

Under saline stress plant growth promoting bacteria affect growth, photosynthesis and antioxidant activities in paddy.

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Abstract

Inoculation of plant growth promoting bacteria (PGPB) was found to be more promising to induce growth of paddy plants under lower and moderate salinity levels. PGPB protects the plants from salinity injury by increasing biomass content, photosynthetic activity and antioxidant enzymes acid phosphatase and glutathione reductase. This study also states that PGPB helps in decrement of lipid peroxidation, plays an important role in regulation of growth for positive adaptation of plants to salt stress. However with increase in soil salinity the glutathione reductase activity decreased in non-inoculated plants. The present study shows that inoculation of paddy (*Oryza sativa*) with root associated bacteria could provide salt tolerant ability to paddy plants as these isolates also remain associated with root and root is the plant part affected first by salinity, so it serve as a useful tool for alleviating salinity stress.

Highlights

- PGPB protects the plants from salinity.
- PGPB increasing biomass, photosynthetic and antioxidant in paddy.
- PGPB helps in decrement of lipid peroxidation under salinity.

Keywords: PGPB, photosynthesis, lipid peroxidation, glutathione reductase, acid phosphatase

Saline soils and saline irrigation constitute a serious production problem for crops as saline conditions are known to suppress plant growth, particularly in arid and semiarid areas (Parida and Das, 2005). The ability of the crop to adjust to salinity is extremely useful. A strategy to acquire much water is essential for plant growth under water deficit conditions due to salinity. To overcome dehydration due to salinity, plants have developed mechanisms of physiological adaptation, such as improvement of water use efficiency by regulation of stomatal closure, development of root system to acquire water, accumulation of osmoprotectants and control of water permeability by aquaporins (Jang *et al.* 2004). Salinity stress also decreases photosynthetic capacity

due to the osmotic stress and partial closure of stomata. Plants can also suffer from membrane destabilization and general nutrient imbalance. Membrane injury induced by salt stress is related to an enhanced production of highly toxic ROS (Shalata *et al.* 2001).

It is already known that cytotoxic ROS, which are also generated during metabolic processes, can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids (McCord, 2000). Therefore, ROS must be scavenged for maintenance of normal growth. Oxidative damage in the plant tissue is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant mechanisms. These mechanisms include β -carotenes, α -tocopherol, ascorbate, glutathione and



enzymes such as superoxide dismutase, peroxidase, catalase, and the enzymes involved in Halliwell-Asada pathway: ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (Halliwell, 1987;). There is good evidence that underlines the intimate relationship between constitutive and/or enhanced antioxidant enzyme activities and increased resistance to environmental stresses (Bor *et al.* 2003). The use of plant growth-promoting bacteria (PGPB) and symbiotic microorganisms, especially arbuscular-mycorrhizal (AM) fungi, may prove useful in developing strategies to facilitate plant growth in saline soils. More specifically, the soil-borne *pseudomonas* have received particular attention because of their catabolic versatility, excellent root-colonising ability and capacity to produce a wide range of enzymes and metabolites that help the plant withstand varied biotic and abiotic stress conditions (Vessey, 2003). The *B. subtilis* strain could also alleviate the effects of salinity stress in soybean and treatment with the bacterium *B. cereus* alleviated the adverse effect of salinity in terms of decrease in growth, number of leaves and shoot and root dry mass (Chakraborty *et al.* 2011).

Nothing is known about the interaction of PGPB and their effect on the physiological response of paddy plants under different conditions of soil salinity. We hypothesize that inoculation with a PGPB, alone or in combination, can confer salinity tolerance to paddy and that such tolerance is correlated with changes in the activity of antioxidant enzymes (glutathione reductase and lipid peroxidation activities), photosynthesis rate, leaf greenness and the acid phosphatase activity.

