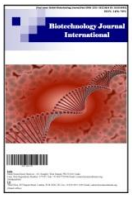

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Microencapsulation: Toward the Reduction of the Salinity Stress Effect on Wheat Plants Using NPK Rhizobacteria

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MMS and HAA conceived the overall study. Authors MMB, IMG and HAA performed the field experiment and phenotyping assays. Authors MMS and HAA performed microbiological work including and bioassays. Authors MMS and HAA wrote the final manuscript.

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ABSTRACT

Salinity is one of the most vicious environmental factors controlling the productivity of crop plants as most of the crop plants are sensitive to salinity affected by high concentrations of salts in the soil. The objective of this study was to evaluate the influence of the inoculation with the encapsulated and liquid culture of three halo-tolerant plants growth-promoting rhizobacteria (PGPR) strains of

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Paenibacillus polymyxa MSRH5, *Bacillus nakamurai* MSRH1 and *Bacillus pacificus* MSR H3 on the growth and yield of wheat (*Triticum aestivum L*.). The three strains MSRH1, MSRH3 and MSR H5 were characterized as salt-tolerant bacteria. *P. polymyxa* MSRH5 had nitrogen fixation ability while *B. nakamurai* MSRH1 and *B. pacificus* MSRH3 were able to solubilize phosphate and K respectively. All strains can produce indole acidic acid (IAA) and exopolysaccharides (EPS) under saline conditions. Encapsulated beads were observed under Scanning Electron Microscope (SEM). Colonization of encapsulated bacteria on the root of the wheat plant was studied by Transmission Electron Microscopy (TEM). Under soil salinity conditions in two consecutive field tries, results cleared that strains in two forms succeed to colonize the plant root, the reduction in shoot proline was 35.8% with capsules inoculation as well as improved relative water content (%) to 60.57% and improved the electrolyte leakage recorded 18.1% respectively compared to control. Generally, halo-tolerant PGPR inoculation increased dehydrogenase activity, acidic and alkaline phosphatase activities compared to control, inoculation with capsules exhibited a reduction in catalase enzymes 46.00%, 37.5% in ascorbate peroxidase and 40% in superoxide dismutase respectively in shoots of the wheat plant. There is a significant increase in all yield parameters, the highest plant height 115.8 cm, spike length 21 cm and 1000 grains 71.3 g respectively recorded with capsules inoculation, it had considerable effects on the content of N, P, K and Na in shoots of wheat plants and reduced the value of Na/K ratio in all treatments inoculated compared to un-inoculated wheat plant.

Keywords: Halo-tolerant bacteria; PGPR; alginate beads; wheat; colonization.

1. INTRODUCTION

Several environmental stresses such as salinity, drought, flooding, heat, cold, and heavy metals harmfully affect the growth, development and productivity of crop plants [1]. Salinity is a major abiotic reason that limits agricultural productivity [2]. Most salty land has to get up from natural causes by the accumulation of salts over extensive periods in arid and semiarid zones [3]. Sodium chloride is the most soluble and abundant salt released; apart from natural salinity, a significant proportion of recently cultivated agricultural land has become saline owing to human activities such as unsuitable agricultural functions [4]. Salts in saline soil happened when the soluble ions in the soil are increasing. Thus, the salt level increases on the surface of soil caused by the appearance of salty soil. However, the dangerous effects of salts depend on many factors e.g. plant type, climatic conditions, and soil-water regulation. The world population is increasing and the world salt-affected area will not be cultivable which would cause famine conditions [5]. The plant growth under salinity decreased because of nutrient disturbances, affecting the accessibility, transport and partitioning of nutrients because this attributed to the competition of Na^+ and Cl^- with nutrients such as K^+, Ca^{2+} and No^{-3} [6]. Thus, the development of salt-tolerant plants is a much-chosen scientific goal. However, efforts have only happened with limited success, and only a few most important genetic determinants of salt tolerance have been identified [7,8].

Wheat is one of the oldest and most important cereal crops. Thousands of varieties are known in common wheat (*Triticum aestivum L*.) which is an important cereal crop in Egypt. It is also the main cash crops for the farmers. Thus; their role in strengthening the economy of the country may not be neglected. For all essential crops, average yields are only a part everywhere between 20% and 50%-of record yields; these losses are mostly caused by high soil salinity and environmental conditions which will worsen in many areas because of global climate change. A wide sort of differences and moderation strategies are required to cope with such effects. Effectual resource management and crop-livestock improvement for evolving better breeds can assist to overcome salinity stress [9]. To start upon this situation**,** along with the old propagation and genetic engineering of plant for salt tolerance, using of plant growth-promoting rhizobacteria (PGPRs) can be practical as an important strategy to improve cultivation in saline soils [10].

The soil in Shal El-Hossynia regions is high salinity conditions, saline (EC > 4 dSm^{-1}) and salt-affected soil is a major environmental issue as it limits plant growth and development, causing loss of productivity [11]. Plant-growth-promoting rhizobacteria (PGPR) are a group of bacteria which colonize the plant roots and increase plant growth either directly or indirectly [12]. PGPRs promote plant growth by changing the selectivity of Na^+, K^+, and Ca^{2+} and tolerate a higher Na+/K+ ratio in plants under salt stress

[13]. Besides that, it significantly enhances our understanding of PGPR-mediated salinity tolerance in host plant and hard work is to complete understanding of the tolerance mechanism at the gene expression level. There are various PGPRs-encouraged changes in plants, and growth promotion possibly results due to a complex combination of various PGPRs-induced mechanisms that affect both plant development as well as plant nutrition [14]. Using mineral nitrogen, phosphors [15] and potassium [16] as fertilizers add to solving the challenge for the world is facing, feeding the human population. High yield production of agriculture was accompanied by an enormous increase in the application of nitrogen fertilizer, so we use nitrogen-fixing bacteria, potassium and phosphates solubilization bacteria as bio-fertilizer [17].

Inoculation methods with PGPR serve to process of colonization, activity and stability of the bacterial cells in the rhizosphere of the plants [18,19]. Encapsulation of living cells is a fine-established technology abroad and an increasing range of different applications [20]. The encapsulated bacteria is considered one of the methods for inoculation, it the feature, which has supported to be more efficient than the liquid form, to their quality of providing defence and stability to the bacterial cells, allowing them to survive for longer in the rhizosphere of the cells [21]. In this regard, capsules supplemented with humic acids, a report providing chemical stability and availability of C and N, generating a greater number of bacterial cells [22]. Thus, the objective of the present work was to alleviate the salinity stress on the growth and yield of wheat (*Triticum aestivum L.*) plant by inoculation with encapsulated NPK rhizobacteria and liquid culture and assist the performance in two seasons at Sahl El-Hossynia filed station.

