FINAL REPORT

Final Project Report:- 27.07.2011 to 27.07.2013
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1. Title of the Project **:- "**Molecular Phylogeny of Plant growth promoting rhizobacteria and its effects in abiotic stress on Rice".

1. INTRODUCTION:-

One of the finest success stories of the post independent era is the green revolution in the 1960s, which transformed the India from a "begging bowl" to a "breadbasket". This has been possible because of the use of chemical fertilizers and hybrid crops. However, in the long run, the use of chemical fertilizers had led to many serious problems, forcing scientists to explore other alternatives. One approach in this direction has been the use biofertilizers, better known as Plant Growth Promoting Rhizobacteria (PGPR). Species of soil bacteria that colonize the rhizosphere of plants and stimulate plant growth by wide array of mechanisms are collectively known as PGPR. Plant growth–promoting rhizobacteria (PGPR) are universal symbionts of higher plants, which enhance the adaptive potential of their hosts through a number of mechanisms, such as the fixation of molecular nitrogen, the mobilization of recalcitrant soil nutrients, the control of phytopathogens, and enhance tolerance against abiotic stress. The rhizobacteria may be present (i) in the soil surrounding roots, utilizing the metabolites leaked from roots as the growth nutrients, (ii) on the root surface or rhizoplane, (iii) in the root tissue, inhabiting spaces between cortical cells and (iv) inside the cells in specialized root structures or nodules.

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 affect the uptake of many indispensable nutrients through competitive interactions and by affecting 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.

1st YEAR'S OBJECTIVES

(i) To investigate the occurrence of plant growth promoting rhizobacteria (PGPR) in some commonly used rice cultivars as GJ-17, GR4, GR1 grown in Gujarat.

(ii) Different biochemical analysis has been done to identify it up to genus level.

(iii) Molecular tool and techniques such as PCR amplification using rDNA specific oligonucleotide primer will be used to determine the phylogeny of PGPR bacteria isolated from rice.

(iv) On the basis of these results, some promising bacteria will be selected for further inoculation studies on the plant against abiotic (salinity) stress and compared to control.

2nd YEAR'S OBJECTIVES

(i) To inoculate the rice cultivar GJ-17 with isolated PGPR.

(ii) To study the effect of the PGPR in plant under saline stress.

(iii) To elucidate the effect of these isolates in induction of various enzymes as nitrate reductase, ascorbate peroxidase, superoxide dismutase, catalase, and peroxidase to overcome salinity.

Experimental design

Two plant growth promoting bacteria, one identified as rhizospheric *Bacillus pumilus* strain (PGPR1) and other as endophytic *Pseudomonas pseudoalcaligenes* strain (PGPR2) were isolated from the root surface and from within the roots of local paddy variety GJ-17 (GR-11) . Their effect on paddy plants was studied following randomized block design (RBD) respectively with the following combinations-

S.No	Combinations
1.	Control (no PGPR, no stress)
2.	Control + PGPR1
3.	Control + PGPR2
4.	Control + PGPR1+ PGPR2
5.	Control + Abiotic stress ie Salinity
6.	Control + PGPR1+ Abiotic stress ie Salinity
7.	Control + PGPR2 + Abiotic stress ie Salinity
8.	Control + PGPR1+ PGPR2 + Abiotic stress ie Salinity

(Control : Paddy plants without PGPRs and biotic)

PGPR1 - *Bacillus pumilus*

PGPR2 - *Pseudomonas pseudoalcaligenes*

Abiotic stress – Salinity.

PROGRESS REPORT

1. Literature Survey:-

Intensive internet survey was carried out for recent papers and developments in the selected field of research. Contacted with the scientists through e-mail and phone working in this area

Visited library and laboratories of Gujarat Agriculture University, and Sardar Patel University Anand many times for referencing and collected many papers.

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 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 30^0C 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 6×10^8 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-DW) × 100/ (TW-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^{o}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).

