3. MATERIAL & METHODS

3.1. Plant material

3.1.1. Collection of wheat cultivars

Seeds of eight wheat (*Triticum aestivum* L.) cultivars – HT 41, HT 17, H715, C306, PBW343, PBW550, Gayetri (GY) and Mohan Wonder (MW) were selected for experimental purposes. The seeds of HT 41, HT 17, HT15, C306, PBW550 were obtained Borlaug institute of South Asia, CYMMIT, PUSA, Bihar, India and Seeds of PBW343, Gayetri (GY) and Mohan Wonder (MW) were collected from UBKV (Uttar Banga Krishi Vishwavidyalaya), Coochbehar, West Bengal, India. Seed viability of eight cultivars was checked before using for experimental purpose.

3.1.2. Maintenance of plants

Wheat seeds were surface sterilized with 1% (w/v) sodium hypochlorite solution and rinsed twice with double distilled water. Seeds were then transferred to plastic pots (Diameter 15 cm) containing sterile soil. Each pot contained five to seven seedlings and were kept in a growth chamber having temperature of 22/16 °C (day/night), photoperiod for 16/8 h at 200 µmol m^{-2} s^{-1} and 70 % humidity and one month old seedlings were taken for further experiments. Plants were watered regularly twice in the morning and evening, till the time of treatment (Fig. 2).

3.2. Preliminary screening of eight cultivars for basal thermotolerance

Pots containing one month old seedlings were exposed to different temperatures (25^0C, 30^0C, 35^0C, 40 ^{0}C) for 12 h and sampling was done at 0, 6 and 12 h. Each treatment had three replicate sets for each cultivars and experiment was conducted in randomized design method. For sampling, leaf samples were collected, immediately flash freezed in liquid nitrogen and subsequently used for biochemical tests, storing where necessary at -80^0C. The fresh weight of seedlings was taken immediately after collecting the leaf samples to avoid any water loss from leaf samples of both control ad treated seedlings. Basal thermotolerance level of eight cultivars were checked on the basis of changes in relative water content, total chlorophyll content, fresh weight, cell viability, malonaldehyde content, electrolyte leakage, H_2O_2 content, osmolyte accumulation and heat susceptibility index of cultivars was also calculated. The detail protocols of which are described in the later sections.

