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ORIGINAL PAPER

Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-P45

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Abstract Production of exopolysaccharides (EPS) can be used as a criteria for the isolation of stress tolerant microorganisms. In the present study, EPS-producing fluorescent pseudomonads were isolated from alfisols, vertisols, inseptisols, oxisols, and aridisols of different semiarid millet growing regions of India and were screened in vitro for drought tolerance in trypticase soy broth supplemented with different concentrations of polyethylene glycol (PEG6000). Out of the total 81 isolates, 26 could tolerate maximum level of stress (−0.73 MPa) and were monitored for the amount of EPS produced under maximum level of water stress. The strain GAP-P45, isolated from alfisol of sunflower rhizosphere, showed the highest level of EPS production under water stress conditions, was identified as *Pseudomonas putida* on the basis of 16S rDNA sequence analysis, and was used as seed treatment to study its effect in alleviating drought stress effects in sunflower seedlings. Inoculation of *Pseudomonas* sp. strain GAP-P45 increased the survival, plant biomass, and root adhering soil/root tissue ratio of sunflower seedlings subjected to drought stress. The inoculated bacteria could efficiently colonize the root adhering soil and rhizoplane and increase the percentage of stable soil aggregates. Scanning electron microscope studies showed the formation of biofilm of inoculated bacteria on the root surface and this, along with a better soil structure, might have protected the plants from the water stress.

Keywords *Pseudomonas putida* GAP-P45 · Exopolysaccharide · Drought stress · Soil aggregate stability · Biofilm

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Introduction

Drought stress is one of the major agricultural problems limiting crop productivity in most of the arid and semiarid regions of the world. The water potential of rhizosphere soil is a key parameter that determines the availability of water, oxygen, and nutrients to plants and microorganisms (Postma et al. 1989; Blum and Johnson 1992; Van Gestel et al. 1993). The complex and dynamic interactions among microorganisms, roots, soil, and water in the rhizosphere induce changes in physico-chemical and structural properties (Haynes and Swift 1990; Tisdall and Oades 1980) of the soil. Microbial polysaccharides can bind soil particles to form microaggregates (<250 μm diameter) and macroaggregates (>250 μm diameter; Oades 1993; Tisdall and Oades 1982; Edwards and Bremmer 1967). Plant roots and fungal hyphae fit in the pores between microaggregates and thus stabilize macroaggregates (Oades and Waters 1991). Maintenance of soil structure is an important feature of sustainable agriculture because it impacts a range of processes influencing crop yield. Drought stress can make physico-chemical and biological properties of soil unsuitable for soil microbial activity and crop yield. Water availability controls the production and consumption of protein and polysaccharides by the bacteria (Roberson and Firestone 1992) and thus indirectly influences soil structure.

Bacteria like *Pseudomonas* can survive under stress conditions due to the production of exopolysaccharide (EPS), which protects microorganisms from water stress by enhancing water retention and by regulating the diffusion of organic carbon sources (Hepper 1975; Wilkinson 1958; Roberson and Firestone 1992; Chenu 1993; Chenu and Roberson 1996). EPS also help the microorganisms to irreversibly attach and colonize the roots due to involvement of a network of fibrillar material that permanently connects the bacteria to the root surface (Bashan and Holguin 1997). Bashan et al. (2004) reported the role of polysaccharides producing *Azospirillum* in soil aggregation. Concentration and composition of microbial EPS dramatically changed under stress conditions. Capsular material of *Azospirillum brasilense* Sp245 was found to contain high molecular weight carbohydrate complexes (lipopolysaccharide–protein (LP) complex and polysaccharide–lipid (PL) complex) that could be responsible for protection under extreme conditions, like desiccation. Addition of these complexes to a suspension of decapsulated cells of *A. brasilense* Sp245 significantly enhanced survival under drought stress (Konnova et al. 2001). The EPS released into soil as capsular and slime materials by soil microbes can be adsorbed by clay surfaces due to cation bridges, hydrogen bonding, Van der Waals forces, and anion adsorption mechanisms, thus forming a protective capsule around soil aggregates (Tisdall and Oades 1982). Plants treated with EPS-producing bacteria display increased resistance to water stress (Bensalim et al. 1998). Alami et al. (2000) observed a significant increase in root adhering soil per root tissue (RAS/RT) ratio in sunflower rhizosphere inoculated with the EPS-producing rhizobial strain YAS34 under drought conditions. Similar results were obtained with wheat plantlets inoculated with *Paenibacillus polymyxa* (Gouzou et al. 1993) and *Pantoea agglomerans* (Amellal et al. 1998) under salt stress. Hartel and Alexander (1986) observed a significant correlation between the amount of EPS produced by cowpea *Bradyrhizobium* strains and their desiccation tolerance. Probably, it may possible to alleviate drought stress in the plants by increasing the population density of EPS-producing bacteria in the root zone.

