Recent Research in Science and Technology 2011, 3(11): 61-70
ISSN: 2076-5061
www.scholarjournals.org

www.recent-science.com

RRST-Biochemistry

Plant Growth Promotion and Amelioration of Salinity Stress in Crop Plants by a Salt-Tolerant Bacterium

Usha Chakraborty[1*], Swarnendu Roy[1], Arka Pratim Chakraborty[1], Pannalal Dey[2] and Bishwanath Chakraborty[2]

[1]*Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Siliguri- 734013, West Bengal, India*
[2]*Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, Siliguri- 734013, West Bengal, India*

Article Info

Article History

Received : 27-06-2011
Revised : 25-08-2011
Accepted : 04-09-2011

*Corresponding Author

Tel : +91-3532776337
Fax : +91-3532699001

Email: chakrabortyusha@hotmail.com

Abstract

Out of a large number of bacteria isolated from the rhizosphere of *Cynodon dactylon*, a facultative halophyte, nine were found to be highly salt-tolerant, growing in nutrient agar medium supplemented with 10% NaCl. Morphological, biochemical, and molecular characterization of all the isolates was done. RAPD analysis to determine relatedness among the nine bacteria were carried out, which revealed that all nine isolates could be grouped into 2 main clusters. In order to determine whether the isolates possess plant growth promoting activities, *in vitro* tests were done initially. Based on the *in vitro* tests the isolate 'S4' was selected as a potential salt-tolerant PGPR. This bacterium was identified as *Bacillus cereus.* For *in vivo* tests, this bacterium was applied as an aqueous suspension to the rhizosphere of 3 plants- *Vigna radiata, Cicer arietinum and Oryza sativa;* a set of each plant was also applied with 200mM NaCl solution 3 times a week after 15 days of growth. Results revealed that the bacterium could promote growth of the seedlings significantly which showed increased height, number and length of leaves, as well as root and shoot biomass. The ability of the bacterium to solubilize soil phosphate was also confirmed. *B.cereus* could also elicit antioxidant responses against salt stress in the plants, as evidenced by increased activities of enzymes such as superoxide dismutase, peroxidase, ascorbate peroxidase and catalase. Besides, it could also induce the activities of defense enzymes such as chitinase, β-1, 3 glucanse and phenyl alanine ammonia lyase associated with induced systemic resistance. Results imply that the bacterium acts through a combination of direct and indirect mechanisms.

Key Words: *Vigna radiata*, *Cicer arietinum*, *Oryza sativa*, salt-stress, PGPR

Introduction

Salinity is one of the most critical constraints which hamper agricultural productions in many areas worldwide. Globally, some 20% of irrigated land (450,000 km^2) is salt-affected, with 2,500–5,000 km^2 of production lost every year as a result of salinity [1]. Therefore, with increasing population and rise in food demand, the utilization of salt-affected soils for agriculture will become necessary to feed the growing population of the world. For this, evolving low cost, easily adaptable technologies become essential and are now considered as a major challenge. Extensive research is going on worldwide to develop strategies for coping with abiotic stresses through development of tolerant varieties, shifting the crop calendars, production of transgenics etc [2].

One of the recently gaining practices of counteracting the adverse effects of salinity on plant growth includes the implementation of salt-tolerant bacteria with natural growth promoting ability in such conditions. Plant growth promoting rhizobacteria, first defined by Kloepper and Schroth [3] include those bacteria, which, on inoculation into the soil, colonize the roots of plants and enhance plant growth. 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 [4]. In comparison to reports of inducing systemic resistance in plants by PGPR, fewer reports have been published on PGPR as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess [4].

Certain soil bacteria can help the plants to avoid or partially overcome a variety of environmental stresses. Yildirim *et al.* [5] reported the mitigation of salt stress in *Raphanus sativus* by plant growth promoting rhizobacteria like *Staphylococcus kloosii* and *Kocuria erythromyxa.* PGPR-elicited induced systemic tolerance (IST) aid the growth of plants under abiotic stresses by producing various antioxidants, which result in the degradation of reactive oxygen species (ROS) [6]. Production of IAA or unknown determinants can increase root length, root surface area and the number of root tips, leading to enhanced uptake of nitrate and phosphorous [7, 8]. PGPRs have also been reported to protect plants from various pathogens by activating defense genes, for example those encoding chitinase, β-1, 3-glucanase (GLU), peroxidase (POX), phenylalanine ammonia lyase (PAL) etc. [9].

The present study aims to isolate salt-tolerant bacteria from the rhizosphere of a facultative halophyte, characterize them by morphological, biochemical and molecular means; and to determine whether any of the isolated bacteria possess PGPR activities. Besides, attempts have been made to determine whether the bacterium can induce plant growth promotion and salinity tolerance *in vivo* and finally, the biochemical mechanisms of induced systemic tolerance have been worked out.

Materials and Methods

Collection of soil samples

Soil samples were collected from the rhizosphere of *Cynodon dactylon* from two different locations of foot hills of Darjeeling [GIS locations – (i) 26^0 42' 35.94"N & $88^0$21' 6.18" E; (ii) 26^0 42' 32.00"N & $88^0$21' 2.60" E].

