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Research Article

Characterization of abiotic stress tolerant *Pseudomonas* spp. occurring in Indian soils

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ABSTRACT: Abiotic stress tolerance of 230 *Pseudomonas* spp. occurring in Indian soils was evaluated for tolerance to temperature, salinity and moisture stresses. Forty seven *Pseudomonas* spp. were characterized as abiotic stress tolerant and were identified as *P. aeruginosa* (24), *P. putida* (14), *P. plecoglossicida* (4), *P. mosselli* (1), *Pseudomonas* sp. (1) and *P. fluorescens* (3). The temperature and salinity tolerance of these bacteria was 45°C and 1 M NaCl respectively. Most isolates (44 out of 47) produced indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and 37 of them showed phosphatase activity. 2, 4-diacetyl phloroglucinol (DAPG) gene was detected in 10 isolates and pyoluteorin gene was detected in 4 isolates. Under water stress, seed treatment with *P. putida* (NBAII-RPF9) and *P. fluorescens* (PFDWD) showed its potential as plant growth promoter. The studies also indicated that stress tolerant *Pseudomonas* spp. may be used as plant protection agents in abiotically stressed soils.

KEY WORDS: *Pseudomonas*, Abiotic stress, tolerance, DAPG, pyoluteorin

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INTRODUCTION

Most of peninsular India experience extreme climatic conditions from very hot (50°C) to very cold (almost 0°C) and surface soils are subjected to such extremes of temperature. About 7.3 million hectares of India's land area is afflicted with the twin problems of alkalinity and salinity coupled with water logging or prolonged drought which seriously reduce agricultural productivity (www.cssri.org). High saline soils deprive plants of water and sodic soils affect nutrient availability. No reports are available on the diversity of *Pseudomonas* spp. occurring in such stressed soils of India. Presence of fluorescent pseudomonads is ubiquitous and some reports are available on their occurrence in stressed environment like saline, sodic and semi arid soils (Djedidi *et al*., 2011; Egamberdieva 2011; Sandhya *et al*., 2010). *Pseudomonas fluorescens* can produce a wide range of enzymes and metabolites that help plants withstand varied biotic and abiotic stresses (Saravanakumar *et al*., 2011). In India commercial preparations of *P. fluorescens* is widely used for disease management in pulses, rice and vegetables. Presently *P. fluorescens*, *P. putida, P. chloraphis, P. aureofacians* and other species are widely used in agriculture as they play a crucial role in soil health and plant development (Weller *et al*., 2007). Hence we undertook a survey to study the diversity of fluorescent pseudomonads that occur in such stressed soils with emphasis on rhizosphere of groundnut, pigeon pea and sorghum as theses crops are mostly cultivated in these soils. We also studied their level of tolerance to high temperature, salinity and drought. Biochemical / antibiotic producing properties were also assessed in order to ascertain their usefulness in plant protection.

MATERIALS AND METHODS

Isolation and testing for abiotic stress tolerance

Rhizospheric soil samples of groundnut, pigeon pea and sorghum were collected from different states (Tamil Nadu, Karnataka, Maharashtra, Gujarat, Rajasthan, Haryana, Uttar Pradesh and Orissa) of India, prone to high temperature and salinity (www.cssri.org). Soils with >4.0mmhos/cm conductivity were considered as saline. Strains were isolated from 400 soil samples collected

from rhizosphere of groundnut, sorghum and pigeon pea by serial dilution and plating on King's B medium of composition (g L^{-1}) as follows: proteose peptone (10), anhydrous K_2HPO_4 (1.5), glycerol (15), $MgSO_4$ (1.5), Agar (20) and 230 isolates were isolated and purified. The 230 isolates were tested for tolerance to high temperature (38-50°C), salinity (0.5 M to 1.5 M NaCl) and pH (4, 7 and 9) with slight modifications (Ruiz-Diez *et al*., 2009). The isolates were inoculated in Luria Bertani (LB) broth and incubated at 28, 37, 40, 45, 48 and 50°C for 48 hours. Viable cell count was determined by serial dilution. Saline tolerant isolates were selected based on the rapid growth at a minimum concentration of 1M (5.8%) NaCl in basal medium (79.42 mmhos/cm) as well as in Luria Bertani (LB) broth (89.93 mmhos/cm) and tolerance was determined based on the number of colony forming units (CFU) in saline treated and normal (Ruiz-Diez *et al*., 2009). Growth under different pH was determined by using LB broth buffered with 30 mM 2, 2-dimethylsuccinic acid (pH 4.5) or 50 mM Tris hydrochloride (pH 9). Osmotic tolerance was evaluated by using polyethylene glycol (PEG 6000) at 0 (control), 30 and 50% concentrations in nutrient broth, incubated at 28°C for 48 hours and viable cell count was determined (Djedidi, 2011). Experiments repeated thrice for confirmation of results.

Identification

Molecular approaches were used to identify the isolated bacteria. Genomic DNA was isolated as per the method earlier adopted (Sambrook *et al*., 1989). 16s ribosomal DNA was amplified using PCR with primers fD1 (51-GAGTTTGATCCTGGCTCA-31) and rP2 (51-ACGGCTACCTTGTTACGACTT-31) (Weisburg *et al*., 1991), synthesized by Sigma (India). PCR cocktail (50 ìl) contained 50 pM of primer, 50 ng of genomic DNA, 1x Taq DNA polymerase buffer, 1U of Taq DNA polymerase, 0.2 mM of each dNTP, and 1.5 mM MgCl2. Amplification was performed in PCR machine (Qantarus, U.K.) with initial denaturation at 94°C for 3 min, followed by 30 cycles of 10 s at 94°C, 1 min at 45°C and 30 s at 72°C with an extension of 72°C for 5 min. A 10ìl aliquot of each amplified product was run on 1.25% agarose gel in 0.5x TAE buffer containing ethidium bromide at 50V for 45 min. Gels were visualized in a gel documentation system (DNR Minilumi, Israel). Sequencing of the PCR fragments was done using automated sequence analyzer (ABI 3500XL Genetic Analyzer, USA). Homology searches with 16S rDNA sequences in GenBank were performed with the BLAST program (version 2.2.1) (Altschul *et al*., 1997) and sequences submitted to GenBank. The identity was also confirmed using BIOLOG version 4.20.05.

