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Research Article

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In vitro screening for salinity and drought stress tolerance in plant growth promoting bacterial strains

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ABSTRACT: The present study was designed to elevate the in vitro bacterial mechanisms related to the plant growth promotion and their tolerance for sodium chloride (NaCl) and polyethylene glycol (PEG) in culture media. Total nine bacterial strains were studied for both stress tolerance under varying concentration of NaCl and PEG. Out of them, three bacterial strains namely *Pseudomonas simiae* AU, *P. koreensis* AK-1 and *Carnobacterium* sp. SJ-5 were found tolerate to stress and further used for biochemical characterization of ACC-deaminase, IAA and Pi-solubilization activities under both stresses. All three strains were exhibited equal amount of Pi-solubilization at each stress levels. The strain P. simiae AU significantly presented the highest ACC-deaminase activity (81 nmol/mg/h and 73 nmol/mg/h) and IAA activity (41.5 µg/mL and 39.08 µg/mL) at 0.4M NaCl and 10% PEG stress respectively.

Keywords: polyethylene glycol (PEG), plant growth promotion, stress tolerance, bacterial strains

INTRODUCTION

Soil is a largest favorable ecological niche for the microbes and their metabolic activities. Root zone area of plants containing huge microbial population and high metabolic activities. Instead of the high microbial population and metabolic activities in the rhizospheric area, these microbes occupy only 5% area of the total space (Chakraborty *et al.,* 2015). However, microbial activity or population is not uniform throughout the soil, but is highly concentrated in the region of the root surface area, known as the rhizosphere (Pinton *et al.,* 2001; Chakraborty *et al.,* 2015). Increased populations of microbes colonize the root zone of plants. Microbes present around the plant roots in higher concentration, the main reason of dense microbial population due to presence of higher level of the nutrient availability including amino acid, sugars, organic acids and flavanoids those are execrated from the roots of plants and are then used by the microbes in the soil (Dimkpa *et al.,* 2009; Ashraf *et al.,* 2013). Many literatures are replete with reports describing the plant growth promotion under abiotic stresses in the occurrence of rhizospheric bacteria. Several approaches have been adopted in order to minimize the adverse effect of abiotic stresses including genetically modified crop, but the use of a diverse species of rhizospheric microorganisms containing ACC-deaminase belonging to various taxonomic groups including *Pseudomonas, Azospirillum, Bacillus, Azotobacter, Klebsiella, Enterobacter, Xanthomonas,* and *Serratia* , shown to promote plant growth is well known and sustainable approach for enhancing plant tolerance to abiotic stresses including drought, salinity and increases growth (Duan *et al.,* 2009; Yang *et al.,* 2008; Egamberdiyeva, 2009; Tilak *et al.,*, 2005). These microorganisms are generally term as plant growth promoting bacteria (PGPB) are well known for enhancing plant tolerance to abiotic stresses and increases growth (Egamberdiyeva, 2007; Yang *et al.,* 2009; Bharti *et al.,* 2013). PGPB having many plant growth promoting (PGP) traits such as ACC-deaminase, volatiles, antioxidants, Indole-3-acetic acid (IAA) etc. maintain reactive oxygen species (ROS) level in the plants by increasing expression of antioxidant enzymes and maintaining the plant phytohormone levels such as ethylene, ABA, IAA etc. under salt and drought stress (Yang *et al.,* 2009; Choudhary *et al.,* 2012; Vaishnav *et al.,* 2013; Kumari *et al.,* 2015). This results in induced systemic tolerance (IST). IST is an important process to overcome the harmful effects of abiotic stress such as soil salinity and severe drought stress. PGPB induced physical and chemical responses in plants that result in enhanced tolerance to drought, salinity, heavy metals, cold, heat shock and fertility stresses (Yang *et al.,* 2009). These PGPB have been enhanced the growth of many different crops grown under stressed agriculture land (Dood *et al.,* 2012). The use of PGPB-containing ACC-deaminase to improve plant growth in many crops has been increased worldwide. PGPB contain an enzyme ACC-deaminase lowers the stress ethylene level by conversion of ACC into ammonia and α-ketobutyrate in the plants (root and seed) (Glick 2012). In addition, PGPB possess several other traits like synthesis of auxins, phosphate solubilization, siderophore production etc. which directly promote plant growth (Zahir *et al.,* 2009; Vaishnav *et al.,* 2013; Kumari *et al.,* 2015). Many research studies have been reported the beneficial effect of bacterial inoculum on the plant growth through direct and indirect mechanisms of PGPB under different