Isolation of bacterial strains

Soil from rhizosphere of *C. dactylon* (1 g) was mixed well with 25ml of sterile distilled water and plated. For isolation of salt-tolerant bacteria, nutrient agar (NA) supplemented with 10% NaCl was used as a selective medium. After the appearance of colonies, isolated colonies were picked up with sterilized loop, transferred to fresh NA slants with 10% NaCl and the pure cultures so obtained were stored in refrigerator at 4^oC. Subsequent sub-culturing was then made in NA and Nutrient Broth media for morphological, biochemical and molecular analyses. Mannitol Salt Agar (MSA) containing 7.5% NaCl [10] was used to confirm the salt tolerance ability of the isolates. Development of halo region due to the fermentation of mannitol was a positive test for salt tolerance.

Morphological studies

Pure cultures of the isolates were streaked up on NA plates with 10% NaCl. The individual colonies were then examined for shape, size, structure of colonies and pigmentation. The Gram reactions of all the isolates were recorded [11]. Scanning electron microscopy (SEM) was also done to study the morphological diversity of the isolates.

Biochemical studies

Biochemical tests viz, starch hydrolysis, catalase, indole production, urease, gelatinase, protease, citrate, nitrate reduction, porphyrin reaction were carried out for biochemical characterization of these isolates.

Molecular variability among the isolates

Genomic DNA extraction

The broth cultures of bacterial isolates were centrifuged at 10,000 rpm at 28^0C for 5 min and the pellets were collected by discarding the supernatant. The pellets were washed thrice with distilled water and resuspended in 0.5ml of lysis solution (100mM Tris HCl, pH 7.5, 20mM EDTA, 250mM NaCl, 2% SDS, 1mg/ml lysozyme). To it 5 µl of RNase (50mg/ml) was added and incubated at 37^0 C for 3 hrs. Then 10 µl proteinase K solution (20mg/ml) was added and it was incubated at 65^0C for 3min. The lysate was extracted with equal volume of Tris and water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 vol of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 min at 4^0C, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4^0C.

Quantification of genomic DNA by gel electrophoresis

The quality of the genomic DNA, isolated from nine different isolates was checked on 0.8% agarose gel electrophoresis. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA. Quantification was done by taking absorbance at 260 & 280 nm in a Coleparmer UV-VIS Spectrophotometer.

PCR amplification

All isolates were taken up for RAPD-PCR amplification. Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100µl, containing 78µl deionized water, 10µl 10X taq polymerase buffer, 1µl of 1U Taq polymerase enzyme, 6µl 2mM dNTPs, 1.5µl of 100mM RAPD primers and 1µl of template DNA. Two random decamers (OPA1- CAGGCCCTTC and OPA4- AATCGGGCTG) were used to determine the RAPD profiles of the isolates. PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. After RAPD-PCR amplifications, all amplified DNA products were resolved by electrophoresis on agarose gel (2%) in TAE (1X) buffer, stained with ethidium bromide and photographed. After that, all reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed in to similarity matrix using NTSYS computer program to prepare a dendrogram.

In vitro tests for the identification of PGPR strains

Phosphate solubilization

Phosphate solubilization by the isolates was checked in Pikovskaya's agar [12].The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

Siderophore production

Production of siderophore was detected by standard method [13] using chrome azurol S (CAS) as indicator. The isolates were spot inoculated at the center of the plate and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot was an indication of siderophore production.

Chitinase production

Production of chitinase was detected by standard method [14]. Colonies showing zones of clearance against the creamy background were regarded as chitinase producing strains.

IAA production

Production of IAA in culture supernatant was assayed by Pillet-Chollet method [15]. For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M H_2SO_4 was added to

1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

Protease production

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates [16].

Selection and identification of the bacterial strain for in vivo plant growth promotion

Selection of the bacterial isolate (S4) was done based on *in vitro* tests for plant growth promotion activity. Identification of the bacterium was done by microscopic and biochemical tests, which was later confirmed by Institute of Microbial Technology, Chandigarh.

Determination of plant growth promoting activity

Aqueous suspension of S4 (10^8 CFU ml^{-1}) was applied as a soil drench to the rhizosphere of *Vigna radiata, Cicer arietinum* and *Oryza sativa.* Plants were grown in natural conditions of light and temperature. For each of the crop, four sets of plants with ten replicate pots were taken- *viz.* control, control+S4, control + NaCl and control+ NaCl + S4. Salinity was imparted to the plants by adding 200mM NaCl solution to the rhizosphere of the plants after 15 days of growth, 3 times a week. The bacterium was also applied after 15 days, once a week.

Extraction and quantification of soil, root and leaf phosphate

Soil sample (1 g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01 g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper [17]. In case of plant leaf and root samples, oven-dried plant material was crushed with extracting solution. Quantitative estimation of phosphate was carried out following ammonium molybdate-ascorbic acid method as described [18].

Biochemical analyses

All biochemical analyses were performed from leaves of the 4 sets of plants after 45 days of growth. Samplings were done within 72 h from the last application of the bacterium.

Defense enzymes

Chitinase

Extraction of chitinase (CHT - EC.3.2.1.14) from leaf samples and assay of its activity was done according to the method described [19] with some modifications. N-acetyl glucosamine (GlcNAc) was used as standard [20]. The enzyme activity was expressed in terms of µg GlcNAc released min^{-1} g^{-1} fresh tissue.

β-1,3-Glucanase

Extraction of β-1, 3-glucanase (GLU- EC.3.2.1.39) from leaf samples and assay of its activity was done following the method described [21]. Laminarin was used as substrate and the enzyme activity was expressed in terms of µg glucose released min^{-1} g^{-1} fresh weight of tissue.