Biochemical characterization

Phosphate solubilizing activity was determined qualitatively by point inoculating on the Pikovskaya's agar medium of composition (g L^{-}) as follows: yeast extract (0.5), dextrose (10), $Ca_3(PO_4)_2$ (5), $(NH_4)_2SO_4$ (0.5), KCl (0.2), $MgSO_4 \cdot 7H_2O$ (0.1), MnSO4.H2O (0.0001), $FeSO_4 \cdot 7H_2O$ (0.0001), agar (15). After 3 days of incubation at 28°C, isolates that induced clear zone around the colonies were considered as positive (Principe *et al*., 2008). ACC deaminase activity was done to detect production of 1-aminocyclopropane 1 carboxylate (ACC) by the method suggested by Husen *et al*., 2009. The production of Indole Acetic Acid (IAA) was determined as described by Bric *et al*., 1991. Single colony was streaked onto LB agar amended with 5 mM L-tryptophan, 0.06% sodium dodecyl sulfate (SDS) and 1% glycerol. Plates were overlaid with Whatman no. 1 filter paper (82-mm diameter) and the bacterial isolate was allowed to grow for 3 days. After 3 days of incubation, the filter paper was removed and treated with Salkowsky's reagent (Gordon and Weber, 1951) in a Petri dish having the formulation of 2% of 0.5 M ferric chloride in 35% perchloric acid at room temperature for 60 min. The production of IAA was identified by the formation of a characteristic red halo on the paper immediately surrounding the colony. IAA was determined quantitatively by colorimetric method. Briefly, the test strains were inoculated in KB without and with tryptophan (500ig/ml) and incubated at 28ºC at 10 rpm/min. After 2, 4 and 6 days of cultivation, aliquots of bacterial cultures were centrifuged at 13,000 rpm for 10 min. Two milliliters of supernatant fluid was added to a tube with 100ìl of 10mM orthophosphoric acid and 4ml of Salkowsky (Gordon and Weber, 1951). The mixture was incubated at room temperature for 25 min. and the absorbance of the developed pink color was read at 530 nm. The IAA concentration in culture was determined by using a calibration curve of pure IAA as a standard (Gordon and Weber, 1951).

Detection of antibiotic coding genes

Detection of genes coding for 2, 4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) was done by PCR using gene specific primers (Raajimakers *et al*., 1997). For DAPG primers Phl2a 51-GAGGACGTCGAAGA CCACCA-31 and Phl2b 51-ACC GCAGCATCG TGTAT GAG-32 were used with initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 1 min, 52.9°C for 1 min and 72°C for 2 min. For PLT PltBf 51-CGGAGCATGGAC CCCCAGC-31 and PltBr 51-GTGCCCGATATTGG TCTTGACCGAG-31 primers were used with initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 1 min,

62°C for 1 min and 72°C for 2 min. PCR amplification was carried out in 25 ìl reaction mixture containing 5ìl of bacterial DNA, 1x PCR buffer, 0.5 g/L bovine serum albumin, 5% dimethyl sulphoxide, 200 ìM each of dATP, dCTP, dGTP and dTTP, 0.4 ìM of each primer and 1.4 U of Taq DNA polymerase. Amplification was performed in PCR machine (Qantarus, UK). The PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide in 0.5x TAE buffer at 75V for 1 hour and visualized with gel documentation system (DNR Mini Lumi, Israel).

Assay for plant growth promotion

The effect of *Pseudomonas* isolates (*P. putida*-NBAII-RPF9, *P. plecoglossicida*- NBAII-BA11D1, *P. fluorescens*-PFDWD) on groundnut (variety TAG-24) growth was tested at 0 and 150 mM NaCl in greenhouse (Principe *et al*., 2008). Test bacteria were grown in 100 mL King's B broth (KB) in 250 ml conical flasks on a rotary shaker (150 rpm/min) (Model – New Brunswick 24KC) for 48 hours at 28 °C. Cells were harvested at 5,000 g for 10 min using a refrigerated centrifuge (Model – Sigma 3-30K) and pellets suspended in 50 mM phosphate buffer (pH 7.0) with 0.1% carboxy methyl cellulose. Seeds (1 kg) were soaked in 10 ml of the bacterial suspension (10^7 CFU/ml) and control seeds were soaked in phosphate buffer for 10 min. Seeds were sown in 20 cm diameter plastic pots containing 350 g of garden soil, sand and coco peat (1:1:1) sterilized at 121°C for 20 min on two consecutive days (Principe *et al*., 2008). Four seeds per pot were sown and thinned to one plant. Pots were watered on alternate days with tap water. Rhizosphere population of bacteria (CFU/g) was determined by serial dilution and plating onto King's B agar. Plant growth parameters like root length, shoot length, fresh and dry biomass was measured at 45 days of growth. The experiment was repeated twice in a randomized block design with three replications. Plant vigour index was calculated as germination % × (shoot length + root length) (Saravanakumar *et al*., 2011). The data obtained were analyzed by ANOVA (AgRes version 3.01), with means compared by using the least significant difference ($p \leq 0.05$).

