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Talc based exopolysaccharides formulation enhancing growth and production of *Hellianthus annuus* under saline conditions

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Abstract

Stress tolerating strain of *Pseudomonas aeruginosa* PF07 possessing plant growth promoting activity was screened for the production of exopolysaccharides (EPS). EPS production was monitored in the cell free culture supernatant (CFCS) and extracted EPS was further purified by thin layer chromatography. EPS producing cells were taken to design talc based formulation and its efficacy was checked on oilseed crop sunflower (*Hellianthus annuus*), under *in vivo* saline conditions (soil irrigated with 125 mM of saline water). Application of bioformulation significantly enhanced the yield and growth attributes of the plant in comparison to control (untreated seeds) under stress and non-stress conditions. Germination rate, plant length, dry weight and seed weight increased remarkably. The above findings suggest the application and benefits of utilizing EPS formulation in boosting early seedling emergence, enhancing plant growth parameters, increasing seed weight and mitigating stress in saline affected regions. Such bioformulation may enhance RAS/RT (Root Adhering Soil to Root Tissue ratio), texture of the soil, increase porosity, improve uptake of nutrients, and hence may be considered as commercially important formulation for renovation of stressed sites and enhancing plant growth.

Key words: Bioformulation, Exopolysaccharides (EPS), *Hellianthus annuus, Pseudomonas aeruginosa, Salinity.*

Introduction

Sunflower (*Helianthus annuus* L.) belonging to the family Asteraceae is an important oil seed crop grown in India (1). Oilseed crops, including sunflower have served as backbone of Indian economy since time immemorial, but nowadays the production is declining due to several abiotic stressors. Salinity is one of the major abiotic stress factor limiting plant growth and productivity (2). The total salt-affected land worldwide is estimated to be 900 million hectares, 6% of the total global land mass (3). In India about ten million-hectares of land is suffering from the constraint of salinization and Uttar Pradesh has about 1.28 million hectare saline soils (4, 5). Nowadays sunflower production is severely affected by several biotic and abiotic stresses. In fact salinity is one of the major stressors resulting in more than 60% loss in the sunflower production around the globe (6).

The extensive use of synthetic organic chemicals in the past decades has led to a number of long-term environmental problems. Repeated use of external inputs destroys the soil biota and reduces the nutritive value of soil, resulting in salinization which causes various stresses in agricultural plants (7). The need of the day is sustainable agriculture without harming the delicate balance of soil ecology as well as unlocking the mystery of biota influencing plant growth by using plant growth promoting rhizobacteria (PGPR) (8). The development of biological products based on beneficial microorganisms can extend the range of options for maintaining the healthy yield of crops under stress conditions such as salinity. In recent years, a new approach has been developed to alleviate salt stress in plants and that is by treating crop seeds and seedlings with tolerant PGPR strains (9).

Soil-borne fluorescent pseudomonads have received particular attention as PGPRs throughout the globe because of their catabolic versatility, excellent root colonizing ability and their capacity to produce a wide range of metabolites that favor the plant withstand under varied biotic conditions (10). Exopolysaccharides (EPS) produced by bacterial cells are instrumental in imparting stress tolerance to bacterial cell, but relatively little attention has been paid on this subject, particularly on EPS-producing fluorescent Pseudomonads and bioformulations developed from them (9). As the saline areas under agriculture have been on the rise every year across the globe, this is a matter of serious concern, but uptill now no possible remedy has been developed as regard to it (11). Thus delivering the stress tolerating PGPR, to the sunflower plant and soil in an effective way can go a long way in enhancing crop yields and remediation of saline soils.

Materials and methods

Bacterial strains

Bacterial strains were isolated from the rhizospheric region of the sunflower crop (*Helianthus annuus)* growing in semiarid conditions of west Kanpur, (20°38′ E and 80°21′ N; temperature maximum 48°C and minimum 1°C) (Uttar Pradesh, Northern India). Isolates were tested for morphological, physiological and biochemical characters according to Bergey's Manual of Systematic Bacteriology (12). On the basis of salinity stress tolerance capacities and plant growth promoting (PGP) qualities, isolate PF07 was selected and further identified by analysis of 1.5 kb 16S rRNA sequences. 16S gene sequence was queried for similarities with

BLAST (13) and with the Ribosomal Database Project (RDP) (14). Bacterial strain PF07 was identified as *Pseudomonas aeruginosa*, purified and maintained on stress tolerant Davis minimal medium (DMM) agar slants at 4˚C for further use (15,16). DMM was selected as it is a minimal media that provides conditions almost similar as faced by bacteria under natural stress environment.

