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ORIGINAL ARTICLE

Bacillus megaterium RTS1 enhances resistance of *Lycopersicon esculentum* to salinity stress through the improvement of antioxidant defenses

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ABSTRACT

Background and Objectives: Plant growth-promoting bacteria (PGPB) may reduce the negative effects of salinity stress. The aim of this study was to optimize *Bacillus megaterium* RTS1 and characterize the effect of the PGPB on the physiological characteristics of tomato (*Lycopersicon esculentum*).

Materials and Methods: The Central composite design (CCD) of response surface methodology (RSM) was used to optimize *Bacillus megaterium* RTS1 to produce maximum cell biomass and spores. Then the effect of the PGPB on the physiological characteristics of tomato (*Lycopersicon esculentum*), including membrane stability, leaf relative water content percentage, anthocyanin and carotenoids content, chlorophyll photosynthetic parameters, sugar and starch level, superoxide anion and antioxidant activity under salt stress conditions. The NFB medium was inoculated with 5% bacterial culture and the fermentation was carried out in a 10-lit fermenter.

Results: After optimization, the amount of cell biomass by the model was 9.45 log10 CFUs/mL, which showed a 1.2-fold increase compared to the non-optimized medium. Usage of bacteria under the optimal conditions of the culture medium may increase the stability of the membrane and improve the relative water content. Bacteria were able to prevent the excessive increase of anthocyanins. Oxidative stress led to an increase in the content of chlorophyll a, while causing the degradation of chlorophyll b. Bacterial inoculation led to an increase in the level of sugar and starch compared to the control. PGPB showed an increasing effect on the amount of superoxide anion production and caused a significant increase in the antioxidant activity under salinity stress conditions.

Conclusion: The PGPB can be a promising way to boost physiological characteristics of tomato plant under salinity stress. Also, sporulation capacity of *Bacillus megaterium* with high bacterial cell density in fermenter produce a sustainable product for tomato plants.

Keywords: Plant growth-promoting bacteria (PGPBs); *Bacillus megaterium*; *Lycopersicon esculentum*; Salinity stress

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is an agricultural product that has gained tremendous popularity in the last century and is cultivated in almost all countries of the world under field and greenhouse conditions (1). Tomatoes contain high amounts of vitamins A and C, minerals, antioxidants, and other nutrients. Vitamin A is important for bone growth, cell division and differentiation, regulation of the immune system, and maintenance of healthy ocular surface epithelium of the eye (2). Vitamin C is im-

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portant in the formation of collagen while helping to maintain capillaries, bones, and teeth and improving iron absorption (3). Lycopene is one of the most important antioxidants in this product, which helps to prevent any type of cancer and reduce the risk of heart disease (4, 5). Therefore, in the last 25 years, the global production and consumption of this product have increased rapidly, with a cultivation area of 4.8 million hectares (6).

Increasing the abiotic stress of soil salinity is a serious problem that severely affects the yield of many agricultural products, including tomatoes (7). Soil salinity hinders the sustainable development of modern agriculture and more than one-third of irrigated land in the world is affected by salinity (8). The main causes of soil salinity are the increase in the level of underground water with high salt content and low-quality drainage and irrigation systems (9). Changes in global weather patterns and insufficient irrigation management cause the area of saline soils to increase by 10% annually (10).

Soil salinity reduces the content of nutrients and microbial diversity severely reduces the amount of nitrogen and dissolved organic carbon and causes many changes in the microbiota pattern of soils (11). A high concentration of salt leads to disturbing the balance of sodium and chlorine ions and creating ionic stress (12). Salt stress also indirectly causes the accumulation of Reactive Oxygen Species (ROS) such as superoxide radicals and H_2O_2, which leads to DNA damage, cell death, and various physiological disorders such as reduced seed germination, seedling growth, flowering, and fruiting (13, 14).

Considering the destructive effects of environmental stress, especially salinity stress, it is critical to provide sustainable solutions to ensure food security and improve the production of plant products (15). The use of plant growth-promoting bacteria (PGPBs) is a promising strategy to improve the tolerance of soil salinity conditions and increase the efficiency of agricultural production (16). PGPBs directly increase the absorption of nutrients from the soil by fixing nitrogen, dissolving phosphate, and producing phytohormones such as indole acetic acid (17). Also, by producing antimicrobial compounds, they prevent the spread of bacterial, viral, fungal, and nematode pathogens and help maintain plant health and growth (10). Today, various bacterial genera are produced and consumed as PGPB, of which the genus *Bacillus* is one of the most important due to its remarkable characteristics (6). *Bacillus* are widely found in the rhizoplane region and due to the production of spores, they can withstand harsh environmental conditions, including salt stress (18, 19).