Induction of abiotic stress and plant response studies

Three stress tolerant *Pseudomonas* isolates *viz*., *P. putida* (NBAII-RPF9), *P. fluorescens* (PFDWD) and *P. plecoglosscida* (NBAII-BA11D1) were tested for effect on Leaf Membrane Stability Index (MSI) and relative Water Content (RWC) in groundnut and pigeon pea (variety TMV-2 and TTB-7) under water stressed conditions as seed treatment as well as foliar spray. The cultures were grown and formulated as described for growth test under saline. Population in spray solution was adjusted to 10^4 CFU/ml. The crop water requirements under 4 different water regimes (100%, 60% and 40%) was calculated on the basis of water holding capacity of soil (Shinde *et al*., 2010) and moisture status maintained for 45 days. The experiment was laid in randomized block design with three replications. Treated seeds were planted in seedling trays with 150 g capacity containing garden soil, sand and coir pith (1:1:1). Leaf Membrane Stability Index (MSI) was determined according to the method of Shinde *et al*. (2010). Leaf discs (0.5g) of uniform diameter were taken in test tubes containing 10ml of double distilled water in two sets. Test tubes in one set were kept at 40ºC in a water bath for 30 min and electrical conductivity (EC) of the sample was measured (C1) using a conductivity meter (Eutech PC2700). Test tubes in the other set were incubated at 100ºC in the boiling water bath for 15 min. and their electrical conductivity was measured (C2). MSI was calculated using the formula: MSI = [1-C1 /C2] x100. Relative Water Content (RWC) was recorded from four leaflets of the second fully expanded leaf form the top of the main stem for each pot. Once leaves were harvested and transported to the laboratory, leaf fresh weight (FW) was recorded. The leaf samples were then soaked in distilled water for 8 hrs and blotted for surface drying and water saturated leaf weight was determined (TW). The samples were oven-dried at 80ºC until reaching constant weight and leaf dry weight could be determined (DW). RWC was calculated based on the formula RWC (%) = (FW-DW) / (TW-DW) × 100.

Estimation of proline

Proline content in leaves was measured as per following protocol. 250 mg of leaf tissues were homogenized with 10 ml of 3% aqueous sulphosalicylic acid. This was centrifuged at 3,000 rpm for 10 min. 2 ml glacial acetic acid and 2 ml acid ninhydrin mixture was added to 2 ml of supernatant. The mixture was kept at 100°C for 1 h. the reaction was terminated by placing it on ice bath for 10 min. 4 ml toluene was added to the reaction mixture and vortexed for 15-20s. The toluene layer was separated from aqueous phase and absorbance was measured at 520 nm. Toluene was used as blank. The proline was determined from a standard curve (Saravanakumar *et al.,* 2011). The data obtained was analyzed by 2 way ANOVA (AgRes version 3.01). Experiment repeated twice for confirmation of results.

RESULTS AND DISCUSSION

The rhizosphere of Pigeon pea, sorghum and groundnut crops yielded a total of 230 *Pseudomonas*

isolates on agar medium. When these isolates were tested for tolerance to abiotic stresses like high temperature, salinity and moisture 47 of them showed tolerance to one or more of the stress parameters tested. All the 47 bacteria were identified by 16s rDNA analysis (sequences deposited in GenBank and further confirmed by BIOLOG) (Table 1). These 47 bacteria were classified as *P. aeruginosa* (24), *P. putida* (14), *P. plecoglossicida* (4), *P. mosselli* (1), *Pseudomonas* sp. (1) and *P. fluorescens* (3). *P. aeruginosa* are generally considered as human pathogens (Knapp *et al*., 2005) and data about them is presented only as academic interest. The temperature tolerance of the 47 bacteria was 45°C, with seven of them showing good growth even at 50°C and 18 isolates were tolerant to 48°C (Table 1). Recent approach by many workers is to use stress tolerant plant growth promoting rhizobacteria (PGPR) in improving crop productivity and such organisms were mostly isolated from a stressed environment (Ali *et al*., 2009; Egamberdieva, 2011; Rangarajan *et al*., 2003). Bacteria growing at 37°C and above are generally considered as temperature tolerant and six temperature tolerant Gram negative bacteria were isolated from arid soils of Uzbekistan (Egamberdieva, 2011). *P. aeruginosa* strain AKM-P6 isolated from pigeon pea rhizosphere grew at 47-50°C and showed plant growth promoting ability (Ali *et al*., 2009). All the 47 isolates were tolerant to 1 M NaCl and 23 isolates could grow even at 1.5 M NaCl (Table 1). Most of the isolates tolerated a high pH of 9.0 and some of them grew even at pH 4.0 (Table 1). These observations are in accordance with other reports. *P. aeruginosa* (TSAU145) grew well in the highest salt concentration tested, 4% NaCl in King's B and 3% NaCl in basal medium (Egamberdieva *et al*., 2008). *P. fluorescens* (MSP-393) was identified as saline tolerant from a collection of 1000 rhizosphere bacteria (Rangarajan *et al*., 2003). Presence of *Pseudomonas* spp. in saline habitat is reported (Egamberdieva, 2011) and also saline tolerant bacteria can occur in both saline and non saline soils and that tolerance can evolve and spread in a bacterial community by genetic means (Trabelsi *et al*., 2009). We also evaluated the 47 bacteria for tolerance to water stress using polyethylene glycol (PEG 6000). Significant differences in growth of the isolates were observed when subjected to different water stresses. At zero osmotic potential (OP) the osmotic conditions are normal and under drought the water is held at a higher osmotic potential making it unavailable to plants similarly PEG 6000 generates osmotic stress measured in terms of OP and at 30% PEG (–10.28 OP) 35 of the isolates were found tolerant (Table 1). At 50% PEG (-26.82 OP) only 23 isolates were found tolerant. *P. aeruginosa* (NBAII-AFP13, NBAII-AFP9 and GR4RAU-A1) were found to be the most tolerant as they showed no significant decrease in population upto 48 hours (Table 1). In one report desiccation tolerance of 19 *Pseudomonas* isolates was studied using 0, 20, 30, 40, 45 and 50% PEG 6000 and it was noticed that tolerance depended upon the isolate and tolerant isolates grew for 24 to 48 hours and thereafter declined (Vyas *et al*., 2009). We did not notice drastic reduction in cell number for tolerant isolates as the population remained above log 7.0 at 48 hours. Other workers (Sandhya *et al*., 2010) also used PEG 6000 to characterize desiccation tolerant bacteria.