Plant growth promoting attributes under saline conditions

PF07 was checked for plant growth promotory (PGP) attributes including phosphate solubilization, indole acetic acid (IAA) production, HCN production, siderophore production, chitinase, β-1-3 glucanase activity and EPS production abilities under saline (0 to 1600 mM NaCl) and non-saline conditions (0 mM NaCl). Phosphate solubilizing activity of PF07 was tested by spot inoculation on Pikovskaya's medium (17) and IAA production was detected in culture filtrate using salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) (18). HCN production was checked by observing the change in color of the filter paper impregnated with 0.5% picric acid in 1% Na_2CO_3 (19), whereas,

siderophore production was determined on Chrome-Azurol S medium according to Schwyn and Neilands (20). Extracellular chitinase activity was determined by spot inoculation on solid chitin minimal medium (CMM) whereas β-1,3 glucanase enzyme was assayed according to Dunne *et al*. (21), and EPS production was monitored by chilled ethanol precipitation method (22, 23).

Chemical mutagenesis for developing EPS-defective mutant

A loopful of PF07 cells were inoculated into 10 ml of DMM broth (16) and incubated at 28°C upto log phase. Subsequently 100 µg ml^{-1} 5-bromouracil was added and further incubated for 02 h. Cells were centrifuged, washed thrice with sterile water, and resuspended in 10 ml of DMM broth and mutants were fixed by overnight incubation at 28°C. The mutant bank was stored in 25% glycerol at -40°C (10). The clones of mutant bank were screened for saline tolerance and EPS production according to Sandhya *et al*. (23). The wild isolate $PF07^{EPS+}$ served as a control in the mutant screening test. Maximum EPS defective mutant was marked as $PF07^{EPS-}$. Mutagenic procedure worked effectively as EPS defective mutant $PF07^{EPS-}$ was repeatedly checked on DMM media for stability.

Salinity Tolerance Assay

The log phase cultures (OD610 = 0.1) of $PF07^{EPS+}$ and $PF07^{EPS-}$ were inoculated in DMM broth, amended with different concentrations of NaCl (0 to 1600 mM) and incubated at 28°C. Optical density was measured at 610 nm by spectrophotometer (GENESYS™6, Model, 335908-02) up to stationary phase (120 rpm). The experiment was conducted in five replicates.

Effect of salinity stress on EPS production

To determine the effect of saline stress (0-1600 mM) conditions on EPS content, selected isolate $PF07^{EPS+}$ and its mutant $PF07^{EPS-}$ were grown in DMM broth, with gradients of NaCl concentrations (0-1600 mM) for seven days. Cells were harvested by centrifugation (10 min at 11,000 x g). The supernatant was filtered through 0.45 µm nitrocellulose membrane, two volumes of cold ethanol were added to culture supernatants and stored overnight at 4°C. Precipitate was collected by centrifugation (20 min at 2,500 x g), suspended in demineralized water, and mixed with 2 volumes of cold ethanol. Samples were centrifuged (2,500 x g) and the pellets were dried at 100°C and weighed. The amount of EPS was expressed as polymer dry mass and expressed in g/l (22, 23).

Components of EPS

The precipitated EPS obtained from $PF07^{EPS+}$ at different levels of salinity was hydrolyzed with 2 volumes of 2.5 M H_2SO_4 at 100°C for 1 h. The solution was neutralized with 1 M sodium carbonate and spotted on the silica gel plate (Silica gel 60F 254; Merck). The plate was developed in a thin layer chromatography (TLC) chamber using n-butanol: acetic acid: water (4:1:5v/v) as the mobile phase at room temperature. The plate was dried, sprayed with alkaline potassium permanganate, and incubated at 100°C for 10 min. The Rf values of the colored spots were measured and compared with those of standard carbohydrates (glucose, mannose, fructose, mannitol, arabinose, xylose, rhamnose, raffinose, galactose) (24).

