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PLANT GROWTH-PROMOTING BACTERIAL MIXTURE ENHANCED GROWTH OF BARLEY UNDER SALT STRESS

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Abstract

Salinization is a primary environmental stress limiting global plant growth and production. Barley (*Hordeum vulgare* L.) is a cereal grain used as important food source in many countries. Inoculation with plant growth-promoting bacteria (PGPB) is a method for alleviating the harmful effects of salt stress on plant growth. This study focused on the impact of salinity on growth parameters, soluble sugars and individual amino acids of barely plants. In addition, to demonstrate the role of the mixture of PGPB (*Pseudomonas putida* and *Bacillus subtilis*) to ameliorate the growth in response to salt stress. There was a significant decrease of growth parameters of barley plants grown in hydroponic cultures supplemented with 100 mM NaCl. This suppression was associated with a significant increase of Na^+ and Na^+/K^+, trehalose and sucrose content compared to control plants. The total amnio acids and free individual amino acids content markedly decreased in response to salt stress. Treatment of barley grains with mixture of PGPB significantly enhanced plant growth in salinized hydroponic cultures. The data suggested that the mixture of PGPB may be induced changes of cellular osmotic potential which enhance the growth of barley plants in response to salt stress.

Key words: Salt stress; Plant growth-promoting bacteria; Barley; *Pseudomonas putida* and *Bacillus subtilis*.

Introduction

In many parts of the world, salinity is a significant environmental stressor that inhibits plant development and yield. Salinity has an impact on more than 800 million hectares of land worldwide (Munns & Tester, 2008). In Egypt, salinity is a significant abiotic factor impacting crops (Mansour & Abolila, 2021). Additionally, according to Noaman (2008), 30% of Egypt's arable land is influenced by salt.

Depending on its degree and duration, salinity stress alters a variety of metabolic pathways before impairing crop output (Rozema & Flowers, 2008). Osmotic stress is exacerbated and ion toxicity is created by a high salt (NaCl) concentration in the root zone. Water uptake, seed germination, photosynthetic rate, nutrient uptake and translocation from root to shoot, and overall plant growth are all adversely affected by osmotic stress (Zelm *et al*., 2020). The intake of K+ ions, a necessary component for growth and development, is inhibited by high Na+ concentrations, which lowers productivity and may even cause death (James *et al*., 2011).

By increasing the concentration of osmoprotectant substances like glucose, fructose, trehalose, sucrose, ammonia compounds, glycine betaine, proline, and amino acids to control the osmotic potential of cells, higher plants can be able to turn off the inhibitory effects of salinity stress (Mark *et al*., 2012). Moreover, the development of enzymatic and non-enzymatic antioxidant defence mechanisms for neutralising and scavenging the produced reactive oxygen species (ROS) to protect the integrity of cellular components and plasma membranes from oxidative damage (Mudgal *et al*., 2010).

Several physicochemical techniques are frequently employed to enhance salty soils (Fita *et al*., 2015). Yet, because they pollute the environment, their widespread and ongoing use is unsustainable (Mishra *et al*., 2021). Applying microbial inoculation with plant growth-promoting bacteria (PGPB) improves soil quality under salinity stress *Via* a variety of processes (Gupta *et al*., 2022). Exopolysaccharides (EPS), siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, phytohormones, antioxidants, induced systemic resistance (ISR), and enhanced nutrient uptake under salt stress are some of the mechanisms involved (Olanrewaju *et al*., 2017).

Several studies investigating the role of plant growth promoting bacterial species in ameliorating the negative effect of salt stress on plants such as *Bacillus megaterium* (Marulanda *et al*., 2010)), *Azospirillum lipoferum* (Abdel Latef *et al*., 2020), *Pseudomonas entomophila* (Fatima & Aora, 2021), However, to date there has been few studies on the synergistic role of plant growth promoting bacterial mixture to reduce the adverse effects of salinity (Jha *et al*., 2011, Orhan, 2016). To our knowledge this work is the first study on the effect of plant growth promoting bacterial mixture on barley under salt stress.

