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Effect of biofilm forming plant growth promoting rhizobacteria on salinity tolerance in barley

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Abstract Formation of biofilm under varying stress conditions is a significant strategy adopted by bacterial strains for their successful survival in plant rhizosphere. In this study, the activity of biofilm formation of 20 isolates and strains of plant growth promoting rhizobacteria (PGPR) was determined under different salt concentrations. The results indicated that all of the 20 PGPRs have the activity of biofilm formation under 0.0, 250, 500 or 1000 mM NaCl which was increased with increasing salt concentration. PGPR strains with the highest activity of biofilm formation were selected and used to coat barley grains. The coated grains were sown in clay/sandy soil and left to grow for 25 days. The results showed that bacterial inoculation was effective in alleviating the deleterious effect of salinity on some growth criteria (seedling length, fresh and dry masses as well as relative water content), compared with the control. The isolate HM6 (B6), which showed the highest activity of biofilm formation at all the studied NaCl concentrations, was identified using 16S ribosomal RNA gene amplification and sequencing of the PCR product. The similarity sequence analysis indicated that HM6 isolate has 97.4% similar sequence identity to *Bacillus amyloliquifaciens*. It could be speculated that the bacterial activity of biofilm formation is helpful for improving salt stress tolerance of barley.

Introduction

Barley (*Hordeum vulgare* L.) is a highly adaptable cereal grain and ranks 5th among all crops for dry matter production in the world. In addition, it is an important food source in many parts of the world (Gupta et al., 2010). Although barley is regarded as salt tolerant among crop plants, its growth and

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development are severely affected by ionic and osmotic stresses in salt-affected soils (Mahmood, 2011).

Saline soils are a major problem for agriculture because salt turns agronomically useful lands into unproductive areas. The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of crop land in the world are salt-stressed (Flowers and Yeo, 1995). Soil salinization is reducing the area that can be used for agriculture by 1–2% every year, hitting hardest in the arid and semi-arid regions (FAO, 2002). Soil salinity induces water stress, nutritional imbalance, specific ion toxicity, hormonal imbalance and generation of reactive oxygen species which may cause membrane destabilization (Omar et al., 2009). Moreover, it decreases the yield of many crops as salt inhibits plant photosynthesis, protein synthesis and lipid metabolism (Paul and Lade, 2014).

Plant growth under stress conditions may be enhanced by the application of microbial inoculation including plant growth promoting rhizobacteria (PGPR). These microbes can promote plant growth by regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients and inducing resistance against plant pathogens (Boostani et al., 2014). Certain strains of PGPR belonging to *Bacillus*, *Enterobacter*, *Burkholderia*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* are now being used worldwide with the aim to enhance crop productivity (Bharti et al., 2013). There has been a great interest in eco-friendly and sustainable agriculture with emphasis on the use of beneficial microorganisms. The benefits of PGPR for plants growing in saline soils were reported as an enhancer of root and shoot growth, nutrient uptake, hydration, chlorophyll content, and resistance to diseases (Qurashi and Sabri, 2012). These PGPRs stimulate plant growth and enhance plant biomass and their beneficial effects have been demonstrated in many agricultural crop species such as wheat, tobacco, *Brassica juncea*, tomatoes, bell peppers, cucumbers and barley as reviewed by Kang et al. (2014).

PGPRs are effective in colonizing the plant root and further multiply into microcolonies and/or produce biofilm as a result of a successful plant-microbe interaction. The plant associated biofilms are highly capable of providing protection from external stress, decreasing microbial competition, and giving protecting effects to the host plant supporting growth, yield and crop quality (Asari, 2015).

The present study aims to do the following: (a) determine the best biofilm forming PGPR isolates and strains by determining their activity of biofilm formation under several salt concentrations, and (b) evaluate the effect of these PGPRs on the growth of two barley cultivars (Giza 123 as salt sensitive and Giza 2000 as salt tolerant) growing under different levels of salinity.

Material and methods

Material

Barley cultivars

Grains of two barley cultivars (*H. vulgare* L.), cv. Giza 123, as salt sensitive (cv.1) and Giza 2000, as salt tolerant (cv.2) were obtained from Barley Department, Agricultural Research Center, Giza, Egypt.

Plant growth promoting rhizobacterial isolates and strains

Twenty different plant growth promoting rhizobacteria (PGPR) isolates and strains B (1–20) were used in this study (Table 1). From these 20 PGPRs eight bacterial isolates (isolates HM1-HM8) were previously isolated from salinized rhizosphere of wheat plant and characterized as salt tolerant isolates by Hewait (2010) and Omar et al. (2013). The other twelve reference strains were obtained from Microbiology Department, Soil, Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC), Giza, Egypt. The isolates and reference strains were maintained for long-term storage at −70 °C in Nutrient Broth (NB) with 30% glycerol.

Soil

The soil used in the present experiment was clay-sandy soil (2:1 w/w). Its characters and composition are illustrated in Table 2. A mineral fertilizer (N, P, K) was applied according to the recommendations of the Egyptian Ministry of Agriculture.

Methods

Selection of the best biofilm forming PGPR under different salt concentrations

The 20 PGPR strains and isolates were screened for their potency of biofilm formation in nutrient broth (NB) which was prepared according to Difco (1985). The qualitative and quantitative biofilm formation assays were carried out as follows:

Qualitative assay

Bacterial cultures of 20 PGPR B (1–20) were grown in NB medium with different NaCl concentrations (0.0, 250, 500 and 1000 mM) for 24 h without agitation. Thereafter, the

Table 1 Names and codes of 20 PGPR isolates and strains (B 1–20).