Membrane stability index (MSI) = [1 - (C1/C2)] × 100

Leaf enzyme extraction and effect of PGPR on enzyme activity

Fresh leaves (2 g) were homogenized with a mortar and pestle at 4 ^{0}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), 0.5mmol 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 (=2.8mM cm^{-1}). One unit of APX was defined as 1mmol ml^{-1} ascorbate oxidized per minute. All tests had been carried out in triplicate.

Superoxide dismutase (SOD) activity

SOD activity was estimated spectrophotometrically as the inhibition of photochemical reduction of NBT at 560 nm. 3 mL of reaction mixture contained 33 mM NBT, 10 mM L-methionine, 0.66 mM EDTA.Na_2 and 0.0033 mM Riboflavin in 0.05M Na-phosphate buffer (pH 7.8) and 0.1 mL enzyme from plant source. One unit of SOD is defined as the amount of enzyme that inhibits 50% NBT photo reduction. Reactions were carried out at 25°C, under light intensity of about 300 micromol m^{-2} s^{-1} for 10 min. Modifications of the original methods are detailed in Costa et al., (2010). All tests were carried out in triplicate.

Catalase (CAT) activity

CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Ramalingam and In-Jung, 2013). The reaction mixture consisted of 3% (v/v) H_2O_2 and 0.1 mM EDTA in 0.05 M Na-phosphate buffer (pH 7) and 0.1 mL enzyme from plant source. The decrease in H_2O_2 was followed as the decline in optical density at 240 nm and activity was calculated as µmol H_2O_2 consumed per min. All tests were carried out in triplicate.

Peroxidase (POX) activity

Leaf sample was homogenized in 1 mL of 0.1 M phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 12, 000 g at 4 °C for 15 min and the supernatant is used as the enzyme source. POX activity was determined as oxidation of 0.1 mM ferulic acid with 1 mM H_2O_2 and 3.6 µg of sample protein in 50 mM K-phosphate buffer, pH 6.0 at 30 °C (Hadži-Tašković Šukalović et al., 2005).

RAPD analysis of GJ-17

The RAPD analysis of GJ-17 was done along with known sensitive (GAUR-100) and resistant (Dandhi) variety for salinity of paddy. The total genomic DNA of all the three varieties was isolated. The isolated DNA was qualified and quantified by agarose gel electrophoresis and UV spectrophotometric methods. RAPD profiles were generated using 4 decameric primer (AH2, AH3, AH4, and AH5) from MWG Bangalore India. The reaction mixture contained 2µl of primer (0.3µM), 1 unit Taq DNA polymerase, 0.5 µL $MgCl_2$, 2µL 4dNTPs, 5µL 10X assay buffer, 2µL DNA sample (100ng), and adjusted to a final volume of 25µL with nuclease free water.

RAPD-PCR reaction was performed in an Eppendorff thermocycler. The standard conditions used for 35 cycles were – initial denaturation temperature at 95 °C for 1 min, denaturation temperature at 95 °C for 30 s, annealing temperature at 36 °C for 1 min, Extension temperature at 72 °C for 1 min and final extension at 72 °C for 5 min. Amplified products were analyzed on 2% agarose gel.

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).