2. MATERIALS AND METHODS

2.1 Microbial Inoculants

Bacterial strains *Paenibacillus polymyxa* MSRH5, *Bacillus nakamurai* MSRH1 and *Bacillus pacificus* MSRH3 were isolated and identified previously by Abo-Koura et al. [17]. The bacterial strain routinely grown in NB media according to Difco [23] for 48 hr at 28°C. For inoculum, the bacterial broth was grown and cell pellets were collected by centrifuging at 6000 rpm for 10 minutes after the cells were washed in sterile distilled water and re-suspended in 400 ml phosphate buffer pH 7 (10^7cfu /ml).

2.2 Salt Tolerance Assays for PGPRs

PGPRs strains MSR H5, MSR H1and MSR H3 were tested for their tolerance to various concentration of sodium chloride, bacteria were re-cultivated in nutrient broth (NB) [23] supplemented with 0,10,15,20,25and 30gL^{-1}NaCl for 24–72 hours and incubated on rotary shaker (150 rpm) at 28°C. Bacterial growth was determined as OD at 660 nm; maximum salt tolerance was tested on to halophilic agar medium plates (containing 60 g L^{-1}NaCl) by spreading 0.1 ml of each bacterium on nutrient agar (NA) as described by Akhtar et al. [24].

2.3 Bioassays for Plant Growth-promoting Characters under Salinity Stress

All of three bacterial strains were grown in the nitrogen-free medium [25] with selected two concentration 0, 25 and 30g NaCl g L^{-1} and incubated at 28°C for 72 hours, to determine the N_2-activity, nitrogenase activity was measured according to Hardy et al. [26] using gas chromatograph according to Somasegaran and Hoben [27]. For phosphate and potassium solubilizing, spot inoculation of a single bacterial colony on the centre of plates with Pikovskaya medium (PVK) [28] and modified Aleksandrov medium [29] supplemented with 0, 25 and 30 g NaCl g L^{-1} respectively. The inoculated plates were incubated at 30±5°C for 48–96h and clearance zone were observed**.** For IAA production, each bacterium was grown in nutrient broth medium supplemented with 1g L^{-1} tryptophan plus 0, 25 and 30 g NaCl gL^{-1} then incubated at 30°C on a shaker at 200 rpm for 72h and IAA production was determined as described by Damery and Alexander [30].

Exopolysaccharides (EPS) production was determined by the assay: flasks 250 ml counting 100 mL of a medium nutrient broth plus different NaCl concentrations (0, 25, 30 NaCl gL^{-1}) and inoculated with each bacterial culture (one colony) and incubated at 160 rpm shaker for 48 h at 28°C, exopolysaccharides production was determined using the method described by Gilickmann and Dessaux [31].

2.4 Preparation of Capsules NPK

The constitutive antagonistic effect was done between three bacteria in Petri dishes as described by Frederiq [32]. For preparation of

capsules, each bacterial strain from MSRH5, MSRH1and MSRH3 earlier grown in 100 mL nutrient broth medium at 28°C for 48h, 33 ml of each strain was taken and mixed with 2% of sodium alginate (ALGOGGL 3001, SG 30- 60, Degussa, France) plus humic acid for 25 min at 350 rpm on a magnetic stirrer (IKA® Modelo C-MAG). Capsules were made by drop formation with a transparent syringe 5 mL capacity, with which the alginate mixture was taken with bacterial culture and drops formed were deposited in a sterile 0.1 M of $CaCl_2$ solution. After that capsules were taken away from $CaCl_2$ solution and washed three times with distilled water. We adjusted the diameter of microcapsules to 4 mm and kept it in sterile 0.85% NaCl solution till use [33]. Scanning electron microscopy (SEM, QUANTA FEG 250) was used to investigate three bacterial cells inside the capsules at the National Research Center, Cairo, Egypt according to the manufacturer protocols.

2.5 Preparation of Inoculums

MSRH5, MSRH1and MSRH3 were grown individually in nutrient broth medium [23] for 48 hours at 28°C to exponential phase ($6x10^7$, $5x10^6$ and $5x10^6$cfu ml^{-1}, respectively). Two forms of bacterial inoculums were used, either in capsules, beads were mixed with seeds as described by Bashan et al. [34] or liquid form, were carried sterilized peat and sterilized sugar solution (10%) with a ratio of 4:5:1 v/v [35] using Arabic gum as cement agent to form slurry. The slurry was then mixed with the seed until it was evenly coated then coated seeds were lifted to dry in the shed for 60 minutes and planted in soil.

2.6 Colonization of the Wheat by Encapsulated PGPR

Wheat seed surface was sterilized in 2% calcium hypochlorite solution for 2 hr under agitation, rinsed thoroughly under aseptic conditions in sterile water and soaked in 1:1 (v/v) H_2O_2 for 20 min. Afterword, the sterilized seeds were kept for germination for 2 days in Petri dishes at 28°C under the aseptic condition to germinate and put it in specially gnotobiotic conditions [36]. The germinated seeds were transferred to sterile tubes containing mineral solution and capsules bacteria were added. The tubes were incubated in a growth chamber at 16-18 h day light/dark cycle and a temperature of 23 /18°C for 7 days. Then, the colonization of wheat roots by capsules was observed by transmission electron microscopy JEOL (JEM-1400 TEM) according to the protocol method described by Bozzola and Russell [37].

2.7 Experimental Design and Treatments

Experiments in two consecutive years were conducted in the field in clay soil at Sahl El-Hossynia Agric. Res. Station Farm in EL-Sharkia Governorate; Egypt during two successive winter seasons to evaluate the effect of inoculation with capsules containing NPK PGPR on growth and productivity of wheat plants under salinity stress. The farm is located at 31°8'12.461" N latitude and 31°52'15.496" E longitude. The physical and chemical characteristics of the studied soil are shown in (Table 1), bulk density and physical and chemical properties of the soil at the experimental site were determined according to [38,39] particle size according to Piper [40] and chemical according to Ryan et al. [41]. Wheat winter cultivar, Gemza 9 (*Triticum aestivum L*.) was used in this study and developed by the national program at Field Crop Research Institute, Agricultural Research Center (ARC), Egypt. Mineral fertilizer was applied as the recommended dose for the Egyptian Ministry of Agriculture. The control plots received 100% from recommended dose from NPK, the experiment was laid out a randomized complete block design (RCBD) with three replications for each treatment as following:- 1-100% mineral NPK, 2-50% mineral NPK, 3- microencapsulation NPK bacteria +50% mineral NPK, 4- Liquid NPK bacteria +50% mineral NPK.

