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RESEARCH PAPERS

Inoculation with Plant Growth-Promoting Bacteria (PGPB) Improves Salt Tolerance of Maize Seedling[1]

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Abstract– Our objective was to evaluate the role of plant growth-promoting bacteria to protect maize (*Zea mays* L.) plants against salt damage. *Bacillus aquimaris* DY-3 based on their 16S rDNA sequences, the most tolerant to salinity and the synthesis of indole acetic acid was selected for further studies. Strain was inoculated on maize roots growing in sterilized sand under salt stress conditions (1% NaCl). After one week, plant growth was promoted by bacterial inoculation regardless of salt stress and non-salt stress. Chlorophyll content, leaf relative water content, accumulation of proline, soluble sugar and total phenolic compound, and activities of superoxide dismutase, catalase, peroxidase and ascorbate peroxidase were enhanced, while lipid peroxidation levels and Na^+ content were decreased. The results showed that *B. aquimaris* DY-3 alleviated the salt stress in maize, likely through the integration of the antioxidant enzymes and the non-antioxidant systems that improve the plant response. Hence, the application of indole acetic acid synthesizing plant growth-promoting bacteria may represent an important alternative approach to decrease the impact of salt stress on crops.

Keywords: Zea mays, *Bacillus aquimaris*, antioxidant, salt stress, non-antioxidant systems

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INTRODUCTION

Excessive salt accumulation in soils is very detrimental to the sustainable development of agriculture, particularly in arid and semi-arid areas. Excessive soil salinity affects the establishment, development, and growth of crops, resulting in serious losses in productivity. Cultivated soils worldwide have become more salt because of suboptimal irrigation water, excessive fertilization, and desertification processes. Currently, more than 800 million hectares of land all over the world are affected by salt stress [1]. Sodium chloride (NaCl) as the predominant form of soil salinity, which can cause crop low productivity and even to death by making the roots water uptake more difficult and causing plant toxicity via accumulation of high concentrations of Na^+ and Cl^- in the plant [2]. Some physiological and biochemical responses such as protein synthesis, photosynthesis, lipid metabolism can be affected by salt [3]. However, most plants have developed the ability to decrease the negative effects of salinity via regulation and compartmentalization of ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathways [4]. Maize, one of the major agricultural crops in the world, is always utilized as food and feed. At the same time, maize is also an important industrial raw material. Therefore, maize is an important strategic material for food security and economic security. However, maize is considered to be a moderately salt-sensitive plant, and under irrigation, it can be subjected to salt toxicity [5]. In order to decrease the toxic effects caused by high salinity on plant growth, some approaches have been developed including the use of salt tolerant plants developed via both conventional breeding and molecular breeding and the application of plant growth-promoting bacteria (PGPB) [6].

PGPB are considered as microorganisms that can grow in, on, or around plant tissues, stimulating plant growth by a variety of mechanisms, such as synthesis of plant hormones, fixation asymbiotic nitrogen, solubilization of inorganic phosphate and mineralization of organic phosphate and/or other nutrients, and antagonism against phytopathogenic microorganisms [7]. Many studies have showed that PGPB could

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Abbreviations: APX—ascorbate peroxidase; CAT—catalase; Chl—chlorophyll; IAA—indole acetic acid; MDA—malondialdehyde; PGPB—plant growth-promoting bacteria; POD—peroxidase; ROS—reactive oxygen species; RWC—relative water content; SOD—superoxide dismutase.

alleviate plant stress caused by salt. For example, *Bacillus subtilis* and *Pseudomonas fluorescens* caused significantly increase in fresh and dry masses of roots and leaves, photosynthetic pigments, proline, total free amino acids and crude protein contents of *Raphanus sativus* compared to non-inoculated ones under salt stress [8]. *Hallobacillus* sp. SL3 and *Bacillus halodenitrificans* PU62 showed more than 90% increase in root elongation and 17.4% increase in dry wt when compared to non-inoculated wheat seedlings at 320 mM NaCl stress [1].