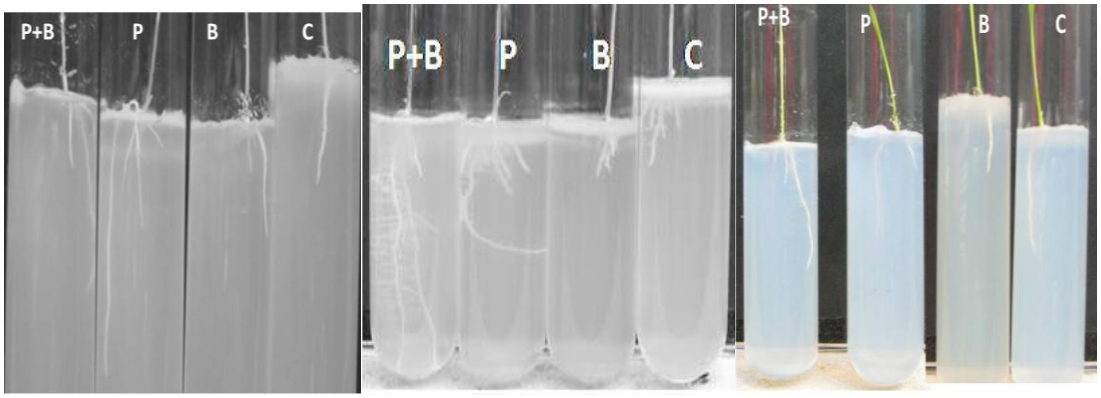

Figure:1 Effect of inoculation of *P. pseudoalcaligenes* and *B. pumilus* on the root morphology(A)at 1.5% salinity, (B) at non saline state and (C) in non inoculated plant after 7 days of plantation. (P= *P. pseudoalcaligenes,* B= *B. pumilus* and C= Control).

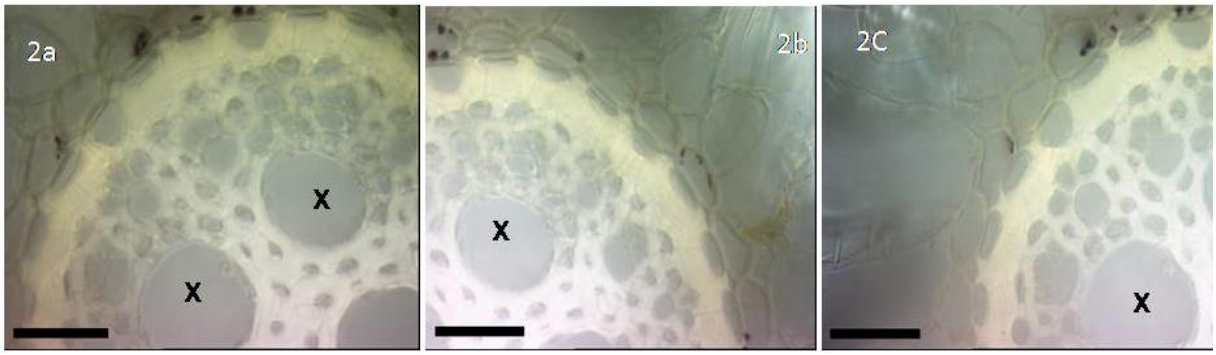

Figure:2 (a) Effect of inoculation of *P. pseudoalcaligenes* and *B. pumilus* on the root xylem (2a) at 1.5% salinity, (2b) at non-saline state, (2c) in non-inoculated at non saline state. The scale bar=25um (micron). Images are taken under 100x and the scales are calculated according to 100x magnification.

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 (Table-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* 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.

Table 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 Chlorop-hyll Content ($mg.m^{-2)}$)	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}	78.1cd	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*	.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}

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 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.

