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EXPERIMENTAL ARTICLES

The Production of Exopolysaccharide by *Pseudomonas putida* GAP-P45 under Various Abiotic Stress Conditions and Its Role in Soil Aggregation[1]

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Abstract—Exopolysaccharides (EPS) production is modified in response to environmental changes and is believed to protect bacteria. In the present study *Pseudomonas putida* strain GAP-P45 identified by 16S rDNA sequence analysis was exposed to stresses such as drought, temperature and salt to test the ability to tolerate and produce EPS. Strain GAP-P45 could tolerate matric stress up to –0.73 MPa, Temperature of 50°C and 1.4 M salt and produced EPS, which increased with increase in stress levels. Among all stress conditions, the production was high under drought stress. HPLC analysis revealed that monosaccharide composition and ratio of sugars in EPS increased under stress that might induce osmotic and thermal tolerance in GAP-P45. Rhamnose was reported as major sugar under all stress conditions. Among the different carbon sources tested, glycerol was found to be best for EPS production under stressed as well as non-stressed conditions. Inoculation with GAP-P45 resulted in better soil aggregation and aggregate stability under different stress conditions. However inoculation effect was more under drought-stress. The significance of these findings shows that abiotic stresses influence the EPS composition and ratios of sugars, which influence the tolerance of the microorganisms which can be employed for improvement in water holding capacity under unfavorable environmental conditions.

Keywords: exopolysaccharide, drought stress, temperature stress, salt stress, *Pseudomonas* spp. GAP-P45, soil aggregation

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Soil bacteria undergo a variety of physiological changes under abiotic stresses like drought, temperature and salinity that help them to adapt and survive under stress conditions. Production of extracellular polymeric substances (EPS), primarily composed of polysaccharides, has been documented as a stress response and survival mechanism for many bacteria [1]. The possible cryoprotective role of EPS in marine Antarctic bacteria inhabiting brine channels of sea ice that pose extremes of high salinity and low temperatures has been described [2]. Microorganisms with EPS have also been identified in arid soil and the role of EPS in water conservation and scavenging of nutrients essential for survival in this extreme environment has been reported [3]. Production of EPS increases in bacterial sand cultures exposed to desiccation [4], which implies that living in EPS-rich habitats confers some competitive advantage on microorganisms during desiccation.

EPS maintain a hydrated microenvironment around microorganisms and holds many times their weight in water at low water potentials, which favor microbial survival. Exopolysaccharides (EPS) also bind to cations including Na^+ [5], thus alleviate the salt stress effect. Increasing population density of EPS producing bacteria in root zone decrease the content of Na^+ available for plant uptake in saline soils and also increase soil aggregation for uptake of nutrients and water thus resulting in better plant growth under saline environments [6]. With respect to temperature adaptation changes occurring in the envelope polysaccharide after exposer to high temperature play a role in thermotolerance [7]. EPS production is modified in response to environmental changes [8]. Changes in environmental conditions elicit modifications in the amount and the synthesis of new types of EPSs in bacteria [7]. This response is induced best under abiotic stress conditions such as drought, temperature and salt. These changes may represent an adaptive mechanism of bacteria against these abiotic stress conditions.

Amendment of soil with microbial EPS results in increased soil aggregation [9]. 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 [10]. The EPS is made

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of fibers, which entangle clay particles creating an intermediate zone in which clay and EPS are mixed which is surrounded by bulk' soil, forming microaggregates (<250 μm diameter) and macroaggregates (>250 μm diameter). Bacterial exopolysaccharides are capable of stabilizing the aggregates from completely dispersed soils. The task of combating erosion is closely linked with the problem of restoring the crumb structure of soil. Polysaccharide networks shrink and swell dramatically with water potential changes and remain water saturated in a wide range of water potentials [11]. An EPS producing strain *Pseudomonas putida* strain GAP-P45 could form biofilm on the root surface of sunflower seedlings and showed improved soil aggregation and root adhering soil stability [12–13] observed a significant increase in aggregate stability of root adhering soil 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* [14] and *Pantoea agglomerans* [15] under salt stress. Plants treated with EPS producing bacteria display increased resistance to water stress, salt stress and heat stress [7, 16, 17]. The present investigation is aimed to determine correlation between EPS production and tolerance and the present investigation is aimed to study correlation between EPS production, soil aggregation and also the stresses tolerance in *Pseudomonas* spp. strain GAP-P45.