Phenylalanine ammonia lyase

Extraction of phenylalanine ammonia lyase (PAL-EC.4.3.1.5) from leaf samples and assay of its activity was done following the method described earlier [22] with some modifications. Enzyme activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The enzyme activity was expressed in terms of µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissue.

Antioxidative enzymes

Peroxidase

Peroxidase (POX- EC 1.11.1.7) was extracted from leaf samples and assayed according to the method described [22] with slight modifications. O-dianisidine was used as a substrate and the enzyme activity was expressed as ΔA_{465} min^{-1} g^{-1} fresh tissue.

Catalase

Catalase (CAT- EC 1.11.1.6) was extracted from leaf samples and assayed according to the method described [23]. The enzyme activity was expressed as ΔA_{240} min^{-1} g^{-1} fresh tissue.

Ascorbate peroxidase

Ascorbate peroxidase (APOX - EC 1.11.1.11) was extracted from leaf samples and assayed [24]. The enzyme activity was expressed as ΔA_{290} min^{-1} g^{-1} fresh tissue.

Superoxide dismutase

Superoxide dismutase (SOD- EC 1.15.1.1) was extracted from leaf samples and assayed [25]. The enzyme activity was expressed as Enzyme Unit (EU) mg^{-1} protein.

Results

Selection of potential isolate and its characterization

Rhizosphere of *Cynodon dactylon*, a facultative halophyte, yielded a large number of bacteria, of which nine were found to be tolerant to NaCl at a concentration as high as 10%. Out of nine isolates, three (S3,S4&S5) were Gram-positive whereas rest six were Gram-negative. All the isolates produced catalase as well as reduced nitrate to nitrite, but showed negative results in urease tests. Only four isolates (S4,N3,N4&N2) could hydrolyse starch, five isolates (S2,S5,N2,N3&N5) liquefied gelatin while all had the ability to produce indole (Table 1). *In vitro* tests such as siderophore production, phosphate solubilization, IAA production, chitinase production and protease production for detection of PGPR activities were carried out. Among all the bacterial isolates, S4 showed positive results in all tests (Table 2, Fig 1A). S4 was found to be Gram-positive and rod shaped. Scanning electron microscopy of S4 was also done (Fig 1 B). The bacterium was identified as *Bacillus cereus* which was further confirmed (No. MTCC 10655) by Microbial Technology Institute, Chandigarh, India and was used for *in vivo* tests.

Molecular diversity analysis

RAPD analysis by two random primers OPA-1 and OPA-4 revealed genetic relatedness among the nine salt-tolerant bacterial isolates. Dendrogram revealed that similarity co-

efficient ranged from 0.65-1.00 (Fig.2). Based on the results obtained all nine isolates can be grouped into two main clusters. One cluster represents S1 and S3 isolates, while the second one represents all the other seven isolates; this is further sub grouped into four- first subgroup with S2, S5 and N5 isolates, second with S4 and N4, third and fourth with N2 and N3 respectively.

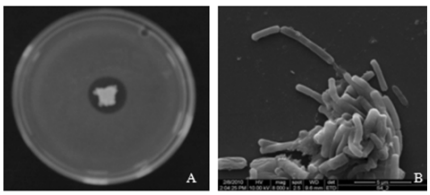

Fig. 1 A- Phosphate solubilization by *Bacillus cereus* in PVK medium ; B- Scanning electron microscopic view of *B.cereus*

Table 1. Morphological and Biochemical tests of nine bacterial isolates

Morphological and Biochemical tests	Bacterial isolates								
	S1	S2	S3	S4	S5	N2	N3	N4	N5
Gram reaction	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Cell shape	Rod with tapering end	Rod	Coccus in chain	Rod	Coccus in chain	Rod	Rod	Rod	Short rod
Catalase	++	+++	+	+	+++	+	+	+	+++
Nitrate Reduction	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	+	-	+	+	+	-
Gelatin liquefication	-	+	-	-	+	+	+	-	+
Indole production	+	+	+	+	+	+	+	+	+
Citrate	-	+	-	+	+	-	+	+	+
Porphyrin	-	-	-	++	-	-	++	-	++

Table 2 . *In vitro* tests of the nine bacterial isolates for determination of PGPR activity

In vitro PGPR tests	Response of bacterial isolates								
	S 1	S2	S3	S4	S5	N2	N3	N4	N5
IAA production	-	-	+	+	-	+	-	+	+
Phosphate solubilization	-	-	-	+	-	-	-	-	-
Protease production	-	-	+	+	+	+	+	+	-
Chitinase production	-	+	-	+	-	+	+	+	-
Siderophore production	-	-	-	+	-	+	-	+	-

+=Positive; - = Negative

Effect of bacterium on growth of ***Cicer arietinum***, ***Vigna radiata*** and ***Oryza sativa***

Results of *in vivo* tests revealed that application of *B. cereus* significantly increased growth of all three crops in terms of height of plants, number of leaves, length of leaves and root and shoot dry mass. In case of plants subjected to PGPR treatment alone or with NaCl treatment in addition to PGPR, all growth parameters were high (Fig 3; Tables 3 and 4).