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environmental stressed conditions (Khalid *et al.,* 2009; Grover *et al.,* 2011; Glick *et al.,* 2012; Jain *et al.,* 2013; Souza *et al.,* 2015; Choudhary *et al.,* 2015).

Here, the main focus of the present work is to study the tolerance level of PGPB and to check the ACC-deaminase activity and other plant growth promoting (PGP) activities of selected drought and salt tolerance bacterial strains in the presence of polyethylene glycol (PEG) and NaCl.

MATERIALS AND METHODS

BACTERIAL STRAINS AND ASSESSMENT OF SALT AND DROUGHT STRESS TOLERANCE

Nine bacterial strains (CBS-1, CSS-1, CCS-2, CHS-1, CJS-2, CKS-2, *Pseudomonas simiae* AU, *P. koreensis* AK-1 and *Carnobacterium* sp. SJ-5) were selected for the study.

The salt and drought stress tolerance of selected strains was tested using NaCl and polyethylene glycol (PEG) respectively. The susceptibility of the selected bacteria in presence of NaCl and PEG was relatively unknown. Take 1 mL of the bacteria-nutrient media suspension added into test-tubes containing 9 mL of nutrient media amended with varying NaCl concentration (0M, 0.2M, 0.4M and 0.6M) for salt stress and PEG concentration (0%, 5%, 10%, and 15%) for drought stress. All test-tubes were kept on shaker at 28± 2 °C for 5 days. Bacteria growth viability under both stresses was monitored over the period of 5 days by measuring the optical density at 600nm.

BIOCHEMICAL ESTIMATION ACC-DEAMINASE AND OTHER PGP TRAITS

Measurement of ACC-deaminase and different other plant growth promoting features of bacterial strains was carried out at varying range of NaCl salt concentration (0M-0.6M) and PEG concentration (0% - 15%).

ACC-DEAMINASE PRODUCTION ASSAY IN BOTH STRESSES

To measure ACC-deaminase activity, bacteria cells were grown in 5 mL of nutrient broth medium at 28°C for 72 h on orbital shaker until they reached at stationary phase. To induce ACC-deaminase activity, after the incubation the cells were harvested by the centrifugation, washed twice with 0.1 M Tris-HCl (pH- 7.5), suspended in 4 mL of modified M9 medium supplemented with 5mM ACC concentration, in the presence of NaCl salt concentrations (0M to 0.6M) and PEG concentration (0% to 15%) and incubated at 28°C for another 48 h on orbital shaker. ACC-deaminase activity was estimated by measuring the amount of α–ketobutyrate generated by the cleavage of ACC (Penrose and Glick 2003).

IAA PRODUCTION ASSAY IN BOTH STRESSES

IAA synthesizing assay in all bacterial strains was measured according to the modified method of Gordon and Weber (1951). Quantitative estimation of IAA was performed by inoculating 0.2 OD overnight grown culture in 50 mL flask containing 10 mL of nutrient broth amended with 200 µg/mL of L- tryptophan in the presence of different salt concentrations (0M to 0.6M) and PEG concentration (0% to 15%) at pH 7.0 ± 0.2. L- tryptophan was filter sterilized with 0.22 µm membrane filter, before adding in nutrient broth.

