Response of Different Cultivars of Wheat Plants (Triticum aestivum L.) to Inoculation by Azotobacter sp. under Salinity Stress Conditions

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Authors’ contributions
This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

ABSTRACT

Salinity is one of the key restraints to agricultural productivity worldwide and is expected to increase further. Therefore, cope with this problem we should develop strategies to enhance salinity tolerance in different crops. One of these modern strategies is to use plant growth promoting rhizobacteria (PGPR) which can help plants to withstand under harsh environmental conditions. The present study was evaluated six isolates of Azotobacter sp. (Az1-Az6) which tested in vitro for growth, PGPR traits such as indole-3 acetic acid (IAA) production and nitrogen fixation, germination indicators for different wheat cultivars i.e. Misr 1, Gemmiza 12 and Sakha 95 under different levels of NaCl. Also, the efficacy of inoculation with two superior isolates in different wheat cultivars in a Gnotobiotic Sand System and greenhouse experiment for improving growth dynamics, physiological attributes, nutrient uptake and antioxidant enzymes under different levels salinity of sandy soil (0, 4, 8 and 12 dS m⁻¹). Out of 6 isolates, two isolates (Az2 and Az6) could show salinity tolerance and exhibited PGPR traits as well as improvement germination tests. Both the bacteria could promote growth in...
Keywords: Wheat; Azotobacter sp.; salt stress; physiological attributes; nutrient uptake; antioxidant enzymes.

1. INTRODUCTION

Worldwide, wheat (Triticum aestivum L.) is one of the most strategic crops for food, feed, and biofuel security [1]. According to FAOSTAT [2], wheat was grown on about 215 M ha with a production of 733.40 Mt worldwide but the cultivated area in Egypt was 1.28 M ha with a production of 9.00 Mt, which is about 20% of the total cultivated agricultural land. However, according to a report from Asseng et al. [3], consumers will require 60% more wheat than today which due to rapid population growth. On the other hand, cultivated soils worldwide have become more salinized due to suboptimal irrigation water, excessive fertilization, and desertification processes. Currently, more than 800 M ha of land all over the world are affected by salt stress [4]. Also, many studies indicated that salt stress lead to diminishes the ameliorative condition of arable lands and suppresses the growth of plants [5], reduces water potential and nutrient deficiency [6], causes osmotic stress, creates ion imbalance and toxicity in plants [7].

For these reasons that increasing wheat productivity is an extremely crucial area of our research and there are several strategies have been developed in order to decrease the toxic effects caused by high salinity on plant growth, including plant genetic engineering [8] and recently the use of plant growth-promoting bacteria (PGPB) [9]. PGPB could play a substantial role in developing crop management strategies that are eco-friendly. However, we need to better exploit their unique attributes of tolerance to extremes, genetic diversity, and cooperation with crop plants, in order to develop methods for their successful utilization in agricultural production [10]. Recently, plant associated microbial communities have received greater consideration for their capability to confer resistance to abiotic stresses either through changes in plant / root growth characteristics or other biochemical changes for inducing systemic tolerance [11]. PGPB are usually defined as microorganisms that can grow in, on, or around plant tissues, stimulating plant growth by a variety of mechanisms [12]. These mechanisms and their effects can be classified as direct or indirect. The direct mechanisms are associated with an increase in availability of nutrients and include biological nitrogen fixation (BNF) [13], phosphate solubilization and mineralization [14], siderophore production [15], and synthesis of plant hormones such as IAA, cytokinins, or GB [16]. Utilization of PGPB has become a promising alternative to alleviate plant stress caused by salinity [17,18,19]. Therefore, inoculation of Azospirillum, Azotobacter, Xanthomonas, Pseudomonas, and Bacillus cereus are collectively known as plant growth-promoting bacteria (PGPB), considerably increases the plant growth and nodulation of legumes [20].

Azotobacter is a free-living nitrogen-fixing rhizobacteria that can promote the growth of various crops by some mechanisms such as the production of GA, IAA and cytokinin [21]. Inoculation effect of free-living Azotobacter species are largely associated with nitrogen fixation, formation of various physiologically active growth hormones, protection against root pathogens, stimulation of beneficial rhizospheric microorganisms and enhancement of plant yield [22,23]. Inoculation of Azotobacter chroococcum and Streptomyces niveus on maize plants grown under different salinity levels were found to influence total soluble sugars, total free amino acids, proline and total soluble proteins DNA and
RNA in shoots and roots, which resulted in higher salt tolerance of the plants [24]. Alikhan et al. [25], reported that inoculation of three salt tolerant bacterial strains i.e., A. chroococcum, A. vinelandii and A. beijerinckii enhanced 75.8% and 56.12% root and shoot dry biomass in Ceriops decandra and Avicennia marina, respectively. Similarly, inoculation of Azotobacter in Brassica oleracea var. italica and wheat resulted in greater plant growth stimulation [26]. Also, inoculation with salinity tolerant Azotobacter strains ST24 under different doses of nitrogen fertilizer caused significant increase in total nitrogen, biomass and grain yield of wheat and their survival in the soil was also highest in all the treatments at 30, 60 and 90 days after sowing [27].

Broetto et al. reported that salt stress decreased chlorophyll content of maize, but inoculation with PGPR strains increased the chlorophyll pigments [28]. Plants infected with IAA-over producing PGPR strains showed high antioxidant enzyme activities that contribute to enhance plant protection against salt stress [29]. Heidari and Golpayegani, suggested that PGPR inoculation enhanced the proline, chlorophyll and relative water content of basil (Ocimum basilicum L.) under stress conditions [30]. Noorieh et al. have reported that PGPR species like Azotobacter and Pseudomonas increased the growth and biomass of canola (Brassica napus L.) by regulating the oxidative stress enzymes and essential nutrient under salinity stress [31]

Babaei et al. showed that application of biofertilizers (A. chroococcum strain 5 (F2), Azospergillum lipoferum strain OF (F3) and Pseudomonas putida strain 186 (F4) and nano Zn-Fe oxide improved grain yield, chlorophyll content, antioxidant enzyme activity, proline and soluble sugars under different levels of salinity (no-salt, salinity 25 and 50 mM NaCl) conditions [32].

A better understanding of wheat physiological responses under salinity conditions may help in programs which the objective is to improve the grain yield. Therefore, the aim of our study was to evaluate the effect of different isolates of Azotobacter on the growth and some physiological responses of different wheat cultivars (Misr 1, Gemmiza 12 and Sakha 95) under different levels of salinity stress conditions.

2. MATERIALS AND METHODS

2.1 Isolates and Culture Conditions

In this study, six isolates of Azotobacter (Az1-Az6) were studied. These isolates were previously selected by their potential as biofertilizers at Bacteriology Laboratory, Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt. The standard culture conditions for bacterial maintenance utilized Ashby medium g L⁻¹: mannitol 10, K₂HPO₄ 0.2, MgSO₄·7H₂O 0.2, NaCl 0.2, CaSO₄ 0.1, CaCO₃ 10.0, agar 15.0, pH 7.5 for 3 days in 28°C under rotary shaker conditions (150 rpm).

2.2 Assessment of Salt Tolerance of Azotobacter Isolates

One mL of the bacterial culture (1x10⁸ CFU ml⁻¹) was inoculated to 150 mL of Ashby medium broth in a 500-mL Erlenmeyr flask supplemented with different sodium chloride concentrations viz, 2, 4, 6 and 8%. Each treatment was performed in triplicate and under standard culture conditions. growth was counted by using standard serial dilution and plated by spread plate count method and expressed in terms of log10.

2.3 Screening of IAA and Nitrogen Fixation by Azotobacter Isolates

All Azotobacter isolates were screened for the production of IAA using the method described by Ivanova et al. [33]. Briefly, one mL of the bacterial suspensions (1x10⁸ CFU ml⁻¹) were inoculated into Ashby medium broth supplemented with 1 g L⁻¹ filter sterilized L-tryptophan as IAA precursor in the presence of previously different concentrations of NaCl and incubated at 30°C on a rotary shaker at 150 rpm for 7 days under the dark conditions. After the incubation period, bacterial cells were centrifuged at 5000 rpm for 10 min and take 0.5 mL supernatant with 2 ml of the Salkowski Reagent (50 mL, 35% perchloric acid +1 ml 0.5 FeCl₃). Development of a pink colour indicates IAA production which read at 540 nm using UV/Visible Spectrophotometer (model 6705). The concentration of IAA produced by the cultures was measured from a calibration curve using a standard IAA and expressed as μg ml⁻¹.

Also, all Azotobacter isolates were estimated for nitrogen fixation using the method described by
Vaddar [34]. To 250 ml conical flasks, 100 ml of the N free malate medium was dispensed for all flasks and autoclaved. One ml of 24 h old culture inoculum was inoculated to each flask. The flasks were incubated at 37°C for seven days. After incubation, the culture was homogenized and 10 ml was digested with 5 ml of concentrated H$_2$SO$_4$ along with 0.2 g digestion catalyst mixture K$_2$SO$_4$:CuSO$_4$:selenium (100:10:1). After cooling, volume was made up to 10 ml with distilled water and determination by microkjeldhal distillation unit. Total nitrogen content of the culture was determined and results were expressed as mg N fixed per g of malate.

