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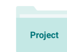

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ORIGINAL PAPER

Effect of osmotic stress on plant growth promoting *Pseudomonas* spp.

V. Sandhya · Sk. Z. Ali · B. Venkateswarlu · Gopal Reddy · Minakshi Grover

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Abstract In this study we isolated and screened drought tolerant *Pseudomonas* isolates from arid and semi arid crop production systems of India. Five isolates could tolerate osmotic stress up to −0.73 MPa and possessed multiple PGP properties such as P-solubilization, production of phytohormones (IAA, GA and cytokinin), siderophores, ammonia and HCN however under osmotic stress expression of PGP traits was low compared to non-stressed conditions. The strains were identified as *Pseudomonas entomophila*, *Pseudomonas stutzeri*, *Pseudomonas putida*, *Pseudomonas syringae* and *Pseudomonas monteilli* respectively on the basis of 16S rRNA gene sequence analysis. Osmotic stress affected growth pattern of all the isolates as indicated by increased mean generation time. An increase level of intracellular free amino acids, proline, total soluble sugars and exopolysaccharides was observed under osmotic stress suggesting bacterial response to applied stress. Further, strains GAP-P45 and GRFHYTP52 showing higher levels of EPS and osmolytes (amino acids and proline) accumulation under stress as compared to non-stress conditions, also exhibited higher expression of PGP traits under stress indicating a relationship between stress response and expression of PGP traits. We conclude that isolation and screening of indigenous, stress adaptable strains possessing PGP traits can be a method for selection of efficient stress tolerant PGPR strains.

Keywords *Pseudomonas* spp. · Drought stress · Osmolytes · PGP traits · 16S rDNA

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Introduction

Drought tolerance and water use efficiency of plants growing in arid and semi arid regions can be improved by inoculation of plants with beneficial plant growth promoting rhizobacteria (PGPR) (Marulanda et al. 2007). The term 'induced systemic tolerance' (IST) has been proposed for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress (Yang et al. 2009). PGPR have a high potential for application in agriculture because they can improve plant growth through phytohormones (IAA, GA) production, solubilization of mineral phosphate, antagonism of plant pathogens etc (Glick 1995) under limited or stressed conditions. IAA act as suitable marker for bacterial effectiveness particularly under osmotic stress (Boiero et al. 2006) and cytokinins are believed to be the signals involved in mediating environmental stresses from root to shoots (Jackson 1993). Bacteria of the species *Azospirillum brasilense* that produces phytohormones, when applied as an inoculant in *Gramineae* promotes root development and the uptake of nutrients and water under severe stress conditions (Bashan and Holguin 1997; Perrig et al. 2007). Interestingly, some of the volatile organic compounds (VOCs) produced by *Bacillus subtilis* GB03 (Ryu et al. 2004) act as determinants involved in tolerance. Timmusk and Wagner (1999) observed that inoculation of *Paenibacillus polymyxa* confers drought tolerance in *Arabidopsis thaliana* through the

induction of drought responsive gene, *ERD15 (EARLY RESPONSE TO DEHYDRATION 15*). Inoculation of *Azospirillum brasilense* Sp245 in wheat (*Triticum aestivum*) under drought stress resulted in a better water status and an additional "elastic adjustment" leading to better grain yield and mineral quality (Mg, K and Ca) (Creus et al. 2004).

Rhizosphere bacteria have the potential to adapt to adverse conditions and may compensate for such detrimental conditions (Marulanda et al. 2009; Ruíz-Lozano and Azcon 2000). Abiotic stress tolerance in bacteria has been studied to provide a biological understanding of the adaptation and survival of living microorganisms in extreme environments. Indigenous microorganisms isolated from stressed sites are reported to exhibit high adaptability and tolerance to stress as compared to those isolated form non-stressed sites (Marulanda et al. 2006). Under drought stress bacterial cells accumulate small compatible solutes called osmolytes that include amino acids like glutamate, glutamine, proline, alanine etc, quarternary amines like glycinebetaine and sugars like sucrose, trehalose and polyglucosyl granules that improve cell growth under adverse osmotic conditions serving as osmoprotectants (Csonka 1989; Potts 1994; Crowe and Crowe 1992). Accumulation of these highly soluble generally non-toxic osmolytes lowers the water potential in the cytoplasm, and maintains the cell turgor thus preventing degenerative processes. Recent studies indicate that osmolytes can also function as free-radical scavengers or chemical chaperones by directly stabilizing membranes and proteins (Diamant et al. 2001). Some bacterial cells produce complex extracellular polysaccharides (EPS) that help in the formation of biofilms and are instrumental in imparting drought tolerance to bacterial cells (Potts 1994). Beneficial rhizobacteria can adapt to specific environmental conditions and develop tolerance to stressful conditions. Therefore the isolation of indigenous rhizobacteria from the stressed soils may help in the selection of stress-adapted strains. Moreover, investigation of stress responsive mechanisms like accumulation of proline, sugars, EPS etc under stress conditions may aid in selecting more stress tolerant microbial strains.

