Isolation and identification of the bacteria
Certified seeds of paddy variety GJ-17 were obtained from Main Rice Research Center, Navagam, Anand, Gujarat. These seeds were planted in pots and maintained for forty days. Bacterial strains were isolated from both the rhizosphere soil and endorhizosphere of rice plants grown in fields. For rhizosphere soil, clumps of soil loosely adhering to the roots were removed and roots with firmly adhering soil were suspended in sterile 0.85 g NaCl/100 ml. Then, roots were removed from these suspensions, washed thoroughly with tap water to remove adhering soil particles; surface sterilized and macerated with a mortar and pestle in sterile 0.85 g NaCl in 100 ml (endorhizosphere). Both samples were serially diluted and inoculated into vials containing 5 ml of semi-solid nitrogen-free medium (NFb) (Do¨bereiner, 1995) with 0.05 g yeast extract/100 ml. After 48 hrs incubation at 30 0C, one loop of pellicle-forming culture was transferred into fresh semisolid NFb medium. Cultures with subsurface pellicle were streaked into solid NFb (supplemented with NH4Cl) and Congo red medium (Ca´ceres, 1982). Distinct colonies were maintained on nutritive agar plates.

Phenotypic characterization of the bacterial isolates
Morphology and Gram staining were determined using a light microscope (1,000X) (Zeiss, Argentina S.A). Pellicle-forming ability and microaerobic N2-dependent growth were assessed in semisolid NFb medium with different carbon sources. For this purpose, malate was replaced by fructose, glucose, glycerol or sucrose (Hartmann and Zimmer, 1994). Motility of bacteria was observed by hanging drop method. A loopful of 2-day-old bacterial culture was suspended in 1 ml of nigrosin solution. A drop of suspension was taken on a cover slip. The cover slip was hung on a hollow slide with Vaseline. The slide was then observed under microscope to test the motility of bacteria.

Biochemical characterization of the bacterial isolates
All the isolates were biochemically characterized for utilization of metabolites and enzymes such as Urease, Lysine Decarboxylase, Ornithine Decarboxylase, Esculin, Adonitol, Rhamnose, Mannitol, Sorbitol, Cellobiose, Melibiose, Glucuronate, Mannose, Maltose, Trehalose, Indole, Malonate, Phenylalanine desaminase, Sucrose,
5-Ketogluconate, Palatinose, Galacturonate, Colistin, Coumarate, Tetra thionate reductase, α-Galactosidase, Indoxylphosphate, Raffinose, o- NitroPhenyl N-Acetyl β-D Glucosaminide, P- Nitrophenyl β - D Galactopyranoside, Oxidase tests as per the standard methods (Cappuccino, 1992).

**Bacterial DNA isolation**

Overnight grown bacterial cultures in yeast extract glucose broth were centrifuged at 10000 g for 10 min and supernatant were discarded. The remaining pellets were used for isolation followed by addition of 5 ml of extraction buffer (250mM NaCl, 25mM EDTA with pH-8.0, 0.5% SDS, and 200mM Tris-HCl). The samples were incubated at room temperature for 1 h and then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected in fresh tubes and equal volume of isopropyl alcohol was added followed by incubation at -20°C for 30 min. The mixture was centrifuged at 10000 g for 10 min at 4°C. Supernatant was discarded and to the pellet an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) was added and mixed properly followed by centrifugation at 10000 g for 10 min at 4°C. The aqueous phase was taken carefully and an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added followed by centrifugation at 10000 g for 10 min at 4°C. Once again the aqueous phase was collected and an equal volume of chilled ethanol and 3M sodium acetate were added followed by incubation for 10-20 min at -20°C. Finally pellet obtained after centrifugation at 10000 g for 10 min at 4 0°C was air dried and redissolved in TE buffer depending upon the size of the pellet.

Concentration and purity of DNA was measured by obtaining the A 260/280 nm ratio with a UV Spectrophotometer (Unicam Alpha, U.K). An aliquot of 10µl of DNA with 2µl of loading dye (40% Sucrose and 0.25% bromophenol blue) was prepared and each sample was loaded in a 0.8% agarose gel to check the purity of DNA samples. DNA concentration was adjusted to 100ng in 100µl of sterile Milli Q water for PCR reactions.

**Molecular identification of bacterial isolates**

16S rDNA analysis was used to identify the isolates. 16S rDNA universal primers 8F:5’AGAGTTTGATCCTGGCTCAG3’ and 1510R:5’GGGCTACCTTGTTACGTA3’ were used for PCR amplification of the DNA with following conditions - initial
denaturation at 95°C for 2 min, denaturation at 95°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 90 sec followed by sequencing of PCR amplicons of 16S rDNA by Applied Bio Systems ABI PRISM 377 DNA Sequencer (GeNei, Bangalore) (Cheng-Hui Xie et al; 2004). The DNA sequences were compared with the sequences obtained from the nucleotide database. The sequences were aligned with the CLUSTALW programme (Thompson et al; 1994) and evolutionary distances and Knuc values (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were not taken into consideration for comparison. Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and the maximum likelihood method in PHYLIP package (Felsenstein, 1989). The topology of the phylogenetic tree was evaluated by bootstrap resampling method of Felsenstein (1989) with 1,000 replicates. The similarity values were calculated using PAUP 4.068 PPC (Swofford, 1998).

