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Paddy physiology and enzymes level is regulated by rhizobacteria under saline stress

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Summary

To investigate the physiological basis of salt adaptation in paddy due to inoculation with plant growth promoting bacteria, we compared their effect in paddy under saline and non-saline condition on root length, chlorophyll content, relative water content, stomatal conductance, membrane stability index and change in ascorbate peroxidase and nitrate reductase activities. In a pot experiment, the effect of endophytic and rhizospheric bacteria was studied in a local paddy rice (*Oryza sativa* L.) variety GJ-17 under salt stress. Our findings suggest that inoculation with *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* resulted in change of ascorbate peroxidase, nitrate reductase activity and plant growth parameters such as root length, chlorophyll content, RWC, stomatal conductance and membrane stability index under salinity. Mixture of both *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* revealed better response in paddy against the adverse effects of salinity.

Introduction

One of the most widespread agricultural problems in arid and semiarid regions is soil salinity, which make fields unproductive and decreases crop yield. Salinity becomes a concern when an excessive amount or concentration of soluble salts occurs in the soil or water. It has been reported that salinity limits plant growth and productivity (Ashaf and Foolad, 2007). The linkage between belowground and above ground sections of ecological system depends mainly on root system (Liang Peng et al., 2007). High concentration of salt in the root zone (rhizosphere) reduces soil water potential and the availability of water. As a result, reduction of the water content leading to dehydration at cellular level and osmotic stress is observed. The increased amounts of Na^+ and Cl^- in the environment effect the uptake of many indispensable nutrients through competitive interactions and by effecting the ion selectivity of membranes. The absorption function of root system is closely related to their morphology and activity. Moreover root systems can interact with the environmental stress under the adverse situation, and adjust the system to build up adaptation responses in morphology and physiology to strengthen its survival chance. The effect of salinity on root (An et al., 2003) of plants had already been reported. Many researchers reported that with an increase in salinity there was a decrease in the development of the xylem. Decrease in development of xylem means decrease in loading of Na+ and also essential nutrients, results in shunted growth of plants. Plants in saline environment can protect themselves from Na+ toxicity through restricting Na+ entry; excluding Na+ from root cells; sequestering Na+ into vacuoles; or retrieving Na+ from the transpirational xylem stream for recirculation to roots (Chinnusamy et al., 2006).

A strategy to acquire much water is essential for plant growth under water deficit conditions. To overcome water deficit, 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. Salinity also affects the chlorophyll pigment, both chlorophyll a and chlorophyll b decreased under salinity in rice seedlings (Djanaguiram and Ramadass, 2004). Plants can also suffer from membrane destabilization and general nutrient imbalance.

An important feature of salinity is the generation of reactive oxygen species (ROS) and free radicals, such as superoxide anion radical (O_2^-), singlet oxygen, hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) which cause oxidative stress to plants. Salinity promotes oxidative damage not only by direct increasing of the cellular concentration of reactive oxygen species but also by the diminution of the cellular antioxidant capacity (Pinto et al., 2002). To minimize the damaging effects of ROS, aerobic organisms evolved non-enzymatic defense systems (ascorbic acid, reduced glutathione, carotenoids, tocopherols, flavonoids, alkaloids) and enzymatic protection mechanisms (superoxide dismutase, peroxidases, catalase).

But in the last decade, there were number of reports on the beneficial effects of microorganisms such as *Pseudomonas*, *Bacillus*, *Pantoea*, *Burkholderia*, *Rhizobium* etc. in enhancing the tolerance of crops such as sunflower, maize, wheat, chickpea, groundnut, spices and grapes to drought, salinity, heat stress and chilling injury under controlled conditions (Arshad et al., 2008). Plant growth promoting bacteria are free-living soil bacteria that can either directly or indirectly facilitate rooting (Mayak et al., 1999). With this aim, the objective of this study to comparatively investigate the role of plant growth promoting rhizobacteria (PGPR) alone and in combination in inoculated rice seedlings under different level of salinity stress. We hypothesise that inoculation with PGPR, alone or in combination, can confer salinity tolerance to paddy and that such tolerance is correlated with changes in plant physiology as chlorophyll content, RWC, stomatal conductance and membrane stability index and activity of enzymes (nitrate reductase and ascorbate peroxidase) by considering that such bacteria help the plant under adverse condition of salinity.