The principal aim of the study was to explore indigenous drought tolerant *Pseudomonas* spp. isolates from soils of different arid and semiarid zones of India and to study growth promoting traits and stress-adaptive mechanisms at cellular level under osmotic stress conditions for selection of efficient drought tolerant strains.

Materials and methods

Isolation and screening

Pseudomonads were isolated from alfisols, vertisols, inceptisols, oxisols and aridisols collected from rhizospheres of different crops (Finger millet, (*Pennisetum glaucum* L); sunflower, *(Helianthus annuus* L); and maize (*Zea mays* L) grown under 25 arid and semi arid locations across India and tested for their ability to grow under water stress conditions in trypticase soya agar (TSA) with different water potentials (−0.05, −0.15, −0.30, −0.49, −0.73 MPa) prepared by adding PEG 6000 as mentioned earlier (Sandhya et al. 2009). The isolates that could grow at the matric potential of −0.73 MPa (25% PEG 6000) were screened for plant growth promoting (PGP) properties like production of IAA (Gordon and Weber 1951), gibberellins (Holbrook et al. 1961), cytokinin (Barbara and Wong 1989), P-solubilization (Mehta and Nautiyal 2001), production of HCN (Bakker and Schipper 1987), ammonia (Dey et al. 2004) and siderophores (Schwyn and Neilands 1987) under no-stress and stress conditions.

Physiological, biochemical and molecular characterization

The isolates possessing multiple PGP traits were further characterized. Growth pattern of the isolates was determined under no-stress (without PEG 6000) and matric stress of −0.73 MPa by inoculating 1 ml of exponential culture (10^9 CFU ml^{-1}) in 100 ml of respective media, and estimating the cell population at 2 h intervals by plating on King's B and recording optical densities with a spectrophotometer (A_{600nm}). The numerical values were log transformed and plotted against time. The isolates were examined microscopically to study cell morphology and capsule formation both under non-stressed and water-stressed conditions by Indian ink staining.

Preliminary biochemical characterization for the selected strains was carried out as per standard methodologies (Holt et al. 1994). For molecular characterization, bacterial genomic DNA was isolated (Chen and Kuo 1993) and subjected to PCR for amplification of 16S rDNA sequence using universal forward (5′ AGAGTTTGATCCTGGCTC AG 3′) and reverse (5′AAGGAGGTGATCCAGCCGCA3′) 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 (~1.5 kb) product was purified using Qiagen QIA quick Gel Extraction Kit following the manufacturer's procedure and sequenced at Ocimum Bio Solutions, India with an Applied Biosystems 3730xl DNA Analyzer using the fluorescence based Big Dye Terminator V 3.1Cycle Sequencing kit.

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 type strain sequence in GenBank; identification at the genus level was defined as a 16S rDNA sequence similarity of ≥97% with that of the type 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. Nucleotide sequences were aligned using CLUSTALX 1.81 algorithm. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the distance matrix from alignment and distances were calculated (Kimura 1980; Tamura and Nei 1993).

Bacterial response to osmotic stress

Exopolysaccharide production

The selected drought tolerant isolates were analyzed for their ability to produce EPS (Fett et al. 1986, 1989) under no stress and matric stress of −0.73 MPa. For extraction of EPS, 3 days old cultures raised in TSB (with and without PEG) were centrifuged at 20,000 g for 25 min and the supernatant was collected. This loose layer was collected and resuspended in 0.85% KCl and recentrifuged as above to separate capsular material from bacterial cells. The pellet was washed twice with 0.85% KCl for complete recovery of EPS. The pooled supernatant was checked for cell disruption by testing the presence of DNA by using DPA reagent (Burton 1956) and protein concentration was determined Bradford method (1976). The supernatant was filtered through 0.45 μm nitrocellulose membrane and dialysed extensively against water at 4°C. The dialysate was centrifuged to remove any insoluble material and mixed with 3 volumes of ice-cold absolute alcohol and kept overnight at 4°C. The precipitated EPS was collected by centrifugation at 10,000*g* for 15 min, suspended in water and further purified by repeating the dialysis and precipitation steps. Total carbohydrate content was determined in the precipitated EPS (Dubois et al. 1956). 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 and spotted on the silica gel plate (Silica gel 60F 254; Merck). The plate was developed in a thin layer chromatography 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 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).