In the rain-fed agriculture systems, moisture stress in soil is a major factor limiting crop production. Therefore, in the present investigation, an attempt was made to isolate drought tolerant *Pseudomonas* strains from cropped soils of different arid and semiarid zones representing rain-fed cropping system of India. An efficient EPS-producing, drought tolerant *Pseudomonas putida* strain GAP-P45 was characterized using biochemical and molecular approaches. The effect of GAP-P45 inoculation on soil aggregation and growth of sunflower seedlings under drought stress conditions was studied.

Materials and methods

Isolation and screening

Pseudomonads were isolated from alfisols, vertisols, inceptisols, oxisols, and aridisols collected from rhizospheres of millets and sunflower plants grown under 25 semiarid locations across India. The plants were uprooted with attached soil, brought to the lab under refrigerated conditions, and immediately processed. Excessive soil from the roots was removed by gentle shaking (Gouzou et al. 1993), and RAS was carefully collected and used for isolation of fluorescent pseudomonads by serial dilution method using King's B (proteose peptone, 10 g; casein enzyme hydrolysate, 10 g; K_2HPO_4, 1.5 g; $MgSO_4$, 1.5 g; and Agar, 15 g; per liter) as selective medium. The isolates able to produce mucoid growth on King's B medium after incubation at 28±2°C for 48–72 h were stained with Indian ink to check the presence of capsular material (Fett et al. 1989) and further screened for their ability to survive under drought stress.

Bacterial growth under water stress

Trypticase soya broth (TSB) with different water potentials (−0.05, −0.15, −0.30, −0.49, and −0.73 MPa) was prepared by adding appropriate concentrations of polyethylene glycol (PEG6000) (Michel and Kaufmann 1973) and was inoculated with 1% of overnight raised cultures of the bacterial isolates in TSB. Three replicates of each isolate and each concentration were prepared. After incubation at 28°C under shaking conditions (120 rpm) for 24 h, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer (Thermospectronic, 336002, USA). The growth of the isolates at various stress levels was recorded.

Extraction and purification of exopolysaccharides

The cultures able to grow at maximum stress level were analyzed for their ability to produce EPS (Fett et al. 1986, 1989) under no stress and maximum stress level (−0.73 MPa). Exopolysaccharide was extracted from 3-day-old cultures raised in TSB (25% PEG was added to TSB for inducing stress). The culture was centrifuged at 20,000×g for 25 min and the supernatant was collected. Highly viscous cultures were diluted with 0.85% KCL before centrifugation. The pellet was washed twice with 0.85% KCl to completely extract EPS. The possible extraction of intracellular polysaccharides was ruled out by testing the presence of DNA in the supernatant by DPA reagent (Burton 1956). Concentration of protein in the supernatant was estimated by Folin's reagent (Lowery et al.

1951). Then, the supernatant was filtered through 0.45 μm nitrocellulose membrane and dialysed extensively against water at 4°C. The dialysate was centrifuged (20,000×g) for 25 min to remove any insoluble material and mixed with 3 v of ice-cold absolute alcohol and kept overnight at 4°C. The precipitated EPS obtained by centrifugation (10,000×g for 15 min) was suspended in water and further purified by repeating the dialysis and precipitation steps. Total carbohydrate content in the precipitated EPS was determined according to Dubois et al. (1956). The isolate GAP-P45 showing maximum production of EPS under moisture stress was selected for further studies

Acid hydrolysis of exopolysaccharides and thin-layer chromatography

The precipitated EPS of isolate GAP-P45 was hydrolyzed with 2 v of 2.5 M H_2SO_4 at 100°C for 1 h, then the solution was neutralized with 1 M sodium carbonate and applied to the silica gel plates (Silica gel 60F 254; Merck) in a thin-layer chromatography chamber using n-butanol: acetic acid: water (4:1:5 *v*/*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 colored spots were measured and compared with those of standard carbohydrates (glucose, mannose, fructose, mannitol, arabinose, xylose, rhamnose, raffinose, galactose, and glucuronic acid; Horborne 1976).

Biochemical and molecular characterization of strain GAP-P45

The GAP-P45 strain was characterized for Gram staining, capsule formation, oxidase activity, catalase activity, urease activity, gelatin hydrolysis, starch hydrolysis, citrate utilization, production of H_2S, production of indole, and acetyl methyl carbinol and fermentation of glucose, sucrose, trehalose, maltose, mannitol, xylose, fructose, galactose, mannose, rhamnose, and sorbitol according to Holt et al. (1994). Antibiotic resistance profile of the strain GAP-P45 was screened by testing resistance of the isolate to various antibiotics such as ampicillin, amoxycillin, bacitracin, cloxacillin, chloramphenicol, carbencillin, ciprofloxacin, erythromycin, gentamycin, kanamycin, methicillin, nalidixicacid, polymyxin B, penicillin G, rifampicin, streptomycin, tetracycline, trimethoprim, and vancomycin on solid medium using antibiotic discs of different concentrations (Himedia, India; Lalucat et al. 2006).