The present study was conducted in an attempt to isolate and characterize halotolerant bacteria from saline soils and evaluate their ability to ameliorate saline stress and improve seedling growth in maize under salt affected conditions.

MATERIALS AND METHODS

Strains isolation and cultivation. Bacterial strains were isolated from the salt-affected soil in Dongying city of Shandong province by the dilution plating technique on beef extract peptone agar plate, purified and checked for their cultural and morphological characteristics by microscopic examination.

Screening for salt-tolerance level. Bacterial strains were purified and grown on beef extract peptone agar plate. Nutrient broth (50 mL) was supplemented with final concentrations of NaCl (5, 10, 15, 16, 17 and 18% (w/v)). Each flask was then added with actively growing selected bacterial strain and incubated on rotary shaker (180 rpm) at 30°C. Bacterial growth was determined as OD600 to find out salt-tolerance.

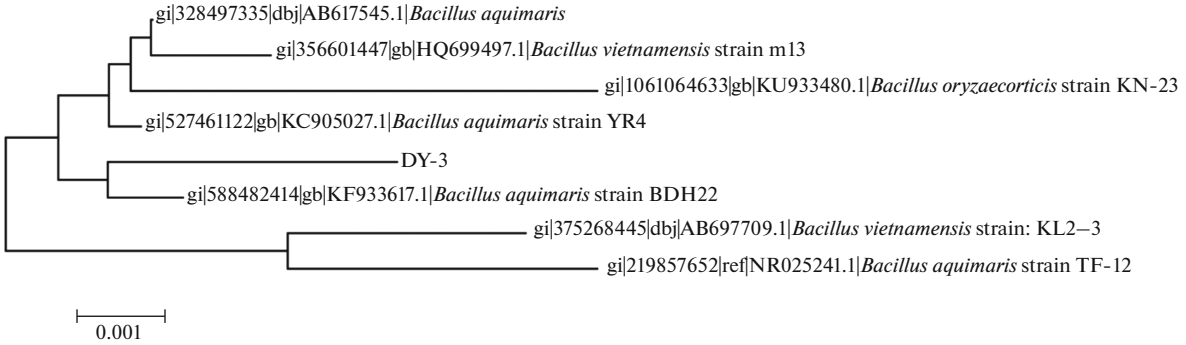
Indole acetic acid (IAA) product in the presence of 10% of NaCl. The IAA production pattern of adapted cultures was studied in the presence of 10% NaCl in King's B medium. The method of IAA production was carried out as described by Glickmann and Dessaux [9] except that the medium was supplemented with final concentrations of 10% NaCl.

Bacterial strain identification by 16S rDNA. On the basis of initial salt-tolerance and IAA production screening, strain DY-3 DNA was extracted by using rapid bacterial genomic DNA isolation kit (Sangon, China) according to the manufacturer's instructions, and was identified on the basis of complete 16S ribosomal DNA sequence. The 16S rDNA was amplified by PCR with the forward 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse 1492R primer (5'-GGTTACCTTGTTACGACTT-3'). The BLAST search program was used to compare the sequence homology of nucleotides. The closely related sequences obtained were aligned through CLUSTALW using MEGA version 5.1 software package, and the phylogenetic tree was constructed using the neighbor joining (NJ) method. The bootstrap replications (1000) were used as a statistical support for the nodes in the phylogenetic tree.

Seedling inoculation with PGPR. Log phase culture of selected isolates DY-3 with an OD600 0.50 was used. The culture was centrifuged at 5000 rpm for 5 min and the pellet was washed three times with sterile distilled water and suspended in sterilized solutions of the treatments prior to seedling inoculation.