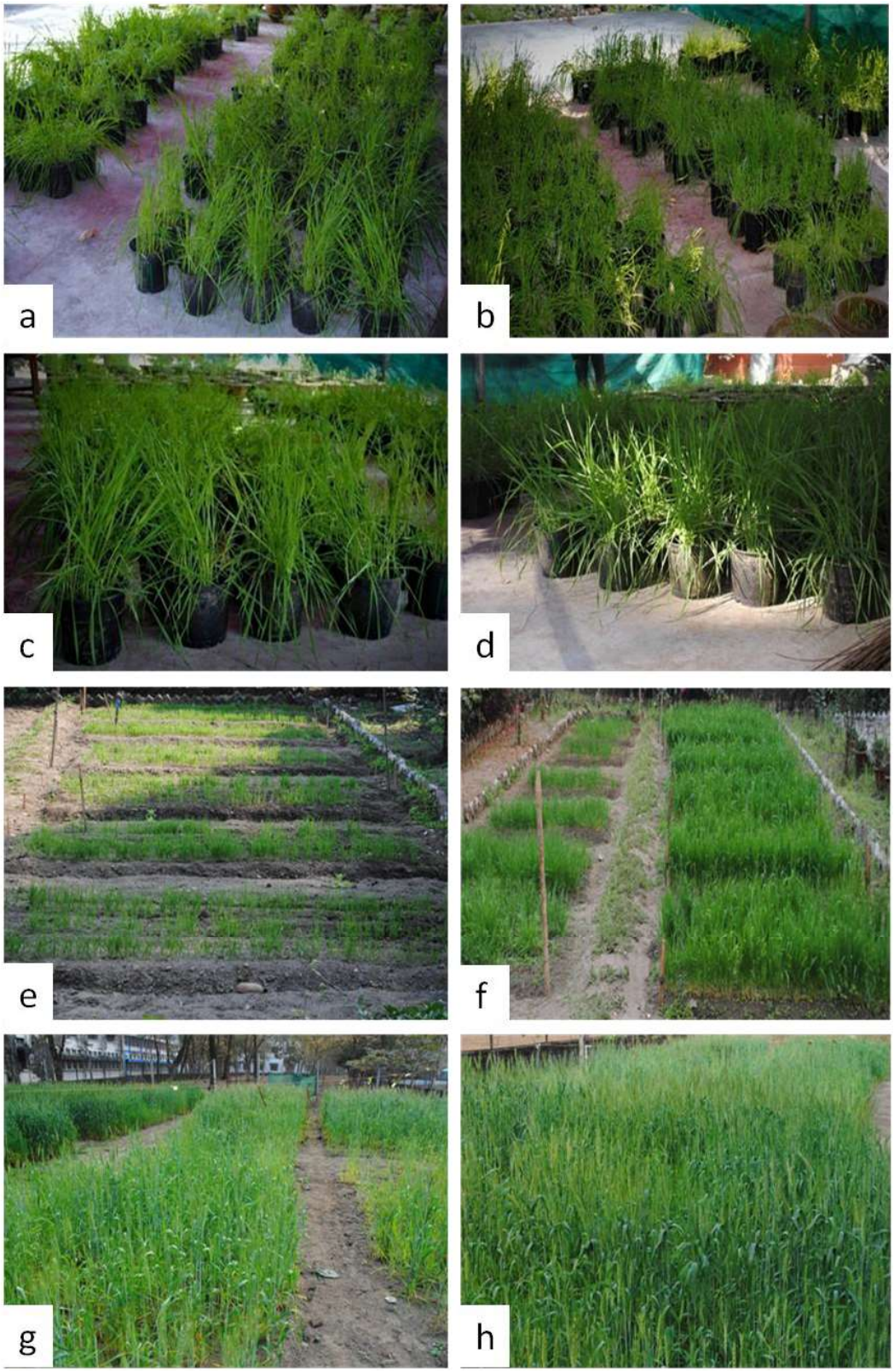

Figure 2: Different cultivars maintained in pot (a, b, c, d) and in field condition (e, f, g, h)

3.3. Selection of 2 cultivars and temperature by cluster analysis

Selection of two most susceptible cultivars for amelioration studies was based on cluster analysis of all eight wheat cultivars differing in sensitivities to high temperature, the data of RWC, proline content, soluble sugar content, MDA, EL and H_2O_2 content after heat treatment (25^0C, 30^0C, 35^0C, 40 ^{0}C) for 6 h and 12 h were chosen for cluster analysis. Hierarchical cluster analysis was performed using the CLUSTER program (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/) by the uncentred matrix and complete linkage method (de Hoon et al., 2004). Resulting tree figure was displayed using the software package, Java Treeview (http://jtreeview.sourceforge.net/) as described by Chan *et al*. 2012.

3.4. Characterization of PGPR isolates

3.4.1. Source

Two PGPR strains, *Bacillus safensis* (NCBI JX660689) and *Ochrobactrum pseudogrignonense* (NCBI JX660688), previously isolated from the wheat (*T. aestivum*) and blady grass (*Imperata cylindrica*) rhizosphere respectively and identified by Chakraborty *et al.* (2013) were selected were selected for the present study.

3.4.2. Morphology

Gram staining technique was followed for microscopic characterization of rhizobacterial cultures. Bacterial isolates were smeared separately on a clean glass slide and heat-fixed after air drying. One drop of crystal violet solution was put onto and the smear was allowed to stand for about minute. Excess stain was then washed off with sterile distilled water. After that one drop of Gram's iodine solution was put and allowed to stand for 45 sec, followed by washing with distilled water and dipping in absolute alcohol for 1 min. Eventually, one drop of safranine (counter stain) was applied, and allowed to stand for 1 min, washed gently with sterile distilled water, air dried and examined under oil immersion.

3.4.3. Scanning electron microscopy

For scanning electron microscopy of the bacterial cells, isolates were grown in Luria Bertani broth for 48 h and collected by centrifugation at 6000 r.p.m. for 15 min. The pellet was collected and washed with 0.1 M phosphate buffer saline. Then the samples

were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) followed by dehydrolysis of the samples with different gradation of ethanol starting from 30%, 50%, 70%, 80%, 90% and 100% for 10 min in each. After serial dehydration samples were subjected to critical drying then mounted on sample stab, coated with gold palladium alloy in a mini sputter coater and examined under a JEOL JSM-6610LV scanning electron microscope.