Total and free amino acids

Bacterial cells were cultured in Luria Bertani broth (LB) (Tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; per liter) media without PEG (non-stressed) and 25% PEG 6000 (−0.73 MPa) (dessication-stressed) at 30°C for 48 h followed by centrifugation at 3,000*g* for 5 min. Cell pellet was boiled at 60°C in water bath with 70–80% ethanol for 45 min, centrifuged at 10,000*g* for 15 min and 1 ml of supernatant was treated with 1 ml of 0.2 M citrate buffer (pH = 5), 1 ml of 80% ethanol and 2 ml of the ninhydrin reagent (1% ninhydrin in 0.006% KCN in acetone). The samples were vortexed and immersed in hot water bath (95°C) for 15 min followed by addition of 5 ml of distilled water. The absorbance was read at 570 nm using UV-visible spectrophotometer (Thermospectronic, 336002, USA) (Moore and Stein 1948). Free proline accumulation was determined in the supernatant using the method of Bates et al. (1973).

Total sugar content

For estimation of total sugars, cell pellet obtained as mentioned above was boiled at 60°C in water bath with a mixture of Methanol: Chloroform (4:1v/v) for 15 min and centrifuged at 10,000*g* for 15 min. The supernatant was used for quantitative estimation of sugars by the method described by Dubois et al. (1956). Different sugars were separated by thin layer chromatography as mentioned above for exopolysaccharide components.

Total protein content

Total cellular protein content was determined by suspending and washing the bacterial pellet (obtained as above) first with 0.02 M $MgCl_2$ and then with sterile distilled water. Each time the cells were obtained by centrifugation at 3,000*g* at 4°C for 15 min. The cells were lysed by incubating with lysis buffer (Banglore Genie India) for 30 min at room temperature followed by centrifugation at 10,000*g* for 15 min at 4°C and the supernatant obtained was used for estimation of protein content by Bradford method (1976).

Results

Isolation and screening

On the basis of cultural, morphological characteristics, a total of 81 isolates were selected tentatively as *Pseudomonas* spp. These isolates were screened for drought tolerance in TSB maintained at different water potentials. Of 81 isolates, 26 could grow up to a minimum water potential of

−0.73 Mpa and were screened in vitro for plant growth promoting traits under stressed and non-stressed conditions. All the isolates could produce IAA and GA under non-stressed (7.36–32.9 and 5.77–38.2 μg mg^{-1} protein, respectively) and stressed (3.41–17.10 and 3.63–14.0 μg mg^{-1} protein, respectively) conditions. Phosphate solubilization was observed in 72% isolates under non-stressed (30–70 ppm) and stressed (30–55 ppm) conditions. Most of the isolates could produce ammonia (96%) whereas siderophores, HCN and cytokinin production was detected in only 64.1, 22, and 24% isolates respectively.

The best five isolates selected on the basis of PGP traits represented three locations with three different soil types (Table 1). PGP properties of these five isolates, BV-P13, GRFHYD-P14, GAP-P45, GRFHYTP52, and WAPP53 under non-stressed and stressed conditions are presented in Table 2. All these five isolates were found positive for all the tested PGP traits under non-stressed and stressed conditions with a few exceptions (isolates GRFHYD-P14 could not produce cytokinin and isolate WAPP53 could not produce HCN). Among these five isolates, GAP-P45 produced highest amount of IAA (17.1 and 32.93 μg mg^{-1} protein under stressed and non-stressed conditions respectively) and gibberellins (14.0 and 38.27 μg mg^{-1} protein under stressed and non-stressed conditions respectively). Isolate GRFHYD-P14 could solubilize maximum amount of P under non-stressed (70.0 ppm) and stressed (55.7 ppm) conditions when compared with other isolates under respective conditions (Table 2).

