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

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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

Posing a great potential of utilizing Plant Growth promoting Rhizobacteria (PGPR) as a promising alternative to alleviate salinity.

Purpose: The aim of the current work is to illustrates the mechanistic basis of PGPRs-triggered salinity tolerance in wheat crops.

Methods: Two experiments were conducted at Agricultural Research Center (ARC), 2021-2022, to evaluate the interaction between wheat plant var.Misr1 and two bacterial strains as PGPR namely, *Azospirillum brasilense* NO40 and *Bacillus thuringiensis* (*Bt*). One experiment were done in the Laboratory and the second was in the greenhouse. In the laboratory, the staining technique utilizing a spermosphere model was employed in order to detect the colonization efficiency of applied bacteria on wheat seedling roots under sterilized and saline conditions.

Results: The date showed the red color of the wheat seedling roots that resulted from the reduction of 2,3,5 triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TPF). Intensity of the red color increased as follow; wheat seedling roots inoculated with mixture of *A. brasilense* and *Bt* > that inoculated with *A. brasilense* > that inoculated with *Bt*. The interaction between wheat seedlings and the PGPR that were used in this work was followed up with transmission electron microscopy (TEM). The greenhouse experiment was conducted on saline soil from Sahl Elhosynia, north of Egypt, to evaluate the effect of these inoculation on growth, photosynthetic pigments and capacity, proline, dry weight of shoots and roots, some antioxidant and rhizosphere enzymatic activities. That data showed increase in all of that parameters in the wheat seedlings that inoculated with mixture of *A, brasilense and Bt* followed by that inoculated with *A. brasilense* alone, then by that inoculated with *Bt*.

In conclusion: our bacterial strains that used in this work as inoculums for wheat plants under saline conditions could alleviate the salt stress in wheat saline soils. The mixture of the two strains *A. brasilense* and *Bt* gave the best results. Thus, we can apply this Mix in open field experiment to confirm this data.

Keyword: wheat, PGPR, salinity, colonization , antioxidant enzymes

Introduction:

. In Egypt, 33% of the cultivated land, which comprises 3% of total land area, is already salinized. As the problem of soil salinity cannot be fixed by agronomical means, the improvement of crop tolerance to salinity and the development of the new techniques are urgently required for agricultural production on salinized land in the near future.

Plant growth promoting rhizobacteria (PGPR) are microbes which inhabit the plant roots and enhance plant development. Since many years ago, researchers have interested in the use of PGPRs to increase the growth and yield of plants. However, the role of these beneficial PGPRs in management of abiotic stresses is acquiring importance in recent years. Indeed, a number of PGPRs have been acknowledged to tolerate salinity stress, posing a great potential utilizing these PGPRs as a promising alternative to alleviate salinity stress in plants. It has been well documented that PGPRs elicit stress tolerance in plants may be due to a variety of direct or indirect mechanisms, including facilitating uptake of water and nutrients, inducing plant anti-oxidative systems, producing phytohormones and osmolytes, and restricting Na^+ uptake by root etc. All these mechanisms are regulated by a complex network of signalling events occurring during the plant-microbe interaction. Unfortunately, still a lot is yet to be explored at physiological, molecular and biochemical level on how PGPRs ameliorate salinity stress in plants.

agriculture

Soil salinity is one of the primary abiotic stresses accountable for loss 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 the secondary-induced salinization (Munns and Tester, 2008). Globally about 3 ha of land are taken out of agricultural production every minute due to continuous land salinization. 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*., 2014), 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. In Egypt, 33% of the cultivated land, which comprises 3% of total land area, is already salinized. On a global scale, the annual losses in agricultural production from salt-affected land are more than US$ 27 billion and rising. Climate changes may lead to even more saline landscapes in many non-irrigated regions since it is accompanied by less rainfall and higher temperatures in most agricultural regions. It will result in a change toward a more arid climate, which is conducive to salt accumulation. Food and Agricultural Organization (FAO), has predicted that by the year 2050, the expanse of 50% of total land mass will be lost due to salinity (Ilangumaran and Smith, 2017). These facts represent a serious threat to sustainable food production and to our natural resources (Ondrasek *et al*., 2009). At the same time, the UN projections suggest that the world population possibly will reach 9.6 billion in 2050 (www.fao.org/economic/esa) raising a crucial demand for boosting agricultural food production up to 70% by then. To achieve this, the improvement of crop tolerance to salinity and the development of the new techniques are urgently required for agricultural production on salinized land in the near future. Mechanisms of PGPRs in mitigating salinity stress in plants: are too far from understanding

Plant growth promoting rhizobacteria (PGPR) elicited stress tolerance in plants may be due to a variety of mechanisms proposed from time to time based on studies done. The major points of these mechanisms are (1) plants treated with PGPR have better root and shoot growth, nutrient uptake, hydration, chlorophyll content, and resistance to diseases; (2) stress tolerance can be explained by nutrient mobilization and biocontrol of phytopathogens in the rhizosphere (3) PGPR produce phytohormones to increase the overall growth and also alter root characteristics (i.e., alteration of root proliferation, metabolism and respiration rate) to facilitate uptake of water and nutrients; (4) PGPRs produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase to decrease the excessive ethylene production in plants caused by salinity stress and thereby eliminate the negative effect of ethylene on roots; (5) PGPRs favor the circulation of plant nutrients in the rhizosphere; (6) PGPRs favor the accumulation of osmo-protectants (i.e., proline, polyamines, glutamate, total free amino acids, etc.) in plants; (7) PGPRs produce exopolysaccharides to bind the toxic Na^+ and restrict Na^+ influx into roots and enhance the uptake of water and nutrients; (8) plants colonized with PGPRs have higher K^+ ion concentration and, in turn, a higher K^+/Na^+ ratio that favor salinity tolerance; (9) Volatile organic compounds (VOCs) can trigger induction of high affinity K^+ transporter (HKT1) in shoots and reduction of HKT1 in roots, limiting Na^+ entry into roots and facilitating shoot-to-root Na^+ recirculation; 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*., 2016; Sáenz-Mata *et al*., 2016; Ilangumaran and Smith, 2017). These mechanisms are regulated by a complex network of signaling events occurring during the plant-microbe interaction (Smith *et al*., 2017), and 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) the morphological, biochemical and physiological characteristics of the interactions between plants and PGPRs under salinity; (2) the information on the metabolically and nutritionally active roles of PGPRs in plants under salinity; (3) the critical signals or chemicals mediating the communications between plants and PGPRs under salinity; (4) the molecular mechanisms by which PGPRs increase plant resistance to salinity and (5) the assessment of key environmental factors that influence the efficacy of PGPRs-mediated amelioration salinity stress in plant.

