapted. The results were summarized in Table 4. It was appeared that None of adaptive isolates returned to the pre-adaptation sensitivity, except $Azto_4$ and $Azos_9$isolates were unstable for gene stability after 60 days at the final concentration of NaCl reached, their growth are suppressed. The results obtained here are in agreement with Braoudaki and Hilton (2004) reported that the adaptive resistances of *Salmonella* serovar Enteritidis, *Salmonella* serovar Typhimurium,*Salmonella* serovar Virchow, and *E. coli* O157 were not lost following more than30 days of passage in antibiotic or biocide-free medium. In the absence of a selective pressure, none of the strains returned to the preadaptation sensitivity. On the other hands, Wu *et al.*(2014) reported that *E. coli* MG1655 and their adaptive isolates ALS1187 showed markedly different in maintenance coefficients under steady-state conditions.

Table 4: Genetic stability of NaCl tolerance in bacterial strains

adapted isolates	Final NaCl %	Growth at final concentration				
		20 day	30 day	40 day	50 day	60 day
$Azto_1$	7.5	+	+	+	+	+
$Azto_2$	8.5	+	+	+	+	+
$Azto_3$	8.5	+	+	+	+	+
$Azto_4$	5.5	+	+	+	+	-
$Azto_5$	9.5	+	+	+	+	+
$Azos_6$	11	+	+	+	+	+
$Azos_7$	10	+	+	+	+	+
$Azos_8$	10	+	+	+	+	+
$Azos_9$	10	+	+	+	+	-
$Azos_{10}$	10	+	+	+	+	+

+,- = Growth and growth suppressed , respectively .

Effect of salinity on IAA production and acetylene-reducing activity of bacterial strains and their adaptive isolates

The data presented in Table5. Determined carefully whether a statistically significant difference existed between

phytohormone (IAA) production and nitrogenase activity of adapted isolates ability in the presence of 5% NaCl concentration at which the parent strains couldn't grow. All adapted isolates appeared significant differences forIAA and nitrogenase production. Isolate $Azos_6$showed the highest produced a significant amount of IAAin the presence of tryptophan (37.1µg /ml) followed by $Azoto_4$(25.6 µg/ml), whereas, isolate $Azos_7$attained the lowest IAA production (4.9µg /ml).The nitrogenase activity of the adapted isolates varied from 16.0 to 3.2 µ moles /C_2H_4/ hr. Adapted isolates$Azto_3$, and Azos $_6$showed the highest significant amount of nitrogenase as compared to other isolates. While, $Azos_9$ showed lowest amount of nitrogenase activity. The results obtained herein are in agreement with Shubhangi *et al*. (2016)found that all salt tolerance isolates have plant growth-promoting activities (nitrogen fixation, phosphate solubilization, IAA, and ammonia production)under salinity conditions. Siddikee *et al*.(2011) reported that different halo tolerant bacteria were able to withstand high salt concentration (1.75 M NaCl) and were able to facilitate plant growth promotion in the presence of growth inhibitory levels of salt. As well as, Ramadoss *et al*. (2013) found that two halo tolerant bacterial isolates (SL3 and J8W) were positive for IAA production.

Two isolates ($Azto_4$and $Azos_6$) had the highest multiple PGP (IAA and nitrogen fixation activities) were chosen to be used with *Saccharomyces cerevisiae* in field experiment**.**

Comparison of IAA and nitrogenase production of different bacterial strains and their adaptive isolates in growth medium containing5% NaCl,. Under these conditions, the wild-type strains showed no growth. In each column, statistically significant differences in IAA and nitrogenase production are denoted by *, $p < 0.05$; **, $p < 0.01$.

Field inoculation experiments

Field results were tabulated comprehensively to clearly indicate the effect of bacterial inoculation alone or mixed with the *S. cerevisiae* during two winter seasons2014/2015 and 2015/2016 under the half doses of fertilizer-N application.