Phytohormones can play a key role in adaptation of plants to environmental stresses (Dimkpa *et al*., 2009) and in the present study 94% of the isolates produced IAA and ACC deaminase (Table 1). Regulation of ACC deaminase activity is a principal mechanism by which bacteria can exert positive effects on abiotically stressed plants (Saleem *et al*., 2007). We feel that that IAA and ACC deaminase producing ability could determine their abiotic stress tolerance. ACC deaminase positive *Pseudomonas* sp. S1 alleviated greatly salt stress and promoted significantly the seedling growth of oat and annual ryegrass under either 5 or 10 g/kg NaCl (Yun-Xiu and Xiao-dong, 2007). We also tested the 47 isolates for protease, HCN, chitinase, cellulase and siderophore properties but no correlation was found with abiotic stress tolerance; however 78% of the isolates had phosphatase activity which could be important in making available nutrients to plants under stress. Supportive evidences be included.

DAPG is a low-molecular-weight polyketide known to inhibit many pathogenic fungi and is responsible for the biocontrol ability of many *P. fluorescens* isolates (Weller *et al*., 2007). We detected 10 DAPG positive isolates (Table 1 and Fig. 1) and partial sequences submitted to GenBank (JQ619857-59, JQ619861-62, JQ619865-66, and JN624295-97). Interestingly these DAPG positive isolates showed enhanced stress tolerance (Table 1). We feel that DAPG could be important in conferring stress tolerance. Pyoluteorin (PLT) is also important in suppression of plant pathogens (Hammer, 1997; Naik *et al*., 2008; Vyas *et al*., 2009) and it was detected in 4 of the isolates (GenBank submissions JX273649 - JX273652) (Table 1) (Fig. 1).

Response of groundnut to seed treatment with saline tolerant bacteria under saline conditions (150 mM NaCl in soil having 12.93 mmhos/cm conductivity) was studied. Plants treated with *P. putida* (RJ26, RJ20), *P. plecoglossicida* (NBAII-BA11D1), *P. mosselli* (CK24C) and *P. fluorescens* (RJ-9) gave significantly higher dry

Table 1. Abiotic stress tolerance exhibited by the *Pseudomonas* species

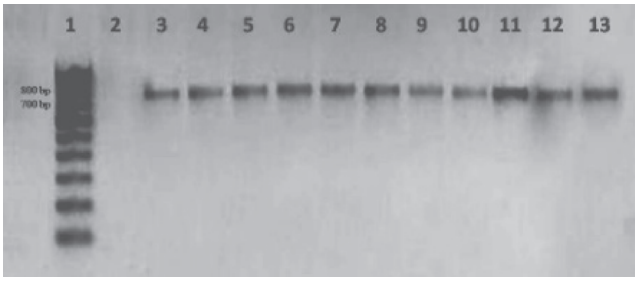
Isolate	Stress tolerance exhibited								Isolate identity and GenBank Accession	
	°C	NaCl (M)	pH	Osmotic Potential (OP) and growth (log CFU/mL at 48 hours)						
				0	–10.28	–26.82	IAA	ACC		
NBAII-AFP13	48	1.0	9	8.96	8.48	8.50	+	+	*P. aeruginosa*	HQ162487
NBAII-AFP3	48	1.0	9	8.78	7.62	6.88	+	+	*P. aeruginosa**	HM439970
NBAII-AFP4	48	1.0	9	8.23	8.23	–	+	+	*P. aeruginosa*	HM439965
NBAII-AFP5	45	1.0	4, 9	8.18	8.18	–	+	+	*P. aeruginosa*	HM439971
NBAII-AFP6	48	1.0	9	8.15	7.27	7.89	+	+	*P. aeruginosa**	HM439961
NBAII-AFP7	48	1.0	9	8.20	7.27	7.92	+	+	*P. aeruginosa*	HM439964
NBAII-AFP8	48	1.0	9	8.68	7.97	7.39	+	+	*P. aeruginosa**+	HM439962
NBAII-AFP9	48	1.0	9	8.97	7.26	8.10	+	+	*P. aeruginosa*	HM439969
NBAII-CK4D1	45	1.5	9	8.81	–	–	+	+	*P. aeruginosa*	HQ162485
NBAII-CK4D2	45	1.5	7	8.93	–	–	–	+	*P. aeruginosa*	HQ162486
CK13C	48	1.5	9	8.51	7.73	–	+	–	*P. aeruginosa*	JF723552
NBAII-CK19E	45	1.5	9	8.32	7.41	–	+	–	*P. aeruginosa*	HQ162480
NBAII-CK21	50	1.5	9	8.86	7.65	7.22	+	+	*P. aeruginosa**	HQ162484
GR4RAU-A1	45	1.0	9	8.77	7.78	8.25	+	+	*P. aeruginosa*	HM011260
NBAII-KM1	45	1.5	4, 9	8.33	7.90	7.52	+	+	*P. aeruginosa*	HQ162483
NBAIIND4IART-B	48	1.0	4, 9	8.26	–	–	+	+	*P. aeruginosa*	HM439975
NBAII-OTN8	45	1.5	9	8.40	8.06	–	+	+	*P. aeruginosa*+	HQ162482
NBAII-PDB1	45	1.0	9	8.60	7.39	7.67	+	+	*P. aeruginosa*	HQ162481
NBAII-PDB11	45	1.5	9	8.15	8.11	–	+	+	*P. aeruginosa*+	HQ162478
NBAII-PDB8	45	1.0	4	8.38	8.17	6.94	+	+	*P. aeruginosa*	HQ162479
NBAII-RPF7	48	1.0	9	8.80	–	–	+	+	*P. aeruginosa*+	HM439972
NBAII-RPF8	48	1.0	9	8.18	–	–	+	+	*P. aeruginosa*	HM439966
PDB9	48	1.0	9	8.62	7.33	–	+	+	*P. aeruginosa*	JN624294
RJ16	45	1.0	4, 9	9.37	8.11	–	+	+	*P. aeruginosa**	JN624286
NBAII- BA14C2	50	1.0	4	9.23	7.80	7.70	+	+	*P. putida*	HQ162488
BA14D-1	45	1.5	4, 9	8.84	7.59	7.58	+	+	*P. putida*	HM011261
BA2D-2	50	1.5	7	8.68	7.30	7.19	+	+	*P. putida*	GU372963
NBAIICK-24E	48	1.0	4,	8.91	7.42	7.34	-	+	*P. putida*	HQ162489
NBAIICK-8C	48	1.5	4, 9	8.30	7.55	7.22	+	+	*P. putida*	HM439953
NBAIIGRIARS-1	50	1.0	9	8.41	7.76	7.70	+	-	*P. putida*	HM439963
NBAII-OTN5E2	45	1.5	9	8.80	–	–	+	+	*P. putida*	HM439974
NBAII-PF4K	45	1.0	4	8.57	–	–	+	+	*P. putida*	HM439958
NBAIIRPF-13	48	1.0	7	8.19	7.60	–	+	+	*P. putida*	HM439957
NBAIIRPF-9	48	1.5	9	9.02	7.43	–	+	+	*P. putida*	HM439967
RJ15	45	1.0	4	8.60	7.11	–	+	+	*P. putida*	JN624285
RJ19	45	1.0	4, 9	8.73	8.29	7.44	+	+	*P. putida**	JN624287
RJ26	40	1.5	4, 9	8.73	–	–	+	+	*P. putida**	JN624290
RJ20	45	1.5	4	8.17	7.63	–	–	+	*P. putida**	JN624293
NBAII-BA11D1	45	1.5	4	9.63	7.86	7.64	+	+	*P. plecoglossicida*	HM439960
NBAII-BA16(2)	50	1.5	7	9.67	8.03	7.15	+	+	*P. plecoglossicida*	HM439959
NBAIIBA3D-1	45	1.5	4, 9	8.59	–	–	+	+	*P. plecoglossicida*	HM439955
NBAIIBA3D-3	50	1.5	9	8.55	7.66	7.00	+	+	*P. plecoglossicida*	HM439954
NBAII-RPF17	50	1.5	4, 9	8.54	–	–	+	+	*Pseudomonas* sp.	HM439973
CK24-C	48	1.5	7	8.76	–	–	+	+	*P. mosselli*	JN624284
NBAII-GR3ARS5	48	1.0	9	8.41	8.03	7.67	+	+	*P. fluorescens*	HM439968
PFDWD (NBAII)	45	1.5	9	8.33	7.52	7.20	+	+	*P. fluorescens**	HM439956
RJ-9	40	1.0	9	9.17	–	–	+	+	*P. fluorescens*	JN624291
CHAO	38	1.5	4, 9	8.74	8.41	–	+	+	*P. fluorescens**+	Reference

