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Research Article

SCREENING OF PLANT GROWTH PROMOTING RHIZOBACTERIA FOR ACC DEAMINASE ACTIVITY

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Abstract

Salinity is one of the most critical constraints which hamper agricultural productions in many areas. One of the recently gaining practices of counteracting the adverse effects of salinity on plant growth includes the application of saline tolerant bacteria with beneficial growth promoting traits. In the present study, twenty different isolates of *Azospirillum*, *Bacillus* and *Pseudomonas* were isolated from salinity affected soils of coastal districts of Tamil Nadu and they were identified. They were screened for their plant growth promoting traits and five isolates were selected from each genus of *Azospirillum*, *Bacillus* and *Pseudomonas*. The selected strains were screened for their ACC (1-amino cyclopropane-1carboxylic acid) deaminase activity. Among the fifteen strains of PGPR, three different strains viz., *A. brasilense* PA-17, *B. subtilis* PB-15 and *P. fluorescens* PP-16 were selected based on the higher production of ACC deaminase activity under salt stress condition. It was also observed that, the above three PGPR strains also produced more proline under salt stress condition. Based on the experimental results, the three PGPR strains viz., *A. brasilense* PA-17, *B. subtilis* PB-15 and *P. fluorescens* PP-16 were selected as saline tolerant PGPR for alleviating the adverse effect of salinity.

Keywords: PGPR, Proline content and ACC deaminase activity.

Introduction

Environmental stresses are major limiting factors for agricultural productivity worldwide. These stresses decrease yields of crops and also represent barriers to the introduction of crop plants into areas that are not suitable for crop cultivation. Abiotic stress factors include high and low temperature (Iba, 2002; Sung *et al.*, 2003), salinity (Hasegawa *et al.*, 2000; Mansour and Salama, 2004), drought (Munns, 2002; Zhu, 2002), flooding (Dat *et al.*, 2000), ultraviolet light (Gyula *et al.*, 2003; Stratmann, 2003), air pollution (ozone) (Langebartels *et al.*, 2002), and heavy metals (Schutzendubel and Polle, 2002). The yield losses associated with abiotic stresses can reach 50% to 82%, depending on the crop (Bray *et al.*, 2000).

Application of plant growth-promoting rhizobacteria (PGPR) increases plant health overall. Precisely how the interaction of plants and PGPR affect plant

physiology and metabolism is unclear. There is much research concerning the effect of PGPR on a plant's resistance or tolerance to different plant pathogens (Bloembergen and Lugtenberg, 2001). Bashan and Holguin (1995) reported that the hormonal effects are the main mechanism in plant growth promotion by *Azospirillum* and so the effects of *Azospirillum* are comparable with the effect of exogenous application of growth hormones GA and NAA.

Plant growth promoting rhizobacteria (PGPR) can promote plant growth indirectly by the reduction or prevention of the action of plant pathogens or directly *via* phosphorus solubilization, nitrogen fixation, iron sequestration by siderophores, phytohormone production (e.g. auxin, cytokinin, or gibberellin) and enzymatic lowering of plant ethylene levels (Glick, 1995; Sivasakthi *et al.*, 2013). Particularly, PGPR containing 1-

aminocyclopropane- l-carboxylic acid (ACC) deaminase were considered to effectively facilitate the growth of variety of plants, especially under stressful conditions such as flooding, heavy metals, phytopathogens, drought and high salt (Grichko and Glick, 2001; Belimov *et al.*, 2005). Ethylene is an important phytohormone, but over-produced ethylene under stressful conditions can result in the inhibition of plant growth or death, especially for seedlings (Ghosh *et al.*, 2004). PGPR containing ACC deaminase can hydrolyze ACC, the immediate precursor of ethylene, to F-ketobuturate and ammonia, and in this way promote plant growth (Glick *et al.*, 1998).

Soils, especially cultivated soils, worldwide are becoming more saline from marginal irrigation water, excessive fertilization, and desertification processes. Impacted soils are a major limiting production factor worldwide for every major crop (Saranraj *et al.*, 2013). Aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme capable of hydrolyzing ACC, the immediate precursor of ethylene was firstly reported in 1978, which was isolated from *Pseudomonas* sp. strain ACP (Honma and Shimomura, 1978). Inoculation of crops with ACC deaminase containing PGPR may assist plant growth by alleviating deleterious effects of salt stress ethylene. Relative to the tolerance of PGPR to saline conditions, none of the most important crops tolerates high levels of salts, and NaCl is the most destructive salt affecting plant growth (Penrose and Glick, 2003).