Table 5: Comparison of IAA and nitrogenase production by different bacterial strains and their adaptive isolates at 5% NaCl

Strains	IAA (µg /ml)	Nitrogenase activity (µmoles /C_2H_4/ hr)/culture
$Azto_1$	24.3	12.9
$Azto_2$	17.2	11.1
$Azto_3$	14.9	16.0
$Azto_4$	25.6	14.9
$Azto_5$	3.0	5.1
$Azos_6$	37.1	13.5
$Azos_7$	4.9	12.2
$Azos_8$	19.0	5.2
$Azos_9$	17.1	3.2
$Azos_{10}$	17.7	12.0
F-test	**	**
LSD 0.05	4.1	7.8
0.01	4.8	9.1

Plant parameters

As shown from the results presented in the Table6indicated that various studied treatments had a significant effect ($P < 0.05$) on growth parameters (shoot Dry weight (g), root dry weight (g), root length (cm) and plant height (cm)during two seasons under soil salinity stress after 60 days. Maximum increase in plant parameters was observed with combined inoculation of$Azto_4$+*S. cerevisiae* during two seasons 2014/2015. Whereas, combined inoculation of$Azos_6$+*S. cerevisiae* inoculation resulted in attaining the highest increase in shoot dry weight (1.6g) during two seasons 2014/2015.Mohamed and Almaroai (2016) found that co-inoculation (*Azotobacter chroococum* with yeast) resulted in significant increase in growth parameters of wheat. Chaudhary *et al.* (2013) found that inoculation wheat with salinity tolerant *Azotobacter* strain ST24 resulted in attaining 89.9 cm plant height, 6.1 g seed yield, 12.0 g shoot dry weight and 0.7 % total nitrogen at fertilization dose of 120 kg N ha^{-1}. Whereas, Pandey *et al*. (1998) reported that the promotion of plants growth inoculated with *Azospirillum* has been obtained in field conditions and greenhouse experiments, resulting in significant changes in several characteristics of the plants. Similar a significant growth improvement of plant growth parameters of wheat and maize were observed by application of microbial inoculants under saline conditions (Mahmoud and Mohamed 2008). Hamdia *et al*. (2004) reported that positive effect of inoculation by *Azospirillum lipoferum*, on plant dry weight and leaf area in maize under high salinity. Creus *et al*., (1997), reported that *Azospirillum brasilense* Sp245can be reversed the damaged effects of salinity stress on the growth of shoots in wheat.

Photosynthetic pigments

Salt stress also had an effect on the biochemical characteristics. Leaf chlorophyll concentration is an indicator of salt tolerance and responds to increasing salinity Percival *et al*. (2003).This decrease was attributed to the salt inducing the weakening protein-lipids complexes and an increased activity of chlorophyllase. Moreover, salinity reduced the biosynthesis of photosynthetic pigments and caused changes in the integrity and composition of the chloroplasts membranes (Günes *et al*., 1996).Data presented in Table 7 indicated that all microbial inoculations with 50% N-fertilizer had a significant effect ($P < 0.01$) on chlorophyll concentrations in the leaves of wheat plants during two seasons under soil salinity stress.

Table 6: Effect of bacterial strains, their adaptive isolates and *S. cerevisiae* on plant growth of wheat at 60 days of plant growth during two seasons

Nfertilization (%)	Inoculants	Growth Parameters							
		Shoot Dry weight (g)		Root dry weight (g)		Root length (cm)		Plant height (cm)	
		Season I	Season II	Season I	Season II	Season I	Season II	Season I	Season II
0	Uninoculated control	0.6	0.7	0.26	0.21	7.5	8.2	65.7	43.7
100	Uninoculated control	1.7	1.9	0.57	0.55	16.6	17.2	62.7	64.7
50	Uninoculated control	0.9	1.4	0.49	0.41	8.3	9.0	73.7	61.0
50	*A.chroococcum* B-14346	1.1	1.6	0.55	0.39	11.1	9.8	56.3	68.7
50	$Azto_4$	0.9	1.6	0.43	0.46	11.2	12.2	67.0	51.7
50	*A. lipoferum*	1.3	1.5	0.53	0.53	11.6	11.6	65.3	63.3
50	$Azos_6$	1.4	1.6	0.49	0.52	11.9	11.9	60.3	63.0
50	*A.chroococcum* B-14346+*S. cerevisiae*	1.2	1.9	0.60	0.48	13.8	13.8	63.0	55.7
50	*A. lipoferum* + *S.cerevisiae*	1.2	1.7	0.50	0.56	13.6	13.6	72.3	59.0
50	$Azto_4$+*S. cerevisiae*	1.0	1.7	0.52	0.54	16.3	16.3	65.0	60.3
50	$Azos_6$+*S. cerevisiae*	1.6	1.6	0.41	0.46	15.1	15.1	72.0	71.7
F-test		**	**	*	**	**	**	*	**
LSD 0.05		0.50	0.59	0.16	0.13	1.76	1.9	9.1	7.4
0.01		0.69	0.80	0.21	0.18	2.39	2.5	12.4	10.02

Significant difference at *, $p < 0.05$; **, $p < 0.01$ by ANOVA.