2.8 Determination of Relative Water Content (RWC %) and Proline Determination

Three leaves of the plant were taken randomly from the stem for each plot and directly weighed (fresh mass, FM). To determine the turgid mass (TM), leaves were floated in distilled water inside. During the imbibition's period, leaves were weighed sometimes after water on the leaf surface was gently distributed with tissue paper. At the end of the imbibition's periods, leaves were placed in a pre-heated oven at 70°C for 48 h, to obtain dry mass (DM). Values of FM, TM and DM were used to calculate leaf RWC according to the equation pronounced by Kaya and Higgs [42]. As well as the proline content in shoots of wheat plants was determined according to Bates et al. [43].

Table 1. Physiochemical properties of the soil

Soil properties	Mechanical properties
Soil type	Clay soil, 0.6% organic matter
Particle size distribution	Coarse Sand: 5.40**%** Fine Sand: 4**%** Silt: 40**%** Clay: 50
	Chemical properties
Chemical properties	pH 8.09 EC dS m^{-1} 10.90
Soluble cations (meq L^{-1})	Ca^{++} =43.30 Mg^{++} =39.70 Na^{+} =79.80 K^{+} =1.70
Soluble anions (meq L^{-1})	$SO4^{-}$ =70.92, Cl^{-} =87.80, HCO3- 5.78, $CO3^{--}$

2.9 Electrolyte Leakage (%) (Membrane Permeability)

To determine the electrolyte leakage, we tacked six leaf discs (10 mm in diameter) from leaves and placed in 50mL glass bottles and washed with distilled water to remove the electrolytes free during leaf disc excision. Bottles were then filled with 30 mL of distilled water to stand in the dark for 24 h at room temperature and the end of the incubation period, the EC1 of the washing solution was determined. Then the bottles were heated in a water bath at 95°C for 20 min and then cooled to room temperature, then we measured the EC2. Electrolyte leakage was calculated as a percentage of EC1/ EC2 [44].

2.10 Antioxidant Enzymes

Catalase (CAT) enzyme was determined according to the method described by Aebi [45]. Ascorbate peroxidase (APX) was determined according to Nakano and Asada [46] and superoxide dismutase (SOD) was determined according to Donahue et al. [47].

2.11 Enzymes Activities

Dehydrogenase activity (DHA) (µg TPF/g dry soil) in rhizosphere soil for each treatment was determined according to Donahue [48]. Alkaline and acidic phosphatases (mg/g dry soil) were determined according to the methods described by Tabatabai [49] respectively.

2.12 Agronomical Data Measurement

We collected plant samples from each treatment 1 m^2 wooden frame to determine wheat yield and its components were recorded following [50,51,52]. Consequently, six plants from each sub-plot were randomly selected at harvesting time for measurement of plant height (cm). 1000 grain weight (g), spike length (cm), straw yield (ton /ha) grain yield (ton/ha), biological yield and harvest index. Samples of straw were oven-dried at 70°C up to a constant dry weight, grounded and prepared for digestion method as described by [39]. The digests were then exposed for measurement of NPK. Nitrogen content was determined by Kjeldahl technique and potassium content was determined by flame photometer as described by Jackson [53]. Phosphorus content was determined by inductively coupled plasma spectrometry (ICPS) (Ultima 2 JY Plasma). Na^{+} was determined according to Wolf [54].

2.13 Statistical Analyses

The study design was a randomized complete block design (RCBD). Least significant difference test was used to compare means using the statistical analysis software; CoStat (CoHort Software, U.S.A) version 6.4. The values of probability $p \leq 0.05$ were considered statistically significant based on the least significant difference test.

3. RESULTS

3.1 Impact of NaCl on the Growth of PGPRs

Data showed that there was variation in the growth of strains (Fig. 1). All three strains were able to grow very well with the variation of NaCl, MSR H5 was able to grow and receive to log

phase entry earlier with 25g/L^{-1}NaCl after 48h from growth, then the decline phase started, where MSRH1 and MSRH3 were able to grow and reach to log phase after 24h with 25gL^{-1}NaCl then the decline phase started. Using NaCl 2% and 2.5% in the medium it improved the growth of all strains, after which a continuous decrease was observed in the growth. It was noticed that strains of MSRH5, MSRH1 were the most tolerant to higher applied NaCl concentrations as 2% and 2.5% while MSR H3 was most tolerant to 2% after 48h and 72h from incubation. Whereas the greater sensitivity to a hyperosmotic medium was observed for MSR H5, MSR H1 and MSRH3 strains which can grow under 30gL^{-1}NaCl.

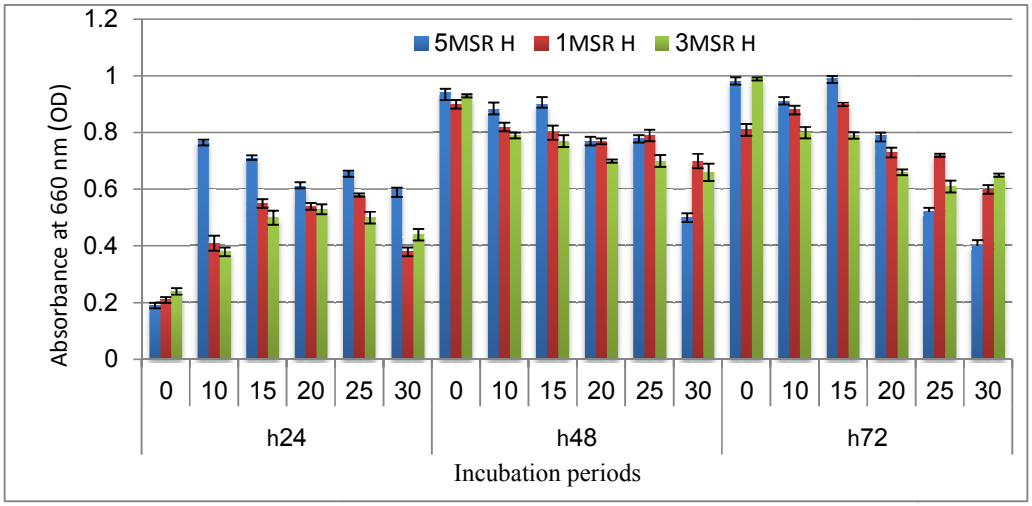

Fig. 1. Optical density (OD) (at 660 nm) as a measure of the activity of growth of three strains cultivated in NB cultures supplemented with six concentrations of NaCl (0, 10, 15, 20, 25 and 30g NaCl L^{-1}). Error bars represent the standard deviation between 3 replicates

Table 2. PGPR properties under salt stress condition

Strains	N2-activity			Phosphate solubilization			Potassium solubilization			IAA production			Exopolysaccharide (EPS) production		
	NaCl g L-1														
	0	25	30	0	25	30	0	25	30	0	25	30	0	25	30
MSRH5	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
MSRH1	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+
MSRH3	-	-	-	-	-	-	+	+	+	+	+	+	++	++	+

