Saline tolerant plant growth promoting diazotrophs from rhizosphere of bermuda grass and their effect on rice

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Saline tolerant plant growth promoting diazotrophs from rhizosphere of bermuda grass and their effect on rice

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ABSTRACT

In this study, beneficial effects of multifaceted growth promoting isolates for rice were investigated under two different salt concentrations in pot culture conditions. Two most salt tolerant isolates (TRY2) *Serratia* sp. and *Bacillus* sp. (TRY4) were selected and their growth promoting characters were studied under slight and moderate NaCl concentration. Isolates *Serratia* sp. and *Bacillus* sp. were able to fix the nitrogen and solubilise phosphate, synthesise IAA, acc deaminase regardless of NaCl concentration in most cases, under conditions of salinity. In pot experiments, plant growth (plant height, dry weight, and chlorophyll content) was promoted by bacterial inoculation with 2.9 and 5.8 g NaCl/kg soil. In this study, uptake nutrients (N^+, P^+, and K^+) were increased regardless of NaCl concentration with inoculation of *Serratia* sp. and *Bacillus* sp. and uptake of Na^+ was reduced with treatments receiving 5.8 NaCl/kg soil with *Serratia* sp. and *Bacillus* sp. isolates used as inoculants as compared to control. The present observations showed that strains *Serratia* sp. and *Bacillus* sp. partially alleviated the saline stress in rice, likely through the integration of several mechanisms that improve the plant response.

Key words: *Bacillus* sp, Plant growth promoting diazotrophs, Rice, Salinity stress, *Serratia* sp.

Salinity is one of the most serious environmental problems influencing crop growth throughout the world. In India, out of an estimated area of 187.7 million ha of total degraded lands, 8.1 million ha are salt affected in which 3.1 million ha are in the coastal regions (Sziderics *et al.* 2007). In most saline soils, sodium chloride is the predominant salt species, and its effect can be observed by decreased productivity or plant death (Munns 2005). Most of the plants possess several mechanisms to decrease the negative effects of salinity including regulation and compartmentalization of ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathways (Parida and Das 2005).

Cynodon dactylon Pers. (Poaceae), a hardy perennial grass, is one of the most commonly occurring weeds in India. It is widely accepted that the rhizosphere of any plant species is a unique niche harboring diversified bacterial communities, which serve as potential resource for bioprospecting. The rhizosphere of plant species growing profusely under stress-conditions harbors novel diazotrophs to meet their nitrogen requirement as observed in salt marsh grasses such as Sp. artina alterniflora, *Juncus roemerianus* (Bagwell and Lovell 2000), ligotrophic habitant *Drosera villosa* (Albino *et al.* 2006) and desert growing *Lasiurus* grass (Chowdhury *et al.* 2009).

The beneficial roles of diazotrophs to plants include nitrogen-fixation, mineral solubilization, production of phyto hormones such as indole acetic acid (IAA) and cytokinins. By virtue of such attributes, pre-treatment of seeds with a suspension of *Azotobacter* was shown to improve seed germination and plant growth (Ravikumar *et al.* 2004). An increasing supply of N through dinitrogen fixation may increase crop production in saline habitats (Yao *et al.* 2010). Diazotrophic bacteria are also PGPR, because of their competitive advantage in C-rich and N-poor environments (Kennedy *et al.* 2004). Several reports revealed that inoculation with free living diazotrophs like *Azotobacter*, *Pseudomonas* and *Azosp. irillum* increased the yield of rice by 20–55% (Mirza *et al.* 2006) and a strain of diazotrophic *Burkholderia* increased the rice plant biomass by 69% (Kennedy *et al.* 2004). Some isolations including genus *Pseudomonas* and *Bacillus* have been shown to have capacity to promote the wheat growth in salinated soils of Uzbekistan (Nautiyal *et al.* 2008). In other work, Palomino *et al.* (2009) reported that *Bacillus subtilis* is a Gram-positive sporulating bacterium able to adapt to wide variations in osmotic and saline strength. Studies

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showed that inoculation with *Azosp.irillum* spp. increased plant growth and the K^+/Na^+ ratio of two maize cultivars (Hamdia *et al*. 2004). Moreover, Yao *et al* (2010) reported that inoculation with *Pseudomonas putida* promoted cotton growth and germination under conditions of salt stress. Considering the efficacy, input cost and environmental safety, use of chemical fertilizers for crop production in saline soil is not a sound proposition.