In a previous study, we isolated *Bacillus megaterium* RTS1 from the rhizoplane region of tomato plants and investigated its PGPB characteristics. The results showed the bacterium having appropriate characteristics as PGPB. The greenhouse experiments also demonstrated that this bacterium improves plant growth (20). In this research, after optimizing bacterial culture and spore production in an industrial medium, we studied its effect on improving plant physiological conditions, including membrane stability, leaf relative water content, plant pigments, carbohydrate storage, superoxide anion level, and antioxidant activity, under salinity stress conditions.

MATERIALS AND METHODS

Bacterial strain and medium culture. *Bacillus megaterium* RTS1 was previously isolated and identified from the rhizoplane of tomato field (Tehran) (20). The modified NFB media (21) (malic acid 5.0 g/l; yeast extract 20 g/l, K_2HPO_4 0.5 g/l; $MgSO_4.7H_2O$ 0.2 g/l; NaCl 0.1 g/l; $CaCl_2.2H_2O$ 0.02 g/l; KOH 4.5 g/l, NH4Cl 1 g/l; FeEDTA 4 mL (solution 16.4 g L^{-1}); vitamin solution (biotin 100 mg/l, and pyridoxal-HCl 200 mg/l) 1 ml/l, and micronutrient solution 2 ml/l, was used for culturing the bacterium. The composition of micronutrient solution was; $CuSO_4.5H_2O$ 0.04 g/l; $ZnSO_4.7H_2O$ 0.12 g/l; H_3BO_3 1.40 g/l; $Na_2MoO_4.2H_2O$ 1.0 g/l; $MnSO_4.H_2O$ 1.175 g/l). All chemicals were from Merck unless specified.

To enumerate bacterial cell numbers, serial dilutions of culture were prepared and cultured in Tryptic Soya Agar (TSA) culture medium (HiMedia Laboratories Pvt. Ltd.). To count the spores, the culture was first heat-treated at 85°C for 15 minutes (21, 22).

Growth optimization in a fermenter. The central composite design (CCD) of Response surface methodology (RSM) was for optimization of the growth *B. megaterium* RTS1. Based on the previous studies, glucose and corn steep liquor (CSL) (Zar fructose Co.) concentration and agitation rate were selected as independent variables (23-26). Each variable was studied at three different levels −1, 0, +1. The experimental plan concerning their coded and uncoded val-

ues is listed in Table 1. The NFB medium was inoculated with 5% bacterial culture and the fermentation was carried out for 48 h at 30°C and an aeration rate of 1 vvm in a 10-lit fermenter.

Table 1. The levels of the three independent variables concerning to their coded and uncoded values

Level	Coded Level (xi)	Uncoded Level		
		Glucose (%)	CSL (%)	Agitation (rpm)
Lowest	-2	0.32	0.65	66.00
Low	-1	1.00	1.50	100.00
Mid	0	2.00	2.75	150.00
High	+1	3.00	4.00	200.00
Highest	+2	3.68	4.85	234.00

The relationship between response (Bacterial growth) and independent factors was described using the CCD quadratic model based on the second-order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j$$

Where Y is the predicted response, Xi and Xj are independent factors, k is the integer of factors, β_0 is the model intercept, βi is the linear constant, and βii is the quadratic constant (27).

The significance of the model and regression coefficient were determined using analysis of variance (ANOVA). The coefficient (R^2) was determined to assess the fit of the model. The response surface and contour structures of the model-expected responses were used to evaluate the mutual corrections between the relevant parameters. and determine their optimum levels for maximal bacterial cell growth production. The RSM-generated mathematical model was validated by performing by experiments using the predicted optimum medium conditions. Design-Expert Version 7 (Stat-Ease Inc., Minneapolis, MN, USA) was employed for experimental design, for regression analyses, and pictorial presentation of the experimental data acquired.

Production of spray-dried powder. The bacterium was cultured in the fermenter under the optimal conditions as determined by the CCD experiment. The culture was sampled at 2-hour intervals as previously reported (28). The spores were separated using a centrifuge after completing the sporulation. The spores were mixed with a protectant solution containing 20% maltodextrin and 0.2% sodium mono glutamate (MSG) (Shandong Linghua Monosodium Glutamate Incorporated Company) and dried in a spray dryer. Then, the number of spores in the resulting dry powder was determined (29).