For molecular characterization, bacterial genomic DNA was isolated (Chen and Kuo 1993) and subjected to polymerase chain reaction (PCR) for amplification of 16S rRNA gene using universal forward (5′-AGAGTTT GATCCTGGCTCAG-3′) and reverse (5′-AAGGAGGT GATCCAGCCGCA-3′) primers under standard conditions (initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 40 s, extension at 72°C for 90 s, and final extension at 72°C for 7 min). The PCR (approximately 1.5 kb) product was purified and sequenced (Oscimum Bio Solutions, India). The partial 16S rDNA sequence was compared with the sequences available in the GenBank, EMBL, and DJB databases using the gapped BLASTN 2.2.21 program through the National Center for Biotechnology Information server and submitted to GenBank. Identification at the species level was defined as a 16S rDNA sequence similarity of ≥99% with that of the prototype strain sequence in GenBank; identification at the genus level was defined as a 16S rDNA sequence similarity of ≥97% with that of the prototype strain sequence in GenBank. A failure to identify was defined as a 16S rDNA sequence similarity score of lower than 97% with those deposited in GenBank at the time of analysis. The strain GAP-P45 was also deposited at the National Bureau of Agriculturally Important Microorganisms, Mau, Utter Pradesh, India.

Plant growth promoting properties of GAP-P45

The isolate GAP-P45 was tested in vitro for plant growth promoting properties. For testing ammonia production, culture was raised in 10 ml of peptone water at 28°C for 4 days, and 1 ml of Nesseler's reagent was added. Development of yellow to brown color indicated production of ammonia (Dey et al. 2004). For siderophore production, 1 μl of overnight raised culture in Luria broth (LB) was spotted on Chrome Azurol S agar plates and incubated at 28°C for 48–72 h. Plates were observed for the appearance of orange halo around the bacterial colony (Schwyn and Neilands 1987). For hydrogen cyanide (HCN) production, the culture was streaked on King's B medium amended with 4.4 g l^{-1} of glycine and Whatman number 1 filter paper disc soaked in 0.5% picric acid (in 2% sodium carbonate) was placed in the lid of Petri plate. The plates were sealed with parafilm and incubated at 28°C for 4 days for the development of deep orange color (Bakker and Schipper 1987). The method of Gordon and Weber (1951) was followed for the estimation indole acetic acid (IAA). One milliliter of the broth culture, raised in LB (amended with 5 mM tryptophan), was centrifuged (10,000 g for 20 min), and supernatant was carefully decanted in a separate test tube; 4 ml of Salkowsky reagent were added to1 ml of supernatant and then the mixture was incubated for 1 h at room temperature for the development of pink color. After incubation, the absorbance was read at 530 nm. Concentration of the proteins in the pellet was determined (Lowery et al. 1951), and the amount of IAA produced was expressed per milligram cell protein. For the estimation of

gibberellic acid, 2 ml of zinc acetate were added followed by 2 ml of potassium ferrocyanide to 15 ml of the culture supernatant raised in minimal media. After centrifugation at low speed for 15 min, 5 ml of the supernatant was taken in a test tube, and 5 ml of 30% HCL were added followed by incubation at 20°C for 75 min; then the absorbance was read at 254 nm (Holbrook et al. 1961). For studying phosphate solubilization, 5 µl of overnight raised culture was spotted on Pikovskaya's agar plates containing 2% tricalcium phosphate. The plates were incubated at 28°C for 24–48 h and observed for the appearance of the solubilization zone around the bacterial colonies. For quantitative analysis, 5 ml of NBRI-BBP medium (Mehta and Nautiyal 2001) in 30 ml test tubes were inoculated in triplicates with 50 µl of bacterial culture (2×10^9 cfu/ml). Test tubes were incubated for 7 days at 28°C on incubator shaker at 180 rpm. The cells were harvested by centrifugation at 2,655×g for 10 min, and the supernatant thus obtained was analyzed for the concentration of unsolubilized phosphate (Fiske and Subbarow 1925). Asymbiotic nitrogen fixation was determined by growing the culture in nitrogen-free malate medium according to Döbereiner and Day (1976).