Clearance index: - not found

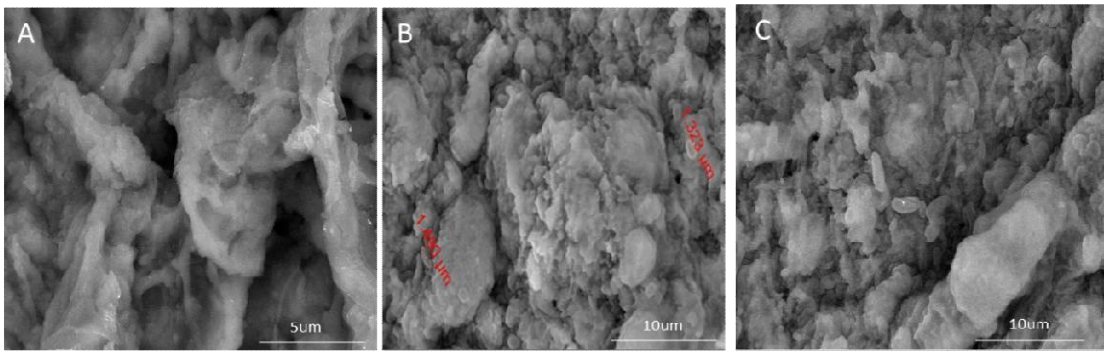

Fig. 2. SEM image of three strains of the encapsulated beads with sodium alginate:- A (an encapsulated media with sodium alginate), B and C encapsulated *P. polymyxa* MSRH5, *B. nakamurai* MSRH1and *B. pacificus* MSRH3 inside the beads of alginate

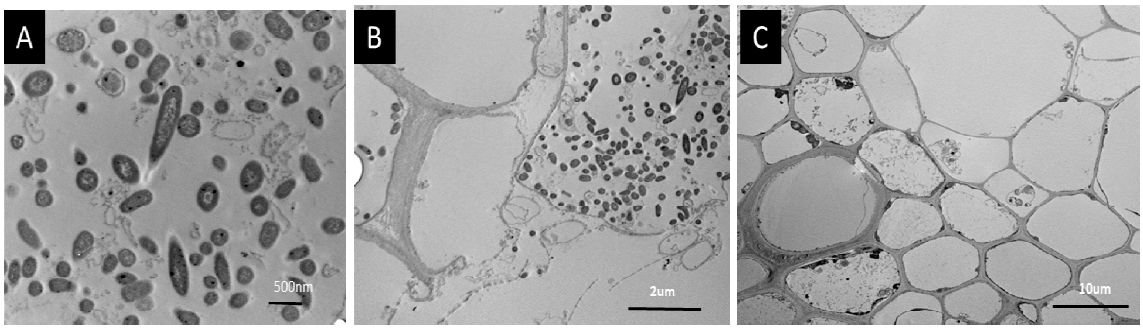

Fig. 3. Transmission electron microscope TEM of wheat root colonization (Root hair zones) by capsules containing *P. polymyxa* MSR H5, *B. nakamurai* MSR H1 and *B. pacificus* MSR H3 (A and B) stains living with the parenchymal cell wall while (C) is the control

3.2 PGPR Properties under Salt Stress

Data presented in Table 2 show that *P. polymyxa* MSRH5 was able to grow and fix nitrogen under 25 and 30 gL^{-1}NaCl compared to other two tested strains. The 30 g L^{-1}NaCl seemed not to affect N_2-fixation by strain MSRH5. *B. nakamurai* MSRH1 was able to solubilize phosphate under 25 and 30 g L^{-1}NaCl, on spit of MSRH5 and MSRH3 could not increase phosphate solubilization under salinity. *B. pacificus* MSRH3 was able to increase K solubilizing ability and gave the largest zone in plate. All strains can produce IAA under different range of NaCl. Production of IAA was found to be a widespread phenomenon among the bacterial strains. There is a gadwall increase in EPS production under salinity compared to medium without NaCl. *B. pacificus* MSRH3 recorded the highest EPS production while. *B. nakamurai* MSRH1 recorded the lowest EPS production with adding 30 NaCl g L^{-1} to the medium.

3.3 Evaluation of Encapsulated *P. polymyxa* MSRH5, *B. nakamurai* MSRH1 and *B. pacificus* MSRH3 by Scanning Electron Microscope (SEM)

SEM analysis (Fig. 2) shows the smallest particle achieved with sodium alginate of 2% the beads always shrivel. A fraction of the beads is likely to result from the liquid-bridge that has been detected during the droplet break-off in the $CaCl_2$; it has a size of range from 13.2 nm to 12.9. Formation of capsule requires techniques that are gentle and non-aggressive towards the cells. The techniques of drop formation have shown their limitations because the cells encapsulated by these techniques are completely available into the product. The three bacterial strains in capsules are clearly and no contamination found inside the capsules.

3.4 Colonization Activity for Microencapsulation on the Root of Wheat by Transmission Electron Microscopy (TEM)

The colonization of bacteria from alginate capsules was investigated by (TEM). Fig. 3 illustrates the location of bacterial micro colonies which were found both on the rod and on the lateral roots. Bacteria strains are well established and colonized on the epidemics and cortex of root wheat plant compared to control (not inoculated), it covering the root surface. The cells of *P. polymyxa* MSR H5, *B. nakamurai* MSR H1and *B. pacificus* MSR H3 were confined on the surface and inside the root using (TEM).

3.5 Physiological Characteristics

Proline content, R.W.C and electrolyte leakage are illustrated in Fig. 4. There is a significant effect ($P < 0.05$) of inoculation on physiological characteristics. The highest proline content in shoots recorded in un- inoculated plants either with 100% or with 50% mineral fertilizer, giving 10.9 and 10.6 (mg/g d.w) respectively ,whereas the best treatment was inoculation with capsulated bacteria followed by inoculation with liquid culture giving 6.8 and 8.2(mg /g d. w) respectively. The reduction in shoot proline was 35.8% in case of inoculation with capsules while the reduction was 22.64% with inoculation with liquid bacteria. RWC % as decreased by increasing salinity intensity in wheat leaves but inoculation with halo-tolerant PGPR improved the RWC in all treatments treated with PGPR in two

forms, the highest RWC was observed with inoculation with capsules containing N_2-fixing, P and K-solubilizing PGPR bacterial (60.57%) compared to the control (46%) while wheat inoculated with liquid culture NPK were (60.19%). Similar pattern of results was obtained when EL leakage was evaluated in wheat grown under salinity stress. EL leakage (membrane permeability) was higher than halo-tolerant PGPR in two forms of inoculation. The reduction in electrolyte leakage was 18.1% in inoculation with capsules while the reduction in liquid inoculation with halo-tolerant PGPR was 16.3%.