*DAPG positive, +Pyoluteorin positive, IAA = Indole acetic acid; ACC = 1-aminocyclopropane-1-carboxylic acid deaminase; CFU = colony forming units. Two way ANNOVA for osmotic (Mpa); CV = 5.55% ; SED = 0.37; CD (P<0.01) = 0.98

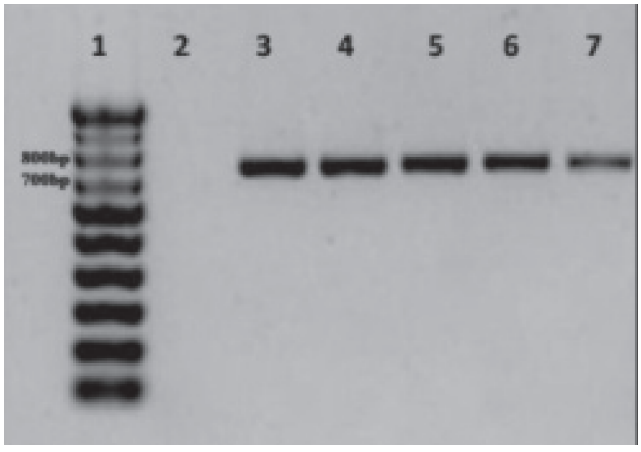
Table 2. Effect of different treatments on relative water content (RWC) and membrane stability index (MSI) of ground nut and pigeon pea under water stress

Treatments	Ground nut						Pigeon pea					
	RWC (%)			MSI (%)			RWC (%)			MSI (%)		
	100	60	40	100	60	40	100	60	40	100	60	40
Control	81	78	78	85	81	79	82	77	61	85	26	26
NBAII-RPF9 ST*	86	87	93	89	98	89	81	74	85	89	73	68
NBAII-RPF9 FS**	86	92	95	91	93	95	85	79	75	91	39	35
NBAII-RPF9 STFS	96	99	87	88	93	97	98	79	65	88	32	31
PFDWD ST	95	96	97	93	95	93	84	82	78	93	90	90
PFDWD FS	79	78	83	79	81	90	78	81	89	79	80	80
PFDWD STFS	87	85	81	89	91	95	95	88	82	89	64	90
NBAII-BA11D1 ST	88	79	83	95	95	97	81	83	75	95	88	78
NBAII-BA11D1 FS	78	84	88	91	93	94	79	83	76	91	92	95
NBAII-BA11D1 STFS	67	67	75	90	92	88	82	85	84	90	80	83
CD (P<0.05) RWC	p (treatment) 1.0; m (water stress) 0.5pm (treatment× water stress) 1.74						p (treatment) 2.2; m (water stress) 1.2 pm (treatment× water stress) 3.9					
CD (P<0.05) MSI	p (treatment) 0.84; m (water stress) 0.46 pm (treatment× water stress) 1.45						p (treatment) 1.02; m (water stress) 0.5 pm (treatment× water stress) 1.78					