Code	PGPR
B1	Isolate HM1
B2	Isolate HM2
B3	Isolate HM3
B4	Isolate HM4
B5	Isolate HM5
B6	Isolate HM6
B7	Isolate HM7
B8	Isolate HM8
B9	*Bacillus megatherium*
B10	*Pseudomonas fluorescens*
B11	*Bacillus circulans*
B12	*Paenibacillus polymyxa*
B13	*Azotobacter chroococcum*
B14	*Azospirillum* sp.
B15	*Paenibacillus polymyxa2*
B16	*Azospirillum brasilense* NO40
B17	*Hyderella* sp.
B18	V1B1C1
B19	Mr16
B20	Ssbr

Table 2 Physical and chemical properties of the soil.

Textural class	Clay Loam
Organic materials (%)	1.43
$CaCO_3$ (%)	0.43
PH (1 soil: 2.5 water)	7.66
EC (dS m^{-1})	36.50
Anions (cmol/kg)	
Ca^{2+}	41.82
Mg^{2+}	16.46
K^{+}	0.58
Na^{+}	62.0
Cations (cmol/kg)	
SO_4^{2-}	59.48
Cl^{-}	58.85
HCO_3^{-}	2.55
CO_3^{2-}	Nil
Macro elements (g/kg)	
N	1.441
P	0.644
K	10.782
Micro elements (g/kg)	
S	0.0196
Fe	12.561
Mn	0.185
Zn	0.131
Cu	0.0742
Se	0.0007

biofilm formation was tested according to Christensen et al. (1985). These high salt concentrations were used only for bacteria in order to make sure that the bacterial strains and isolates can tolerate the tested salt concentrations applied to barley cultivars.

Quantitative assay

For bacterial biofilm formation under different NaCl concentrations (0.0, 250, 500 and 1000 mM), the microliter plate assay was used according to Auger et al. (2006) where the biofilm formation was quantified by measuring the absorbance at 570 nm in a microliter plate reader.

From the results of this screening experiment, the bacterial strains B (5–13) were selected as the best biofilm forming bacteria at 500 mM NaCl (Table 3). Therefore, they were used as inoculants for barley grains to test their effect on salt tolerance of barley seedlings of both cultivars in the greenhouse experiment.

Effect of bacterial biofilm on salt tolerance of barley seedlings

A pot experiment was carried out to evaluate the effect of the selected biofilm forming PGPR on salt tolerance of barley seedlings. It was conducted during the winter season of 2013/2014 at the experimental greenhouse of Microbiology Department, SWERI, ARC, Giza, Egypt. Grains of the two barley cultivars, Giza 123 (cv.1) and Giza 2000 (cv.2) were inoculated with the previously selected 9 biofilm forming PGPR isolates and strains (B 5–13) or NB media as control.

The 9 PGPR strains and isolates were streaked on nutrient agar (NA) plates. Then, according to Ibrahim and Omar (2015), a single colony was picked to inoculate 250 ml of NB medium and left to grow overnight at 30 °C with shaking. This culture was used for coating barley grains and as liquid inoculation. Barley grains were selected for uniformity and size and grouped in equal numbers. Each group was coated with 26 ml of each culture which was loaded on definite vermiculite carrier in small plastic bag (30 g/bag) and incubated for 24 h at 37 °C. To improve the adhesion of the carrier particles to the grains, 5 ml of the Arabian gum solution was added and the mixture was left to dry for 2 h, and then six of these coated grains were sown in each plastic pot (9 cm depth × 8 cm width), which was previously filled with 300 g of clay-sandy soil. After that, 5 ml of fresh culture as liquid inoculation was mixed with irrigation water and added to each pot. Nutrient broth was used as control. The grains were left to grow for 25 days at 25 ± 2 °C and 16/8 h day/night and irrigated every other day with either 150 (S1), 200 (S2), 250 (S3) or 350 (S4) mM NaCl, or with tap water as control. These concentrations were selected for only for barley and they were different from those used for bacteria. The pots were leached with water once a week. Each treatment was represented with three pots.

Table 3 List of the best biofilm forming PGPR, B (5–13) at 500 mM NaCl.

Code	PGPR
B5	Isolate HM5
B6	Isolate HM6
B7	Isolate HM7
B8	Isolate HM8
B9	*Bacillus megatherium*
B10	*Pseudomonas fluorescens*
B11	*Bacillus circulans*
B12	*Paenibacillus polymyxa*
B13	*Azotobacter chroococcum*

From each treatment, six seedlings were used for measurements of growth criteria: root depth, shoot height, fresh mass (FM) and dry mass (DM) of roots and shoots. The relative water content (RWC) of shoots and roots was determined according to Sharp et al. (1990) as follows:

$$\mathrm{RWC} = \left(\frac{\mathrm{FM} - \mathrm{DM}}{\mathrm{TM} - \mathrm{DM}}\right) \times 100$$

where the turgid mass (TM) was estimated as the weight after holding shoots and roots in 100% humidity conditions in the dark at 4 °C for 4 h. Then, the samples were dried at 60 °C to estimate the dry mass (DM).