***In-vivo* plant growth promotion assay under salinity stress.** Soil samples were analyzed for their physicochemical properties. Tomato seeds were disinfected by soaking in 30% sodium hypochlorite solution containing 0.1% Triton X-100 for 15 min and rinsed with sterile water. The seeds were cultivated in pots in 3 groups (n=10):

- Negative control group (Ctrl-): without PGPB inoculation and salt stress
- Stress group: without PGPB inoculation and under salt stress conditions
- PGPB group: with PGPB inoculation and under salinity stress conditions

The soil of all the pots had been sterilized in an autoclave at a temperature of 121°C for 15 minutes. The spry dried powder of bacterium was added to the final concentration of 1×10^7 CFUs of spore/g of soil. The pots were incubated in a phytotron under 16 h at 27°C and 22°C during day and night, respectively (27). All the pots were irrigated with sterile distilled water with an electrical conductivity of 0.043 dS/m, until reaching the 4-leaf stage. After that, the pots of the Ctrl+ and PGPB groups were irrigated with distilled water, and electrical conductivity was raised to 6 dS/m using sodium chloride. While the Ctrl- group was irrigated with the same conditions as before for three weeks. At the end of the experiment, the physiological characteristics of the plants were evaluated.

Membrane stability index. The membrane Stability Index was determined according to Sairam's method (30). Briefly, 0.1 g leaf discs were taken in test tubes containing double distilled water. They were kept in a water bath at 40°C. After keeping it 30 minutes, the EC was measured (C1). The same leaf sample was then kept at 100°C for 10 minutes in a water bath and the EC was again recorded (C2). The formula used was:

$$Membrane\ Stability\ Index = \left(\frac{1 - C_1}{C_2}\right) \times 100$$

Relative water content (RWC). For evaluation of relative water content, the leaf's fresh weight was calculated. Then, the saturated weight was after dipping leaves in water for about 24 hours. The leaves were then placed in an oven at 60°C until the weight became constant (31). The RCW is calculated as below:

$$RWC\% = \frac{Fresh\ weigh - Dry\ werigh}{Saturated\ weight - Dry\ weight} * 100$$

Pigments Quantification. The chlorophylls, anthocyanin, and carotenoid pigments were extracted by grinding 1 g of fresh leaves with 80% acetone. After adding 1 mL of the extraction buffer, tubes were centrifuged (10 min) at 10,000× g at 4°C. Supernatants were transferred to falcon tubes, and pellets were mixed with the extraction buffer (1.5 mL), and centrifuged again. Supernatants were added to previous ones. Subsequently, the absorbance of the extract at 663.3 nm, 647 nm, 537 nm, and 470 nm was analyzed by a UV-1800 spectrophotometer, and pigment contents were calculated according to the Sims and Gamon method (32).

Chlorophyll a= $0.01373A_{663}$- $0.000897A_{537}$- $0.003046A_{647}$

Chlorophyll b= $0.02405A_{647}$ - $0.004305A_{537}$ - $0.005507A_{663}$

Anthocyanin=$0.08173A_{537}$ - $0.00697A_{647}$ - $0.00228A_{663}$

$$Carotenoids = \frac{(A_{470} - (17.1 \times (chl_a + Chl_b) - 9.479 \times Anthocyanin))}{119.26}$$

Carbohydrates content (TSS). TSS content was quantified by the anthrone method (33), and the starch content according to Osaki et al. (34). For TSS determination, 0.5 g of two top mature frozen leaves from each plant were macerated with 80% ethanol. After incubation at 80°C for 1 h, tubes were kept in ice, and centrifuged (10 min) at 10,000× g, at 4°C. Supernatants were transferred into new tubes and the pellets were preserved. 30 µL of supernatant was mixed with 750 µL of the anthrone solution (40 mg anthrone in 1:20 v/v (H_2O: H_2SO_4)). Reaction tubes were incubated (10 min) at 100°C and cooled in ice. Absorbances were read at 625 nm using a UV-1800 spectrophotometer. A standard curve for glucose was used. For starch content quantification, 1g pellets reserved from the TSS quantification protocol were mixed with 5 mL of 30% perchloric acid. Tubes were incubated for 1 h at 60°C, and then kept in ice, and centrifuged (10 min) at 10,000×g, at 4°C. Supernatant (30 µL) was mixed with 750 µL of anthrone solution. Reaction tubes were incubated at 100°C (10 min), and vortexed. The same process for TSS quantification was used here.

Superoxide anion ($O_2^{\bullet-}$). Superoxide anion ($O_2^{\bullet-}$) was measured according to Gajewska and Sklodowska (35). Briefly, 100 mg of frozen leaves were mixed with the extraction buffer (2 mL), containing 0.01 M phosphate buffer (pH = 7.8), 0.05% Nitro-blue tetrazolium (NBT) diluted in 100 µL Dimethyl sulfoxide (DMSO), and 10 mM sodium azide, incubated (1h) at room temperature. The solution was centrifuged at 13,000× g for 2 min, at 4°C. The supernatant was then incubated at 85°C (15 min) and kept in ice (10 min). Absorbances were analyzed at 580 nm using a UV-1800 spectrophotometer.