Materials and methods

Isolation and Identification of PGPR

Certified seeds of rice variety GJ-17 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 $HgCl_2$ 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 surface were

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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. The 16S rDNA Universal primers 8 F to 1510 R were used for amplification of the DNA followed by sequencing for identification of isolates (JHA et al., 2011b). The isolated endophytic *Pseudomonas pseudoalcaligenes* and rhizospheric *Bacillus pumilus* have good ability for phosphate solubilization as well as other plant growth promoting abilities (data already communicated), were selected for further studies.

Rice cultivation and inoculation

Seeds of rice variety GJ-17 were washed thoroughly with distilled water followed by surface sterilization with 0.1 % $HgCl_2$ 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 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 10^8 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 x 10^8 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 kg^{-1} available nitrogen 200 mg dm^{-2}, available Ca: 12.1 cmol, available P_20_5: 9.5 mg dm^{-2}, available K_20, 265 mg kg^{-1}, Fe, 3.1 mg kg^{-1}, Zn, 285 mg kg^{-1}, Mn, 3.7 mg kg^{-1}, Cu, 2.2 mg kg^{-1} 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 of PGPR on morphology and anatomy of plant root

Plants from each treatment after 35 days of sowing the seeds were collected carefully with plant root and cross sections of roots were examined under image analyzer microscope (Carl Zeiss) to analyze the effect of salinity on xylem in inoculated as well non-inoculated plant.

Effect of PGPR on growth parameters under different salinity level

The observation on physical parameters i.e., root length, chlorophyll content, RWC, stomatal conductance and membrane stability index were recorded from thee replicate from each treatment after 35 days of sowing the seeds.

Total Chlorophyll was extracted from fresh leaves by 80 % acetone (v/v) and its contents were determined at 663 nm and 645 nm by a Hitachi U-2000 dual length spectrophotometer (ARNON, 1949). Stomatal conductance of plants was measured using fresh leaves by Li-Cor 6400. The N concentration was determined by colorimetric methods, after the Kjeldahl digestion.

Relative Water Content

Fresh weight (FW) and dry weight (DW) of leaves of each plant were determined after counting the leaf number. Leaf relative water content (RWC) was measured in the second or third youngest fully expanded leaf from top of the plant, using the following equations (SCHONFELD et al., 1988).

$$RWC\ (\%) = (FW\text{-}DW) \times 100/\ (TW\text{-}DW)$$

where, FW is leaf fresh weight, DW is leaf dry weight after 24 h drying at 70 °C, and TW is leaf turgid weight after submergence in distilled H_2O for 4 h. Plant dry weight was determined after oven drying at 70 °C until they reached constant weight.

Membrane stability index

Membrane stability index (MSI) was estimated as per SAIRAM et al., (1997). Fresh leaves (0.1 g) were taken in 10 cm^3 of double distilled water in two sets. One set was subjected to 40 °C for 30 min and its electrical conductivity was recorded using an electric conductivity meter (C1). Second set was kept in a boiling water bath (100 °C) for 10 min and its conductivity was also recorded (C2).

$$\text{Membrane stability index (MSI)} = [1 - (C1/C2)] \times 100$$

Leaf enzyme extraction and effect of PGPR on enzyme activity

Fresh leaves (2 g) were homogenized with a mortar and pestle at 4 °C 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 14,000 × *g* for 20 min and the supernatant fraction was filtered though Sephadex G-25 columns equilibrated with the same buffer used for homogenisation. Protein concentration was determined by taking optical density at 595 nm by spectrophotometer (Hitachi-220), using bovine serum albumin as a standard (BRADFORD, 1976).

Nitrate reductase activity

Nitrate reductase activity in leaves, roots and nodules was determined using the method of SYM (1983). Fresh plant material (0.5 g) of the plant was homogenized in 5 ml phosphate buffer (pH, 7.0). After treating the sample extracts with 1 % sulphanilamide in 3 N HCl and 0.02 % N(1-Naphthylethylene diamine dihydrogenchloride), the optical density was read at 542 nm on a spectrophotometer (Hitachi-220) and the NRA was calculated from a standard curve established with $NaNO_2$ concentrations and expressed in produced µmol NO_2 h^{-1} g^{-1} FW. All tests had been carried out in triplicate.