Materials and Methods

Isolation and Identification of PGPB

Certified Seeds of rice variety GR-11 were obtained from Main Rice Research Center, Navagam, Anand, Gujarat. These seeds were planted in pots and maintained for forty days. Microorganisms were isolated from the root tissue as well as rhizospheric soil. For isolation of endophytic bacteria from roots, fresh roots of paddy were surface sterilized with 70% alcohol and HgCl₂ for 5 min each, followed by washing with sterile distilled water. The root tissues were then homogenized in a sterile 4% sucrose solution in mortar and pestle and the extract was used for

isolation of bacteria. For isolation of rhizospheric bacteria, soil adhere with root were collected and subjected to serial dilution, then both sample were plated on YEMA (Yeast Extract Mannitol Agar) medium. Various biochemical tests were performed (data not shown here) followed by 16S RNA ribo-typing to identify the isolates (Jha and Subramanian, 2011). Biochemical and PCR amplification of 16S rDNA indicate that isolated organisms are *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* respectively having NCBI data bank accession nos. EU921258 and EU921259 respectively.

Rice cultivation and inoculation

Seeds of rice variety GR-11 were washed thoroughly with distilled water followed by surface sterilization with 0.1% HgCl₂ solution for 4 min and 70 % ethanol for 10 min. The seeds were washed thoroughly with sterile distilled water and kept in a shaker for 5 – 6 h in autoclaved distilled water on a rotary shaker. Later the seeds were transferred to Petri dishes containing tryptone glucose yeast extract agar medium (Bashan *et al.* 2004) and incubated in dark at 30°C to test for possible contamination. The germinated seedlings devoid of any contamination were used for inoculation experiments.

To study the effect of the isolated bacteria on the physiological and biochemical parameters selected, 4 days old germinated seedlings devoid of any contamination were transferred to culture tubes containing 400 µl Hoagland's nutrient medium, 400 µl micronutrients and 1% agar in 40 ml distilled water. Before the transfer, bacterial inoculums of the isolated bacteria *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* were added with the medium at a concentration of 6 x 10⁸ cfu ml⁻¹. To obtain a mixture of both bacterial cultures, an equal volume of both the cultures were mixed in the medium to give a concentration of 6 × 10⁸ cfu ml⁻¹. The tubes were incubated at 27°C in a 12 h light – dark cycle in a growth chamber. Seven days old rice plants were carefully removed from different test tubes inoculated with the strain of bacterium, and planted in a pot. Similarly the control plants (un-inoculated) were also transferred to a fresh pot. The quantity of the soil possessing the following physio-chemical properties; pH: 7.79, electrical conductivity 1063 µS/cm, CEC:3 cmol, organic carbon:

5500 mg per kg, available nitrogen 200 mg per square decimeter, available Ca: 12.1cmol, available P 205 : 9.5 mg per square decimeter, available K 20 : 265 mg per kg, Fe: 3.1 mg per kg, Zn: 285 mg per kg, Mn: 3.7 mg per kg, Cu : 2.2 mg per kg was maintained as 5 Kg per pot. Rice seedlings were planted at the rate of 4 plants per transplant and 6 transplantations per pot. Pots were watered at the time of transplantation of the rice seedlings. All experiments were carried in 5 replicates.

Maintenance of saline stress condition

The saline condition was maintained at five different salinity levels by adding (5g, 10g 15g, 20g and 25g NaCl L⁻¹) saline solution to the pots. To avoid osmotic shocks, NaCl concentration was gradually increased for four consecutive days. A plastic bag was put underneath each pot to collect excess water due to drainage. This water was reapplied to the respective pot. All seedlings were grown for 5 weeks without any fertilizer treatment. The experiment was conducted in a greenhouse at 20 to 25°C and the relative humidity 70 to 80%.

Effect on growth Parameters under salinity

The observation on physical parameters i.e., plant height, dry weight, leaf greenness and photosynthetic rate were recorded from three replicate from each treatment after 45 days of sowing the seeds. For dry weight (DW) determination, the leaves and roots were dried at 70°C for 48 h and weighed. Leaf greenness was measured by portable chlorophyll meter (SAPD- 502) and photosynthetic rate by an open-system portable photosynthesis meter (Li-Cor 6400).

