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Effectiveness of exopolysaccharides and biofilm forming plant growth promoting rhizobacteria on salinity tolerance of faba bean (*Vicia faba* L.)

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This study aimed to investigate the production of biofilm and exopolysaccharides by plant growth promoting rhizobacteria (PGPR) under different salt concentrations. In this study, the activity of biofilm formation and exopolysaccharides production by 20 strains of PGPR which previously isolated and identified from root samples of different crops were determined under different salt concentrations. Out of 20 strains, only 12 PGPR strains have the ability to form biofilm at 0.0, 50, 100 and 150 mM NaCl concentration. PGPR strains with the highest activity of biofilm formation and exopolysaccharides production were selected to check them on the faba bean plants under different concentration of salt stress. Inoculation with PGPR strains increased plant growth at higher level of salt concentrations compared with their corresponding uninoculated ones. The strain *Pseudomonas anguilliseptica* SAW 24 showed the highest activity of biofilm formation and exopolysaccharides production at different NaCl concentrations furthermore, gave the highest records of plant height (cm), fresh and dry weight (g/plant) of faba bean plants. The activities of biofilm formation and exopolysaccharides production of plant growth promoting bacteria enhance faba bean plants against different salt concentrations.

Key words: Biofilm, faba bean, exopolysaccharides, salinity, plant growth promoting rhizobacteria (PGPR).

INTRODUCTION

Biotic and abiotic stress factors have a major effect on plants which cause major damages to crop production around the world (Nemati et al., 2011). Salt stress considers one of the major problems that cause a decrease in fertile land productivity. Salinity not only affects in agriculture but also has other problems that effect on biodiversity of that environment. Unfortunately, leguminous crop is most salt-sensitive which severely affected by soil salinity throughout the world, to enhance its yield and productivity using safe biological measures to improve the soil management and symbiotic relationships (Sarker and Erskine, 2006; Fernandez-Aunión et al., 2010). Beneficial bacteria that enhance growth promotion and prime stress tolerance of plants

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have great chance to improve crop production and environmental friendly resource management. Aggregation of biofilm formation is commonly detected in bacteria. Biofilm is the aggregation of microbial cells that are irreversibly connected with biotic and abiotic surface and usually enclosed in the self-secreted extracellular polymeric substances.

Microorganisms within biofilms have a lot of advantages (Wilson, 2005; De Beer and Stoodley, 2006; Vu et al., 2009; Afrasayab et al., 2010; Nawaz and Ashraf, 2010; Asari, 2015) such as: protect the plant from external stress, Increase adhesion to surfaces, high population densities, high tolerance to antimicrobial agents and higher level of nutritional competition between microorganisms.

The main objective of this research was to check the biofilm formation and exopolysaccharides production by PGPR strains at different salt concentration and study the most efficient PGPR strains in terms of their ability to produce biofilm and exopolysaccharides under salt stress conditions on faba bean plants.

MATERIALS AND METHODS

Microorganisms and growth condition

Selection of the best biofilm forming bacteria was under different salt concentrations (0.0, 50, 100 or 150 mM). 20 strains of plant growth promoting rhizobacteria (PGPR) used in this study are presented in Table 1. These strains were previously isolated and identified by Fathalla et al. (2015).

The strains were screened for the potential 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 for biofilm detection

A loopfull of bacterial strains were inoculated in 10 ml of NB with different NaCl concentrations (0, 50, 100 and 150 mM) in test tubes. The test tubes were incubated at 28°C for two days. Then, the supernatant was thrown away and the test tubes were washed with phosphate buffer saline (pH 7). The dried glass tubes were stained with 0.1% of crystal violet for 15 min and stain was removed by washing the tubes with distilled water. Biofilm formation in tubes was detected when a visible film lined the wall and the bottom of the tube.