3.4.4. Assessment of growth at high temperature

The ability of the bacterial isolates to grow at different temperatures of 25^0C -60^0C was tested. For this, the two bacterial isolates were separately grown in nutrient broth at various temperatures (25^0C, 30^0C, 40^0C, 50^0C, 60^0C). Absorbance was noted in colorimeter at 600nm after 24, 48, 96, 144 h incubation following growth. Absorbance was converted into CFU/ml from a standard where known concentration of bacterial suspension was used. The CFU values were counted to log whenever needed and optimum incubation time that is when bacterial growth was found to be highest at various temperature was observed. Bacterial isolates grown at various temperatures were then transferred to nutrient agar plates with 10X dilution by pour-plating after optimum period of incubation.

3.5. *In vitro* characterization of plant growth promoting traits

3.5.1. Phosphate solubilisation

Screening of two PGPR, for phosphate solubilization was done by inoculating the two strains separately in Pikovskaya (PVK) agar medium (Pikovskaya, 1948) supplemented with tricalcium phosphate (TCP) and pH of the medium was adjusted to 7.0 before autoclaving. The plates were kept at 40±2ºC for 7 days. Appearence of transparent (halo) zones of around the colonies of microorganisms indicate phosphate solubilisation.

3.5.2. Siderophore production

Siderophore production of bacterial isolates was detected following the method of Schwyn and Neiland (1987) using indicator blue dye, chrome azurol S (CAS). To prepare CAS agar, CAS (60.5 mg) was dissolved in 50 ml water and blended with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl) and volume made up to 1L. With constant stirring this solution was added to 72.9 mg hexadecytrimethyl

ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile Petri plates (20 ml /plate). The plates were inoculated with the bacteria and incubated for 10-15 days till any change in the color of the medium was observed.

3.5.3. Protease production

Protease production was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh *et al.* (1995). Isolates were inoculated and kept at 40±2ºC and clear region around the bacterial colony signified the enzymatic degradation of protease.

3.5.4. IAA production

10 ml of Davis Mingoli's broth containing 0.1% tryptophan was inoculated with the isolates and incubated at 37º C for one week. The culture was then centrifuged at 10,000 r.p.m for 15 min and supernatant was taken for analysis. The supernatant was layered carefully with 2 ml of Ehrlich- Bobme (Pdimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the supernatant- reagent interface indicating the production of indole.

3.5.5. Starch hydrolysis

The bacteria were streaked on starch agar plates (NA + 0.1% soluble starch) and incubated for 5 days at 37°C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.6. Fungal Culture

3.6.1. Isolation

The fungal pathogen was isolated from naturally infected wheat leaf tissue. For that infected leaf were thoroughly cleaned in sterile water and then wiped with 80% ethanol, followed by exposure to an alcohol flame for a few seconds. The outer layer of tissues was quickly removed by a flame sterilized scalpel. Small pieces from the central core of tissue in the area of the advancing margin of infection were removed by a sterilized scalpel and sterilized by dipping into 90% alcohol then flamed for a few seconds. The sterilized tissues were transferred to potato dextrose agar in Petri dishes and incubated at

28ºC for 1 week. The fungal mycelium grown was transferred to PDA slants and kept for further identification. The isolated fungi were allowed to grow in Petri plates (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspension was prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lacto phenol- cotton blue, covered with cover slip and sealed with wax. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

3.6.2. Completion of Koch's Postulate

Fresh healthy wheat leaves were inoculated with conidial suspension of the isolated fungal pathogen following detached leaf inoculation technique. After 96 h of inoculation, the infected leaves were cleaned thoroughly, cut into small pieces, disinfected with 0.1% $HgCl_2$ solution for 3-5 min washed several times with sterile distilled water and transferred aseptically into Potato Dextrose Agar (PDA) slants and incubated at 28ºC for 7 days. Nature of mycelia growth, rate of growth and time of sporulation of these isolates were observed and identity of the organism was confirmed by comparing with the stock culture.

3.6.3. Scanning electron microscopy

Spores of fungal pathogens were examined under scanning electron microscopy (SEM). Selected fungal spores were sonicated under 35 MHz to followed by washing five times in sterile distilled water, surface disinfected with 4% (wt/vol) chloramine-T and 300 ppm of streptomycin for 1 h, and then rinsed a further five times in sterile distilled water and were stored in eppendorf's tube in room temperature. Each sample was placed within separate aluminium "disc cup" (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-r5 Kev.