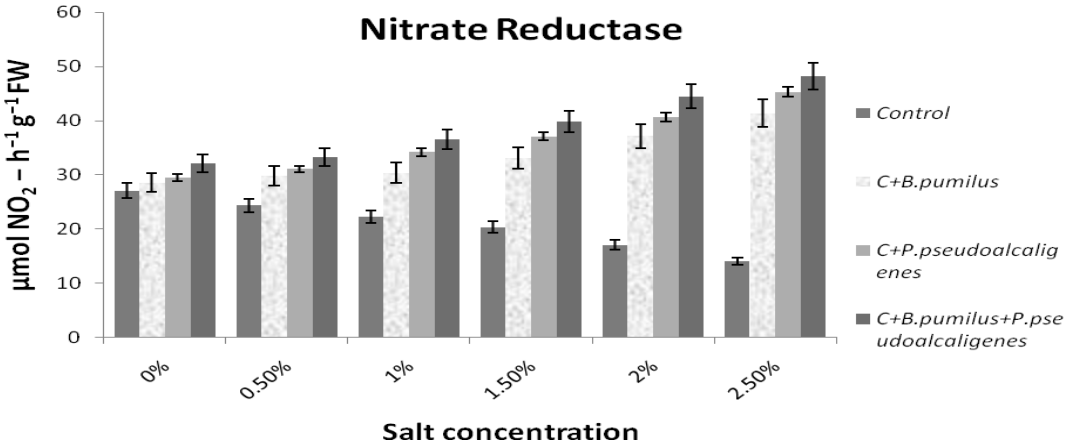

Figure 3: The effect of inoculation with *B.pumilus, P. pseudoalcaligenes* alone and in combination on nitrate reductase activity in paddy rice variety GJ-17 at five different levels of salinity (n=5).

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.

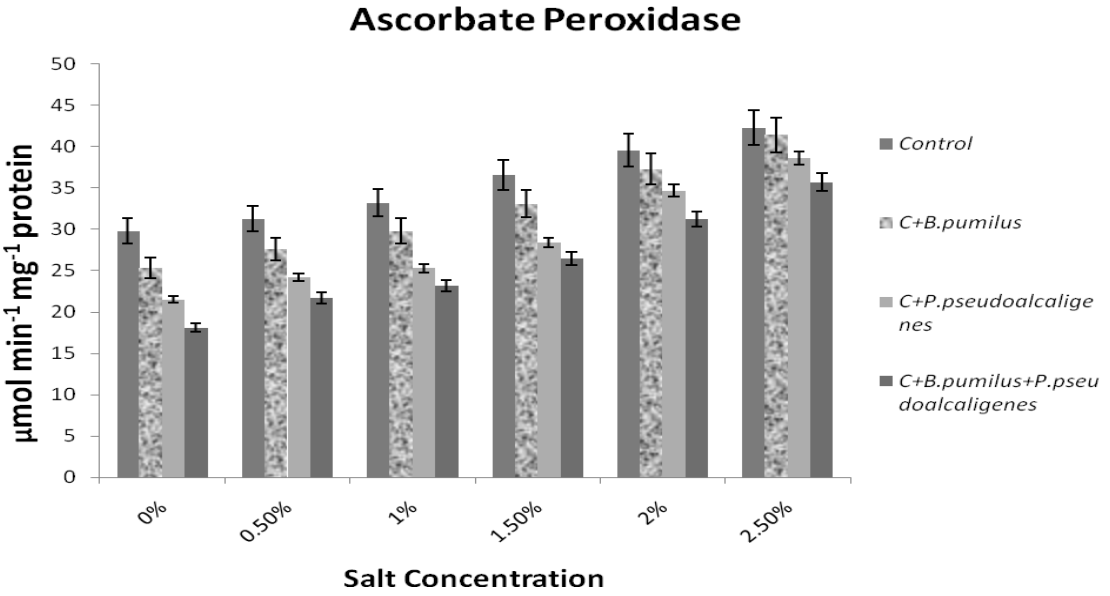

Figure4: Effect of inoculation with *B.pumilus, P. pseudoalcaligenes* alone and in combination on ascorbate peroxidase activity in paddy rice variety GJ-17 at five different levels of salinity (n=5).

Catalase activity decreased in PGPR inoculated plants at non saline state and in non-inoculated plants under salinity. Highest CAT activity was observed in the plants inoculated with both the PGPR grown at 2.5% NaCl. There was 4 -36% increased CAT activity in plants inoculated with PGPR at salinity condition (Figure-5).