The values are mean of three replicates ± SE (P<0.05). *ST = Seed Treatment; **FS = Foliar Spray



A

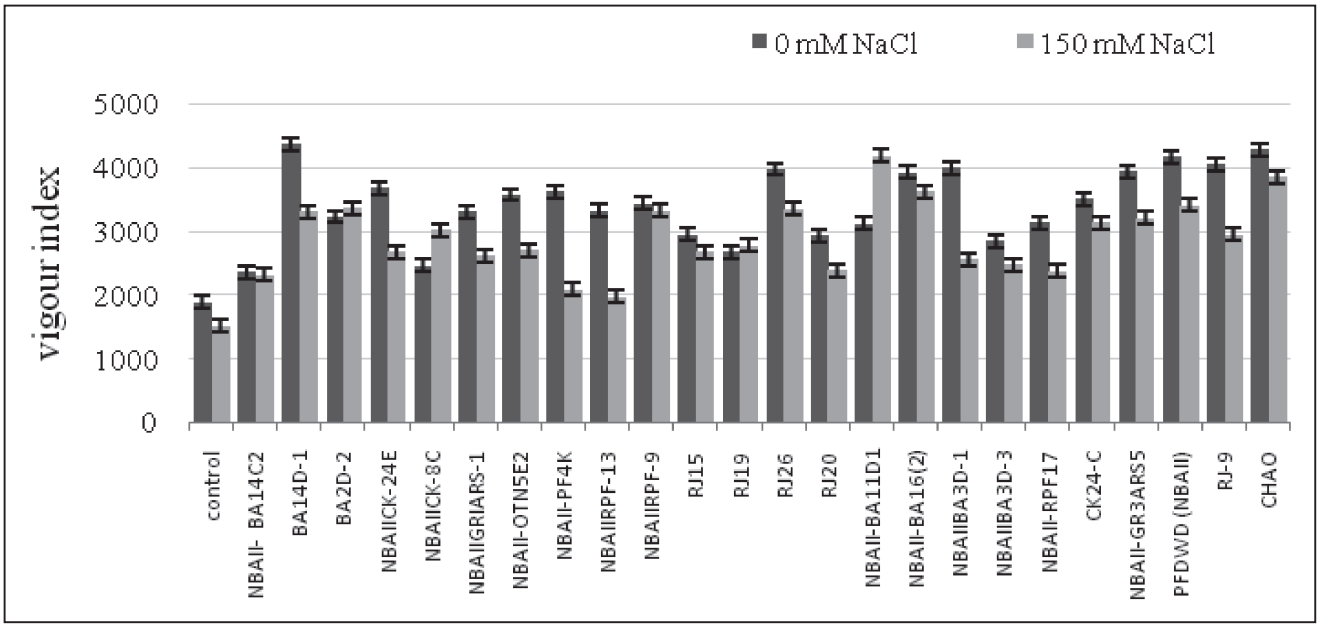


B

Fig. 1. Detection of 2,4-diacetylphloroglucinol (DAPG) (A) and pyoluteorin (PLT) (B) genes in the stress tolerant *Pseudomonas* spp.

matter content under saline conditions as compared to control (non saline / 2.17mmhos/cm) (Fig. 2). Dry weight was 39-43% more than control in RJ-26 and RJ-20 treated plants and intriguingly these two were DAPG positive. The vigour index was also significantly higher in bacteria treated plants (Fig. 2) and highest vigour index of 4182 was exhibited by *Pseudomonas* sp. (NBAII-BA11D1) followed by *P. fluorescens* (CHAO). The observations are in accordance with other studies. The soil bacterium MEP211a was able to significantly increase the root dry weight only under saline stress conditions (Principe *et al*., 2008). *P. putida* (RS-198) isolated from saline soil (Yao *et al*., 2010) could protect cotton against salt stress and plant stand was increased by 30.7%. *Pseudomonas* strains were able to colonize the rhizosphere of bean at the highest saline condition (12.5 dS/m) and rhizos-phere population was $3.9 \times 10^3 \pm 0.8$ CFU/g of fresh root with *P. extremorientalis* TSAU20 (Egamberdieva, 2011).

Bacteria found in association with plants grown under chronically stressful conditions may confer a certain degree of resistance to drought (Mayak *et al*., 2004; Saravanakumar *et al*., 2011). High RWC content is a resistance mechanism to drought that helps in enhanced osmotic regulation or less elasticity of tissue cell wall by the presence of membrane - compatible solutes (Keyvan 2010; Ritchie *et al*., 1990). We found significant variations