Material and Methods:

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

1 Bacteria and inoculant preparation:

Two bacterial strains namely, *Azospirillum brasilense* NO40 and *Bacillus thuringiensis* (*Bt*) were obtained by 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. The bacteria were cultured on nutrient broth media

according to (Atlas 2010). inoculum of each strain was freshly prepared Broth culture (10^9 cells/ml). These bacterial strains used in the treatments Under saline (100 Mm NaCl) semi agar medium of Watanabe (Watanabe, 1979); T1 = *Azospirillum brasilense* NO40. T2= *Bacillus thuringiensis* (*Bt*), T3= Mixture of both strains and T4= Control without bacteria

2- Spermosphere model experiment:

The staining technique utilizing a spermosphere model was employed in order to detect the colonization efficiency of applied bacteria on wheat seedlings roots under sterilized conditions. This model was elaborated at the Center of Pédologie, Nancy, France (Omar *et al*. 1989). It consists of a long tube supplied with a short lateral tube. The lateral tube can act as a continuous trap for CO_2 released by the small seedling as it contains 2 ml of 1N NaOH fig. (1). The wheat seeds were washed under distilled water and surface was sterilized using ethanol alcohol for 30 second then rinsed thoroughly under aseptic condition in sterile distilled water following by soaking in 20% sodium hypochlorite for 15 minutes. Finally washed three times in sterile distilled water and put them in a Petri dish on a filter paper 10 minutes to dry until seed culture. The sterilized seeds were aseptically germinated on Petri dish contains Watanabe medium devoid of any carbon sources and any growth regulators. After seeds had been germinated, it transplanted into the long tube that contains 7 ml of Watanabe semi solid medium devoid of any carbon sources. When coleoptiles were 1cm high, the spermosphere models were either inoculated by bacteria or not as control, followed by an incubation period (7 days) to be sure that the colonization occurred. In the last day of the incubation period, the roots of wheat in spermosphere model treated with 2,3,5 triphenyl tetrazolium chloride (2 ml TTC solution /sample) for 3 h to detect the colonization by formation of triphenyl formazan (TPF) which indicated by change the root color to red color.

3- Greenhouse experiment:

Surface soil samples (0-30 cm) were collected from Sahel El-Hosainiya at north east of the Delta region, Sharqueya Governorate, Egypt, to represent saline sodic. The samples were air dried, crushed, sieved to pass through a 2.0 mm sieve and analyzed for their physical and chemical properties according to the standard methods proceeded by (Page *et al.* 1982). Physical and chemical characteristics of the used soil are outlined in table 1

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**

Table 1. Physical and chemical characteristics of the used soils.

Seeds of bread wheat, a carrier-based inoculum of each strain was freshly prepared. Broth culture (10^9 cells/ml) was mixed with sterilized vermiculite contained 10% Irish peat up to 60% of water holding capacity and packed into polyethylene bags to be used as seed inoculants. Thirty minutes before sowing, wheat seeds were coated with different carrier-based inocula using a sucrose solution as adhesive. Coated seeds were air-dried for 15 min under shade and sown immediately. The experiment was conducted under greenhouse condition using saline soil (the pots 20 cm - 25 cm were filled with 3 kg saline soil) and bacterial inoculation. Before sowing, wheat seeds were primed with *Bt* and *Azospirillum* individually or in mixture. Seeds without bacterial inoculation was assigned as control treatment. The treatments prepared in three replicates. Wheat seedlings were sampled at 30 days after sowing as a vegetative stage in order 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 the analyses

4- For each treatment, the leaves and roots image (fig 4) were captured with 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 2003). The leaf area and root area were measured using the number of pixels (Baker *et al*. 1996)**.**

5- 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 TEM lab FA-CURP, Fac. OF Agri., Cairo Univ. Research Park.

6- The photosynthetic pigments chlorophyll a, b and total were determined quantitatively as described by (Arnon, 1949). A known fresh weight of leaves was homogenized in pure methanol for 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 666 and 653 nm, taking into consideration the dilution of the sample. The concentrations of pigment fractions (chlorophyll a, chlorophyll b and total chlorophyll) were calculated as mg/g fresh weight by using equations of (Lichtenthaler and Wellburn, 1983).

7- Free proline was determined according to the method described by (Bates *et al*. 1973). A 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 ninhydrine 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.