Physiological, biochemical and molecular characterization

Growth pattern of the selected five isolates studied under normal and matric stress indicated low cell population under stress condition than under normal conditions (Fig. 1). Mean generation time of the isolates BV-P13, GRFHYD-P14, GAP-P45, GRFHYTP52, and WAPP53 were 40.9, 40.7. 39.66, 39.51, 40.1 min respectively under non-stressed conditions and min 48.8, 46.8, 43.6, 45.1, 45.9 respectively under stressed conditions. The bacterial cells accumulated conspicuous amount of capsular material under osmotic stress conditions, as indicated by staining with Indian ink.

General biochemical and morphological characteristics of the selected strains are illustrated in Table 3. For molecular identification 16S rDNA was amplified and the product (~1,500 bp) was sequenced. Partial 16S rDNA sequences of the strains BV-P13 (1,260 bp), GRFHAP-P14 (1,251 bp), GAP-P45 (1,081 bp), GRFHYTP52 (1,244 bp) and WAPP53 (1,008 bp) percent identity to *Pseudomonas entomophila* (97%), *Pseudomonas stutzeri* (99%), *Pseudomonas putida* (99%), *Pseudomonas syringae* (99%), *Pseudomonas monteilli* (98%) of the existing database of

Table 1 Geographical locations of rhizosphere soil samples used for isolation of drought tolerant plant growth promoting *Pseudomonas* spp.

Drought tolerant PGP *Pseudomonas* spp.	Crop	Location	District	State	Soil type	Climate	Available water capacity (cm)	Water holding capacity (%)	pH	EC ms
BV-P13	Maize (*Zea mays L.*)	DFRS	Bhilwara	Rajasthan	Aridisol	Arid	5–12	28.2	6.6	0.127
GRFHAP-P14	Pearl millet (*Pennisetum glaucum L.*)	Gunegal	Hyderabad	Andhra Pradesh	Alfisol	Semi arid	5–20	31.6	7.0	0.113
GAP-P45	Sunflower (*Helianthus annuus L.*)	Gunegal	Hyderabad	Andhra Pradesh	Alfisol	Semi arid	5–13	37.9	7.0	0.103
GRFHYTP52	Sunflower (*Helianthus annuus* L.)	Gunegal	Hyderabad	Andhra Pradesh	Alfisol	Semi arid	5–13	37.9	7.0	0.103
WAPP53	Sunflower (*Helianthus annuus L.*)	Warangal	Warangal	Andhra Pradesh	Vertisol	Semi arid	5–15	34.5	7.2	0.082

Table 2 Plant growth promoting properties of *Pseudomonas* spp. under non-stressed conditions drought-stressed conditions

Isolates	IAA production (μg mg^{-1} protein)		GA production (μg mg^{-1} protein)		Cytokinin		P-solubilization (ppm)		HCN		Siderophore production		Ammonia production	
	NS	DS	NS	DS	NS	DS	NS	DS	NS	DS	NS	DS	NS	DS
BV-P13	14.64 ± 1.49	8.20 ± 2.10	12.47 ± 1.00	7.70 ± 0.61	+	+	60.0 ± 6.6	41.7 ± 4.0	+++	+	+	+	+++	+
GRFHYD-P14	24.33 ± 1.69	13.07 ± 2.65	19.13 ± 102	9.10 ± 1.10	−	−	63.7 ± 2.1	44.3 ± 1.5	+++	+	+	+	++	+
GAP-P45	32.93 ± 1.00	17.10 ± 1.28	38.27 ± 2.12	14.0 ± 1.25	+	+	70.0 ± 2.3	55.7 ± 2.5	+++	+	+	+	+	+
GRFHYTP52	28.26 ± 3.52	15.53 ± 3.57	26.10 ± 1.31	12.7 ± 0.19	+	+	69.3 ± 6.7	50.3 ± 7.4	+	+	+	+	+	+
WAPP53	27.53 ± 2.14	13.23 ± 1.56	23.32 ± 2.25	11.7 ± 0.75	+	+	66.0 ± 6.0	46.7 ± 5.7	−	−	+	+	+	+

Numerical values are mean ± SD of six independent observations; *NS* non-stressed; DS, drought-stressed, *IAA* Indole acetic acid, *GA* gibberllins, *HCN* hydrogen cyanide; +, good; ++, very good; +++, excellent

IAA, GA cyrokinin, HCN, siderophore and ammonia were detected after 48 h of incubation; *P* was estimation after 7 days of incubation

National Center of Bioinformatics respectively (NCBI) and were submitted to GenBank under the accession no. GU120097, GQ160905, GQ221267, GQ160904 and FJ905913, respectively. Phylogenetic analysis of 16S rRNA gene sequences was carried out using neighbor-joining method, which showed significant boot strap values. The dendogram constructed from the 16S rDNA sequence data (Fig. 2) to investigate the relationship among the pseudomonad strains, showed five clusters with bootstrap values of 58, 98, 87, 96, 97% along with reference strains from NCBI database.