From the results of these two preliminary experiments, B6 was selected as the best isolate of the tested 9 PGPR isolates and strains that showed the highest activity of biofilm formation and induced the highest salt tolerance for sensitive barley cultivar (Giza 123) grown at 250 mM NaCl.

Bacterial identification

Gram staining and colony and cell morphology were carried out to characterize the selected isolate HM6 (B6). Moreover, the molecular characterization of the bacterial isolate was carried out with the help of SolGent Company, Daejeon, South Korea. Fresh bacterial cultures were cultivated on nutrient agar at 28 °C for 3 days. A small amount of the bacterial

isolate was scraped and suspended in 100 µl autoclaved distilled water in 2 ml sterile vials and boiled at 100 °C for 15 minutes. The non-living cells were sent to SolGent Company for rRNA gene PCR amplification and sequencing. Firstly, the bacterial DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the 16S ribosomal rRNA gene was amplified using PCR technique in which two universal 16S bacterial primers 27F (forward) and 1492R (reverse) were incorporated in the PCR reaction mixture. Primers used for gene amplification were having the following sequences: 27F (5′-AGA GTT TGA TCC TGG CTCAG-3′), and 1492R (5′-TAC GGY TAC CTT ACG ACT T-3′). Secondly, the purified PCR products were confirmed (using size marker) by electrophoresis on 1% agarose gel. Finally, the PCR product was eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) into the reaction mixture.

Sequences were further analyzed using BLAST software on the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of partial 16s rRNA gene sequences was done using MegAlign software of DNA Star package version 7.01.

Statistical analysis

All data of the experiments were replicated three times and the presented data are the mean values. The obtained results were subjected to analysis of variance (ANOVA) (type of analysis depended on the factors affected the experiment) to determine the significance between treatments using Costat software (CoHort software, California, USA).

Results

Selection of the best biofilm forming PGPR under different salt concentrations

Fig. 1 shows the values of absorbance at 570 nm as a measure of optical density (OD) which reflected the activity of biofilm formation of 20 isolates and strains of PGPR -B (1–20)- in NB cultures supplemented with four concentrations of NaCl (0.0, 250, 500 and 1000 mM).

The results indicated that the activity of biofilm formation was increased with increasing NaCl concentration, specifically at 500 mM NaCl. The highest significant increase was recorded in nine strains of PGPR treated with 500 mM NaCl. These PGPR(s) were the isolates HM (5–8) and the reference strains (*Bacillus circulans*, *Pseudomonas fluorescens*, *Paenibacillus polymyxa*, *Bacillus megatherium* and *Azotobacter chroococcum*). However, the data indicated also that the biofilm formation was inhibited in all the tested PGPRs treated with 1000 mM NaCl, except in *Azospirillum brasilense* (NO40), V1B1C1, *B. megatherium* and *A. chroococcum*.

Effect of bacterial biofilm on salt tolerance of barley seedlings

The effect of bacterial inoculation with the selected nine biofilm forming PGPR B (5–13) on some growth criteria of 25-days-old barley seedlings cv.1 (Giza 123) and cv.2 (Giza 2000) grown in clay-sandy soil (2:1 w/w) under four salt concentrations (150, 200, 250 and 350 mM NaCl) is represented in Fig. 2 for shoots height and roots depth; Fig. 3 for fresh mass (FM); Fig. 4 for dry mass (DM) and Fig. 5 for relative water content (RWC).

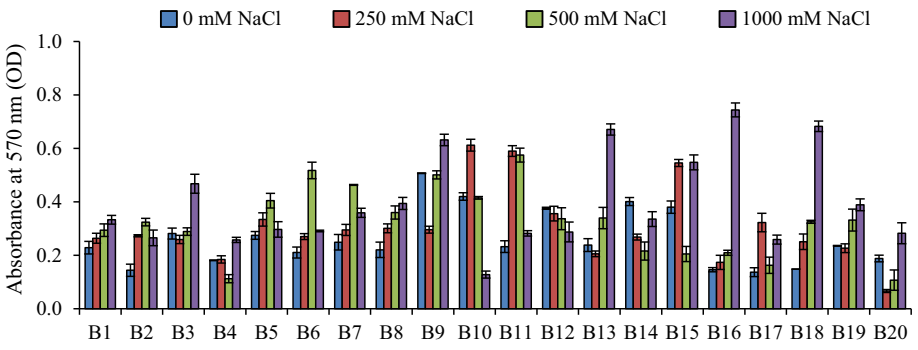
Fig. 2 shows that shoot height and root depth of the uninoculated-salt stressed seedlings decreased significantly with increasing NaCl levels. However, cv.1 was more affected by salt stress, compared with cv.2, where in cv.1, under 250 mM NaCl, the shoot height and root depth were significantly decreased by 35% and 10%, respectively compared with the uninoculated unstressed corresponding control. Concerning bacterial inoculation, it showed significantly better responses than cv.2; where it was found that the inoculation with B6 significantly enhanced the shoot height and root depth in cv.1 by 10 and 14%, respectively, compared with their uninoculated counterparts. On the other hand, in the stressed-B8 treated seedlings of cv.1, shoot height was significantly increased by 15% while root depth was increased by 6% at 250 mM NaCl (Fig. 2).

The results in Fig. 3 showed that the salt stress significantly reduced the fresh mass (FM) of shoot and root; however, the application of PGPRs significantly alleviated this reduction, compared with the uninoculated salt-stressed samples. Under the treatment with 250 mM NaCl, the inoculation with B8 enhanced the shoot and root FMs by 64 and 35%, respectively, compared with their uninoculated counterparts.