3.6.4. Molecular identification

3.6.4.1. Extraction of DNA

Total genomic DNA from fungal mycelia was extracted by following method of Kuramae Izioka (1997). 6-7 days old fungal mycelia grown in potato dextrose broth was harvested and ground into the fine powder using liquid nitrogen and suspended in 700 µL extraction buffer (1M Tris-HCl pH 8.0, 5M NaCl, 0.5 mM EDTA, pH 8.0 ,10% SDS). Upon homogenization, the tubes were incubated for 30 min at 65^0C. DNA samples were purified with equal volumes of chloroform: isoamyl alcohol (24:1) mixture (1X), and precipitated with isopropanol. The tubes were centrifuged at 10000 r.p.m for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried. The pellets was resuspended in 100 µl 1 X TE buffer and incubated at 37^oC for 30 min with RNAse (60µg/ml). The sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol after incubation. The mixture was centrifuged at 10,000 r.p.m. for 5 min at 4ºC, the pellet was air dried and finally dissolved in 40 µl TE buffer (pH 8.0) and stored at 4ºC.

3.6.4.2. Quantification

The genomic DNA of selected isolates was checked for their purities by A260/A280 ratio. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The reading at 280 nm gives the amount of protein in the sample. Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 to 2.0, respectively.

The quality of the genomic DNAs were also checked on 0.8% agarose gel electrophoresis. For this purpose, 1X TAE buffer was prepared from 50X TAE buffer by dilution. 0.8% agarose was prepared in 100 ml of 1X TAE buffer by melting in a microwave oven. 2 µl ethidium bromide was then added after cooling the agarose solution. After cooling, the solution was poured in to a casting tray with a comb and allowed to solidify at room temperature. DNA sample was prepared by adding 1 µl of tracking dye to a volume of 5 µl sample.

The gel was horizontally placed on the tray and the electrophoresis chamber was filled with fresh running buffer (TAE) to cover the gel. The wells were loaded with DNA samples with the help of the pipette and 60V current was applied for the purpose of electrophoresis.

3.6.4.3. ITS- PCR analysis

For 18S rDNA amplification in a total volume of 100 µl reaction mixture, 78 µl deionized water, 10 µl 10X Taq polymerase buffer, 1 µl of 1U Taq polymerase enzyme, 6µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

3.6.4.4. Sequencing of rDNA and Identification

The PCR products were then sent for sequencing to Scigenome, Kerala, India. . DNA sequencing was done bidirectionally using the ITS primer pairs. For the confirmation of identification, sequences obtained from PCR products were subjected to BLAST analyses and aligned with extype isolates sequences from NCBI GenBank. The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the sequence after complete annotation and given accession numbers. Phylogenetic analysis was done in MEGA 4.1 software (Tamura *et al.* 2004).

ITS- PCR primers: The following primers were used to amplify ITS regions

Name	Primer Sequence (5'-3')	GC %	Length	TM Value
ITS-1	TCTGTAGGTGAACCTGCGG	57	19	63.0 ^{0}C
ITS-4	TCCTCCGCTTATTGATATGC	45	20	61.5 ^{0}C

3.7. Serology

3.7.1. Preparation of antigen

3.7.1.1. Leaf antigen

Antigens from healthy and infected leaves were prepared following the method of Chakraborty and Saha (1994). Fresh, young leaves were weighed and crushed in mortar and pestle with 0.05M Sodium phosphate buffer supplemented with 10mM Sodium metabisulphite, 2 mM PVP (soluble) and 0.5 mM magnesium chloride (pH 7.2). At the time of crushing with sea-sand was used. The leaf slurry was strained through a muslin

cloth and then centrifuged (10,000 r.p.m) for 30 min at 4°C. The supernatant was used as healthy leaf antigen and was kept at -20°C until required.

3.7.1.2. Fungal antigen

Mycelial protein was prepared following the method as outlined by (Chakarborty and Saha, 1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4 ºC and then centrifuged at 10,000rpm for 30 min at 4 ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

3.7.2. Raising polyclonal antibody

3.7.2.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal antigens in New Zealand white male rabbits of approximately 2 kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera reserves for plant pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. After each bleeding they were given saline water for three consecutive days and kept in proper hygienic conditions.