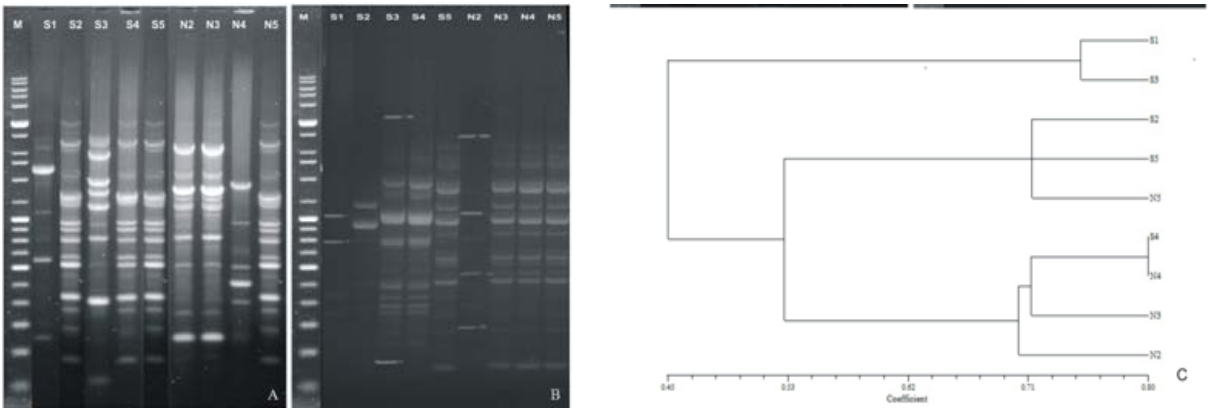

Fig. 2 RAPD profiles of nine isolates using OPA-4 (A) and OPA-1 (B) universal primers and dendrogram of salt tolerant isolates created by UPGMA method (C).

Table 3. Effect of application of NaCl (200 mM) and *Bacillus cereus* on growth of *V.radiata, C. arietinum* and *O. sativa*

Average of 10 replicates; ±=SE; Differences between control and treated significant at P=0.01(b) or P==0.05(c) (Student's t-test) where superscript of control and treated are different; where superscript same (a), difference insignificant.

Test plants	Treatments	Avg. height/plant (cm)	Avg. no of leaves/plant	Avg. leaf length / plant (cm)	Avg. root length/plant (cm)
V. radiata	Control	15.5± 0.90^{a}	21±1.20^{a}	02.8± 0.04^{a}	03.2±0.03^{a}
	NaCl	14.7±0.80^{a}	20± 0.97^{a}	02.7± 0.04^{a}	04.7± 0.04^{b}
	B. cereus	19.5 ±1.02^{a}	35±1.16^{b}	03.2± 0.01^{b}	07.7± 0.02^{b}
	NaCl+*B. cereus*	20.0 ±0.7^{c}	37±1.24^{b}	04.2±0.03^{b}	05.5± 0.01^{b}
C. arietinum	Control	07.0±0.06^{a}	42±1.98^{a}	0.80±0.05^{a}	01.2± 0.01^{a}
	NaCl	07.1±0.05^{a}	42±2.08^{a}	0.70±0.03^{a}	03.5± 0.02^{b}
	B. cereus	14.0±0.86^{b}	52±1.54^{c}	01.0±0.01^{c}	04.0± 0.03^{b}
	NaCl+*B. cereus*	15.2± 0.93^{b}	55±2.32^{c}	01.1±0.01^{b}	03.7± 0.02^{a}
O. sativa	Control	15.0± 0.54^{a}	09±0.92^{a}	07.5±0.02^{a}	07.0±0.03^{a}
	NaCl	14.4±0.66^{a}	08±0.89^{a}	06.8±0.03^{b}	07.7±0.05^{b}
	B. cereus	26.0±1.08^{b}	12±0.65^{a}	15.0±0.01^{b}	11.5±0.09^{b}
	NaCl+*B. cereus*	23.5±0.96^{b}	11±0.97^{a}	16.0±0.08^{b}	08.0±0.04^{b}

Table 4. Effect of application of NaCl (200 mM) and *Bacillus cereus* on root and shoot dry wts. (g) of *V. radiata, C. arietinum* and *O. sativa*
Average of 10 replicates; ±=SE; Differences between control and treated significant at P=0.01(b) or P==0.05(c) (Student's t-test) where superscript of control and treated are different; where superscript same (a), difference insignificant.

Test plants	Treatments	Root dry wt./plant(g)	Leaf and shoot dry wt./plant (g)
V. radiata	Control	0.42± 0.02^{a}	1.32±0.02^{a}
	NaCl	0.32± 0.03^{a}	1.24± 0.03^{a}
	B. cereus	0.54± 0.02^{c}	2.63± 0.03^{b}
	NaCl+*B. cereus*	0.86±0.04^{b}	4.79± 0.04^{b}
C. arietinum	Control	1.28±0.05^{a}	2.79± 0.02^{a}
	NaCl	0.79±0.02^{b}	2.17± 0.03^{a}
	B. cereus	1.58±0.01^{b}	5.68± 0.04^{b}
	NaCl+*B. cereus*	1.47±0.02^{b}	6.40± 0.05^{b}
O. sativa	Control	1.84±0.03^{a}	3.06±0.03^{a}
	NaCl	2.32±0.02^{b}	2.86±0.02^{c}
	B. cereus	2.94±0.02^{b}	4.18±0.05^{b}
	NaCl+*B. cereus*	3.24±0.07^{b}	8.48±0.06^{b}

Fig. 3 Effect of application of *Bacillus cereus* and NaCl on growth of *Vigna radiata* (A) and *Cicer arietinum* (B).