Materials and Methods

Effect of NaCl on the accumulation of proline content by selected PGPR stains

The Selected *Azospirillum*, *Bacillus* and *Pseudomonas* strains were grown in 100 ml of Yeast extract glucose broth with different concentrations of NaCl *viz.*, 0.0%, 1.0%, 2.0%, 3.0% and 4.0% in 250 ml Erlenmeyer flasks. A quantity of 2 ml of the culture was mixed thoroughly with 5 ml of 3 per cent aqueous sulpho salicylic acid. The solution was filtered through Whatman No.2 filter paper. A quantity of 2 ml of the filtrate was reacted with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid for 1 hour at 100°C and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml of toluene, mixed vigorously in a test tube and stirred for 15 to 20 sec. The toluene phase was aspirated from the aqueous phase warmed to room temperature and the intensity of pink colour was determined at 420 nm in a spectrophotometer 1001 using toluene as blank. The proline concentration was determined using pure proline standards.

Effect of NaCl on ACC deaminase activity of selected PGPR strains

Hundred ml volumes of Yeast extract glucose broth supplemented with different concentrations of NaCl *viz.*, 0.0%, 1.0%, 2.0%, 3.0% and 4.0% and ACC at 3.0 mM concentration was taken into 250 ml Erlenmeyer flasks separately, and sterilized by autoclaving. Then, the flasks were individually and separately inoculated with 1 ml (1×10^7 CFU/ml) 48 hrs old culture of selected *Azospirillum*, *Bacillus* and *Pseudomonas*, respectively. After the inoculation, the flasks were incubated for 18 hrs at $30 \pm 2^\circ\text{C}$ under static condition. After the incubation period, the cells were collected by centrifugation at $5000 \times g$ for 10 min, resuspended in 0.1M Tris-HCl and ruptured by three freeze - thaw cycles, each consisting of immersion in liquid nitrogen for 1min and in a 25°C water bath for 5 min. The lysate was centrifuged at $10,000 \times g$ for 1 hour and the supernatant was assayed for ACC-deaminase activity. ACC-deaminase activity was quantified by monitoring the amount of - ketobutyrate that was produced by the deamination of ACC and measured following the derivitization of - ketobutyrate with 2,4-dinitrophenylhydrazine as described by Honma and Shimomura (1978).

Results and Discussion

Microbial inoculation enhanced proline accumulation in the roots and provides tolerance to plants under salinity stress. The higher proline contents in roots might be due to the fact that roots are the primary sites of water absorption and must maintain the osmotic balance between the water absorbing cells and external media as reported by Sharifi *et al.* (2007). Proline protects membranes and proteins against the adverse effects of high concentration of inorganic ions and temperature extremes. It also functions as a protein-compatible hydrotope, and as a hydroxyl radical scavenger (Smirnof and Cumbes, 1989).

The proline production of *Azospirillum brasilense* on various concentrations of NaCl was studied in the present research. The maximum production was observed at 4.0% NaCl concentration and the isolates are PA-17 (270.56 mg g^{-1}), PA-16 (232.10 mg g^{-1}), PA-18 (210.20 mg g^{-1}) and PA-1 (175.65 mg g^{-1}). Minimum production was found in *Azospirillum brasilense* PA-14 (141.90 mg g^{-1}). The various concentrations of NaCl and proline production of *Bacillus subtilis* were tested. Among the five isolates, maximum production was recorded by the isolate *Bacillus subtilis* PB-15 (242.60 mg g^{-1}) at 4.0% NaCl concentration followed by PB-11 (215.36 mg g^{-1}), PB-17 (183.90 mg g^{-1}) and PB-5

(155.46 mg g⁻¹). Least production was observed in the isolate *Bacillus subtilis* PB-8 (131.90 mg g⁻¹). The *Pseudomonas fluorescens* isolates (PP-3, PP-9, PP-13, PP-16 and PP-19) were tested against various concentration of NaCl. Among the various concentrations, *Pseudomonas fluorescens* PP-16 recorded highest proline production at 4.0% NaCl of

228.00 mg g⁻¹ followed by PP-13 (210.78 mg g⁻¹), PP-19 (178.45 mg g⁻¹) and PP-3 (140.10 mg g⁻¹). The least production was recorded by *Pseudomonas fluorescens* PP-9 (118.15 mg g⁻¹). Next to 4.0% NaCl concentration maximum production was recorded at 3.0%, 2.0%, 1.0% and 0.0% (Table – 1).