Determination of the carbohydrate content in the EPS

The carbohydrate concentrations were determined according to Gaudy's method (25). The precipitated EPS obtained from $PF07^{EPS+}$ at different levels of salinity (0 to 1600 mM NaCl) was hydrolyzed with 2 volumes of 2.5 M H_2SO_4 at 100°C for 1 h. Briefly, cell free culture supernatant (CFCS) was dissolved in phosphate buffer (1ml) and added to 10 ml sterile test tubes. Freshly prepared Anthrone solution (1ml) was added in each test tube. The mixture was incubated in a water bath at 95˚ C for 15 min. After incubation, the mixture was allowed to cool to room temperature. Cooled aliquots (200 µl) were transferred to micro plate wells and read at 620 nm using an Elisa plate reader (Thermo scientific Multiskan Ex Type 355). Glucose was used as a standard to construct a standard curve.

Determination of the protein content in the EPS

Protein content, were determined by the modified method of Lowry (26). EPS (10 µl) was dissolved in phosphate buffer and inoculated into wells of a micro titter plate. Control wells were inoculated with phosphate buffer. Comassie plus reagent (300µl) was added to each well. The plate was incubated at room temperature for 10 min. After incubation, absorbance was read at 595 nm using an Elisa plate reader, (Thermo scientific Multiskan Ex Type 355). Bovine serum albumin (BSA) was used as a standard to construct the standard curve.

Talc-Based Formulation from EPS producing bacterial Cells

The talc-based formulation from $PF07^{EPS+}$ and $PF07^{EPS-}$ were developed by following the method described by Vidhyasekaran and Muthailan (27). In brief, one kilogram of talc powder was taken and pH adjusted

to neutral by adding $CaCO_3$ at the rate of 15 g/kg. The 400 ml of 7 days grown bacterial suspension was mixed separately with carrier-cellulose mixture under aseptic conditions. After drying (approximately 35% moisture content) overnight under sterile conditions, it was packed in polypropylene bag, sealed, and stored at room temperature to determine population density for 180 days. The population density was measured by mixing 1 g of the bioformulation in 10 ml of distilled water aseptically and serially diluted to 10^{-6} and 10^{-7}. The prepared bioformulation from $PF07^{EPS+}$ and $PF07^{EPS-}$ were taken for coating sunflower seeds.

In vitro PGP activity

The study was conducted to detect PGP response ability of $PF07^{EPS+}$ and $PF07^{EPS-}$, taking sunflower as the test crop under saline and non-saline conditions. Seeds of sunflower were surface sterilized for 2 min with 70% ethanol followed by 2% sodium hypochlorite (10 min). Surface sterilized seeds of sunflower were soaked in suspension of talc-based formulations in sterile distilled water (SDW) (1:1 w/v) for 01 h, and then dried under shade (28). The seeds were germinated in glass tubes of 50 ml capacity (2 seeds per tube), 1/3 filled with autoclaved plant growth media (PGM) (containing 1.2 mM K_2HPO_4, 0.4 mM KH_2PO_4, 5 mM $CaCl_2$, 3.35 mg ferric citrate l^{-1}, 2.5 Mm Mg SO_4, 2.5 mM K_2SO_4, 10 µM $MnSO_4$, 20 µM H_3BO_3, 5µM $ZnSO_4$, 0.2 µM $CuSO_4$, 1.5 µM $CaSO_4$, 1.0 µM $NaMoO_4$, 1% agar, with pH 6.8) (29) supplemented with 125 mM NaCl (as sunflower seedlings displayed germination only up to 125 mM salinity level) and without NaCl in following sets of treatments: (i) non bacterized seeds (untreated seeds, control); (ii) seeds coated with talc formulation (unbacterized formulation) (iii) seeds treated with talc based formulation of $PF07^{EPS+}$; (iv) seeds treated with talc based formulation of $PF07^{EPS-}$.

In vivo (pot study) PGP Activity

Experiment was conducted in small plastic pots (24×12×12 cm) during the month of March-June in year 2012 and 2013 (for two consecutive years). The experiment was conducted in sterilized soil supplemented with 125 mM NaCl (as sunflower seedlings displayed germination only up to 125 mM salinity level) and without NaCl in similar sets of treatments as mentioned in *in planta* studies.