3.7.2.2. Immunization

Method of Alba and Devay (1985) and Chakraborty and Saha (1994) were followed for immunization. Before immunization, normal sera were collected from each rabbits. For developing antisera, mixture of 1 ml antigen (protein extracted) and 1ml of Freund‘s complete adjuvant (Genei) was injected into each rabbit in intramuscular region 7 days after pre- immunization bleeding and doses were repeated every 7 days intervals for consecutive weeks. From the second week, mixture of 1 ml antigen (protein extracted) and 1ml of Freund‘s incomplete adjuvant (Genei) was injected every 7 days interval upto 12-14 consecutive weeks as required.

3.7.2.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. To grip during blood collection, the rabbits, they were put on their back on a wooden plank fixed at an angle of 60º, and held the rabbits tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 -10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at 37ºC for 1hr for clotting. After clotting; the colt was loosened with a sterile needle. Finally, the serum was classified by centrifugation. (2000g for 10 minute at room temperature) and distributed in 1 ml vials and stored at -20ºC as crude antisera. The serum was used for double diffusion analysis, dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.7.3. Immuno assay

3.7.3.1. Dot immuno binding assay (DIBA)

Method suggested by Lange *et al.* (1989) was followed to perform Dot immune binding assay. Following buffers were used for dot immunobinding assay.

I. **Coating buffer** - Carbonate - bicarbonate (0.05 M, pH 9.6)

II. **Washing) buffer (TBST)** -Tris buffer (10 Mm, pH 7.4) with 0.9% NaCl and 0.5% Tween 20

III. **Blocking solutions**- 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3)

IV. **Alkaline phosphatase buffer** - (100mM Na phosphate buffrer, 100 mM tris HCl, 100 mM NaCl, 5mM $MgCl_2$, pH 7.4)

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate- bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Antigen (5µl) was loaded on to nitrocellulose membrane and allowed to dry for 30 min at room temperature. Blocking of nitrocellulose membrane was done with

19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker after removing the template. Respective polyclonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at 4ºC for overnight. The membrane was then washed gently in running tap water for three min, thrice followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2 h at 37ºC. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.7.3.2. Enzyme linked immuno sorbent assay (ELISA)

Plate trapped antigen coated (PTA)-ELISA was performed following the method as described by (Chakraborty *et al.* 1995) with modifications. Antigen were diluted with coating buffer and the antigens were loaded (200µl per well) in ELISA plate (Coaster EIA/RIA, strip plate, USA) arranged in12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25 ºC for 4 h. Then the plate was washed four times under running tap water and twice with PBS-Tween and each time shaken well to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and plate was incubated at 25ºC for 1h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200 µl per well) and incubated at 4ºC overnight. After washing, antirabbit IgG goat antiserum labeled with alkaline phosphatase diluted 10000 times in PBS, was added to each well (100 µl per well) and incubated at 37 ºC for 2 h. The plate was washed, dried and loaded with 200 µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 1 h. color development was stopped by adding 50 µg per well of 3 N NaOH solution and the absorbance was determined in an Multiscan Ex (Thermo Electron) ELISA Reader (Multiskan, Thermo Labsystems) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.7.3. 3. Immunofluroscence

Immunolocalization of disease as well as chitinase and β 1, 3 glucanase enzymes was detected by indirect immunefluorescence staining of healthy and infected leaf tissues following the method of Chakraborty and Saha, 1994. Cross section of healthy, infected

and treated roots were cut and immersed in PBS (pH 7.2). These sections were treated with primary antibody raised against *B. sorokiniana*, β 1,3 glucanase and chitinase enzyme specific antibody which was diluted (1:50) in PBS and incubated for 1 hour at room temperature. After incubation, section were washed thrice with PBS- Tween pH 7.2 for 15 min and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescence (FITC).The sections were incubated for 30 minutes in dark. After that sections were washed thrice with PBS- Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Fluroescence of the tissue sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.7. 3. 4. Immuno gold localization