The effect of inoculation on dry masses (DMs) of the salt-stressed barley seedlings is represented in Fig. 4. Generally, under salt stress, the bacteria-inoculated seedlings showed higher accumulation of DMs of shoots and roots, compared with their corresponding uninoculated ones. The inoculation with B9, B6 and B8 resulted in the highest increase in root DMs, which were 0.015, 0.013 and 0.012 g, respectively comparing to 0.011 g in the uninoculated-stressed treatment at 250 mM NaCl in cv.1. However, at 350 mM NaCl, the inoculation with these bacteria also increased the DMs of roots higher than those in the uninoculated-stressed ones and the inoculation with B6 was the most effective, where it increased the DM of root by 140%, relative to the control. The same pattern was recorded for shoots of cv1, where B8 and B6 enhanced the accumulation of DMs of shoots by 41% and 43%, respectively at 250 mM NaCl, while at 350 mM, they were 58% and 51%, respectively relative to the control.

As shown in Fig. 5, salt stress induced a massive decrease in the RWC of shoot and root of cv.1 by 48% and 16%, respectively relative to uninoculated unstressed counterparts. On the other hand, the bacterial inoculation significantly diminished these reductions, where the RWC of shoots of B6 and B5 treated seedlings were increased by 7%, while those of roots of cv.1 inoculated with B11, B6 and B8 were increased by 15%, 13%, 12%, respectively in cv.1 under 250 mM NaCl compared with the uninoculated stressed counterparts.

Depending on results of the present experiments, the isolate HM6 (B6) was selected for further study because it showed the highest activity of biofilm formation at the three used NaCl concentrations. Also, it enhanced the growth of barley seedlings under salt stress and induced significant recovery for them which was represented by the greatest shoot height, root depth, fresh and dry masses and relative water contents (Figs. 2–5).



F values of 2-way ANOVA at $P \leq 0.01$ (*= Significant)

Treatments	Absorbance at 570 nm	
	F value	significancy
PGPR	138.59	*
Salt	343.22	*
PGPR×Salt	99.32	*

Fig. 1 The optical density (OD) (at 570 nm) as a measure of the activity of biofilm formation of 20 isolates and strains of PGPR B (1–20) in NB cultures supplemented with four concentrations of NaCl (0.0, 250, 500 and 1000 mM). Error bars represent the standard deviation between 3 replicates.

Molecular identification of the best PGPR isolate (B6) using 16S rRNA sequencing

Morphological tests conducted on nutrient agar plates of the selected isolate HM6 (B6), which showed the highest biofilm activity and enhanced the growth of barley seedling under salt stress, showed that this isolate is a gram positive bacillus which produced white wrinkled colonies with irregular form and lobed margin (Fig. 6).

For the isolate HM6, the total sequence length of the PCR product was 1484 nucleotides. Comparison of the HM6 nucleotide sequence with those in NCBI database using BLAST tool revealed that HM6 has a highest sequence similarity (97.4% sequence identity) to *Bacillus amyloliquifaciens* strain (ACC No.: KC692163.1).

Phylogenetic analysis of partial 16s rRNA gene sequences using MegAlign software of DNAStar revealed that HM6 was grouped in one cluster with two strains (JF460736 and KC692163) of *B. amyloliquifaciens*, while the *Bacillus cereus* (DQ923487) represented the outgroup cluster (Fig. 7).