After passed through a 2 mm sieve to remove large particles of stones and debris, sand was sterilized and put in plastic pots (volume 150 mL), 100 g of sterilized sand per pot. Hoagland nutrient solution content in sterilized sand was 10% (v/w). Salt treatment was performed by supplementing the nutrient solution with final concentrations of 1% NaCl. Healthy maize (*Zea mays* L.) seeds var. Jundan20 were surface sterilized with 0.5% sodium hypochlorite for 10 min, and rinsed with distilled water and germinated under dark conditions at 25°C on moist filter paper for 3 days. Then 3 days old seedlings selected for uniform growth were transplanted into sand culture. The solutions were renewed every 2 days. The treatments were as follows: CK—seedlings in sand with Hoagland nutrient solution only; T1—seedlings in sand with Hoagland nutrient solution containing 1% NaCl; T2 —seedlings in sand with Hoagland nutrient solution inoculation with bacterial; and T3 – T1 inoculation with bacterial. There were 3 replications for each treatment and 10 seedlings per replication. After 10 days, the seedlings were harvested, washed carefully in tap water and surface dried with filter paper. Then plants were separated into two parts: one part was at 105°C for 30 min then oven at 80°C up to constant weight, another part was used for determination of biochemical index.

Assay for biochemical index. The relative water content (RWC) of the leaf sample was estimated by the method of Ghahfarokhi et al. [10] and expressed in percentage. Chlorophyll content was measured with a SPAD 502 Plus meter [11]. The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content, the proline content was determined after extraction at room temperature with 3% 5-sulfosalicylic acid, soluble sugar content was determined using the anthrone–sulphuric acid method [12]. Na^+ content was measured in dry plant material with concentrated H_2SO_4 with a flame photometer and expressed in percentage. Total phenolic content was determined calorimetrically using folin-ciocalteau reagent as described by Rojas-Tapias et al. [4] with a little modification. The extract (0.5 mL) was mixed with 1.0 mL of folin-cioalteau reagent and allowed to stand at room temperature for 3 min. A 1.5 mL sodium bicarbonate solution (10%) was added to the mixture. The tubes were incubated in darkness for 1 h at room temperature. After incubation absorbance of the mixtures at 725 nm was read. The content of total phenolic compound was calculated from calibration curve (standard: *p*-hydroxybenzoic acid) and estimated as μg/g fr wt.



Phylogenetic tree of DY-3.

A fresh sample (500 mg) was homogenized in 10.0 mL of 0.05 M phosphate buffer (pH 7.8) solution and centrifuged at 10000 *g* for 10 min. The supernatant was collected and stored at 4°C for assay of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). SOD activity was determined according to the method of Zhu et al. [12]. Catalase activity was measured as the decrease of absorbance at 240 nm as a consequence of H_2O_2 consumption, POD activity was determined according to optical density change per minute at 240 nm [10]. APX activity was determined as the decrease in absorbance of ascorbate at 290 nm as oxidized ascorbic acid [13].

In all the experiments, analysis of variance (ANOVA) was conducted using Statistics SPSS 17.0 software. The means of each treatment were compared using Duncan′s multiple range test at 0.05 level. The data are shown as the means and their standard errors.

RESULTS

According to morphological trait, a total of 12 bacterial strains were isolated as it showed the best performance with 17% NaCl tolerance and the synthesis of IAA among all isolated strains. IAA production of the bacteria in the presence of 10% NaCl (10.53 ng/mL) in the king's B medium was lower than that of without NaCl (62.01 ng/mL). Results of BLAST search of the national center of biotechnology information (NCBI) and phylogenetic analysis revealed that bacteria have 99.0% sequence homology with *Bacillus aquimaris* (Accession No. KF933617). Therefore, the strain was identified as *B. aquimaris* DY-3 (accession no. KU167484) (figure).

Compared to the normal condition, dry weight of salt-treated maize seedlings decreased significantly by up to 33.63%. However, inoculation with *B. aquimaris* DY-3 alleviated the inhibitory effect of salt stress and improved maize seedling dry weight by up to 31.73% at the 1% NaCl (Table 1), but dry wt of maize seedlings was low to the normal condition. Inoculation with *B. aquimaris* DY-3 also improved maize seedling dry wt by up to 12.29% at the normal condition.