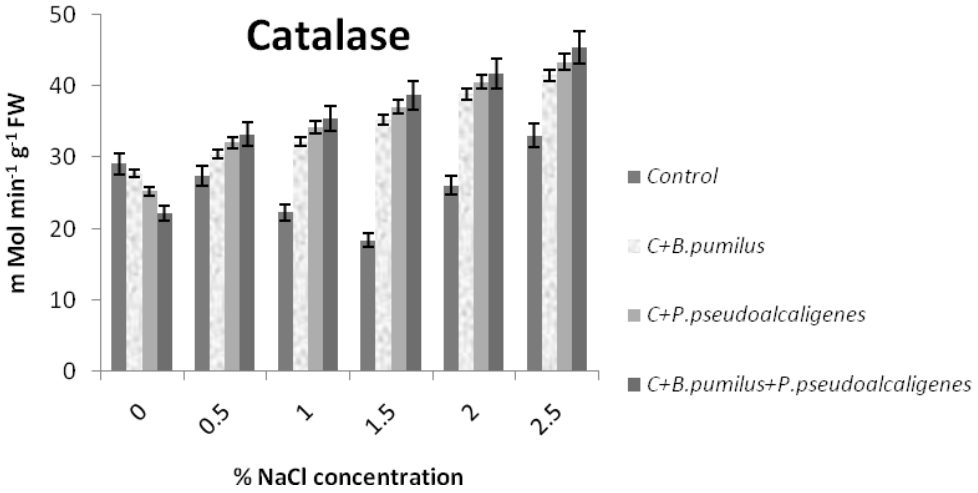

Figure- 5: Effect of *B.pumilus, P. pseudoalcaligenes* on catalase activity in paddy variety GJ-17 at five different levels of salinity (n=5).

The pattern of SOD activity was similar to that of catalase enzyme. Salinity resulted in gradual and significant increase in SOD activity in the PGPR inoculated plants. However, inoculation of PGPR resulted in decrease in SOD activity at non-saline state. Unlike catalase enzyme activity, highest increase in SOD activity at each NaCl concentration was observed in plants inoculated PGPR. PGPR inoculated plants showed increase of SOD activity by 7-69% in plants inoculated with PGPR at different salinity compared to plants at non saline condition (Figure-6).

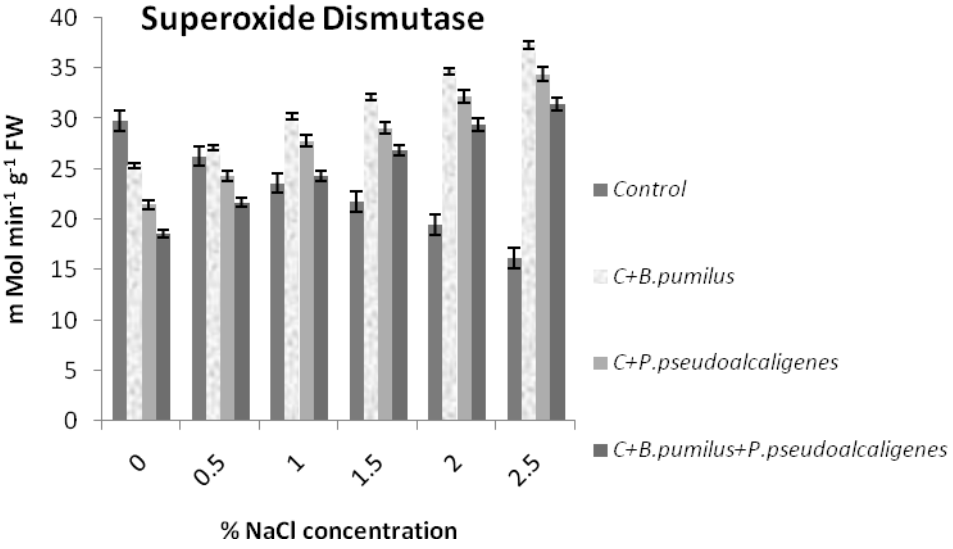

Figure- 6: Effect of *B.pumilus, P. pseudoalcaligenes* on superoxide dismutase activity in paddy variety GJ-17 at five different levels of salinity (n=5).

Peroxidase (POX) activity showed significant changes due to both salinity and PGPR inoculation. There was a direct correlation between salt concentration and POX

activity.The POX activity increased by 15-55% in plant inoculated with PGPR at non saline condition and 17-90% in non-inoculated plants at different levels of salinity compared to pure control (non-inoculated plants at non- saline condition) (Figure-7).