Quantitative assay

The biofilm production was quantitatively assayed according to the method described by Arciola et al. (2002). Single colonies of strains from nutrient agar plates were inoculated in 10 mL of NB in separate test tubes, with different NaCl concentrations (0, 50, 100, 150 mM) through broth incubated for 2 days at 28°C and diluted (1 in 100) with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 0.2 ml aliquots of the diluted cultures. Negative control wells contained sterile broth only.

Table 1. Plant growth promoting rhizobacteria strains.

Code	Strain
SAW19	*Pseudomonas putida* SAW19
SAW1	*Pseudomonas mosselii* SAW1
SAW7	*Pseudomonas entomophila* SAW7
SAC1	*Pseudomonas corrugata* AC1
SAW10	*Pseudomonas plecoglossicida* SAW10
SAB12	*Pseudomonas putida* SAB12
SAW23	*Pseudomonas corrugata* SAW23
SAW24	*Pseudomonas anguilliseptica* SAW24
SAW5	*Pseudomonas entomophila* SAW5
SAW9	*Pseudomonas argentinensis* SAW9
SAW22	*Pseudomonas putida* SAW22
SAB15	*Pseudomonas palleroniana* SAB15
SAW15	*Pseudomonas plecoglossicida* SAB1
SAB1	*Pseudomonas palleroniana* SAB17
SAB17	*Pseudomonas parafulva* SAB14
SAB14	*Pseudomonas putida* SAB12
SAB12	*Pseudomonas plecoglossicida* SAB8
SAB8	*Pseudomonas putida* SAW3
SAW3	*Pseudomonas putida* SAW3
SAB10	*Pseudomonas putida* SAB10

The plates were incubated at 28°C for two days. After incubation period, content of each well was gently decanted, then washed with phosphate buffer saline (pH 7) and stained by crystal violet (0.1%). Stain was removed by washing the wells with distilled water and dried. To release the crystal violet, 75% ethanol was added to the wells and OD of stained biofilm was determined by using micro ELISA auto reader at 570 nm. Experiments for each strain were carried out in triplicate and repeated three times.

Exopolysaccharide production assay

For exopolysaccharide determination, flasks (250 mL) including 100 mL of a medium proposed by Verhoef et al. (2003) were supplemented with different NaCl concentrations (0, 50, 100, 150 mM) and inoculated with 24 h old prepared bacterial culture (1000 µl) then incubated at 160 rpm shaker for 48 h at 28°C. To extract exopolysaccharide, the method of De Vuyst et al. (1998) was used. Bacterial cultures were centrifuged at optimized conditions (10000 rpm for 15 min). Supernatant was carefully remove, then wash the cells by 50 mM NaCl, centrifuge at 10000 x g for 5 min and remove supernatant. Repeat washing four additional times. Re-suspend cells in 1 ml of 50 mM EDTA, and incubated at 28°C for 60 min, centrifuged at 14,500 x *g* for 5 min, carefully removed the supernatant and transfer to fresh eppendorf tubes. Exopolysaccharides were quantified in terms of total carbohydrates and measured by the phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1956).

Effect of PGPR biofilm on salt tolerance of faba bean growth

Healthy seeds of faba bean (Nubaria 3 variety) were obtained from Agricultural Research Center, Giza, Egypt. Faba bean seeds were disinfected using 1% sodium hypochlorite solution for 10 min,

Table 2. Physical properties of the experimental soil.

Particle size distribution	(%)
Sand	97.65
Silt	1.51
Clay	0.84
Textural class	Sand
Field capacity %	17.0
pH	7.80

rinsed thrice in sterile distilled. After surface sterilization, seeds were inoculated with selected bacterial strains. Suspension of bacterial cultures (24 h old) was centrifuged for 2 min at 13400 rpm.

Cells were suspended in saline solution (NaCl 1%) to get bacterial suspension (OD 600 nm adjusted 10^8 mL^{-1} CFU). Sterilized seeds were inoculated for 30 minu before sowing. For control, seeds were steeped in sterile water for the same period of time. The PGPR populations were detected by adding suitable amounts of saline solution to obtain 10^8 CFU / 200 ml. These dilutions served as bacterial inoculum for plant experiment, 2.8 kg of winnowed, sterilized and air dried soil was thoroughly mixed with 200 ml diluted bacterial suspension for 2 min. The characters and composition of soil are presented in Table 2.