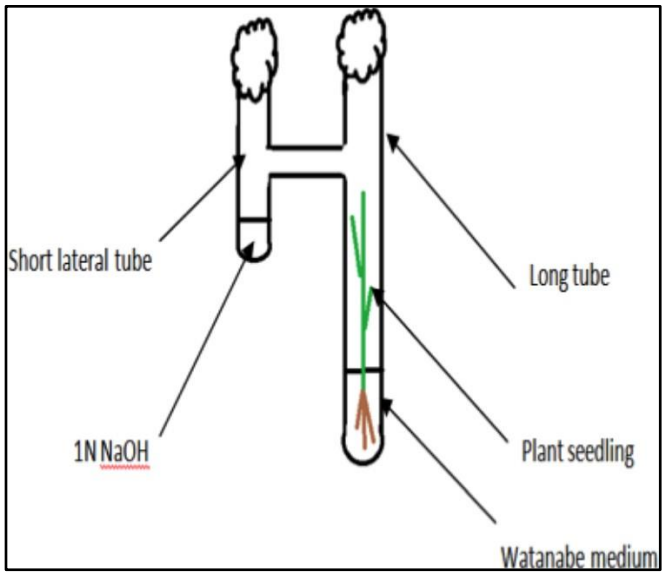
8- Rhizosphere analysis

- Rhizosphere soil pH was measured by taking 20 g of the air dried soils in 50 ml distilled water (1: 2.5) into covered beakers with shaking at regular intervals for about 1 hour. Then, the pH was detected by immersing a glass electrode in the suspension.

- The 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, then the suspension was left for 24 hours before filtrations to measure the soil EC in the filtrate using a glass electrode.

- The dehydrogenase (EC 1.1) activity in the rhizosphere soil was carried out according to (Casida *et al*. 1964). Two gm. of the soil was transferred to test tubes, then 2 ml aliquots of 0.5 % of 2,3,5 triphenyl tetrazolium chloride (TTC) solution was added and mixed thoroughly. Tubes were then sealed with rubber silicon stopper and incubated at 30 ^{o}C for 24 h in dark. Thereafter, 8 ml of pure methanol were added to each tube, shake and left for two hours in dark with shaking at regular intervals to extract the formed pink colored triphenyl formazan (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 wave length 485 nm using a spectro-colorimeter. Concentrations of formazan were calculated from standard curve and presented in µg TPF/g dry soil / 24 h. A blank treatment included all additives without soil sample should be considered and subtracted.

Fig 1: Spermosher model



- Peroxidase (EC 1.11.1.7) was assayed as described by (Kumar and Khan, 1982). 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 H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C, after that 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.

Results and discussion

Soil salinity has emerged as a major obstacle to meet world food demands. Halotolerant plant growth promoting rhizobacteria (PGPR) are potential bioinoculants to enhance crop productivity in saline agriculture (Aniqa *et al*., 2020).

The present study aimed to assess to what extent the halotolerant PGPR could improve individually or synergistically the wheat (cultivars Misr 1) growth and yield under saline conditions. The inoculants strain *Azospirillum brasilense* (*A. brasilense*) (NO40) and *Bacillus thuringiensis* (*Bt*) are used in the present study; they were isolated from hyper saline soil and they are able to survive up to 1,800 mM NaCl *in* the basal media. A green house experiment was conducted to evaluate the effect of these strains as inoculums on wheat growth under saline conditions. Photosynthetic pigments, free proline, dry weight of shoots and roots, some antioxidant and rhizosphere enzymatic activities are determined.

1- Seed germination is an initial and critical stage in the plant's life cycle which outline the other stages of plant development and yield production. During such stage the bacterial colonization of the roots was started in response to their exudates. So that, the study of microbial colonization on roots using staining technique under salt stress during initial stage of plant growth is being useful and could predict efficiency of microorganisms for alleviating such stress. The colonization of bacterial strains on wheat seedlings (10 days old) under aseptic and saline conditions (fig 2). Fig 2 showed the microbial colonization in the spermospher of wheat seedling. The assays here based on the reduction of 2,3,5 - triphenyle tetrazolium chloride (TTC) to the red coloured formazan (TPF). The seedlings inoculated with mixture of *A. brasilense* & *Bt* showed more density red color > that inoculated with *A. brasilense* > that inoculated with *Bt* > control (were no inoculation). The effect of inoculation causes the interactions between some complex mechanisms such as hormonal effect, 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 1993; Han and Lee 2005). This notion is further supported by the findings of (Ashraf *et al*., 2004) that increased the population density of PGPR in the root zone and could decrease the Na available for plant uptake, thus helping to alleviate salt stress in plants.

2- Transmission electron microscopy (TEM):

The interaction between wheat seedlings and the PGPR that were used in this work was followed up with transmission electron microscopy (TEM) Figure 3 (A, B, C and D). In the current work the TEM analysis showed no any distortion in cell wall in the control (fig 3 A). While in the seedlings that inoculated with salt tolerant PGPR *A. brasilense*, *Bt* and mixture of both of them (fig 3 B, C and D respectively) showed clear differences from the control. These data agreed with that obtained by (Zhen *et al*., 2022). The TEM images of inoculated wheat roots showed differences in the wheat roots before and after inoculation with salt tolerant PGPR. They attribute

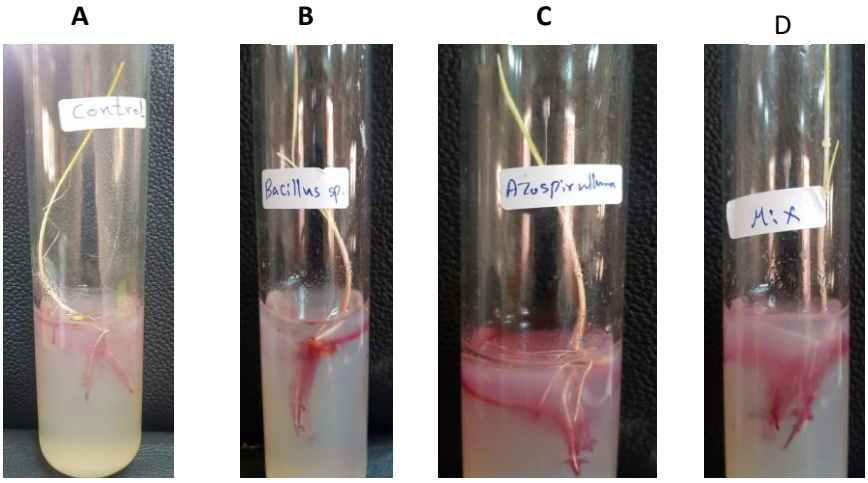


Fig 2: Showing the microbial colonization in the spermosphere of wheat seedling under salinity conditions (fig 5 A is the control, fig 5 B is the *bacillus thuringiensis* (*Bt*) , fig 5 C is the *Azospirillum (A) brasilense* and Fig 5 D is mixsture between *Bt* and *A. brasilense*)