Antioxidant activity. The scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was determined according to Harkat-Madouri et al. (36). 100 mg Frozen leaves were macerated with 3 mL of methanol at 4°C. Extracts were vacuum-filtered and collected in falcon. Suspension was centrifuged at 2500 rpm (10 min). Each sample (150 µL) was diluted with 100 µL of methanol and 1.25 mL of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) 0.1 mM was added. Tubes were incubated for 30 minutes in a dark place. Absorbances were analyzed at 517 nm using a UV-1800 spectrophotometer and antiradical capacity was determined using a standard curve of gallic acid.

Statistical analysis. The statistical analysis of quantitative variables was performed using GraphPad Prism 8 software with one-way ANOVA and t-test at a significance level of P-value = 0.05.

RESULTS

Optimization of medium components by RSM. The interaction between the relevant parameters was further studied with CCD and their optimum levels were determined. Table 2 shows the experimental matrix of CCD giving the actual values for Bacterial growth by strain *Bacillus megaterium* RTS1. Multiple regression analysis was used to create the second-or-

Table 2. Experimental central composite design (CCD) data of Bacterial growth by strain *Bacillus megaterium* RTS1

Run No	Glucose (g/l)	CSL (g/l)	Agitation (rpm)	Bacterial growth Log NO. (CFUs/ml)
1	20.00	30.00	234.09	8.34
2	20.00	30.00	150.00	8.22
3	30.00	40.00	100.00	6.34
4	10.00	20.00	100.00	4.23
5	20.00	30.00	150.00	8.12
6	36.82	30.00	150.00	4.23
7	20.00	30.00	150.00	8.09
8	30.00	20.00	200.00	6.12
9	30.00	40.00	200.00	6.50
10	10.00	40.00	200.00	6.23
11	20.00	46.82	150.00	7.45
12	10.00	40.00	100.00	5.34
13	20.00	30.00	150.00	8.13
14	20.00	30.00	150.00	8.32
15	30.00	20.00	100.00	6.12
16	20.00	30.00	150.00	8.44
17	3.18	30.00	150.00	2.35
18	20.00	30.00	65.91	7.12
19	20.00	13.18	150.00	7.12
20	10.00	20.00	200.00	6.23

der polynomial equation:

$Y = +8.22 + 0.4548A + 0.1659B + 0.3736C - 0.0638 AB - 0.3413 AC - 0.1188 BC - 1.76 A^2 - 0.3484 B^2 - 0.1911 C^2$

Where Y is the Bacterial growth ($\log_{10}$ CFUs/mL), A is Glucose (g/l), B is CSL and C is (Agitation). Analyses of variance confirmed that the model was lack of fit for prediction contained by the range of the employed variables (Table 3).

As indicated by the three-dimensional response surface plots for the interaction of glucose and agitation determined in the production of live bacterial cells, the interaction of glucose and agitation to some extent increases the production of bacterial biomass. Agitation has a significant effect on the growth of bacteria in a manner which, with its increase, the amount of live bacterial cell production increases and reaches its maximum at 200 rpm. This also increases the concentration of glucose from 2% and above and reduces the positive effect of the agitation cycle on the production of live bacterial cells (Fig. 1A).

The three-dimensional surface plot of the interaction of glucose and CSL in (Fig. 1B) also demonstrated that increasing the concentration of CSL up to 3.5% increases the production of live bacterial cells, but this effect is more pronounced in the presence of a glucose concentration of 2%. The results (Fig. 1C) indicated that increasing the amount of glucose and agitation to a certain extent may increase growth. In addition, the interaction of these two factors does not have a significant effect on the growth of bacteria.

The model was validated by performing a test experiment using conditions of glucose 28.138 g/l, CSL 32.867 g/l, and 174.638 (rpm), as predicted by RSM. Under these optimized conditions, a significant improvement in bacterial growth of 9.45 log10 CFUs/mL was predicted, further supporting the validity of the model.

Growth kinetics of *Bacillus megaterium* RTS1 bacteria. Analysis of bacterial growth kinetics showed that after the 2-hr lag phase, the bacteria entered the logarithmic growth phase, which continued until 16-hr. Based on counts of live bacteria, the maximum number of live bacteria cells at the end of the logarithmic phase was equal to 2.82e9 CFUs/ml (Fig. 2). The counts of spores showed that the sporulation phase started at 22-hr and reached the maximum at 30-hr, which was equal to 8.48e9 CFUs/ml. In other words, after 30 hours, the sporulation efficiency was equal to 48% and almost half of the vegetative cells completed the sporulation phase.