Compared to the normal condition, leaf RWC and Chl content decreased significantly by up to 14.29% and 13.01% by salt stress. However, inoculation with *B. aquimaris* DY-3 improved significantly RWC and Chl content in salt-treated maize seedlings by up to 3.23 and 4.27%, but RWC and Chl content were low to the normal condition. Inoculation with *B. aquimaris* DY-3 also improved RWC and chlorophyll content by up to 2.50 and 5.20% at the normal condition. Compared to the normal condition, MDA content in salt-treated maize seedlings increased significantly by up to 39.42% (Table 1). However, inoculation with *B. aquimaris* DY-3 caused a 9.55% decrease in MDA content in salt-treated maize seedlings, but MDA content was higher to the normal condition. Inoculation with *B. aquimaris* DY-3 also decreased MDA content by up to 7.68% at the normal condition (Table 1).

Compared to the normal condition, proline, soluble sugars, total phenolic compound and Na^+ contents

Table 1. Effects of salt stress and *Bacillus aquimaris* DY-3 on dry wt, RWC, Chl content and MDA content of maize seedlings

Treatments	Dry wt, mg/plant	RWC, %	Chl, SPAD	MDA, μmol/g fr wt
CK	181.4 ± 0.4b	86.66 ± 0.54b	26.9 ± 0.2b	5.86 ± 0.31c
T1	120.4 ± 0.8d	74.28 ± 0.33d	23.4 ± 0.5c	8.17 ± 0.38a
T2	203.7 ± 0.7a	88.83 ± 0.71a	28.3 ± 0.8a	5.41 ± 0.43c
T3	158.6 ± 0.5c	76.68 ± 0.68c	24.4 ± 0.7c	7.39 ± 0.13b

Lowercase letters in the same column represent differences in different treatments

Table 2. Effects of salt stress and *Bacillus aquimaris* DY-3 on proline, soluble sugars, total phenolic compound and Na^+ content of maize seedlings

Treatments	Proline, mg/g fr wt	Soluble sugars, mg/g fr wt	Total phenolic, mg/g fr wt	Na^+, %
CK	3.49 ± 0.48c	10.62 ± 0.11c	43.57 ± 4.97c	0.64 ± 0.04c
T1	4.76 ± 0.31b	12.07 ± 0.67b	53.09 ± 2.70b	1.91 ± 0.09a
T2	4.34 ± 0.28b	11.54 ± 0.28b	48.22 ± 4.61bc	0.63 ± 0.07c
T3	6.16 ± 0.25a	13.43 ± 0.52a	72.97 ± 3.47a	1.55 ± 0.13b

Lowercase letters in the same column represent differences in different treatments

Table 3. Effects of salt stress and *Bacillus aquimaris DY-3* on the activities of SOD, POD, CAT and APX of maize seedlings

Treatments	SOD, U/g fr wt	POD, U/g fr wt	CAT, U/g fr wt	APX, U/g fr wt
CK	18.29 ± 0.76d	14.49 ± 0.38b	9.79 ± 0.57c	1.90 ± 0.26c
T1	22.05 ± 0.70b	16.77 ± 0.12a	11.55 ± 0.44b	2.33 ± 0.03b
T2	20.64 ± 0.58c	12.77 ± 0.34c	10.68 ± 0.40bc	2.07 ± 0.13bc
T3	27.99 ± 0.48a	14.18 ± 0.40b	13.88 ± 0.47a	3.13 ± 0.04a

Lowercase letters in the same column represent differences in different treatments

in salt-treated maize seedlings increased significantly by up to 36.39%, 13.65%, 21.85% and 198.44% (Table 2). Inoculation with *B. aquimaris* DY-3 further significantly increased proline, soluble sugars and the total phenolic compound contents in salt-treated maize seedlings by up to 29.41, 11.27 and 37.45%. However, Na^+ content was reduced significantly by 18.85%. In normal condition, *B. aquimaris* DY-3 also increased proline, soluble sugars and the total phenolic compound contents by up to 24.36, 8.66 and 10.67%, and decreased Na^+ content by 1.56% (Table 2).