Plant growth under drought stress

The *Pseudomonas* sp. strain GAP-P45, isolated from sunflower rhizosphere grown in alfisol of Gunegal Research Farm (GRF), Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad, India was an efficient EPS producer under drought stress (−0.73 MPa) and possessed plant growth properties (Table 1). Therefore, it was selected for inoculation of sunflower plants under drought stress conditions. Soil was collected from homogeneous horizon (0–20 cm) of GRF, CRIDA, a semiarid region under rain-fed production system. The soil was air-dried and sieved (<2 mm) before being analyzed for the physico-chemical properties. The soil contained 71% sand, 3% silt, and 26% clay with 1.60 Mg m^{-3} bulk density, 39.9% total porosity, and 37.9% water holding capacity; it had pH 7.0 and electrical conductivity of 0.103 ms. Organic C, total N and total P content of soil were, 0.62, 0.12, and 0.05 g/kg, respectively. Soil water content, determined by drying the initially saturated soil at different matric potentials by pressure plate apparatus (Santra Barbara, CA, USA), was 16.5% (−0.3 MPa). Seeds of sunflower (var. Sunbred) were surface sterilized with 0.1% $HgCl_2$ and 70% ethanol, washed with sterile distilled water, and coated with talc based formulation (10^8 cells/g) of GAP-P45 using 1% carboxy methyl cellulose as adhesive. For the control treatment, the seeds were treated with plain talc. The coated seeds were shade dried and sown in plastic cups (surface sterilized) filled with 950 g of sterile soil (sterilized for three consecutive days). Both inoculated and uninoculated treatments were replicated twenty times, and each treatment had three plants per pot. The pots were incubated in a controlled environmental chamber at 28/18°C day/night temperature and a 16/8 h light/dark cycle (350 µmol $m^{-2}s^{-1}$ light intensity). The soil moisture was adjusted at 75% of water holding capacity (WHC). Soil moisture (12.5% of dry weight of soil) was maintained constant during the experiment by daily sprinkling with sterile distilled water.

After 11 days of germination, water stress was induced in ten out of 20 replicates by discontinuing watering. After 15 days of germination (after 4 days of water stress), the seedlings were harvested, and soil moisture in the pots was measured using a HH_2 moisture meter (Theta probe type ML2X Delta-T-device, England).

Harvesting, determination of RAS, RAS/RT ratio, and aggregate water stability

Twelve plantlets per treatment were sampled. Roots with adhering soil were carefully separated from bulk soil by gentle shaking. RAS was removed by washing roots in distilled water, and its EPS content was estimated (Šafařík and Šantrůčková 1992). Shoot and root dry mass was recorded after drying the samples at 105°C, and RAS/RT ratio was calculated. Water stability of RAS was determined by the wet sieving method. Root system with adhering soil was passed through a set of sieves (2, 1, 0.5, and 0.25 mm) and immersed in water and shaken. Amounts of water stable aggregates (>0.25 mm) were calculated by substracting coarse sand and root fragments remaining on the sieve. Oven-dried soil aggregates were transferred into dispersion cups and stirred for 10 min with 10% sodium hexameta phosphate to remove clay particles from microaggregates,

Table 1 Plant growth promoting properties of *P. putida* strain GAP-P45 under non-stressed and drought-stressed conditions

Plant growth promoting properties	Non-stressed conditions	Drought-stressed conditions
Ammonia production	+++	++
Hydrogen cyanide	++	+
Indole acetic acid	329.33 (±10.12) µg mg^{-1} protein	171 (±2.60) µg mg^{-1} protein
Gibberellins	382.7 (±2.1) µg mg^{-1} protein	105.0 (±13.5) µg mg^{-1} protein
P-solubilization	69.3 (±6.7) µg ml^{-1}	41.7 (±4.0) µg ml^{-1}
Siderophore	+	+

+, presence; +, fair; ++, good; +++, excellent

and aggregate stability and mean weight diameter were recorded (Chaudhary and Kar 1972; Bartoli et al. 1991). Relative water content (RWC) of leaves was determined by recording fresh weight, saturated weight, and dry weight of leaves (Teulat et al. 2003).

Enumeration of rhizobacteria

Total counts of inoculated strain GAP-P45 in bulk soil, RAS, and rhizoplane were determined on the 15th day of sowing using King's B medium containing antibiotic (trimethoprim and vancomycin, 30 μg each). The entire root system with adhered soil was removed from the pot and agitated gently, to get the bulk soil fraction. The RAS samples were obtained as mentioned earlier. Serial dilutions of roots and soil suspensions were prepared, and appropriate dilutions were plated on King's B medium supplemented with trimethoprim (30 μg/ml) and vancomycin (30 μg/ml). After incubation at 28°C for 24–48 h, fluorescent colonies were counted.