Enzyme extractions and enzymes assay

Leaves (2 g) were homogenized with a mortar and pestle in 4 ml of ice-cold 50 mM Tris-acetate buffer pH 6.0, containing 0.1mM ethylene diamine tetraacetic acid (EDTA), 5 mM cysteine, 2% (w/v) polyvinylpyrrolidone (PVP), 0.1mM phenyl methyl sulphonyl fluoride (PMSF) and 0.2% (v/v) Triton X 100. The homogenate was centrifuged at 12,000 g for 20 min and the supernatant was filtered through Sephadex G-25 column equilibrated with the same buffer used for homogenization. The column elute were used as enzyme source for determination of

enzyme activity. All operations were performed at 4°C. Protein concentration was determined by taking OD at 595 nm according to Bradford, (1976) using bovine serum albumin as a standard.

Estimation of Lipid peroxidation activity

The level of lipid peroxidation in leaf samples were determined in terms of malondialdehyde (MDA) content according to the method of Wang *et al.*, 2013. Content of MDA, which is an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. MDA concentration was calculated from the absorbance at 532 nm and measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM cm⁻¹. All tests were carried out in triplicate.

Estimation of Acid phosphatase activity

Acid phosphatase activity was estimated according to the method of Hadjiivanova and Tzvetkov (2010). The reaction mixture, in a final volume of 2 ml, contained 100 mM sodium acetate buffer (pH 5.0), 5 mM *p*NPP as substrate and enzyme. After 10 min of incubation at 37°C, the reaction was stopped by the addition of 1 ml of 1 M NaOH, and the *p*-nitrophenol (*p*NP) released was monitored at 405 nm, using a molar extinction coefficient of 18 000 M⁻¹ cm⁻¹. One unit of AP was defined as μ mol PNP g⁻¹ h⁻¹. All tests were carried out in triplicate.

Estimation of Glutathione reductase activity

Glutathione reductase activity was measured according to method of Yannarelli *et al.*,(2007). The assay medium contained 0.025 mmol L⁻¹ Na-phosphate buffer (pH 7.8), 0.5 mmol L⁻¹ GSSG, 0.12 mmol L⁻¹ NADPHNa₄ and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was followed at 340 nm. Activity was calculated using the extinction coefficient (C=6.2mM⁻¹cm⁻¹) for GSSG. One unit of GR was defined as 1mmol ml⁻¹ GSSG reduced per minute. The specific enzyme activity for all enzymes was expressed in unit's nmol min⁻¹ mg⁻¹ protein FW.

Table 1: Effect of PGPR on dry weight, plant height, leaf greenness and photosynthetic rate under saline condition.

