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Wheat plant –Plant Growth Promoting Rhizobacteria (PGPR) interaction to alleviate the salt stress *in vitro* and *in vivo*

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Abstract

The current work studied the mechanistic basis of salt tolerant PGPR bacteria in alleviating salinity stress in wheat.Two experiments were conducted at the Agricultural Research Center (ARC), 2021-2022, to evaluate the interaction between wheat var. Misr1 and two salt tolerant PGPR bacterial strains, *Azospirillum* (*A.*) *brasilense* NO40 and *Bacillus thuringiensis* (*Bt*) under saline conditions. The staining technique used to detect the colonization effectiveness of applied bacteria. Images of 30-day wheat seedlings were captured and subjected to the following measurements: root area, leaf area, root length, shoot length, and dark green color index (DGCI). TEM was used to look for bacterial colonization in wheat seedling. Chlorophyll, proline, peroxidase activity, rhizosphere dehydrogenase activity and electrical conductivity (EC) were all quantified. Results revealed that the wheat seedling roots inoculated with the salt tolerant bacterial strains produced a highly intense red colour. The colonized bacteria appeared on the outer surface of the root and intercellular space of root tissue. The data showed a significant rise in all measured parameters in the inoculated wheat seedlings with the salt tolerant bacterial strains compared to the non-inoculated wheat seedlings. Additionally, the wheat seedlings inoculated with a mixture of NO40 and *Bt* showed the best results as compared to that inoculated with only NO40 or with only *Bt*. Finally, EC values before and after planting with the inoculated wheat seeds revealed a decrease in the salinity of the soil rhizosphere (9.27 mmhos/cm vs. 5.1 mmhos/cm). In conclusion: Salt stress in wheat saline soils was alleviated by bacterial strains studied. Optimum results were obtained from the mixture of *A. brasilense* and *Bt*. Results must later be verified in field trials.

Keywords: Wheat seedlings; PGPR; Salinity; Colonization; Antioxidant enzymes

1. Introduction

Soil salinity is one of the primary abiotic stresses causing reduction of plant growth, crop yield, and productivity.Over 950 million ha of the Earth's surface (~10%) are affected by salinity, and over half of all irrigated agricultural land in the world suffers from secondary-induced salinization (Munns and Tester) [1].In Egypt, 33% of the cultivated land, which comprises 3% of total land area are affected by salinity.

Plant growth promoting rhizobacteria (PGPR) are microbes which inhabit plant roots and enhance plant development. Many years ago, researchers studied the use of PGPRs to increase the growth and yield of plants. However, with new analytical methods the study of beneficial PGPRs in management of abiotic stresses is once again receiving renewed interest. Numerous researchers are now proving the hypothesis that PGPRs enable agricultural plants to maintain productivity under different stressed conditions (Sharma *et al* [2]). It has been well documented that PGPRs elicit stress tolerance in plants due to a variety of direct or indirect mechanisms (Minakshi *et al* [3]). These effects include facilitating

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uptake of water and nutrients, inducing plant anti-oxidative systems (Hidri *et al* [4]), producing phytohormones and osmolytes (Sai *et al* [5]), and restricting Na^+ uptake by root (SaifUllah [6]) etc. A complex network of signalling events occurring during the plant-microbe interaction (Kaushal and Wani [7]) regulates all these mechanisms.

In China, more than 100 million hectares of soil are saline or sodic and is increasing at a rate of 1.4% - 3% annually (Liu *et al* [8]), making China the 3rd most affected country by salinity in the world.Likewise, Egypt is also one of the countries that suffers severe salinity problems (Al-Naggar *et al* [9]). The Food and Agricultural Organization (FAO), has predicted that by the year 2050, 50% of the total land mass will be lost due to salinity (Ilangumaran and Smith [10]). These facts represent a serious threat to sustainable food production and to our natural resources (Ondrasek *et al* [11]). At the same time, UN projections suggest that the world population may possibly reach 9.6 billion in 2050 (www.fao.org/economic/esa) needing up to 70% more food by then. To achieve this, tolerance to salinity and the development of the new techniques are urgently required for agricultural production on salinized land (Ajay [12]).

Plant growth promoting rhizobacteria (PGPR) elicited stress tolerance in plants due to a variety of mechanisms. The major points of these mechanism are (1) plants treated with PGPR have better root and shoot growth, nutrient uptake, hydration, chlorophyll content, and resistance to diseases (Diby and Harshad [13]); (2) stress tolerance can be explained by nutrient mobilization and biocontrol of phytopathogens in the rhizosphere (Backer *et al* [14]); (3) PGPR produce phytohormones increase the overall growth and alter root characteristics (i.e. alteration of root proliferation, metabolism and respiration rate) to facilitate uptake of water and nutrients (Kudoyarova *et al* [15]); (4) PGPRs produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase to decrease excessive ethylene production in plants caused by salinity stress and thereby eliminate the negative effect of ethylene on roots (Liang *et al* [16]); (5) PGPRs favor the circulation of plant nutrients in the rhizosphere (Jordan *et al* [17]); (6) PGPRs favor the accumulation of osmo-protectants (i.e. proline, polyamines, glutamate, total free amino acids, etc.) in plants (Egamberdieva *et al* [18]); (7) PGPRs produce exopolysaccharides to bind toxic Na^+ and restrict Na^+ influx into roots (Morcillo and Manzanera [19]**);** (8) plants colonized with PGPRs have higher K^+ ion concentration and, in turn, higher K^+/Na^+ ratio that favors salinity tolerance (Gupta [20]); (9) volatile organic compounds (VOCs) can trigger induction of high affinity K^+ transporter (HKT1) in shoots and reduction of HKT1 in roots thereby limiting Na^+ entry into roots and facilitating shoot-to-root Na^+ recirculation (Naveen *et al* [21]); and (10) PGPRs induce plant anti oxidative systems for reactive oxygen species (ROS) scavenging such as enzymatic components of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), and glutathione reductase (GR) and non-enzymatic components of cysteine, glutathione and ascorbic acid to degrade reactive oxygen species generated upon salt shock (Qin *et al* ; Sáenz-Mata *et al*; Ilangumaran and Smith [22, 23, 10]).