Colonization and biofilm formation by GAP-P45

After harvesting, the root samples were fixed in 2.5% Gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4°C and post fixed in 2% aqueous osmium tetraoxide in the same buffer for 2 h. After the post fixation, samples were dehydrated with alcohol and mounted over the stubs with double-sided conductivity tape. A thin layer of gold metal was applied over the samples using an automated sputter coater (JEOL JFC-1600). The samples were scanned under scanning electron microscope (Model: JOEL-JSM 5600, JAPAN) at various magnifications (RUSKA Lab, Hyderabad, India; Bozzola and Russell 1999). The root samples were also stained with acridine orange (0.5% *w/v* in PBS) for 5 min at room temperature and immediately observed under fluorescent microscope using green filter (800 nm).

Statistical analysis

Comparisons between treatments were carried out by one-way analysis of variance (ANOVA). Tukeys test was applied after ANOVA for heterogeneity of variance.

Results

Isolation and screening

A total of 212 fluorescent pseudomonads were isolated on King's B media, of which, 81 showed mucoid growth on King's B media and the presence of capsular material under microscope. These EPS-producing isolates were screened for drought tolerance on solid media at varying water potential. Isolates, which could tolerate higher levels of drought stress, were used for EPS production under both no stressed conditions as well as under minimum water potential (−0.73 MPa). The strain GAP-P45 produced a significant amount of EPS (4.06 mg mg^{-1} protein) under minimum water potential (−0.73 Mpa) when compared to other strains and also showed higher cell viability at −0.73 Mpa.

Exopolysaccharide produced by GAP-P45 was acid hydrolyzed, and component sugars were qualitatively identified by thin-layer chromatography. The Rf values of the yellow colored spots developed were recorded, compared with those of standard sugars. The hydrolysate of EPS of GAP-P45 strain produced spots on silica gel plates, with Rf values of 0.18, 0.28, corresponding to glucose and mannose, respectively, under no stress and spots with Rf values of 0.172, 0.26, and 0.39, corresponding to glucose, mannose, and rhamnose, respectively, under stress conditions.

Biochemical and molecular characterization of the isolate GAP-P45

Microscopic studies revealed strain GAP-P45 as Gram-negative, motile, capsulated rods having pale yellow, entire, convex, opaque, and mucoid colony morphology. The isolate showed presence of oxidase activity, catalase activity, urease activity, and could utilize citrate as carbon source; it produced H_2S and acid from glucose and fructose. Production of acetyl methyl carbinol was not observed. Based on biochemical characterization, the strain GAP-P45 was identified as *Pseudomonas* sp. Molecular characterization of the strain was done on the basis of 16S rDNA gene sequence that showed 99% homology with that of *Pseudomonas putida* in the existing database of National Center of Bioinformatics. The sequence was submitted to GenBank under the accession no GQ221267. The strain GAP-P45 was also deposited at the National Bureau of Agriculturally Important Microorganisms, Mau, Utter Pradesh, India.

P. putida GAP-P45 strain showed resistance to amoxycillin (25 μg/disc), cloxacillin (10 μg/disc), kanamycin (30 μg/disc), methicillin (30 μg/disc), vancomycin (30 μg/disc), penicillin (6 μg/disc), and trimethoprim (30 μg/disc). The isolate GAP-P45 also exhibited plant growth promoting properties (Table 1) like production of ammonia, siderophore, HCN, IAA, gibberellic acid, and P-solubilization both under no stress and drought stress conditions; significant reduction in plant growth promoting traits was observed under drought stress. The production of IAA and gibberellic acid under no stress conditions was

329.33 μg mg^{-1} protein and 382.7 μg mg^{-1} protein, respectively, whereas under drought stress conditions, the production was 171.0 μg mg^{-1} protein and 105.0 μg mg^{-1} protein, respectively. Phosphate solubilization was 69.3 and 41.7 ppm ml^{-1} under no stress and stress conditions, respectively.

Growth studies

Drought stress drastically affected the growth of sunflower seedlings as reflected by stunted growth, less vigor, and drying of leaves. After 15 days of germination, soil moisture in pots subjected to drought stress was 8.2% (49.6% of WHC) as compared to 12.37% (75% of WHC) in pots without stress. Inoculation of EPS-producing *P. putida* strain GAP-P45 significantly increased total root, shoot length (Fig. 1a), and dry biomass in sunflower seedlings, both under no stress and drought stress conditions, and the effects of inoculation on root dry biomass were higher (53.92% and 45.1% under no stress and drought stress conditions, respectively) than on shoots (Fig. 1b). An increase in total dry biomass by 64.6% and 23% due to strain GAP-P45 inoculation was observed under drought stress and no stress conditions, respectively. Leaves of inoculated seedlings under drought stress had RWC compared to that of uninoculated seedlings maintained under no stress conditions.