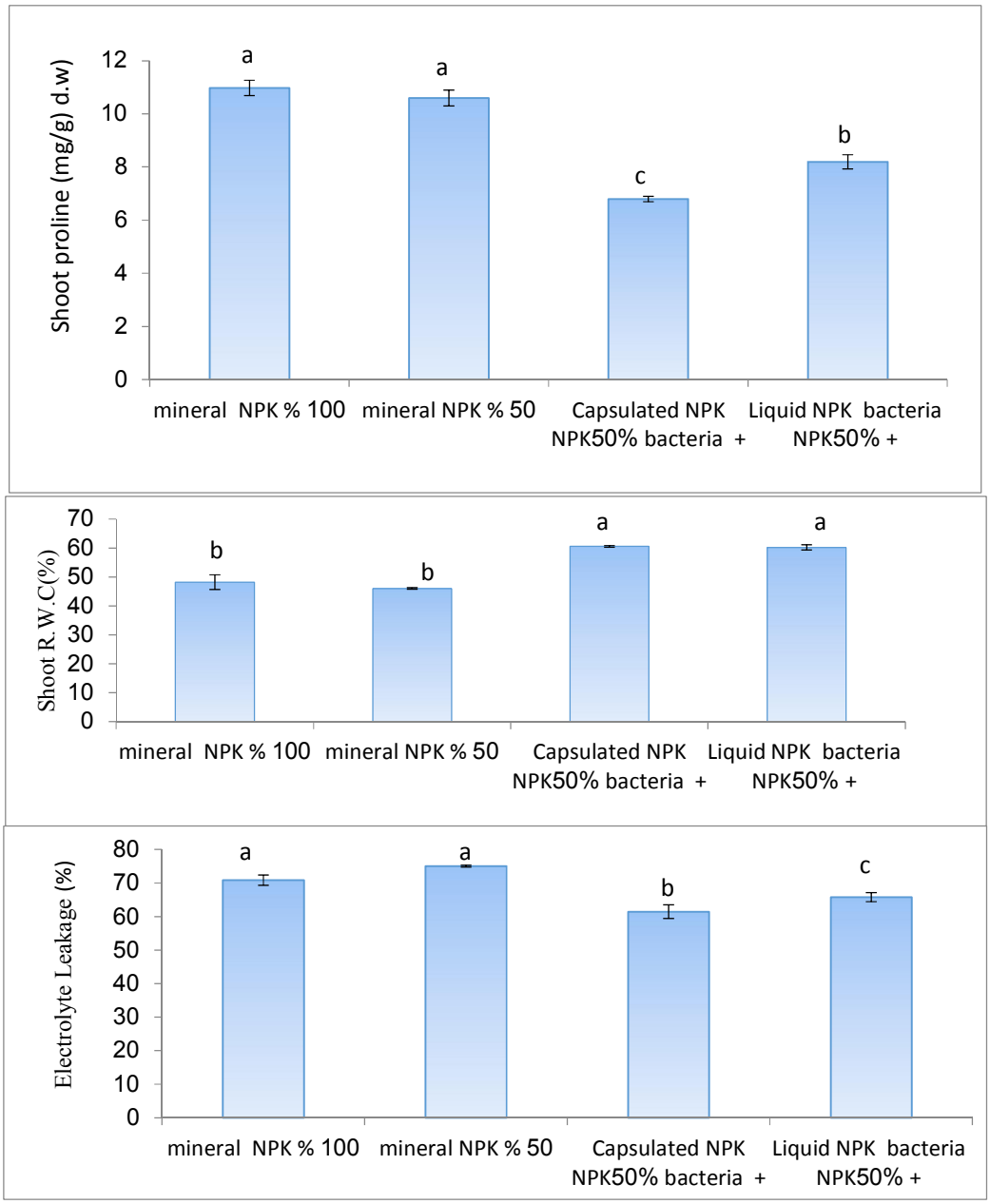

Fig. 4. Effects of halo tolerant PGPR on shoot proline content, relative water content (R.W.C %) and electrolyte leakage (%) of wheat plants grown under salinity stress. Data presented are means of three repeats for 2 years. Error bars represent the standard deviation between 3 replicates

3.6 Effect of Saline Conditions on Enzymatic Activities

Results indicated that there was a gradual decrease in DHA, acidic and alkaline phosphatase due to the presence of salinity affected growth of wheat plants (Table 3). Inoculation with capsules containing N_2-fixing, P and K-solubilizing PGPR bacteria gave the highest significantly DHA and acidic phosphatase (61.9 µg TPF g dry soil^{-1} day^{-1}) and (99.9 µg pnp g^{-1} soil h-^{1}) respectively compared to the control with the lowest DHA (41.7 µg TPF g dry soil^{-1} day^{-1}) and acidic phosphatase (42.8 µg pnp g^{-1} soil h-^{1}) respectively, inoculation with liquid bacteria gave the highest alkaline phosphatase (59.4µg pnp g^{-1} soil h^{-1}) followed by capsules (51.64 µgpnp g^{-1} soil h^{-1}) compared to the control (38.8 µg pnp g^{-1} soil h^{-1}).

3.7 Effect of Saline Conditions on Antioxidant Enzyme

The activity of antioxidant enzymes increased in response to salinity stress treatments in the absence of bacterial (Fig. 5). Under salinity stress plants had significantly higher activities of peroxidase, catalase and super oxide dismutase than un- inoculated plants. Results clearly suggest the positive role of N_2-fixing, P and K-solubilizing PGPR in two forms applied up regulating the CAT, APX and SOD activities in wheat plants under salinity stress, inoculation with capsules exhibited reduction in CAT enzymes 46.00%, 37.5% in APX and 40% in SOD while when inoculation with liquid culture the reduction was 36.1% in CAT, 33.5% in APX and 30.1% in SOD activities. Generally, inoculation with halo-tolerant PGPR in two forms under salinity stress enhances enzyme activity.

3.8 Yield Components

Results indicated that there was a significant increase in all yield parameters (Table 4) such as the highest plant height 115.8 cm, spike length (21 cm) and weight of 1000 grains (71.3 g) respectively recorded with inoculation by capsules and followed by wheat treated with liquid culture such as the plant height 112.1 cm, spike length (13.7 cm) and weight of 1000 grains (69.3 g) respectively. PGPR either capsules or liquid culture significantly increased yield parameters of wheat plants compared to the non-inoculated control. The lowest plant height, spike length and weight of 1000 grains were recorded with wheat un-inoculated (76.3 cm), (9.7 cm) and (50.6 g) respectively. As well as N_2-fixing, P and K-solubilizing PGPR inoculation significantly increased straw yield (10.8 ton ha^{-1}), grain yield (9.4 ton ha^{-1}) with capsules inoculated compared to the control plants under salinity stress. The results showed that the highest biological yield 20.2 and harvest index was 46.5% recorded with inoculation with of halo-tolerant PGPR in form of capsules plus 50% mineral fertilizer as compared to un-inoculated wheat.