These mechanisms are regulated by a complex network of signalling events occurring during the plant-microbe interaction (Smith *et al* [24]), but still a lot is yet to be explored at physiological, molecular and biochemical level on how PGPRs ameliorate salinity stress in plants. In this regard, several research issues should be fully addressed (1) Morphological, biochemical and physiological characteristics of the interactions between plants and PGPRs under salinity; (2) Information on the metabolically and nutritionally active roles of PGPRs in plants under salinity; (3) Critical signals or chemicals mediating the communications between plants and PGPRs under salinity;(4) Molecular mechanisms by which PGPRs increase plant resistance to salinity; and (5) Assessment of key environmental factors that influence the efficacy of PGPRs-mediated amelioration of salinity stress in the plant. The present study aimed to assess to what extent salt tolerant PGPR could improve, individually or synergistically wheat growth and yield under saline conditions. The inoculant strains *Azospirillum brasilense* (*A. brasilense*)(NO40) and *Bacillus thuringiensis* (*Bt*) were used in this study.

2. Material and methods

Seeds of bread wheat (*Triticumaestivum L.*) cultivar Misr 1 was provided by Wheat Research Department, Field Crop Research Institute, Agricultural Research Center, Giza, Egypt.

2.1 Bacteria and inoculants preparation

Two salt tolerant bacterial strains, *A. brasilense*NO40 and *Bt*, were obtained from Agriculture Microbiology Department, Soils, Water and Environment Research Institute (SWERI), and Microbial Molecular Biology Lab, Agricultural Genetic Engineering Res., Inst. (AGERI), Agricultural Research Center, Giza, Egypt. NO40 of *A. brasilense* was isolated from rice rhizospere soil at the Moshtohoor Experimental Station in North Egypt (Omar *et al* [25]).This strain can survive in 10.53 % NaCl (Omar *et al* [26]). *Bt* was isolated from hypersaline environments along the costal ridge, mallahat Maryut, Bourg El-Arab, along the Mediterranean (Nahed *et al* [27]). It could grow in salt medium up to 45% NaCl and showed the maximum proline concentration at 5.8 % NaCl (Labib *et al* [28]). These bacteria were cultured on nutrient broth media

according to Atlas [29]. 0.1 ml of each bacterial strain that was freshly prepared in broth culture (10^9 cells/ml) was added to saline (5.8 %NaCl{100 mM}) semi agar medium of Watanabe (Watanabe [30]); Treatments were as follows:

- T1 = *A. brasilense* NO40
- T2= *Bt*,
- T3= Mixture of both strains (equal amounts of 10^9 cells/ml broth culture of *Bt* and *A. brasilense*)
- T4= Control without bacteria.

2.2 Spermosphere model experiment

The staining technique utilizing a spermosphere model was conducted in order to detect colonization patterns of applied bacteria on wheat seedlings roots under sterilized conditions.This model was adapted from the Center of Pédologie, Nancy, France (Omar *et al* [31]). Wheat seeds were washed under distilled water and the surface was sterilized using ethanol 70 % for 30 seconds then rinsed thoroughly under aseptic conditions in sterile distilled water followed by soaking in 20% sodium hypochlorite for 15 minutes. Seeds were washed three times again in sterile distilled water and put in a Petri dish on a filter paper for 10 minutes to dry until seed culturing. The sterilized seeds were aseptically germinated on Petri dish containing Watanabe medium devoid of any carbon sources and any growth regulators. After the seeds had been germinated, they were transplanted into the long tube containing 7 ml of saline Watanabe semi solid medium without any carbon sources. When seedlings were 1cm height, the spermosphere models were either inoculated by bacteria or not as a control, followed by an incubation period (7 days) to be sure that the colonization occurred. On the last day of the incubation period, the roots of wheat in spermosphere model treated with 2,3,5 triphenyltetrazolium chloride (2 ml TTC solution /sample) for 3 h to detect the colonization by the formation of triphenylformazan (TPF) which can be indicated by changing the root color to red (fig 1).