There was a positive effect of inoculation on RAS/RT ratio, which increased by 12% and 49.8% under no stress and drought stress conditions, respectively, and the effect was positively correlated with the content of water insoluble saccharides in RAS (Table 2).The effect of *P. putida* GAP-P45 on aggregation of rhizosphere soil was assessed by determining the percentage of water stable aggregates in RAS. Inoculation significantly enhanced percentage of water stable aggregates (diameter >0.25 mm) from 30±2 to 51±4% and from 28±1% to 67±1% under no stress and drought stress, respectively. Mean weight diameter of soil aggregates was also significantly increased due to GAP-P45 inoculation under both no stress and drought stress conditions.

After 15 days of germination, the population of strain GAP-P45 in bulk soil, RAS, and rhizosplane was 6×10^3 (±5.279), 6×10^6 (±8.085), 2×10^7 (±12.29) CFU g^{-1} soil/root under no stress and 4×10^2 (±5.203), 5×10^5 (±5.657), 3×10^6 (±9.94) CFU g^{-1} soil/root, respectively, under drought stress conditions, respectively. The population of uninoculated bulk soil, RAS, and rhizoplane was 4×10^2 (±6.129), 3×10^1 (±13.21), 3×10^1 (±16.01) CFU g^{-1} soil/root under no stress and 2×10^1 (±3.110), 2×10^1 (±6.112), 2×10^1 (±11.25) CFU g^{-1} soil/root, respectively, under drought stress conditions, respectively.

Scanning electron microscope studies also showed bacterial colonization on the root surface both under normal and drought stress conditions (Fig. 2) and revealed that the strain GAP-P45 produced biofilm like communities, where bacteria were connected together by an extracellular polymeric matrix with micro colony formation under water stress conditions. Staining of the roots with acridine orange (DNA staining dye) further confirmed root surface colonization by the bacteria.

Discussion

The management of drought-affected soils is essential to meet the ever increasing food demands. Introduction of EPS-producing microorganisms in the drought-stressed soils can alleviate stress in the crop plants. In the present investigation of the 212 fluorescent pseudomonads isolated from soils of different semiarid regions of the country, only 81 isolates (38.2%) produced mucoid

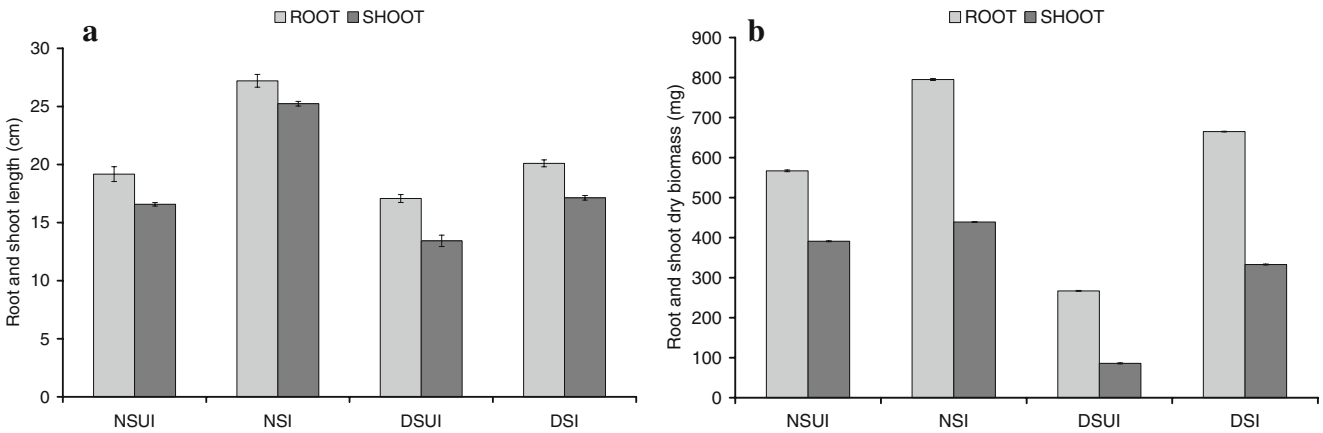

Fig. 1 Growth promotion of sunflower seedlings inoculated with *P. putida* strain GAP-P45 ((**a**) root and shoot length; (**b**) root and shoot dry biomass). *NSUI* non-stressed uninoculated, *NSI* non-stressed inoculated, *DSUI* drought-stressed uninoculated, *DSI* drought-stressed inoculated. Values with different letters are significantly different at $P<0.05$ in all the treatments

Table 2 Effect of *Pseudomonas* sp. strain GAP-P45 inoculation on soil structure and physiology of sunflower seedlings