Barley (*Hordeum vulgare* L.) is an important crop species, is the fourth most important cereal crop produced globally (Anon., 2019). It is one of the main crops grown in Egypt.

The current investigation intends to assess the effectiveness of a mixture of *Pseudomonas putida* and *Bacillus subtilis* in mitigating the adverse effect of salinity on barley plants.

Material and Methods

The Agricultural Research Center (GARC), Giza, Egypt, graciously provided grains of barley (*Hordeum vulgare* L. cv Giza 123). *Pseudomonas putida* was obtained from the Department of Plant Pathology at the Faculty of Agriculture, Alexandria University. Bacterial

cultures were adjusted 48 hours before inoculation. A spectrophotometer method of Nabti *et al*., (2014) was used to adjust the mixed bacterial suspension of *Bacillus subtilis* and *Pseudomonas putida* to an absorbance of 1 at 600 nm, which is equal to 1×10^9 CFU/ml.

Grains inoculation: Barley grains were sterilized by soaking for two minutes in 4% sodium hypochlorite, followed by numerous washes in distilled water. The sterilized barley grains were inoculated by immersing them for two hours at room temperature in a mixed bacterial slurry that had been supplied with 2% sucrose (Mohamed & Gomaa, 2012).

Hydroponic cultures: In a dark growing chamber, control (non-infected) and inoculated grains were allowed to germinate for two days in plastic containers containing 0.1 mM $CaSO_4$ solution. The light was switched on at a temperature of 25°C with aeration, a photoperiod of 16/8 light/darkness, and a light intensity of 250 mol m^{-2} s^{-1}. After germination grains were moved to 2L hydroponic cultures of Hoagland nutrient solution supplied with 0 and 100 mM NaCl six days after the experiment started (Abdel-Latif *et al*., 2004). Four different treatments were applied to the cultures: (T0), non-inoculated plants (control); (S), salt-treated non-inoculated plants; (M), inoculated-treated plants in mixed bacterial suspension and (M+S), salt-treated inoculated plants. Each treatment was represented by three replicates and placed in a controlled growth chamber.

Estimation of growth parameters: Fifteen days after the beginning of the experiment, ten plants from each treatment were selected, washed with distilled water, dried through tissue papers, and dissected into shoots and roots. To calculate dry biomass (D. M.), fresh samples of shoots and roots were weighed and placed in an oven at 60°C until they reached a constant weight. The shoot length (cm) was measured.

Determination of Na^+ and K^+ ions, soluble sugars and amino acid content

Na^+ and K^+ ions, soluble sugars and amino acid content were extracted as a method by Handa *et al*., (1986). According to Kubrak *et al*., (2015), sodium and potassium ions were measured using a capillary electrophoresis (CE) equipment (Agilent Technologies, Santa Clara, CA, USA). The sodium and potassium ions were expressed as mmol g^{-1}D.M. The quantity of soluble sugars was determined using Gas chromatography/Mass Spectrometry (GC/ MS Shimazu GC-2014, GLS Cat. No. 1010-18642, Niigata, Japan). Data processing according to Que'ro *et al*., (2012) using Chromato-PRO software. The content of sugars was expressed as µmol g^{-1} D.M. The amino acids profile was determined utilizing ultra-high-pressure liquid chromatography (UPLC) according to Nagumo *et al*., (2009). Chromatography Data Software (Waters, Milford, MA, USA) was used to collect data, process it, and manage the UPLC system. All amino acids were expressed as µmol g^{-1} D.M.

Statistical analysis

Results are presented as the mean after three replications of each treatment. All attribute data were subjected to a one-way analysis of variance and using the Least Significant Difference (LSD) method proposed by Steel & Torrie (1980), the mean values were compared.