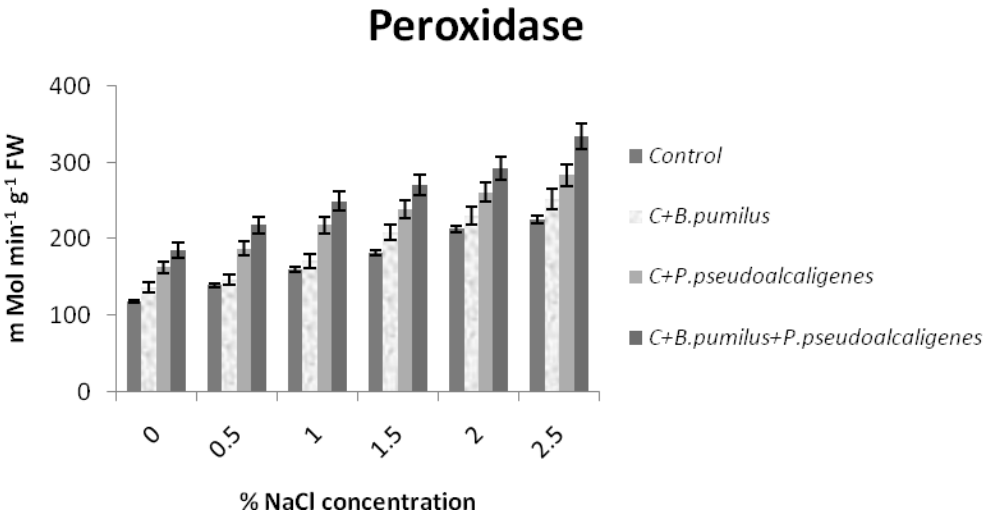

Figure- 7: Effect of *B.pumilus, P. pseudoalcaligenes* on peroxidase activity in paddy variety GJ-17 at five different levels of salinity (n=5).

RAPD analysis

RAPD analysis of GJ-17 was done with known salinity sensitive (GAUR-100) and resistant (Dandhi) varieties of paddy (Figure-8). Phylogenetic tree was constructed by using Tree explorer 2.12 clearly indicated that the paddy variety GJ-17 is related with salinity sensitive variety as shown in Figure-9.

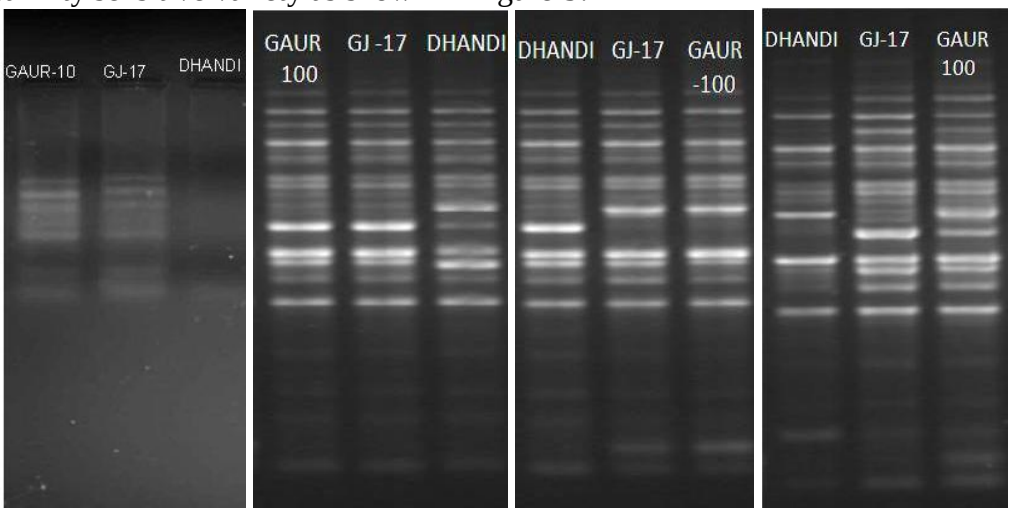

Figure-9: Agarose gels of RAPD analysis with primer AH2, AH3, AH4 and AH5 for GJ-17 with the known sensitive (GAUR-100) and resistant (dandhi) variety of paddy for salinity.