2.3 Greenhouse experiment

Surface soil samples (0-30 cm) were collected from Sahel El-Hosainiya in the Sharqueya Governorate of Egypt, which is located northeast of the Delta region to represent saline sodic soils. The samples were air dried, crushed, sieved to pass a 2.0 mm sieve and analyzed for their physical and chemical properties according to standard methods (Page *et al* [32]). Physical and chemical characteristics of the used soil are outlined in table 1

Table 1 Physical and chemical characteristics of saline soils collected from Sahel El-Hosainiya, northeast of the Delta region, Sharqueya Governorate, Egypt

Character	Unit	Value
Particle size		
Clay	%	65.52
Silt	%	25.33
Fine sand	%	6.23
Coarse Sand	%	2.92
Texture	-	Clay
EC (Paste Extract)	dS/m	9.27
pH (Paste Extract)	-	8.13
SP (Solubility product)	%	92
Organic matter	%	0.26
Exchangeable Sodium Percentage (ESP)		21.41

Sterilized vermiculite carrier mixed with 10 % Irish Peat has a water holding capacity up to 60 %. Then bacterial broth cultures were prepared at a concentration of 10^9 cells/ml. 250 gm carrier was mixed with sterile 50 ml bacterial broth culture of 10^9 cells/ml and packed into polyethylene bags to be used as seed inoculum. Thirty minutes before sowing, wheat seeds were coated with the different seed inoculum (*A. brasilense* NO40, *Bt* and a mixture of equal amounts {25 ml of 10^9 cells/ml each} of both *A. brasilens* NO40 and *Bt*) using sucrose solution as the adhesive medium. Coated seeds were air-dried for 15 min. under shade and sown immediately. The experiment was conducted under greenhouse conditions using saline soil (the pots 20 cm - 25 cm were filled with 3 kg saline soil). Seeds without bacterial inoculation

were assigned as control treatment. Each treatment was prepared in three replicates. Five wheat seedlings were sampled at 30 days after sowing to measure morphological and biochemical growth criteria. The rhizosphere soil was collected by uprooting the wheat seedlings carefully from the pots and preserved at 4°C to be used for analysis.

- For each treatment, the leaf and root images (fig 3) were captured in 300 dots per inch (dpi) resolution using a flatbed scanner (HP Scan jet G2710). The images were processed to extract values of RGB (red, green, blue light brightness) using Adobe Photoshop CS6 Ver. 13-Extended. The dark green color index (DGCI) was calculated from hue, saturation, and brightness (HSB) levels, according to (Karcher and Richardson[33]). The leaf area and root area were measured using the number of pixels (Baker *et al* [34]).
- The interaction between wheat seedlings and the PGPR that were used in this work was followed up with transmission electron microscopy (TEM). The work was done in the TEM Lab FA-CURP, Fac. OF Agri., Cairo Univ. Research Park. The method according to Bozzola and Russell [35] was used. Tissue samples were sliced into ~ 1 mm portions. Sliced tissue was processed for TEM by fixation in glutaraldehyde and osmium tetroxide, dehydrated in alcohol and embedded in an epoxy resin. Microtome sections were prepared at approximately 500-1000 µm thickness with a Leica Ultracut UCT ultramicrotome. Thin sections were stained with tolodin blue (1X) then examined by camera (Lica ICC50 HD). Ultra-thin sections were prepared at approximately 75-90 µm thickness and were stained with uranyl acetate and lead citrate, then examined by transmission electron microscope JEOL (JEM-1400 TEM). Images were captured by CCD camera model AMT with 1632 x 1632 pixel as side mount configuration. This camera uses a 1394 fire wire board for acquision (Dykstra and Reuss [36])
- Photosynthetic pigments chlorophyll a, b and total chlorophyll were determined quantitatively as described by Arnon [37]. A known fresh weight of leaves was homogenized in pure methanol overnight in the dark. The homogenate was centrifuged at 4000 rpm. The supernatant was made up to known volume with pure methanol. The absorbance was measured calorimetrically against a blank of pure methanol at two wavelengths 666nm and 653 nm, taking into consideration the dilution of the sample. The concentration of pigment fractions (chlorophyll a, chlorophyll b and total chlorophyll) was calculated as mg/g fresh weight by using the equations of (Lichtenthaler and Wellburn [38]).
- Free proline was determined according to the method described by Bates *et al*. [39]. 50 mg of the dry plant martial was homogenized in 10 ml of 3 % aqueous sulphosalicylic acid and centrifuged at 4000 rpm. Then, 2 ml of supernatant were taken with 2 ml of acidic ninhydrin reagent and 2 ml of glacial acetic acid in a test tube and left for 1 h at 100 °C. The reaction was terminated in an ice bath. The reaction mixture was extracted using 5 ml toluene. The absorbance of the toluene layer was measured at 520 nm using toluene as a blank. The seedling proline content was presented as µmoles /g fresh weight.
- Rhizosphere analysis
  - Rhizosphere soil pH was measured on 20 g of the air-dried soil in 50 ml distilled water (1: 2.5).The mixture was shaken for 1 hr. then pH was detected by immersing a glass electrode in the suspension.
  - Electrical conductivity (EC) of the soil was measured by adding 10 g of the air-dried soils into 50 ml distilled water (1:5) and shaking it continuously for 1 h. The suspension was left for 24 hours before filtration to measure the soil EC in the filtrate using a glass electrode (Page *et al* [32]).
  - The dehydrogenase (EC 1.1) activity in the rhizosphere soil was carried out according to (Casida *et al* [40]).Two gm of the soil was transferred to a test tube, then 2 ml aliquots of 0.5 % of 2,3,5 triphenyltetrazolium chloride (TTC) solution were added and mixed thoroughly.Tubes were then sealed with a rubber silicon stopper and incubated at 30°C for 24 h in dark. Thereafter, 8 ml of pure methanol were added to each tube, shaken and left for 2 hrs in the dark with shaking at regular intervals to extract triphenylformazan (TPF). The suspension was then filtered through Whatman filter paper No.1. The intensity of the developed pink color of the filtrate was measured at wavelength of 485 nm using a spectro-colorimeter. Concentrations of formazan were calculated from a standard curve and presented in µg TPF/g dry soil /24 h. A blank treatment included all additives without soil.
  - Peroxidase (EC 1.11.1.7) was assayed as described by Kumar and Khan [41]. The assay mixture of POX contained 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.01 M pyrogallol, 1 ml of 0.005 M H2O2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25°C, after which the reaction was terminated by adding 1 ml of 2.5 N H_2SO_4. The amount of the formed pyrogallin was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time.