**Characterization of plant growth-promoting mechanism**

The isolate from rhizosphere was identified as *Bacillus pumilus* strain and endorhizosphere as *Pseudomonas pseudoalcaligenes*. Their growth promotion efficiency was analyzed by their ability to solubilize phosphate, produce siderophore, indoleacetic acid (IAA), gibberellins and utilize ACC as sole nitrogen source as well as to overcome or suppress infection by producing β-1, 3-glucanases and chitinases.

**Quantitative estimation of phosphate solubilization**

Phosphate was estimated by using freshly prepared 10% ascorbic acid mixed with cold 0.42% ammonium molybdate in 1N H₂SO₄ in a ratio of 1:6 and incubated on an ice bath for at least 1 h (Ames, 1964). Potassium dihydrogen phosphate (0.1mg ml⁻¹) was used for the preparation of standard curve. This method allows the estimation of phosphate within a range 500-1500 ng ml⁻¹. The absorbance was determined at 820 nm. Cultures grown in Pikovskaya’s broth at 30°C, were centrifuged at 12,000 g. The supernatant was collected and 1 ml of the diluted sample was used to perform the assay. To the sample, 2.3 ml of the above freshly prepared reagent mixture was added and the tubes were incubated for 20 min at 45°C in a water bath. The amount of phosphate solubilized was estimated from the standard curve and expressed in mg L⁻¹. The readings were taken at an interval of 3 days.
Estimation of titratable acidity

Titratable acidity (TA) was determined by titrating 1 ml of culture filtrate against 10 mM NaOH in presence of phenoephthelein (Whitelaw et al; 1999).

Estimation of gluconic acid production

For estimation of gluconic acid released by cultures, 1 ml of culture supernatant was used. The volume was made to 5 ml with distilled water. To this 0.05 ml ammonia ammonium chloride, 0.05 ml magnesium sulphate (0.5 M) and a pinch of Eriochrome T dye were added. This solution was titrated with 0.05 M ethylene diamine tetraacetic acid (EDTA). The end point of the reaction was given by the appearance of blue color. The result was expressed in mmol L\(^{-1}\) (Welcher, 1958).

Estimation of Indole acetic acid production

Overnight grown cultures were inoculated in N-broth containing 0.2% yeast extract and 1% glucose and incubated for 24 hrs. The cultures were then grown in N-broth containing 100µl L-tryptophan stock solution (1%) for 48 hrs. After the incubation period, the cultures were centrifuged and supernatant was used for the estimation of IAA. To the supernatant, ferric chloride-perchloric acid reagent was added in 1:2 ratio for estimation of IAA produced by the cultures. The tubes were incubated at room temperature for 25 min. The absorbance was measured at 530 nm (Gordon and Weber, 1951).

Estimation of Gibberellic acid

Gibberellic acid production was estimated by colorimetric method of Hohlbrook et al; (1961). Bacterial cultures were inoculated in N-broth and allowed to grow for 24hrs at 30 °C at 150 rpm on shaker. After growth was obtained, broth was centrifuged at 10000 g for 15 min and the culture supernatant was used for estimation of GA\(_3\). The reaction mixture comprised of 15 ml of culture supernatant and 2 ml of zinc acetate. The mixture was then incubated at room temperature for 2 min and 2 ml of potassium ferrocynide was added. The mixture was then centrifuged at low speed for 15 min. To 5 ml of supernatant, 5 ml of 30 % HCl was added. The reaction mixture was incubated at 20 °C for 75 min. Absorbance was measured at 254nm.
Estimation of β-1, 3-glucanase

Bacterial cultures were inoculated in N-Broth and allowed to grow for 24 hrs at 30 °C on shaker at 150 rpm. The bacterial culture was centrifuged at 10,000 g for 20 min and the supernatant was used as enzyme source. The reaction mixture comprised of 62.5 µl of crude enzyme extract and 62.5 µl of laminarin (4%). The mixture was then incubated at 40 °C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent and heated for 5 min in a boiling water bath. The resulting dark brown colored solution was diluted with 4.5 ml of water. The enzyme activity was expressed as nmo1 min⁻¹ mg⁻¹ (Pan et al; 1991).

Estimation of Chitinase

Bacterial cultures were inoculated in N-Broth and allowed to grow for 24 hrs at 30 °C at 150 rpm on shaker. The bacterial culture was centrifuged for 20 min at 10,000 g at 4 °C and the supernatant was used for the enzyme assay. Colloidal chitin was prepared according to Berger and Reynolds (1958) method, from crab shell chitin (Sigma). The commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was desalted as described by Boller and Mauch, (1988). For the colorimetric assay of chitinase 0.01 ml of 1M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipette into 1.5 ml microfuge tubes. After 2 hrs incubation at 37 °C, the reaction was stopped by sedimentation of the content by centrifugation at 1300 g for 3 min. An aliquot of the supernatant (0.3ml) was pipetted into a glass tube containing 0.03 ml 1M potassium phosphate buffer (pH 7.1) and incubated with 0.02 ml desalted snail gut enzyme for 1h. The resulting monomer N-acetyl-glucosamine (GlcNAc) was determined following the method of Reissig et al, (1959). Enzyme activity was expressed as µmol GlcNAc equivalents’s⁻¹·g⁻¹.