MATERIALS AND METHODS

Microorganism. *Pseudomonas putida* strain GAP-P45 used was earlier isolated 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 from sunflower rhizosphere grown in alfisol [12]. The 16S rRNA gene sequence of the strain has been submitted to GenBank under the accession number GQ221267.

Bacterial growth under drought, temperature and salt stress conditions *Pseudomonas* GAP-P45 tested for its ability to grow under water stress conditions in trypticase soya agar (TSB) (pancreatic digest of casein, 17 g; papaic digest of soyabean, 3 g; NaCl, 5 g; K_2HPO_4, 2.5 g; dextrose, 2.5 g; per liter) maintained at different water potentials (−0.05 to −1.03 MPa) by adding PEG6000 as mentioned earlier [12]. For temperature stress TSB inoculated with 1% of overnight raised bacterial culture and incubated at different temperatures (28°C, to 55°C) and for salt stress, TSB was prepared with different molar concentrations of NaCl (0.2 M to 1.6 M) and was inoculated with 1% of overnight raised culture and incubated at 28°C. After incubation under shaking conditions (120 rpm) for 24 h, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer (Thermospectronic, 336002, United States). The growth of the isolates at various stress levels was recorded.

Effect of Drought, Temperature and Salt Stress on EPS Production and Monomer Composition of EPS

Extraction and purification of exopolysaccharides. The strain GAP-P45 was analyzed for its ability to produce EPS under different water, temperature and salt stress levels according to the method of [12].

Exopolysaccharide was extracted from 3 days old cultures raised in TSB. The culture was centrifuged at 20000 *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 [18]. Concentration of protein in the supernatant was estimated by Folin's reagent [19]. Then the supernatant was filtered through 0.45 μm nitrocellulose membrane and dialysed extensively against water at 4°C. The dialysate was centrifuged (20000 *g*) for 25 min to remove any insoluble material and mixed with 3 volumes of ice-cold absolute alcohol and kept overnight at 4°C. The precipitated EPS obtained by centrifugation (10000 *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 [20] and uronic acid (D-glucuronic acid) was determined according to [21] in the EPS. The effect of various carbon sources (glucose, glycerol, mannitol, sucrose, fructose,) on EPS production was studied both under non-stress and stressed conditions by adding 5% each of the sugar source to TSA medium and sugar source for maximum EPS production was defined.

The precipitated EPS of the isolates was hydrolyzed with 2 volumes of 2.5 M H_2SO_4 at 100°C for 1 h, and then the solution was neutralized with 1 M sodium carbonate. The monosaccharide composition of exopolysaccharide samples was determined by HPLC (LaChrom HPLC system, Merck, Dietikon, Switzerland) with a refractive index (RI) detector (Bio-Rad, Switzerland), using Aminex HPX-87H ion exclusion column (Bio-Rad, Rheinach, Switzerland) and acetonitrile : milli Q water (75 : 25) as mobile phase. Column temperature was maintained at 30°C. 20 μL sample and standards were injected and chromatographed at a constant flow rate of 1 mL min^{-1} (mobile phase). Monomer analyses of EPS were carried out by Vimta analytical laboratory, R and D Centre, India.). Peak integrations were carried out with the Millenium HPLC software package (Waters).

Aggregation of Soil by EPS Producing Pseudomonas spp. Strain GAP-p45

Soil used for aggregation studies was collected from homogeneous horizon (0–20 cm) of Gunegal research