2.4 Germination Indicators

This experiment aims to investigate the ability of studied bacterial isolates to ameliorate the seed germination of some wheat (Triticum aestivum L.) cultivars under elevated salt stress. For this purpose, 3 different wheat cultivars (MISR 1, Gemmiza 12 and Sakha 95) which provided by the Field Crops Research Institute, Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt were subjected to 4 different NaCl doses (0, 50, 100 and 150 mM) with four replicates. Before germination experiment, 10 seeds of each cultivar were first rinsed with 70% (v/v) ethanol and surface sterilized with diluted sodium hypochlorite (5% v/v) for 3 min and then washed five times with sterile distilled water. Seeds were soaked in bacterial suspensions (1x10$^8$ CFU ml$^{-1}$) overnight before germinated in a sterile 15-cm Petri plates containing 1% distilled water agar supplemented with different NaCl doses and left for germination at full-dark environment at 25°C for 10 days. Petri plates were arranged in a randomized complete block design and sealed well with polyethylene sheet to prevent from drying across the experiment period. For control, seeds were soaked in autoclaved inocula. Germinated seeds were counted every day for each replicate. Germination indicators were calculated as follows:

a. Final germination percentage (FGP, %) was calculated using the formula:

$$FGP, \% = \left( \frac{TNG}{TNP} \right) \times 100$$

Where FGP, % is the final germination percentage, TNG is the total number of germinated seeds, and TNP is the total number of planted seeds according to Ranal and Santana [35].

b. Mean germination time (MGT) was used to evaluate seedling emergence and computed by the formula:

$$MGT = \sum \left( \frac{n_i \times t_i}{n_i} \right)$$

Where MGT is the mean germination time, ni is the number of germinated seeds on germination days, and ti is the number of days during the germination period (between 0 and 10 days), cited by Mauromicale and Licandro [36].

2.5 Plant Growth in a Gnotobiotic Sand System

Upon the results of the above experiment, this experiment aims to study the effect of inoculation with two superior isolates of Azotobacter (Az2 and Az6) on the growth parameters and root colonization of different wheat cultivars exposed to different NaCl doses (0, 50, 100 and 150 mM) was preliminarily studied in glass tubes (2.5 cm in diameter, 20 cm in length) as described by Simons et al. [37], with 10 replicates (N=10).

Each tube contains 60 g of a sterilized mixture of washed sand and vermiculite (1:1) soaked with 6 ml of nitrogen-free Jensen’s nutrient solution [38], supplemented with different NaCl doses. Sterilized grains of different wheat cultivars were planted into sterile tubes, one grain per tube and inoculated with 0.2 mL from the bacterial suspensions (1x10$^8$ CFU ml$^{-1}$), whereas control grains were inoculated with autoclaved inocula. All tubes were grown in a growth cabinet with a photoperiod of 16:8 h at 20°C. After one month, the fresh and dry weights of plants, length of shoots and roots, were measured. Also, from each treatment, 3 plants were taken randomly to determine root colonization, which roots were washed thoroughly to remove soil then weighted and kept in test tube containing 9 mL sterile saline solution (0.85% NaCl) then shaking on a vortex every 10 min for 60 min. Serial dilutions were done and 0.1 mL from each diluent was spread on Ashby agar medium plates and incubated at 30°C for 72 h. The experiment was replicated three times, and the number of viable cells was counted and calculated as CFU g$^{-1}$ root [39].

2.6 Greenhouse Experiment

This experiment was conducted to study the effect of inoculation with Azotobacter (Az2 and Az6) on vegetative growth, nutrient uptake, biochemical properties and antioxidant enzymes.
of different wheat cultivars grown in sandy soil salinized with NaCl and CaCl₂ according to Manual of salinity research methods [40].

The experiment was carried out as 2 x 4 x 3 x 6 split-plot designed, i.e. 2 inoculation treatments (Azo 2 and Azo 6), 4 different soil salinity treatments (0, 4, 8 and 12 dS m⁻¹) and 3 different wheat cultivars (Misr 1, Gemmiza 12 and Sakha 95) with 6 replicates. Sandy soil was washed several times with 0.1 N HCl solution followed by washing with distilled water in order to remove minerals then autoclaved twice at 130 °C. After that, polyethylene bags were filled with 3 Kg sandy soil (15 cm in diameter and 20 cm in depth). Wheat grains were surface sterilized as mentioned above, then each bag was sown with 10 grains at a depth of 2 cm and after one week of germination, inoculation 2.0 ml (1.1 x 10⁸ CFU ml⁻¹) was done. Uninoculated treatment (control), grains were inoculated with autoclaved inocula. Irrigation was carried out twice weekly by nitrogen-free Jensen’s nutrient solution [38].

2.7 Measurements and Analysis

At 90 days from sowing, random selection of five plants were made from each replication to determine plant height (cm plant⁻¹), dry weight (g plant⁻¹), root length (cm) and estimation of nitrogen, potassium, sodium and potassium sodium ratio contents (%) in Plants. Also, measure biochemical analysis i.e. total chlorophyll content, carotenoids, proline content and antioxidant enzymes.

Chlorophyll content and carotenoid: According to Mousa et al. [42]. 0.1 g of fresh leaf tissue was grinded with 5 ml acetone 80% then centrifugation at 13,000 rpm for 10 min. The absorbance of the supernatant was read at 645, 663 and 470 nm. The amount of chlorophyll and carotenoid (mg g⁻¹ FW) in the extract was calculated as below:

\[
\text{Total Chl} = 20.21 \times A_{645} + 8.02 \times A_{663}
\]

Carotenoids = (1000 \times A_{470} – 2.27 \times (\text{Chl a}) – 81.4 \times (\text{Chl b})) / 227

Proline content: According to Bates et al. [43]. proline content was measured with colourimetric assay at 520 nm. Briefly, 0.01 g fresh plant were ground with 0.4 mL sulfosalicylic acid (3%) and placed overnight at 5°C. The suspension was centrifuged at room temperature at 3000 rpm for 5 min. The supernatant was mixed with 0.4 mL of acidic ninhydrin reagent then heated in a boiling water bath for 60 min. Thereafter, the content in the tubes was cooled and the mixture was extracted with 0.4 mL of toluene. The absorbance of the formed complex was determined using UV/Visible Spectrophotometer (model 6705) and the proline concentrations were measured using a standard curve and calculated as mg g⁻¹ fresh weight.

Antioxidant Enzymes activity: In order to estimate the ascorbate peroxidase (APX) and catalase (CAT) enzyme activities, flag leaves of wheat were collected at 45 and 90 days after sowing and homogenized in a cooled 0.1 mol L⁻¹ Tris-HCl buffer at pH 7.8 containing 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ dithiothreitol and 5 mL of 4% polyvinyl pyrrolidone per one gram of fresh weight. Two mL reaction mixture consisting of 20 μL crude leaf extract, 660 μL potassium phosphate buffer (pH 7.0), 660 μL ascorbic acid solution, and 660 μL H₂O₂ was used to measure ascorbate peroxidase (APX) activity. Enzyme activity was tested by observing the ascorbate reduction through H₂O₂ at 290 nm for 3 min [44].

On the other hand, catalase (CAT) activity was extracted by grinding 1 g of leaf tissues in 0.1 M sodium phosphate buffer at pH 7.1 in a porcelain mortar. Reaction mixture contained 25 mM L⁻¹ Tris-acectate buffer (pH 7.0), 0.8 mM L⁻¹ EDTANa, and 20 mM L⁻¹ H₂O₂ at 25°C. Enzyme activity was tested by observing H₂O₂ consumption at 240 nm for 3 min [45].

2.8 Statistical Methods

Using two-way and split-plot analysis variances (ANOVA), data were analysed by software SPSS 14.0 for windows and Duncan’s multiple range test was used for comparison among the treatment means [46].

3. RESULTS

3.1 Assessment of Salt Tolerance of Azotobacter Isolates

To determine tolerance level of different Azotobacter isolates (Az1-Az6) to salt stress conditions, cells were cultured in Ashby medium broth suplemented with different concentrations of NaCl 2, 4, 6 and 8%. After incubation conditions (72 h, 28°C and 150 rpm), viable cells numbers were determined (Fig. 1).
Initially, the bacterial growth was comparatively slow with increasing of NaCl concentrations, as compared to low concentrations. Among the 6 isolates under study, two isolates (Az2 and Az6) were capable of tolerating up to 8% NaCl concentration as compared to other isolates.