% Salinity of irrigation water	Treatment	Dry weight (g plant ⁻¹)	Plant Height (cm)	Leaf greenness (SPAD)	Photosynthetic rate (μmol CO ₂ m ⁻² s ⁻¹) (Li-Cor)
0.0 % NaCl Control	1. No inoculation	2.43 ^d	62.1 ^d	43.3 ^d	23.4 ^{cd}
	2. Inoculation with <i>B. pumulis</i>	2.68 ^c	69.3 ^c	44.1 ^c	25.1 ^{bc}
	3. Inoculation with <i>P.pseudoalcaligenes</i>	2.87 ^{ab}	74.2 ^{ab}	44.9 ^{ab}	27.7 ^b
	4. Inoculation with <i>B.pumulis</i> + <i>P. pseudoalcaligenes</i>	2.93 ^a	78.4 ^a	45.2 ^a	29.3 ^a
0.5 % NaCl	1. No inoculation	2.03 ^d	54.2 ^{cd}	39.8 ^{cd}	19.2 ^{cd}
	2. Inoculation with <i>B. pumulis</i>	2.23 ^c	61.4 ^c	40.3 ^{bc}	20.8 ^{bc}
	3. Inoculation with <i>P.pseudoalcaligenes</i>	2.41 ^b	68.5 ^b	41.2 ^b	21.7 ^b
	4. Inoculation with <i>B.pumulis</i> + <i>P. pseudoalcaligenes</i>	2.58 ^a	71.2 ^a	43.1 ^a	22.5 ^a
1.0 % NaCl	1. No inoculation	1.98 ^d	42.3 ^d	36.1 ^{cd}	18.6 ^d
	2. Inoculation with <i>B. pumulis</i>	2.21 ^c	48.4 ^c	37.2 ^c	19.7 ^c
	3. Inoculation with <i>P.pseudoalcaligenes</i>	2.34 ^b	55.2 ^b	38.3 ^b	20.5 ^{ab}
	4. Inoculation with <i>B.pumulis</i> + <i>P. pseudoalcaligenes</i>	2.46 ^a	58.6 ^a	39.2 ^a	21.3 ^a
1.5 % NaCl	1. No inoculation	1.73 ^d	34.1 ^d	32.2 ^{cd}	16.2 ^{cd}
	2. Inoculation with <i>B. pumulis</i>	1.86 ^{bc}	37.5 ^{bc}	33.3 ^c	17.2 ^{bc}
	3. Inoculation with <i>P.pseudoalcaligenes</i>	1.98 ^b	43.7 ^b	34.1 ^{ab}	18.4 ^b
	4. Inoculation with <i>B.pumulis</i> + <i>P. pseudoalcaligenes</i>	2.12 ^a	47.1 ^a	34.9 ^a	19.3 ^a
2 % NaCl	1. No inoculation	1.52 ^d	28.6 ^d	29.3 ^{cd}	13.4 ^{cd}
	2. Inoculation with <i>B. pumulis</i>	1.74 ^c	31.4 ^c	30.1 ^c	14.2 ^c
	3. Inoculation with <i>P.pseudoalcaligenes</i>	1.86 ^b	37.2 ^{ab}	31.3 ^b	15.3 ^b
	4. Inoculation with <i>B.pumulis</i> + <i>P. pseudoalcaligenes</i>	1.92 ^a	41.3 ^a	32.2 ^a	16.5 ^a
2.5 % NaCl	1. No inoculation	1.21 ^d	21.7 ^d	25.4 ^{cd}	10.4 ^{cd}
	2. Inoculation with <i>B. pumulis</i>	1.42 ^{bc}	26.3 ^c	26.3 ^c	11.2 ^c
	3. Inoculation with <i>P.pseudoalcaligenes</i>	1.56 ^b	32.4 ^b	27.2 ^{ab}	12.3 ^b
	4. Inoculation with <i>B.pumulis</i> + <i>P. pseudoalcaligenes</i>	1.75 ^a	37.1 ^a	27.9 ^a	13.4 ^a

Values are mean of three replications. Means within columns sharing the same letters are not significantly different ($P \leq 0.05$; LSD test).