2.4 The statistical analysis

One way analysis of variance (ANOVA) was used for data analysis **(**Snedecor and Cochran [42]**)**.

3. Results and discussion

Seed germination is an initial and critical stage in a plant's life cycle (Elzbieta *et al* [43]). During this stage, bacterial colonization of the roots starts in response to root exudates (Haichao *et al* [44]; Bashan and de-Bashan [45]).

Study of microbial colonization on roots using the staining technique under salt stress in the spermosphere during the initial stage of plant growth is useful and could predict the efficiency of the microorganisms that were used in this work for alleviating such stress. Fig 1 showed the colonization of salt tolerant (ST) bacterial strains (*A. brasilense* and *Bt*) on wheat seedlings (10 days old) under aseptic and saline conditions in the spermosphere. The assays here were based on the reduction of 2, 3, 5-triphenyletetrazolium chloride (TTC) to the red coloured formazan (TPF) (Praveen and Tarafdar [46]). The seedlings inoculated with a mixture of *A. brasilense* and *Bt*, those inoculated with *A. brasilense* and those inoculated with *Bt* showed high intensity in red color as compared to the control (where there was no inoculation). In this experiment, we relied only on visual quantification as a preliminary assessment. Reduction of TTC by seedling tissue is directly linked to the activity of mitochondrial respiratory chain (Markus and Brunner [47]; Omar and Basilious [48]). Only living tissues reduces TTC to TPF. This finding describes why the inoculated treatments showed higher intensity in color due to colonization of bacteria. The effect of inoculation causes the interactions between some complex mechanisms such as hormonal effects, N_2 fixation, proton extrusion and/or mineral uptake. Moreover, PGPR strains were shown to produce bacterial exopolysaccharides which bind with some cations including Na (Geddie and Sutherland [49]; Han and Lee [50]). This notion is further supported by the findings of Ashraf *et al* [51] that increased population density of PGPR in the root zone and could decrease Na available for plant uptake, thus helping to alleviate salt stress in plants.

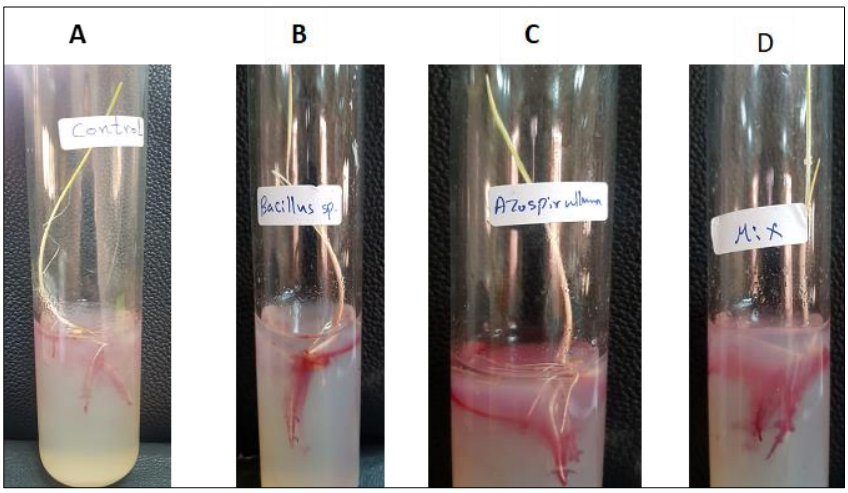

Figure 1 The microbial colonization of wheat seedlings in the spermosphere under salinity conditions, **fig 1 A** is the control, **fig 1 B** wheat seedlings inoculated with *bacillus thuringiensis* (*Bt*), **fig 1 C** wheat seedlings inoculated with *Azospirillum* (*A*.) *brasilense* and **Fig 1D** wheat seedlings inoculated with mixture of *Bt* and *A. brasilense*