3.2 Assessment of Azotobacter Isolates for Production of IAA and Nitrogen Fixation under Salt Stress Conditions

On the basis of the above screening growth strategy, all isolates (Az1, Az2, Az3, Az4, Az5 and Az6) were tested for production of IAA and nitrogen fixation under salt stress conditions (Fig. 2). The amount of indole acetic acid varied between the isolates, the results showed that the highest IAA produced by Az6 isolate was 12.47 µg ml⁻¹ followed by Az2 isolate was 11.49 µg ml⁻¹. While, Az1 isolate gave recorded the lowest IAA amount of 6.36 µg ml⁻¹ at 8% NaCl concentration. For nitrogen fixation, the maximum values was recorded by Az6 isolate (2.84, 2.74, 1.94 and 0.74 mg g⁻¹ malate), for 2, 4, 6 and 8% NaCl concentrations, as compared to other tested isolates, respectively (Fig. 2).

3.3 Assessment of Azotobacter Isolates for Final Germination Percent (%) and Mean Germination Time (day) of Different Wheat Cultivars under Salt Stress Conditions

Effect of different isolates of Azotobacter (Az1-Az6) on final germination percent (FGP %) and mean germination time (MGT day) for different wheat cultivars Misr1 (M 1), Gemmiza 12 (Ge 12) and Sakha 95 (S 95) under different concentrations of NaCl (0, 50, 100 and 150 mM) are depicted in Table 1. Generally, increasing NaCl stress drastically affected the final germination percentage of different wheat cultivars seeds. However, among all tested Azotobacter isolates, Az2 and Az6 improved FGP of all studied wheat cultivars which recorded higher values by 35.00 and 36.66% for Misr1 cultivar, 16.66 and 26.66% for Gemmiza 12 cultivar and 21.66 and 28.33% for Sakha 95 cultivar when the germination medium was supplemented with 150 mM NaCl concentration as compared to other isolates, respectively.

![Fig. 1. Viable cells numbers of different Azotobacter isolates growing in Ashby medium broth supplemented with different concentrations of NaCl (%)](image)
On the other hand, all *Azotobacter* isolates led to a decrease of mean germination time at high NaCl concentrations up to 150 mM (Table 1). Among all treatments, the shortest MGT was found when wheat cultivars seeds treated with Az6 under 0, 50, 100 and 150 mM NaCl concentrations recording 1.8, 2.06, 2.89 and 3.13 days for Misr1 cultivar, and 1.85, 2.25, 3.04 and 3.44 days for Gemmiza 12 cultivar and 1.79, 2.17, 3.01 and 3.41 days for Sakha 95 cultivar compared to other isolate, respectively. But, inoculation treatment with Az6 was more efficient for Misr1 cultivar which recorded 0.044 and 0.038 g plant⁻¹, dry weight, root and shoot length as well as root colonization of all studied wheat cultivars over uninoculated control both under unstressed and salt-stressed conditions.

In respect to different NaCl concentrations (Table 2), data showed that highly significant effect of fresh weight for studied wheat cultivars at 50 mM NaCl concentration which attained 0.045, 0.029 and 0.034 g plant⁻¹ for Misr1, Gemmiza 12 and Sakha 95, compared to other different NaCl concentrations, respectively. On the other hand, inoculation treatments showed that inoculation treatment with Az6 was more efficient for Misr1 and Sakha 95 cultivars which recorded 0.044 and 0.038 g plant⁻¹ fresh weight, compared to other inoculation treatment and control, respectively. But, inoculation treatment with Az2 was more efficient for Gemmiza 12 cultivar which recorded 0.034 g plant⁻¹ fresh weight. Similar trend was observed for dry weight, root and shoot length as well as root colonization under both of different NaCl concentrations and inoculation treatments (Table 2).

**3.4 Plant Growth Parameters in a Gnotobiotic Sand System**

Response of salt-affected wheat cultivars to the inoculation with *Azotobacter* isolates (Az2 and Az6) under a gnotobiotic sand system are showed in Tables 2 and 3. Generally, *Azotobacter* isolates increased the fresh weight, dry weight, root and shoot length as well as root colonization of all studied wheat cultivars over uninoculated control both under unstressed and salt-stressed conditions.

![Figure 2: Effect of different concentrations of NaCl on production of IAA (µg ml⁻¹) and nitrogen fixation (mg g⁻¹ Malate) by different *Azotobacter* isolates](image)
Table 1. Effect of different *Azotobacter* isolates on final germination percent (%) and mean germination time (day) of different wheat cultivars Misr1 (M1), Gemmiza 12 (Ge 12) and Sakha 95 (S 95) under different concentrations of NaCl (0, 50, 100 and 150 mM)

| Isolates | NaCl conc. (mM) | Final germination percent (%) | Mean germination time (day) |
|----------|----------------|------------------------------|----------------------------|
|          | M1  | Ge 12   | S 95  | M1  | Ge 12   | S 95  |
| Az1      | 0   | 100     | 100   | 100 | 1.92 ± 0.010 | 1.89 ± 0.011 | 1.97 ± 0.011 |
|          | 50  | 100     | 100   | 100 | 2.20 ± 0.020 | 2.09 ± 0.015 | 2.33 ± 0.005 |
|          | 100 | 48.33 ± 7.63 | 23.33 ± 5.72 | 33.33 ± 5.70 | 2.83 ± 0.005 | 2.92 ± 0.010 | 2.95 ± 0.015 |
|          | 150 | 26.66 ± 2.80 | 16.66 ± 5.77 | 21.66 ± 2.84 | 3.23 ± 0.032 | 3.32 ± 0.020 | 3.47 ± 0.010 |
| Az2      | 0   | 100     | 100   | 100 | 1.89 ± 0.011 | 1.97 ± 0.011 | 1.97 ± 0.015 |
|          | 50  | 100     | 100   | 100 | 2.09 ± 0.015 | 2.33 ± 0.005 | 2.29 ± 0.015 |
|          | 100 | 63.33 ± 5.22 | 33.33 ± 5.12 | 36.66 ± 5.34 | 2.92 ± 0.010 | 2.95 ± 0.015 | 2.97 ± 0.010 |
|          | 150 | 35.00 ± 5.32 | 16.66 ± 6.10 | 21.66 ± 5.42 | 3.32 ± 0.020 | 3.47 ± 0.010 | 3.41 ± 0.010 |
| Az3      | 0   | 100     | 100   | 100 | 1.92 ± 0.010 | 1.89 ± 0.011 | 1.97 ± 0.011 |
|          | 50  | 100     | 100   | 100 | 2.20 ± 0.020 | 2.09 ± 0.015 | 2.33 ± 0.005 |
|          | 100 | 48.33 ± 5.70 | 23.33 ± 5.77 | 33.33 ± 7.63 | 2.83 ± 0.005 | 2.92 ± 0.010 | 2.95 ± 0.015 |
|          | 150 | 26.66 ± 2.88 | 16.66 ± 5.69 | 21.66 ± 2.82 | 3.23 ± 0.032 | 3.32 ± 0.020 | 3.47 ± 0.010 |
| Az4      | 0   | 100     | 100   | 100 | 1.92 ± 0.010 | 1.89 ± 0.011 | 1.97 ± 0.011 |
|          | 50  | 100     | 100   | 100 | 2.20 ± 0.020 | 2.09 ± 0.015 | 2.33 ± 0.005 |
|          | 100 | 48.33 ± 5.72 | 23.33 ± 5.70 | 33.33 ± 5.71 | 2.83 ± 0.005 | 2.92 ± 0.010 | 2.95 ± 0.015 |
|          | 150 | 26.66 ± 2.88 | 16.66 ± 2.88 | 21.66 ± 2.83 | 3.23 ± 0.032 | 3.32 ± 0.020 | 3.47 ± 0.010 |
| Az5      | 0   | 100     | 100   | 100 | 1.92 ± 0.010 | 1.89 ± 0.011 | 1.97 ± 0.011 |
|          | 50  | 100     | 100   | 100 | 2.20 ± 0.020 | 2.09 ± 0.015 | 2.33 ± 0.005 |
|          | 100 | 48.33 ± 7.63 | 23.33 ± 5.77 | 33.33 ± 5.73 | 2.83 ± 0.005 | 2.92 ± 0.010 | 2.95 ± 0.015 |
|          | 150 | 26.66 ± 2.88 | 16.66 ± 5.77 | 21.66 ± 2.83 | 3.23 ± 0.032 | 3.32 ± 0.020 | 3.47 ± 0.010 |
| Az6      | 0   | 100     | 100   | 100 | 1.80 ± 0.010 | 1.85 ± 0.010 | 1.79 ± 0.015 |
|          | 50  | 100     | 100   | 100 | 2.06 ± 0.015 | 2.25 ± 0.015 | 2.17 ± 0.026 |
|          | 100 | 68.33 ± 5.71 | 46.66 ± 7.66 | 53.33 ± 5.77 | 2.89 ± 0.036 | 3.04 ± 0.030 | 3.01 ± 0.017 |
|          | 150 | 36.66 ± 2.88 | 26.66 ± 2.80 | 28.33 ± 5.73 | 3.13 ± 0.023 | 3.44 ± 0.030 | 3.41 ± 0.025 |