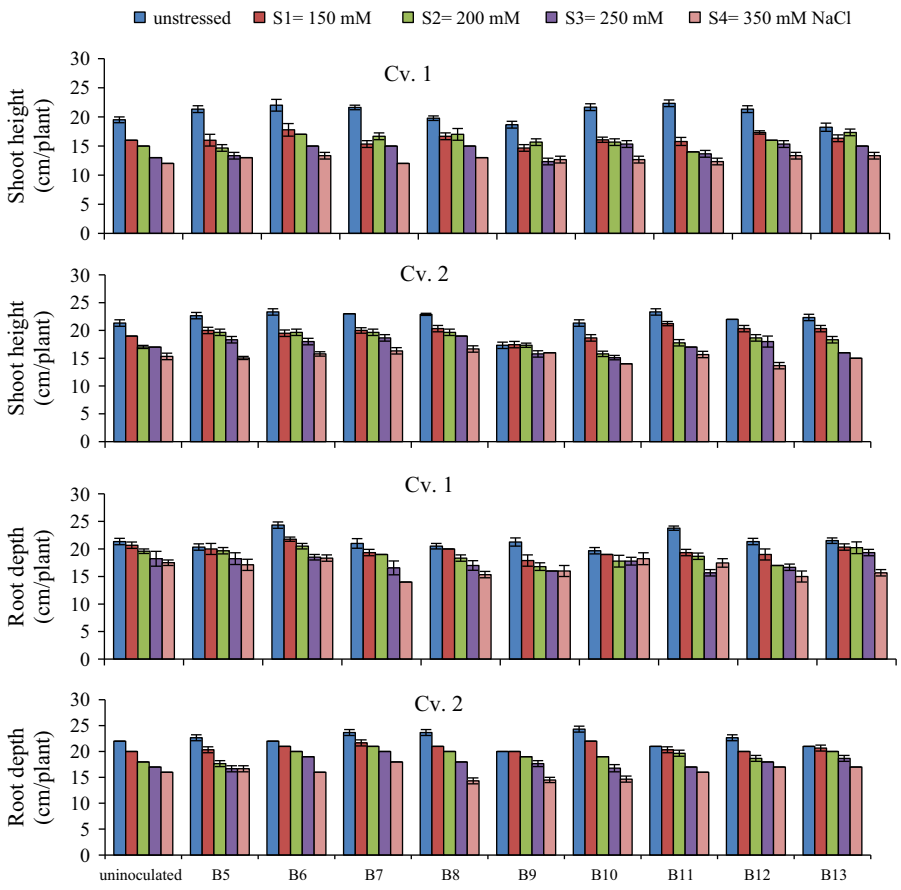
Discussion

The results showed that the activity of biofilm formation was increased with increasing NaCl concentration, especially at 500 mM NaCl. These results were in accordance with those of Qurashi and Sabri (2012) who reported that biofilm development protected the bacterial cells at elevated stress of nutrients and salt with increasing time. They added also that, environmental stress such as nutrients and osmotic stress poses increased bacterial competition for available nutrients, and bacteria revert from the planktonic stage to sessile assemblages at various biotic and abiotic surfaces to protect them in the rhizosphere. Moreover, increasing production of exopolysaccharides against higher salt stress also favors the biofilm formation and protects them in assembly by retaining a water layer around the cells.

The results also showed that salinity stress significantly reduced the growth of the salt-sensitive (Giza 123) and the salt-tolerant (Giza 2000) cultivars of barley. This reduction in growth was represented as an ultimate outcome of the salt-induced reduction in shoot height, root depth, fresh masses and relative water content (RWC) and consequently, in the accumulation of dry matter in the shoots and roots. Such effects were comparatively more pronounced in the sensitive than in the tolerant cultivar. These results are in agreement with those of Omar et al. (2009) who found that, salinity stress inhibited the growth and yield of two barley cultivars (Giza 123 and 2000). They also added that inoculation with *A. brasilense* significantly ameliorated these adverse effects of salinity on the barley growth and the PGPR-induced salt tolerance is more pronounced in the salt sensitive cultivar.

However, PGPR are effective in colonizing the plant root and further multiply into microcolonies and/or produce biofilm as a result of a successful plant-microbe interaction and these plant associated biofilms are highly capable of providing protection from external stress, decreasing microbial competition, and giving beneficial effects to the host plant supporting growth, yield and crop quality as reported by Ramey et al. (2004), Saleh-Lakha and Glick (2006) and Lugtenberg and Kamilova (2009).

The present results were also in agreement with those of Abd El-Daim (2015) who reported that the beneficial effects of rhizobacteria isolate AZP2 seem to be connected with its ability to protect drought stressed wheat roots through biofilm formation which resulted in better utilization of soil water contents and overall improved drought stress tolerance in wheat. In general, it was observed in this study that PGPRs bacterial inoculation had ameliorative effects on the growth criteria of barley plants. These strains improved the



F values of 3-way ANOVA at $P \leq 0.01$ (*= Significant)

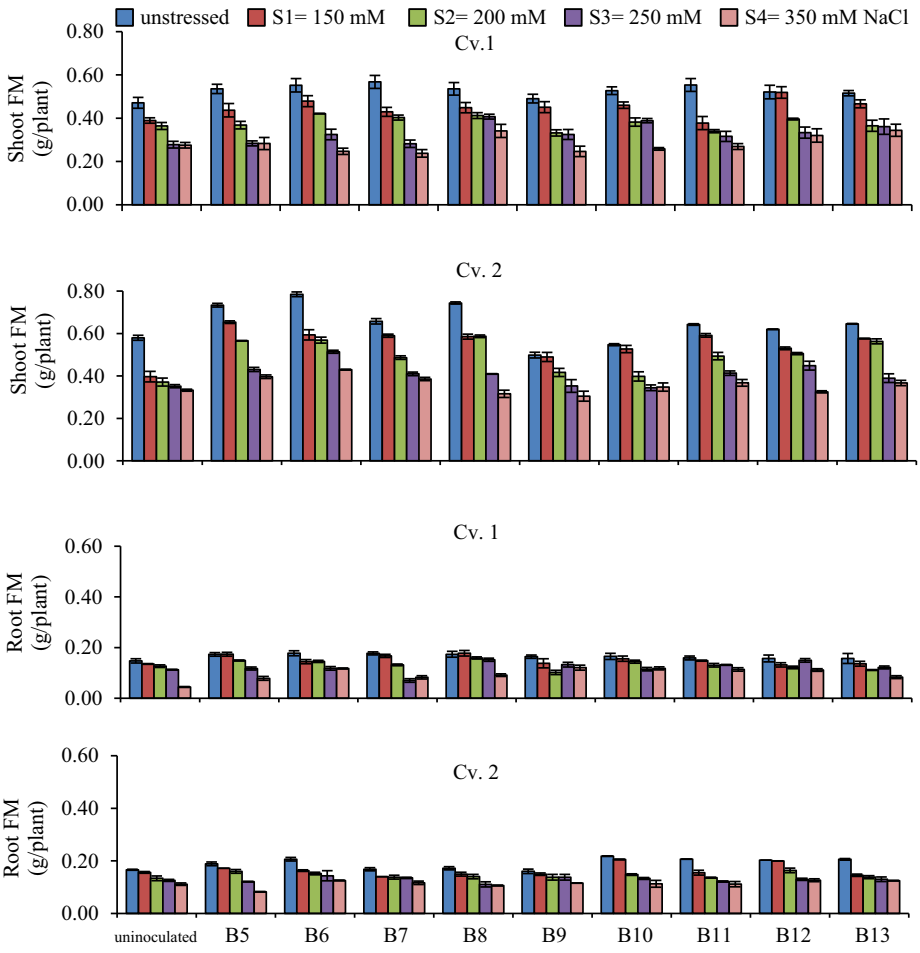
Treatments	Shoot length (cm/plant)		Root depth (cm/plant)	
	F value	significancy	F value	significancy
Cv.	1654.4	*	44.1	*
PGPR	60.8	*	55.1	*
Salt	1570.8	*	935.6	*
Cv.×PGPR	24.1	*	14.5	*
Cv.×Salt	31.7	*	13.9	*
PGPR×Salt	11.9	*	5.8	*
Cv.×PGPR×Salt	6.7	*	3.9	*