The objective of this study was to identify the multi-faceted growth promoting salt tolerant diazotrophs from the rhizosphere of *Cynodon dactylon* and their effect on rice seedling in the presence and absence of salt stress under pot culture conditions.

MATERIALS AND METHODS

Isolation and screening of growth promoting diazotrophs

The soil samples were collected from the rhizosphere of *C. dactylon* at salinity affected agricultural fields of Trichy district, Tamilnadu on March, 2012. Diazotrophic microorganisms isolated using serial dilution technique (10^{-6} dilution) on selective N-free malate medium (NFM) (Piao *et al*. 2005) with 1% (w/v) NaCl concentration. After required incubation period, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies from rhizosphere soil samples picked from NFM plates and sub-cultured several times in same medium to obtain pure cultures and stored as glycerol stocks at -20º C. The salt tolerances of diazotrophic bacterial isolates were determined on free nitrogen media supplemented with different NaCl concentrations of 0-10%.

Identification of diazotrophs by 16S rRNA gene sequencing

Nearly full-length of 16S rRNA gene was amplified from elite isolates as described earlier using universal eubacterial primers, FD1 and RP2 (Weisburg *et al*. 1991) and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using PGMT vector supplied with TA cloning kit (Promega, USA) prior to sequencing. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/BLAST).

Plant growth promoting features of isolates

Plant growth-promoting capabilities of the selected strains were studied in the presence and absence of NaCl (nitrogen fixation, phosphate solubilization, indole acetic acid, acc d synthesis and siderophore production).The nitrogen fixing capacity of the diazotrophic isolates was evaluated by estimating the acetylene reduction activity (ARA) (Bergersen 1980) using gas chromatograph (Chemito-7610) equipped with FID detector and Porapak N column (2m x 1/8"). Temperatures such as injector, detector, and column were maintained at 110°C, 120°C and 73°C, respectively. Nitrogen gas was used as carrier gas at the flow rate of 30 ml/min. After completion of ARA, the bacterial cells in the medium were evenly mixed and the protein concentration was measured (Lowry *et al*.1951). ARA results were expressed as n mole ethylene /mg/protein/h. An aliquot was taken from each pure culture for evaluation of plant growth promoting characteristics indole acetic acid (IAA) production was done using the method of Chandramohan and Mahadevan (1968). Solubilization of insoluble phosphates (Bunt and Rovira 1955) were also assayed. Siderophore production was checked using the Chrome Azurol S (CAS) agar plates (Dubey and Maheshwari 2004). ACC deaminase activity was measured by measuring the production of á-Ketobutyrate as described by Honma and Shimomura (1978).

Effects of NaCl and bacterial inoculation on rice growth

Pot experiments were conducted in order to evaluate the effect of NaCl and bacterial inoculation on growth of rice. Pots containing 400 g of dry-sterilized soil were supplemented to reach 0, 2.9 and 5.8 g NaCl/kg soil, which was prepared by adding 0, 1.08 and 2.25 g NaCl dissolved in 100 mL water. The treatment without exogenous addition of NaCl was considered as 0 g NaCl/kg soil concentration. Characteristics of the soil without added salt were pH (1:5 water)- 6.5, EC- 1.50 dS /m, organic matter -15 g /kg, available N-289 kg/ha, P-18.92 kg/ha, potassium - 134.34 kg/ha. Dehulled rice seeds var. *ADT43* were disinfected by soaking in 30% hydrogen peroxide and 70% ethanol for 10 min, and followed by rinsing several times in sterilized distilled water. The seeds were then pre-germinated in sterilized plain agar at room conditions for five days. For inoculum preparation, bacteria were grown in nutrient broth for 24 h at 37^0C, rinsed twice, and finally resuspended to the same initial volume using phosphate buffer pH7.0. Roots of seedlings, with the same size, were submerged three times in bacterial suspension adjusted to OD_{600} = 1 and planted in each pot supplemented or not with NaCl. Seedlings submerged in sterilized water were used as a control. Biometric observations such as plant height and dry matter production were taken at 4 weeks after the inoculation. In addition, chlorophyll content of rice leaves was estimated by the method of Hiscox and Israelstam (1979).

Inorganic elements

Leaf tissues were separated after harvesting and air-dried at 70°C for 5 days. Dried materials were ground and then digested in H_2SO_4 for the determination of total nitrogen (Kjeldahl method) or in a ternary solution (HNO_3: H_2SO_4 : $HClO_4$ = 10:1:4 with volume) for the determination of P, K and Na (Mani *et al.* 2007).