Results

Growth parameters: After 15 days in hydroponic culture, (S) treatment showed a significant reduction in growth parameters compared to (T_0) control plants (Table 1). Plant shoots' fresh and dry biomass reduced by about 41 and 21%, respectively, while plant roots' fresh and dry biomass declined by 76 and 62%, respectively. Inoculation of barley plants with a mixture of PGPB (M) treatment) caused significant enhancement in F.M. in shoot and root by 26% and 18%, respectively, compared to (T_0) control plants. In contrast the improvement of F.M. in shoots and roots of plants treated with PGPB mixture (M+S) treatment was 43 and 82%, respectively, in comparison to salt stressed plants (S) treatment. Similar to the trend of F.M., the D.M. of (M+S) treatment for both shoot and root was significantly increased by 19% and 39%, respectively, compared to (S) treatment plants.

Na^+ and K^+ ions: As shown in (Fig. 1) salt stress caused a significant increase of sodium and Na/K % in the shoots and roots in comparison to non-salinized control plants. This was followed by a significant [decrease in potassium content. Treatment of barley grains with bacterial mixture caused a significant increase in K^+ content and decrease of Na^+ content in salinized and non-salinized barley plants. In comparison to the salinized control, the Na+ content of the shoots and roots decreased by 12.9 and 24%, respectively.

Table 1. Changes in fresh (F.M.) and dry biomasses (D.M.) of shoots and roots, shoot length (SL) of non-inoculated and inoculated barley grains with a mixture of PGPB in response to 100 mM NaCl.

Treament	Shoot (mg plant^{-1})		Root (mg plant^{-1})		Shoot length SL
	F.M	D.M.	F.M.	D.M.	OO (cm)
T_0	$306.6^a \pm 2.44$	$85.1^a \pm 2.44$	$148.9^a \pm 1.63$	$39.3^a \pm 2.40$	$16.2^a \pm 0.97$
S	$181.5^b \pm 3.30$	$66.9^b \pm 2.49$	$35.6^b \pm 1.64$	$14.9^b \pm 2.44$	$11.8^b \pm 0.81$
M	$385.8^c \pm 3.26$	$98.6^c \pm 1.63$	$176.2^c \pm 1.63$	$40.1^a \pm 3.26$	$22.7^c \pm 1.63$
M+S	$259.7^c \pm 4.89$	$79.7^d \pm 1.65$	$64.9^d \pm 3.26$	$20.\ 7^c \pm 0.81$	$14.3^a \pm 0.81$
LSD	**8.28**	**4.80**	**4.98**	**5.56**	**2.56**

(T_0), Non-inoculated plants (Control); (S), Salt-treated non-inoculated plants; (M), Inoculated-treated plants in mixed bacterial suspension and (M+S), Salt-treated inoculated plants. Different letters within the columns show a significant difference between treatments at p = 0.05 according to LSD test