3.8.1 PGPR in two forms effects on plant mineral contents in shoots of wheat plants

Table 5 indicated that considerable effects of the inoculation of two forms of PGPR on the different mineral contents in shoots of wheat plants especially N, P, K and Na under salinity stress as compared to the control under salinity stress. Conversely, the control plants had significantly lower N, P, K contents in the shoot of wheat. Highest N, P, K (28.4, 3.1 and 48.6 mg^{-1} d.w)

Table 3. Enzymatic activity of rhizosphere of wheat plants grown under salinity stress. Data presented are means of three repeats 2 years

Treatments	Dehydrogenase activity (µg TPF g dry soil^{-1} day^{-1})	Acidic Phosphatase activity (µg pnp g^{-1} soil h^{-1})	Alkaline phosphatase activity (µg pnp g^{-1} soil h^{-1})
100 % mineral NPK	41.7	42.8	38.8
50 % mineral NPK	37.9	34.2	25.4
Capsulated NPK bacteria+50% NPK	61.9	99.9	51.6
Liquid NPK bacteria50% NPK	59.1	64.9	59.4
L.S.D at 0.05	1.00	1.56	3.09

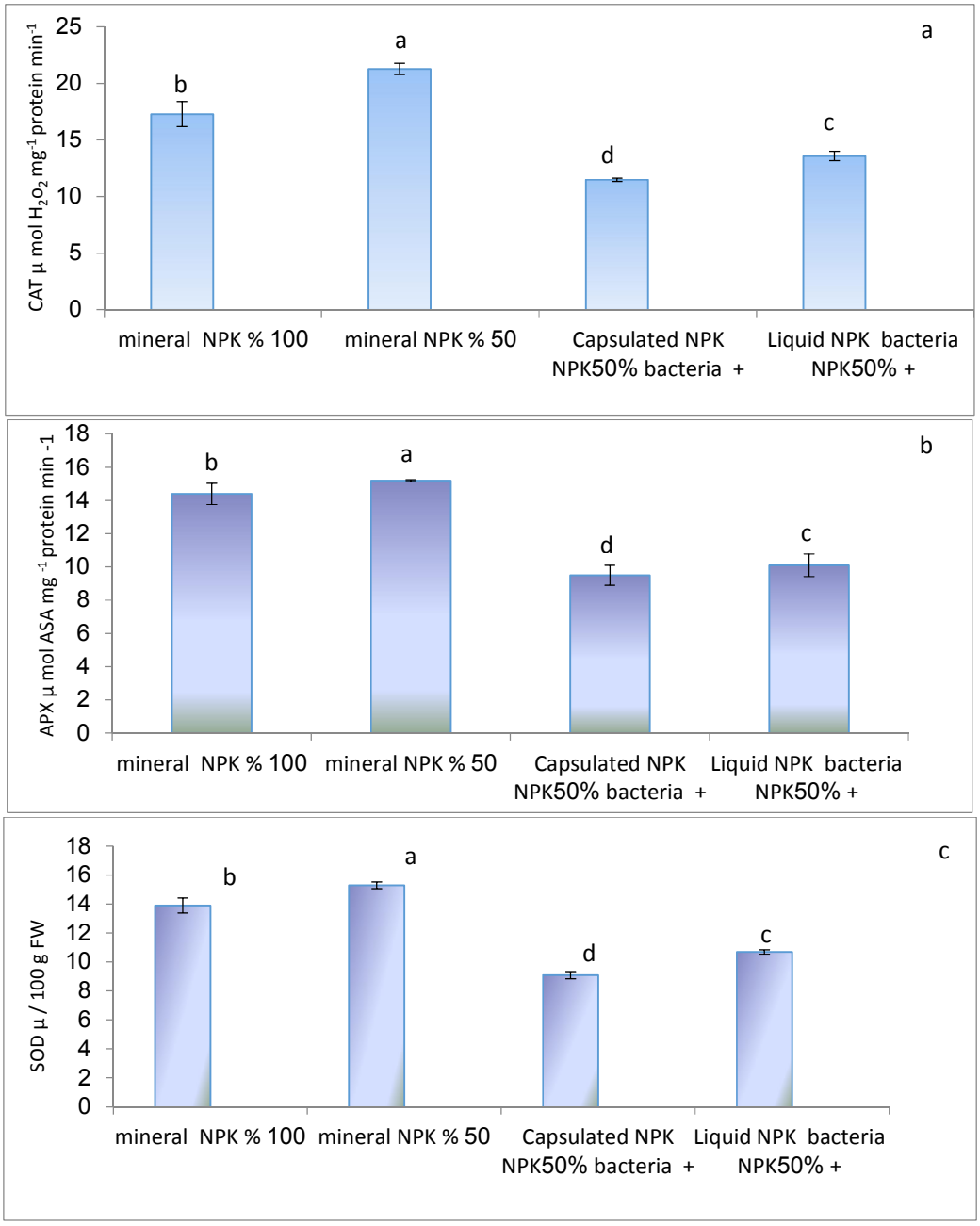

Fig. 5. Antioxidant enzyme (a, b and c) in shoots of wheat plants inoculated with two forms from PGPR and grown in saline soil. Data presented are means of three repeats for 2 years. Error bars represent the standard deviation between 3 replicates

respectively, recorded with inoculation halo-tolerant PGPR compared to un-inoculated wheat. On the other hand, inoculation with PGPR had significant effect on Na content and gave the lowest values, compared to un-inoculated wheat, the inoculation reduce the Na in shoots of wheat.

3.8.2 Effect of Na/K ratio on shoots of wheat under saline soil

Na/K ratio was affected by inoculation with capsules as illustrated in Fig. 6. N_2-fixing, P and K-solubilizing halo-tolerant PGPR reduced the value of Na/K ratio in all treatments inoculated

compared to un-inoculated wheat. The highest reduction of Na/K was 0.03 in case of inoculation with capsules and followed by inoculation with liquid culture recorded 0.05.On the other hand un-inoculated wheat plus 50% mineral fertilizer recorded 0.12 in saline soil and was 0.10 in wheat amended 100% mineral fertilizer NPK.