Ascorbate peroxidase activity

Ascorbate peroxidase activity was estimated according to the method of NAKANO and ASADA (1981). The reaction mixture consisted of 0.05 mol L^{-1} Na-phosphate buffer (pH 7), 0.5 mmol L^{-1} ascorbate, 0.1 mmol L^{-1} EDTA.Na_2, 1.2 mmol L^{-1} H_2O_2 and 0.1 ml enzyme

extract in a final assay volume of 1 ml. The increase in absorption at A_{290} was recorded for 3 min. The concentration of oxidized ascorbate was calculated using extinction coefficient (= 2.8 mM cm^{-1}). One unit of APX was defined as 1 mmol ml^{-1} ascorbate oxidized per minute. All tests had been carried out in triplicate.

Data analysis

Data was subjected to analysis of variance (ANOVA) using a statistical computer package SAS to determine whether the treatments effects were significant. The treatment and variety means were separated using the least significant differences (LSD) test.

Results

Identification of Isolated isolates

Biochemical and PCR amplification of 16S rDNA indicate that isolated organisms are *P. pseudoalcaligenes* and *Bacillus pumilus* respectively having NCBI data bank accession nos. EU921258 and EU921259 respectively.

Effect o PGPR on plant growth parameters under salinity

In control plants (without NaCl), combination of both *P. pseudoalcaligenes* and *B. pumilus* enhanced the root length as well as root hair development (Fig. 1), but no anatomical change was observed in the xylem vessels under salinity (Fig. 2).

The overall results obtained from the present study indicate that inoculation of the two PGPRs viz. *P. pseudoalcaligenes* and *B. pumilus* either alone or in combination led to recovery of the plants from the saline stress (Tab. 1).

In control plants (without NaCl) the combination of both *P. pseudoalcaligenes* and *B. pumilus* enhanced the root length to 39 %, even upto 1 % NaCl root length increased by 22-29 % compared to non-inoculated plants.

Chlorophyll content under non-saline state increased by 4.3 %, but under salinity it decreased both in inoculated as well as non-inoculated plants. Under salinity it decreased from 0.4-35 % in plant inoculated with both *P. pseudoalcaligenes* and *B. pumilus* in compared to pure control plants (at non-saline and non-inoculated), while in non-inoculated plants under salinity a decrease by 72 % was observed.

Stomatal conductance also increased by 57 % at non-saline state and 19 % at 0.5 % NaCl, while a marginal decrease of 5.6 % was recorded at 1 % NaCl and 65 % decreased at 2.5 % NaCl in plant inoculated with both *P. pseudoalcaligenes* and *B. pumilus* in pure control plants.

The concentration of N-substance was always higher in the plants inoculated with both the PGPRs in saline condition and a gradual increase of 3 % in N_2 concentration was recorded at 1 % NaCl. In non-inoculated plants a decreased of 18 % - 54 % was recorded at higher salinity state compared to plants at non-saline state. While in inoculated plants 7.6 % enhanced N_2 concentration was recorded at 1 % NaCl and gradually decreased upto 46 % compared plants at non-saline state.

RWC (relative water content) in inoculated plants, increased by 29-57 % at non saline state but under salinity, it increased by 10-14 % in plant inoculated with both *P. pseudoalcaligenes* and *B. pumilus*