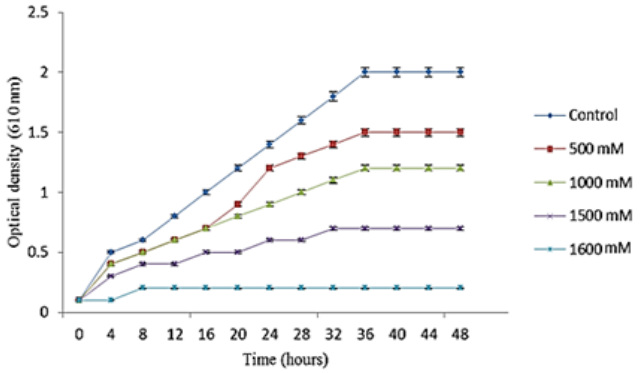

Figure 1. Salinity tolerance assay of PF07. Error bars show the standard deviation of the mean values of five replicates. Five samples were analyzed for each replication, and each treatment consisted of five replications.

The effect of treatments was determined under control (received only normal irrigation water) and saline (irrigated as per requirement with 125 mM NaCl solution) conditions (30). Five plants from each set were taken randomly to determine root associated soil / root tissue ratio (RAS/RT). Plant watering was stopped 6 days before harvesting to facilitate the separation of root associated soil from bulk soil. Roots with adhering soil were carefully separated from bulk soil by gentle agitation for 1 min. RAS was removed from RT by washing them in sterile water. RAS dry mass (dm) and RT dm were measured after 24 h at 105°C, to calculate RAS/RT. Other plant growth parameters including (shoot and root length, fresh weight, dry weight, head diameter and seed yield) and were measured after 120 days.

Statistical analysis

The data generated during quantitative evaluation of EPS and plant growth parameters were analyzed by means of analysis of variance (ANOVA), and means were compared by the Duncans Multiplicity Test Range (DMRT) using the SPSS software (ver. 10.1, SPSS Inc., www.spss.com). The significance level for the analysis was p=0.05.

Results

Bacterial strains

Isolate PF07 was selected from the collection of pseudomonads, as it displayed maximum salinity tolerance capacity and high EPS production. Isolate PF07 was fluorescent, Gram negative, motile rod, oxidase and catalase positive indicating according to Bergey's Manual of Systematic Bacteriology (12) to be a member of the genus *Pseudomonas*. Based on biochemical, physiological characteristics, and nucleotide homology, isolate PF07 displayed maximum relatedness to *Pseudomonas aeruginosa*.

Plant growth promoting attributes

PF07 was positive for siderophore, IAA production but negative for phosphate solubilization, HCN production, chitinase and β-1-3 glucanase. The production of IAA (in supernatant) and siderophore (on CAS agar plate) got drastically reduced with increase in salt concentration and was completely inhibited at 100 mM NaCl concentration. On the other hand an increase in EPS production was recorded with progressive increase in salinity in precipitated supernatant upto 1600 mM NaCl.

Chemical mutagenesis for developing EPS - defective mutant

Of the 100 mutant clones, 5 were identified as defective for EPS production. One of these mutants had stable mutation. EPS defective mutant $PF07^{EPS-}$ showed reduction of 92% in EPS production in comparison to wild strain $PF07^{EPS+}$. However, all the other PGP characters including IAA and siderophore were significantly similar to the wild strains.

Salinity tolerance Assay

Isolate PF07 $^{EPS+}$ could tolerate salinity level upto 1600 mM (9.6 %). Salt shock with 100 mM NaCl did not

Table 1. Effect of saline stress on EPS production, carbohydrate content and protein content.

Salt stress (mM)	Dry weight of EPS (g/l)	Sugar components present in EPS	Carbohydrate content (µg/ml^{-1})	Protein content (µg/ml^{-1})
0	0.821 ±0.01^{a}	Glucose	120±0.01^{a}	2230±0.01^{a}
100	0.834 ±0.02^{a}	Glucose, galactose	130±0.01^{ab}	2398±0.01^{b}
500	0.999 ±0.03^{ab}	Glucose, galactose	149±0.01^{b}	2471±0.01^{c}
1000	1.197 ±0.01^{b}	Glucose, rhamnose	152±0.01^{cd}	2507±0.01^{d}
1500	1.252 ±0.02^{c}	Glucose, rhamnose, trehalose	163±0.01^{de}	2596±0.01^{e}
1600	1.298 ±0.01^{cd}	Glucose, rhamnose, trehalose	175±0.01^{e}	2660±0.01^{f}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range test Two samples were analyzed for each replication, and each treatment consisted of five replications.