Estimation of hydroxymate siderophores production

Estimation of hydroxymate type siderophores was carried out by following Mayer and Abdallah’s method (1978). 100 ml of media was inoculated with 1 ml of fresh culture and kept for incubation at 30 °C for 24 hrs in incubator. After the incubation period, the culture was centrifuged at 10,000 g for 10 min. The supernatant was diluted with
0.5 M phosphate buffer, pH 7 and absorbance was read at 400 nm. Phosphate buffer was used as a blank. Concentration of hydroxymate type siderophore was calculated by the following formula:

\[
\text{Siderophore concentration} = \frac{\text{OD} \times 1500 \times 1000}{16500} \text{ mg L}^{-1}
\]

**Estimation of catechol type siderophore**

To determine whether the siderophore contains catechol groups, Arnow’s colorimetric assay method was followed (Arnow, 1937) using pyrocatechol as a standard. The assay was performed by mixing the following in order: 1 ml culture supernatant, 1 ml 0.5 M HCl, 1 ml nitrite-molybdate reagent and 1 ml 1M NaOH. These reaction mixtures were incubated for 5 min and absorbance was measured at 500 nm.

**ACC deaminase activity assay**

To determine the ACC deaminase activity of bacterial isolates, the amount of F-ketobutyric acid (F-KA) generated from the cleavage of ACC was monitored using spectrophotometer (Penrose et al; 2001). The amount of F-KA produced during this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of F-ketobutarate. The ACC deaminase activity was expressed as the amount of F-ketobutarate produced mg \( \text{L}^{-1} \) of protein h \( \text{h}^{-1} \).

**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 hrs in sterile 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.

To study the effect of the isolated bacteria on the biochemical parameters selected, rice seedlings were transferred to culture tubes containing 400 µl Hoagland’s nutrient medium, 400 µl micronutrients and 1% agar in 40 ml distilled water.
transfer, bacterial inoculum of the isolated bacteria was added to the medium at a concentration of $6 \times 10^8$ 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 $6 \times 10^8$ cfu ml$^{-1}$. All experiments were carried out in 5 replicates. The tubes were incubated at $27^\circ$C in a 12 hrs light – dark cycle in a growth chamber.

**Visualization of association of bacteria in the paddy root**

Association of isolates within the root was confirmed by TTC staining (2, 3, 5-triphenyl-2H-tetrazolium chloride) which consisted of maleic acid and 1.5 gm of TTC in sterile potassium phosphate buffer (pH 7). Paddy roots inoculated with isolates were surface sterilized with sodium hypochlorite and were incubated overnight in the TTC stain and cross sections of root were examined under an image analyzer microscope (Carl Zeiss).

**Greenhouse study**

Plants were transferred to plastic pots containing sterilized sand-perlite (1:1) and kept in a greenhouse. The plants were irrigated with tap water and with Hoagland nutrient solution once a week. Fresh and dry weights of shoot and root were determined after 4 weeks.

**Effect of PGPRs on plant under biotic stress**

**Compatibility between bacterial isolates and fungus**

Bacterial isolates were tested for their compatibility with each other following the methods of Fukui et al; (1994). Bacterial isolates were streaked parallel to each other in Yeast Extract Glucose Agar medium and incubated at $28^\circ$C in an incubator. Compatibility was tested by overgrowth or inhibition of growth and observations were made for a period of 72 hrs. Antagonism between bacteria and fungus was also determined by inoculating both organisms on the same Yeast Extract Glucose Agar plate at $28^\circ$C in incubator for a week and antagonistic effects were tested by inhibition of growth of fungus by bacteria.
Preparation of soil for pot experiment

Soil samples were collected from wet rice fields and various physio-chemical properties; as pH, electrical conductivity, CEC, organic carbon, available nitrogen per square decimeter, available Ca, available P per square decimeter, available K per kg, Fe per kg, Zn per kg, Mn per kg, Cu per kg and salinity were analyzed by Multi Parameter Analyzer Eutech (SICART). Seven-day old rice plants were carefully removed from different test tubes inoculated with the bacterial isolates and planted in pots. Similarly, the control plants (non-inoculated) were also transferred to a fresh pot. The quantity of the soil was maintained at 5 kg per pot. Rice seedlings were planted at the rate of 4 plants per transplant and 6 transplantations per pot. Pots were watered with tap water at the time of transplantation of the rice seedlings. Five days after transplantation, they were watered again and a 2 cm water level was maintained in pots throughout the experiment.

Biocontrol under greenhouse conditions

The bacterial isolates, either alone or as a mixture, were assessed for their efficiency in suppressing rice blast disease under greenhouse conditions. The spore suspension of *Magnaporthe grisea* with a spore load of $10^4$ conidia ml$^{-1}$ was sprayed on the plants, which caused more than 75% infection under greenhouse conditions. Observations on the percent disease incidence of rice blast were recorded. In addition, growth parameters like plant height, root length, tiller number and biomass production were recorded 45 days after planting (maximum tillering) as grades 0 to 5 (Sriram et al; 1999). Disease index was calculated using the formula:

\[
\text{Disease index} = \frac{\text{Total grade} \times 100}{\text{No. of sheaths observed} \times \text{maximum grade}}
\]

Extraction of plant enzymes

Paddy leaves (1 g) were homogenized in 5 ml of 0.1 M borate buffer, pH 7.0 containing 0.1 g poly-vinylpolypyrrolidone (PVP). The homogenate was centrifuged at 10,000 g for 35 min at 4 °C. The supernatant was collected and used for the enzyme assays.