Compared to the normal condition, activities of SOD, CAT, POD and APX in salt-treated maize seedlings significantly increased. Compared to salt stress alone, activities of SOD, CAT and APX in salt-treated maize seedlings inoculated with *B. aquimaris* DY-3 have a significant enhancement by up to 26.94%, 20.17% and 34.33% (Table 3). Meanwhile, *B. aquimaris* DY-3 also enhanced activities of SOD, CAT and APX in normal maize seedlings. Compared to salt stress alone, POD activity decreased by up to 15.44% in salt-treated maize seedlings inoculated with *B. aquimaris* DY-3. Meanwhile, *B. aquimaris* DY-3 also decreased POD activity in normal maize seedlings (Table 3).

DISCUSSION

Salt has been shown to inhibit plant growth and interfere with important cellular processes, leading to reduced biomass. A possible factor of the inhibitory effect of salt on plant dry weight is that salt stress causes nutrient imbalances and increases ion-deficiency, which decreases the ability of plant to take up water and nutrient [2]. In this study, our results also showed salt stress decreased significantly dry weight of maize seedlings. Some microorganisms, particularly PGPR, can improve plant performance under stressful environments by providing with fixed nitrogen, phytohormones, soluble phosphate, and etc. [14]. Therefore, an isolate of *B. aquimaris* DY-3 was tested for effectiveness on maize seedlings treated with 1% NaCl. The results clearly indicate that the *B. aquimaris* DY-3 significantly improved seedling biomass by improving salt tolerance through the bacteria production of IAA, which are in line with the previous reports. For example, the IAA producing bacterial strains *Hallobacillus* sp. SL3 and *Bacillus halodenitrificans* PU62 showed more than 17.4% increase in dry wt when compared to non-inoculated wheat seedlings at 320 mM NaCl stress indicating a significant reduction of the deleterious effects of NaCl [1]. The IAA producing bacterial strain *Pseudomonas putida* TSAU1 significantly increased soybean seedling dry wt in non-salt condition and at 100 mM NaCl compared to control plants [15]. Leaf chlorophyll content is an important physiological parameter which can serve as an indicator of plant stress. Some studies have shown that salt stress decreases chlorophyll content in many plants, such as green bean [16], cucumber [17], basil [18]. In this work, salt stress also decreased chlorophyll content in maize seedlings. Reduced chlorophyll had a negative effect on plant photosynthesis, which may be a reason for a decrease in maize seedling dry wt. This may be attributed to higher salt inhibited synthesis of a precursor of chlorophyll of 5-aminolevulinic acid, causing a decrease in chlorophyllase [19]. However, it was observed that inoculation with *B. aquimaris* DY-3 significantly increased the chloro-

phyll content of maize plants compared with and without salt treatments. Rojas-Tapias et al. [4] also observed enhanced chlorophyll content in maize upon inoculation with *Azotobacter* strains. Jha and Subramanian [20] also suggested that inoculation with *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* enhanced chlorophyll content of paddy rice compared with and without salt control. The increase in chlorophyll content may also be the result of an increase in chlorophyll biosynthesis.

Water deficit caused by high salt, as one of the serious factors to limit plant growth, has negative effects on almost all metabolism in plants. Leaf relative water content has been proposed as an important indicator of water status [21]. In this study, maize seedlings inoculated with *B. aquimaris* DY-3 under non-salt as well as salt has greater relative water content which is accordance with Jha and Subramanian [20], who suggested that inoculation with *P. pseudoalcaligenes* and *B. pumilus* resulted in an increase of relative water content. The increase of leaf relative water content in maize seedlings under salinity suggests the role of osmoprotectants in preventing cell dehydration from salt stress.

MDA, a lipid peroxidation product, is considered to be an indicator of cell membrane oxidative damage by ROS and is accumulated in tissues when plants are exposed to salt stress [22]. Our results showed a marked increase in MDA content was induced by salt stress, indicating that the presence of salt stress might enhance membrane lipid peroxidation, consequently causing an increase in membrane permeability, exosmosis of electrolytes and, finally, injury to cell membrane systems. However, a significant reduction of up to 9.55% in MDA with *B. aquimaris* DY-3 compared to salt treatment. The lower MDA content indicates that seedlings inoculated with *B. aquimaris* DY-3 are more tolerant to oxidative stress under these conditions, which is consistent with the decrease of the Na^+ content. These suggest *B. aquimaris* DY-3 reduced the ion toxicity caused by Na^+. Similar results were obtained in maize plant under abiotic stress and inoculated with mycorrhiza [12], where MDA content in mycorrhizal plants was lower than that in non-mycorrhizal plants.