3.1 Transmission electron microscopy (TEM)

The colonization of wheat seedlings by the ST PGPR that were used in this work was followed up with transmission electron microscopy (TEM) (figure2). TEM analysis showed no dissolution in cell wall in the control (fig2A) while in the seedlings inoculated with ST PGPR *A. brasilense*, *Bt* and a mixture of both (fig 2 B, C, D, E, F and G) showed clear differences from the control. The colonized bacteria were visible on the root surface of the wheat seedling that had been inoculated with the ST PGPR strains NO40, *Bt*, and a combination of NO40 and *Bt* (fig 2B).This result was consistent with (Su-Jung and Kremer [52]) when plant root seedlings were colonized with the root colonizing rhizobacteria. Additionally, TEM of the inoculated root wheat seedlings with the ST PGPR strains NO40, *Bt* and a mixture of both NO40 and *Bt* revealed the colonized bacteria in the intercellular and intracellular spaces of plant root cells (fig 2 C, D, E. F and G). The same finding was obtained by (Bashan and de-Bashan [45]) who used *A. brasilense* as PGPR. Our findings also agreed with those of (Zhen *et al* [53]) whose TEM pictures of inoculated wheat roots showed variations in the wheat roots before and after inoculation with ST PGPR. They attribute these variations to the connection of the ST PGPR with the root of wheat. Su-Jung and Kremer [52] had also used TEM to study the relationship of PGPR bacteria with plant roots. TEM revealed considerable alterations of root cells including partial cell wall degradation and cytoplasm disorganization. They attribute that to the fact that bacterial attachment to plant surfaces begins with attraction by seedling root exudates. This leads to higher bacterial colonization of roots. The picture in (fig 2 D) depicted the shape

of the *A. brasilense*and it appeared to be a replica of the electron micrographs picture of *A. brasilense* that were published by (Doumit *et al* [54]). Eman *et al* [55] demonstrated the ST PGPR bacterial colonization of root plant seedlings was consistent with TEM evidence from the present study.

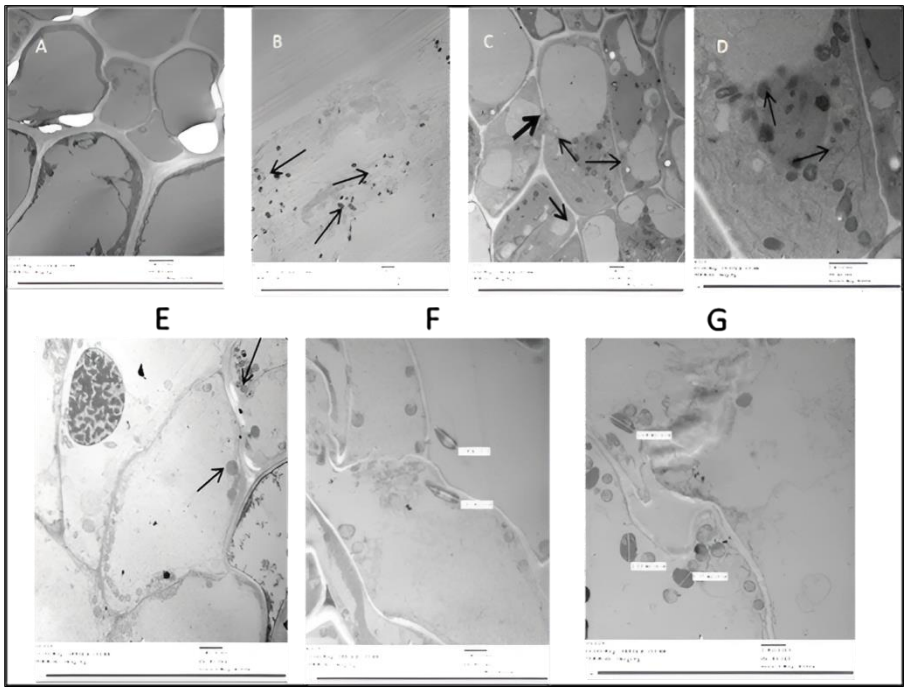

Figure 2 Colonization of the mixture of ST PGPR *A. brasilense* and *Bt* with the root of wheat seedlings through transmission electron microscopy (TEM). **A:** control which is not inoculated with bacteria. **B:** bacteria on the outer surface of the root cortex of inoculated seedlings (the arrows refer to the bacteria). **C:** arrows refer to the partial plasma membrane and cell wall dissolution as a result of bacterial colonization. **D:** colonized bacteria of *A. brasilense* (the arrows refer to the bacteria). **E:** bacteria in the intercellular and intracellular spaces**. F:** *Bt* intracellular. **G:** *A. brasilense* intracellular

3.2 Image of the various wheat seedling treatments.

Figure 3 (A, B, C and D) showed the captured image of salt stressed wheat seedlings inoculated with the ST PGPR *A. brasilense*, *Bt* and a mixture of both compared to the non-inoculated control.