Estimation of Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) activity was measured following the method of Dickerson et al; (1984). The assay mixture containing 100 µl of plant extract, 500µl
of 50mM Tris HCl (pH 8.8) and 600 µl of 1 mM L-phenylalanine was incubated for 60 min at room temperature, and the reaction was arrested by adding 0.5 ml 2N HCl. The assay mixture was extracted with 1.5 ml of toluene by vortexing for 30 sec. Toluene was recovered after centrifuging at 1300 g for 5 min and the absorbance of the toluene phase containing Trans-cinnamic acid was measured at 290 nm against the blank of toluene. Enzyme activity was expressed as nmol trans-cinnamic acid released min⁻¹ g⁻¹ fresh weight. All tests were carried out in triplicate.

**Estimation of β- 1, 3-glucanase**

Enzyme activity was assayed by the laminarin-dinitrosalicylic acid method (Pan et al; 1991). The reaction mixture was prepared by mixing 62.5 µl of 4 % laminarin and 62.5 µl of plant extract and incubating at 40 ℃ for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent (DNS, prepared by adding 300 ml of 4.5 % NaOH to 880 ml containing 8.8 g of dinitrosalicylic acid and 22.5 g potassium sodium tartarate) with subsequent heating for 5 min in a boiling water bath. The resulting dark brown colored solution (0.5 ml) was diluted with 4.5 ml of distilled water and vortexed. Products released were estimated for reducing groups at 500 nm. The enzyme activity was expressed as 1 nmol of reducing substances min⁻¹ mg⁻¹ of fresh weight. All tests were carried out in triplicate.

**Estimation of chitinase**

One g of leaf tissue was homogenized in 5 ml of 0.1 mol L⁻¹ sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 g at 4 ℃ and the supernatant was used in the enzyme assay. Colloidal chitin was prepared by taking 2 g of crab–shell chitin (Sigma, USA). It was slowly added into 35 ml of cold concentrated hydrochloric acid with vigorous stirring and incubated at 4 ℃ for 24 hrs. The mixture was filtered through glass wool into ethanol (200 ml) at -20 ℃ with rapid stirring. The resultant chitin suspension was centrifuged at 10,000 g for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral (Roberts and Selitrennikoff, 1988). The commercial lyophilized snail gut enzyme was desalted as described by Boller and Mauch, (1988). For the colorimetric assay of chitinase 10µl of 1 mol L⁻¹ sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml microfuge tube.
After 2 hrs at 37 °C, the reaction was stopped due to sedimentation of the content by centrifugation at 1000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass tube containing 30 µl of desalted snail gut enzyme for 1 h. After 1 h the reaction mixture was brought to pH 8.9 by adding 70 µl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in boiling water bath for 3 min and then rapidly cooled in an ice bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 min at 37 °C and immediately thereafter the absorbance was measured at 585 nm. N-acetyl glucosamine (GlcNAc) was used as a standard. The enzyme activity was expressed as nmol GlcNAc equivalents min⁻¹ mg⁻¹ of protein (Boller and Mauch, 1988). All tests were carried out in triplicate.

**Estimation of Peroxidase (PO)**

Leaf sample (1g) was homogenized in 1 ml of 0.1 M phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 12000 g at 4 °C for 15 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 % H₂O₂. The reaction mixture was incubated at room temperature. The change in O.D was recorded at 420 nm at 30 sec intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min⁻¹ g⁻¹ protein (Hammerschmidt et al; 1982). All tests were carried out in triplicate.

**Estimation of Polyphenol Oxidase (PPO)**

PPO activity was determined by the method of Mayer et al; (1965). Leaf sample was homogenized in 1 ml of 0.1 M sodium phosphate buffer, pH 6.5 and centrifuged at 12000 g at 4 °C for 15 min. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µl of enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5. To start the reaction, 200 µl of 0.01M catechol was added and the change in absorbance was recorded at 30 sec interval up to 3 min at 495 nm. The enzyme activity was expressed as changes in absorbance at 495 nm min⁻¹ g⁻¹ protein. All tests were carried out in triplicate.
Field study
Field experiments were conducted to determine whether selected PGPR treatments could elicit ISR activity against target pathogen Magnaporthe grisea. Field trials were conducted for three rainy seasons from July to October 2007-2009 at a local sick agricultural field near department of biosciences, S,P University. The experimental design was a randomized complete block with three different treatments and three replications. Plots with 6 m$^2$ of area were separated by 1m; with plants in 4 rows spaced at 40cm. Plots were periodically watered and were covered with white polyethylene plastic film. Weeds were periodically eradicated either mechanically or by hand. Treatments consisted of a non bacterized control, individual PGPR strains (rhizospheric and endophytic) and mixtures of both.

Effect of PCPR on abiotic stress
Five different salinity levels were maintained by adding NaCl solution (0.5 g, 1.0 g, 1.5 g, 2.0 g and 2.5 g NaCl kg$^{-1}$ soil) to the pots. To avoid osmotic shocks, NaCl concentration was gradually increased for four consecutive days, until the desired concentration was attained. A plastic bag was kept under each pot to collect excess water due to drainage. This water was reapplied to the respective pot. All seedlings were grown for 4 weeks without any fertilizer treatment. The experiment was conducted in a greenhouse at 20 to 25 $^0$C with a relative humidity of 70 to 80 %.