Antioxidant activity. Salt stress causes an increase in reactive oxygen species (ROS). An increase in antioxidant capacity is one of the mechanisms of resistance in plants. Superoxide dismutase and catalase are the most important enzyme antioxidants that prevent the oxidation of intracellular compounds by neutralizing ROS. As expected, in plants exposed to stress, the antioxidant activity increased significantly and reached 310.70 mg/l which is responsible for detoxifying ROS and protecting intracellular compounds. The use of PGPB caused a significant increase in antioxidant capacity up to a concentration of 368.30 (P value=0.012) (Table 4).

Superoxide anion. Watering the plants with sodium chloride solution with an electrical conductivity of 6 ds/m caused oxidative stress to start in the plant and the amount of superoxide anion reached 18.27 nmol/g per gram of fresh leaf weight (P value=0.024). It was expected that by increasing the amount of antioxidant

Table 3. Analysis of variance (ANOVA) of Bacterial growth by strain *Bacillus megaterium* RTS1

Source	Sum of Squares	df	Mean Squares	F-value	p-value	
Model	51.30	9	5.70	121.85	<0.0001	Significant
A	2.83	1	2.83	60.39	<0.0001	
B	0.3756	1	0.3756	8.03	0.0177	
C	1.91	1	1.91	40.74	<0.0001	
AB	0.0325	1	0.0325	0.6950	0.4239	
AC	0.9316	1	0.9316	19.91	0.0012	
BC	0.1128	1	0.1128	2.41	0.1515	
A2	44.68	1	44.68	955.09	<0.0001	
B2	1.75	1	1.75	37.39	0.0001	
C2	0.5260	1	0.5260	11.24	0.0073	
Residual	0.4678	10	0.0468			
Lack of fit	0.3744	5	0.0749	4.01	0.0769	Not significant
Pure Error	0.0934	5	0.0187			
Cor Total	51.77	19				
Std. dev.	0.2163		R2	0.9910		
Mean	6.65		Adjusted R2	0.9828		
C.V%	3.25		Predicted	0.9364		
Press			Adequate Precision	38.1397		

A: Glucose, B: CSL, C: Agitation; *: P < 0.001

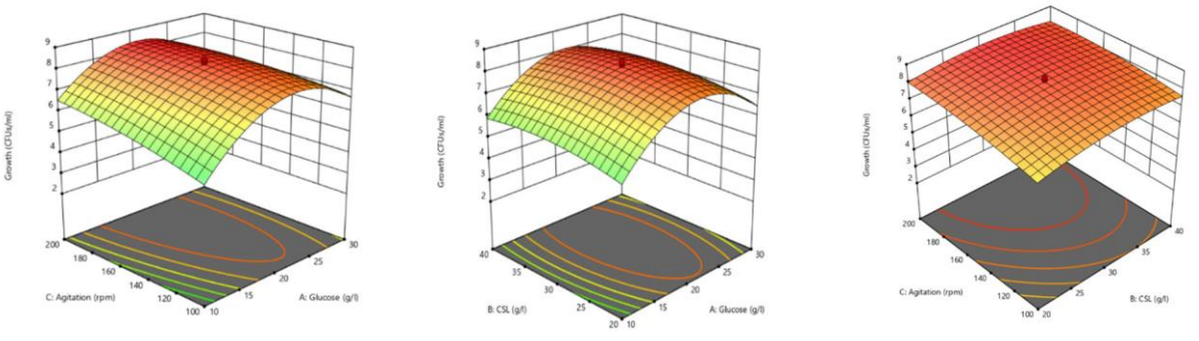

Fig. 1. Three-dimensional response surface plot for interactions between (A) Glucose e and Agitation, (B) CSL and Glucose, and (C) CSL and Agitation, illustrating their effect on Bacterial growth by strain *Bacillus megaterium* RTS1

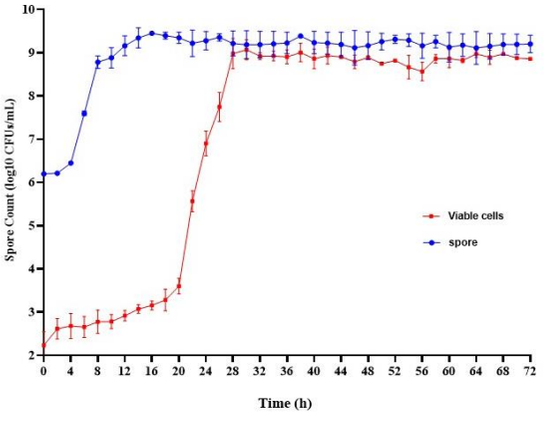

Fig. 2. Cell Growth and sporulation kinetics, of *B. megaterium* RTS1 (log10 CFUs/mL)

capacity, dismutation of superoxide anion would take place and its amount would decrease significantly (P value=0.009). In the plant sample treated with PGPB, the amount of this anion reached 15.40 nmol/g, which indicates an increase in plant resistance to salt stress.