Transverse sections (1m of healthy and infected plant leaf tissues were sliced into 2 x 2 mm and fixed in 0.1M sodium phosphate buffer (pH 7.4) containing 2.5% Glutaraldehyde in eppendorf tubes for 2-12 h at room temperature. In the next step dehydration was done in ascending grades of alcohol at intervals of 30 mins in 4°C (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 h interval each at 4° C in PLT-272(M) Fume Hood (Tanco). Then infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1 h each at 4° C. The samples were dipped in LR White and kept overnight at 4° C. They were kept at room temperature for 3 h. A fresh change of LR white was done and kept at 56° C for36 hrs. Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM60) fitted with a rotating milling cutter. Ultrathin sections (60nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labelling. The grids containing ultrathin sections were then floated in blocking solution containing 2% skimmed milk agar for 30 min. Primary antibody was diluted in 1% fish gelatin in the ratio 1:20. Grids were incubated the primary antibody raised against *B. sorokiniana*, β 1, 3 glucanase and chitnasse enzyme specific antibody for 24 h at 4°C. Grids were washed on drops (100 µl) of fish gelatin pipetted on to parafilm 10X2 min. Grids were incubated with anti-rabbit IgG (Whole Molecule) gold antibody produced in goat affinity isolated antibody (Sigma-G7402) diluted in 1:5 in fish gelatin at room temp for 3 h. Sections were stained with 2% uranyl acetate for 15 min. The sections were washed in double distilled water. Post stain was done in 0.2% lead acetate for 5 min. Washed again in double distilled water. Ultrastructural analysis of the section was

performed with Morgagni 268D with iTEM Imaging System. Specificity of labeling was assessed by the control test by incubating sections with rabbit pre-immune serum instead of the primary antibody.

3.8. Assessment of plant growth promotion following PGPR priming

Growth promotion in seedlings was assessed by comparing the increase in height, root length, shoot length, relative water content and dry biomass as well as yield of the treated plants to the untreated control plants. The experiments consisted of at least three replicates in each treatment incompletely randomized design. Estimation of total phosphate content in soil, leaf and plant tissues was carried out following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988).

3.9. Antagonistic test of PGPR against fungal pathogen

For antagonistic tests the bacterial isolates were streak at a distance of 3.5 cm from rim of individual Petri plate containing PDA medium. A 6 mm mycelial disc from a 7 day old PDA culture of fungal pathogen was then placed on the other side on the Petri dish and the plates were incubated ay 28^0C for 7 days (Rabindran *et al.* 1996). Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria.

3.10. Partial characterization of antifungal compounds

3.10.1. Extraction of cell free culture filtrate

The antagonistic isolate was grown in nutrient broth medium for 48 h at 37°C in shaking condition. Centrifugation was done at 15000 r.p.m. for 20 min and supernatant was collected and passed through the micro filter (0.22 μm pore size). The cell free culture filtrate (200 ml) was extracted separately with equal volumes of ethyl acetate. The culture filtrate was mixed with equal volumes of organic solvent and taken in separating funnel. The mixture was shaken vigorously and allowed to stand for few minutes and observed for the separation of two liquid phases. The ethyl acetate ethyl acetate fraction was collected in beakers. The extraction procedure was repeated thrice. The ethyl acetate fraction was evaporated to complete dryness in a rotary evaporator at room temperature and used for GC-MS analysis.

3.10.2. GC-MS analysis of crude cell free extract

Identification of the antimicrobial metabolites was done by Gas Chromatography-Mass Spectrometry (GC-MS) analysis with GCMS-QP2010 SE GC System. 1µL of sample solution was injected into the GC system provided with HP 5 Ms column at 220°C and high pure helium was used as a carrier gas at a flow rate of 1.21ml/min. For GC–MS the GC oven was held at 20°C and then ramped from 20°C to 280°C at 10°C/min. ACQ top double focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-3333 software was used for all analyses. High resolution mass spectra were taken at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 40 to m/z 650 at 1 second per scan. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum of NIST (National Institute for Standards and Technology) library of the corresponding organic compound.

3.11. PGPR application and experimental design

Two PGPR, *Bacillus safensis* (NCBI JX660689) and *Ochrobactrum pseudogrignonense* (NCBI JX660688) were grown separately in nutrient broth (Himedia for 48 h at 35^0C. The cultures were centrifuged at 5,000 rpm for 15 mins, supernatants were discarded and the pellets were dissolved in equal amount of distilled water to make bacterial suspension concentration 1x 10^8 CFU/ ml. Few drops of tween 20 was added to each as adhesive. Then surface sterilized seeds were dipped in the aqueous suspension of each PGPR strains for 12 h. After that both primed and control seeds were sown in 15 cm black plastic pots containing sterilized soil allowed to grow for one month in green house condition having temperature of 22/16 °C (day/night), photoperiod for 16/8 h at 200 µmol m^{-2} and 70 % humidity and one month old seedlings were used for experiments. From initial trials using different temperatures, 40°C proved to provide irreversible damaging impact on plants with less than 50% survival rate after 24 h in plants and therefore 40^0C was selected as treatment temperature for further observation.Seedlings of both primed and unprimed plants were exposed to 40^0C for 12 h and sampling was done at 0, 4, 8 and 12 h. Each treatment had three replicate sets and each replicate set had 10 pots of replications in randomized design. For sampling, leaf samples were collected, immediately flash freezed in liquid nitrogen and subsequently used for biochemical tests, storing where necessary at -80^0C.