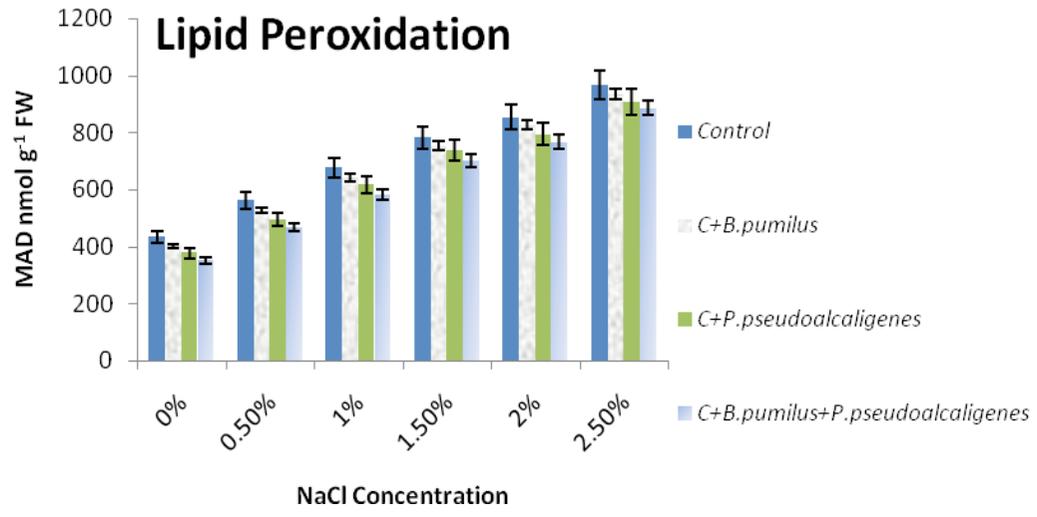


Figure 1: MDA content in paddy variety GR-11 at five different levels of salinity (n=5).

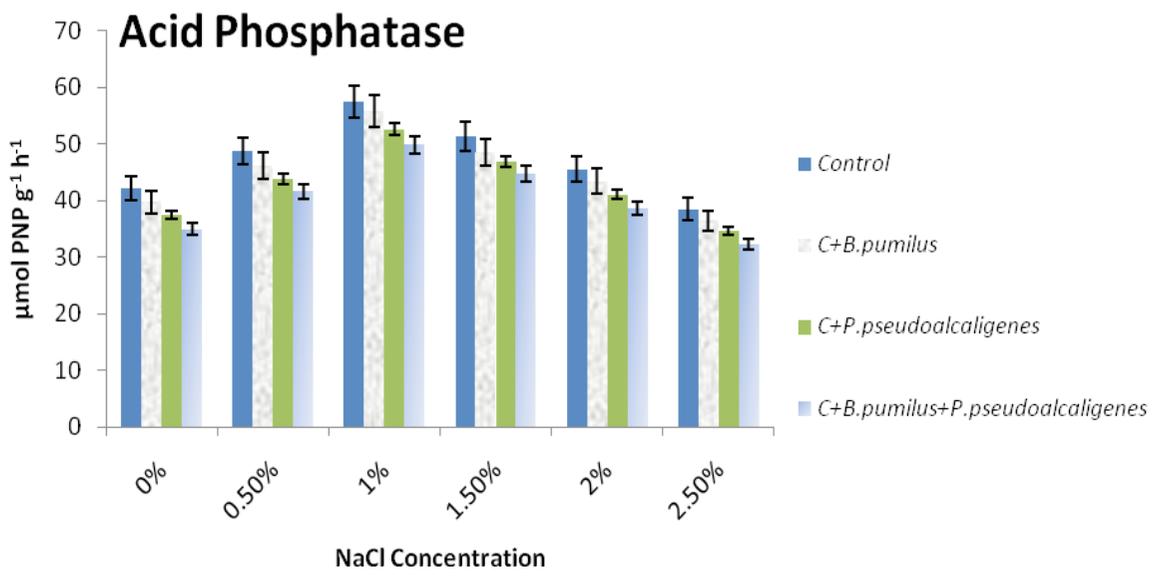


Figure 2: Acid Phosphatase activity in paddy variety GR-11 at five different levels of salinity (n=5).