RESULTS AND DISCUSSION

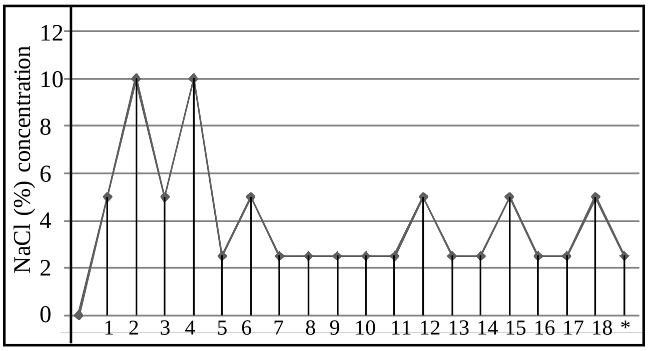
In the present study 18 pure cultures of diazotrophic organisms were isolated from saline soils and tested for tolerance of NaCl. Among the isolates tested, isolates C2 and C4 tolerated a higher content of NaCl (10%) than other isolates (Fig. 1). Due to their high tolerances, TRY2 and TRY4 were selected for further studies. Identification of isolates based on phenotypical and physiological criteria however was difficult, if the features displayed by a particular isolate are not fully identical with a described species. Utilization of PGPB has become a promising alternative to alleviate plant stress caused by salinity (Fu *et al.* 2010). Thus the molecular based method, 16S rDNA sequence analysis, was therefore chosen to identify the selected isolates. The isolates were identified as *Serratia* sp. (TRY2) and *Bacillus* sp. (TRY4) within the order Enterobateriales and Firmicutes, respectively (Table 1).

Table 1. Molecular characteristics of selected isolates

Code of the isolate	Length of 16sr DNA sequence (bp)	Most closely related organism	Similarity (%)
TRY2	1540	*Serratia* sp.	99
TRY4	1469	*Bacillus* sp.	99

Plant growth-promoting features

The isolates *Serratia* sp. and *Bacillus* sp. were able to reduce acetylene in both the presence and absence of NaCl. In the present study, the nitrogenase enzyme activity of the isolates ranged from 114 ± 4.63 to 136 ± 7.92 n moles of ethylene/mg of protein/h. The highest nitrogenase activity was exhibited by isolate *Bacillus* sp. with 2.9g NaCl concentration (136±7.92 n moles of ethylene/ mg of protein/ h) (Table 2).



TRY- 1 to 18; *Az 2 04

Fig. 1. Maximum salt tolerance level of diazotrophic isolates

The microbial synthesis of plant growth regulators is an important factor in soil fertility. Salt-tolerant IAA-producing bacterial strains *P. aureantiaca* and *P. extremorientalis* alleviated quite successfully the reductive effect of salt stress on percentage of germination (up to 79%), probably through their ability to produce IAA (Egamberdieva *et al.* 2008). They were able to produce indole-3-acetic acid (IAA) in saline conditions. In the present study, the maximum amount of IAA was produced by *Bacillus* sp. (18.8 ±1.0 µg/ml of sample) followed by *Serratia* sp. (14.8±0.8 µg/ml of sample) with 2.9 g/l NaCl concentration (Table 2).

Diazotrophic microorganisms showing phosphate solubilizing activity have beenreported. (Mayak *et al.* 2004). Phosphate solubilization activity was exhibited by both strains *Serratia* sp and *Bacillus* sp (Table 2). In the present study, the maximum amount of siderophore was produced by isolate *Bacillus* sp and *Serratia* sp with 2.9 NaCl concentration which produced 16.5±0.9 and 15.5±0.8 µg/ml of sample respectively. In other work, direct use of *P. putida* siderophores by plants has been demonstrated in many species, including dicot legumes such as peanut or monocots such as sorghum (Albino *et al.* 2006).