Seeds were sown in plastic pots for 45 days and irrigated regularly by different concentrations of NaCl (0, 50,100 and 150 mM) per gram weight of soil. After 45 days, the seedlings were harvested and different growth parameters that is, plant length (cm), dry weight (g/plant) and fresh weight (g/plant) were measured.

Statistical analysis

Collected data were statistically analyzed using the appropriate analysis of variance according to Steel and Torrie (1981). The experiment date designated in two ways was completely randomized with three replicates. Computer program software CoStat version 6.311 was used to analyses the data of experiment. Least significant difference (LSD) at 5% level was used separately to evaluate the response of each character.

RESULTS

Out of 20 strains, 12 strains had the ability to produce biofilm. Biofilm producing strains were confirmed by various methods. In the current study, qualitative and quantitative estimation of biofilm production by PGPR strains was performed.

Biofilm qualitative screening

Obtained results observed that thick film was formed inside the wall and bottom of the tube. Twelve strains (SAW 7, SAC1, SAW 10, SAB12, SAW 23, SAW 24, SAB 8, SAW 19, SAW 5, SAW 9, SAW 15 and SAW 1) had thick film inside the wall of the tube denoting that strains could produce biofilm while eight strains could not produce biofilm. The results showed that the purple ring appeared under different levels of NaCl concentrations (0, 50, 100 and 150 mM).

Biofilm quantitative screening

Twelve *Pseudomonas* were assayed for production of biofilm. The results indicated that the activity of biofilm formation was increased with increasing NaCl concentration. The highest significant increase was recorded in seven strains treated with 150 mM NaCl. These antagonistic bacteria were strains (SAW 1, SAB 12, SAW 15, SAW 24, SAW 19, SAW 7 and SAW 9). However, no biofilm ring was formed either in the absence of NaCl in six strains (SAW 24, SAW 1, SAW 5, SAW 9, SAW 23 and SAW 19).

The results in Figure 1 showed the values of absorbance at 570 nm as a measure of optical density (OD) which reflected the activity of biofilm formation of 12 strains in nutrient broth cultures supplemented with four concentrations of NaCl (0, 50, 100 and 150 mM).

Exopolysaccharide production under different salt concentrations

Fxopolysaccharides (EPS) production of PGPR strains under different NaCl concentrations (0, 50, 100 and 150 mM) was tested. The results showed that there was a general direction of gradual increase in EPS with increasing salt concentrations (Figure 1). However, at no salt stress or low concentration of NaCl exopolysaccharide production was reduced (Figure 2).

Effect of inoculation with selected PGPR on plant growth of faba bean

The effect of inoculation with selected PGPR (SAW 1, SAW 24 and SAW 19) on growth of faba bean at four salt concentrations (0, 50, 100 and 150 mM) was studied. The results in Figure 3 showed that the increasing salt stress affect plant height, shoot fresh weight and shoot dry weight. Also, results indicated that the increasing of salinity stress was significantly reduced all growth parameters of non-inoculated plants. Results denoted that in non-inoculated plants, there was a little bit impact on NaCl stress up to 50 mM. The effect of inoculation on dry masses of the salinity stress on faba bean is represented in Figure 3.