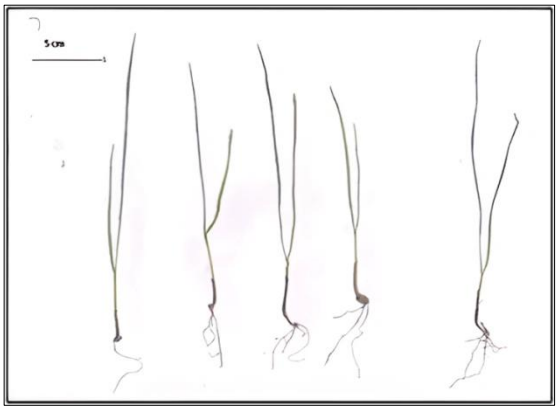

Figure 3 A wheat seedlings without inoculation (Control)

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Figure 3 B Wheat seedlings inoculated with ST PGPR *A. brasilense* compared to control

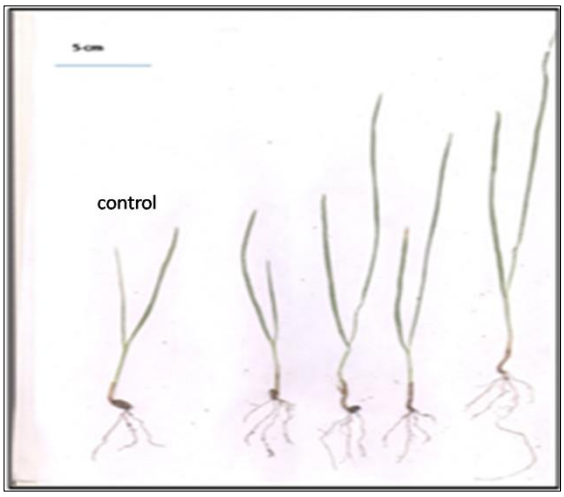

Figure 3 C Wheat seedlings inoculated with ST PGPR *Bt* compared to control

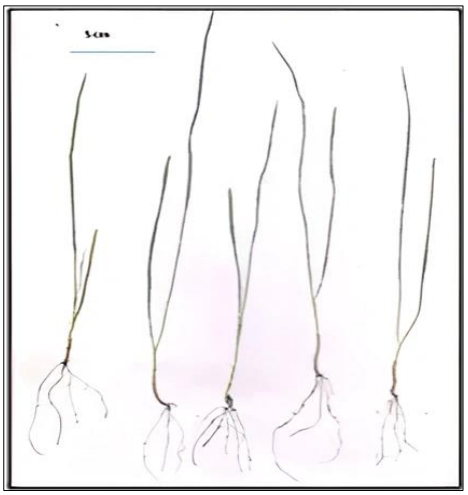

Figure 3 D Wheat seedlings inoculated with ST PGPR mixture of *Bt* and *A. brasilense*

Figure 3 The captured images of wheat seedlings inoculated with ST bacteria under saline conditions. **A**: control, where wheat are not inoculated with any bacteria **B**: wheat inoculated with *A brasilense* NO40, control is on the left. **C**: wheat inoculated with *Bt*, control is put on the left **D**: wheat inoculated with mixture of both of the two bacterial strains NO40 & *Bt*.

3.3 Shoots and roots feature

Table 2 shows shoot length, root length, shoot dry weight, root dry weight and root area of the wheat seedlings that were inoculated with ST PGPR *A. brasilense*, *Bt* and a mixture of *A. brasilense* and *Bt*. Wheat seedlings inoculated with a mixture of *A. brasilense* and *Bt* showed higher values than those inoculated with *A. brasilense* and those inoculated with *Bt*. All the inoculated wheat seedling showed higher values than the control. These data agreed with that of Zhen *et al* [53].

Wheat seedlings under stress of 300 mM (17.4 %) NaCl were inoculated with ST PGPR strains showed an increase in plant height, root length, dry weight and fresh weight over the control (no inoculation). Additionally, they revealed that the mixed inoculation gave the best results. They were attributed to the complementary strengths of the strains.

Table 2 Shoots and roots measurements of wheat seedlings inoculated with salt tolerant bacteria under saline conditions

Parameters / **Treatments**	**Shoot length (cm)**	**Root length(cm)**	**Shoot dry weight (mg/plant)**	**Root dry weight (mg/plant)**	**Root Area (cm2)**
Control	15.65±0.69	4.43±0.22	13±1.00	72±14	0.76±0.24
Bt	16.7±1.58	4.6±0.502	24±7.00	107±2	1.23±0.26
A. brasilense	17.3±1.69	5.95±0.32	49±11.0	183±11	1.41±0.37
Mixture	18.38±2.49	6.1±0.28	113±39.0	199±52	2.48±0.75
LSD0.05	2.22	0.65	38.72	51.77	0.6

3.4 Plant leaf characterization

Plant leaves are the main organ responsible for photosynthesis, through which the plant produces its energy and food. Salinity is amongst the most limiting factors affecting crop growth and yield through negative impacts on crop leaves (Hnilickova *et al* [56]).The impact of salinity of several leaf assays is shown in Table 3 and Table 4.

Table 3 Wheat seedling leaves inoculated with Salt tolerant PGPR *A. brasilense*, *Bt* and a mixture of both under saline conditions

Parameters / Treatments	Leaf Area(cm2)	DGCI	Chlorophyll-A (mg/g fw)	Chlorophyll-B (mg/g fw)	Chlorophyll-Total (mg/g fw)	Proline content (µmoles /g fresh weight)
Control	3.47±0.72	0.47±0.05	1.43±0.06	0.33±0.02	1.77±0.08	1.54±0.16
Bacillus th.	4.36±1.6	0.48±0.036	1.99±0.18	0.34±0.02	2.33±0.16	1.68±0.13
Azospirillum	5.73±1.46	0.53±0.042	1.91±0.14	0.33±0.03	2.23±0.17	1.77±0.01
Mixture	6.39±1.01	0.55±0.04	2.37±0.19	0.44±0.03	2.82±0.22	2.15±0.03
LSD0.05	1.66	0.057	0.284	0.048	0.313	0.199