Membrane stability. Salinity stress causes instability and dysfunction of the membrane by disrupting the homeostasis of Ca+ and Na+. Our results showed that irrigation with salt water causes the membrane stability index to decrease significantly from 92.85 to 65.42. This in turn causes severe damage to plant metabolism and disrupts photosynthesis (P value=0.0002). Increasing the stress resistance capacity in the plant through

soil inoculation with PGPB increased the membrane stability index to 73.79. However, the damage to the membrane was evident even with the treatment of the plant with bacteria (P value=0.0089) (Table 4).

Leaf relative water content. Salinity stress caused a significant decrease in leaf relative water content from 87.43 to 65.51% (P value=<0.001). Salinity stress causes the relative water content of the leaf to decrease by increasing the osmotic pressure. This in turn causes the interference of the enzyme action inside the cells, which manifests itself in metabolic processes. However, the use of PGPB causes a significant increase in the percentage of leaf relative water content by improving the resistance mechanisms under these conditions (72.35%) (P value=0.005) (Table 4).

Leaf pigments content. Salinity stress causes the destruction of cellular structures, including chlorophyll, by creating ROS. Enzymes (catalase, peroxidases, reductases, etc.) and non-enzymatic compounds create resistance in plants. Carotenoids and anthocyanins are two important groups of plant pigments whose levels increase strongly during abiotic stresses.

The results showed that stress decreased the amount of chlorophyll a and b to 0.23 mg/g and 0.16 mg/g of the corresponding leaf weight, respectively. The use of PGPB caused the decomposition of chlorophyll a not occurred (P value=0.184). Surprisingly, the treatment could not prevent the decomposition of chlorophyll b (0.18 mg/g) so there was a significant difference between the concentration of this pigment in the treated sample and the control (P value=0.018) (Table 4).

The results demonstrated that the levels of carotenoid and anthocyanin pigments increase significantly in plants under salinity stress. Treatment with PGPB caused the amount of these two pigments to increase more than the stress group, which resulted in the reduction of oxidative compounds and the increase of antioxidant capacity (Table 4).

Levels of soluble sugars and starch in leaves. As expected, due to the destructive effects of stress on pigments, the amount of total sugars as well as starch stored in leaves showed a significant decrease compared to the control group under salinity stress (5.20 mg/g and 12.41 mg/g, respectively) (Table 4). The application of PGPB prevents the decomposition of chlorophyll and improves ion stress and osmotic pressure resulting from salinity stress, leading to a situation where there was no difference in the amount of carbohydrates (starch and TSS) between the test group and the control group (21.44 mg/g and 27/03 mg/g respectively). (Table 4). The results showed that this bacterium increases the access of plants to nutrients and thus increases photosynthesis and catabolism, as well as improving the physiological conditions of the plant.

DISCUSSION

Salinity stress has caused a decrease in the yield of plant products and is one of the increasing problems of sustainable and modern agriculture (37). A soil with an electrical conductivity (EC) of more than 4

Table 4. Effect of *Bacillus megaterium* RTS1 on membrane stability index, RWC, anthocyanins, chlorophyll a, ahlorophyll b, total soluble sugar, starch, superoxide, antioxidant and carotenoids content of tomato leaves under salinity stress

Compound	Mean ± SEM		
	Ctrl-	Stress	PGPB
Membrane Stability Index	92.85 ± 5.160	65.42 ± 2.012	79.73 ± 2.454
Relative water content (%)	87.43 ± 1.172	65.51 ± 0.586	72.35 ± 1.286
Anthocyanins (mg/g of FW)	0.20 ± 0.009	0.26 ± 0.009	0.29 ± 0.010
Carotenoids (mg/g of FW)	0.21 ± 0.008	0.25 ± 0.026	0.28 ± 0.028
Chlorophyll a (mg/g of FW)	0.31 ± 0.011	0.23 ± 0.016	0.32 ± 0.017
Chlorophyll b (mg/g of FW)	0.21 ± 0.012	0.16 ± 0.003	0.18 ± 0.010
Total soluble sugar (mg/g of FW)	24.48 ± 1.048	12.41 ± 0.966	27.03 ± 3.260
Starch (mg/g of FW)	24.00 ± 14.690	2.50 ± 2.055	21.44 ± 1.187
Superoxide (nmol/g of FW)	11.91± 0.754	18.27 ± 0.988	15.40 ±1.010
Antioxidant activity (mg/L)	254.70 ± 19.140	310.70 ± 11.680	368.30 ± 20.230