these differences to the connection of the salt tolerant PGPR with root of wheat. Su-Jung and Kremer (2005) had also used TEM to deduce the relation of PGPR *B. megaterium* GP4 with ivy leaf morningglory. TEM showed considerable alterations of root cells including vesiculation, partial cell wall degradation and cytoplasm disorganization. They attribute that to; when bacterial attachment to plant surfaces begins with attraction by seedling root exudates. This lead to higher bacterial colonization of roots.

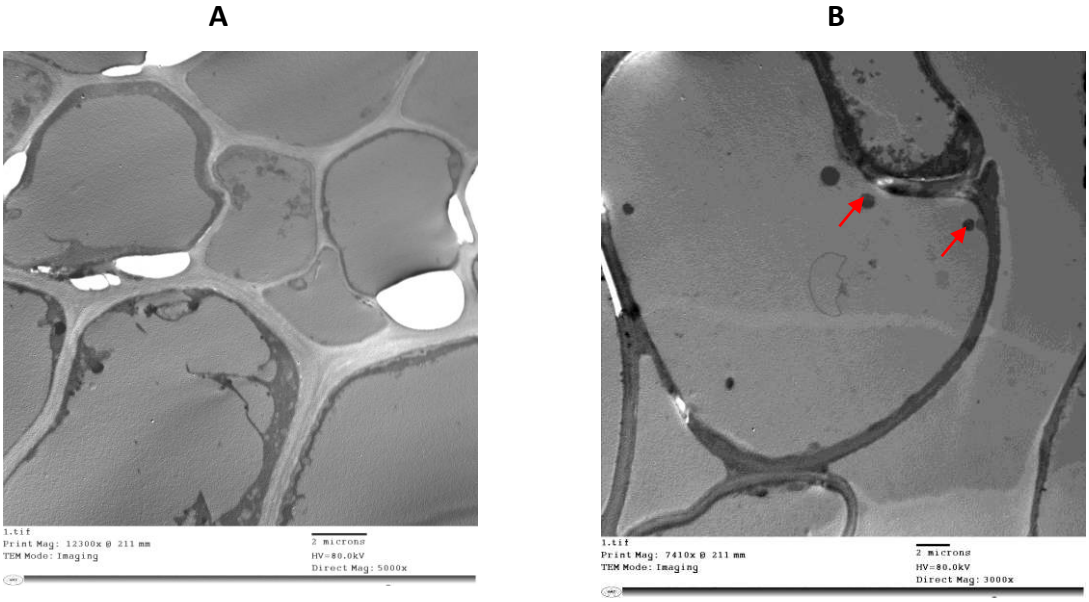
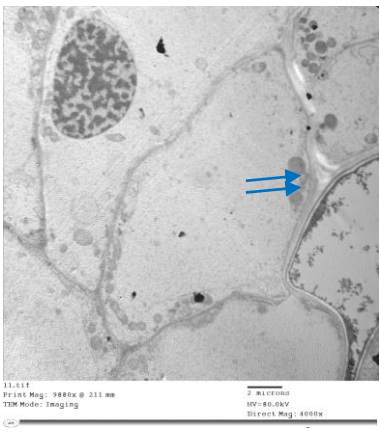
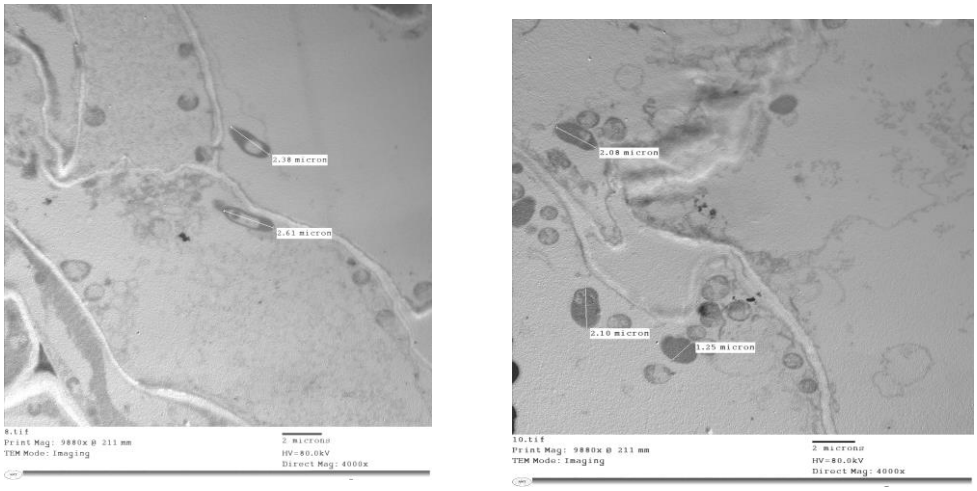