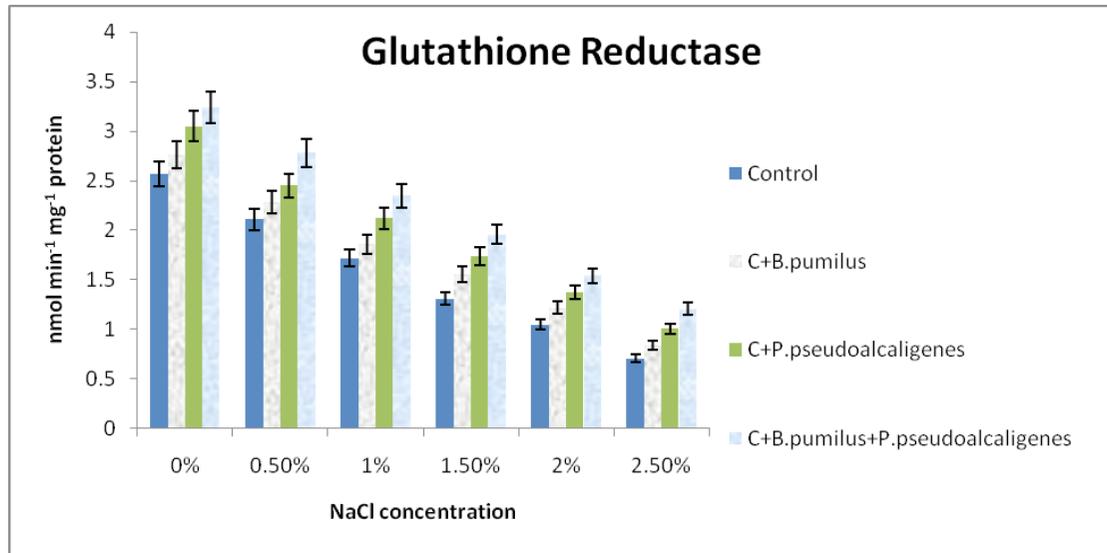


Figure 3: Glutathione Reductase activity in paddy variety GR-11 at five different levels of salinity (n=5). Vertical bar represents $SME \pm$ of three replicates.

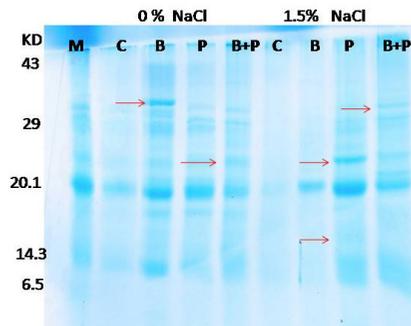


Figure 4: Polyacrylamide gel (12%) of total soluble protein showing the differential expression of small protein due to inoculation of PGPB and salinity. Loaded samples were adjusted to a constant amount of protein (15 μ g), M= marker (KD), C= Control, B= Plant inoculated with *B.pumilis*, P= *Ppseudoalcaligenes* and B+P= inoculated with both the isolates.

Protein profiling by SDS-PAGE

10% SDS-PAGE was performed from the total soluble protein of the paddy seedling exposed to two different concentration of NaCl.

Statistical analysis

Each pot was considered as replicate and all of the treatments were repeated five times. A two-way analysis of variance (ANOVA) was performed using STATISTICA program. The means and calculated standard errors are reported. The significance was tested at 5% level.

Results and discussion

Soil salinity is an important factor affecting the soil health and crop productivity. Soil salinity in arid regions is frequently an important limiting factor for cultivating agricultural crops. The occurrence of *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, *Azospirillum*, *Pseudomonas* and *Bacillus* has been reported from desert ecosystems, acid soils, saline soils, alkaline areas and highly eroded hill slopes (Selva kumar *et al.* 2009, Upadhyay *et al.* 2009). Salinity adversely affected the growth of the selected paddy plant, regardless of the biological treatment and salt stress level, as the reduction of plant growth is the result of the alteration of many physiological activities in the plant, such as photosynthetic activity, mineral uptake and antioxidant activity. However, when the plants were inoculated with PGPB, the extent of growth suppression decreased and the inoculated plants showed greater dry weight than non-inoculated plants as shown in (table 1) and also reported by Han and Lee (2005) and Kohler *et al.* (2009). In the present study leaf greenness and photosynthetic rate was also significantly high in plants inoculated with *P. pseudoalcaligenes* and *B. pumilus* at non saline condition, as well as at different level of salinity compared to non-inoculated control plants may be because these isolates help the plants in water absorption and retention, finding are similar to those of Han and Lee (2005). However, mixture of both isolates was more effective only upto 1% salinity level, at higher salinity, none of the above treatments were helpful to the plants to overcome the deleterious effects of salinity.