Apparently, the inoculation of saline adapted isolates with or without *S. Cerevisiae* appeared significant increasein chlacompared with uninoculated control with 50 % N fertilization during first season after 60 days. Whereas, plants inoculated with the adapted isolates withor without *S. Cerevisiae* had higher Chl b concentrations than controls and control with 50 % N fertilization during two seasons. Similar to our study, (Galleguillos *et al*. 2000).reported that combined inoculation of bacterial strains with AM fungi produced growth-stimulating effects that surpassed those of individual inoculations on non-legumeplant species .Whereas, Habib *et al*. (2016) reported that PGPR bacterial strains appeared higher germination percentage, growth parameters, and chlorophyll content than control of okra plants. Rojas-Tapias *et al*. (2012) reported that the content of chlorophyll of maize was enhanced by inoculation with salinity tolerant *Azotobacter* strains C5 and C9.Ma *et al*. (2012). reported that inoculation of *A. brasilence* considerably improved the leaf chlorophyll content in white clover plant, cultivated under salt concentrations of 40, 80, and 120 mM NaCl. Zhang *et al*. (2008) found that the inoculation by *B. subtilis*GB03 enhances photosynthetic efficiency in Arabidopsis.

Table 7: Effect of salinity tolerant PGPR strains and *S. cerevisiae* on chlorophyll concentration of wheat at 60 days of plant growth during two season

N fertilization (%)	Inoculants	Chlorophyll content(mg/g F.W)							
		Chl a		Chl b		Total Chl		Chla/b	
		Season I	Seasone II	Season I	Seasone II	Season I	Seasone II	Season I	Seasone II
0	Uninoculated control	0.38	0.37	1.79	1.82	2.36	2.19	0.36	0.21
100	Uninoculated control	0.51	0.27	3.47	1.35	3.98	1.62	0.15	0.20
50	Uninoculated control	0.16	0.21	2.94	1.00	3.40	1.22	0.06	0.22
50	*A.chroococcum* B-14346	0.37	0.27	2.51	1.22	2.88	1.50	0.15	0.23
50	$Azto_4$	0.54	0.26	3.25	1.41	3.79	1.67	0.17	0.18
50	*A. lipoferum*	0.53	0.25	3.68	1.12	4.21	1.37	0.14	0.22
50	$Azos_6$	0.31	0.31	3.12	1.85	3.43	2.16	0.10	0.17
50	*A.chroococcum* B-14346+*S. cerevisiae*	0.46	0.37	3.37	1.92	3.83	2.29	0.14	0.19
50	*A. lipoferum* + *S.cerevisiae*	0.55	0.34	3.19	1.48	3.73	1.82	0.17	0.23
50	$Azto_4$+*S. cerevisiae*	0.49	0.31	3.20	1.54	3.69	1.84	0.15	0.20
50	$Azos_6$+*S. cerevisiae*	0.57	0.31	3.60	1.70	3.98	2.01	0.10	0.18
F-test		**	*	**	**	**	**	*	NS
LSD 0.05		0.19	0.09	0.51	0.16	0.44	0.18	0.13	
0.01		0.25	0.14	0.69	0.21	0.59	0.24	0.18	

Significant difference at *, $p < 0.05$; **, $p < 0.01$ by ANOVA.

The results obtained clearly established that, plants inoculated with microbial inoculations had a significant effect ($P < 0.01$) on growth parameters (plant height(cm), tillers/plant, spikelet / plant and spikelet length cm)of wheat plants during second seasons after 130 daysin salt-affected soil. The microbial inoculation *A.chroococcum* B-14346, $Azto_4$, $Azto_4$+*S. Cerevisiae* and $Azos_6$+*S.cerevisiae* appeared significant increase in plant height compared to uninoculated control under soil salinity stress during season 2013/2014after 130 days. However, combined inoculation of$Azto_4$with *S. Cerevisiae* and $Azos_6$ with *S. Cerevisiae* appeared the same result as compared to uninoculated control during the winter growing season of 2014/2015in plant height. Moreover, all microbial inoculants appeared significant increase tillers/plant, spikelet/ plant and spikelet length (cm) as compared to uninoculated control under soil salinity stress during two seasons. This results agreed with Nia *et al*. (2012) saline adapted *Azospirillum* inoculation significantly increased total plant dry weight, total number of tillers, and ears; earlier heading and flowering time; number of spikes and grains per spike. The increase population density of PGPR in the root zone could decrease