Fig 3: The figure showing the colonization of the bacteria with wheat seedlings through transmission electron microscopy (TEM). **A**: is the control which not inoculated with bacteria. **B**: the red arrows refer to the colonized bacteria *A. brasilense* with wheat seedlings. **C:** the blue arrows refer to the colonized bacteria *Bt* with wheat seedlings. **D:** showed the colonized of *Bt* and *A. brasilense* with wheat seedlings that inoculated with mixture from *Bt* and *A. brasilense*

C



D



3- **Greenhouse experiment**

Wheat seedlings were sampled at 30 days after sowing as a vegetative stage in order to measure morphological and biochemical growth criteria. The morphology of seedlings was described as Shoot length, Root length, shoot dry weight, leaf and root area, and dark green color index (DGCI). For each treatment, the leaves and roots image were captured. The images were processed to extract values of (red, green, blue light brightining) RGB. The dark green color index (DGCI) was calculated from hue, saturation, and brightness (HSB) levels. The leaf area and root area were measured using the number of pixels. DGCI were used by (Abdelaziz and Moha, 2020). They reported that nitrogen (N) fertilization is crucial for maximizing crop yield, but optimizing the amount of N to apply for crop must also taken in consideration for the potential impact on the environment. Thus, the DGCI technology determines the greenness of plant leaves to assess plant N status.

A

Fig 4 A: Control (wheat seedlings without inoculation)

B

Fig 4 (B): Wheat seedlings inoculated with PGPR *A. brasilense*

C

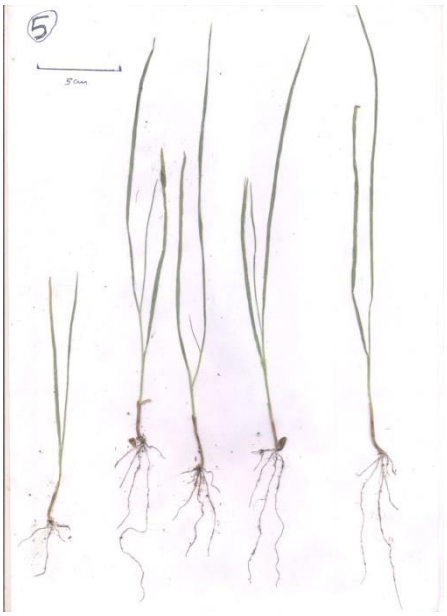


Fig 4 (C): Wheat seedlings inoculated with PGPR *Bt*

D

Fig 4 (D): Wheat seedlings inoculated with PGPR mixture of *Bt & A. brasilense*

Fig 4: The captured images of wheat seedlings inoculated with bacteria under saline conditions. A: control, where wheat are not inoculated with any bacteria B: wheat inoculated with *A brasilense* NO40. C: wheat inoculated with *Bt*. D: wheat inoculated with mixture of both of the two bacterial strains NO40 & *Bt*.

3-A Shoot and root features

Shoot and roots of wheat seedlings showed how much the PGPR inoculation have affected it. The obtained data from fig 4 have been deduced in table 2. Shoot length, root length, shoot dry weight, root dry weight and root area of the wheat seedlings that inoculated with salt tolerant PGPR *A. brasilense*, *Bt* and mixture of *A. brasilense* & *Bt*. That inoculated with mixture of *A. brasilense* & *Bt* gave more increased values > *A. brasilense* > *Bt* > control. These data agreed with that released by Zhen *et al*., (2022). wheat seedlings under stress 300 mM NaCl were inoculated with salt tolerant PGPR strains showed increase in plant height, root length, dry weight and fresh weight over the control (no inoculation). Moreover, they revealed that the mixed inoculation gave the best results. They were attribute that to the complementary strengths of the strains

Parameters / Treatments	**Shoot length (cm)**	**Root length(cm)**	**Shoot dry weight (mg/plant)**	**Root dry weight (mg/plant)**	**Root Area (cm^2)**
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. brasilinse	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

Table 2: Some criteria of shoot and root of wheat seedlings inoculated with salt tolerant bacteria under saline conditions.