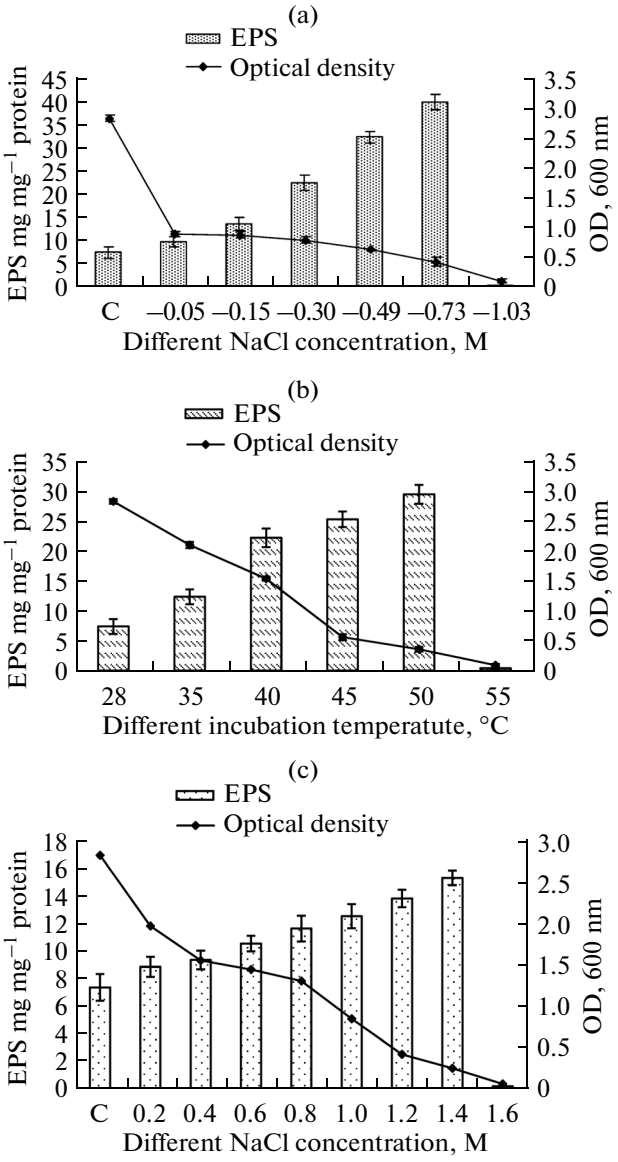

Fig. 1. Tolerance and EPS production of *Pseudomonas* spp. strain GAP-P45 under control and different levels of (a) drought, (b) temperature and (c) salt stress. Error bars are mean of ± standard deviation, $n = 6$.

farm (GRF), CRIDA, Hyderabad, India, 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, United States), was 16.5% (–0.3 MPa).

The EPS producing, *P. putida* strain GAP-P45 culture was grown in TSB and 1% (10^9 CFU/mL) bacterial culture inoculated in 100 g sterilized soil and left undisturbed for 4 weeks at 28°C, but for temperature stress incubated at 50°C. During incubation periods, soil samples were kept under near the field capacity and 40% (6.5% of field capacity) water holding capacity for drought stress conditions. For salt stress soil samples mixed with 1.4 M NaCl respectively. For control treatment, flasks with soil samples were treated with sterile distilled water. At the end of the incubation period, aggregate stability percentage of the samples was determined using wet sieving method. Soil samples were 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 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 and aggregate stability was recorded [22].

EPS was extracted from soil by heating the samples at 120°C with 5 N H_2SO_4 for 30 minutes and filtered through glass fiber filter. Samples were further extracted once with boiling water. The extracts were pooled, and the amount of carbohydrates the method of [20].

Statistical analysis. Results of each representative experiment were analyzed by one-way ANOVA. *P* values less than 0.05 are considered significant.

RESULTS

Pseudomonas spp. strain *P. putida* strain GAP-P45 could tolerate upto –0.73 MPa of water stress, 50°C of temperature and 1.4 M of NaCl concentration (Fig. 1), was screened for EPS production under non-stressed and stressed-conditions. A significant increase in EPS production was observed in strain GAP-P45 with increase in drought stress (from –0.05 to –0.73 MPa), temperature stress (28 to 50°C) and salt stress (0.2 to 1.4 M NaCl) as compared to non-stressed conditions (Fig. 1). *Pseudomonas* spp. strain GAP-P45 produced higher amount of EPS under drought stress condition (–0.73 MPa) (40 mg mg^{-1} protein), which is followed by that under temperature stress (50°C) (29.6 mg mg^{-1} protein) and salt stress (1.4 M) (15.4 mg mg^{-1} protein). Uronic acid production was also observed under drought stress (10.62 μg mL^{-1}) and temperature stress (6.6 μg mL^{-1}) respectively.

The effect of various carbon sources on EPS production revealed that glycerol act as best carbon source, which yielded higher amount of EPS than other carbon sources used both under non-stress and higher levels of (–0.73 MPa, 50°C, 1.4 M) all the three stress conditions (Fig. 2).

Sugar monomers makeup of the strain GAP-P45 under no stress and stressed conditions (drought, –0.73 MPa; temperature, 50°C; and salt stress, 1.4 M) was characterized and quantified by HPLC (Table 1; Fig. 3). Abiotic stress had an influence on the monomer composition of EPS. Under non-stressed condition and salt stress, EPS of strain GAP-P45 was composed of glucose (44.40 and 41.70%) and mannose (9.60 and 10.75%), rhamnose (40.70 and 42.10%) whereas under drought and temperature stress EPS was composed of glucose (10.10 and 9.90%), mannose (15.70 and 14.73%), rhamnose (43.60 and 41.80%) and raffinose (28.10 and 27.92%) respectively.