Table 4. Yield components of wheat plants grown in saline soil and inoculated with two forms of halo-tolerant PGPR. Data presented are means of three repeats for 2 years

Treatments	Plant height (cm)	Spike length (cm)	1000 grains wt. (g)	Straw yield (ton ha $^{-1}$)	Grain yield (ton ha $^{-1}$)	Biological yield	Harvest index (%)
100% mineral NPK	99.2	11.1	54.9	9.4	7.4	16.8	44.0
50% mineral NPK	76.3	9.7	50.6	8.8	7.2	16.0	45.0
Capsulated NPK bacteria +50% NPK	115.8	21.0	71.3	10.8	9.4	20.2	46.5
Liquid NPK bacteria +50% NPK	112.1	13.7	69.3	10.6	8.6	19.2	44.8
L.S.D at 0.05	33.73	18.61	1.60	0.68	0.18	0.64	--

Table 5. Effects of inoculation with halo-tolerant PGPR on nutrient contents in shoots of wheat plants. Data presented are means of three repeats for 2 years

Treatments	Available of macronutrients mg $^{-1}$d.w			
	N^{-3}	P^{-3}	K^{+}	Na^{+}
100 % mineral NPK	24.3	2.2	32.9	3.4
50 % mineral NPK	22.1	1.8	35.5	4.1
Capsulated NPK bacteria +50% NPK	28.4	3.1	48.6	2.1
Liquid NPK bacteria +50% NPK	26.9	2.6	42.6	7.4
L.S.D at 0.05	5.46	1.03	13.32	1.96

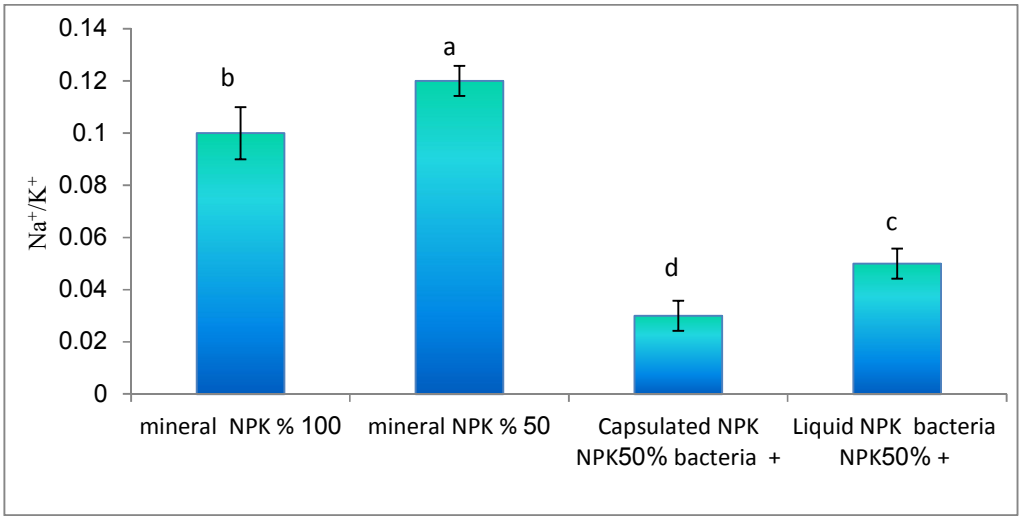

Fig. 6. Na^{+}/K^{+} ratio in shoots of wheat plants inoculated with two forms of PGPR and grown in saline soil. Data presented are means of three repeats for 2 years. Error bars represent the standard deviation between 3 replicates

4. DISCUSSION

Salinity has a direct influence on the physiochemical and biological properties of soil led to detrimental effects on growth as well as the productivity of the plants [55]. The PGPR intermediated plant's salinity tolerance is a well-known phenomenon [56] and offers carefully possible approach for combating salinity at large scale. In the current study we used three tolerant PGPR (*P. polymyxa* MSR H5, *B. nakamurai* MSR H1and *B. pacificus* MSR H3) and evaluated their behaviors under the high concentration of salt (NaCl) in growth medium to establish and confirm their tolerance and the adaptability to saline stress. MSRH5, MSRH1and MSRH3 have been found to grow in wide range of the high concentration of NaCl and increased concentration of NaCl leads to the increase in plant growth. Therefore, these bacteria could be classified as halo-tolerant bacteria [57].

The three strains play a role in nitrogen fixing, phosphate and potassium solubilization and EPS and IAA production under salt stress up to 30 gL^{-1}. Nitrogen fixing, phosphate and potassium solubilizing microorganisms make bio-available phosphate, potassium and nitrogen for plant growth and are dynamic traits for bio-fertilizer production. IAA is an auxin required by most plant cells for division and root origination [58] who cleared that maximum IAA-production significantly increased plant growth under salt stress. Auxin production of *Azospirillum brasilense* spp was up to 200 mM [59] but *P. polymyxa* MSRH5 and *B. nakamurai* MSRH1 showed higher tolerance and IAA production up to 300 mM which are selective for biofertilizer production in salt-affected soils. In addition, phosphate and potassium solubilizations were important characteristics due to their role in bio-fertilization [60].

Particularly in saline soil, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized in the soil quickly and then becomes unavailable to the plant [61]. Previous research showed that exopolysaccharides production by halo-tolerant PGPR strains significantly increased by increasing salinity; these results are in agreement with those obtained by [62]. SEM analysis demonstrate that the immobilization had no significant effect on bacterial morphology. This means that this method can be used as a capable tool for protection and enhancement of bacterial cells in a harsh environment without prompting the metabolic activity. This result is harmony with [63].

This provides a new protocol for statement the limitations associated with the future application of sustainable bio self-healing material. The cells of *P. polymyxa* MSRH5 and *B. nakamurai* MSRH1 and *B. pacificus* MSRH3 were localized on the surface and inside the root of wheat using TEM. The cells are attached to the root surface. Twisting and deformation of root hairs were induced by bacteria [64].

Assumed that these salt-tolerant PGPR strains are free-living bacteria and exhibited a potential for PGP stimulates, their effects on rhizosphere of wheat in Sahl El-Hossynia of Egypt. The proline content could keep the growth of PGPR isolates up to higher salinity level because it could act as intermediary of osmotic adjustment protects macromolecules during dehydration and help as a hydroxyl radical scavenger [65]. The accumulation of proline in saline-stressed plants was significantly higher than non-saline-stressed plants, a similar result was reported by Heidari et al. [66]. The mechanism by which proline reduces ROS destruction and helping plant resistance is that proline reduces saline stress by detoxification of ROS produced as end of saline deficit. In our study halo-tolerant PGPR improved the RWC in all treatments than un-inoculated wheat; the increase in RWC up to 31.6% than un-inoculated wheat, this result is in agreement with [67] who found that RWC was decreased by increasing saline stress intensity in basil leaves. Similarly, the increased RWC may indirectly or directly contribute to the increase in photosynthetic pigments of inoculated alfalfa, in agreement with [68]. The highest reduction in electrolyte leakage (%) was achieved by the PGPR inoculation plants as compared to saline-stressed plants (control). These results prove the productivity of PGPR in alleviation of the cell membrane injury. The preservation of cellular membrane integrity under stress is considered to be an essential part of the saline tolerance mechanism. It was proposed that PGPR can stimulate a PGPR-intermediate encouraged systemic resistance and stimulate accumulation of the sending molecules of salicylic acid and jasmonate [69].