The soluble sugars are important osmotic adjustment substances in plant cells, accumulation of which contribute to regulate osmotic stress in plant cells and leads to preservation of biological molecules and membranes [23]. Salt stress usually results to soluble sugars increase in plants. For example, Gandonou et al. [24] reported that soluble sugars concentrations increased significantly in leaves and roots of sugarcane under salt stress. In this work, our results showed soluble sugar content increased in maize seedlings at 1% NaCl. Proline is also an important osmotic adjustment substance in plant cells, accumulation of which in plants is a general response to various abiotic stresses [25]. Actually, increased proline in plant stress is a defensive mechanism to salt stress. Our results show that proline content in salt-treated maize seedlings has a significant increase, which are agreement with previous reports. Studies on osmoregulation in plants treated with bacteria showed that bacteria made a major contribution to osmotic adjustment in plants under abiotic stress [26]. In present study, the increase in osmotic solutes in the presence of *B. aquimaris* DY-3 compared to salt treatment alone could possibly provide an adaptive mechanism to maintain a favorable osmotic potential under salt toxicity. Similar results were observed by Feng et al. [27] who found colonization with the arbuscular mycorrhizal fungus could improve significantly soluble sugars content in maize seedlings treated with salt, suggested a higher osmoregulating capacity of these plants.

Various abiotic stresses, including salinity, lead to the overproduction of reactive oxygen species (ROS) in plants, such as the superoxide radical, hydrogen peroxide and hydroxyl radical, which causes damage to biochemical process and ultimately results in oxidative stress. However, evolution has equipped plants with a wider range of defense measures including the antioxidant enzymes and the non-antioxidant metabolites to eliminate ROS [28]. Several major antioxidant enzymes, including SOD, POD, CAT and APX, are involved in this process. In this work, activities of SOD, POD, CAT and APX significantly increased in maize seedlings with salt stress, this might counter the toxicity generated by salt stress. It is discovered that inoculation with some beneficial bacteria can alleviate negative effect on plant growth via increasing the antioxidant enzyme activity under abiotic stress, such as *Bacillus subtilis* SU47, *Glomus etunicatum*, and etc. [12, 29]. In our study, the observed increase in antioxidant enzyme activity might be sufficient to explain the vital role of *B. aquimaris* DY-3, which alleviated the toxic effect of salt on maize seedlings also via increasing the antioxidant enzyme activity. Phenolic compounds have been studied as non-enzymatic antioxidants that protect plants against the damaging effects of increased ROS levels due to salt stress [30]. In this study, salinity significantly increased total phenolic compounds content in maize leaves and bacterial inoculation also improved the total phenolic compounds content compared with the respective non-inoculated control. Our results are agreement with Rojas-Tapias et al. [4] who found that inoculation with PGPB *Azotobacte*r strains C5 and C9 could enhance content of polyphenol in maize leaves and alleviated the saline stress in maize.

We demonstrated that inoculation with *B. aquimaris* DY-3 protected plants against the inhibitory effects of NaCl stress. We argue on the basis of our findings that bacterial amelioration of salt stress could be the integration of several aspects including increasing plant antioxidant capacity, improving relative water content and chlorophyll content, proline, solu-

ble sugars and total phenolic compound contents, reducing Na^+ content by inoculation with *B. aquimaris* DY-3. The results indicated that bacteria could ameliorate negative effects on maize seedlings caused by high levels of NaCl via increasing the antioxidant enzymes and the non-antioxidant systems. Therefore, application of *B. aquimaris* DY-3 may be the promising technique to decrease the deleterious effects of salt stress.

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