Results are expressed as the mean of three replicates. ± = Standard error
Table 2. Effect of different NaCl concentrations (mM) and Azotobacter isolates on some growth parameters and root colonization of different wheat cultivars Misr1, Gemmiza 12 and Sakha 95 at 30 days after sowing in a Gnotobiotic sand system

| Treatment | Fresh Weight (g) | Dry Weight (g) | Root length (cm) | Shoot length (cm) | Root colonization (CFU×10^5 g^-1 root) |
|-----------|------------------|----------------|------------------|-------------------|-------------------------------------|
| **Misr 1** |                  |                |                  |                   |                                     |
| NaCl concentrations (S) |                  |                |                  |                   |                                     |
| 0 | 0.043 b | 0.010 a | 12.33 a | 17.88 a | 16.33 a |
| 50 | 0.045 a | 0.011 a | 12.77 a | 16.55 b | 13.33 b |
| 100 | 0.033 c | 0.007 b | 10.77 b | 15.00 c | 11.33 bc |
| 150 | 0.027 d | 0.004 c | 9.777 b | 14.11 d | 12.00 c |
| Azotobacter isolates (Az) |                  |                |                  |                   |                                     |
| 0 | 0.031 c | 0.005 c | 7.583 c | 12.417 c | 0.000 c |
| Az2 | 0.038 b | 0.009 b | 11.833 b | 16.750 b | 18.667 b |
| Az6 | 0.044 a | 0.011 a | 14.833 a | 18.500 a | 21.083 a |
| **Gemmiza 12** |                  |                |                  |                   |                                     |
| NaCl concentrations (S) |                  |                |                  |                   |                                     |
| 0 | 0.033 a | 0.007 a | 9.111 a | 12.555 | 15.44 a |
| 50 | 0.029 b | 0.007 a | 8.888 a | 10.000 | 12.77 b |
| 100 | 0.028 b | 0.003 b | 8.555 b | 11.777 | 10.88 b |
| 150 | 0.023 c | 0.003 b | 7.555 ab | 10.888 | 10.66 b |
| Azotobacter isolates (Az) |                  |                |                  |                   |                                     |
| 0 | 0.028 c | 0.007 a | 8.417 ab | 10.917 | 0.00 c |
| Az2 | 0.034 a | 0.006 a | 9.333 a | 12.083 | 17.58 b |
| Az6 | 0.030 b | 0.004 b | 7.833 b | 10.917 | 19.75 a |
| **Sakha 95** |                  |                |                  |                   |                                     |
| NaCl concentrations (S) |                  |                |                  |                   |                                     |
| 0 | 0.039 a | 0.007 a | 10.500 a | 13.888 a | 15.77 a |
| 50 | 0.034 b | 0.006 a | 10.111 a | 13.666 a | 13.11 b |
| 100 | 0.030 c | 0.005 b | 9.666 b | 12.888 b | 11.11 c |
| 150 | 0.027 d | 0.004 b | 9.333 b | 12.375 c | 10.66 c |
| Azotobacter isolates (Az) |                  |                |                  |                   |                                     |
| 0 | 0.027 c | 0.004 b | 9.367 c | 14.000 a | 0.00 c |
| Az2 | 0.034 b | 0.007 ab | 9.664 b | 13.500 b | 18.00 b |
| Az6 | 0.038 a | 0.008 b | 10.583 a | 12.091 c | 20.00 a |

Data were analyzed by two-way ANOVA analysis followed by Tukey’s multiple comparison test.

The interaction effect between the different NaCl concentrations and the inoculation with Azotobacter isolates showed significant effects on different wheat cultivars (Table 3). Under the high concentration of NaCl (150 mM), increasing rate of inoculation treatments with Az6 and Az2 reached to 70 and 23% fresh weight of plant for Misr 1 cultivar, 15 and 4% fresh weight of plant for Gemmiza 12 cultivar, and 15 and 14% fresh weight of plant for Sakha 95 cultivar over control, respectively. Similar trend was observed for dry weight, root and shoot length.

Regarding root colonization, data showed that inoculation with Azotobacter isolates increased the number of bacteria on the root surface of wheat plants under unstressed compared to salt-stressed conditions and control (Table 3). Under the high concentration of NaCl (150 mM), the highest bacterial CFU g^-1 root was observed with Az6, 18 × 10^5 g^-1 root for Misr1 cultivar and 17 × 10^5 g^-1 root for both of Gemmiza 12 and Sakha 95 cultivars compared to other inoculation treatment and control.

From above results, positive response of salt-affected wheat cultivars to the inoculation with Azotobacter isolates (Az2 and Az6) due to enhancement some vegetative parameters and root colonization.

3.5 Plant Growth Parameters in a Greenhouse Experiment

Salinity tolerant Azotobacter isolates (Az2 and Az6) were used for inoculation studies on
different wheat cultivars (Misr1, Gemmiza 12 and Sakha 95) grown in sandy soil salinized with different levels (0, 4, 8 and 12 dS m⁻¹) under greenhouse conditions for 90 days (Table 4). There was significant increase in height plant, dry weight and root length of different wheat cultivars on inoculation with selected salinity tolerant Azotobacter isolates. At different levels salinity (Table 4), results showed that highly significant effect of height plant recorded 77.178, 72.002 and 74.609 cm plant⁻¹ and dry weight recorded 3.858, 3.606 and 3.721 g plant⁻¹ and root length recorded 22.022, 20.507 and 21.307 cm plant⁻¹ for Misr1, Gemmiza 12 and Sakha 95 at 4 dS m⁻¹, compared to other levels salinity, respectively.

Also, inoculation treatment with Az6 showed maximum increase for Misr1, Gemmiza 12 and Sakha 95 cultivars which recorded 78.098, 72.328 and 75.428 cm plant⁻¹ for height plant and 3.899, 3.620 and 3.762 g plant⁻¹ for dry weight and 22.317, 20.641 and 21.528 cm plant⁻¹ for root length, compared to other inoculation treatment and control, respectively (Table 4).

For the interaction effect between the main plot (different levels salinity) and sub main plot (inoculation treatments), data showed that an increase in plant growth parameters was observed with Azotobacter isolate Az6 treatment at 8 dS m⁻¹ resulted in attaining 79.033, 73.000 and 76.470 cm plant height, 3.946, 3.656 and 3.816 g dry weight, 22.586, 20.900 and 21.746 cm root plant⁻¹ for Misr1, Gemmiza 12 and Sakha 95 cultivars, respectively. Another Azotobacter isolate Az2 attained 77.913, 73.293 and 75.026 cm plant height, 3.893, 3.663 and 3.750 g dry weight, 22.260, 20.966 and 21.500 cm root plant⁻¹ for Misr1, Gemmiza 12 and Sakha 95 cultivars at 8 dS m⁻¹, respectively, compared to other inoculation treatments under different levels of salinity and control (Table 4).

3.6 Nitrogen, Potassium, Sodium and Potassium Sodium Ratio

Results of nitrogen, potassium and sodium as well as potassium sodium ratio percent of different wheat cultivars (shoot) after 90 days from sowing in the presence of inoculation with Azotobacter isolates and different levels of sandy soil salinity are presented in Table 5.

The highest N% from wheat plants attained 2.64, 2.51 and 2.43% at 4 dS m⁻¹ when plants inoculated with Az6 isolate for Misr1, Sakha 95 and Gemmiza 12 cultivars, respectively. Also, the highest K⁺, K⁺/Na⁺ percent and the lowest Na⁺ percent were obtained from plants that inoculated with Az6 isolate and grown in soil salinized with 8 and 12 dS m⁻¹ for all studied wheat cultivars as compared to other different levels of soil salinity (Table 5).

In respect to the interaction effect between soil salinity and inoculation treatments showed significant effects on different wheat cultivars (Table 5). Az6+ S4 treatment attained an increase of 13.30, 10.95 and 11.06% in nitrogen percent, 11.19, 9.12 and 7.27% in potassium percent, 2.41, 3.22 and 1.6% in K⁺/Na⁺ percent but reached to the reduction of 10, 8.38 and 8.51% in sodium percent for Misr1, Gemmiza 12 and Sakha 95 cultivars as compared to Az0+S0 treatment (control), respectively.

3.7 Total Chlorophyll, Carotenoids and Proline Contents

Changes in total chlorophyll, carotenoids and proline contents in different wheat cultivars were shown as response to both salinity stress and bacterial inoculation (Table 6). The amount of proline was enhanced with increasing concentrations of salinity. However, the content of total chlorophyll and carotenoids was reduced with increasing salinity stress. In addition, when bacteria were present, an increase in total chlorophyll, carotenoids and proline were also observed regardless of salt concentration. Among bacterial treatments, Azotobacter isolate Az6 caused the greatest effect over Azotobacter isolate Az2 and control (Table 6).