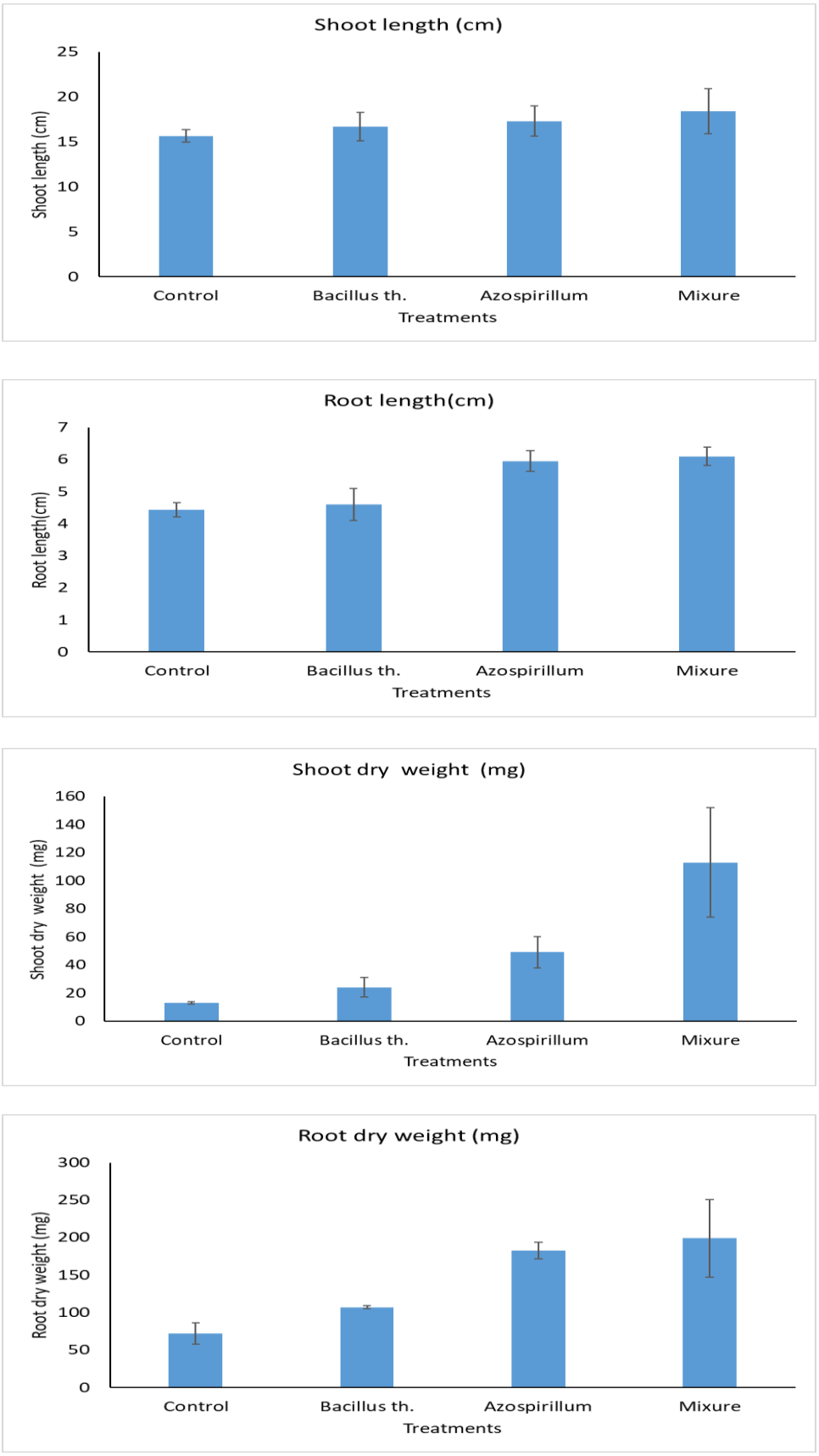


Fig 5: Some criteria of shoot and root of wheat seedlings inoculated with salt tolerant bacteria under saline conditions.

3 B Plant Leaves characterization

Plant leaf is actually called 'the kitchen of the plant. This is because they are the main organ responsible for photosynthesis, through which the plant produce its energy and food beside they help the plant breathe and storage of food. They obtain their green color due to the presence of chlorophyll. Salinity is amongst major limiting factors affecting crop growth and yield through their negative impacts on their leaves. Thus, some characters of wheat seedling leaves that inoculated with our bacterial strains under saline conditions are represented in Table 3 & 4.

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

Table 3: Character of leaves of wheat seedling inoculated with Salt tolerant PGPR *A. brasilense*, *Bt* and mixture of both of them 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

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

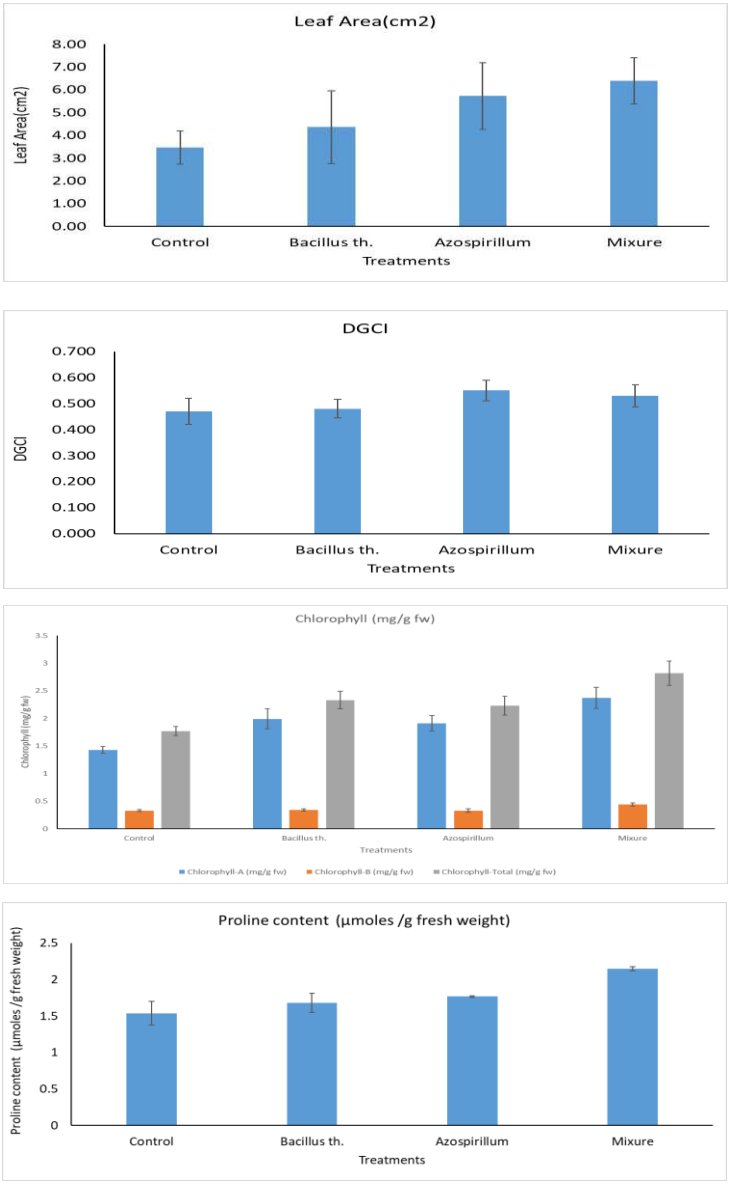


Fig 6: Character of leaves of wheat seedlings inoculated with Salt tolerant PGPR *A. brasilense*, *Bt* and mixture of both of them under saline conditions.