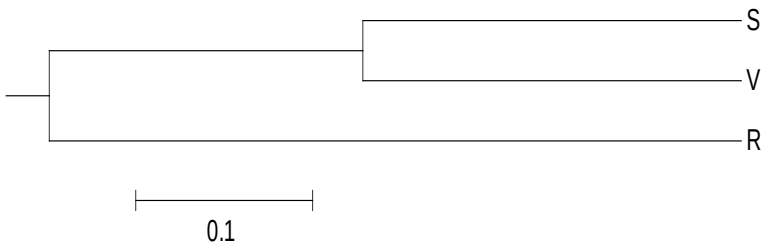

Figure-9: The phylogenetic tree constructed using Tree explorer 2.12 software indicated that the paddy variety GJ-17 (V) is closely related with sensitive variety GAUR-100 (S).

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_2 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).

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 peroxidase (POX), catalase (CAT), and membrane stability index (MSI) are common and important indices for evaluating the redox status of plants. The increased activities of antioxidant enzymes act as a damage control system and thus provide protection from oxidative stress, which otherwise could cause lipid peroxidation resulting in damage to the cell membrane and organelles, protein and DNA structure and inhibit photosynthesis and other enzyme activities. Increasing salinity stress significantly affects CAT, POX and SOD activity in the PGPR inoculated and non-inoculated plants. Han and Lee, (2005b) reported that under salinity non-inoculated plants had an increased antioxidant activity compared to the PGPR inoculated plants. The induction of antioxidant enzymes such as catalase, peroxidase and superoxide dismutase can be considered as one mechanism of salt tolerance in paddy plants. These antioxidant enzymes are involved in eliminating H_2O_2 from salt–stressed plants. In the present study, reduction in CAT activity under salt stress condition in the non-inoculated paddy plants and also in inoculated plants under non saline condition may be due to the free radical scavengers which could be attributed to the decreased H_2O_2 levels being not sufficient to activate the antioxidative property of the enzyme. Highest CAT activity was observed in the plants inoculated with both the PGPR grown at 2.5% NaCl. Kumar et al.(2003) reported that salt stress inhibited the

activities of CAT and POX but the activities of these enzymes were significantly higher in the presence of PGPR than in their absence. The POX activity increased in plant inoculated with PGPR at non saline condition and in non-inoculated plants at different levels of salinity.

The increased CAT activity at high salinity and POX activities in PGPR inoculated plants at different salinity level, point to a signaling role of H_2O_2 in the induction of H_2O_2 detoxifying enzymes in rice leaves, as reported for other abiotic stresses by Sairam et al.,(2005).

In the present study, SOD activity decreased under salinity in PGPR inoculated paddy plants may be for its recovery under free radical scavengers' due to the generation of oxidative stress by H_2O_2 and the possible inactivation of SOD as reported by Panda and Patra,(2000). PGPR inoculated plants showed increase of SOD activity at different salinity. In the PGPR inoculated paddy, decline in SOD activity were observed in this present study suggests a lesser O_2^- scavenging and dismutating capacity in this salt-sensitive cultivar and signifies a possible involvement of this enzyme in salt-tolerance, which are in accordance with Dureja,(2003).

In RAPD analysis of paddy variety GJ-17 showed more closeness with a known salt sensitive variety GAUR-100, beside that inoculation of PGPR provide ability to this paddy to survive very well upto 1% salinity level.

Salt stress may induce a combination of negative effects on salt-sensitive paddy variety including osmotic stress, ion toxicity and oxidative stress. Present study indicates that regulation of antioxidant enzymes involved in the greater effectiveness in the PGPR inoculated plant with respect to increasing the tolerance of paddy variety GJ-17 to severe salt stress.

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