Our findings for the interaction effect indicated that there was a statistically significant positive relationship (p<0.05) between different levels salinity and inoculation treatments. Data showed that an increase in total chlorophyll and carotenoids was observed with Azotobacter isolate Az6 at 4 dS m⁻¹ resulted 3.28 and 1.08 mg g⁻¹ FW for Misr 1 cultivar, followed by 3.18 and 0.98 mg g⁻¹ FW for Sahka 95 cultivar and 3.08 and 0.78 mg g⁻¹ FW for Gemmiza 12 cultivar, as compared to other inoculation treatments and control, respectively. However, the content of proline was increased by bacterial inoculation and different levels of salinity. Proline content in bacterial-inoculated wheat plants was enhanced with Azotobacter isolate Az6 treatment at 12 dS m⁻¹ which recorded 8.76, 7.83 and 8.16 mg g⁻¹ FW for Misr1, Gemmiza 12 and Sakha 95 cultivars, respectively. (Table 6).
3.8 Activity of APX and CAT Enzymes

At 90 days from sowing, bacterial inoculation treatments reduced antioxidant enzymes APX and CAT activity in leaves of wheat significantly over the control under non-stressed conditions, but an increased amount of these enzymes was observed under salinity stress (Table 7). Under salinity-stressed conditions (12 dS m\(^{-1}\)), Az6 + S12 treatment efficiently increased the APX content by 6.01, 6.56 and 6.37%, but at 4 dS m\(^{-1}\) of soil salinity the increased rate reached to 41.11, 46.25 and 44.33% for Misr1, Gemmiza 12 and Sakha 95 cultivars, over the uninoculated control, respectively.

Table 3. Interaction effect of inoculation with Azotobacter isolates (Az2 and Az6) and different concentrations of NaCl (0, 50, 100 and 150 mM) on some growth parameters of different wheat cultivars Misr1, Gemmiza 12 and Sakha 95 at 30 days after sowing in a Gnotobiotic sand system

| Treatment     | 0.039 d | 0.007 c | 9.66 fg | 15.00 d | 0.00 e |
|---------------|---------|---------|---------|---------|--------|
| Az0 + S0      | 0.037 e | 0.006 c | 8.33 g  | 12.66 e | 0.00 e |
| Az0 + S50     | 0.025 g | 0.003 d | 6.66 h  | 11.66 ef| 0.00 e |
| Az0 + S100    | 0.021 h | 0.001 e | 5.66 h  | 10.33 f | 0.00 e |
| Az2 + S0      | 0.044 bc| 0.012 ab| 12.00 de| 18.33 bc| 23.00 b|
| Az2 + S50     | 0.048 a | 0.013 ab| 13.66 bcd| 17.66 bc| 18.66 c|
| Az2 + S100    | 0.032 f | 0.006 c | 11.33 ef| 15.66 d | 15.00 d|
| Az2 + S150    | 0.026 g | 0.003 d | 10.33 ef| 15.33 d | 18.00 c|
| Az6 + S0      | 0.045 b | 0.012 ab| 15.33 ab| 20.33 a | 26.00 a|
| Az6 + S50     | 0.049 a | 0.014 a | 16.33 a | 19.33 ab| 21.33 b|
| Az6 + S100    | 0.042 c | 0.011 b | 14.33 bc| 17.66 bc| 19.00 c|
| Az6 + S150    | 0.036 e | 0.008 c | 13.33 cd| 16.66 cd| 18.00 c|

| Treatment     | 0.031 cd| 0.004 b | 7.33 bc | 11.00 a-e| 0.00 e |
|---------------|---------|---------|---------|-----------|------|
| Az0 + S0      | 0.036 ab| 0.009 a | 9.66 ab | 7.33 e    | 0.00 e|
| Az0 + S50     | 0.036 ab| 0.008 a | 10.66 ab| 15.33 a   | 0.00 e|
| Az0 + S100    | 0.026 e | 0.003 b | 6.00 bc | 10.00 b-e | 0.00 e|
| Az2 + S0      | 0.035 cde| 0.010 b | 10.66 ab| 13.66 abc | 21.66 b|
| Az2 + S50     | 0.040 a | 0.011 a | 12.66 a | 14.66 ab  | 18.66 c|
| Az2 + S100    | 0.018 f | 0.001 b | 4.33 c  | 8.66 cde  | 15.00 d|
| Az2 + S150    | 0.027 e | 0.002 b | 9.66 ab | 11.33 a-e | 15.00 d|
| Az6 + S0      | 0.032 bc| 0.008 a | 9.33 ab | 13.00 a-d | 24.66 a|
| Az6 + S50     | 0.013 g | 0.001 b | 4.33 c  | 8.00 de   | 19.66 bc|
| Az6 + S100    | 0.017 f | 0.001 b | 7.66 abc| 11.33 a-e | 17.66 cd|
| Az6 + S150    | 0.030 de| 0.004 b | 10.00 ab| 11.33 a-e | 17.00 cd|

| Treatment     | 0.037 a | 0.007 a | 9.00 d  | 13.66 c  | 0.00 e |
|---------------|---------|---------|---------|---------|------|
| Az0 + S0      | 0.041 a | 0.010 a | 10.00 c | 15.33 a  | 0.00 e|
| Az0 + S50     | 0.040 a | 0.008 a | 11.33 b | 15.00 b  | 0.00 e|
| Az0 + S100    | 0.028 a | 0.005 a | 8.33 e  | 12.00 d  | 0.00 e|
| Az2 + S0      | 0.041 a | 0.011 a | 12.00 a | 15.66 a  | 22.66 a|
| Az2 + S50     | 0.045 a | 0.008 a | 11.66 ab| 15.00 b  | 19.00 bc|
| Az2 + S100    | 0.020 a | 0.003 a | 7.33 f  | 10.33 f  | 15.33 d|
| Az2 + S150    | 0.031 a | 0.005 a | 11.33 b | 13.00 cd  | 15.00 c|
| Az6 + S0      | 0.038 a | 0.006 a | 9.33 d  | 12.33 d  | 24.66 a|
| Az6 + S50     | 0.017 a | 0.001 a | 7.33 f  | 10.66 e  | 20.33 b|
| Az6 + S100    | 0.022 a | 0.002 a | 9.33 d  | 13.33 c  | 18.00 bc|
| Az6 + S150    | 0.032 a | 0.004 a | 12.33 a | 12.33 d  | 17.00 cd|

Data were analyzed by two-way ANOVA analysis followed by Tukey’s multiple comparison test

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Table 4. Effect of different sandy soil salinity (dS m\(^{-1}\)) and Azotobacter isolates and their interactions on some growth parameters of different wheat cultivars Misr1, Gemmiza 12 and Sakha 95 at 90 days after sowing in greenhouse conditions

| Treatment | Height (cm plant\(^{-1}\)) | Dry weight (g plant\(^{-1}\)) | Root length (cm) |
|-----------|-----------------------------|-------------------------------|------------------|
|           | M1 | Ge 12 | S 95   | M1 | Ge 12 | S 95   | M1 | Ge 12 | S 95   |
| Soil salinity (S) |     |       |       |     |       |       |     |       |       |
| 0         | 73.934 c | 68.214 b | 71.337 b | 3.691 c | 3.412 b | 3.557 b | 21.071 c | 19.467 b | 19.353 b |
| 4         | 77.178 a | 72.002 a | 74.609 a | 3.858 a | 3.606 a | 3.721 a | 22.022 a | 20.507 a | 21.307 a |
| 8         | 75.510 b | 71.303 a | 73.698 a | 3.771 b | 3.566 a | 3.680 a | 21.549 b | 20.378 a | 21.036 ab |
| 12        | 71.854 d | 66.882 b | 69.876 b | 3.595 d | 3.3644 b | 3.497 b | 20.538 d | 19.077 b | 19.983 ab |
| Azotobacter isolates (Az) |     |       |       |     |       |       |     |       |       |
| 0         | 69.422 c | 65.675 c | 67.835 c | 3.467 c | 3.292 c | 3.388 c | 19.811 c | 18.715 c | 18.624 b |
| Az2       | 76.336 b | 70.798 b | 73.876 b | 3.820 b | 3.547 b | 3.690 a | 21.756 b | 20.215 b | 21.106 a |
| Az6       | 78.098 a | 72.328 a | 75.428 a | 3.899 a | 3.620 a | 3.762 a | 22.317 a | 20.641 a | 21.528 a |
| Interactions |     |       |       |     |       |       |     |       |       |
| Az0 + S0  | 70.363 e | 66.086 d | 68.100 e | 3.5133 f | 3.300 c | 3.400 d | 20.096 d | 18.826 d | 16.433 b |
| Az0 + S4  | 74.206 d | 69.740 a-d | 71.773 cd | 3.710 e | 3.500 ab | 3.583 bc | 21.183 c | 19.900 a-d | 20.486 a |
| Az0 + S8  | 69.583 e | 67.616 cd | 69.546 de | 3.473 f | 3.376 bc | 3.473 cd | 19.800 d | 19.266 cd | 19.860 ab |
| Az0 + S12 | 63.536 f | 59.256 e | 61.920 f | 3.173 g | 2.993 d | 3.096 e | 18.166 e | 16.866 e | 17.716 ab |
| Az2 + S0  | 74.956 cd | 67.966 cd | 72.590 bcd | 3.743 de | 3.410 bc | 3.626 abc | 21.266 c | 19.440 cd | 20.726 a |
| Az2 + S4  | 77.700 ab | 72.933 ab | 75.326 abc | 3.886 abc | 3.653 a | 3.760 ab | 22.150 ab | 20.753 ab | 21.500 a |
| Az2 + S8  | 77.913 ab | 73.293 a | 75.076 abc | 3.893 abc | 3.663 a | 3.750 ab | 22.260 ab | 20.966 a | 21.500 a |
| Az2 + S12 | 74.776 cd | 69.000 bcd | 72.513 bcd | 3.756 de | 3.463 abc | 3.626 abc | 21.350 c | 19.700 bcd | 20.700 a |
| Az6 + S0  | 76.483 bc | 70.590 abc | 73.320 abc | 3.816 cd | 3.526 ab | 3.643 abc | 21.850 bc | 20.133 abc | 20.900 a |
| Az6 + S4  | 79.626 a | 73.333 a | 76.726 a | 3.976 a | 3.663 a | 3.820 a | 22.733 a | 20.866 a | 21.933 a |
| Az6 + S8  | 79.033 a | 73.000 a | 76.470 ab | 3.946 ab | 3.656 a | 3.816 a | 22.586 ab | 20.900 a | 21.746 a |
| Az6 + S12 | 77.250 ab | 72.390 ab | 75.196 abc | 3.856 bc | 3.636 a | 3.770 ab | 22.100 ab | 20.666 ab | 21.533 a |