Fig. 2 Effect of bacterial inoculation with the selected nine biofilm forming PGPR B (5–13) on shoot height and roots depth of 25-days-old barley seedlings cv.1 (Giza 123) and cv.2 (Giza 2000) grown in clay-sandy soil (2:1 w/w) under four salt concentrations (150, 200, 250 and 350 mM NaCl). Error bars represent the standard deviation between 3 replicas.

barley plant growth to maintain its growth during salinity stress. The results were in agreement with many other reports dealing with the role of bacterial inoculations in improving plant growth, increasing root and shoot dry weights and water content in stressed plants and reducing the antagonistic effects of abiotic stress (Wu, 2009; Chookhampaeng, 2011; Franco, 2015).

The results indicated that RWC of PGPR-treated barley plants was found to be higher than that of control during salinity stress. This increase may be due to that the beneficial association can undermine such stresses and the PGPR-inoculation of plants not only reduces stress but also helps to fetch higher water quantity from sources inaccessible to control plants as reported by Rakshapal et al. (2013).

The genus *Bacillus* was characterized based on the distinguishing features such as Gram positive, aerobic, endospore producing, rod-shaped bacteria observed in the tested isolate. The identification of the isolate was eventually confirmed through sequencing of 16S rRNA gene. The use of 16S rRNA gene sequences to study bacterial phylogeny has been by far the most common housekeeping genetic marker used for a number of reasons. The 16S rRNA gene sequences of the selected isolate showed 97.4% similarity to *Bacillus amyloliquefaciens*. These results of characterization and identification were in accordance with those of Sushil et al. (2013) who reported that the characterization features which indicate that his isolate belonging to the genus *Bacillus* include the following; the colonies morphology which are small, fast growing,



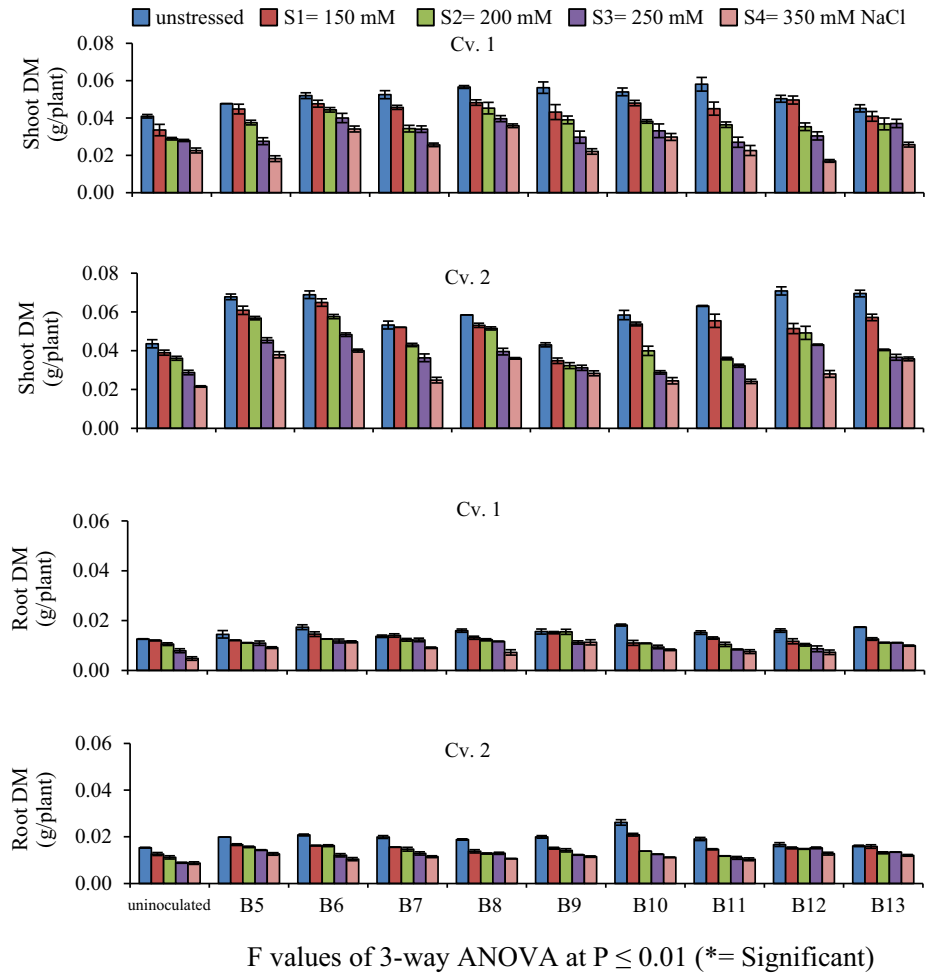
F values of 3-way ANOVA at $P \leq 0.01$ (*= Significant)