PGPR inoculated plants showed higher accumulation of shoot dry mass than their corresponding uninoculated ones under salt stress. However, the inoculation with SAW 24, SAW 1 and SAW 19 gave the highest value in

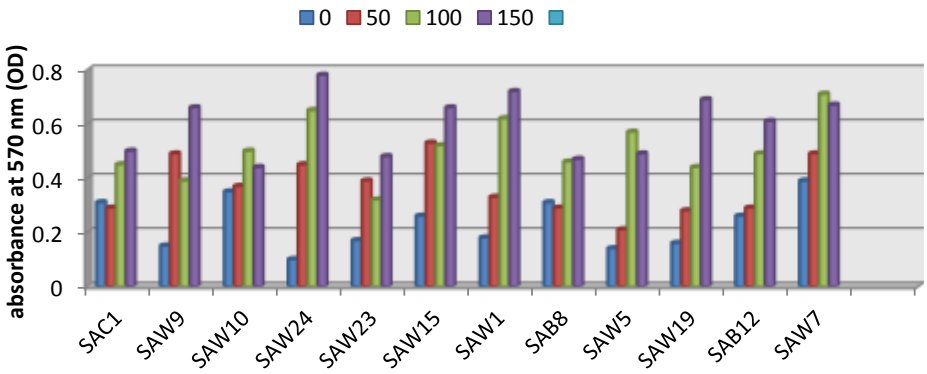

Figure 1. The optical density (OD) (at 570 nm) as a measure of the activity of biofilm formation of 12 strains in nutrient broth cultures supplemented with four concentrations of NaCl (0.0, 50, 100 and 150 mM).

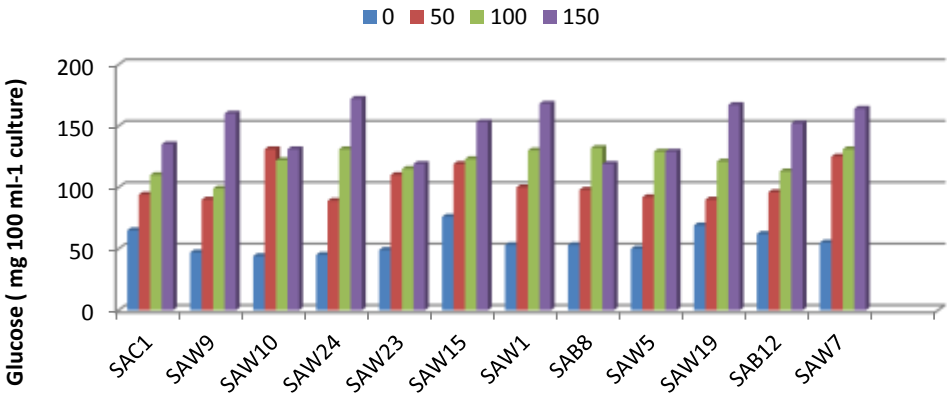

Figure 2. Effect of different concentrations of NaCl (0.0, 50, 100 and 150 mM) on glucose content (mg 100 ml^{-1} culture) of bacterial strains in EPS media.

shoot fresh mass with 7.2, 6.1 and 4.8 g, respectively comparing to 3.6 g in the uninoculated stressed treatment at 100 mM NaCl.

At 100 mM NaCl, the inoculation with SAW 24, SAW1 and SAW 19 PGPR strains enhanced the plant height by 43, 40 and 36%, respectively compared to their uninoculated plants. The PGPR SAW 24 and SAW1 strains enhanced the accumulation of dry mass of shoots by 47 and 42%, respectively at 100 mM NaCl. Maximum increase under stress in the plant height, fresh weight and dry weight of shoot was observed at 50 mM NaCl stress. Depending on obtained results of the present experiment, the results showed that the strain SAW 24 showed the highest activity of biofilm formation under the different levels of NaCl concentrations. Also, the same strain *Pseudomonas anguilliseptica* SAW 24 showed the highest growth measurements of faba bean plants under salt stress.

DISCUSSION

The results show that increasing NaCl concentration leads to the increase in exopolysaccharide production. Moreover, increasing the production of exopolysaccharide against higher salt stress leads to support biofilm formation (Ishii et al., 2004; Fujishige et al., 2006). These results are in agreement with those obtained by Arora et al. (2010), Qurashi and Sabri (2012), Deng et al. (2015) and Kasim et al. (2016).

It was reported that formation of biofilm and exopolysaccharide kept the viability of bacterial cells under salt stress to protect them in the rhizosphere. Previous research showed that biofilm formation and exopolysaccharide production by PGPR strains significantly increase soil fertility and enhance plant growth (Ashraf et al., 2005; Liaqat et al., 2009).