Means in the same column followed by the same letter are not significantly different according to Duncan’s test at 0.05 level

M1: Misr 1; Ge 12: Gemmiza 12 and S 95: Sakha 95
Table 5. Effect of different sandy soil salinity (dS m\(^{-1}\)) and *Azotobacter* isolates and their interactions on N, K\(^+\) and Na\(^+\) as well as K\(^+\)/Na\(^+\) ratio percent of different wheat cultivars Misr1, Gemmiza 12 and Sakha 95 at 90 days after sowing in greenhouse conditions

| Treatment | N (%) | K\(^+\) (%) | Na\(^+\) (%) | K\(^+\)/Na\(^+\) ratio |
|-----------|-------|-------------|-------------|----------------------|
|           | M1    | Ge 12       | S 95        | M1                   | Ge 12 | S 95 | M1 | Ge 12 | S 95 |
| Soil salinity (S) |       |             |             |                      |       |      |    |       |      |
| 0         | 2.45 b | 2.27 b      | 2.36 b      | 2.89 b               | 2.71 b | 2.80 b | 2.29 b | 2.11 b | 2.20 b | 1.26 b | 1.28 a | 1.272 b |
| 4         | 2.56 a | 2.40 a      | 2.46 a      | 3.00 a               | 2.84 a | 2.90 a | 2.40 a | 2.24 a | 2.30 a | 1.25 c | 1.26 b | 1.260 c |
| 8         | 2.51 a | 2.37 a      | 2.44 a      | 2.95 a               | 2.81 a | 2.88 a | 2.35 a | 2.21 a | 2.28 a | 1.25 c | 1.27 b | 1.260 c |
| 12        | 2.39 c | 2.24 b      | 2.33 b      | 2.83 c               | 2.68 b | 2.77 b | 2.23 c | 2.08 b | 2.17 b | 1.27 a | 1.29 a | 1.278 a |
| Azotobacter isolates (Az) | | | | | | | | |
| 0         | 2.30 c | 2.19 c      | 2.25 c      | 2.74 c               | 2.63 c | 2.69 c | 2.14 c | 2.03 c | 2.09 c | 1.28 a | 1.29 a | 1.28 a  |
| Az2       | 2.54 b | 2.36 b      | 2.45 b      | 2.98 b               | 2.80 b | 2.89 b | 2.38 b | 2.20 b | 2.29 b | 1.25 b | 1.27 b | 1.26 b  |
| Az6       | 2.59 a | 2.41 a      | 2.49 a      | 3.03 a               | 2.85 a | 2.93 a | 2.43 a | 2.25 a | 2.33 a | 1.24 c | 1.26 c | 1.25 c  |
| Interactions | | | | | | | | |
| Az0 + S0  | 2.33 e | 2.19 c      | 2.26 d      | 2.77 e               | 2.63 c | 2.75 cd | 2.40 abc | 2.27 a | 2.35 ab | 1.24 a | 1.24 d | 1.25 d  |
| Az0 + S4  | 2.44 d | 2.33 ab     | 2.38 bc     | 2.88 d               | 2.77 ab | 2.98 a | 2.47 ab | 2.27 a | 2.38 a | 1.24 a | 1.26 d | 1.25 d  |
| Az0 + S8  | 2.32 e | 2.24 bc     | 2.31 cd     | 2.76 e               | 2.68 bc | 2.95 ab | 2.48 a | 2.27 a | 2.35 ab | 1.24 a | 1.26 d | 1.25 d  |
| Az0 + S12 | 2.10 f | 2.00 d      | 2.06 e      | 2.54 f               | 2.44 d | 2.86 abc | 2.38 bc | 2.18 ab | 2.26 abc | 1.25 a | 1.27 cd | 1.26 cd |
| Az2 + S0  | 2.49 cd | 2.27 bc    | 2.41 abc    | 2.93 cd              | 2.71 bc | 2.85 abc | 2.33 cd | 2.14 b | 2.25 abc | 1.25 a | 1.27 cd | 1.26 cd |
| Az2 + S4  | 2.59 ab | 2.43 a     | 2.50 ab     | 3.03 ab              | 2.87 a | 2.93 ab | 2.42 abc | 2.28 a | 2.33 ab | 1.24 a | 1.26 d | 1.25 d  |
| Az2 + S8  | 2.58 abc | 2.44 a    | 2.49 ab     | 3.02 abc             | 2.88 a | 2.94 ab | 2.43 ab | 2.27 a | 2.34 ab | 1.24 a | 1.26 d | 1.25 d  |
| Az2 + S12 | 2.49 cd | 2.30 b     | 2.41 abc    | 2.93 cd              | 2.74 b | 2.85 abc | 2.33 cd | 2.11 bc | 2.25 abc | 1.25 a | 1.28 bc | 1.26 cd |
| Az6 + S0  | 2.54 bc | 2.34 ab    | 2.42 abc    | 2.98 bc              | 2.78 ab | 2.50 e | 1.94 f  | 1.84 d | 1.90 e | 1.30 a | 1.32 a | 1.31 a  |
| Az6 + S4  | 2.64 a | 2.43 a     | 2.51 ab     | 3.08 a               | 2.87 a | 2.95 ab | 2.16 e  | 2.08 bc | 2.15 cd | 1.27 a | 1.28 bc | 1.27 bc |
| Az6 + S8  | 2.63 ab | 2.43 a    | 2.54 a      | 3.07 ab              | 2.87 a | 2.82 bc | 2.28 d  | 2.17 ab | 2.22 bc | 1.26 a | 1.27 cd | 1.26 cd |
| Az6 + S12 | 2.56 abc | 2.43 a   | 2.51 ab     | 3.00 abc             | 2.87 a | 2.70 d | 2.17 e  | 2.03 c | 2.10 d | 1.27 a | 1.29 b | 1.28 b  |

Means in the same column followed by the same letter are not significantly different according to Duncan's test at 0.05 level

M1: Misr 1; Ge 12: Gemmiza 12 and S 95: Sakha 95
Table 6. Effect of different sandy soil salinity (dS m\(^{-1}\)) and *Azotobacter* isolates and their interactions on total chlorophyll, carotenoids and proline contents (mg g\(^{-1}\) FW) of different wheat cultivars Misr1, Gemmiza 12 and Sakha 95 at 90 days after sowing in greenhouse conditions