Treatments	Shoot FM (g/plant)		Root FM (g/plant)	
	F value	significancy	F value	significancy
Cv.	2244.6	*	325.7	*
PGPR	120.3	*	39.1	*
Salt	2031.1	*	911.6	*
Cv.×PGPR	60.6	*	25.9	*
Cv.×Salt	21.3	*	12.4	*
PGPR×Salt	10.1	*	13.1	*
Cv.×PGPR×Salt	14.2	*	16.3	*

Fig. 3 Effect of bacterial inoculation with the selected nine biofilm forming PGPR B (5–13) on fresh mass (FM) of shoot and root of 25-days-old barley seedlings cv.1 (Giza 123) and cv.2 (Giza 2000) grown in clay-sandy soil (2:1 w/w) under four salt concentrations (150, 200, 250 and 350 mM NaCl). Error bars represent the standard deviation between 3 replicas.

wrinkle, dull and dry, white, irregular, lobed and flat on NA medium colonies and the cell morphology, under microscopic examination was Gram positive, endospore-forming and the cells were rod shaped and in chains. In addition, the identification using the nucleotides sequence of 16S rRNA gene of the isolate showed 98.7% similarity to *B. amyloliquefaciens*.

B. amyloliquefaciens strains are also often known to serve as plant growth promoting bacteria (PGPB). The known mechanisms of plant growth promotion are as follows: (a) bacterial synthesis of plant growth hormones, such as indole-3-acetic acid (IAA), cytokinin and gibberellins,(b) reduction in the volatile plant hormone, ethylene by production of 1-amino



F values of 3-way ANOVA at $P \leq 0.01$ (*= Significant)

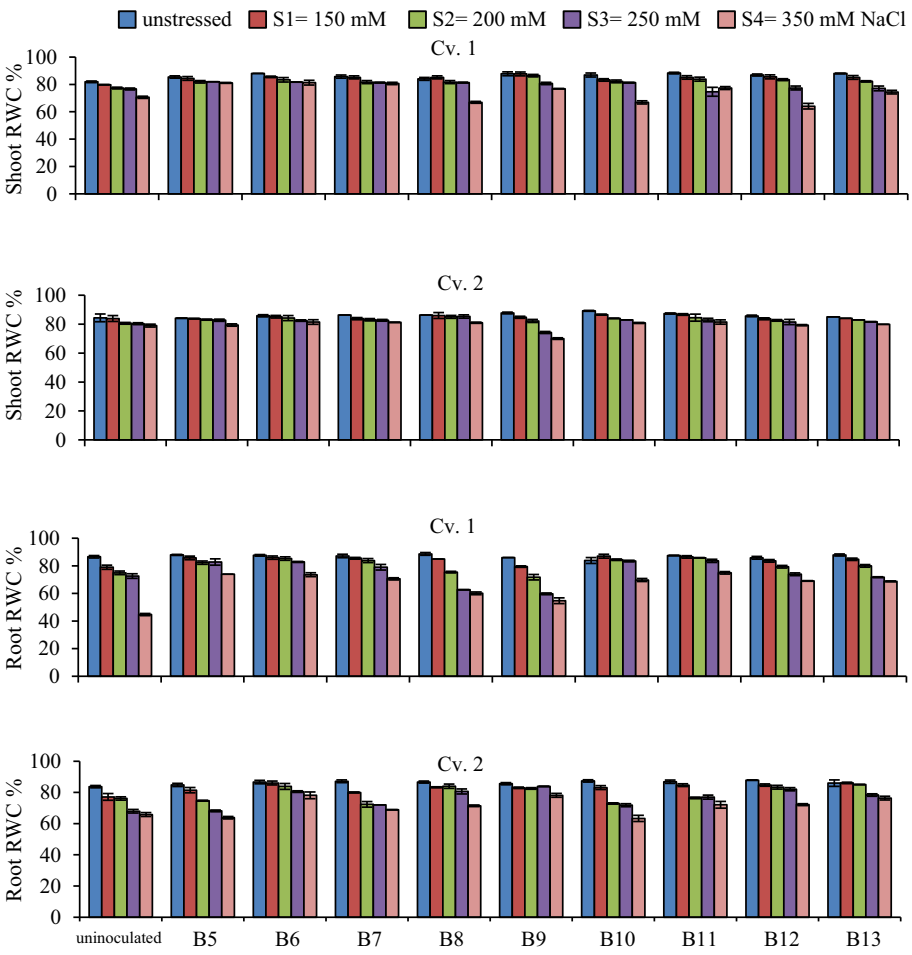
Treatments	Shoot DM (g/plant)		Root DM (g/plant)	
	F value	significancy	F value	significancy
Cv.	836.6	*	1105.6	*
PGPR	205.9	*	83.6	*
Salt	1978.9	*	1051.7	*
Cv.×PGPR	96.3	*	32.4	*
Cv.×Salt	12.5	*	10.1	*
PGPR×Salt	15.5	*	12.3	*
Cv.× PGPR×Salt	12.9	*	12.6	*

Fig. 4 Effect of bacterial inoculation with the selected nine biofilm forming PGPR B (5–13) on dry mass (DM) of shoot and root of 25-days-old barley seedlings cv.1 (Giza 123) and cv.2 (Giza 2000) grown in clay-sandy soil (2:1 w/w) under four salt concentrations (150, 200, 250 and 350 mM NaCl). Error bars represent the standard deviation between 3 replicas.

cyclopropane-1-carboxylate deaminase and (c) through an increased uptake of nutrient from the soil (Wang et al., 2009).