affect the growth, but higher osmotic stress of 500 mM, 1000 mM and 1500 mM brought significant reduction in the optical density by 33.33%, 66.66% and 185.71% respectively, in comparison to non-stress conditions (0 mM NaCl) (Fig. 1). No growth was observed above 1600 mM salt concentration. On the other side EPS defective strain PF07^{EPS-} displayed 82% growth reduction in presence of 150 mM NaCl, suggesting it to be a non-salt tolerating strain.

Effect of saline stress on EPS production

Increase in salinity brought increment in the EPS production up to a certain limit. Under saline conditions PF07^{EPS+} brought increase in EPS production by 21.68 %, 45.79 %, 52.49% and 58.09% at salinity level of 500 mM, 1000 mM, 1500 mM and 1600 mM, respectively (Table 1). PF07^{EPS+} produced 0.821 g/l of EPS at 0 mM salinity. PF07^{EPS-} displayed 0.016 g/l of EPS production at 0 mM salinity, which drastically reduced above 100 mM NaCl, and finally got diminished beyond 200 mM NaCl, respectively.

Components of EPS

Analysis of EPS constituents by TLC revealed differences in the sugar components of salinity tolerant strain PF07^{EPS+} under non-stressed and stressed conditions. Under normal conditions (0 mM NaCl) glucose (Rf 0.42) was present as the saccharide unit in the EPS hydrosylate, whereas, EPS obtained under salt stress was composed of various units including glucose (Rf 0.42), galactose (Rf 0.37), rhamnose (Rf 0.74) and trehalose (Rf 0.32) (Table 1).

Determination of the carbohydrate content in the EPS

There was an increase in the concentration of carbohydrates in EPS with the corresponding increase in NaCl level upto 1600 mM NaCl. Under saline conditions PF07^{EPS+} brought increment in carbohydrate content by 8.3%, 24.16%, 26.7%, 35.83%, 45.83% at 100 mM, 500 mM, 1000 mM, 1500 mM and 1600 mM NaCl, respectively in comparison to control (0 mM NaCl) (Table1).

Determination of the protein content in the EPS

Protein content increased remarkably with progressive increase in salinity. Protein content varied from 2130 µg/ml^{-1} at 0 mM NaCl to 3160 µg/ml^{-1} at 1600 mM NaCl. There was 7.88%, 16%, 18.29%, 35.96% and 48.35% increment in protein content (in PF07 $^{EPS+}$) at 100 mM, 500 mM, 1000 mM, 1500 mM and 1600 mM NaCl, respectively, in comparison to control (0 mM NaCl) (Table 1).

Talc-Based Formulation from EPS producing Bacterial Cells

Talc based formulation showed 12.5%, 18.75%, 25%, 31.25% and 37.5% reduction in bacterial population after 30, 60, 90, 120 and 150 days of formulation storage, respectively colony forming units (cfu) count after six month storage showed 42.5% reduction in PF07^{EPS+} population in talc based formualtion. Whereasmutant strain PF07^{EPS-} brought 71.4% reduction in population density of bacterial cells, after six months storage (Fig. 2).

In planta PGP activity

Treatment of seeds with talc based bioformulation of EPS producing cells (PF07^{EPS+}) showed significant increase in germination % in comparison to EPS mutant cells (PF07^{EPS-}) especially under saline (125 mM NaCl) conditions. Talc based bioformulation of PF07^{EPS+} brought 30% and 50% increment in germination % in comparison to mutant PF07^{EPS-} cells under non - saline and saline conditions, respectively. Treatment by PF07^{EPS+} formulation brought increment in root length, shoot length, fresh weight and dry weight by 163.1%, 69.3%, 102.5% and 198.4% respectively, in comparison to PF07^{EPS-} cells under saline conditions. Under non-saline conditions it was observed that PF07^{EPS+} formulation brought 37.5% and 90.32% enhancement in root length and dry weight, respectively in comparison to PF07^{EPS-} bioformulation (Table 2). Whereas, under saline conditions, untreated (control) seeds and bioformu-