| Treatment         | Total chlorophyll content | Carotenoids | Proline |
|-------------------|---------------------------|-------------|---------|
|                   | M1 | Ge 12 | S 95 | M1 | Ge 12 | S 95 | M1 | Ge 12 | S 95 |
| **Soil salinity (S)** |     |       |       |     |       |       |     |       |       |
| 0                 | 3.09 b | 2.89 b | 2.99 b | 0.89 b | 0.59 b | 0.79 b | 5.73 d | 4.80 d | 5.13 d |
| 4                 | 3.20 a | 3.00 a | 3.10 a | 1.00 a | 0.70 a | 0.90 a | 6.68 c | 5.75 c | 6.08 c |
| 8                 | 3.15 a | 2.95 a | 3.05 a | 0.95 a | 0.65 a | 0.85 a | 7.53 b | 6.60 b | 6.93 b |
| 12                | 3.03 c | 2.83 c | 2.93 c | 0.83 c | 0.53 c | 0.73 c | 8.12 a | 7.19 a | 7.52 a |
| **Azotobacter isolates (Az)** |     |       |       |     |       |       |     |       |       |
| 0                 | 2.94 c | 2.74 c | 2.84 c | 0.74 c | 0.44 c | 0.64 c | 6.75 c | 5.82 c | 6.15 c |
| Az2               | 3.18 b | 2.98 b | 3.08 b | 0.98 b | 0.68 b | 0.88 b | 7.00 b | 6.07 b | 6.40 b |
| Az6               | 3.23 a | 3.03 a | 3.13 a | 1.03 a | 0.73 a | 0.93 a | 7.30 a | 6.37 a | 6.70 a |
| **Interactions**  |     |       |       |     |       |       |     |       |       |
| Az0 + S0          | 2.97 e | 2.77 e | 2.87 e | 0.77 e | 0.47 e | 0.67 e | 5.70 g | 4.77 g | 5.10 g |
| Az0 + S4          | 3.08 d | 2.88 d | 2.98 d | 0.88 d | 0.58 d | 0.78 d | 6.50 f | 5.57 f | 5.90 f |
| Az0 + S8          | 2.96 e | 2.76 e | 2.86 e | 0.76 e | 0.46 e | 0.66 e | 7.20 d | 6.27 d | 6.60 d |
| Az0 + S12         | 2.74 f | 2.54 f | 2.64 f | 0.54 f | 0.24 f | 0.44 f | 7.60 c | 6.67 c | 7.00 c |
| Az2 + S0          | 3.13 cd | 2.93 cd | 3.03 cd | 0.93 cd | 0.63 cd | 0.83 cd | 5.80 g | 4.87 g | 5.20 g |
| Az2 + S4          | 3.23 ab | 3.03 ab | 3.13 ab | 1.03 ab | 0.73 ab | 0.93 ab | 6.66 ef | 5.73 ef | 6.06 ef |
| Az2 + S8          | 3.22 abc | 3.02 abc | 3.12 abc | 1.02 abc | 0.72 ab | 0.92 ab | 7.53 c | 6.60 c | 6.93 c |
| Az2 + S12         | 3.13 cd | 2.93 cd | 3.03 cd | 0.93 cd | 0.63 cd | 0.83 cd | 8.00 b | 7.07 b | 7.40 b |
| Az6 + S0          | 3.18 bc | 2.98 bc | 3.08 bc | 0.98 bc | 0.68 bc | 0.88 bc | 5.70 g | 4.77 g | 5.10 g |
| Az6 + S4          | 3.28 a | 3.08 a | 3.18 a | 1.08 a | 0.78 a | 0.98 a | 6.90 de | 5.97 de | 6.30 de |
| Az6 + S8          | 3.27 ab | 3.07 ab | 3.17 a | 1.07 ab | 0.77 a | 0.97 a | 7.86 bc | 6.93 bc | 7.26 bc |
| Az6 + S12         | 3.20 abc | 3.00 abc | 3.10 abc | 1.00 abc | 0.70 abc | 0.90 abc | 8.76 a | 7.83 a | 8.16 a |

Means in the same column followed by the same letter are not significantly different according to Duncan’s test at 0.05 level

*M1: Misr 1; Ge 12: Gemmiza 12 and S 95: Sakha 95*
4. DISCUSSION

Abiotic stresses such as salinity, drought and high temperature profoundly influence agro-ecosystems by limiting crop productivity and, in worst cases, creating unproductive areas. To overcome these problems, soil microbes having multifunctional stress amelioration attributes have been suggested [17,18,19]. However, microbes vary in tolerance to abiotic stress in vitro and performance may not be consistent under field conditions due to multiple fluctuating stress conditions. Therefore, selection of rhizobacteria with multifunctional PGP traits and tolerance to abiotic stress provide protection to plants [19]. Considering the above facts, we attempted to screen six rhizobacteria associated (Azotobacter sp.) with different wheat cultivars (Misr1, Gemmiza 12 and Sakha 95) based on their growth tolerance, PGP traits such as IAA production and nitrogen fixation, germination indicators such as final germination percent and mean germination time as well as plant growth parameters in a Gnotobiotic sand system under different concentrations of NaCl. After the initial screening of rhizobacterial isolates, we selected two potential isolates Az2 and Az6 for further studies in greenhouse experiment.

Therefore, assessments were conducted to evaluate the functional activities of Azotobacter isolates under stress conditions in vitro. We found that high salinity (6 and 8% NaCl) can also negatively influenced by salinity which can synthesize compatible solutes such as sugars and amino acids that act as osmolytes and help

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Table 7. Effect of different sandy soil salinity (dS m\(^{-1}\)) and Azotobacter isolates and their interactions on ascorbate peroxidase (μM H\(_2\)O\(_2\) min\(^{-1}\) g\(^{-1}\) FW) and catalase (μM H\(_2\)O\(_2\) min\(^{-1}\) g\(^{-1}\) FW) contents of leaves of different wheat cultivars Misr1, Gemmiza 12 and Sakha 95 at 90 days after sowing in greenhouse conditions

| Treatment | Misr 1 | Gemmiza 12 | Sakha 95 |
|-----------|--------|------------|----------|
| **Soil salinity (S)** | APX    | CAT        | APX      | CAT        | APX     | CAT     |
| 0         | 334.33 c | 14.97 d    | 300.33 c | 12.87 d    | 311.33 c | 13.37 d |
| 4         | 361.88 b | 18.53 c    | 327.88 b | 16.43 c    | 338.88 b | 16.93 c |
| 8         | 372.11 b | 21.46 b    | 338.11 b | 19.36 b    | 349.11 b | 19.86 b |
| 12        | 414.33 a | 22.24 a    | 380.33 a | 20.14 a    | 391.33 a | 20.64 a |
| **Azotobacter isolates (Az)** |        |            |          |            |          |          |
| 0         | 407.75 a | 21.84 a    | 373.75 a | 19.74 a    | 384.75 a | 20.24 a |
| Az2       | 385.75 b | 21.10 b    | 351.75 b | 19.00 b    | 362.75 b | 19.50 b |
| Az6       | 318.50 c | 14.97 c    | 284.50 c | 12.87 c    | 295.50 c | 13.37 c |
| **Interactions** |        |            |          |            |          |          |
| Az2 + S0  | 256.33 d | 10.36 g    | 222.33 d | 8.26 g     | 233.33 d | 8.76 g  |
| Az2 + S4  | 287.66 d | 12.52 f    | 253.66 d | 10.42 f    | 264.66 d | 10.92 f |
| Az2 + S8  | 330.66 c | 16.98 e    | 296.66 c | 14.88 e    | 307.66 c | 15.38 e |
| Az2 + S12 | 399.33 ab | 20.03 d   | 365.33 ab | 17.93 d    | 376.33 ab | 18.43 d |
| Az2 + S0  | 350.00 bc | 16.95 e   | 323.00 bc | 14.85 e    | 334.00 bc | 15.35 e |
| Az2 + S4  | 393.00 ab | 21.15 cd   | 359.00 ab | 19.05 cd   | 370.00 ab | 19.55 cd |
| Az2 + S8  | 372.66 abc | 23.59 ab | 338.66 abc | 21.49 ab | 349.66 abc | 21.99 ab |
| Az2 + S12 | 420.33 a  | 22.71 abc  | 386.33 a  | 20.61 abc  | 397.33 a  | 21.11 abc |
| Az2 + S0  | 389.66 ab | 17.61 e    | 355.66 ab | 15.51 e    | 366.66 ab | 16.01 e |
| Az2 + S4  | 405.00 ab | 21.92 bc   | 371.00 ab | 19.82 bc   | 382.00 ab | 20.32 bc |
| Az2 + S8  | 413.00 ab | 23.83 a    | 379.00 ab | 21.73 a    | 390.00 ab | 22.23 a |
| Az2 + S12 | 423.33 a  | 24.00 a    | 389.33 a  | 21.90 a    | 400.33 a  | 22.40 a |

Means in the same column followed by the same letter are not significantly different according to Duncan’s test at 0.05 level. APX: ascorbate peroxidase; CAT: catalase
organisms to survive when there is extreme osmotic stress as shown in Figs. 1 and 2 [19,47,48]. So, Azotobacter isolates (Az2 and Az6) revealed better survival in terms of CFU counts under all different NaCl concentrations. For bacterial traits, such as IAA synthesis and nitrogen fixation have exhibited an influence on plant growth by increasing nutrient availability and by influencing plant development [49]. Therefore, growth promotion by isolates Az2 and Az6 may be mediated by these traits. Azotobacter isolates (Az2 and Az6) were able to IAA production and nitrogen fixation, indicating plant growth-promoting characteristics.