PHOSPHATE SOLUBILIZATION (Pi-SOLUBILIZATION) ASSAY IN BOTH STRESSES

For quantification determination of Pi-solubilization the method of chlorostannus reduced chloromolybdic acid blue described by Diby *et al.,* (2005) was used with some modification. A 72 h old bacterial culture of 0.2 OD was inoculated in 50 mL flask containing 10 mL of PVK broth (HiMedia, India) having different salt concentrations (0M to 0.6M) and PEG concentration (0% to 15%) at pH 7.0 ± 0.2 was centrifuged at 8,000 for 15 min at 4°C and the supernatant was used for Pi quantification. The amount of phosphate solubilized by the bacterial cells was calculated using the standard curve of KH_2PO_4.

RESULTS

SURVIVAL RATE OF BACTERIA IN RESPONSE TO NaCl AND PEG

All selected bacterial strains were tested for survival rate in the presence of different NaCl concentration (0M to 0.6M) and PEG concentration (0%, to 15%). Based on the growth pattern it was shown that the bacterial strains CBS-1, CSS-1, CCS-2, CHS-1, CJS-2 and CKS-2 were able to grow only in 0.2M NaCl concentrations and 5% PEG concentration, whereas bacterial strains AU, AK-1 and SJ-5 were able to tolerate and grow in all NaCl salt and PEG stress conditions (Table 1). On the basis of results of stress tolerance, we have selected three bacterial strains AU, AK-1 and SJ-5 for further characterization of different PGP features.

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Table 1: Effect of varying concentration of NaCl and PEG stress on growth of selected bacterial strains

Bacterial strains	Non-stress	NaCl stress			PEG stress		
		0.2M	0.4M	0.6M	5%	10%	15%
P. simiae AU	+	+	+	+	+	+	+
P. korensis AK-1	+	+	+	+	+	+	+
C. sp. SJ-5	+	+	+	+	+	+	+
CBS-1	+	+	-	-	+	-	-
CSS-1	+	+	-	-	+	-	-
CCS-2	+	+	-	-	+	-	-
CHS-1	+	+	-	-	+	-	-
CJS-2	+	+	-	-	+	-	-
CKS-2	+	+	-	-	+	-	-

+ represents growth of bacterial strain in culture medium after 5 days and – represents no bacterial growth.

BIOCHEMICAL CHARACTERIZATION OF PGP FEATURES UNDER SALT AND PEG STRESSES

PGP capabilities of the three selected bacterial strains AU, AK-1 and SJ-5were studied (ACC-deaminase activity, IAA synthesis, Pi-solubilization, EPS production, siderophore production and proline synthesis) at all NaCl and PEG concentrations. For the biochemical characterization of PGP activities all three strains were grown on the specified medium.

ACC-DEAMINASE ACTIVITY

The ability of all three salt and drought tolerant bacterial strains AU, AK-1 and SJ-5 to utilize ACC as a sole source of N was determined in the presence of NaCl and PEG concentration. Results of ACC-deaminase activity in the form of α-ketobutyrate formation revealed that all the three strains metabolized ACC in presence or absence of NaCl and PEG (positive for possessing ACC-deaminase activity); whoever under control conditions (without stress), we observed no statistical difference in ACC-deaminase activity of all strains. Activity was decreased with increasing NaCl and PEG concentration. We observed only significant difference at 0.4M NaCl (81 nmol/mg/h) and 10% PEG (73 nmol/mg/h) with strain AU (Fig 1).