Phosphate solubilization

Root and shoot phosphate contents showed increase following application of *B. cereus*. Application of NaCl also increased root and shoot phosphate contents, with simultaneous decrease in total soil phosphate contents (Table 5).

Table 5. Phosphate contents of rhizosphere soil, root and leaves of *V. radiata, C. arietinum* and *O. sativa* following different treatments Average of 10 replicates; ±=SE; Differences between control and treated significant at P=0.01$^{(b)}$ or P==0.05$^{(c)}$ (Student's t-test) where superscript of control and treated are different; where superscript same $^{(a)}$, difference insignificant.

Test plants	Treatments	Soil phosphate (µg g soil^{-1})	Leaf phosphate (µg gtissue^{-1})	Root phosphate (µg gtissue^{-1})
V. radiata	Control	56±1.15^{a}	80±2.30^{a}	95±1.15^{a}
	NaCl	45±0.87^{b}	101±1.80^{b}	135±0.57^{b}
	B. cereus	53±0.58^{a}	150±2.80^{b}	123±2.88^{b}
	NaCl+*B. cereus*	48±1.73^{c}	175±0.57^{b}	148±0.86^{b}
C. arietinum	Control	98±1.10^{a}	120±0.86^{a}	88±0.23^{a}
	NaCl	47±1.01^{b}	176±0.11^{b}	133±1.73^{b}
	B. cereus	80±1.30^{b}	150±0.80^{b}	135±0.17^{b}
	NaCl+*B. cereus*	55±2.31^{b}	185±0.57^{b}	155±1.15^{b}
O. sativa	Control	110±2.00^{a}	210±1.04^{a}	156±0.69^{a}
	NaCl	105±1.08^{a}	250±2.20^{b}	165±1.15^{b}
	B. cereus	111±2.00	246±3.60^{b}	159±1.73^{a}
	NaCl+*B. cereus*	90±1.00^{b}	295±2.30^{b}	177±2.30^{b}

Effect of NaCl and B. cereus on defense enzymes

Chitinase, β-1,3 glucanase and phenyl alanine ammonia lyase activities were enhanced by *B.cereus*. CHT and β-1,3 GLU activities showed a certain decline but in case of PAL, NaCl alone also enhanced activity in *C. arietinum* and *O. sativa*, but not in *V. radiata* (Fig.4).

Effect of NaCl and B. cereus on antioxidative enzymes and antioxidants

Activities of antioxidative enzymes- POX, SOD, CAT and APOX were assayed after the different applications. POX activities increased following application of *B. cereus* or *B. cereus*+NaCl. In case of NaCl treatment alone, in *V. radiata* and *C. arietinum* there was a decrease in activity in relation to control, but in *O. sativa* all three treatments led to an increase (Fig.5A). Similar results were also obtained for CAT activities where all treatments induced increased activities in both *C. arietinum* and *O. sativa*. However, in *V. radiata*, application of NaCl showed a decline in activity while *B. cereus* alone or in combination with NaCl enhanced activity (Fig 5B). APOX activities increased significantly in *O. sativa*, while superoxide dismutase was found to increase most significantly in *C. arietinum*. However, treatment of *B. cereus* + NaCl increased activities in all three test plants (Fig.6 A &B).

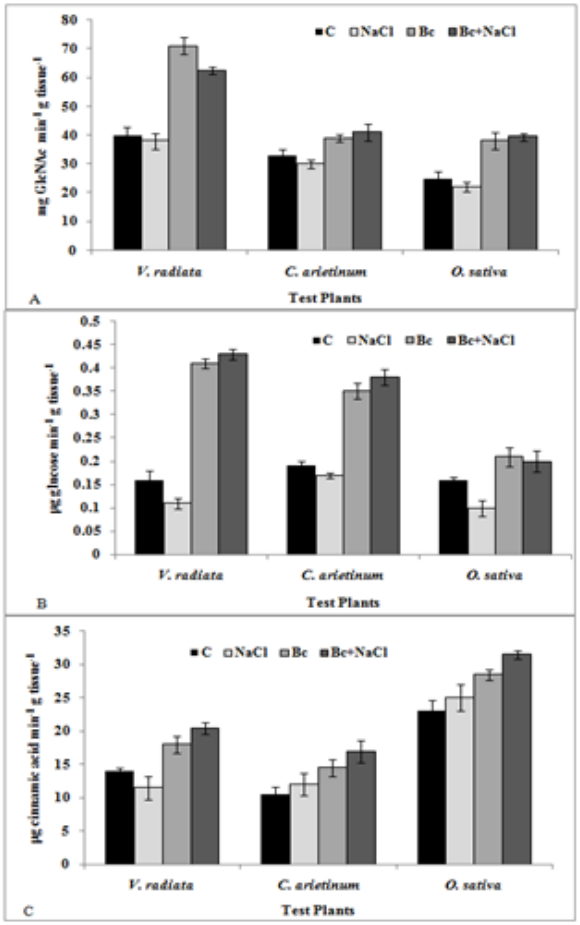

Fig. 4 Activities of chitinase (A), β-1,3 glucanase (B) , and phenyl alanine ammonia lyase (C) enzymes in *V.radiata, C.arietinum* and *O.sativa* following application of *B. cereus* and NaCl .