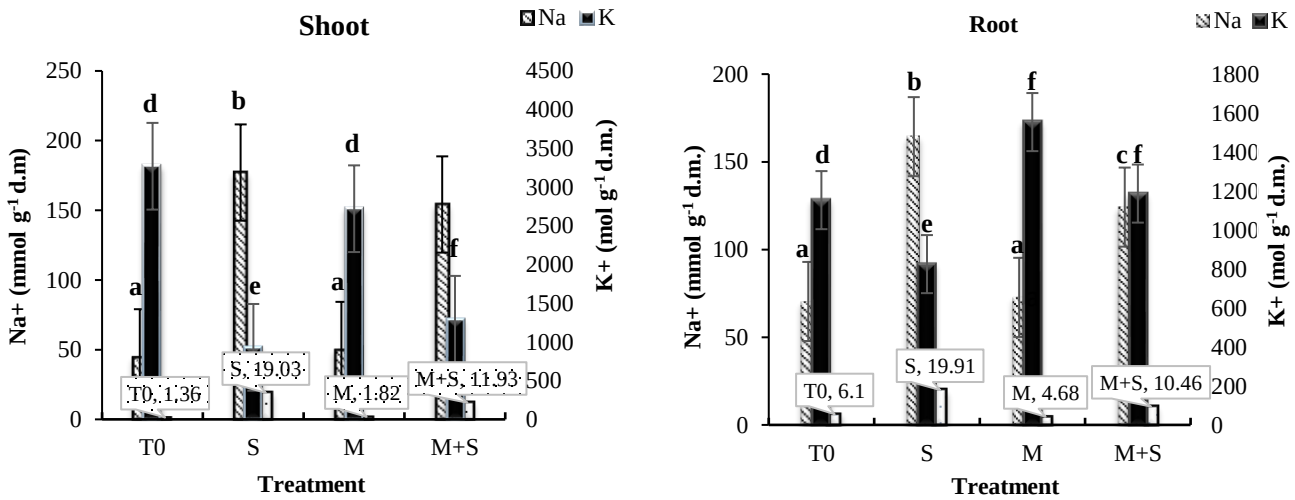

Fig. 1. Change in Na^+, K^+ contents and Na/K ratio in shoots and roots of non-inoculated and inoculated barley grains with a mixture of PGPB in response to 100 mM NaCl. (T_0), Non-inoculated plants (Control); (S), Salt-treated non-inoculated plants; (M), Inoculated-treated plants in mixed bacterial suspension and (M+S), Salt-treated inoculated plants. Different letters show a significant difference between treatments at p = 0.05 according to LSD test.

Table 2. Change in sugars content of non-inoculated and inoculated barley grains with a mixture of PGPB in response to 100 mM NaCl.

Treatment	Shoot				Root			
	µmol g^{-1} d.m							
	GLU.	FRU.	TRE.	SU.	GLU.	FRU.	TRE.	SU.
T_0	3.13^{a}±0.54	4.72^{a}±0.29	0.49^{a}±0.10	17.84^{a}±3.46	1.42^{ab}±0.17	1.78^{a}±0.61	1.28^{a}±0.28	13.77^{a}±1.04
S	4.18^{a}±1.18	14.04^{b}±1.81	2.17^{b}±0.04	27.93^{b}±0.45	1.89^{b}±0.43	2.32^{a}±0.62	4.10^{b}±0.63	46.57^{b}±4.99
M	1.26^{b}±1.18	3.29^{a}±0.56	0.53^{a}±0.11	10.04^{c}±1.42	2.92^{c}±0.12	1.34^{a}±0.38	1.16^{a}±0.20	11.30^{a}±1.99
M+S	1.74^{b}±0.40	13.04^{b}±1.80	3.44^{c}±1.13	57.21^{d}±3.9	1.28^{a}±0.37	1.69^{a}±0.18	6.94^{c}±1.07	31.21^{c}±3.70
LSD	**1.31**	**1.94**	**1.07**	**5.13**	**0.57**		**1.21**	**6.22**

(T_0), non-inoculated plants (Control); (S), salt-treated non-inoculated plants; (M), inoculated-treated plants in mixed bacterial suspension and (M+S), salt-treated inoculated plants. GLU. Glucose; FRU. Fructose; TRE. Trehalose; SU. Sucrose. Different letters within the columns show a significant difference between treatments at p = 0.05 according to LSD test.

Soluble sugar fractions: Salinization of the hydroponic culture with 100 mM NaCl of inoculated and non-inoculated barley grains with a mixture of PGPB significantly increased the trehalose and sucrose compared to the control (Table 2). Trehalose and sucrose increased 6.5 and 1.5 fold in NaCl-salinized shoots than in the control, whereas the equivalent values in the roots were 3 and 3.8-fold, respectively. Whereas the enhancement of trehalose in the PGPB-treated salt stressed roots and shoots was 1.69 and 1.58 compared to non-treated salinized plants, the increase of sucrose in the shoot was 2- fold.

Amino acid content: The results in Table 3 revealed that the application of 100 mM NaCl with the nutrient culture markedly decreased the total amino acids in barley plants. When compared to the control, the total amino acid content of salinized roots and shoots decreased by 66 and 64%, respectively. However, barley grains inoculated with a mixture of PGPB markedly induced accumulation of amino acids in the shoots and roots under normal and salt stress conditions. The total amino acids in inoculated salt stressed roots and shoots was 2.8 and 3.8 -fold, respectively, of non-inoculated salt stressed plants. In addition, Ala, Arg, Asp, Aspr, Cyst, Glu, Glun, Gly, Meth, Phen, Pro and Trp were markedly increased under inoculation treatment.