Enzyme activities can be reduced due to lower contents of microbial biomass emission and less amounts of enzymes under stress condition as shown in Table 3. Salt stress induced reduction in dehydrogenase activity; it is a measure of the

amount of microbial metabolism in soil as O_2 is accepted from the soil, PGPR can colonize the rhizosphere which leads to increase in CO_2 evolution and carbonic acids formation that reduces soil pH and consequently increases mineral absorption and improves plant growth [70]. The addition of humic acids in the capsules providing chemical stability and an organic nutritious sources of C and N, which keep a higher number of living bacterial cells [71]. They reported that the increase of salinity as measured by increased conductivity dissolves clay minerals and stable enzymes thus remain unprotected and more disposed to denaturation. The lowest values of phosphatase activity in wheat rhizosphere was observed in saline soil with 50% mineral fertilizer (control). *P. polymyxa* MSRH5, *B. nakamurai* MSRH1 and *B. pacificus* MSRH3 succeeded to increase the plant development and the development depended on the beneficial role of the used microorganisms while the increase of phosphatase (acid and alkaline) as noted with capsules beads of PGPR was due to the act of *B. nakamurai* individuals to produce organic acids which are careful as a solubilizing agents of phosphorus compounds in soil leading to an increase of phosphorus rates in soil [72]. The peroxidase (POX), catalase (CAT), and super oxide dismutase (SOD) are important common indices to evaluate the changes redox status of plants. Antioxidant enzymes act as a destruction control system and thus make available protection from oxidative stress which can cause lipid peroxidation resulting in damage to the cell membrane, protein, DNA structure and other enzyme activities [73]. In the present study, encapsulated PGPR or liquid culture inoculation under 50% from mineral fertilizer significantly induced effects on CAT, POX, and SOD activities in wheat plants under salt stress condition, the stimulation of antioxidant enzymes, such as catalase, peroxidase and superoxide dismutase, can be measured as a salt-tolerance mechanism in wheat plants. The antioxidant can be decreased due to the free radical scavengers, which can be attributed to reduced H_2O_2 levels that are not enough to activate the enzyme's antioxidant property and the reduction can be considered as a salt-tolerance mechanism in wheat plants. Results support those of Gururani et al. [74], who also cleared that the activities of ROS scavenging enzymes, such as APX, CAT and SOD, were enhanced in PGPR-inoculated potato plants exposed to various stressors (salt).In our study wheat inoculated with encapsulated PGPR or liquid culture was observed to decrease SOD activity, due to a lower O_2-scavenaging and suppressing capacity in the wheat plants, this indicates a possible involvement of this enzyme in salt tolerance in accordance with [75].

Use of rhizobacterial strains of *P. polymyxa, B. nakamurai* and *B. pacificus* among others, have proven to be efficient in plant growth promotion, particularly for their quality to promote vegetative growth and productivity in wheat crops [76], maize [77] alfalfas [78] and potatoes [79]. The stimulating activity is related to the ability of rhizobacteria in the present study to produce enzyme activities, including IAA production, phosphate and potassium solubilization. These producers increased growth and yield-contributing traits such as plant height, spike length, weight of 1000 grain, straw yield and grain yield, direct stimulation on plant growth was observed in the present study. In general encapsulation of rhizobacteria is to conserve high cell density with maximum survival even after protracted storage [80]. Alginate is one of the most frequently used polymers for encapsulation of plant growth promoting bacteria in agriculture [81]. In our study we detected that salt stress reduced the shoot length (51.7 %) and spike length (116 %) and weight of 1000 grains (40%) of wheat grown in saline soil. These results are in agreement with those obtained by Egamberdieva [82].

Inoculation of wheat with halo tolerant PGPR is suggested as a sustainable way to increase crop yields due to the plant growth promoting substances produced by the bio-fertilizer [83], besides to the reasonable quantity of atmospheric nitrogen fixed by *P. polymyxa*, phosphate and potassium solubilizing by *B. nakamurai* and *B. pacificus* as reported by [17] so the general physiological status of the wheat exhibit positive reaction to use of bio-fertilizer. Puccini et al. [84] who found that the grain yield and harvest index of wheat were improved when wheat plants were grown in saline soil plus a combination of chemical N and bio-fertilizer inoculation. Earlier studies have found that salinity conditions slightly reduced the plant nutrient element content in the leaves of wheat plants through the exclusion of Na, which increased under salt stress [85]. Results indicated that the inoculation with either encapsulated or liquid PGPR had considerable effects on the different mineral contents in shoots of wheat plants especially N, P and K are harmony with [86] who found that the application of encapsulated of *Enterobacter sp*. increased

the growth and phosphorus uptake in lettuce plants. In contrast with increasing Na content, K content decreased with increasing salinity levels. A similar result was informed in wheat by Grieve and Poss [87] who confirmed antagonistic absorption between Na and K under salinity stress conditions. In this regard [88], encapsuleted *Pseudomonas fluorescens* and *Serratia* sp. inoculated with wheat plants, significantly promote foliar content of P, attributing that encapsulates increase the effect of rhizobacteria by acting as mini-reactors that provide bacterial cells stabilization, protection, population increase and advanced release around the rhizosphere of the plants where they are applied. Rhizobacteria *P. polymyxa*, *B. nakamurai* and *B. pacificus* producing EPS have to match against salt stress by making rhizo-sheaths everywhere in the roots of plants by attaching the EPS with Na^+ ions and decreasing the toxicity of Na^+ which makes it unavailable for absorbance by Abbas [89].

5. CONCLUSION

Encapsulated PGPR: *P. polymyxa*, *B. nakamurai* and *B. pacificus* succeed to colonize the rhizosphere of wheat plants and protect wheat plants from destruction to salt stress. These PGPR had ameliorative effect on reduction of proline accumulation in shoots, improved RWC, electrolyte leakage and enzymatic activity as well as improvement of the antioxidant enzymes, growth and yield of wheat under saline stress. Consequently, it could be applied in supporting wheat plants to tolerate against salt stress beside in increasing crop production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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