theNa available for plant uptake, thus helping to all eviate salt stress in plants Ashraf (2004). Furthermore, the stimulatory effect of bacterial inoculation may be played important role in decrease salinity negative effect due to the growth-promoting substrates production by PGPR bacterial such as nitrogen, phosphorus, nitrite and indole-3 acetic acid in the plant rhizosphere (Akhter *et al*., 2004; Rothballer *et al*., 2005). Also, (Zahiroddini *et al*., 2004) found that the microbial inoculation increased the plant nutritional as simulation and improved soil properties such as organic matter and total N-content.

Table 8: Effect of salinity tolerant PGPR strains and *S. cerevisiae*on growth parameters of wheat at 130 days of plant growth in both seasons

N fertilization (%)	Inoculants	Growth Parameters							
		Plant height (cm)		Tillers/ plant		Spikelet / plant		Spikelet length (cm)	
		Season I	Seasone II	Season I	Seasone II	Season I	Seasone II	Season I	Seasone II
0	Uninoculated control	66.4	66.6	2	1	1	2	7.7	8.2
100	Uninoculated control	87.7	82.3	4	5	3	4	13.1	9.9
50	Uninoculated control	72.9	75.1	3	4	3	2	9.6	11.3
50	*A.chroococcum* B-14346	71.4	66.8	3	4	3	3	10.3	12.3
50	$Azto_4$	75.1	67.9	4	3	3	3	11.3	11.6
50	*A. lipoferum*	73.2	68.6	2	3	2	4	10.0	10.9
50	$Azos_6$	73.0	63.3	3	4	3	5	10.9	12.2
50	*A.chroococcum* B-14346 +*S. cerevisiae*	79.3	69.1	5	5	4	5	12.2	13.5
50	*A. lipoferum* + *S.cerevisiae*	76.5	77.6	4	5	4	5	12.1	14.8
50	$Azto_4$+*S. cerevisiae*	79.0	81.7	5	5	4	5	14.2	14.2
50	$Azos_6$+*S. cerevisiae*	86.0	81.8	5	6	5	6	14.5	15.3
F-test		**	**	**	**	*	**	**	**
LSD 0.05		7.1	9.5	1.4	1.3	1.6	0.96	1.85	2.2
0.01		9.6	12.9	1.9	1.8	2.2	1.30	2.51	2.95

Significant difference at *, $p < 0.05$; **, $p < 0.01$ by ANOVA.

Yield parameters

In addition to enhancing soil biological and nutrient availability, adapted PGPR bacterial strains are known to improve plant growth and nutrient uptake under soil salinity stress. Data presented in Table 9 show statistically significant increases in 1,000-grain weight (g) by various microbial inoculations with the maximum increase by combined inoculation of $Azos_6$ with *S. Cerevisiae* relative to the uninoculated control during two seasons. Inoculation with bioinoculants *A. lipoferum* + *S.cerevisiae* and $Azos_6$+*S. cerevisiae* appeared significant increase in compared to uninoculated plants with 50% N fertilizers.The increase in grain yield was resulted from increasing the number of fertile tillers per plant as reported by Ozturk*et al*. (2003) and Salantur *et al*. (2006). Inoculation with *A.chroococcum* B-14346 and all combined inoculant mixtures provided the statistically highest grain yield(Ton/fed) in compared with uninoculated plants and uninoculated plants with 50% N fertilizers in both seasons. However, all microbial inoculants appeared significantly increase in straw yield (Ton/fed) than uninoculanted plants in second season. The inoculants *A.chroococcum* B-14346 and all combined inoculant mixtures appeared significant increase in comparison with uninoculated plants and uninoculated plants with 50% N fertilizers in first season in Biological yield (Ton/fed). The same inoculants appeared the same effect in second season except *A.chroococcum* B-14346. Data regarding harvest index under soil salinity stress indicated that revealed that all inoculants except (*A. lipoferum*, $Azos_6$) gave significant increase in comparison with uninoculated plants in both seasons. However, all combined inoculant mixtures showed significantly increase in comparison with uninoculated plants with 50% N fertilizers in both seasons. Similar to our findings, Nia *et al*. (2012) found that inoculation with saline adapted *Azospirillum* improved wheat growth under salt-stress conditions. Inoculated wheat plants with salt stress decreased the negative effects caused by salinity stress, such as reduction of straw weight, grain yield, harvest index, plant and spike height, tiller number, spike weight, seed number and 1000-seedweight of wheat. The inoculation of a liquid formulation of *A. brasilense* increased the numberof harvested wheat grains by 6% and grain yield by 8% (Zorita andCanigia 2009).Similar enhancement in wheat grain yieldwas reported byinoculation with PGPR strains under salinity stress Nadeem *et al.* (2013).Also, (Bashan *et al.* 2000) noticed that the salttolerant *Azospirillum* spp. promote the growth of the oilseed halophyte *Salicornia bigelovi*