Discussion

The present study showed a significant decrease in the growth parameters of salinized barley plants compared to the control. These results are consistent with those of other studies and suggest that salt stress has negative effect on plant growth (Ansari *et al*., 2019; Mansour& Aboulila, 2021; Corti *et al*., 2023). The suppression of growth parameters in salt-stressed barley plants might be related to an increase of osmotic stress around the root zone and ionic toxicity that results in an imbalance of water and nutrient uptake leading to disturbance of photosynthetic machinery, metabolites translocation and metabolic reactions (Munns &Tester 2008; Zelm *et al*., 2020). Increasing the intercellular Na^+ might down-regulate the expression genes of aquaporin transcript-associated with plasma membranes (Jia *et al*., 2020), leading to a decrease in water uptake. Moreover, the increase in Na^+/K^+ ratio could be leading to the depolarization of plasma membranes and competition between Na^+ and K^+ on specific transporters, which restricted K^+ influx (Dogan *et al*., 2010). In addition, Miller *et al*., (2010) mentioned that increasing Na^+ accumulation as toxic ions might enhance ROS generation and oxidative damage of plant structures, plasma membranes and cellular components.

Table 3. Change in amino acid content in shoots and roots of non-inoculated and inoculated barley grains with a mixture of PGPB in response to 100 mM NaCl.

Amino acid	Shoot				Root			
	(µmol g^{-1} D.M.)							
	T0	S	M	M+S	T0	S	M	M+S
Ala	0.11	0.21	0.08	0.46	0.06	0.14	0.16	0.29
Arg	0.38	0.66	0.43	0.92	0.15	0.36	0.19	0.77
Asp	4.08	0.46	5.59	3.19	3.23	0.27	4.11	1.51
Aspr	1.94	0.33	2.02	2.87	1.71	0.18	1.79	0.25
Cyst	0.27	0.47	0.28	0.82	0.19	0.29	0.34	0.86
Glu	4.98	0.65	8.62	4.13	4.11	0.49	6.31	4.07
Glun	0.44	0.29	2.49	2.08	0.86	0.22	1.55	0.38
Gly	0.39	0.50	0.44	0.93	0.32	0.41	1.98	0.71
His	0.19	0.06	0.19	0.09	0.08	0.09	0.11	0.01
Isoleu	0.22	0.14	0.22	0.14	0.12	0.11	0.17	0.12
Ieu	0.13	0.07	0.13 0.11	0.05	0.03	0.05	0.04	0.04
Lys	0.04	0.04	0.11	0.05	0.06	0.03	0.15	0.14
Meth	0.09	0.13	0.23	0.34	0.14	0.25	0.17	0.48
Phen	0.36	0.28	1.93	0.51	0.17	0.14	0.12	0.21
Pro	0.18	0.47	0.20	0.64	0.21	0.66	0.28	0.46
Ser	0.65	0.38	0.78	0.96	0.40	0.19	0.51	0.33
Thr	0.12	0.06	0.12	0.15	0.06	0.09	0.15	0.11
Trp	0.17	0.03	0.21	0.08	0.18	0.04	0.37	0.12
Tyre	0.03	0.01	0.07	0.06	0.05	0.04	0.18	0.05
Val	0.11	0.12	0.16	0.13	0.34	0.17	0.37	0.14
Total	**14.88**	**5.36**	**25.39**	**18.63**	**12.48**	**4.22**	**19.06**	**11.95**

(T_0), Non-inoculated plants (Control); (S), Salt-treated non-inoculated plants; (M), Inoculated-treated plants in mixed bacterial suspension and (M+S), Salt-treated inoculated plants. Different letters within the columns show a significant difference between treatments at p = 0.05 according to LSD test

On the contrary, a significant increase in fresh biomass of salt-stressed shoots and roots originated from PGPB-inoculated barley grains compared to those of salt-stressed plants alone. These findings were accompanied by a significant increase in K^+ content and a decrease in Na^+ content. It has been reported that PGPB under salinity stress might regulate the expression of aquaporins, and that enhances water status and growth. Marulanda *et al*., (2010) stated that inoculation maize with PGPB *Bacillus megaterium* could upregulate the expression of aquaporin.