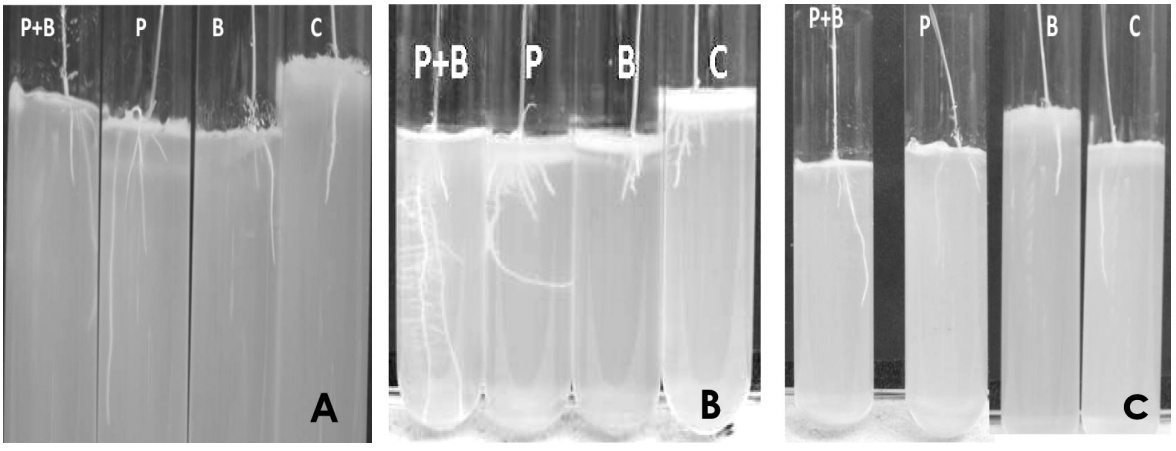

Fig. 1: Effect of inoculation of *P. pseudoalcaligenes* and *B. pumilus* on the root morphology (A) at 1.5% salinity, (B) at non saline state and (C) in non inoculated plant after 7 days of plantation. (P = *P. pseudoalcaligenes,* B = *B. pumilus* and C = Control).

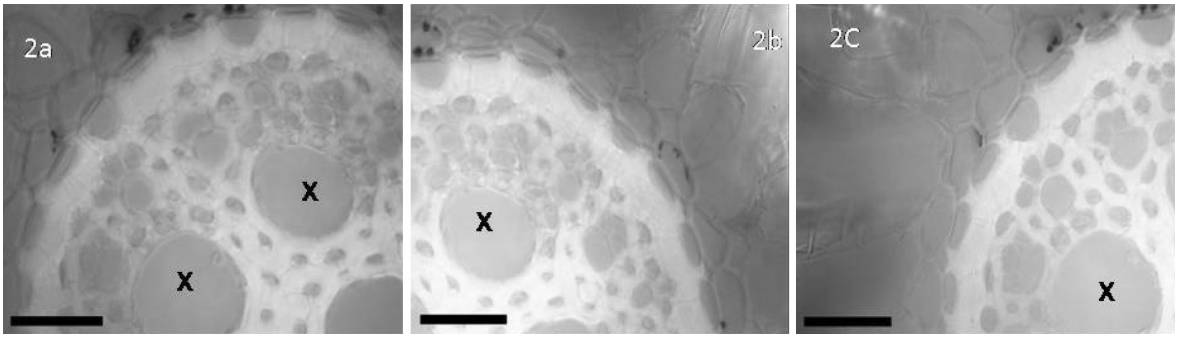

Fig. 2: (a) Effect of inoculation of *P. pseudoalcaligenes* and *B. pumilus* on the root xylem (2a) at 1.5% salinity, (2b) at non-saline state, (2c) in non-inoculated at non saline state. The scale bar = 25um (micron). Images are taken under 100x and the scales are calculated according to 100x magnification.

Tab. 1: Effect of PGPR on the root length, chlorophyll content, stomatal conductance, RWC and membrane stability Index of paddy variety GJ-17 at five different levels of salinity (n = 5).