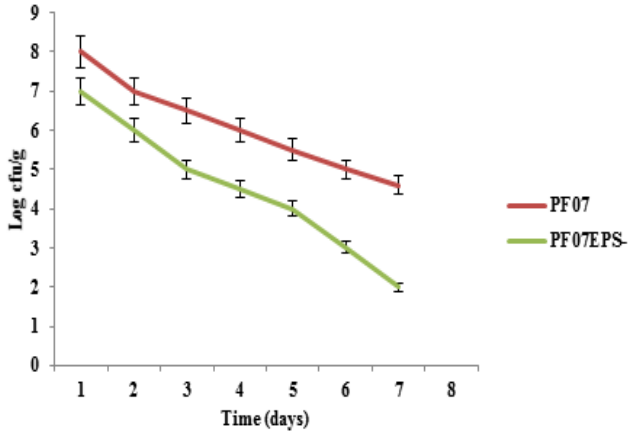

Figure 2. Population density of PF07 in talc based bioformulations on six month storage. Error bars show the standard deviation of the mean values of five replicates. Five samples were analyzed for each replication, and each treatment consisted of five replications.

Table 2. Influence of *P. aeruginosa* PF07 treatment on growth of sunflower plants in non-saline (control) and saline (*in planta*) conditions.

	Non-stress condition (0 mM NaCl)					Stress condition (125 mM NaCl)				
Treatment	**Germination %**	**Root length (cm)**	**Shoot length (cm)**	**Fresh weight (g)**	**Dry weight (g)**	**Germination %**	**Root length (cm)**	**Shoot length (cm)**	**Fresh weight (g)**	**Dry weight (g)**
Control (untreated seeds)	60±0.01[a]	3.0 ±0.02[a]	11.5 ±0.01[a]	1.44±0.02[a]	0.24 ±0.03[a]	40 ±0.02[a]	1.7±0.01[a]	7.6±0.02[a]	0.75±0.02[a]	0.126 ±0.01[a]
Talc coated seeds	60±0.01[a]	2.9 ±0.02[a]	11.4 ±0.01[a]	1.42±0.02[a]	0.23 ±0.03[a]	40 ±0.02[a]	1.6±0.01[a]	7.5±0.02[a]	0.74±0.02[a]	0.125 ±0.01[a]
PF07^{EPS-} formulation	70±0.02[b]	4.0 ±0.01[b]	12.1±0.03[b]	1.56±0.02[b]	0.31 ±0.01[b]	40 ±0.01[a]	1.9±0.03[a]	7.8±0.01[a]	0.77±0.01[a]	0.128 ±0.03[a]
PF07^{EPS+} formulation	100 ±0.02[c]	5.5±0.01[c]	14.3±0.03[c]	1.79 ±0.01[c]	0.59±0.01[c]	90±0.01[b]	5.0±0.03[b]	13.2±0.03[b]	1.56±0.01[b]	0.382±0.01[b]

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range testTwo samples were analyzed for each replication, and each treatment consisted of five replications.

Table 3. Influence of *P. aeruginosa* PF07 treatment on growth of sunflower plants in under non-saline (control) and saline (*in vivo*) conditions.

	Non stress conditions (0 mM)						Saline stress conditions (125 mM)					
Treatments	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (gm)**	**Head diameter (cm)**	**Seed yield (g/pot)**	**RAS/RT**	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (gm)**	**Head diameter (cm)**	**Seed yield (g/pot)**	**RAS/RT**
Control (untreated seeds)	7.11±0.03[a]	48.5±0.01[a]	1.47±0.02[a]	5.71±0.01[a]	09.38±0.01[a]	0.756±0.02[a]	4.82±0.02[a]	37.2±0.01[a]	1.29±0.02[a]	4.78±0.01[a]	7.88±0.01[a]	0.579±0.02[a]
Talc coated seeds	7.10±0.03[a]	48.6±0.01[a]	1.48±0.02[a]	5.72±0.01[a]	09.39±0.01[a]	0.753±0.02[a]	4.81±0.02[a]	37.0±0.01[a]	1.30±0.02[a]	4.77±0.01[a]	7.86±0.01[a]	0.577±0.02[a]
PF07^{EPS-} formulation	7.39±0.01[b]	51.6±0.02[b]	1.50±0.01[b]	6.18±0.03[b]	10.03±0.02[b]	0.771±0.01[b]	4.85±0.03[a]	38.5±0.02[a]	1.30±0.01[a]	4.80±0.03[a]	7.90±0.02[a]	0.581±0.03[a]
PF07^{EPS+} formulation	8.61±0.02[c]	56.9±0.03[c]	1.59±0.03[c]	6.87±0.02[c]	11.56±0.03[c]	0.999±0.03[c]	6.72±0.01[b]	43.4±0.03[b]	1.40±0.03[b]	5.59±0.02[b]	10.91±0.01[b]	0.859±0.01[b]