Plant-associated *B. amyloliquefaciens* strains belonging to subsp. *plantarum* are distinguished from other representatives of endospore-forming *B. amyloliquefaciens* by their ability to colonize plant rhizosphere, to stimulate plant growth, and to suppress competing phytopathogenic bacteria and fungi as reported by Borriss et al. (2011). Due to their biofertilizer and biocontrol properties, they are becoming increasingly important as a natural alternative to chemical pesticides and other agrochemicals (Qiao et al., 2014).

In this concern, Cao et al. (2011) and Qiu et al. (2012) stated that *B. amyloliquefaciens* SQR9, a beneficial bacterium, has been used as an exogenous strain in a commercial bio-organic fertilizer for plant growth promotion and the suppression of soil-borne diseases in the field. However, as reported by Xu et al. (2013) and Shao et al. (2014), *B. amyloliquefaciens* is able to produce phytohormones and antibiotics, such as



F values of 3-way ANOVA at $P \leq 0.01$ (*= Significant)

Treatments	Shoot RWC (%)		Root RWC (%)	
	F value	significancy	F value	significancy
Cv.	192.3	*	21.6	*
PGPR	43.7	*	208.4	*
Salt	771.9	*	2563.6	*
Cv.×PGPR	54.8	*	263.8	*
Cv.×Salt	72.1	*	85.1	*
PGPR×Salt	20.9	*	25.7	*
Cv.×PGPR×Salt	15.5	*	52.3	*

Fig. 5 Effect of bacterial inoculation with the selected nine biofilm forming PGPR B (5–13) on the relative water content (RWC) of 25-days-old barley seedlings cv.1 (Giza 123) and cv.2 (Giza 2000) grown in clay-sandy soil (2:1 w/w) under four salt concentrations (150, 200, 250 and 350 mM NaCl). Error bars represent the standard deviation between 3 replicas.

indole-3-acetic acid (IAA) and bacillomycin D, that promote plant growth directly or indirectly. In addition, it is able to colonize plant roots and establish close interactions with the hosts (Liu et al., 2014; Shao et al., 2014).

Our findings are in the same trend with those reported by Chen et al. (2016) who found that PGPR strain *B. amyloliquefaciens* SQR9 could help maize plants to tolerate salt stress for 20 days and significantly promoted the growth of maize seedlings, enhanced the chlorophyll content and was able to colonize plant roots and establish close interactions with the hosts. *B. amyloliquefaciens* may confer plant salt tolerance through sequestering Na^+ into vacuoles, expelling Na^+ from roots, improving the accumulation of total soluble sugar and enhancing antioxidant content in maize under salt stress as suggested by Chen et al. (2016). They added also that it upregulates the expression of genes related to salt tolerance and downregulates the expression of genes related to ABA in plants.

Finally, it could be concluded that the inoculation with the biofilm forming PGPR (*B. amyloliquefaciens*) had ameliorative effects on the growth of barley plants growing under salinity stress. Consequently, it may be applied in assisting barley plants to tolerate salt stress beside its role in growth promotion.

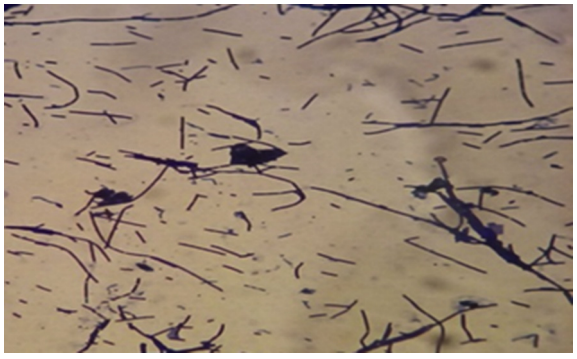


Fig. 6 Photomicrograph of morphological characterization of the bacterial isolate in the HM6 (B6) with Gram stain.

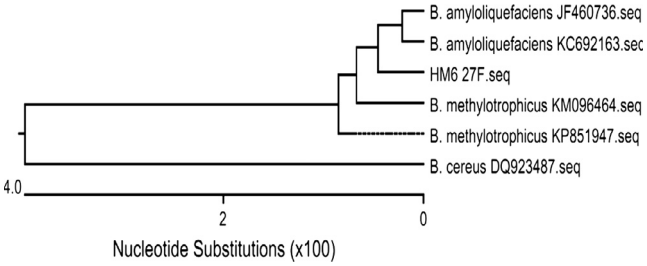


Fig. 7 Phylogenetic tree of the bacterial isolate in the HM6 (B6) compared with closely related strains accessed from the GenBank. *Bacillus cereus* is included as an out-group strain. The HM6 bacterial isolate is more related to *Bacillus amyloliquifaciens*.

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