Treatments	Root adhering soil dry weight per root tissue ratio (mg/mg)	Exopolysaccharide (mg/plant)	Aggregate stability (%)	Mean weight diameter (mm)	Relative water content (%)	Soil moisture (%)
Non-stressed uninoculated	18.23a (±0.49)	14.37a (±0.51)	30.00a (±2.00)	0.170a (±0.02)	53.70a (±1.60)	12.3a (±0.04)
Non-stressed inoculated	20.60b (±0.52)	54.00b (±2.26)	51.33b (±4.50)	0.356b (±0.05)	60.20b (±1.06)	12.3ba (±0.04)
Drought-stressed uninoculated	24.80c (±0.32)	15.67ca (±0.30)	28.40ca (±0.69)	0.140ca (±0.02)	40.60c (±0.85)	8.16c (±0.65)
Drought-stressed inoculated	10.36d (±0.76)	63.30db (±9.95)	70.80d (±0.80)	0.389db (±0.09)	51.30da (±1.17)	8.20dc (±0.25)

Mean values with different letters are significantly different at 5% probability level (mean±standard deviation)

growth. Of these 81 isolates, 26 could tolerate maximum level of drought stress (−0.73 Mpa). The EPS production of these selected isolates was higher under stressed than under no stress conditions, and it increased by increasing stress level, indicating that EPS production in bacteria occurs as a response to the stress (Roberson and Firestone 1992).

Role of capsular material has been suggested in the protection of *A. brasilense* Sp245 cells against desiccation (Konnova et al. 2001). Probably EPS can provide a