% Salinity of irrigation water	Treatment	Root Length	Total Chlorophyll Content [%]	Stomatal Conductance [mol m^{-2} s^{-1}]	N [g kg^{-1}]	RWC %	Membrane Stability Index
0.0 % NaCl Control	1. No inoculation	0.192 ^{cd}	43.3^{d}	0.71^{d}	19.1 ^{d}	90.2^{d}	82.4^{cd}
	2. Inoculation with *B. pumulis*	0.211^{c}	44.1^{c}	0.84^{bc}	20.4^{c}	93 ^{bc}	84.1^{bc}
	3. Inoculation with *P. pseudoalcaligenes*	0.254^{ab}	44.9^{ab}	0.92^{b}	23.8^{ab}	94.7^{ab}	84.7^{b}
	4. Inoculation with *B. pumulis*+ *P. pseudoalcaligenes*	0.263^{a}	45.2^{a}	1.12^{a}	24.2^{a}	96.3^{a}	86.3^{a}
0.5 % NaCl	1.No inoculation	0.184^{cd}	39.8^{cd}	0.54^{cd}	22.4^{cd}	78.1^{cd}	74.5^{cd}
	2. Inoculation with *B. pumulis*	0.197^{c}	40.3^{bc}	0.62^{bc}	23.1^{c}	81.4 ^{bc}	76.2^{bc}
	3. Inoculation with *P. pseudoalcaligenes*	0.229^{b}	41.2^{b}	0.76^{b}	26.3^{b}	83.1b	77.1^{b}
	4. Inoculation with *B. pumulis*+ *P. pseudoalcaligenes*	0.235^{a}	43.1^{a}	0.85^{a}	27.3^{a}	86.1^{a}	79.3^{a}
1.0 % NaCl	1.No inoculation	0.167^{d}	36.1^{cd}	0.36^{d}	23.1^{cd}	65.4 ^{cd}	59.1 ^{cd}
	2. Inoculation with *B. pumulis*	0.187^{c}	37.2^{c}	0.45^{c}	24.3^{c}	67.2 ^{bc}	61.2 ^{bc}
	3. Inoculation with *P. pseudoalcaligenes*	0.231^{b}	38.3^{b}	0.58^{b}	27.2^{b}	68.7 ^{ab}	62.1 ^{ab}
	4. Inoculation with *B. pumulis*+ *P. pseudoalcaligenes*	0. 248^{a}	39.2^{a}	0.67^{a}	29.4^{a}	69.8 ^{a}	64.1 ^{a}
1.5 % NaCl	1.No inoculation	0.154^{cd}	32.2^{cd}	0.23^{cd}	18.3^{cd}	43.1 ^{d}	38.1 ^{cd}
	2. Inoculation with *B. pumulis*	0.161^{c}	33.3^{c}	0.31^{c}	19.5^{c}	45.2 ^{bc}	39.4 ^{bc}
	3. Inoculation with *P. pseudoalcaligenes*	0.189^{ab}	34.1^{ab}	0.39 ^{b}	22.3^{ab}	46.7 ^{b}	40.7 ^{ab}
	4. Inoculation with *B. pumulis*+ *P. pseudoalcaligenes*	0.203^{a}	34.9^{a}	0.42^{a}	23.2^{a}	49.1 ^{a}	41.6 ^{a}
2 % NaCl	1.No inoculation	0.145 $^{c\ d}$	29.3^{cd}	0.15^{d}	14.4^{d}	31.1 ^{cd}	31.0 ^{cd}
	2. Inoculation with *B. pumulis*	0.151c	30.1^{c}	0.22^{bc}	15.3^{c}	32.4 ^{bc}	32.4 ^{bc}
	3. Inoculation with *P. pseudoalcaligenes*	0.179^{b}	31.3^{b}	0.28^{b}	17.1^{ab}	33.8 ^{ab}	33.6 ^{ab}
	4. Inoculation with *B. pumulis*+ *P. pseudoalcaligenes*	0.187^{a}	32.2^{a}	0.31^{a}	17.9^{a}	35.4 ^{a}	34.1 ^{a}
2.5 % NaCl	1.No inoculation	0.113^{d}	25.4^{cd}	0.07^{d}	10.2^{cd}	21.2 ^{cd}	22.1 ^{cd}
	2. Inoculation with *B. pumulis*	0.127^{c}	26.3^{c}	0.13^{bc}	11.3^{c}	22.1 ^{bc}	22.8 ^{bc}
	3. Inoculation with *P. pseudoalcaligenes*	0.149^{ab}	27.2^{ab}	0.18^{ab}	13.6^{b}	23.6 ^{ab}	23.4 ^{ab}
	4. Inoculation with *B. pumulis*+ *P. pseudoalcaligenes*	0.157^{a}	27.9^{a}	0.23^{a}	14.5^{a}	24.2 ^{a}	24.5 ^{a}

For each Parameter, values in columns followed by the same letter are not significantly different at (P≤0.05).

compared to non-inoculated control.

Membrane stability index significantly increased in inoculated plant both under non-saline as well as under salinity. MSI increased by 2.5-5 % at non-saline state and 6.7-9 % under salinity in the plant inoculated with PGPR compared to non-inoculated control plants.