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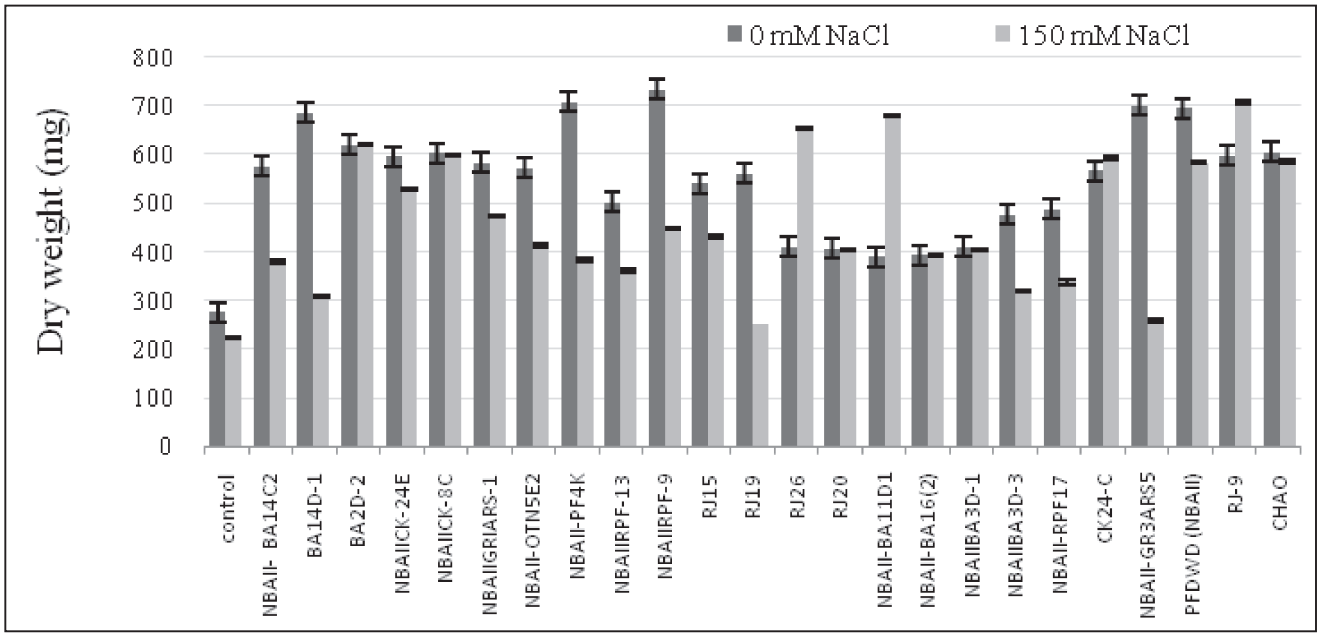
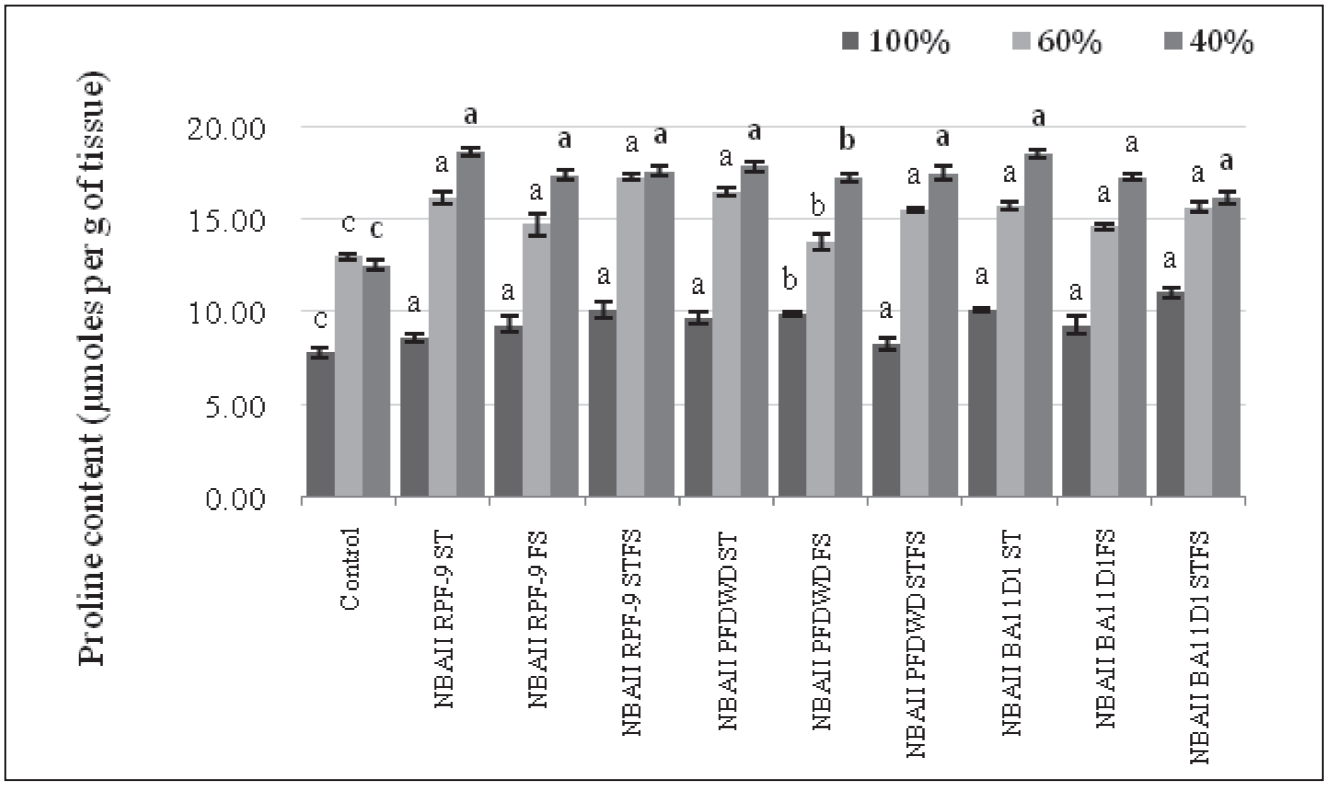