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range test. Five samples were analyed for each replication, and each treatment consisted of five replications.

lation of EPS mutant PF07^{EPS-} gave significantly similar results.

In vivo (pot study) PGP Activity

Results of *in vivo* study showed that treatment of seeds with PF07^{EPS+} bioformulation brought significant increase in root length, shoot length, dry weight, RAS/RT ratio and head diameter under saline and non - saline conditions over untreated seeds and EPS mutant cells PF07^{EPS-}. Bioformulation of PF07^{EPS+} brought increment in root length, seed yield and head diameter by 39.42%, 26.96% and 16.94% respectively in comparison to untreated seeds under saline conditions**.** RAS/RT ratio increased by about 47.84 % and 32.14 % under saline and non- saline conditions respectively, in comparison to unbacterized seeds (Table 3). Untreated seeds and bioformulation of EPS mutant PF07^{EPS-} gave significantly similar results under saline stress conditions.

Discussion

Isolate PF07^{EPS+} selected from the collection of fluorescent pseudomonads and identified as *P. aeruginosa*, displayed high salt tolerance and EPS production. EPS defective mutant PF07^{EPS-} didn't display salt tolerance above 150 mM and showed 92% reduction in EPS production in comparison to wild strain. There was remarkable increment of about 58% in EPS production with progressive increase in salinity from 100 mM to 1600 mM NaCl. Similarly there was significant increment in carbohydrate and protein content with increase in salinity. Protein content of PF07^{EPS+} increased by 48.35%, suggesting the induction or over expression of stress proteins in strains PF07^{EPS+} that assist in membrane stabilization. Several workers reported the accumulation of stress proteins under saline conditions, that protect the cells by balancing osmotic strength of bacterial membrane (31, 32, 33). It may also be speculated that as protein content increases it upsurges osmotic regulatory mechanisms, which in turn cause decreased sodium toxicity in cytoplasm, thereby protecting cells from salt shock. Sandhya *et al*. (34) reported accumulation of free amino acids and protein molecules in bacterial cells under osmotic stress. Prokaryotic cells respond to environmental stress by inducing specific sets of proteins characteristic to each stress. The proteins in each set of their coding genes constitute a stimulon, such as in oxidative and ionic stress (35).

Addition of NaCl (above 500 mM) in the medium also stimulated the mucoid, slimy growth (profuse spreading of the EPS) of the PF07^{EPS+} up to 1600 mM. Increase in EPS production with increase in salinity suggests that under stress condition energy flow of the PF07^{EPS+} is directed towards protective mechanism, and synthesis of EPS is opted as a defensive strategy for maintaining its survivability and ameliorating salt stress by bacterial cells (36). At low salt concentration (upto 500 mM NaCl) EPS mainly constituted glucose and galactose as its components. Whereas, further increase in salinity resulted in glucose, rhamnose and trehalose as major subunits. These subunits function as a carbon reservoir, which protect microorganisms from saline stress and fluctuations in water potential by enhancing water retention and regulating the diffusion of carbon sources in microbial environment (23). Thus, a strong relationship could be observed in between EPS production, protein content and salinity tolerance in PF07^{EPS+} cells.

For a bioformulation to have a high shelf life and be consistent, it must tolerate the constantly changing and frequently stressful environmental conditions. Developed talc-based bioformulation of PF07^{EPS+} was able to support population density of about 57.5% after six month storage whereas there was steep reduction in cell number of PF07^{EPS-} mutant by 71.4%. This also proved the impact and role of EPS in maintaining the cfu and protecting the cells in the formulation. Many microorganisms produce EPS as a strategy for growing, adhering to solid surfaces, and surviving adverse conditions.