Nitrogen and protein content

Results in Table 10 showed that the nitrogen concentration in leaves at 60 days on both winter seasons 2013/2014-2014-2015showed that the effect of microbial inoculants were significantly increased compared to the uninoculated control (Table 10).

Only the inoculant *A. lipoferum* + *S.cerevisiae* recorded significant increase in compared with uninoculated plants with 100% N fertilizers in both seasons under soil salinity stress. The data also indicated that inoculation with all microbial inoculants except *A. Lipoferum* significantly increased wheat N uptake compared with the uninoculated control and uninoculated plants with 50% N fertilizers in both seasons with soil salinity stress.

Where, all microbial inoculants appeared significantly increase in protein in the leaves in compared with uninoculated plants and uninoculated plants with 50% N fertilizers. However, the combined inoculant mixtures *A. lipoferum* + *S.cerevisiae* and $Azto_4$+*S. cerevisiae* resulted in

the highest increase, compared with the uninoculated control with 100% N fertilizers in both seasons. Plants inoculated with *A. lipoferum*, $Azos_6$and all combined inoculant mixtures showed significant increase in protein content in seeds in compared with uninoculated plants and uninoculated plants with 50% N fertilizers in both seasons. Plants inoculated with salt-adapted$Azos_6$ andco- inoculation $Azos_6$+*S. cerevisiae* had higherprotein contents in seeds than uninoculated plants, uninoculated plants with 50% N and the uninoculated control plants with 100% N fertilizers in both seasons. These results in agreement with Nabila *et al.* (2007) found that the inoculation by biotreatments of yeast + Azo showed significant increases in all wheat yield traits except harvest index. It is also evident from the work of (Kaya *et al*., 2009) reported that plants inoculated with saline-adapted *Azospirillum* had higher N concentrations in compared with uninoculanted plants.

Table 9: Effects of salinity tolerant PGPR strains and *S. cerevisiae*on yield parameters of wheat at 130 days of plant growth in both seasons

N fertilization (%)	Inoculants	Yield Parameters									
		1,000-grain weight (g)		Grain yield (Ton/fed)		Straw yield (Ton/fed)		Biological yield (Ton/fed)		Harvest index	
		Season I	Season II	Season I	Season II	Season I	Season II	Season I	Season II	Season I	Season II
0	Uninoculated control	18.7	22.5	1.2	1.4	2.9	1.8	4.1	3.2	28.6	47.0
100	Uninoculated control	32.2	37.7	2.8	3.7	3.5	4.2	6.2	7.9	43.7	47.4
50	Uninoculated control	26.2	25.0	1.8	1.7	3.7	2.6	5.5	4.3	31.5	39.3
50	*A.chroococcum* B-14346	30.0	26.3	2.8	2.0	3.5	2.5	6.3	4.4	44.6	44.9
50	$Azto_4$	29.3	30.0	1.8	2.2	2.1	2.4	3.9	4.5	45.6	51.3
50	*A. lipoferum*	30.3	29.3	1.9	1.8	2.7	2.5	4.6	4.3	42.0	41.8
50	$Azos_6$	34.0	36.3	1.8	1.9	3.1	3.2	4.9	5.1	36.7	36.4
50	*A.chroococcum* B-14346 + *S. cerevisiae*	37.2	27.0	3.0	2.6	3.1	3.2	6.1	5.8	49.4	45.0
50	*A. lipoferum* + *S.cerevisiae*	32.5	37.0	3.3	4.0	4.2	5.0	7.5	9.0	44.3	45.0
50	$Azto_4$+*S. cerevisiae*	32.7	30.3	3.1	2.9	3.2	3.5	6.3	6.3	49.1	44.8
50	$Azos_6$+*S. cerevisiae*	35	44.5	3.1	3.5	2.9	4.9	6.0	8.3	55.4	41.7
F-test		*	**	**	**	NS	**	*	**	*	NS
LSD 0.05		8.7	9.7	0.9	0.9		1.2	1.8	1.95	14.7	
0.01		11.9	13.2	1.2	1.3		2.0	2.4	2.66	20.0	