Table - 1: Effect of NaCl on the accumulation of proline content by PGPR strains

PGPR isolates	Isolate number	Proline content (mg g ⁻¹ of dry weight of cells)				
		0.0%	1.0%	2.0%	3.0%	4.0%
<i>Azospirillum brasilense</i>	PA-1	110.60	132.10	150.20	164.75	175.65
	PA-14	61.36	86.70	110.62	125.40	141.90
	PA-16	135.20	150.36	188.70	215.56	232.10
	PA-17	225.50	238.10	253.18	265.32	270.56
	PA-18	123.30	139.12	180.30	202.10	210.20
SEd		2.671	2.470	2.353	2.363	2.224
CD(p=0.05)		5.343	4.941	4.707	4.726	4.449
<i>Bacillus subtilis</i>	PB-5	88.56	102.79	115.79	138.88	155.46
	PB-8	61.72	75.70	92.20	110.74	131.90
	PB-11	133.49	150.82	175.65	193.30	215.36
	PB-15	165.80	188.06	206.12	225.60	242.62
	PB-17	100.74	121.50	142.98	160.22	183.90
SEd		1.805	1.941	2.038	2.014	1.991
CD(p=0.05)		3.610	3.883	4.078	4.028	3.983
<i>Pseudomonas fluorescens</i>	PP-3	80.30	99.71	112.30	128.25	140.10
	PP-9	71.56	83.20	94.40	103.34	118.15
	PP-13	112.75	132.78	150.46	188.20	210.78
	PP-16	120.85	145.79	166.20	195.56	228.00
	PP-19	104.65	120.50	138.88	166.20	178.45
SEd		0.947	1.124	1.295	1.767	2.067
CD(p=0.05)		1.895	2.249	2.590	3.535	4.135

Chen *et al.* (2007) correlated proline accumulation with drought and salt tolerance in plants. Introduction of proBA genes derived from *Bacillus subtilis* into *A. thaliana* resulted in production of higher levels of free proline resulting in increased tolerance to osmotic stress in the transgenic plants. Increased production of proline along with decreased electrolyte leakage, maintenance of relative water content of leaves and selective uptake of potassium ions resulted in salt tolerance in *Zea mays* co-inoculated with *Rhizobium* and *Pseudomonas* (Bano and Fatima, 2009; Usharani *et al.*, 2014).

In the present study, ACC deaminase activity by saline tolerant PGPR strains (*Azospirillum*, *Bacillus* and *Pseudomonas*) was studied at various NaCl concentrations (0.0%, 1.0%, 2.0%, 3.0% and 4.0%). At various concentrations of NaCl, the ACC deaminase activity *Azospirillum brasilense* was tested. Among the five isolates, maximum production was recorded by the isolate PA-17 (6.80 μ mol -ketobutyrate mg⁻¹

protein h⁻¹) at 4.0% NaCl concentration and least production was observed in the isolate *Azospirillum brasilense* PA-14 (5.90 μ mol -ketobutyrate mg⁻¹ protein h⁻¹) (Table – 2).

The ACC deaminase activity of *Bacillus subtilis* on various concentrations of NaCl was studied. The maximum production was observed at 4.0% NaCl concentration and the isolates are *Bacillus subtilis* PB-15 (5.12 μ mol -ketobutyrate mg⁻¹ protein h⁻¹), PB-11 (5.03 μ mol -ketobutyrate mg⁻¹ protein h⁻¹), PB-17 (4.95 μ mol -ketobutyrate mg⁻¹ protein h⁻¹) and PB-5 (4.50 μ mol -ketobutyrate mg⁻¹ protein h⁻¹). Minimum production was found in *Bacillus subtilis* PB-117 (4.95 μ mol -ketobutyrate mg⁻¹ protein h⁻¹). The *Pseudomonas fluorescens* isolates were tested against various concentration of NaCl. Among various concentrations, *Pseudomonas fluorescens* PP-16 recorded highest ACC deaminase activity at 3.0% NaCl of 5.05 μ mol -ketobutyrate mg⁻¹ protein h⁻¹ and the least production was recorded at