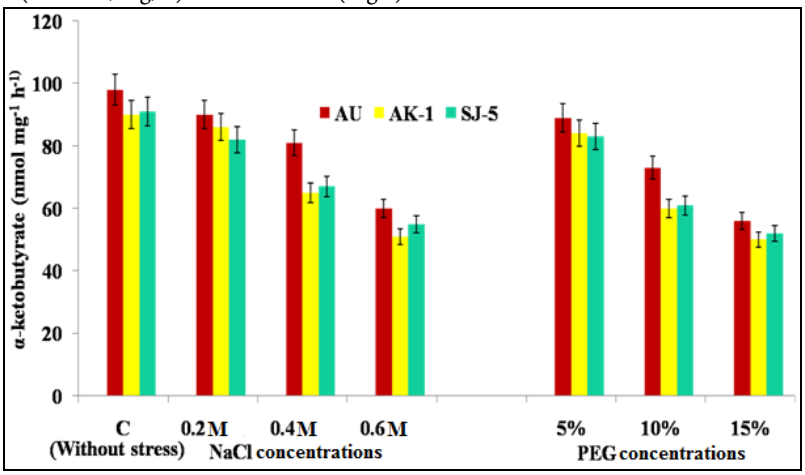

Fig 1: Effect of varying concentration of NaCl and PEG on ACC-deaminase activity of AU, AK-1 and SJ-5 bacterial strains. Figure shows *P. simiae* strain AU is produced more significant different ACC-deaminase activity, 81 nmol/mg/h and 73 nmol/mg/h at 0.4M and 10% PEG respectively. Bars are mean of 3 replicates and error bars show ± standard errors

IAA PRODUCTION

All three bacterial strains were able to produce IAA in control conditions as well as in the presence of NaCl and PEG concentrations. Bacterial strains produced IAA only in presence of L-tryptophan i.e. tryptophan independent pathway for IAA production was absent in bacterial strains. Upon increasing NaCl and PEG concentration IAA production decreased. All three bacterial strains AU, AK -1and SJ-5 were able to synthesized sufficient IAA production at all NaCl and PEG concentration, whereas we found only significant IAA production value at 0.4M (41.5 µg/mL) and 10% PEG (39.08 µg/mL) with *P. simiae* strain AU respectively (Fig. 2).

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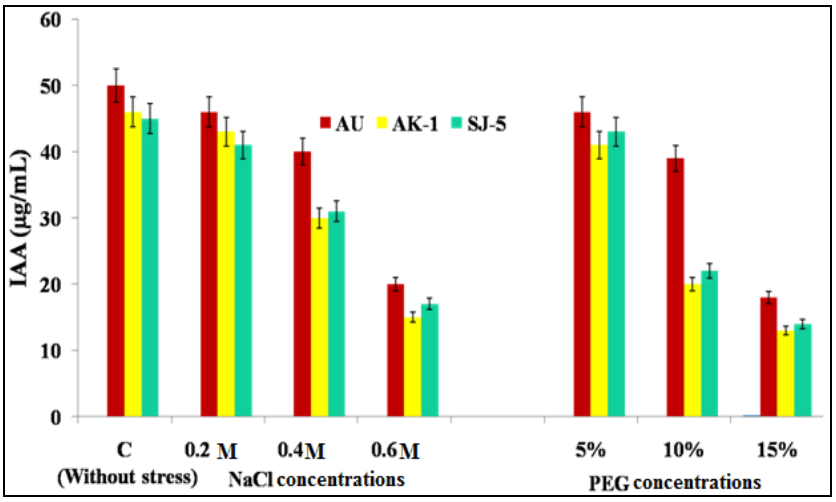

Fig 2: Effect of varying concentration of NaCl and PEG on IAA production of AU, AK-1 and SJ-5 bacterial strains. Figure shows *P. simiae* strain AU is produced more significant different IAA production, 0.4M (41.5 µg/mL) and 10% PEG (39.08 µg/mL). Bars are mean of 3 replicates and error bars show ± standard errors.

QUANTITATIVE ESTIMATION FOR Pi-SOLUBILIZATION

All the selected bacterial strains were checked for inorganic Pi-solubilization in different salt and PEG concentration quantitatively. All bacterial strains were able to solubilize inorganic phosphate in control and stress condition. The tendency to solubilize Pi decreased as the concentrations of NaCl and PEG increased (Fig. 3). All strains give almost equal quantity of Pi-solubilization.