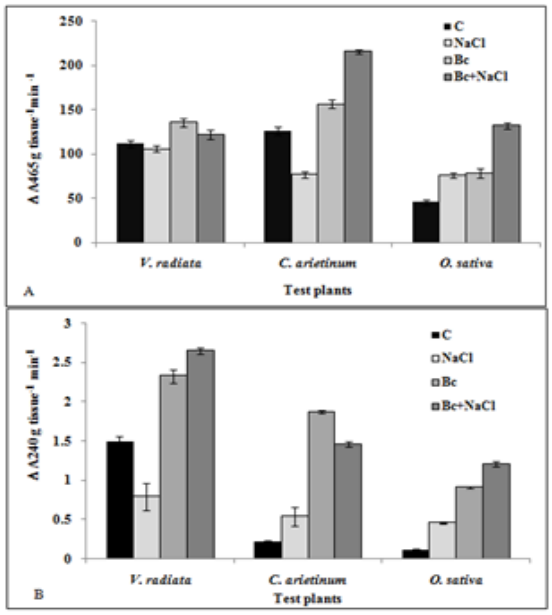

Fig.5 Changes in activities of peroxidase (A) and catalase (B) in *V.radiata, C.arietinum* and *O.sativa* following application of *B. cereus* and NaCl .

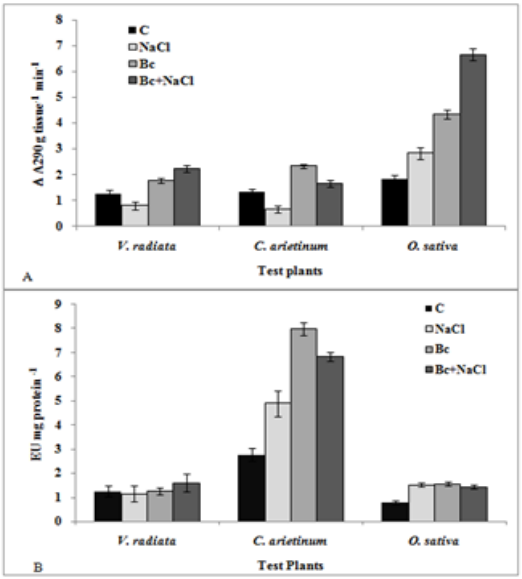

Fig.6 Activities of ascorbate peroxidase (A) and superoxide dismutase (B) in *V.radiata, C.arietinum* and *O.sativa* following application of *B. cereus* and NaCl .

Discussion

Out of the large number of bacteria isolated from the rhizosphere of *Cynodon dactylon*, nine were tolerant to high concentration of NaCl. I*n vitro* tests were carried out to determine whether these have any characteristics attributed to plant growth promoting rhizobacteria (PGPR) since it is quite probable that bacteria isolated from the rhizosphere of salt tolerant plants would have better ability to withstand saline conditions.

It has also been reported previously that bacteria isolated from such soil are more likely to withstand salinity conditions [26, 27]. On the other hand, if such bacteria also possess plant growth promoting traits they would be ideal for use in sustainable agriculture. Results of the present study revealed that one of the bacterium, identified as *Bacillus cereus* S4 exhibited all the *in vitro* PGPR characteristics. It has been shown that genera such as *Bacillus* and *Pseudomonas* tend to be pre-dominant in saline soils [28-31]. In the present study, RAPD analysis of the nine genera was done by using two random primers in order to determine their relationships. The nine genera formed two clusters and dendrogram revealed that *B.cereus* was most closely related to N4. It was previously reported that RAPD analysis of salt-tolerant bacterial isolates obtained from the rhizosphere of rice yielded four different patterns and the dendrogram constructed revealed strain specific differences [32].Besides, use of RAPD analysis for determining relationships among bacterial genera such as *Pseudomonas*, *Bacillus* and *Arthrobacter* has also been reported by a number of previous workers [27, 33].

B. cereus was further used for *in vivo* studies. It was observed that the adverse effect of salinity in terms of decrease in growth, number of leaves and shoot and root dry mass, could be overcome by treatment with the bacterium. *B.cereus* alone, or in combination with NaCl could increase growth significantly. There are also several previous reports on the induction of tolerance to salinity stress by PGPR [34-38]. *B. cereus* could solubilize phosphate and make absorption by the plant easier as evidenced by increased leaf and shoot phosphate contents, along with a decrease in soil total phosphate. An interesting aspect of the observed result was that, in comparison to control, better solubilaztion of phosphate was found to occur even in NaCl alone treated plants and much higher solubilization was recorded due to application of *B.cereus*. It is well known that one of the direct mechanisms by which PGPR promote growth is through solubilization of insoluble phosphates in the soil [39-41]. Besides, since PGPR are known to induce defense responses in the host, activities of three important defense enzymes - chitinase, □1, 3- glucanase and phenyl alanine ammonia lyase were determined. Activities of all the three enzymes were significantly enhanced by *B.cereus*. Increased activity was also evident in the treatment with *B.cereus* and NaCl. It is now known that inoculation with non-pathognic root zone bacteria can trigger signaling pathways that lead to higher resistance to biotic stresses. Some of the bacteria that have been used to study beneficial effects under abiotic stress conditions, such as *Bacillus* sp. have been shown to induce ISR [42-44]. Huang et al [45] reported that *B.cereus* C1L could induce ISR and control southern corn blight in maize. Though PGPR are more commonly known to induce resistance against pathogen infection, reports are now available on their ability to elicit 'induced systemic tolerance' against abiotic stresses.