Protection against oxygen radical damage appears to be vital in times of stress. Such stress conditions are frequently

found to induce increases in the extractable activities of free radical-metabolizing enzymes such as glutathione reductase, acid phosphatase and other protective enzymes. Peroxidation of lipid membranes of plants reflects free radical-induced oxidative damage at the cellular level under salt stress conditions (Sudhakar *et al.* 2001). In the present study with increase in soil salinity there was simultaneous increase in MDA content was recorded in non-inoculated plant, while inoculated plant showed decreased MDA activity (Fig.1). The results indicates strong agreement with the results of Chen *et al.* (2000) who found decreased level of lipid peroxidation in maize cells when exposed to chilling stress. And high induced activities of antioxidant enzymes and lower levels of lipid peroxidation were found in tolerant rice cultivar under salt stress (Dionisio-Sese and Tobita, 1998), so inoculation of PGPB helps in developing tolerance in paddy under salinity.

Acid phosphatase is known to act under stress by maintaining a certain level of inorganic phosphate which can be co-transported with H⁺ along a gradient of proton motive force in plant cells (Olmos and Hellin, 1997). Increased acid phosphatase activity has been reported in plants grown under salt stress by Stephen *et al.* (1994). The enhanced AP activity at mild stress may be related with increased pool of antioxidant metabolites which may act as scavengers of various reactive species and influence the gene expression as proposed by Panda and Upadhyay (2003). In the present study AP activity also increased at moderate salinity and decreased at high salinity in non-inoculated plants. While plants inoculated with *P. pseudoalcaligenes* and *B. pumilus* has decreased acid phosphatase activity (Fig. 2). Phosphatase enzymes are also used by soil microorganisms to access organically bound phosphate nutrients may be the reason for decreased acid phosphatase activity in inoculated plant.

Salt tolerance is often correlated with a more efficient antioxidative system (Bor *et al.* 2003), so induced activities of acid phosphatase and glutathione reductase in this study suggest improved salt tolerance in paddy plant under salinity in presence of PGPB. Since NaCl-induced enzyme activity indicates a specific role in coping with the stress (Gueta-Dahan *et al.* 1997). In the present study with increase in soil salinity the glutathione reductase activity decreased in non-inoculated plant, while



inoculated plant showed increased glutathione reductase activity (Fig. 3). Our results were supported by Bor *et al.* (2003), have showed that protection against salinity has been achieved by higher constitutive and induced activities of glutathione reductase in leaves of wild sugar beet species. Glutathione reductase - indicated that the tolerant plant exhibit a more active ascorbate-glutathione cycle than the non-tolerant cultivar. This cycle has been implicated in mitigating the effect of reactive oxygen species (Molina *et al.* 2002) and like other environmental stresses, salt stress affects the activities of these enzymes. The role of glutathione and glutathione reductase in the H₂O₂ scavenging in plant cells has been well established in the Halliwell–Asada pathway (Noctor and Foyer, 1998). Tepperman and Dunsmuir (1990) propose a similar conclusion that the plant always produces sufficient level of antioxidative enzymes in order to maintain the balance of cellular compounds.

Few new bands of low molecular weight protein were observed in the SDS gel, which may be having some function in helping the plants in adverse condition. Hettema *et al.* 1998 reported that low molecular weight polypeptides may act as molecular chaperones. It is thought that the molecular chaperones help to bind denatured proteins and to maintain them in a state that allows for refolding (Lee and Vierling 2000).

The results suggested that inoculation of salt-stressed plants with PGPB strain could alleviate salinity stress and improved tolerance.

Conclusions

The present study shows that *P. pseudoalcaligenes* in combination with *B. pumilus* was able to protect the paddy plants by regulating antioxidant proteins. So the isolated PGPB enhance salt tolerant ability to paddy as these isolates also remain associated with paddy root and root is the plant part affected first by salinity, so it serve as a useful tool for alleviating salinity stress.

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