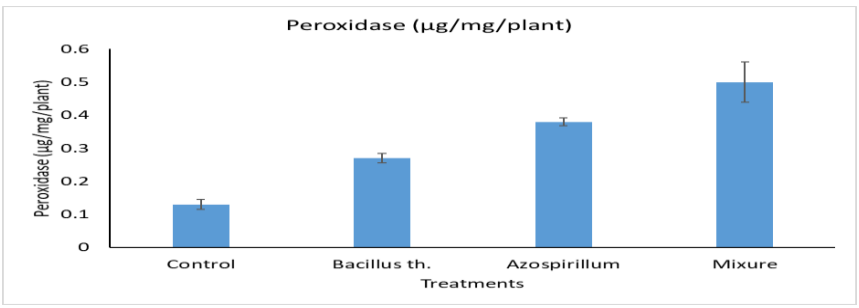


Fig 7: Peroxidase activity in leaves of wheat seedling inoculated with Salt tolerant PGPR *A. brasilense*, *Bt* and mixture of both of them under saline conditions.

3- B.1 Leaf area

As shown in fig 6, the leaf area of the wheat seedlings that inoculated with mixture of *A. brasilense* and *Bt* showed the highest value followed by that inoculated with *A. brasilense* then by that inoculated with *Bt*. This results were agreed with that obtained by (Gholami *et al*., 2009). They revealed that the inoculation with bacterial strains had significant effect on leaf surface area under sterile or non sterile soils. They also, showed that the wheat seedlings that inoculated with bacteria *A. brasilense* increased up to 65% over the non inoculated (control).

3- B.2 DGCI and chlorophyll

The data in fig 6 and table 3 showed that The dark green color index (DGCI) of both seedlings that were inoculated with bacterial mixture or with *A. brasilense* were nearly the same value followed by that inoculated with *Bt*. The fig 6 and table 3 also showed the chlorophyll measurements. Wheat seedlings that inoculated with mix. of *A. brasilense* and *Bt* showed the best results of chlorophyll content. Its chlorophyll A, B and A+B were about 65% over the control and nearly 30% over that inoculated with *A. brasilense* or *Bt*. In the same research area, Nathalie *et al*., (2020), released the data that inoculation with PGPR improved resistance to salinity by increasing their growth parameters. Where, chlorophyll and proline contents were increased in the inoculated plants under salt stress.

The literatures proved a linkage between DGCI & chlorophyll content and between nitrogen (N) content of plant leaves. Jennifer *et al*., (2016) proved by experiments that leaf color charts (LCCs) were highly correlated to chlorophyll content and chlorophyll meter 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 (2020), 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 were obtained. The finding by (El Azazy, 2018), also, revealed that leaf color image analysis technique could be used for determination of nitrogen content of plant seedlings chlorophyll of leaves; the main cause of greenish of leaves. That is proved to be highly correlated with nitrogen. That is essential for chlorophyll formation and the basic color component (RGB). Consequently it is important to indexes NRGB and DGCI. Thus, for plant seedlings, the RGB, values of a color leaf image could be used to estimate the leaf chlorophyll. In wheat plant (Bojovic and Markovic, 2009) a linear correlation between nitrogen content and chlorophyll (a), chlorophyll (B) and total chlorophyll were obtained.

3- B.3 Proline

Fig 6 and table 3 showed an increase in the values of proline in the salt stressed wheat seedlings that inoculated with mixture of the salt tolerant *A. brasilense* & *Bt* by 21% more than that only inoculated with *A. brasilense* or with *Bt* and by 40% over the control. This finding agreed with (Rameesha *et al*., 2019). In salt stress, PGPR produce compatible osmolytes to help plants promote their growth. During stress

condition, proline are accumulated in plants. Proline is the key of osmolytes, that formed in plant by the hydrolysis of proteins to reduce osmotic stress (Krasensky and Jonak, 2012). Reports declered by (Shamsul *et al*., 2012) 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 exposed to various stress conditions. Besides acting as an excellent osmolyte, proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule. 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 turgor or 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. Reports indicate enhanced stress tolerance

3- B.4 Peroxidase

The results of fig7 and table 4 showed the high increase in peroxidase activity in the wheat seedlings that inoculated with mixture of salt tolerant *A. brasilense* & *Bt* under salt stress. It raised to 0.5 ug /mg /plant while the control was 0.13 ug /mg / plant. i.e. showed ~ very high percentage over the control. It was also higher than that inoculated with only *A. brasilense* (0.38 ug /mg /plant) and than that inoculated only with *Bt* (0.27 ug /mg /plant). these results were agreed with that obtained by (Aniqa *et al*., 2020). They found that peroxidase was higher in wheat inoculated with salt tolerant PGPR under saline conditions than control (not inoculated). Yulmira, (2015) revealed that the increased activity of peroxidase in shallots seedling that were inoculated with PGPR ; was an indicator of induced resistance to pathogens. Thus, peroxidase is an enzyme that plays a role in the resistance of plants to biotic or abiotic stresses.

3 C Rhizosphere

Salinity-induced changes in rhizosphere of wheat plants inoculated with bacteria including pH, EC and microbial activity

3- C.1 pH and EC

Table 5 and fig 8 showed the values of the pH and EC in the rhizosphere soil of wheat seedlings that inoculated with bacteria *A. brasilense* and *Bt* and a mixture of both. The pH of the soil before planting (table 1) was 8.13, which it is nearly the same of the pH of the soil after planting for all the inoculated seedlings and the control. Table 1 showed that the most component of the used soil was the clay (65.52 %). The charged surfaces of clay make them more resistant to pH changes.