Measurement of growth Parameters under different salinity level
Observations on physical parameters i.e., percentage of germination, survival and relative water content (RWC), membrane stability index and plant height were recorded. Other parameters such as dry weight, leaf greenness and photosynthetic rate were also recorded from three replicates from each treatment after 45 days of sowing the seeds. For dry weight (DW) determination, the leaves and roots were dried at 70 $^0$C for 48 hrs in hot air oven and weighed until it became constant. For standardizing data, the results were expressed as the relative reduction in comparison to the control using the following formula:

Relative reduction (%) = [(1− (salinized/control)] ×100
Estimation of Relative water content (RWC)
Fresh weight (FW) and dry weight (DW) of shoots 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; 1998).
\[ \text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100 \]
where, FW is leaf fresh weight, DW is leaf dry weight after 24 hrs drying at 70 °C, and TW is leaf turgid weight after submergence in distilled H2O for 4 hrs.

Estimation of Membrane stability index
Membrane stability index (MSI) was estimated as per the method of Sairam et al, (1997). Plant material (0.1 g) was taken in equal volume of double distilled water in two sets. One set was subjected to 40 °C for 30 min and its conductivity was recorded using a conductivity meter (C1). A 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) = \[1 - \left(\frac{\text{C1}}{\text{C2}}\right)\] \times 100

Estimation of foliar inorganic ion concentrations in paddy
Paddy plant leaf was homogenized for chemical analysis. The foliar phosphorus content was determined, after digestion in nitric–perchloric acid (5:3 v/v) for 6 hrs, by colorimetry (Murphy and Riley, 1962) and the plant potassium was estimated by flame photometry. The nitrogen concentration was determined by colorimetry after the Kjeldahl digestion. Chlorophyll was extracted by 80% acetone and its contents were determined at 663 nm and 645 nm by uv-visible spectrophotometer (Hitachi U-2000).

Estimation Glycine betaine like quarternary ammonium compounds (QACs)
Quarternary ammonium compounds (QACs) were extracted and measured as glycine betaine (GB) equivalents according to the method of Grieve and Grattan, (1983). Dried and finely ground plant leaves (0.5 g) were mechanically shaken with 20 ml of deionised water for 24hrs at 25 °C. The samples were then filtered and the filtrates were diluted (1:1) with 2N H2SO4. Aliquots (0.5ml) were taken into centrifuge tubes and cooled in ice for 1h. Cold (0.2ml) KI-I2 reagent (20 % KI-8.5 % I2) was added in
it and the reactants were gently stirred. The tubes were stored at 4 °C for 16 hrs and then centrifuged at 10,000 g for 15 min at 0 °C. The supernatant was carefully aspirated with a fine tipped glass tube. The per-iodide crystals were dissolved in 9.0 ml of 1, 2-dichloroethane and mixed vigorously. After 2 hrs the absorbance was measured at 365 nm by using a spectrophotometer. Reference standards of glycine betaine (5-200 µg ml⁻¹) were prepared in 1N H₂SO₄. All tests were carried out in triplicate.

**Estimation of Proline**

Proline was determined following the method of Bates et al; (1973). Fresh plant leaves (0.5-1.0 g) were homogenized in 10 ml of 3% sulfosalicylic acid and the homogenate was filtered. The filtrate (2 ml) was treated with 2 ml acid (3 % v/v) ninhydrin and 2 ml of glacial acetic acid, followed by 4 ml of toluene. Absorbance was read at 520 nm. All tests were carried out in triplicate. A reference standard of proline was prepared and enzyme activity was expressed in m mol g⁻¹. All tests were carried out in triplicate.

**Enzyme extraction**

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 according to Bradford, (1976) using bovine serum albumin as a standard.

**Estimation of Superoxide dismutase (SOD) activity**

SOD activity was estimated spectrophotometrically as the inhibition of photochemical reduction of NBT at 560 nm (Beauchamp and Fridovich, 1971). 3ml of reaction mixture contained 33 mmol⁻¹ NBT, 10 mmol L⁻¹ L-methionine, 0.66 mmol L⁻¹ EDTA.Na₂ and 0.0033 mmol L⁻¹ Riboflavin in 0.05 mol L⁻¹ Na-phosphate buffer (pH
7.8) and 0.1 ml enzyme from plant source. One unit of SOD was 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 mmol m⁻² s⁻¹ for 10 min. All tests were carried out in triplicate.

**Estimation of Catalase (CAT) activity**

CAT activity was assayed by measuring the initial rate of disappearance of H₂O₂ (Bergmeyer, 1970). The reaction mixture consisted of 3% H₂O₂ and 0.1 m mol L⁻¹ EDTA in 0.05 mol L⁻¹ Na-phosphate buffer (pH 7) and 0.1ml enzyme from plant source. The decrease in H₂O₂ was followed as the decline in optical density at 240 nm and activity was calculated as mmol H₂O₂ consumed per min. All tests were carried out in triplicate.

**Estimation of Peroxidase (POX) activity**

POX activity was determined according to Hammerschmidt and Kuc (1982) method. Leaf sample was homogenized in 1 ml of 0.1 M phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 12000 g at 4 °C for 15 min and the supernatant used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 % H₂O₂. The reaction mixture was incubated at room temperature. The change in O.D at 420 nm was recorded at 30 sec intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min⁻¹ g⁻¹ protein. All tests were carried out in triplicate.

**Estimation of Lipid peroxidation activity**

The level of lipid peroxidation in leaf samples was determined in terms of malondialdehyde (MDA) content according to the method of Madhava Rao and Sresty, (2000). Content of MDA, which is an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. MDA concentration was calculated from 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 (AP) activity
Acid phosphatase activity was estimated according to the method of Ferreira et al; (1998). The reaction mixture, in a final volume of 2 ml, contained 100 mM sodium acetate buffer (pH 5.0), 5 mM pNPP 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 (pNP) 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 (GR) activity
Glutathione reductase activity was measured according to Foyer and Halliwell, (1976) method. 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 NADPH, Na₄ 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 min. The specific enzyme activity was expressed in unit’ nmol mg⁻¹ protein min⁻¹ FW. All tests were carried out in triplicate.