The present results are similar with several previous

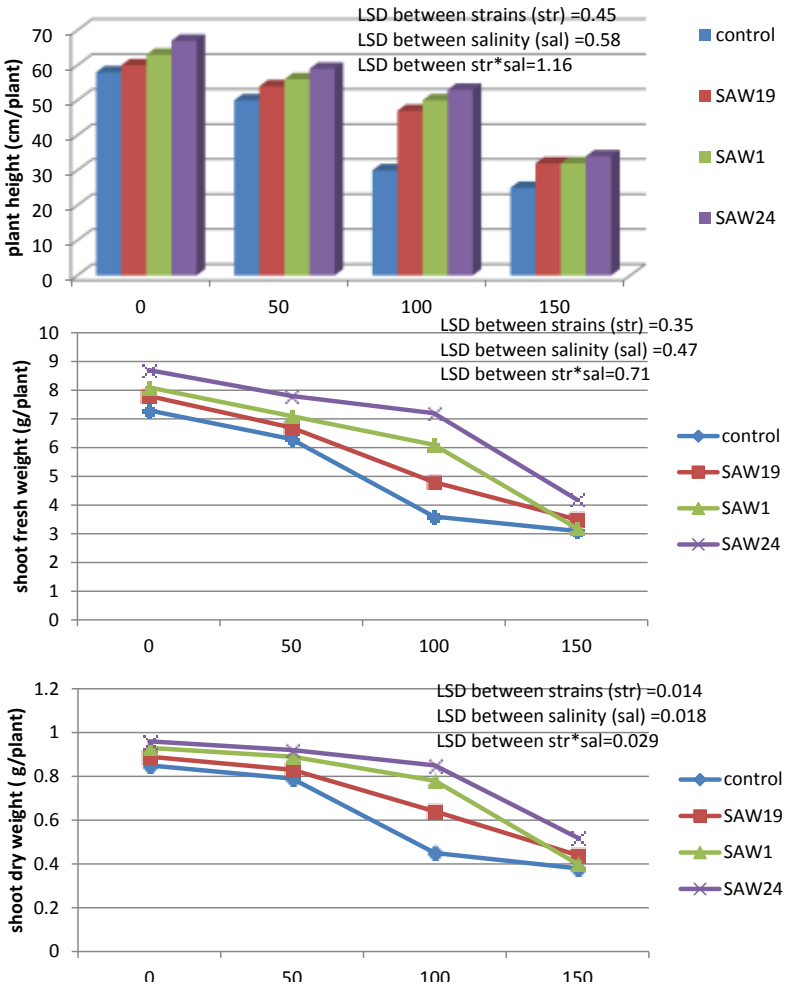

Figure 3. Effect of the three selected PGPR strains biofilm forming and exopolysaccharides producing on plant height, shoot fresh weight and shoot dry weight of faba bean under different salt concentrations.

reports that, salinity stress significantly reduced leguminous crops (Soussi et al., 1998; Chookhampaeng, 2011). However, inoculation with PGPR encouraged the plant growth of *Leucaena esculenta* with and without salt stress by releasing phytohormones which lead to increase bacterial root colonization and biofilm formation (Lugtenberg and Kamilova, 2009; Arora et al., 2010; Ahmed and Shahida, 2010).

Previous studies showed that Bacterial EPS under salt stress can bind sodium ions and reduces its toxicity in the soil (Arora et al., 2010). These may be in the same line with the current study showing the role of EPS to bind Na^+ and reduce its toxic effect. In general, the present results illustrate the effect of PGPR strains on enhancement of faba bean plant growth with and without salinity stress. In addition, the results show that the PGPR strain *P. anguilliseptica* SAW 24 have the highest biofilms formation and exopolysaccharides production under different salt stress. Also, gave the best records of plant height, fresh and dry weight of faba bean plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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