Drought responsive parameters

Production of EPS, sugars, amino acids and proteins by the selected stress tolerant strains was determined under non-stressed as well as stressed conditions. A significant increase in the levels of EPS, total free amino acids, proline and total soluble sugar content was observed under stressed conditions as compared to non-stressed conditions. Whereas, concentration of cell proteins was significantly lower under stressed conditions as compared to non-stressed conditions (Table 4). Isolate GAP-P45 showed maximum production of EPS, total free amino acids, proline and total soluble sugars under non-stressed and stressed conditions when compared with other isolates under respective conditions.

Component analysis of total sugars in cell free extracts by thin layer chromatography revealed differences in the sugar components of drought tolerant strains under non-stressed and stressed conditions. Under non-stress conditions strains BV-P13, GRFHYD-P14, GAP-P45 and WAPP53 produced glucose and GRFHYTP52 produced sucrose whereas under drought-stress, strain GRFHYD-P14 produced trehalose and other four strains produced mannitol. (Table 5).

Sugar components of the exopolysaccharide produced by the isolates were analyzed by acid hydrolysis followed by thin-layer chromatography (Table 5). Under non-stress conditions glucose, mannose and rhamnose were detected in EPS hydrolysate of the strains except in strain GRFHYTP52 which produced glucose and mannose whereas under drought-stress conditions glucose, raffinose and rhamnose were detected in all the strains except that in strain WAPP53 mannose was detected instead of rhamnose. The results indicate that raffinose is produced as stress response in these strains.

Discussion

Crop plants are constantly exposed to different abiotic stresses like drought, heat waves, cold waves, floods, frost etc.

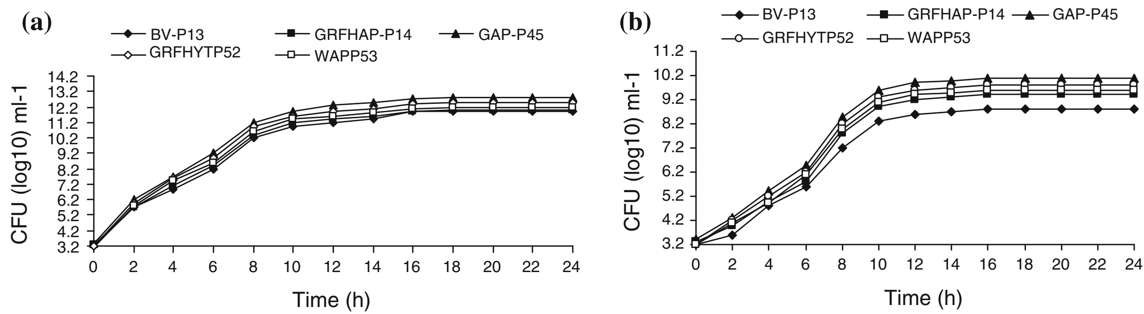

Fig. 1 Growth pattern of *Pseudomonas* spp. strains BV-P13, GRFHAP-P14, GAP-P45, GRFHYTP54 and WAPP53 under **a** non-stressed and **b** drought-stressed conditions

Table 3 Biochemical and microbiological characters of *Pseudomonas* spp. strains

Test	BV-P13	GRFHAP-P14	GAP-P45	GRFHYTP52	WAPP53
Margin	Entire	Entire	Entire	Wrinkled	Entire
Elevation	Convex	Convex	Convex	Convex	Convex
Consistency	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Opacity	Opaque	Translucent	Opaque	Translucent	Opaque
Gram nature	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Indol	−	−	−	−	−
Methyl red	−	−	−	−	−
Voges-Praoskauer	−	+	+	+	−
Citrate	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Starch hydrolysis	−	−	−	−	−
Urease test	+	+	+	+	+
Gelatin Hydrolysis		+	−		
D-Glucose	+	+	+	+	+
D-Fructose		+	+		
Maltose	+	+	−	+	+
Glycerol	−	+	+	−	−
Arabinose		−	−		
Sucrose	−	−	−	+	−
Erythritol	−	−	−	−	−
Ribose	−	−	−	−	−
Xylose	−	−	−	−	−
Galactose	−	−	−	−	−
Mannose	−	−	−	−	−
Rhamnose	−	−	−	−	−
Mannitol	+	+	+	+	+
Sorbitol	+	+	+	+	+
Raffinose	−	−	−	−	−
Lactose	−	−	−	−	−
Arginine	−	−	+	+	−