The results obtained demonstrated that the selected salt-tolerant bacterium containing ACC deaminase. *Pseudomonas fluorescens* strain TDK1 containing ACC deaminase activity enhanced the saline resistance in groundnut plants and increased yield as compared to plants inoculated with *Pseudomonas* strains lacking ACC deaminase activity (Saravanakumar and Samiyappan 2007). In the present study, maximum amount of ACC observed in *Bacillus* sp. (89.8 ± 1.1 nmoles of á-ketobutyrate /mg/h) followed by *Serratia* sp. (76.6 ± 2.3 nmoles of á -ketobutyrate/mg/h) with 5.8g/l NaCl concentration (Table

Table 2. Plant growth promoting rhizobacteria (PGPR) features of strains

Strain	NaCl (g/l)	[1]ARA	[2]IAA	[2]Phosphate solubilzation	[2]Siderophore production	[3]acc deaminase activity
Serratia sp.	0	117(±9.2)^{c}	11.7(±1.2)^{ab}	12.0(±0.9)^{ab}	11.0(±1.0)^{b}	13.9 (±1.0)^{d}
	2.9	122(±8.1)^{ab}	14.8(±0.8) ^{ab}	11.8(±0.7)^{ab}	15.5(±0.8)^{a}	34.8 (±1.4)^{c}
	5.8	118(±7.1)^{c}	10.3(±0.9)^{b}	14.8 (±1.2)^{a}	11.0(±0.6)^{b}	76.6 (±1.3)^{b}
Bacillus sp.	0	114(±4.6)^{ab}	11.8(±0.8) ^{b}	11.7 (±0.9)^{ab}	12.5(±0.8)^{b}	14.9 (±1.2)^{d}
	2.9	136 (±7.9)^{a}	18.8(±1.0)^{a}	12.8 (±0.7)^{ab}	16.5 (±0.9)^{a}	45.8 (±1.1)^{c}
	5.8	123 (±5.2)^{ab}	10.8(±1.0)^{b}	10.8 (±1.0)^{b}	10.5 (±1.0)^{b}	109.8(±1.2)^{a}

ARA: Acetylene reduction activity; IAA: Indole acetic acid; Values are mean (± standard error) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$). [1]n moles of ethylene/ mg of protein/h, [2]µg/ml of sample, [3]n moles of a-ketobutyrate/mg/h

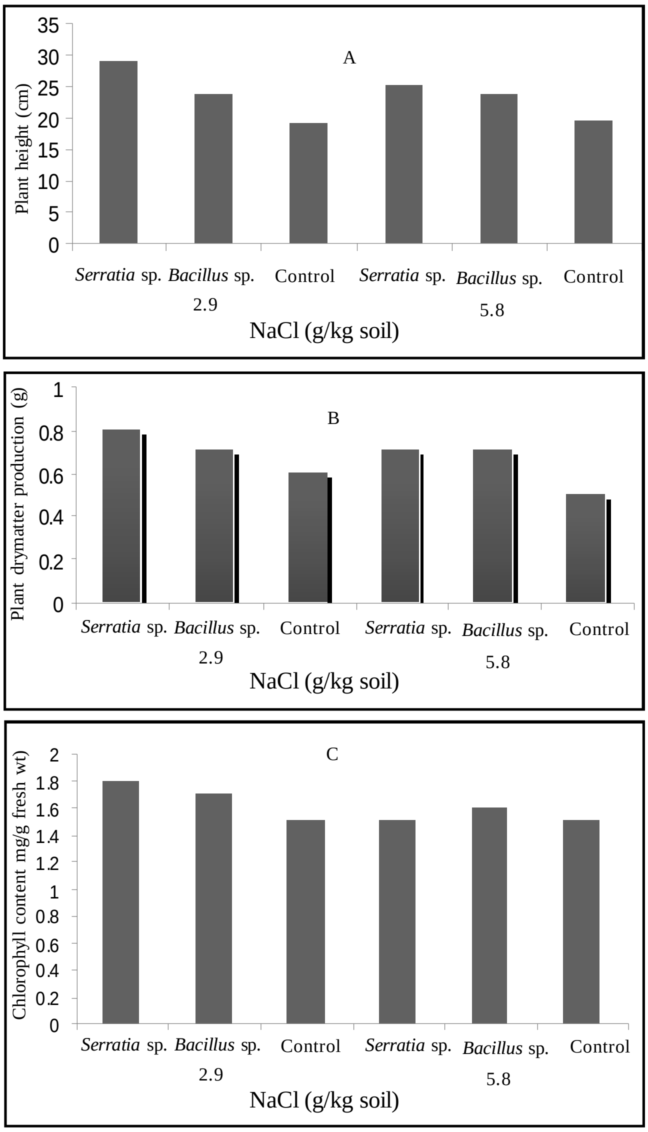


Fig 2. Effect of NaCl and diazotrophic PGPR on a) plant height, b) plant dry matter production and C) chlorophyll content of rice seedlings