Effect of PGPR on enzymes activity

Nitrate reductase activity was significantly ($P \leq 0.0001$) inhibited by NaCl treatment in non-inoculated control plant and inhibition increased progressively with an increase in NaCl concentration (Fig. 3). A decrease of 10-48 % was recorded in non-inoculated plants at different salinity compared to pure control (non-inoculated plants at non-saline condition). While plants inoculated with PGPR result in increased nitrate reductase activity under saline as well as non saline condition. The highest nitrate reductase activity was

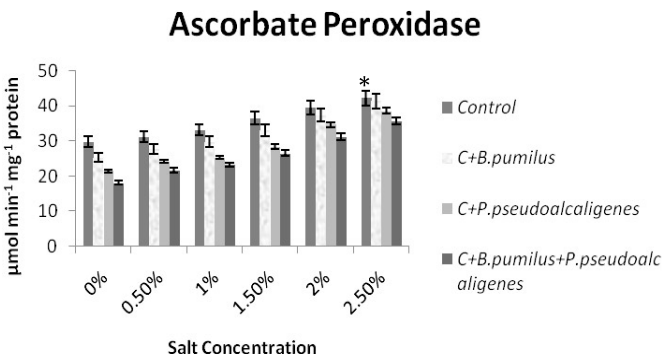

Fig. 3: Effect of inoculation with *B. pumilus*, *P. pseudoalcaligenes* alone and in combination on ascorbate peroxidase activity in paddy rice variety GJ-17 at five different levels of salinity (n = 5).

recorded in the plants grown in soil having 2.5 % NaCl and inoculated with both the isolates. An increase of 6-18 % was observed in the plants inoculated with the PGPR at non saline condition. The PGPR inoculated plants showed increase in nitrate reductase activity by 4-45 % in plant inoculated with *B. pumilus,* 5-53 % in plants inoculated with *P. pseudoalcaligenes* and 4-50 % in plants inoculated with both the PGPR at different salinity level compared to plants at non saline condition.

Ascorbate peroxidase activity showed that there is a gradual increase in activity as the concentration of NaCl in the soil increased (Fig. 4). The highest ascorbate peroxidase activity was observed in leaves of non-inoculated plants exposed to 2.5 % NaCl. The highest of ascorbate peroxidase activity was recorded in the non-inoculated control plant leaves at highest salinity level of 2.5 % which was 40 % high compared the plants grown under at non-saline condition. Inoculation of plants with PGPR resulted in 15-40 % decrease in activity compared to non-inoculated plants under non-saline condition. Among the two PGPR applied, *P. pseudoalcaligenes* showed reduction of 9-27 % and *B. pumilus* showed reduction of 2-15 %, but combination of both showed reduction of 16-40 % ascorbate peroxidase activity in all treatment compared to non-inoculated control.

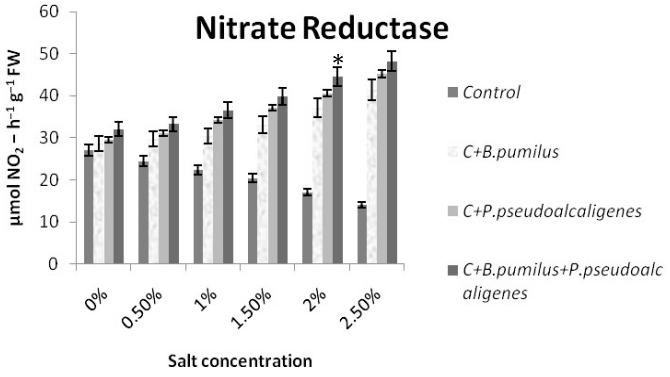

Fig. 4: The effect of inoculation with *B. pumilus*, *P. pseudoalcaligenes* alone and in combination on nitrate reductase activity in paddy rice variety GJ-17 at five different levels of salinity (n = 5).

Discussion

Water stress caused by high salinity, is one of the serious factors to limit crop productivity. Osmotic stress caused by salinity is one of the major abiotic factors limiting crop productivity because it affects almost plant's all functions. In the present study, plants inoculated with PGPR under non saline as well as at different salinity levels have greater root length and denser root hair is supported by Bashan et al., 2004. *Bacillus* is very consistent in improving different root parameters such as rooting performance, root length and dry matter content of root in mint is reported by Kaymak et al. (2008). The presence of denser root hairs has increased the surface area of root, which enhance water as well as mineral uptake (Raven and Edwards, 2001). An improved root growth was proposed as a possible mechanism by which PGPR affects plant growth (Fallik et al., 1994). Salinity rapidly decreases stomatal conductance, resulted in reduced transpiration rate. Stomata closure is known to be an effective mechanism for economical water utilization under salt stress and for limitation of the harmful salt ions uptake (Hasegawa et al., 2000). However inoculation of PGPR increased stomatal conductance under saline and non saline state to improve leaf water potential in adverse condition, observation is supported by Mia et al. (2010).