In the present study, in order to determine whether *B. cereus* could elicit antioxidant activities in the plants, activities of four antioxidative enzymes- POX, APOX, CAT and SOD. It was quite clear that the bacterium could elicit antioxidant responses in the plants, though the particular enzyme which was up-regulated varied from plant to plant. In *V.radiata*, CAT was found to be induced maximum, whereas in *C. arietinum* it was APOX and SOD. On the other hand, in *O.sativa*, APOX seemed to be most over-expressed. It is evident that there is no single antioxidative enzyme responsible for inducing tolerance against salinity by *B. cereus* but more than one may be functional.

Our studies have established that *Bacillus cereus*, isolated from the rhizosphere of a facultative halophyte was not only salt-tolerant, but also had plant growth promoting attributes, which was first demonstrated *in vitro* and subsequently *in vivo*. While determining its mechanism of action, it was found to induce plant growth promotion as well as tolerance to salt-stress through an array of mechanisms, some of which such as phosphate solubilization and hormone secretion, were direct, whereas most of them were induced in the host. The use of such microorganisms which can induce tolerance to abiotic stresses in the host as biofertilizers may be a boon to agriculture since urbanization and industrialization are fast depleting our cultivable lands. Under such conditions, agriculture will gradually shift towards hitherto uncultivable areas such as coastal areas and waste lands and these microorganisms can contribute to sustainable agriculture under adverse conditions.

Acknowledgement

Financial assistance received from Council of Scientific and Industrial Research, New Delhi, India, is gratefully acknowledged.

References

[1] UNEP., 2009. The Environmental Food Crisis: The Environment's role in averting future food crisis.

[2] Venkateswarulu, B., A.K. Shanker. 2009. Climate change and agriculture: adaptation and mitigation strategies. Ind. J. Agron. 54: 226-230.

[3] Kloepper, J.W., M.N.Schroth. 1978. Plant growth promoting rhizobacteria on radishes. IV. International Conference on Plant Pathogenic Bacteria. Angers. France, 2*:* 879-882.

[4] Yang, J., J.W. Kloepper and C.M. Ryu. 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends. Plant. Sci*.* 14:1-4.

[5] Yildrim, E., M. Turan and M.F. Donmez. 2008. Mitigation of salt stress in radish (*Raphanus sativus*) by plant growth promoting rhizobacteria. Romanian. Biotechnol. Lett. 13 : 3933-3943.

[6] Figueredo, M.V.B., H.A Burity, C.R. Martinez, and C.P. Chanway. 2008. Alleviation of drought stress in common bean (*Phaseolus vulgaris* L.) by co-inoculation with *Paenibacillus polymyxa* and *Rhizobium tropici*. Appl. Soil. Ecol. 40:182-188.