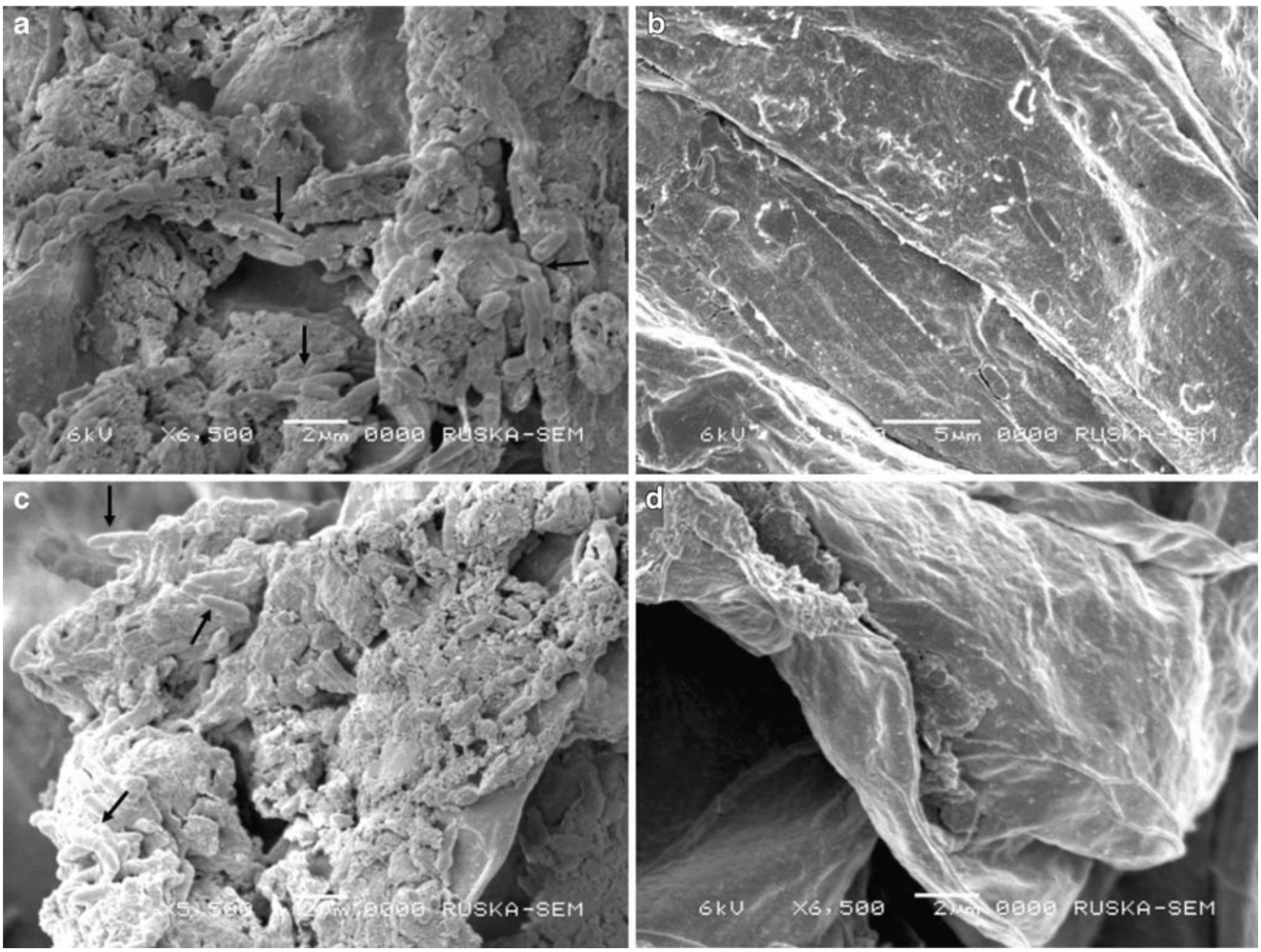

Fig. 2 Scanning electron microscopic micrographs of sunflower roots colonized by *P. putida* strain GAP-P45. (**a**) inoculated, non-stressed (**b**) uninoculated, non-stressed (**c**) inoculated, drought-stressed (**d**) uninoculated, drought-stressed

microenvironment that holds water and dries more slowly than the surrounding microenvironment, thus protecting bacteria from drying and fluctuations in water potential (Hepper 1975; Wilkinson 1958). Isolate GAP-P45 produced significant amount of EPS under minimum water potential, exhibited plant growth promoting properties like P-solubilization, produced of ammonia, siderophore, HCN, IAA, and gibberellic acid both under no stressed and drought-stressed conditions (Table 1) and was identified as *P. putida* on the basis of 16S rRNA gene sequence analysis. Seed inoculation of sunflower with the *P. putida* GAP-P45 could help the plants in tolerating drought stress as indicated by significantly high root and shoot dry biomass (Fig 1b), the RAS/RT ratio, the aggregate stability, and mean weight diameter of the inoculated plants under stressed conditions (Table 2). Plants with higher root biomass had higher microbial biomass and percent aggregation in RAS (Haynes and Francis 1993). Production of EPS by bacteria improved RAS permeability by increasing soil aggregation and maintaining higher water potential around the roots; in this way, there was an increase in the uptake of nutrients by plant, with an increase in plant growth; in addition, the bacteria protected the seedlings from drought stress (Alami et al. 2000; Miller and Wood 1996). A high insoluble saccharide content of RAS of the inoculated seedlings indicated an enhanced EPS synthesis in the root zone. There was also an increased mass of soil aggregated around roots of the inoculated seedlings with a highly significant positive correlation ($P<0.01$) between water insoluble saccharides and RAS/RT ratio; these data indicated the role of bacterial EPS in aggregating the soil around roots (Watt et al. 1993; Alami et al. 2000; Bezzate et al. 2000). Higher EPS content and better aggregation of RAS could help the plants to take up a higher volume of water and nutrients from rhizosphere soil (Miller and Wood 1996), resulting in a better growth of plants, and also, this was useful to counteract the negative effects of drought stress (Munns 2002). Probably other factors like mechanical impedance (the axial root pressure exerted per unit area) and gaseous and moisture contents of the RAS-root association could also have influenced the plant growth and crop yield of the inoculated seedlings. Under dry conditions, the increased root biomass and the rhizobacterial population probably increased root and soil microbial respiration with influences on the composition of soil atmosphere. All these RAS factors can control and regulate growth and functioning of roots (Mohr and Schopfer 1996; Clarke et al. 2003; Kuzyakov and Larionova 2005; Wittenmayer and Merbach 2005). In uninoculated seedlings, due to the absence of EPS-producing bacterial populations, most of roots was devoid of RAS and thus was more susceptible to stress effect. Moreover, a higher population of EPS-producing bacteria on roots of inoculated plants may have stimulated root exudation (Tisdall 1994; Miller and Wood 1996; Wittenmayer and Merbach 2005), with stimulation of growth of inoculated bacteria with higher EPS production in the rhizosphere (Fischer et al. 2003). Scanning electron microscope confirmed the colonization of bacteria and biofilm formation on the surface of the roots (Fig. 2). Most of the nutrients and water taken up by the plants passes through the interfacial region, that is the soil adhering strictly to plant roots (McCully and Canny 1988; Watt et al. 1994).

Conclusion

The EPS-producing *Pseudomonas strain* GAP-P45 act as a plant growth promoting rhizobacteria and can alleviate the effect of drought stress in sunflower plants possibly through improved soil structure and plant growth promoting substances. The moisture sorption and colloidal stabilization properties of EPS are important and should be considered in combination with other factors like spread of bacteria along the root system and physical properties of RAS. Good soil structure in the rhizosphere could improve growth of the seedlings, as mediated by efficient uptake of nutrients and water.

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