Soil EC is often used as a measure of salinity. Salinity is an indication of the amount of salts in the soil. The saline soils are those that have an EC higher 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 in the soil. Sodium can be leached from the soil,

thereby reducing the EC levels, through the addition of elemental sulphur or gypsum (Marno Fourie, Trace & Save).

Table 5 and fig 8 showed the measurements of EC values in the rhiyzosphere soil of the wheat seedlings that inoculated with the salt tolerant *A. brasiliense*, *Bt* and a mixture of both of them under saline conditions. The EC of the soil rhizosphere that inoculated with mixture of *A. brasilense* and *Bt* was 5.1 < that inoculated with *A. brasiliense* 6.4 < that inoculated with *Bt* 6.75. The all values were lower than control 7.59. The EC of that soil before planting was 9.27 (table 1). Thus the data showed lowering in the salinity of the soil rhizosphere as a result of bacterial colonization as shown in (fig 2). This finding agreed with (Stefan, 2020), who revealed that *A. brasilense* and *Bacillus* were among other type of bacteria that improved plant growth and enhanced the tolerance to sodium chloride in wheat. Wang et al (2020) found also 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.

3- C.2 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, dehydrogenase activity (DHA) of soil is supposed to reflect microbial activity (Skujins 1976). DHA estimation is based on use of redox-sensitive tetrazolium dye which is reduced to insoluble formazan inside cells due to respiratory activity. Table 5 and fig 8 showed the dehydrogenase activity of the rhizospher soil of wheat seedlings inoculated with salt tolerant PGPR *A. brasilense*, *Bt* and mixture of both of them under saline conditions. The mixture of *A. brasilense* & *Bt* gave activity 27.9 mg TPF g^{-1} soil day^{-1} which were higher> than *A. brasilense* 23.4 mg TPF g^{-1} soil day^{-1}> *Bt* 20.5 mg TPF g^{-1} soil day^{-1}> control 20.3 mg TPF g^{-1} soil day^{-1}

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

Table 5: measurements of pH, EC, and dehydrogenase activity of rhizosphere soil of wheat seedlings inoculated with PGPR under saline conditions.

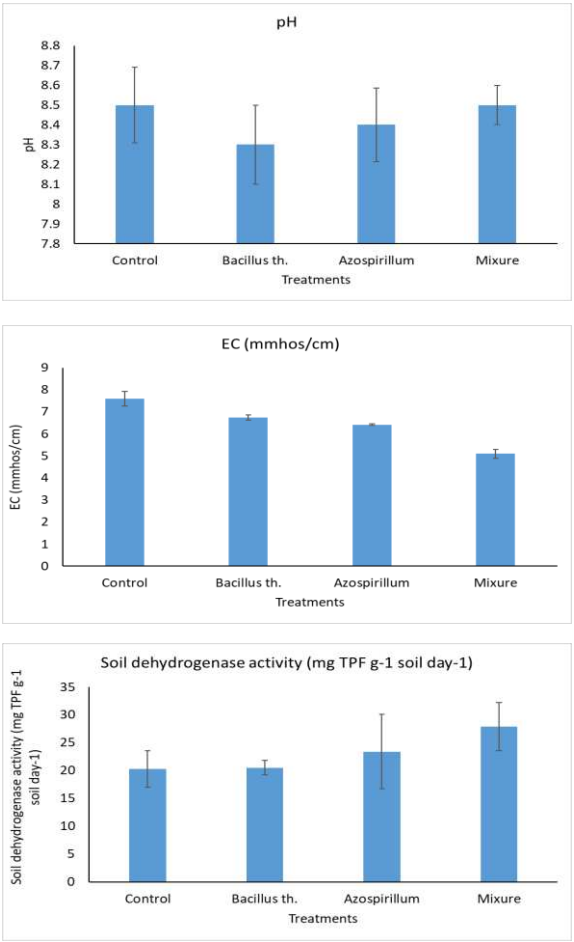


Fig 8: Rhizosphere soil pH, EC, and dehydrogenase activity of wheat seedlings inoculated with PGPR under saline conditions.

Conclusion

In this work, some important functions that bacteria exhibit in order to compete, colonize, and establish themselves in the rhizosphere of wheat plants. Outcomes of the work was showed that the salt tolerant PGPR *Azospirillum* (*A*) *brasilense* and *Bacillus thurigiensis* (*Bt*) employed beneficial impact on physio-chemical attributes of inoculated wheat plants under saline conditions. Consequently, leading toward alleviation of salinity induced damages. our bacteria that used in this work showed 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. It showed their ability of root colonization through the dehydrogenase reduction effect of TCC to TPF in the spermoshpere model (fig 2), and the soil dehydrogenase activity in mg /TPF g^{-1} soil day^{-1} and through the TEM. Moreover, the mixture of *A. brasilense* and *Bt* gave the highest and best results of all the parameters mentioned. This convoy us to do a great field experiment using a mixture of salt stress *A. brasilense* and *Bt* in inoculation of wheat in saline soils.

Declarations

ETHICAL STATEMENT:

Ethics approval and consent to participate:

This study did not violate ethics, and all participants agreed to publish the paper

Consent for publication:

All authors declared that consent for publication

Availability of data and materials:

Data transparency.

Competing interests:

The authors declared that they have no conflict of interests.

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we applied APS waiver as grant

Authors' contributions:

Nabil omar, designed and supervised the experiment and took overall responsibility for the research and its funding. Nahed ibrahim, shared the discussion of the data and she wrote the manuscript. Yasser, Hend, Soad and Monira who are set up the experiments in the lab and green house and they did the soil and plant data analysis. The final manuscript was read and approved by all the authors.

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