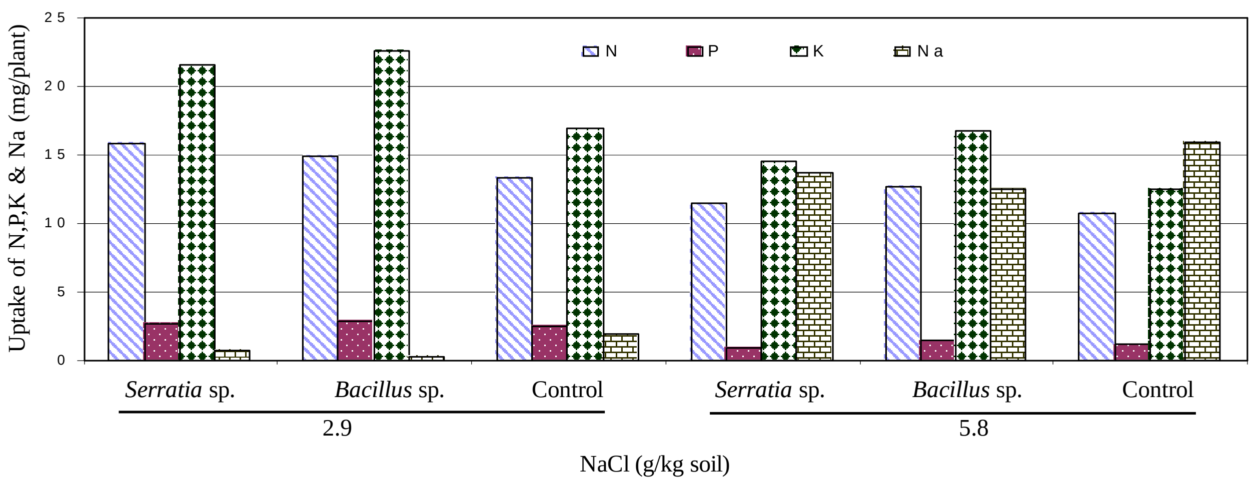
2). In other work, *Pseudomonas putida* UW4, which produces IAA and ACC deaminase, protected canola seedling from growth inhibition by high levels of salt (Mayak *et al.* 2004).

Rice growth and nutrient uptake

Electrical conductivities were 3.05 and 6.00 dS/m for 2.9 and 5.8 g NaCl/kg soil, respectively. Inoculation with strains *Serratia* sp. and *Bacillus* sp. increased plant growth, both normal and under saline stress. Similar work conducted by Mayak *et al.* (2004) that *Achromobacter piechaudii* having ACC deaminase activity significantly increased the fresh and dry weights of tomato seedlings grown in the presence of NaCl salt (up to 172 mM). In the present study, the treatment inoculation with strain *Bacillus* sp. in 1.5 g NaCl/kg soil, the increase in plant height (28.9± 1.3cm) and dry matter production (0.8 g) (Fig. 2). Salinity decreases carbon uptake by limiting photosynthesis, causing an over-reduction of photosynthetic electron chain, and redirecting the photon energy into processes that favour the production of reactive oxygen species (ROS) (Hichem *et al.* 2009). In our study, the maximum total chlorophyll (1.8± 0.3 mg) was observed in *Serratia* sp. with 2.9 NaCl g/kg. Diazotrophs may become selectively enriched to promote plant growth because of their competitive advantage in C-rich and N poor environments (Kennedy *et al*. 2004). Hence inoculation with diazotrophic bacteria might improve crop growth and productivity in such soils.

The PGPR strains varied greatly in their effect on the concentration of major mineral nutrients in rice leaves under soil salinity conditions. The N, P, K, and Na uptake per plant in the soil salinity treatment were significantly decreased compared to the non-salinity treatment (Fig. 3). The concentration of major cations in the non-salinity treatment was increased more with the PGPR treatment (*Serratia* sp. and *Bacillus* sp.) than the control, but Na^{+}

Fig. 3 Effect of NaCl and diazotrophic PGPR on uptake N, P, K and Na



uptake under soil salinity were decreased in the treatment inoculated with (*Serratia* sp.) and (*Bacillus* sp.) strains. Among the isolates, *Bacillus* sp. caused a lower uptake of Na (22.5 ± 0.6 mg) compared to *Serratia* sp. (Fig. 3). Our findings clearly showed that two isolates of *Serratia* sp. and *Bacillus* sp. were capable of exerting multifaceted beneficial plant-growth promoting activities under moderate saline conditions. These isolates could serve as potential bioinoculants for meeting the nutritional requirement of the crop plants in an eco-friendly and cost effective manner in saline soil conditions.

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