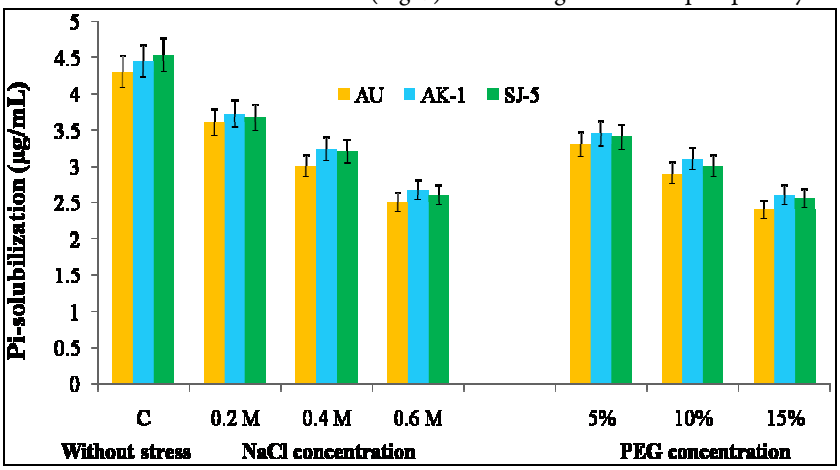

Fig 3: Quantitative Pi-solubilization estimation in all three bacterial strains in different NaCl and PEG concentration and control conditions. Values of bars are mean of 3 replicates and error bars show ± standard errors.

DISCUSSION

ASSESSMENT OF SALT AND DROUGHT TOLERANCE LEVEL

The introduction and perseverance ability of a strain are affected by a many abiotic factors like high salt, severe drought, high pH, and high temperature (Rojas-Tapias *et al.,* 2012). Therefore, tolerance to high salt and water stresses may be important to identify the most suitable salt and drought tolerant bacteria, containing ACC-deaminase as a biofertilizers for sustainable plant growth under such harsh environmental conditions. Many researchers have been documented the assessment of tolerance level of ACC-deaminase containing bacteria against heavy metal, drought, extreme temperature and different salts, were also characterized for plant growth promotion under extreme environmental conditions (Siddikee *et al.,* 2011; Egamberdieva *et al.,* 2007). It is very likely that microbial species exhibiting their optimum growth and ACC-deaminase activity at extreme environmental conditions might be useful in sites occurring at environmental extremes. In this work, Tolerance of the nine isolated bacteria to salinity and drought stresses has been observed by using different NaCl and PEG concentrations. High salinity and drought stress, by adding varying NaCl and PEG concentrations, resulted in a growth reduction of all strains tested in this investigation. Among the strains, significant reaction differences to both stresses were

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obtained. The results tolerance assessment showed that out of nine strains only three strains namely AU, AK-1 and SJ-5 were able to significantly grow in NB supplemented with all NaCl and PEG concentrations at all incubation time. Therefore, the three bacteria could be classified as halotolerant bacteria. Several other studies showed the influence of salinity and drought conditions on the survival of rhizobia in culture medium, using non-permeating substances NaCl and PEG respectively. Naz *et al.,* (2009) also demonstrated that salinity and drought reduced viability of bacterium.

ACC-DEAMINASE AND OTHER PGP ACTIVITIES UNDER NaCl AND PEG CONCENTRATIONS

After salt and drought tolerance assay three above selected bacterial strains *P. simiae* strain AU, *P. koreensis* strain AK-1 and *Canobacterium* sp. strain SJ-5 were further screened for diffent PGP activities under varying concentration of NaCl ranging from 0M to 0.6M and 0 to 15% PEG concentrations.