Coating of seeds with cell free talc formulation reduced germination and plant growth parameters under saline conditions. Several workers reported decline in germination rate and plant growth parameters with increase in salinity due to reduction of the water passage into the seeds during imbibition (37, 38, 39, 40, 41) and due to slowing down the water absorption by the plant (42, 43, 44). Under non-stress conditions, formulation of PF07^{EPS-} brought significant increase in root length, shoot length and seed yield, suggesting the role of PGP metabolites like siderophore and IAA in growth promotion and yield enhancement, as the mutant was positive for these PGP characteristics. However, PF07^{EPS-} was ineffective and insignificant in enhancing plant growth parameters under saline conditions. Talc based formulation of PF07^{EPS+} was effective both under saline and non-saline conditions suggesting absence of PGP activity under saline stress conditions, and thus confirming the involvement of EPS in plant growth promotion under saline stress. In fact isolate PF07^{EPS+} was much better in enhancing the growth parameters of sunflower in *in vitro* and in *in vivo* studies under non-saline conditions, which was significantly higher than the mutant. Under saline conditions the enhancement in dry weight and yield was even starker as compared to control, the mutant being almost insignificant. Ashraf *et al.* (36) reported the significant enhancement in plant growth parameters in sunflower seedling by treating with EPS producing bacterial strains. The most conceivable reason for such heightened yield may be due to the fact that introduction of designed bioformulation brought significant increase in mass of RAS/RT in comparison to uninoculated control, as was clearly observed. Increased RAS/RT upsurges adhesion of soil particles, intensify soil aggregation, enhances soil texture, increase water holding capacity of soil and, reduce water loss during stress conditions (45). Increased root associated soil on PF07^{EPS+} application showed that hydrophilization of soil leads to improved supply of nutrients that is responsible for plant growth promotion (9).

Presence of carbohydrates and proteins in EPS provides a self-protective strategy to bacterial cells that help them to maintain their population density in soil after inoculation. Proteins released by PF07^{EPS+} generally deposit in plants grown under stress conditions and they may supply a storage form of nitrogen which play a vital role in osmotic adjustment, that is utilized during saline stress (46). Bioformulation designed from EPS producing *P. aeruginosa* PF07^{EPS+} displayed significant

enhancement in plant growth producing attributes. EPS can bind soil particles to form microaggregates and macroaggregates. Plant roots fit in the pores between microaggregates and thus stabilize macroaggregates. Plants treated with EPS producing bacteria display increased resistance to saline stress due to improved soil structure (9, 23). EPS can also bind to cations including Na^+ thus making it unavailable to plants under saline conditions. Several workers reported the role of EPS produced by *Paenibacillus polymyxa* in increasing the aggregation of RAS/RT ratio on wheat (47, 9, 48). RAS forms the immediate environment where plants take up water and nutrients for their growth (9).

It has been already assessed that more than 800 million hectares land throughout the world is suffering from salinization, and is responsible for 60% loss in sunflower yield (49). *P. aeruginosa* PF07 $^{EPS+}$ along with an important oil seed crop can thus also be used in reclamation of barren saline soils. Increase in production and reclamation of semiarid regions utilizing EPS producing microbes can make great contribution in enhancing yield and production of sunflower in stressed soils around the globe.

Talc based formulation of the isolate PF07 $^{EPS+}$ brought more significant enhancement in plant growth parameters in comparison to non-formulated seeds and control seeds. The present investigation may be a step towards field application and commercialization of talc as a carrier for long-term sustenance and storage of stress tolerating PF07^{EPS+}, which may minimize salinity disposal in the environment. Conclusively, the talc provides prolong shelf life, and sustain the efficacy of the PF07^{EPS+} suggesting the carrier is stable for the bacteria. Talc based formulation was effective under saline conditions as it increased, yield of sunflower crop under saline conditions.

Thus, salt - tolerating PGPR PF07^{EPS+} and its bioformulation can serve as a nonpolluting and more cost-effective way to improve production in a saline stressed habitat. EPS helped not only in protecting the bacterial cells under saline conditions but also helped in maintaining high cfu in talc based formulation. The EPS based talc based formulation is thus recommended for enhancing the yield of sunflower in arid saline soils.

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Other articles in this theme issue include references (50-65).

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