Protein separation by SDS-PAGE
Reagents and gel (for SDS PAGE) preparation were prepared as per the method of Lamelli, (1970).

Stock Reagent Preparation:
1) Acrylamide/Bisacrylamide:

<table>
<thead>
<tr>
<th>Acrylamide</th>
<th>146.0gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisacrylamide</td>
<td>4.0gm</td>
</tr>
</tbody>
</table>

Distilled water was added to make up the volume to 500ml. The solution was filtered into a brown bottle and stored at 4°C in dark.

2) 1.5M Tris HCL, pH 8.8:

<table>
<thead>
<tr>
<th>Tris Base</th>
<th>54.45gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/W</td>
<td>300ml</td>
</tr>
</tbody>
</table>
Tris Base was dissolved in approximately 200ml of distilled water and pH was adjusted to 8.8 with 0.1N HCl and final volume was made up to 300ml. The buffer was stored at 4°C.

3) 0.5M Tris HCl, pH 6.8:

<table>
<thead>
<tr>
<th>Tris Base</th>
<th>6gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/W</td>
<td>60ml</td>
</tr>
</tbody>
</table>

Tris Base was dissolved in 50ml of distilled water and pH was adjusted to 6.8 with 0.1N HCl and final volume was made up to 60ml with the help of D/W.

4) 10% Sodium Dodecyl Sulphate:

<table>
<thead>
<tr>
<th>SDS</th>
<th>10gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/W</td>
<td>100ml</td>
</tr>
</tbody>
</table>

10 g SDS was added in 60ml of distilled water with gentle stirring and volume was made up to 100 ml with distilled water and stored at 4°C.

5) 100mg of Ammonium per sulphate was dissolved in 1ml of distilled water.

6) 2X Sample Buffer:

<table>
<thead>
<tr>
<th>D/W</th>
<th>3.0ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M Tris HCl pH 6.8</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.6ml</td>
</tr>
<tr>
<td>0.5% (w/v) Bromophenol blue in water</td>
<td>0.4ml</td>
</tr>
<tr>
<td>β Mercaptoethanol</td>
<td>0.4ml</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulphate</td>
<td>1.6ml</td>
</tr>
</tbody>
</table>

7) 5X Running Buffer:

<table>
<thead>
<tr>
<th>Tris Base</th>
<th>45.0gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>216.0gm</td>
</tr>
<tr>
<td>SDS</td>
<td>15 gm</td>
</tr>
<tr>
<td>D/W</td>
<td>3.0litre</td>
</tr>
</tbody>
</table>

Tris Base and glycine were added in an approximate volume of water and final volume was made up to 3.0 liter for preparation of 5X stock solution of running buffer. 300ml of 5X stock was diluted with 1.2 liter distilled water for one electrophoretic run.
Sample Preparation
To 50µl of protein sample 10µl of sample buffer was added and incubated at 95°C for 4 min.

Gel Preparation

<table>
<thead>
<tr>
<th>Components</th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer concentration</td>
<td>10% 12% 15% 5%</td>
<td></td>
</tr>
<tr>
<td>Acrylamide/Bisacylamide</td>
<td>8.3ml 10.0ml 12.5ml 1.0ml</td>
<td></td>
</tr>
<tr>
<td>D/W</td>
<td>9.9ml 8.2ml 5.75ml 4.1ml</td>
<td></td>
</tr>
<tr>
<td>1.5M Tris HCL pH 8.8</td>
<td>6.3ml 6.3ml 6.25ml -</td>
<td></td>
</tr>
<tr>
<td>0.5m Tris HCL pH 6.8</td>
<td>- - - 0.750ml</td>
<td></td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.250ml 0.250ml 0.250ml 0.06ml</td>
<td></td>
</tr>
<tr>
<td>10% APS</td>
<td>0.250ml 0.250ml 0.187ml 0.06ml</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01ml 0.01ml 0.0125ml 0.006ml</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>25.0ml 25.0ml 25ml 6.0ml</td>
<td></td>
</tr>
</tbody>
</table>

1) Two plates were taken and spacers were kept between the edges of the plates and were joined with the help of clamps.

2) For gel preparation, the components of separating gel as described in the table above were mixed in a glass beaker and poured between the two glass plates.

3) Gel was allowed to polymerize under a layer of water.

4) After the polymerization of gel the water layer was removed and comb was adjusted in such a way that it remains above the gel.

5) All ingredients of stacking gel as described above were mixed in a glass beaker and poured on the polymerized separating gel with the help of a pipette and was allowed to polymerize.

6) The comb was removed from the polymerized gel and samples were loaded into the wells.

7) One well was loaded with the standard molecular weight marker.

8) Electrophoresis was carried out with diluted 1X running buffer until the tracking dye reached the end of the gel.
9) Gel was removed and used for staining procedure.

**Protein detection through colloidal coomassie stain**
The gel was kept in staining solution overnight for staining and de-staining was carried out with double distilled water.