The role of ACC-deaminase in decreasing stress ethylene level by the enzymatic hydrolysis of ACC into α-ketobutyrate and ammonia has been presented as one of the critical mechanism of PGPB in promoting plant growth under extreme environmental conditions (Fig. 2). ACC-deaminase activity as measured in all three bacterial strains AU, AK-1 and SJ-5 under NaCl and PEG concentrations, however out off three strains AU showed significant quantity of ACC-deaminase at 0.4M NaCl and 10% PEG concentration (Fig. 1), reflects its ability to utilize ACC as a sole source of nitrogen which can efficiently enhance root length and plant growth under salt and drought stresses. Many researchers have been documented the ACC deaminase containing PGPB improving the salinity and drought tolerance in plants (Siddikee *et al.,* 2011; Nadeem *et al.,* 2009; Nautiyal *et al.,* 2013; Kumari *et al.,* 2015; Singh *et al.,* 2015; Glick *et al.,* 2013; Belimov *et al.,* 2009). Sharp increases in ACC levels and consequently, ethylene synthesis in plants under drought stress conditions have been frequently reported (Arshad *et al.,* 2008). Therefore, the inhibitory effects of ethylene induced by salinity and drought stresses might have been eliminated through ACC-deaminase activity of the PGPR.

IAA production is widespread among PGPB and its positive effects on plant growth have been well documented (Spaepen *et al.,* 2007). Bacterial IAA has been shown to enhance the development of the host plant root system (Patten *et al.,* 2002, Spaepen *et al.,* 2008; Kasotia *et al.,* 2013), which increases the surface area through which soil nutrients are absorbed. Various reports have been published on IAA production by PGPB strains either with or without the tryptophan supplement in culture media, correlated with direct effects on plant growth (Naz *et al.,* 2009). Here, we demonstrate directly that bacterial IAA plays a major role in promotion of root elongation when a bacterium is associated with its host plant under salt and drought stress. In this study, all 3 bacterial isolates able to produce IAA when tryptophan (500ppm) was added in media supplemented with different NaCl and PEG concentrations, however out of them AU isolates was found significantly higher ability to convert tryptophan to IAA than other isolates at 0.4M NaCl and 10% PEG (Fig. 2). When these bacteria inoculated with soybean and mungbean seeds, root length, no. of lateral roots and fresh weight were increased in each individual treatment (Kumari *et al.,* 2015, 2016). Belimov *et al.,* (2015) proposed that rhizobacteria that produce IAA and contain ACC-deaminase decrease amino acid concentrations in the rhizosphere and improve *Solanum tuberosum* growth and yield under water deficient conditions.

Phosphate solubilizing bacteria (PSB) solubilize inorganic phosphate through directly synthesis of organic acids such as gluconic and citric acid etc. or indirectly solubilize P by decreasing the soil pH, thus converting insoluble phosphate in to soluble form that plants can absorb (Bashan *et al.,* 2013). The unavailability of phosphate to the plants under adverse environmental conditions limits the plant growth, means this element is essential for plant growth. Thus solubilization and mineralization of phosphate by PSB is an important trait of PGPB (Khan *et al.,* 2006; Rodriguz *et al.,* 2004). Here, we were reported the Pi-solubilization of all three strains AU AK-1 and SJ-5 in the Pikovskaya medium supplemented with NaCl and PEG concentrations as well as non-stress (Fig 3). The capacity to solubilize inorganic phosphate is a good characteristic for the selection of bacteria capable of increasing P content in the rhizosphere and their application to increase plant protection against adverse abiotic factors has now been an upcoming strategy (Vassilev *et al.,* 2012). PSB isolated from canola plants were tested for growth-promoting effects and found able to promote plant growth (Singh *et al.,* 2014).

CONCLUSION

The findings of the present study are much useful to the agriculture sector for utilizing these potential beneficial bacteria as a bio-inoculant which can successfully improve the stress tolerance and enhance the crop production under salinity and drought stresses. Three bacterial strains belonging to the genera *Pseudomonas* and *Bacillus* have been isolated in this study. All three bacterial strains were salt and drought resistance and they can produce high levels of ACC deaminase, IAA and Pi-solubilization. The strain *Pseudomonas simiae* strain AU showed the possible ability to promote the growth of Vigna radiata under drought stress conditions (Kumari *et al.,* 2016).

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CONFLICTS OF INTEREST
"The authors declare no conflict of interest".