+, Positive; −, negative

leading to poor performance and yield losses. Recently, role of microorganisms in the management of abiotic stresses has been reported (Ali et al. 2009). A variety of mechanisms have been identified behind microbial response to abiotic stresses (Marulanda et al. 2009). Microorganisms have unique property of adapting to changing environmental conditions (Potts 1994). Therefore, isolation of microorganisms from stress-affected soil may result in selection of efficient stress tolerant microorganisms. In the present investigation, a total of 81 pseudomonads were isolated

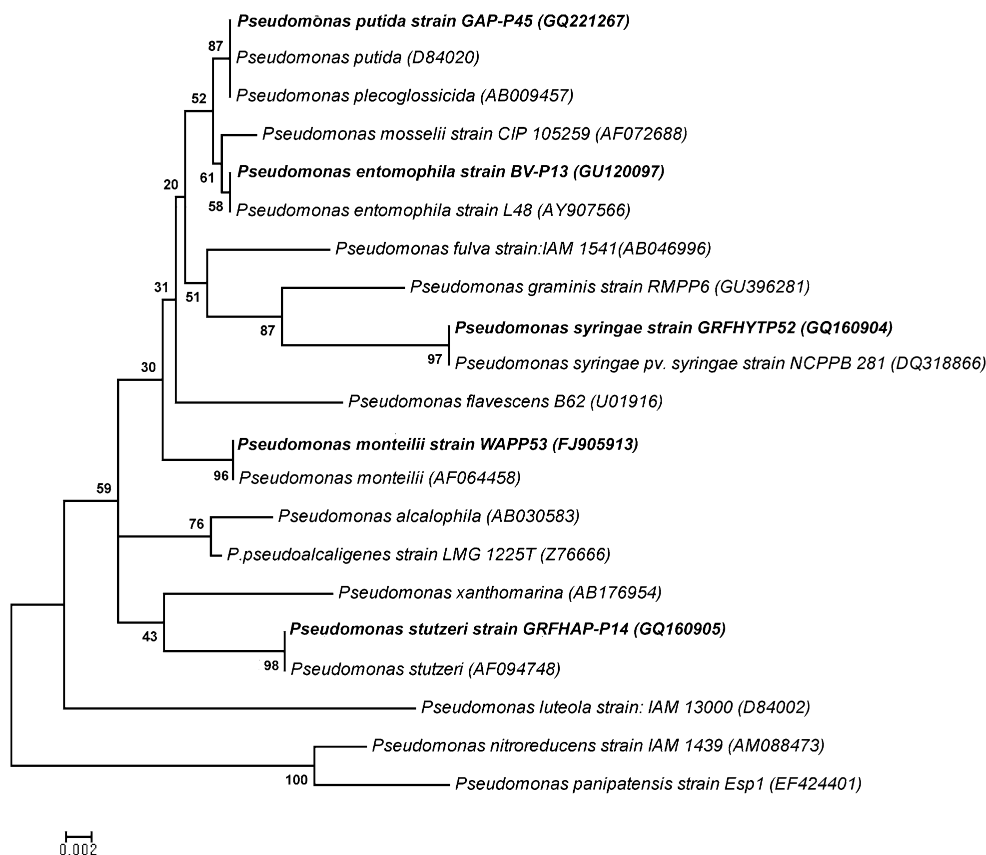

Fig. 2 Neighbour-joining tree showing the phylogenetic relationship between *Pseudomonas* spp. (with GenBank accession numbers). The *bars* represent 0.002 substitutions per site, bootstrap values ($n = 500$)

Table 4 Physiological parameters in *Pseudomonas* spp. strains under drought stress