**Composition of Colloidal coomassie stain**
Solution A: 16 ml concentrated H$_2$SO$_4$ in 768ml water and 80g of (NH$_4$)$_2$SO$_4$.
Solution B: 5% CBB G-250 in 16ml of above solution (solution A).
200ml methanol was added to solution B just before use.
Final concentration of colloidal coomassie stain:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CBB</td>
<td>0.08%</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>1.6%</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>8.0%</td>
</tr>
<tr>
<td>Methanol</td>
<td>20.0%</td>
</tr>
</tbody>
</table>

Total soluble protein of the paddy seedling exposed to different concentration of NaCl was separated on 10% SDS-PAGE for the expression of low molecular weight proteins.

**Statistical analysis**
All data obtained was subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by a Lowest Standard Deviations (LSD) test. Each data point was a mean of six replicates ($n = 6$) and comparisons with $P$ values $\leq 0.05$ were considered as significantly different. Statistical analysis of the data was made with SPSS for Windows: Release 11.0-standard version.

**Molecular analysis of differential gene expression**

**RNA extraction from plant tissue**
All apparatus used were first washed with soap, then in 10 % (w/v) sodium dodecyl sulfate (SDS) and finally rinsed in water that was previously treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) to destroy RNases. Apparatus treated with 0.1% (v/v) DEPC were left in the fume hood overnight and autoclaved for 25 min at 125 °C the
next morning. The frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle that was baked at 260 °C. The total RNA was extracted from the harvested plant material following the method of by Ildiko et al; (1999).

All individual solutions used, except Tris-HCl, were prepared with Millipore purified water, treated with diethylpyrocarbonate (DEPC) (Sambrook et al; 1989) and autoclaved. RNA extraction buffer consisted of: 2% CTAB (w/v) (Sigma), 2% polyvinylpyrrolidone K 25 (w/v) (Fluka Chemie), 100 mM Tris-HCl (pH 8.0), 25 mM sodium-EDTA (pH 8.0) and 2.0 M NaCl. Just before use, β-mercaptoethanol was added to a final concentration of 2% (v/v).

A pre-warmed microfuge tube (65 °C) was taken, containing 700 µl of RNA extraction buffer. 0.1 g of plant leaves was ground in a mortar using liquid nitrogen. The powder was transferred into the pre-warmed tube consisting 700 µl of RNA extraction buffer and shaken vigorously. Extraction was done twice with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and the phases were separated by spinning the tubes in a microcentrifuge at 10,000 g for 10 min at 4 °C. To the aqueous phase, 0.25 volumes of 10 M LiCl was added and mixed well. The RNA was precipitated at 4 °C overnight. RNA was pelleted by spinning at 10,000 g at 4 °C for 20 min and RNA pellet was dissolved in 50 ml of DEPC-treated water. Re-precipitation of the RNA was done with 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volume 96% ethanol by incubating for at least 30 min at -70 °C. RNA was pelleted by spinning at 10,000 g, at 4 °C for 20 min. Pellet was washed with 70% ethanol and RNA was dissolved in 30 ml of DEPC-treated water and stored at -20 °C for further use.

The concentration of the RNA was determined (Sambrook et al; 1989). To evaluate the quantity and quality of the extracted RNA, 500 ng total RNA of each treatment was separated on a 1% (w/v) agarose gel prepared in 0.5 X TAE (20 mM Tris(hydroxymethyl)-amino-methane-hydrochloric acid (Tris-HCl), 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.28% (v/v) acetic acid) (Sambrook et al; 1989). Ethidium bromide (EtBr) was added to a final concentration of 0.5 µg ml⁻¹ to the gel to allow the visualization of the separated RNA. The running buffer used was a 0.5 X TAE solution. RNA was diluted in loading buffer having bromophenol blue 0.04% (w/v), ficol 2.5% (w/v), loaded on the gel and separated at 10 Vcm⁻¹ for 1 h.
RNA was visualized under ultra violet light illumination (302 nm) and the gel was photographed using a gel documentation system (Alpha Digi Doc RT, USA).

**Reverse Transcriptase polymerase chain reaction**
Reverse transcription polymerase chain reaction (RT-PCR) was performed using a RT PCR Kit (GeNei, Bangalore) according to manufacturer’s specifications with certain modifications. 100 nanogram mRNA was incubated at 65°C for 10 min at room temperature, subsequently with oligo (T) primers, 0.25 mM deoxynucleotide triphosphates (dNTP’s), 5 mM 1,4-dithiothreitol (DTT), 4 mM Tris-HCl pH 7.5, 20 mM KCl, 0.02 mM EDTA, 0.1% (v/v) Polyoxyethylene sorbitan monolaurate (Tween 20), 0.5µl M-MULV reverse transcriptase and 9µl nuclease free water for the synthesis of cDNA. The mixture was incubated at 37°C for 1 h. Temperature was then raised to 95°C for 2 min to denature RNA-DNA hybrid. In order to amplify β-1, 3 glucanase and Rab 18 genes two gene specific primers for RAB18, forward 5’- AGCTACGACTGCTCCCTC-3’ and reverse5’- CTGGCACATTCAGCACAC-3’; and β-1,3 glucanase forward 5’-GTGTCTGCTATGGCGTTGTCG-3’ and reverse 5’-GGTTCTCGTTGAACATGGCGA -3’; were designed. PCR amplification of cDNA was carried out in the next step of RT-PCR reaction with 2µl each of forward and reverse primers, 1.25 µl of 10x assay buffer, 1µl dNTP mix, 0.25µl Taq DNA polymerase, 3 µl cDNA and 3µL nuclease free water. The PCR conditions were: initial denaturation at 94°C for 2 min, denaturation 94°C for 1 min, annealing of primer for 1 min, extention at 72°C for 4 min and 72°C for 10 min for 30 cycles. All the PCR products were analyzed by electrophoresis in a 0.8% agarose gel (GeNei, Bangalore) with 1XTAE buffer, diluted from a 50X stock solution which consisted of Tris Base (242 gm), Glacial acetic acid (57.1 ml), 0.5 M Na- EDTA (100 ml) and 0.05% ethidium bromide and run at a constant voltage (100V) in 1X tank buffer. Documentation and photography of the gel was carried out using a Gel Documentation System (Alpha Digi Doc RT, USA). All PCRs with different primer combinations were performed in duplicate.