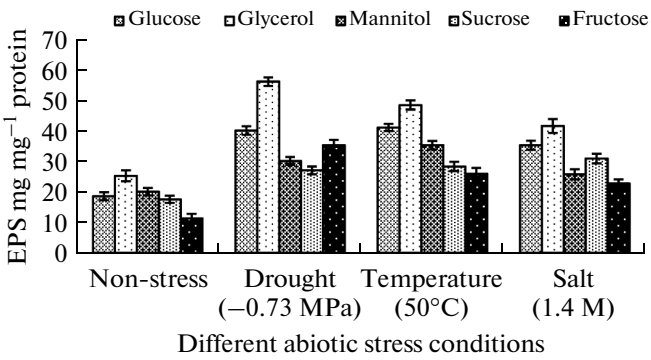

Fig. 2. EPS production by *Pseudomonas* spp. GAP-P45 with different carbon sources under highest level of drought stress (–0.73 MPa) (a) temperature stress (50°C) (b) and salt concentration (1.4 M).

Aggregation of Soil by Pseudomonas spp. Stra in GAP-p45

The effect of inoculation with *Pseudomonas* spp. strain GAP-P45 on aggregation stability and EPS concentration of soil under non-stressed and stressed conditions is given in Table 2. Effect of inoculation on percentage aggregate stability and EPS was more under drought followed by that under temperature and salt stress. Control non-stressed soil when exposed to different abiotic stresses showed non-significant increase in aggregate stability and EPS production whereas inoculation with GAP-P45 resulted in more than two fold increase in EPS concentration and showed significant improvement in percent aggregate stability under non-stressed as well as drought-stress conditions.

DISCUSSION

In this work, we aimed to quantify and characterize the EPS synthesized by *P. putida* strain GAP-P45 during growth under abiotic stress conditions such as drought, temperature and salt stress. Strain GAP-P45 could tolerate matric stress of –0.73 MPa, temperature stress of 50°C and NaCl concentration of 1.4 M indicating the presence of stress responsive mechanisms [23]. The total yield of EPS produced by strain GAP-P45 was greatly influenced by all the three stress factors and production increased with increasing stress levels. Many different environmental stresses increase production of extracellular carbohydrates [24]. An increase in the production of EPS with increasing matric stress has been reported in *Pseudomonas* spp. strain [4]. Sheng et al. [25] reported that the amount of EPS produced by *Rhodopseudomonas acidophila* increased at high NaCl concentrations and high temperature induced changes in exopolysaccharide production of heat resistant mutants of *Rhizobium* sp. and *Xanthomonas campestris* was observed [7, 26] respectively. Moreover under stress conditions, microorganisms generate EPS to act as diffusion barrier between the cell wall and extreme environments [27] a stress responsive mechanism.

The strain GAP-P45 showed the presence of glucuronic acid in EPS under stress condition, indicating relation between EPS composition and the stress tolerance. It is reported that exopolysaccharides produced by marine bacteria generally contain 20–50% of the polysaccharide as uronic acid [28]. In a study on bacterium *Halomonas* isolated from hyper saline habitats contain high uronic acid with acidic carboxyl group that is ionisable at saline pH conditions, this

Table 1. Monosaccharide composition expressed in percentage (%) of the single monosaccharide of the EPS contents of the strains of GAP-P45 under non-stressed, drought, temperature and salt stress conditions

Isolates	Monosaccharide composition of exopolysaccharide[a, b] (EPS) (%) under non-stressed and stressed condition									
	EPS[b] mg mg^{-1} protein	treatment	glucose	mannose	fructose	arabinose	xylose	rhamnose	raffinose	galactose
GAP-P45	7.4 ± 0.85	NS	44.40	9.60	–	–	–	40.70	–	–
	40.0 ± 1.10	DS	10.10	15.70	–	–	–	43.60	28.10	–
	29.6 ± 1.21	TS	9.90	14.73	–	–	–	41.80	27.92	–
	15.4 ± 1.47	SS	41.70	10.75	–	–	–	42.10	–	–

[a] Determined by HPLC; [b] values represent averages ± standard deviations for triplicate experiments; – not detected; NS = non-stress; DS = drought-stress; TS = temperature stress; SS = salt stress.