ds/m^2 at a temperature of 25°C and a salt accumulation ratio (SAR), and exchangeable sodium percentage (ESP) less than 13 and 15, respectively, is considered as saline soil (38). Sodium enters plant cells mainly through non-selective channels including glutamate receptor-like (GLRs) and HKT2 high-affinity K+ transporters and to a lesser extent through channels such as PIP aquaporins. With the entry of high amounts of sodium into the plant cell, first the osmotic pressure increases, and then the ionic balance inside the cell is destroyed, which in turn results in several physiological disorders, ultimately leading to a decrease in agricultural productivity. Among the most important of these processes may include seed germination, photosynthesis, electron transfer in the membrane, levels of antioxidants, and production of plant hormones (38, 39). With the increase of intracellular sodium concentration, increased osmotic pressure, caspase-like proteases and intracellular endonucleases are activated, leading to the start of programmed cell death (PCD) (39). Further, when sodium enters the cell, the process of K+ absorption is disturbed and the ratio of $\frac{Na^+}{K^+}$ decreases (40). Potassium plays a key role in controlling the activity of many intracellular enzymes (such as enzymes involved in the primary metabolism of the cell, including glycolysis, Kelvin cycle, and starch synthesis). During salinity stress, sodium replaces potassium ions and finally, the metabolic activities of the plant are severely disrupted (41).

The increase in osmotic pressure caused by salinity stress reduces the absorption of water in the plant and as a result, closes the stomata (42). The reduction of oxygen absorption in these conditions causes a sharp drop in photosynthesis. This in turn provides for the absorption of higher light levels in the leaf cell than needed and eventually, reactive oxygen species accumulate in the cell. Excessive accumulation of ROS causes lipid peroxidation of the cell membrane, damage to DNA, denaturation of proteins, decomposition of pigments, oxidation of carbohydrates, and disruption of enzyme activities (43, 44).

This research was aimed to investigate *Bacillus megatrium* isolated by Jafari et al. as PGPB, to improve tomato plant growth under stress conditions. Therefore, cell production with high biomass was highly considered. Response surface method and CCD design were applied to optimize factors affecting bacterial growth. The results showed that the sporulation phase in bacteria starts and reaches its maximum at 30-hr under the assumed growth conditions after 22-hr. A period of 8 hours was required to complete sporulation, and during this period, more than 48% of vegetative bacteria were transformed into spores (8.48e9 CFUs/ml). Our target phase was to produce a spore-based product because the stability of these products in the following environment is higher and they may easily tolerate unfavorable environmental conditions, including salinity stress. Next, the effect of produced PGPB on salinity stress amelioration in tomatoes was evaluated. Salinity stress was induced by watering the plants with a saline solution with an electrical conductivity of 6 dS/m.

Salinity stress causes the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS) in plants, which severely damages nucleic acids, enzymes, and metabolic processes (45). Plants respond to oxidative conditions by activating enzymatic (catalase, superoxide dismutase, peroxidase, etc.) and non-enzymatic mechanisms. The most important non-enzymatic compounds include ascorbate, flavonoids, carotenoids, anthocyanins, and phenolic compounds (46). In this study, stress increased the amount of superoxide anion by 53%, and at the same time, the antioxidant activity also increased by 22%. The use of *Bacillus megaterium* caused the antioxidant activity of the plant to increase by 45% compared to the control group, and subsequently, the superoxide concentration increased by only 30% compared to the control group. Santos also reported a decrease in ROS production and an increase in antioxidant defense after inoculation of *Bacillus megaterium* 1PB1 to tomato plants (47). Nikolaos Katsenios et al. also showed that the inoculation of PGPB strains increases the antioxidant activity. They stated that this antioxidant activity is directly related to carotenoid production and phenolic content (47, 48). Our results also demonstrated that PGPB increased the concentration of anthocyanins and carotenoids by 45 and 33% compared to the control group. Carotenoids have a double bond in their structure and are known as the most effective natural O_2 quencher. Anthocyanin is also one of the important phenolic compounds whose expression is increased during stress and prevents damage to the plant by scavenging oxidative compounds (49).