**DNA elution**
DNA band of interest was excised from the agarose gel using a sharp sterile blade. Gel piece was weighed and larger pieces were crushed into smaller pieces. 2.5
volumes of sodium iodide solution were added and the gel was incubated in a water bath at 50 °C - 55 °C for 2 to 3 min to solubilize it. To the solubilised gel, 15µl of glass solution was added to samples containing 5µl or less DNA. The contents were mixed thoroughly and spun at 12000 g for 30 sec. Supernatant was discarded and the pellet containing DNA was taken and wash buffer was added to remove the glass solution, vortexed and spun at 12000 g for 30 sec and again the supernatant was discarded. The tubes were incubated at 37 °C in the water bath for 10 min. For elution of DNA, the pellet was re-suspended in 1X TE buffer, vortexed and incubated at 45 °C for 5 min. This step was repeated thrice; all the fractions were pooled and finally centrifuged at 5000 g for 2 min to remove any traces of glass solution. Efficiency of elution was checked on 1.5% Agarose gel. The eluted DNA was sequenced by Applied Bio Systems ABI PRISM 377 DNA Sequencer (Xplorigen Technologies Pvt. Ltd) and sequence obtained was subjected to multiple sequence alignment using CLUSTALW software. The sequences were then translated to amino acid sequences using the ExPASy translate tool (http://au.expasy.org/tools/dna.html). The longest open reading frame (ORF) was used for a BLAST search on the GenBank website (http://www.ncbi.nlm.nih.gov/Genbank) to search for similarities at DNA and protein level with all known genes.

RNA Dot-blot assay

Preparation of labeled probes

Biotinylated-labeled probes were prepared from RNAs by incorporation of biotinylated nucleotide analogs during cDNA amplification. The first-strand reverse transcription reaction (50 mL) consisted of 1 mg of RNA, 2 mg of oligo(T) primers, 0.25 mM deoxynucleotide triphosphates (dNTP’s), 5 mM 1,4-dithiothreitol (DTT), 4 mM Tris-HCl pH 7.5, 20 mM KCl, 0.02 mM EDTA, 0.1% (v/v) Polyoxyethylene sorbitan monolaurate (Tween 20), 0.5µl M-MULV reverse transcriptase and 9µl nuclease free water for the synthesis of cDNA. The mixture was incubated at 37 °C for 1 h. Temperature was then raised to 95 °C for 2 min to denature RNA-DNA hybrid. PCR amplification of cDNA was carried out in next step of RT-PCR reaction which consisted of 2µl each of forward and reverse primers for β-1, 3 glucanase and Rab 18 genes, 1.25 µl of 10X assay buffer, 0.25µl Taq DNA polymerase, 3 µl cDNA, 0.5 mM each of biotin-dATP using SuperScript II (Gibco-BRL) dATP, dCTP, and
dGTP, 0.2 mM dTTP, and 3µL nuclease free water. The PCR conditions were: 94 °C for 2 min, 94 °C for 1 min, annealing (55 °C for β-1, 3 glucanase and 50 °C for Rab 18 genes) for 90 sec, 72 °C for 1 min and 72 °C for 5 min for 30 cycles. The biotinylated probes obtained were stored at -20°C for further use.

RNA dot blot assay

For RNA dot blots, 6 µg of RNA was applied directly to nylon membrane. The membrane was then vacuum baked for 2 hrs at 80 °C. Membrane was prehybridized in a mixture containing 50 mm NaPi, pH 6.5, 5x SSC (20x SSC is sodium citrate, pH 7.0, and 3 m NaCl), 5x Denhard’s reagent (100x Denhard’s is 2% (w/v) bovine serum albumin, 2% (w/v) polyvinylpyrrolidone, and 2% (w/v) ficoll 400), 0.1 mg ml⁻¹ denatured salmon sperm DNA, and 50% (w/v) deionized formamide at 42 °C for 3 hrs. The hybridization procedure was the same as for pre-hybridization, except that the mixture additionally contained the Biotin labeled probe. The membranes were hybridized with PCR-labeled gene specific biotinylated DNA probes and incubated overnight. The membrane was washed at 62 °C in 2x SSC containing 0.2% (w/v) SDS for 10 min, then in 1x SSC, 0.2% (w/v) SDS for 10 min, then twice 0.5xSSC, 0.2% (w/v) SDS for 10 min followed by 0.1x SSC, 0.2% (w/v) SDS for 10 min. Detection was done by incubating the membrane at room temperature with diluted HRP-Streptavidin conjugate followed by further washing. The substrate solution was added to it with gentle swirling until a blue coloured band developed. The reaction was stopped by adding distilled water. The detection reaction was according to the manufacturer’s instruction (GeNei, Bangalore). The spot density of the RNA dot blot was measured by Alpha Ease FC Imaging software (Alpha Innotech).