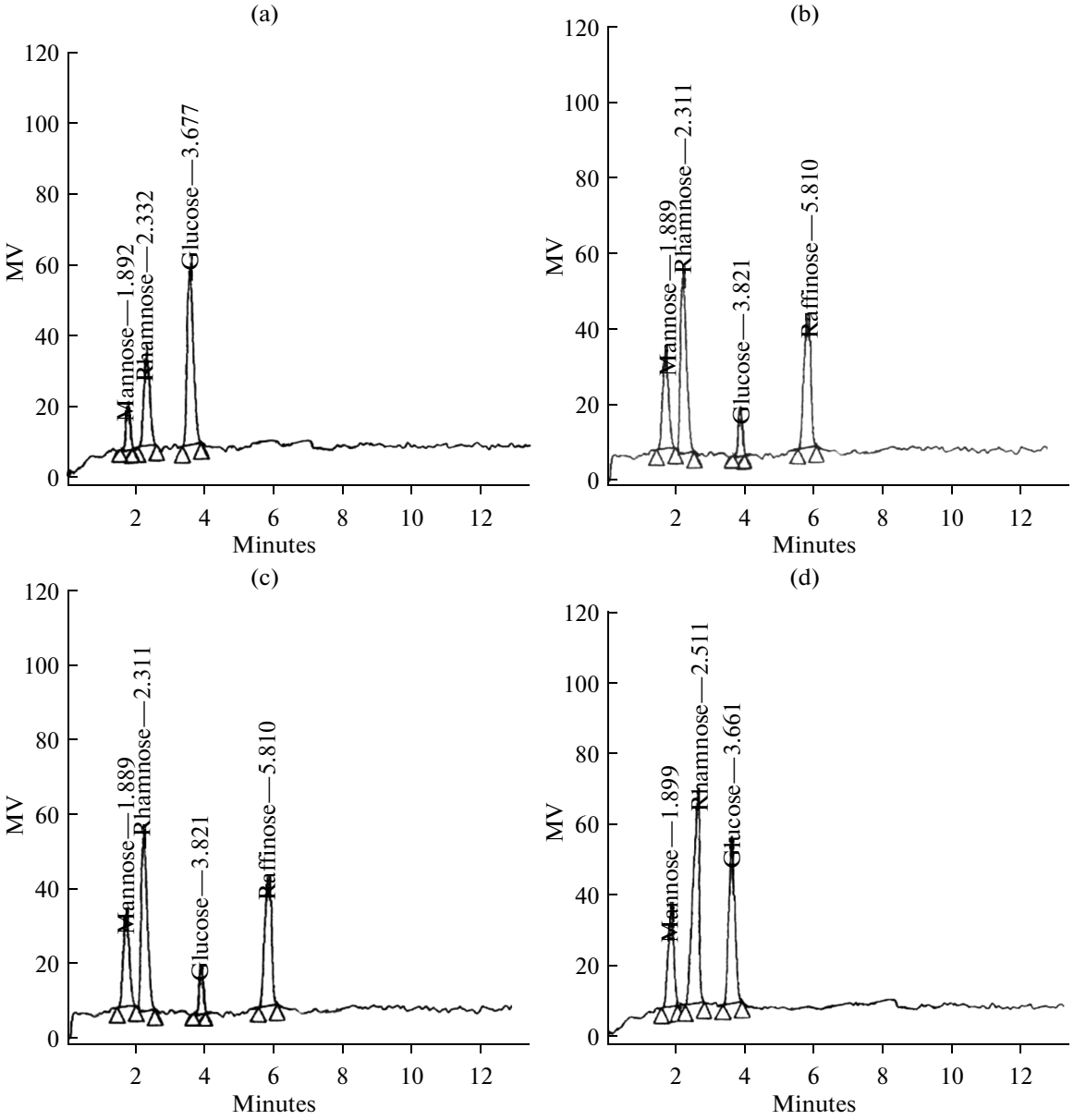

Fig. 3. HPLC chromatograms of carbohydrate composition of exopolysaccharides produced by *Pseudomonas* spp. strains under non-stress and stressed conditions. (a), non-stress; (b), drought-stress; (c), temperature stress; (d), salt stress.

contributes a negative charge to the overall polymer counteracting negative effect of salt [27, 29].