Table 4 Peroxidase activity in wheat seedling leaves inoculated with Salt tolerant PGPR *A. brasilense*, *Bt* and a mixture of both under saline conditions

Parameters / Treatments	Peroxidase (µg/mg/plant)
Control	0.13±0.015
Bacillus th.	0.27±0.014
Azospirillum	0.38±0.012
Mixture	0.5±0.061
LSD0.05	0.0638

3.5 Leaf area

Total counts of green leaf pixels and red calibration pixels were used to estimate leaf area (leaf area= green pixel count x calibration area / red pixel count) (Hsien and Bloom [57]). As shown in Table 3, the leaf area of wheat seedlings that were inoculated with a mixture of *A. brasilense* and *Bt* showed the highest value followed by that inoculated with *A. brasilense* and then by that inoculated with *Bt*. This result agreed with that obtained by (Gholami *et al* [58]). They revealed that inoculation with bacterial strains had a significant effect on leaf surface area under sterile or non-sterile soils. They also showed that the wheat seedling leaf area inoculated with bacteria *A. brasilense* increased by up to 65% compared to those that were not inoculated (control).

3.6 DGCI and chlorophyll

The data in table 3 showed similar values of dark green color index (DGCI) of seedlings that were inoculated with the bacterial mixture or with *A. brasilense* (0.55 and 0.53, respectively).While the DGCI value of those that were inoculated with *Bt* were the lowest (0.48). Table 3 also showed chlorophyll measurements. Wheat seedlings that were inoculated with a mixture of *A. brasilense* and *Bt* showed the highest chlorophyll content. Chlorophyll A, B and A+B were about 65% over the control and nearly 30% over that inoculated with *A. brasilense* or *Bt.* Nathalie *et al* [59] published data in the same field showing that plants inoculated with PGPR under salt stress increased their resistance to salinity by increasing their growth parameters chlorophyll and proline. The literature established a link between DGCI and chlorophyll content as well as nitrogen (N) content of plant leaves. Jennifer *et al* [60] reported that leaf color charts (LCCs) were highly correlated to chlorophyll content and chlorophyll metre values.Chlorophyll is highly related to plant nitrogen (N). That is because a large amount of leaf N is used for photosynthetic enzymes. Abdelaziz and Moha [61] released the data of the processed leaf image by computer software to obtain intensity of three main colors red, green and blue (RGB). Consequently, the two indexes, DGCI and NRGB (nitrogen) were calculated. A linear relationship between DGCI and leaf N content was obtained. El Azazy [62] discovered that the leaf color image analysis technique could be used for determination of nitrogen content of plant seedlings. That is essential for chlorophyll formation and the basic color component (RGB). Consequently, it is important to index NRGB and DGCI. Thus, for plant seedlings, the RGB values of a

color leaf image could be used to estimate leaf chlorophyll. In wheat plants (Bojovic and Markovic [63]), a linear correlation between nitrogen content and chlorophyll (a), chlorophyll (B), and total chlorophyll was obtained.

3.7 Proline

Table 3 shows a 21% increase in proline concentration in salt stressed wheat seedlings that were inoculated with a mixture of the ST *A. brasilense* and *Bt* compared to those only inoculated with *A. brasilense* or with *Bt*, and by 40% *compared to* the control. This finding agrees with Rameesha *et al* [64] who reported similar results. In salt stressed plants, PGPR produced compatible osmolytes to help plants promote their growth. During stress conditions, proline is accumulated in plants. Proline is the key osmolyte that is formed in plants by the hydrolysis of proteins to reduce osmotic stress (Krasensk and Jonak [65]). Reports by Shamsul *et al* [66] said: "When exposed to stressful conditions, plants accumulate an array of metabolites, particularly amino acids. Amino acids have traditionally been considered as precursors to and constituents of proteins and play an important role in plant metabolism and development". A large body of data suggests a positive correlation between proline accumulation and plant stress. Proline, an amino acid, plays a highly beneficial role in plants which are exposed to various stress conditions. Besides acting as an excellent osmolyte, proline plays three major roles during stress: as a metal chelator, an anti-oxidative defense molecule, and a signaling molecule. A review of the literature indicates that a stressful environment results in an overproduction of proline in plants, which in turn imparts stress tolerance by maintaining cell osmotic balance, stabilizing membranes, thereby preventing electrolyte leakage and bringing concentrations of reactive oxygen species (ROS) within normal ranges, thus preventing oxidative burst in plants.

3.8 Peroxidase

Table 4 shows an increase in peroxidase activity in the wheat seedlings that were inoculated with a mixture of salt tolerant *A. brasilense* and *Bt* under salt stress. It rose to 0.5 ug /mg /plant while the control was 0.13 ug /mg / plant. This increase was also 37% higher than the control. It was also higher than those inoculated with only *A. brasilense* (0.38 ug /mg /plant) (12 %) and those inoculated only with *Bt* (0.27 ug /mg /plant) (23 %).These results agreed with those obtained by Aniqa *et al* [67]. They found that peroxidase was higher in wheat inoculated with ST PGPR under saline conditions than the control (not inoculated). Yulmira [68] revealed that the increased activity of peroxidase in shallot seedlings inoculated with PGPR was an indicator of induced resistance to pathogens. Mansour *et al* [69] reported a significant interaction between peroxidase activity in the roots and leaves of inoculated plants with PGPR under water deficit stress (WDS). Increasing the WDS caused a marked increase in total peroxidase activity of both roots and leaves in PGPR treated plants. Thus, peroxidase is an enzyme that plays a role in the resistance of plants to biotic or abiotic stresses.