Table – 2: Effect of NaCl on ACC deaminase activity of PGPR strains

PGPR isolates	Isolate number	ACC deaminase activity mol -ketobutyrate mg ⁻¹ protein h ⁻¹) (μ				
		0.0%	1.0%	2.0%	3.0%	4.0%
<i>Azospirillum brasilense</i>	PA-1	5.02	5.36	5.53	5.82	6.10
	PA-14	4.75	5.05	5.31	5.63	5.90
	PA-16	5.38	5.72	6.08	6.27	6.55
	PA-17	5.66	5.95	6.28	6.53	6.80
	PA-18	5.20	5.50	5.82	6.08	6.32
SEd		0.015	0.015	0.017	0.015	0.016
CD (P = 0.05)		0.030	0.031	0.035	0.031	0.033
<i>Bacillus subtilis</i>	PB-5	3.33	3.65	3.96	4.25	4.50
	PB-8	3.18	3.42	3.75	4.03	4.22
	PB-11	3.87	4.12	4.48	4.72	5.03
	PB-15	3.95	4.26	4.54	4.86	5.12
	PB-17	3.76	4.05	4.33	4.60	4.95
SEd		0.015	0.016	0.015	0.015	0.017
CD (P = 0.05)		0.031	0.032	0.030	0.031	0.035
<i>Pseudomonas fluorescens</i>	PP-3	3.33	3.30	3.82	4.07	4.38
	PP-9	2.82	3.04	3.32	3.76	4.02
	PP-13	3.70	4.03	4.22	4.56	4.80
	PP-16	3.79	4.10	4.39	4.70	5.05
	PP-19	3.62	3.85	4.04	4.33	4.68
SEd		0.017	0.020	0.018	0.016	0.017
CD (P = 0.05)		0.035	0.041	0.037	0.032	0.034

Pseudomonas fluorescens PP-9 (4.02 μ mol -ketobutyrate mg⁻¹ protein h⁻¹). Next to 4.0% NaCl concentration, minimum activity was recorded at 3.0%, 2.0%, 1.0% and 0.0%.

The results of the present study was in line with the studies of Mayak *et al.* (2004); Kausar and Shahzad (2006) in which the effectiveness of rhizobacteria containing ACC deaminase for plant growth promotion under salinity stress was recorded. The results also coincided with the studies on effects of ACC deaminase in promoting plant growth cultivated under axenic conditions (Arif *et al.*, 2010) and in drying soil (Belimov *et al.*, 2008), flooded soil (Grichko and Glick, 2001), and normal soil (Ghosh *et al.*, 2003; Dey *et al.*, 2004; Shaharoona *et al.*, 2006).

Several strains of PGPR that produce ACC deaminase have been shown to increase root elongation, improve seedling survival, and enhance stress tolerance (Grichko and Glick, 2001). Glick *et al.* (1998) proposed that root elongation results when ACC deaminase produced by such strains break down ethylene, which inhibits root elongation. Our results with *Pseudomonas* strain 89B-61 support this model, as this strain

produced ACC deaminase. However, none of the *Bacillus* strains produced ACC deaminase. Therefore, some unknown mechanism that affects root morphology is operable with these strains.

Under salinity stress, ethylene is produced at higher concentration and this higher concentration of ethylene is inhibitory to plant growth. It influences various phases of vegetative growth in plants resulting in overall reduced growth (Smalle and Van der Straeten, 1997). In many instances, removing or blocking the effect of stress ethylene results in alleviation of the stress effect. It is very likely that the PGPR strains promoted root growth by lowering the endogenous inhibitory levels of ethylene in roots because of their ACC metabolizing ability. This may imply that the inoculation with rhizobacteria could result in the development of much better root system, which subsequently affects shoot growth positively.

Shaharoona *et al.* (2006) reported a significantly positive correlation between ACC deaminase activity and root elongation in maize due to inoculation with rhizobacteria containing ACC deaminase activity under axenic conditions.

The data in this study revealed that inoculation with rhizobacterial strain S20 proved to be the most effective at all salinity levels followed by S5, which increased root growth and other parameters even at high salt stress i.e. 12 dS m⁻¹. This may be attributed to its intensive root colonization ability and ACC-deaminase activity compared to other strains which made it more competitive under stress conditions. Similar findings were also obtained by Shaharoon *et al.* (2006) where strain having good root colonization ability showed more promising results than others. Upadhyay *et al.* (2009) found that PGPR loose PGP traits with increasing salinity *in vitro*. Thus, the selection and use of ACC deaminase-producing halotolerant bacteria based on both high salt tolerance and efficiency in producing plant growth promoting compounds is potentially of enormous impact in facilitating the growth of crops in a wide range of saline environments.

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