In the present study, chlorophyll content was also significantly higher in plants inoculated with *P. pseudoalcaligenes* and *B. pumilus* at non saline condition, and different level of salinity, because these isolates influence better root development which enhance water absorption and retention, findings are accordance with Han and Lee (2005). Decrease of chlorophyll content in non-inoculated plant under salinity has been considered to be a typical symptom of oxidative stress and may be the result of pigment photo-oxidation, chlorophyll degradation or lack of chlorophyll synthesis (Smirnoff, 1993).

Inoculations of plants with PGPR always have higher N-concentration under saline and non-saline states as present study is supported by Bashan et al. (2004). *Azospirillum* could result in significant changes in various growth parameters, such as increase in plant biomass, nutrient uptake, tissue N content, plant height, leaf size and root length of cereals (Bashan et al., 2004).

An interesting observation of this study was that plants inoculated with PGPR under non saline as well as at different salinity levels has greater RWC and membrane stability index which is accordance with Sandhya et al. (2010). An increased level of leaf RWC in paddy under salinity suggests the role of osmoprotectants in preventing cell injury from salt stress-induced dehydration, as suggested by Yancy et al. (1982). Non-inoculated plants has decreased RWC and membrane stability index with increased salinity. Membranes are main loci affected under water stress conditions. The lower membrane stability index reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to saline stress conditions.

The nitrate and nitrogen nutrition is very important for plant growth (Raab and Terry, 1994). Nitrate reductase is not sensitive to osmotic effect, but sensitive to Na^+ ions (Gouia et al., 1994), the nitrate reductase activity (NRA) was used as an indicator of the damaging effects of NaCl. In present study, nitrate reductase activity increased in inoculated plant may be due to high concentration of nitrogen under saline and non-saline conditions, resulted in production of high amount of NH_3. Our results are accordance with the findings of Hamdia et al. (2002) showed that nitrate reductase activity decreased with increasing salt stress. Nitrate reductase activity increased the concentration of NH_3, used by a-ketoglutarate to form glutamic acid

(El-Komy et al., 2003). High glutamic acid concentration may be used as a sink for the synthesis of other amino acids and proteins (Wang et al., 1999) or perhaps it may be directly used in osmoregulation (Hsu et al., 1999). In our previous study, we also observed that these PGPR help in accumulation of osmoprotectants in paddy plants under salinity (Jha et al., 2011).

Ascorbate peroxidases also play a vital role in plant defense against oxidative stress like superoxide reductase. Ascorbate peroxidases are the key enzymes for scavenging hydrogen peroxide in chloroplast and cytosol of plant cells (Asada, 1992). They catalyze the oxidation of ascorbate by hydrogen peroxide and give monodehydroascorbate radical. In present study inoculation of PGPR decreased the ascorbate peroxidase activity under saline and non-saline state are accordance with the Sandhya, who reported that *Pseudomonas* spp. treated seedlings showed decrease in ascorbate peroxidase activity, glutathione peroxidase activity, and catalase activity was higher compared to un-inoculated seedlings (Sandhya et al., 2010). The decreased ascorbate peroxidase activity indicates that plants inoculated with PGPR were more relaxed under salinity. These enzymes proportions paralleled the changes in electrolyte leakage and were accordance with membrane stability function of these enzymes. Inoculation probably protected the seedlings where leaves could reduce water transpiration by curling and stoma regulation.

The present study showed that *P. pseudoalcaligenes*, an endophytic

bacterium in combination with a rhizosphere *B. pumilus* in paddy was able to protect the paddy from saline stress by physiological changes in plant and regulation of antioxidant proteins than bacteria alone. In our previous study, induction of defense related enzymes such as catalase, peroxidase and polyphenol oxidase in presence of *P. pseudoalcaligenes* and *B. pumilus* alone and in combination were observed in paddy under biotic stress (JHA and SUBRAMANIAN, 2011a).

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