3.9 PH and EC

Table 5 shows pH and EC in rhizosphere soil of wheat seedlings that are inoculated with bacteria *A. brasilense* and *Bt* or a mixture of both. The pH of the soil before planting (table 1) was 8.13, which is nearly the same as all inoculated seedlings and the control. Table 1 shows that the most common component of the soil was clay (65.52 %).The charged surfaces of clay make them more resistant to pH changes (Yuan and Xiong [70]).

Soil electrical conductivity (EC) is often used as a measure of salinity. Salinity is an indication of the amount of salt in the soil. Saline soils are those with an EC greater than 4 dsm^{-1} at 25 °C. The cation that most significantly influences salinity is sodium, especially when it is in excess of 100 mg/kg soil. Through the addition of elemental sulphur or gypsum, sodium can be leached from the soil, thereby reducing EC levels (MarnoFourie, Trace & Save [71]). The EC of the soil rhizosphere of wheat seedlings that were inoculated with a mixture of *A. brasilense* and *Bt* was 5.1 mmhos/cm, which was lower than those inoculated with *A. brasiliense* at 6.4 mmhos/cm. Wheat seedlings that were inoculated with *Bt* showed an EC value of 6.75 mmhos/cm (Table 5). The overall values were lower than those obtained from the control at 7.59 mmhos/cm. The EC of that soil before planting was 9.27 mmhos/cm (table 1).Thus, the data showed a decrease in the salinity of the soil rhizosphere as a result of bacterial colonization as shown in (Fig 1). This finding was supported by Stefan [72], who reported that *A. brasilense* and *Bacillus* those that improved plant growth and enhanced the tolerance to sodium chloride in wheat. Wang *et al* [73] also found that EC of soils was the most influential driving force of bacterial community composition, while the second most important factor was suggesting a clear separation of bacterial communities in accordance with the EC. Additionally, Saifullah *et al* [6] published that the PGPR inoculations significantly decreased EC and sodium adsorption ratio of rhizosphere soil of inoculated plants over that of uninoculated soil.

3.10 Soil dehydrogenase activity

Dehydrogenase assays based on the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) to the red-coloured formazan (TPF) were used to determine microbial activity in the rhizosphere and wheat seedlings. Biological oxidation of organic compounds is a dehydrogenation process. Therefore, the dehydrogenase activity (DHA) of soil reflects microbial activity (Skujins [74]). DHA estimation is based on the use of redox-sensitive tetrazolium dye, which is reduced to insoluble formazan inside cells due to respiratory activity. Table 5 shows the dehydrogenase activity of the rhizosphere soil of wheat seedlings inoculated with ST PGPR *A. brasilense*, *Bt* and a mixture of both of them under saline conditions. The mixture of *A.brasilense* and *Bt* had an activity of 27.9 mg TPF g^{-1} soil day^{-1}, *A. brasilense* 23.4 mg TPF g^{-1} soil day^{-1} and *Bt* 20.5 mg TPF g^{-1} soil day^{-1}.The control activity was 20.3 mg TPF g^{-1} soil day^{-1}

Table 5 pH, EC, and dehydrogenase activity (DHA) of rhizosphere soil of wheat seedlings inoculated with ST PGPR under saline conditions

Parameters / Treatments	pH	EC (mmhos/cm)	Soil dehydrogenase activity (mg TPF g-1 soil day-1)
Control	8.5±0.19	7.59±0.34	20.3±3.3
Bt	8.3±0.2	6.75±0.12	20.5±1.3
A, brasilense	8.4±0.186	6.42±0.04	23.4±6.7
Mixture	8.5±0.1	5.1±0.2	27.9±4.3
LSD0.05	0.327	0.389	8.204

4. Conclusion

In this work, we demonstrated important functions that bacteria exhibit in order to compete, colonize, and establish themselves in the rhizosphere of wheat plants. Results showed that the salt tolerant PGPR *A. brasilense* and *Bt* enhanced physio-chemical attributes of inoculated wheat plants under saline conditions, consequently leading to the alleviation of salinity-induced damages. Bacteria that were used in this work showed an increase in shoot length, root length, shoot dry weight, root dry weight, root area, leaf area, chlorophyll A, B, and A + B (total), proline, peroxidase and lowering in EC. They also demonstrated that root colonization resulted in dehydrogenase reduction of TCC to TPF in the spermoshpere. Moreover, the mixture of *A. brasilense* and *Bt* gave the highest and best results of all the parameters mentioned. This study will continue in the field to assess whether lab-based results translate into more real-world application.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have declared that no competing interests exist.

Statement of ethical approval

The present research work does not contain any studies performed on animals / human subjects by any of the authors.

Statement of informed consent

All authors declared consent for publication and data transparency.

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