[7] Mantelin, S., B.Touraine. 2004. Plant growth-promoting bacteria and nitrate availability impacts on root development and nitrate uptake. J. Exp. Bot. 55: 27-34.
[8] Gyaneshwar, P., G.N. Kumar, L.J. Parekh and P.S. Poole. 2002. Role of soil microorganisms in improving P nutrition of plants. Plant. Soil. 245: 83-93.
[9] M'Piga, P., R.R. Belanger, T.C. Paulitz and N. Benhamou 1997. Increased resistance to *Fusarium oxysporium* f.sp. *radicis- lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 6328. Physiol. Mol. Plant Pathol. 50: 301-320.
[10] Hitchens., T., McCarron. 1995. FDA bacteriological analytical manual, 8th ed. AOAC International, Gaithersburg, Md.
[11] Buchanan, R.E., N.E. Gibbson. 1974. Bergeys Manual of Determinative Bacteriology. 8th ed. Williams and Wilkins Co. Baltimore.
[12] Pikovskaya, R.I. 1948. Mobilization of phosphorous in soil connection with the vital activity of some microbial species. Microbiologiya. 17: 362-370.
[13] Schwyn, B., J.B. Neiland. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem.160: 47-56.
[14] Hsu, S.C., J.L. Lockwood. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl. Microbiol. 29: 422-426 .
[15] Dobbelaere, S., A. Croonenberghs, A. Thys, A. Vande Broek and J. Vanderleyden 1999. Photostimulatory effects of *Azospirillum brasilense* wild type and mutant strain altered in IAA production on wheat. Plant. Soil. 212: 155-164.
[16] Walsh, G.A., R.A. Murphy, G.F. Killen and DR.RF. Headon Power 1995. Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. J. Animal. Sci. 73: 1074-1076.
[17] Mehlich, A. 1984. Mehlich 3 soil test extractant: A modification of the Mehlich 2 extractant. Commun. Soil Sci. Plant Anal. 15: 1409-1416.
[18] Knudsen, D., D. Beegle. 1988. Recommended phosphorous tests. In: W.C. Dahnke (Ed), Recommended chemical soil tests procedures for the north central region. Bull North Dakota Agric Exp Stn. North Dakota, USA; No 499, pp 122-115.
[19] Boller, T., F.Mauch. 1988. Colorimetric assay for chitinase. Meth. Enzymol. 161: 430-435.
[20] Ressig, J.L., J.L. Strominger and L.F.Leloir.1959. A modified colorimetric method for the estimation of N-acetyl sugars. J. Biol. Chem. 217: 959-962.
[21] Pan, S.Q., X.S. Ye and J. Kuc.1991. A technique for detection of chitinase, β-1, 3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis and isoelectric focusing. Phytopathology. 81: 970- 974.
[22] Chakraborty, U., B.N. Chakraborty and M. Kapoor. 1993. Changes in the levels of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. Folia. Microbiol. 38: 491-496.
[23] Beers, R., I. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol.Chem. 195: 133.
[24] Asada, K. 1984. Chloroplasts: Formation of active oxygen and its scavenging. Methods. Enzymol. 105: 422-429.
[25] Dhindsa, R.S., P.L. Dhindsa and T.A.Thorpe.1981. Leaf senescence: Correlated with increased level of membrane permeability lipid peroxidation and decreased superoxide dismutase and catalase. J. Expt. Bot. 32: 93-101.
[26] Hua, S.S.T., V.Tsai, G.M. Lichens and A.T. Noma. 1982. Accumulation of aminoacids in *Rhizobium spp*. WR 10001 in response to sodium chloride salinity. Appl. Environ. Microbiol. 44: 135-140.
[27] Upadhyay, S.K., D.P. Singh and R. Saikia.2009. Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition. Curr. Microbiol. 59: 489-496.
[28] Egamberdiyeva, D., K.R. Islam. 2008. Salt-tolerant rhizobacteria: Plant growth promoting traits and physiological characterization within ecologically stressed environments. Plant Bact. Interact. 14: 257-280.
[29] Tank, N., M. Saraf. 2010. Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. J. Plant. Interact. 5 : 51-58.
[30] Tran, H., M. Kruijt and J.M. Raaiimakers. 2008. Diversity and activity of biosurfactant-producing *Pseudomonas* in the rhizosphere of black pepper in Vietnam. J. Appl. Microbiol. 104: 839-851.
[31] Zahran, H.H., M.S. Ahmed and E.A. Afkar. 1995. Isolation and characterization of nitrogen-fixing moderate halophilic bacteria from saline soils of Ezypt. J. Basic. Microbiol.35: 269-275.
[32] Tripathi, A.K., S.C. Verma and E.Z. Ron. 2002. Molecular characterization of a salt-tolerant bacterial community in the rice rhizosphere. Res. Microbiol. 153: 579-584.
[33] Kumar, N.R., V.T. Arasu and P. Gunashekaran. 2002. Genotyping of antifungal compounds producing plant growth promoting rhizobacteria *Pseudomonas fluorescens*. Curr. Sci. 82: 1463-1466.
[34] Ashraf, M., S. Hasnain, O. Berge and T. Mahmood. 2004. Inoculating wheat seedling with exopolysaccharide - producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. Biol & Fert Soils.40:157-162.
[35] Barasssi, C.A., G. Ayrault, C.M Creus, R.J. Sueldo and M.T. Sobrero. 2006. Seed inoculation with *Azospirillum* mitigates NaCl effects on lettuce. Sci. Hort. 109: 8-14.
[36] Dardanelli, M.S., F.J. Fernandez de Cordoba, M. Rosario Espuny, M.A. Rodriguez Carvajal, M.E. Soria Diaz, A.M. Gil Serrano, Y. Okon and M. Megias. 2008. Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress. Soil. Biol. Biochem. 40: 2713-2721.
[37] Nadeem, S.M., Z.A. Zahir, M. Naveed and M. Arshad. 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Can. J .Microbiol.53: 1141-1149.
[38] Saravanaskumar, D., R. Samiyappan. 2007. ACC deaminase from *Pseudomonas fluorescens* mediated

saline resistance in ground- nut (*Arachis hypogaea*) plants. J. Appl. Microbiol.102 :1283-1292.
[39] Bojinova, D., R. Velkova, I. Grancharov and S. Zhelev. 1997. The bioconversion of Tunisian phosphorite using *Aspergillus niger*. Nutr. Cyc. Agroecosyst. 47: 227-232.
[40] Chakraborty, U., B.N. Chakraborty and A.P.Chakraborty 2010. Influence of *Serratia marcescens* TRS-1 on growth promotion and induction of resistance in *Camellia sinensis* against *Fomes lamaoensis*. J. Plant. Interact. 4: 261-272.
[41] Nahas, E., Banzatto, D.A. and Assis, L.C., 1990. Fluorapatite solubilization by *Aspergillus niger* in vinasse medium. Soil. Biol. Biochem. 22: 1097-1101.
[42] Barriuso, J., B.R. Solano, R.G.Fray, M.Camara, A. Hartmann and F.J.G. Manero. 2008. Transgenic tomato plants alter quorum sensing in plant growth promoting rhizobacteria. Plant Biotech. J. 6:442-452.
[43] Chakraborty, U., B.N. Chakraborty and M. Basnet. 2006. Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. J. Basic. Microbiol. 46: 186-195.
[44] Dimkpa, C., T. Weinand and F.Asch. 2009. Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ. 32: 1682-1694.
[45] Huang, C.J., K.H. Ken-Haow Yang, Y.H. Liu, Y.J. Lin and C.Y. Chen. 2010. Suppression of southern corn leaf blight by a plant growth- promoting rhizobacterium *Bacillus cereus* C1L. Ann. Appl. Biol. 157: 45–53.