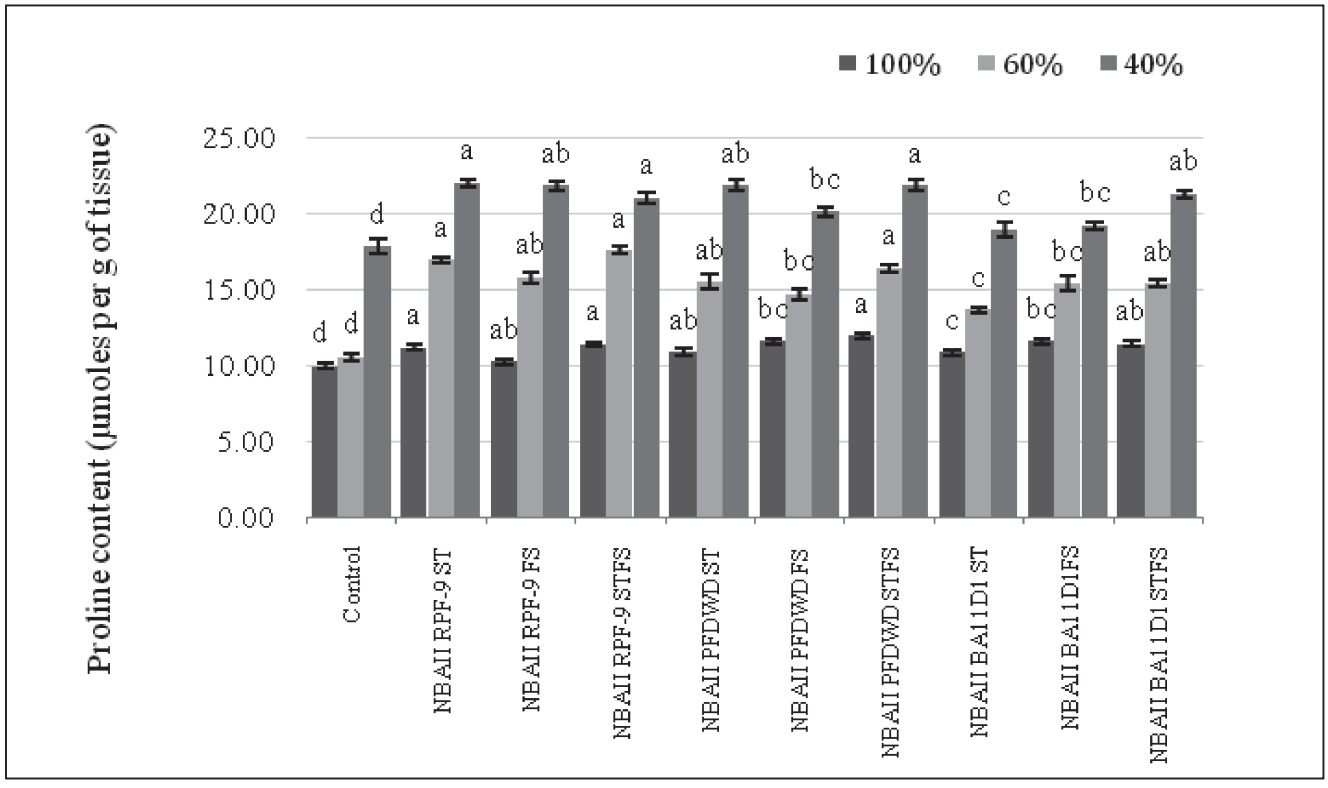
B

Fig. 2. Effect of abiotic stress tolerant *Pseudomonas* (non *aeruginosa* isolates) on vigour index (A) and dry weight (a) CD (P<0.05)=99.8 (0mMNacl); CD (P<0.05) = 255.8 (150 mMNaCl) (B) of 45 day old groundnut seedlings grown under saline conditions (b) CD (P<0.05) = 501.1 (omMNaCl); CD (P<0.05)=461.2 (150 mMNaCl)

in RWC and MSI in bacteria treated plants (Table 3). In groundnut when water availability was reduced to 40%, RWC was maximum (97%) in plants raised from *P. fluorescens* (PFDWD) treated seeds. MSI was also high (97%) in both *P. putida* (NBAII-RPF9) and *P. plecoglossicida* (NBAII-BA11D1) treatments. Pigeon pea responded well to *P. fluorescens* (PFDWD) and *P. plecoglosscida* (NBAII-BA11D1) treatments. Studies show that plants treated with *P. fluorescens* Pf1 conferred greater resistance to water stress (Saravanakumar *et al*., 2011). Accumulation of proline in plants is a physiological response to drought / salinity and proline helps in the stability of membranes and proteins (Delauney and Verma, 1993). We detected significantly high proline content (Fig. 3) in all bacteria treated plants (when grown under water stress) and seed treated with *P. putida* (NBAII-RPF9) induced maximum proline content of 18.70 µmoles/g in pigeon pea and 21.99 µmoles/g in groundnut which



A



B

Fig. 3. Bacteria induced proline accumulation in pigeon pea (A) and groundnut (a) CD (P<0.05) = 1.24 (100%); CD (P<0.05) = 0.68 (60%); CD (P<0.05) = 2.16 (40%), (B) at 35 days under 100%, 60% and 40% water availability (ST - seed treatment; FS - Foliar spray; STFS - seed treatment + foliar spray). Different letters on the histograms indicate that the means differ significantly (P<0.05) (b) CD (P<0.05) = 1.09 (100%); CD (P<0.05) = 0.60 (60%); CD (P<0.05) = 1.90 (40%)

indicated that this bacteria induced/ enhanced proline content in water stressed plants in comparison to control (12.58 and 17.79 µmoles/g respectively). Rhizobacteria caused an increase in proline of banana leaves (49.7 ì g/g FW compared to control at 35.0 µg/g FW) (Mahmood *et al*., 2010). The ultimate aim of this study is to use these stress tolerant *Pseudomonas* spp. as plant protection agents in agriculture especially for stressed soils. Hence we also evaluated the isolates for *in vitro* antagonism against *Sclerotium rolfsii, Rhizoctonia solani, Macrophomina phaseolina, Colletotrichum gloeosporioides* and *Fusarium oxysporum* using only potato dextrose agar (PDA). All the isolates showed antagonism against one or more pathogens (data not shown).

Hence our observations indicate that abiotic stress tolerant PGPR can occur in a stressed habitat and significant enhancement in plant growth was observed when some of these bacteria were used as seed or foliar inoculants under saline or water stressed conditions. However the role of DAPG, ACC deaminase and IAA genes in conferring stress tolerance needs to be further studied. *P. aeruginosa* are generally considered as human pathogens (Knapp *et al*., 2005) and the occurrence of *P. aeruginosa* in high numbers (as evidenced in this study) is of concern as they are opportunistic pathogens of multicellular organisms including human beings (Knapp *et al*., 2005); whether they are normal rhizosphere microflora or contaminants from human activity needs to be ascertained.

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