Salinity stress causes the stomata to close and reduce the amount of available CO_2. Further, the damage to photosynthetic systems greatly reduces the amount of stored carbohydrates in the plant

(50). In this research after salinity stress, there was a significant decrease in the levels of chlorophyll (a and b) and subsequent reduction of starch and TSS. Treatment with PGPB caused a significant increase in these pigments which revealed in productivities.

PGPB microorganisms may improve salinity stress in various ways. The osmolytes produced by them and increasing the expression of aquaporins (PIPs) may improve the water potential and hydraulic conductivity and prevent stomatal closure under these conditions (51, 52). Chauhan et al. showed that *Bacillus amyloliquefaciens* inoculation increases proline osmolyte and TSS in rice plants (53). The research showed that PGPB, in addition to osmolyte-synthesis, may also induce the expression of osmolyte synthesizing plant genes (6). In this research, the use of PGPB bacteria caused the relative water content of the leaves to improve by about 11% compared to the stress group, which may lead to the production of osmolytes.

PGPBs can produce plant hormones such as abscisic acid, gibberellin, and auxin. These hormones improve plant growth by improving food and water absorption, and control of stomata (54).

Exopolysaccharide (EPS) produced by PGPB may bind to (Na+) around the root zone, remove it from the reach of the plant, and thus help maintain ion balance. EPS also helps in soil accumulation, water retention, and chelation of metal ions in the plant under salinity conditions (55, 56). The tests conducted on *B. megaterium* RTS1 also showed that this bacterium has a high ability to produce EPS, which may be one of the effective factors in tolerating salinity stress and improving tomato plant growth under these conditions. The positive effect of this bacterium on salinity tolerance was indicated in the membrane stability index. The replacement of calcium with sodium in the wall structures is one of the reasons for membrane instability under salinity stress. The EPS produced by this bacterium has likely prevented its entry into plant cells by absorbing sodium, resulting in membrane instability.

It has been proven that some PGPBRs reduce the need for nitrogen supplementation required for plant growth directly through nitrogen fixation and mobilization or indirectly by stimulating nitrogen-fixing bacteria or changing plant root anatomy. It has also been proven that PGPBs also lead to higher absorption of nutrients by increasing the expression of arabidopsis nitrate and ammonium transporters which are vitally important in stressful conditions.

Plants produce ethylene and reactive oxygen species under salinity stress conditions, which leads to a decrease in plant growth. The plant directly uses aminocyclopropane-1-carboxylic acid (ACC) as a nitrogen source and produces ethylene. Some PGPB bacteria have the ability to produce ACC deaminase, which hydrolyzes ACC to ammonia and α-ketobutyrate, thereby reducing the amount of ethylene production. This has reduced the production of ethylene gas and ROS, thus preventing the destructive effects of salinity stress. Research has shown that these bacterial isolates contain the *acdS* gene that produces ACCD, which increases the activity of antioxidant enzymes such as SOD (superoxide dismutase) and CAT (catalase), APX (ascorbate peroxidase), POD (peroxidase), LPX (peroxidase lipid) and GPX (glutathione peroxidase). These enzymes play an important role in protecting cells from the harmful effects of ROS under salt stress conditions.

Most PGPR have the ability to produce hormones similar to plant hormones, including indoleacetic acid (IAA), gibberellic acid, cytokinins, and abscisic acid. IAA is the most important hormone produced by these bacteria, including bacilli. IAA can improve plant growth and yield under salinity stress. IAA increases root length and surface and indirectly increases nutrient absorption and plant growth in nutrient deficiency conditions. In fact, this hormone increases the formation of lateral roots, absorption of minerals and production of root secretions

A number of PGPB bacteria have the ability to produce abscisic acid. Abscisic acid (ABA) controls a wide range of physiological processes, including plant growth and resistance to adverse environmental conditions such as drought, cold, and salinity. This plant hormone, by increasing the amount of cytosolic calcium, caused the plant stomata to close and prevents the reduction of cell water during salt stress.

CONCLUSION

It is predicted that the increase of saline soils in the coming years will cause many problems in the development of sustainable and modern agriculture. Conventional methods for rehabilitating saline lands are mostly not economically feasible. Therefore, the use of PGPB microorganisms is a new and dynamic method to improve the tolerance of plants to salin-

ity. The results of this research showed that *B. megaterium* RTS1 is a suitable candidate for improving tomato growth in saline soils. This bacterium is isolated from the rhizoplane region of the plant and can create a suitable symbiosis with this plant. Further, the growth of bacteria with high cell density and high sporulation ability promises the possibility of producing an economic product with proper stability.

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