PGPB was found to upregulate plasma lemma and tonoplast-associated SOS pathway genes responsible for maintaining the ion homeostasis (JI *et al*., 2013), Na^+/H^+ antiporter (Hanin *et al*., 2016) and high-affinity K^+ transporter (Arora *et al*., 2020a). Several studies showed that PGPB under salinity stress could produce bacterial polysaccharides to the external medium bind with Na^+, resulting in a marked suppression of Na^+ uptake and reduction of Na^+ level, thereby maintaining the ionic balance and improving the growth (Fatima & Arora, 2021). Following these views, the mixture of PGPB *Pseudomonas putida* and *Bacillus subtilis* might ameliorate the impact of salt stress on barley plants, in this study, *via* modulating the expression of the related genes the expression of the related genes for enhancing water uptake and maintaining Na^+ and K^+ homeostasis. Moreover, PGPB could improve the plasma membranes from oxidative damage through the induction of non-enzymatic and enzymatic antioxidant systems (Arora *et al*., 2020b; Cruz *et al*., 2023).

Several glycophytes can accumulate low molecular mass, and soluble osmoprotectant components, including sugars (glucose, trehalose, sucrose, raffinose and sugar alcohols), ammonia compounds and amino acids to maintain osmotic balance in plant cells to overcome the external osmotic stress (Singh *et al*., 2015). In the current research, there was a significant accumulation of trehalose and sucrose and decreased total estimated amino acids in the roots and shoots of NaCl-stressed barley plants in comparison to the control. The decrease of total amino acids in the salt-stressed shoots and roots might be related to the decrease of NO^-_3 uptake and nitrate assimilating activity (Ashraf *et al*., 2018) besides the oxidation of amino acids (Kapoor *et al*., 2019). However, the increase in Arg, Cyst and Gly levels in the shoots and roots of salinized barley plants in comparison to control might reflect their involvement in the biosynthesis of polyamines and glutathione as an osmoprotectant and non-enzymatic antioxidants.

There was a marked increase of total and individual free amino acids in the PGPB mixture inoculated shoots and roots under salinity stress, which could be related to the improvement of the activity of nitrate assimilation enzymes and nitrate uptake, and accelerated rate of amino acids metabolism (Kang *et al*., 2014). PGPB mixture of

Pseudomonas putida and *Bacillus subtilis* are well-known N_2-fixing rhizobacteria that can improve plant growth by supplying N_2 components.

Furthermore, increased the level of Phe and Trp might indicate the induction of phenolic compounds and IAA biosynthesis, whereas the increase in Arg, Cyst and Gly could interfere with the biosynthesis of polyamines and glutathione. These findings were associated with an increase in the growth parameters of barley plants. Jha (2017) stated that PGPB *Pseudomonas aeruginosa* increased phenolic compounds in salt-stressed maize plants. Arora *et al*., (2020a) reported that *Piriformospora indica* and *Azotobacter chroococcum* could reduce the oxidative damage of plasma membrane and increase phenolic and carotenoids in salt stressed *Artimisia* species plants. In addition, the increase of soluble sugars and free amino acids accumulation in PGPB -mixture inoculated shoots and roots of barley under salt stress, in this study, might be considered as osmoprotectant agents for maintaining the osmotic potential of cells as well as act as scavengers for generated ROS and hence improve the plasma membranes integrity and various cellular components from oxidative damage. Nishizawa *et al*., (2008) suggested that sucrose and soluble oligosaccharides could eliminate the generated hydroxyl radicals.

Conclusion

The current hydroponic study indicated that salinity stress negatively affected growth parameters, soluble sugars and individual amino acids of barely plants. This investigation also showed that inoculation of barely grains with growth-promoting bacterial mixture (*Pseudomonas putida* and *Bacillus subtilis*) significantly improved barley growth under salinity stress.

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