The production of EPS is influenced not only by environmental factors and but also with sugars as carbon sources [30]. EPS production of strain GAP-P45 was high when glycerol used as carbon source, this indicates that bacterial EPS production is substrate dependent. The strain, the culture conditions, and the type of carbon source influence the amount and the composition of microbial EPS that is produced by a certain species [31]. With glucose as a carbon source, *P. putida* and *P. fluorescens* synthesized an EPS composed of glucose, galactose and pyruvate [32]. When glucose or gluconate was used as the carbon sources different strains of *Pseudomonas* spp. produces alginate and with sucrose as carbon source levan (polyfructan) alone or alginate and levan together were produced [32]. Similarly, EPS isolated from *Pseudomonas caryophylli* CFR 1705 grown on lactose containing medium was composed of rhamnose, mannose and glucose [34]. This information indicates that one specific sugar may not be the most efficient carbon source for EPS production for different types of bacteria.

Microbial exopolysaccharides due to high C content can be an important factor affecting soil aggregation [11]. Aggregation is an important part of soil formation because it influences the soil infiltration, aeration, root penetration, and reducing runoff. The soils

Table 2. EPS concentration and aggregate stability percentage and percentage increase under drought, temperature, and salt stress with *Pseudomonas* spp. strain GAP-P45

Treatments	EPS mg mg^{-1} protein	AGS %	PI %
Control non-stress	1.58 ± 0.21	41 ± 1.06	
Inoculated non-stress	3.68 ± 0.14	45 ± 3.03	9.75
Control drought-stress	1.61 ± 0.26	41 ± 1.21	
Inoculated drought-stress	4.98 ± 0.22	52 ± 2.21	26.8
Control temperature-stress	1.76 ± 0.32	42 ± 1.40	
Inoculated temperature-stress	4.64 ± 0.12	49 ± 4.23	16.6
Control salt-stress	1.73 ± 0.43	42 ± 3.46	
Inoculated Salt-stress	4.18 ± 0.30	47 ± 3.23	11.9
LSD ($P = 0.05$)	1.08	1.964	
CV, %	2.67	0.96	

EPS = exopolysaccharides, AGS = aggregate stability, PC = percentage change. Numerical values are mean ± SD of six independent values. PI is percentage over control.

when inoculated with *Pseudomonas* spp. GAP-P45 and incubated showed the formation of aggregates of varying sizes, on sieving these aggregates, it was observed that the aggregates recovered from sand ranged in size from about 0.25–2.5 mm. Bashan et al. [35] reported the role of polysaccharides producing *Azospirillum* in soil aggregation. *Microbacterium arborescens* have been shown to cement soil particles together by forming polysaccharide substances [36]. Increase in aggregate stability was observed both under inoculated no stress and all the three stressed conditions, however increase was higher under stressed conditions due to increase in EPS production. Among all the three stressed conditions, drought stress could show higher aggregate stability due to higher production of EPS under drought stress conditions. It was observed that inoculation with EPS producing *Rhizobium* spp. increased aggregate stability under drought stress [13].

Determination of the monomer composition of the exopolysaccharide isolated from *Pseudomonas* spp. strain GAP-P45 is the first step towards understanding its role in stress tolerance. Under stress (–0.73 MPa, 50°C, 1.4 M NaCl) monomer composition and ratios of sugars changed. Stress conditions increased the ratio and composition of monosaccharides in the EPS compared to non-stress. Glucose, rhamnose and mannose were common monosaccharides detected both under non-stress and all the three stressed conditions but the ratio of these sugars increased on exposure of the strain GAP-P45 to drought, temperature and salt stress. Under all the three stress conditions ratios of rhamnose and mannose increased compared to non-stress whereas ratio of glucose decreased in case of drought and temperature stress and increased in case of salt stress compared to non-stress. In case of drought and temperature stress an additional sugar raffinose was also detected. Rhamnose was a major sugar under all stress conditions. These sugars are typically found in bacterial EPS [37]. Hung et al. [38] concluded that galactose, mannose and arabinose were main composition of EPS produced by *P. fluorescens* Biovar II. Glucose dominated in the EPS material produced by *P. aeruginosa* under starvation conditions [39] and to survive under chlorinated water *P. aeruginosa* synthesized EPS alginate [40]. The presence of mannose in the exopolymers produced by Antarctic marine *Pseudomonas haloplanktis* TAC 125 was observed [41]. Manca et al. [42] reported a sulphated heteropolysaccharide, composed exclusively of mannose and glucose in *Bacillus thermoantarcticus* strain. Differences in the monosaccharide composition and ratios of EPS may influence abiotic stress tolerance of the microorganisms [43].

EPS molecules play ecological roles by channeling energy and nutrients into polysaccharide production and protecting the *Pseudomonas* spp. cells against water limited environments and contribute to the improvement of soil structure.

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