Significant difference at *, $p < 0.05$; **, $p < 0.01$ by ANOVA.

El-Kholy and Gomaa (2000)noticed that the inoculation by biofertilizer could alternate 50% of the chemical fertilizer recommended for miltplants without adverse effect on the green and dry fodder, this is due to plant growth promoting substances produced by the biofertilizer. Furthermore, the inoculation with rhizobacterial strains containing ACC-deaminase activity realized significant increase in growth parameters such as root, shoot dry weight, the biomass of plants and alsohad a significant impact on the total N in plant tissues when compared to uninoculated control of wheat at all salinity levels under pot conditions(Yim *et al*. 2009).

5. Conclusion

The property of salinity tolerance is not a simple attribute. The results indicate that the symbiotic association between the saline-adapted isolates and wheat plants especially with *S. cerevisiae* improved the growth of wheat under salinity stress. We conclude that the mechanism underlying salt-tolerant rhizobacteria and *Saccharomyces cerevisiae*-inoculated plant growth improvement in wheat in salinity soil was associated with growth parameters, photosynthetic pigments and yield parameters. In the present study, results suggested that *Azotobacter* and *Azospirillum* strains which are adapted to higher salinity environments may more efficient in alleviating salinity toxicity effect on wheat at soil salinity and have great ability to improve the growth of and yield with *Saccharomyces cerevisiae*.

Table 10: Evaluation ofsalinity tolerant PGPR strains and *S. cerevisiae* on content of nitrogen and protein in wheat in both seasons

Nfertilization (%)	Strains			Yield Parameters					
		N%		Nitrogen uptake		Protein in leave		Protein in seeds	
		Season I	Season II	Season I	Season II	Season I	Season II	Season I	Season II
0	Uninoculated control	1.0	1.04	1.11	1.40	5.62	6.04	11.00	13.1
100	Uninoculated control	2.3	2.42	6.52	9.11	13.31	14.09	17.79	14.41
50	Uninoculated control	1.2	1.20	2.04	2.06	7.02	7.02	12.10	13.96
50	*A.chroococcum* B-14346	1.8	1.81	5.11	3.61	10.53	10.53	13.82	13.82
50	$Azto_4$	1.6	1.63	2.79	3.52	9.52	9.52	13.77	13.77
50	*A. lipoferum*	1.9	2.01	3.64	3.55	11.12	11.70	13.10	16
50	$Azos_6$	2.1	2.05	3.63	3.85	11.97	11.97	14.41	17.97
50	*A.chroococcum* B-14346 + *S. cerevisiae*	2.6	2.57	7.63	6.87	14.96	14.96	15.06	15.43
50	*A. lipoferum* + *S.cerevisiae*	3.2	3.20	10.76	12.84	18.66	18.66	16.76	16.76
50	$Azto_4$+*S. cerevisiae*	2.9	2.93	9.08	8.59	17.10	17.10	16.98	16.98

50	Azos$_6$+*S. cerevisiae*	2.2	2.19	6.77	7.65	12.75	12.75	18.13	18.8
F-test		**	**	**	**	**	**	**	*
LSD 0.05		0.59	0.56	2.55	3.24	3.43	3.28	3.09	3.71
0.01		0.80	0.76	3.46	4.41	4.66	4.45	4.20	5.04

6. Acknowledgement

We are thankful to Microbiology Dep., Soil , Water and Environmental Research Institute, Agriculture Research Center and National center for Agriculture Utilization Research, USDA and for kindly provided the strains used in this study.

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