Strains	Exopolysaccharide (mg mg^{-1} protein)		Protein (mg g^{-1} DW)		Total free amino acids (μmol g^{-1} DW)		Proline (μmol g^{-1} DW)		Total soluble sugars (μmol g^{-1} DW)	
	NS	DS	NS	DS	NS	DS	NS	DS	NS	DS
BV-P13	2.4 ± 0.02	14.9 ± 0.03	21.67 ± 0.94	6.89 ± 0.55	11.56 ± 0.52	39.53 ± 0.41	3.45 ± 0.21	17.5 ± 0.75	7.6 ± 0.50	31.6 ± 1.60
GRFHAP-P14	3.7 ± 0.41	20.7 ± 0.14	21.23 ± 0.61	6.47 ± 1.02	13.03 ± 0.14	43.10 ± 0.24	6.66 ± 0.51	18.4 ± 1.24	9.0 ± 0.72	36.3 ± 1.50
GAP-P45	7.4 ± 0.32	40.0 ± 0.14	23.57 ± 1.08	10.95 ± 0.03	18.87 ± 0.82	53.73 ± 0.16	8.12 ± 0.01	23.4 ± 1.37	16.5 ± 0.50	46.3 ± 1.89
GRFHYTP52	4.2 ± 0.02	25.4 ± 0.31	22.56 ± 1.90	9.68 ± 0.15	16.03 ± 0.52	48.73 ± 0.56	7.74 ± 0.01	21.7 ± 1.48	13.3 ± 0.11	42.9 ± 1.50
WAPP53	3.9 ± 0.09	21.8 ± 0.45	21.13 ± 1.00	7.34 ± 0.25	14.13 ± 0.50	47.32 ± 0.29	7.33 ± 0.05	20.6 ± 1.00	11.6 ± 0.03	40.3 ± 1.12

Numerical values are mean ± SD of six independent values; *NS* non-stressed, *DS* drought-stressed

from soils of different arid and semiarid regions across India, of which 26 (32%) could grow up to a minimal water potential of −0.73 MPa, were screened for plant growth promoting properties like production of phytohormones (IAA, GA, cytokinin), P-solubilization, production of siderophores, HCN and ammonia under normal as well as drought stress (−0.73 MPa). Production of PGP substances under drought stress conditions can be helpful in maintaining better nutritional status of the plant thus influencing plant-microbe interaction, hence can be used as criteria for selecting efficient PGP strains for harsh conditions. In addition to general plant growth, IAA stimulates stress tolerance in plant (Marulanda et al. 2009). Inoculation with cadmium resistant strains *Pseudomonas tolaasi* and *Pseudomonas fluorescens* enabled *Brassica napus* to grow under cadmium stress because of the production of indole acetic acid, siderophores, and ACC deaminase (Dell' Amico et al. 2008). Similarly cytokinins are believed to be signals involved in mediating of environmental stresses (Jackson 1993) and their balance is influenced by environmental cues (Timmusk 2003). Five drought tolerant isolates BV-P13, GRFHYD-P14, GAP-P45, GRFHYTP52, WAPP53 exhibiting multiple PGP traits under normal and stress conditions were selected and identified as *P. entomophila*, *P. stutzeri*, *P. putida*, *P. syringae*, *P. monteilli* respectively on the basis of biochemical and 16S rDNA sequence analysis. The phylogenetic analysis by neighbor-joining method revealed the diversity among the drought

Table 5 Sugar and exopolysaccharide components of drought tolerant *Pseudomonas* spp. under non-stress and drought-stress

Isolates	Sugar components		Exopolysaccharide components	
	Non-stress	Drought-stress	Non-stress	Drought-stress
BV-P13	Glucose	Mannitol	Glucose, mannose and rhamnose	Glucose, raffinose and rhamnose
GRFHAP-P14	Glucose	Trehalose	Glucose, mannose and rhamnose	Glucose, raffinose and rhamnose
GAP-P45	Glucose	Mannitol	Glucose, mannose and rhamnose	Glucose, raffinose and rhamnose
GRFHYTP52	Sucrose	Mannitol	Glucose and mannose	Glucose, raffinose and rhamnose
WAPP53	Glucose	Mannitol	Glucose, mannose and rhamnose	Glucose, mannose and raffinose