On the other hand, salinity tolerance mainly relies on plant cultivar, and thus, genetic variability exists between cultivars [50]. Germination parameters such as FGP and MGT could be attributed to inhibition of water absorption by seeds of wheat as a result of increasing the osmotic potential of soil solution and/or ionic effect in which NaCl concentrate in plant tissues, causing an inequity in the uptake of nutrients and poisonous effect [51]. To overcome the previously discussed adverse effect of elevated NaCl concentrations on the germination of different wheat cultivars seeds were treated with different isolates of Azotobacter which can be utilized to reduce the adverse effect. In our current experiment (Table 1), high NaCl concentration (150 mM), reduced the FGP and increased MGT of all different wheat cultivars seeds. This may indicate that increasing NaCl decreased relative water content in seedlings. However, FGP enhanced when using Azotobacter Az6 isolate reached to 36.66% for Misr1 cultivar, 26.66% for Gemmiza 12 cultivar and 28.33% for Sakha 95 cultivar as compared to other isolates, respectively. While, the lowest MGT recorded 3.13 days for Misr1 cultivar, 3.44 days for Gemmiza 12 cultivar and 3.41 days for Sakha 95 cultivar by using Az6 isolate compared to other isolate, respectively. These results were in harmony with those obtained by other authors. For instance, Cokkizgin [52], showed that high NaCl concentrations postponed the initiation of seed germination of common bean and induced a reduction in its FGP. Also, Mena et al. [51], reported that seed germination of common bean was reduced by increasing NaCl above 8775 mg L⁻¹. In the same context, Alom et al. [53], stated the negative effect of NaCl (15 dS m⁻¹) on seed germination of different wheat genotypes.

Our findings clearly demonstrate that rhizobacteria-inoculated wheat cultivars positively impacted growth characteristics even under salt stress conditions for up to 30 days of treatment (Tables 2 and 3). Among all the tested isolates under salt stress, Az2 and Az6 enhanced maximum vegetative growth attributes of wheat cultivars such as fresh weight, dry weight, root and shoot length as well as root colonization. For example, under 150 mM concentration, fresh weight of plant and root colonization increasing by inoculation treatment with Az6 isolate reached to 70% fresh weight of plant and 18 × 10⁵ g⁻¹ root for Misr 1 cultivar, 15 % fresh weight of plant and 17 × 10⁵ g⁻¹ root for Gemmiza 12 cultivar, 15% fresh weight of plant and 17 × 10⁵ g⁻¹ root for Sakha 95 cultivar over control, respectively. This might be due to the ability of bacteria to survive and colonize roots effectively through biofilm development and expression of properties relevant to stress tolerance such as EPS production [17,18,48,54,55,56].

In our study (pot trials), high salinity levels caused inhibitory effects on the growth of different wheat cultivars (Table 4). Salinity affects the growth of the plants by obstructing the cell division and enlarging in meristematic region; water deficit due to osmotic effect, inhibition of nutrient absorption or other metabolic disorders due to salt [57]. However, an increase in plant growth parameters was observed with Azotobacter isolate Az6 treatment at 8 dS m⁻¹ resulted in attaining 79.033, 73.000 and 76.470 cm plant height, 3.946, 3.656 and 3.816 g dry weight, 22.586, 20.900 and 21.746 cm root length, fresh and dry weights were recorded in agreement with our results, [58], observed increased tomato plant biomass under 120 and 207 mM NaCl stress inoculated with Achromobacter piechaudii ARV8. Similarly, [54], observed that inoculation of wheat (Triticum aestivum L.) with ACC deaminase-producing Klebsiella spp. SBP-8 under salt stress results in increased root length, shoot length, fresh and dry weight. Also, Bhise et al. [59], showed that maximum shoot length, root length, fresh and dry weights were recorded in Vigna radiata L. when supplemented with E. cloacae KBPD in the presence of 0, 50, 100 and 150 mM of NaCl after 14 days. In addition, many reports showed that plant growth promotion can be induced by beneficial soil bacteria under salinity stress which have been demonstrated in several plant species such as wheat [60], maize [61], lettuce [62], alfalfa [63], and cowpea [64].

Inhibition of plant growth occurring due to soil salinity is often associated with the accumulation
of higher Na⁺ content and a low K⁺/Na⁺ ratio [65]. In our results we observed that the inoculation of *Azotobacter* isolate Az6 significantly decreased the level of Na⁺, and increased N and K⁺/Na⁺ ratio in the leaf of the different wheat cultivars (Table 5). Likewise, [66], showed that increased levels of K⁺ in plants may minimize the adverse effects of salinity on plant growth and yield. Potassium is widely known to maintain water stress and also acts as a cationic solute for stomatal function in leaves under water scarcity [67]. The reduced Na⁺ concentration in wheat plants under imposed salt stress, due to Az6 inoculation, might have helped plants prevent the accumulation of cellular Na⁺ to a toxic concentration. Similar to our observation, the Arabidopsis plant inoculated with the PGPB *Bacillus subtilis* GB03 showed a 54% decrease in Na⁺ compared with control plants. This decrease in saline-induced Na⁺ content could be differential expression of ionic transporters. Earlier studies reported that the decline of Na⁺ accumulation takes place due to decreased expression of HKT1 (high affinity potassium transporter) in roots to decrease root Na⁺ uptake and upregulation of HKT1 leading to enhanced shoot-to-root Na⁺ recirculation, respectively [56, 65, 68]. Also, bacterial inoculation treatments can reduce plant Na⁺ uptake by the excretion of IAA and bacterial exopolysaccharide, which could bind Na⁺ and prevent its uptake in plants.

For photosynthetic pigments, salinity stress leading to decreased chlorophyll and carotenoids due to suppression of specific enzymes responsible for synthesis of photosynthetic pigments or also via membrane damage [69]. The role of *Azotobacter* isolates in reducing the negative impact on photosynthetic performance of different wheat cultivars has been documented during stress conditions (Table 6). Also, improved nutritional status of wheat plants by bacterial inoculation can lead to increase in carotenoid and chlorophyll contents and consequently specific stimulation of photosynthetic capacity through stomatal conductance. Carotenoids, which are well-known singlet oxygen quenchers, can also scavenge other ROS and protect chlorophylls from photo-oxidative damage [70]. Therefore, better growth of *Azotobacter* isolates-inoculated plants compared to control under soil salinity conditions may be attributed to higher contents of chlorophyll and carotenoid pigments in the leaves. Our findings are in accordance with [71], where microbial treatment was reported to improve chlorophyll and carotenoids concentration under salt stress of 10 and 20 dS/m, and maximum improvement shown by the combination (*Arthrobacter protophormiae, Rhizobium* and *Glomus mosseae*) treatment with respect to untreated stressed pea plants. On the other hand, proline one of the most important osmolytes, increases under salt stress and has a strong correlation with the extent of exposure to salt [72]. Accumulation of proline enables the plants to maintain the proper osmotic balance under salinity-induced low water potentials. It not only protects the plants against salinity stress, but also stabilizes membranes, proteins, and enzymes like Rubisco [73]. In our study, we observed a significant increase in the accumulation of proline in Az6-treated plants with respect to untreated plants grown under salt stress. This increase illustrates that Az6-inoculated plants experienced less stress than uninoculated counterparts [72]. Antioxidant enzymes play a key role in the defense system of the plant against oxidative stresses induced by salinity. Change in the activity of antioxidant enzymes is a defense mechanism of plants under oxidative stress induced by environmental stresses [74]. Our results showed that increased of APX and CAT content by *Azotobacter* Az6 treatment at 12 dS m⁻¹ for different wheat cultivars over the uninoculated control (Table 7). Therefore, antioxidative enzymes like APX and CAT are the most important components in the scavenging system of ROS [75]. Also, [32], reported that the activities of the antioxidative enzymes such as CAT and APX increased under salt stress in plants and a correlation of these enzyme levels and salt tolerance exist. It has been found that plants inoculated with bacterial strains showed high antioxidant enzymes activity which contributed to enhance plant protection against salt stress by eliminating hydrogen peroxide from salt-stressed roots [31].

5. CONCLUSION

This study revealed that the rhizosphere engineering is a good option to produce environmental friendly stress tolerance in crop plants. Our results showed that the inoculation of different wheat cultivars with the bacterium *Azotobacter* increased vegetative parameters, improving physiological attributes and nutrient uptake as well as alleviation of salt stress. Among all tested *Azotobacter* isolates, Az2 and Az6 isolates showed that the highest growth and exhibit positive PGPR traits. Both the bacteria could promote growth in three cultivars of wheat tested (Misr 1, Gemmiza 12 and Sakha 95) in
terms of increase in germination indicators, growth dynamics as well as increase in total chlorophyll, carotenoids and proline content. Besides, wheat cultivars could withstand salt stress more efficiently in presence of the bacteria which arranged as follows: Misr1 > Sakha 95 > Gemmiza 12. In addition, inoculation treatment enhanced antioxidant enzymes such as catalase and ascorbate peroxidase. Therefore, the improved growth dynamics, plant physiological and antioxidant activity ultimately leads to enhanced crop yield and quality. Thus, inoculation with Azotobacter isolates Az2 and Az6 could be efficiently used to partially or completely eliminate the effects of salt stress on growth and yield of different wheat cultivars. Further investigations are required to identify Azotobacter isolates Az2 and Az6 by 16S rRNA and to assign their role in other crops during other stresses.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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