tolerant strains of genus *Pseudomonas* that formed five clusters. Although all the five strains could tolerate matric stress up to −0.73 MPa, growth pattern of the strains was affected under stress as indicated by increased mean generation time. Under stress conditions energy flow of the cells is directed toward protection mechanisms, which might affect the growth pattern of the cells (Räsänen et al. 2004). Soil bacteria synthesize osmolytes to protect themselves against fluctuations in osmotic conditions (Timmusk 2003; Csonka 1989) and accumulate to higher levels to alleviate stress effects (Räsänen et al. 2004). The accumulated osmolytes enhance the stability of proteins and membrane under water limiting environments (Kogut and Russell 1987). All the five *Pseudomonas* spp. strains which could tolerate minimal water potential tested (−0.73 Mpa) showed the accumulation of more proline, free amino acid, sugars and produced EPS under stress conditions than under non-stressed conditions, indicating the role of these metabolites in stress tolerance. However the concentration of protein was reduced significantly under stress indicating the degeneracy under stress condition. Under stress, proteins are used for polysaccharide production (Roberson and Firestone 1992). All the *Pseudomonas* spp. strains BV-P13, GRFHYD-P14, GAP-P45, GRFHYTP52, and WAPP53 secreted conspicuous amounts of exopolysaccharide under stress conditions. These results are in line with the findings of Roberson and Firestone (1992) that EPS production in *Pseudomonas* increases with increase in matric stress. Exopolysaccharides produced by the bacterial cells forms an organo-mineral sheath around the colonies thus creating a micro-environment that decrease the water loss from the cells and help in their survival as water potential declines (Kilbertus et al. 1979). Raffinose was detected as EPS components of all the five strains under drought stress and not under non-stressed conditions. Many species of Gram-positive bacteria are able to increase the proline pool size and accumulate total free amino acids under osmotic stress (Measures 1975; Halverson et al. 2000). Proline in high concentrations protects proteins from denaturation and helps in osmotic potential of cells (Tempest et al. 1970) indicative of osmotic cellular adaptation and helping the cells to produce PGP phytohormones under stress conditions. The level of glutamate increases in response to osmotic stress in Gram-negative bacteria (Brown and Stanley 1972; Tempest et al. 1970). Desiccation tolerant cells also accumulate high levels of disaccharides such as trehalose, sorbitol, fructose, glucose and sucrose (Crowe and Crowe 1992), which protect cellular enzymes by replacing water around macromolecules (Webb 1965) and also stabilize cell membranes during dessication (Crowe and Crowe 1986, 1992). Sugars serve as carbon source and osmoprotectants (Räsänen et al. 2004), thus helps in the better growth and production of PGP compounds by the *Pseudomonas* strains BV-P13, GRFHYD-P14, GAP-P45, GRFHYTP52 and WAPP53. A difference in the sugar components of the *Pseudomonas* strains raised under non-stressed and stressed conditions was observed. Under non-stressed conditions four of the five strains produced glucose whereas under stressed conditions four strains accumulated mannitol, one strain accumulated trehalose, indicating a differential response of the bacterial strains to stress. Accumulation of low molecular weight carbohydrates has been reported in cyanobacteria as a response to water stress (Hershkovitz et al. 1991). The results indicate that physiological changes occur at different levels in bacterial cells as a response to osmotic stress. In general it was observed that the strains GAP-P45 and GRFHYTP52 accumulating higher levels of EPS and osmolytes under stress, also exhibited higher levels of expression of PGP traits whereas the strain BV-P13 exhibiting lower levels of EPS and osmolytes (proline, sugar and amino acid) under stress, exhibited lower levels of expression for PGP traits, indicating some sort of relationship between stress tolerance response and expression of PGP traits. More over, the ability of these strains to produce IAA, gibberellins and cytokinins under osmotic stress could account for their tolerance and help in alleviating drought stress Rhizobacteria inhabiting the sites exposed to frequent dry periods or where water is limited, are likely to serve as better plant growth promoters as compared to those isolated from sites where water resources are abundant (Lifshitz et al. 1986) due to stress adaptation mechanisms. It has been reported that *P. polymyxa* inoculation is more effective in relatively harsh and poor quality conditions (Chanway and Holl 1994). These results are in agreement

with findings that drought-exposed barley plants when inoculated with *P. polymyxa* could tolerate stress 2 weeks longer than uninoculated control plants (Timmusk 2003). The strains selected in the present study were drought tolerant, and exhibited drought responsive mechanisms as well as PGP traits under osmotic stress, indicating that these strains have the potential to be developed as bioinoculants for drought stress management.

From the study we conclude that the isolation of indigenous microorganisms from the stress affected soils and screening on the basis of their ability to tolerate stress and effect of stress responsive mechanisms on PGP traits may be useful in the rapid selection of efficient PGPR strains that could be used as bioinoculants for stressed crops.

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