To evaluate effect of high temperature on seed quality of PGPR primed and unprimed plants in field condition, plant material was examined in two sowing date's i.e. normal planting (20^{th}October) and late planting (20^{th} January) during growing season of 2015-17. The effect of heat stress was assessed on three yield related traits (spike length, spikelets spike^{-1}, grains spike^{-1} and 1000-grain weight). Soluble protein and starch content in seeds was measured. SEM analysis of seeds was perfromed. The current experiment was carried out in randomized complete block design having three replications. Monthly average humidity, minimum and maximum temperature was recorded throughout three consecutive years. All the required cultural operations were adopted uniformly in both the plots throughout the growing period as and when required.

3.12. Disease assessment

3.12.1. Inoculum preparation

Bipolaris sorokiniana strain WH.PBW.IP.04 (NCBI - KM06649) previously isolated form naturally infected PBW 343 cultivar and identified was used for screening of wheat cultivar against spot blotch disease. For sporulation the fungal was grown on potato dextrose agar medium (PDA) for 7 days at 30^0C. Spore suspension was prepared following method of Shamim *et al.* (2010) and one drop of tween 20 was added per 100 ml of spore suspension as adhesive.

3.12.2. Pathogen inoculation and experimental design

One month old seedlings of both PGPR primed and unprimed seedlings were inoculated by uniformly spraying spore suspension containing 1×10^3 conidia/ml. For making adequate moisture which is needed for infection development, pots were enveloped with polyethylene bags right away after inoculation till symptom development. There are two sets of experiment each having 6 treatment combinations (unprimed healthy, *B. sorokinina* infected, *B. safensis* primed healthy, *B. safensis* primed infected, *O.pseudorignonense* primed healthy, *O.pseudorignonense* infected). Each treatment had three replicate sets and each replicate set had 10 pots of replications in randomized design. One of the sets was exposed to 38 ± 2^0C and other set was kept at normal temperature (25 ± 2^0C) simultaneously for 96 h. Disease incidence was measured at 48, 72 and 96 h. For biochemical analysis, leaf samples were collected at 12, 24, 48 and 72 h interval and immediately flash frozen in liquid nitrogen and subsequently used for biochemical tests, storing where necessary at -80^0C.

3.12.3. Assessment of spot blotch disease

Disease assessment was conducted after 48, 72, 96 h of inoculation on the basis of appearance of infection on leaves. The disease severity was estimated in terms of lesion number per leaf and percentage disease index was calculated using 0-5 scale [0%= no infection/ immune; 0-10%= resistant response (R); 10.1-20.0%= moderately resistant (MR); 20.1-30.0%=moderately susceptible (MS); 30.1-50.0%= susceptible (S) and >50.0%= highly susceptible (HS)] as described by Adlakha *et al.* (1984). Percentage disease index (PDI) was calculated as follows-

$$\text{Percent disease index (PDI)} = \frac{\text{(Class rating x class frequency)}}{\text{(Total no.of leaves x maximum rating)}} \text{ x } 100$$

3.13. Assessment of biochemical changes in wheat

3.13.1. Relative water content

Relative water content (RWC) was assessd following the method described by Barr and Weatherley (1962). Fresh leaf samples from control and different treatment sets was weighed to obtain fresh weight (F.W.) and placed in a glass vial, slightly longer than the sample, with its basal part to the bottom. The samples were then immediately hydrated to full turgidity for 4 h under normal room light and temperature. After 4 h, the samples were taken out of water and well dried of any surface moisture with filter paper and immediately weighed to obtain fully turgid weight (T.W.). Samples were then dehydrated at 80^0C for 24 h in oven and weighed after being cooled down to determine dry weight (D.W.). RWC was then calculated by the following equation:

$$\textbf{RWC (\%) = [(F.W. - D.W.) / (T.W. - D.W.)] × 100}$$

3.13.2. Heat susceptibility index (HSI)

HSI was calculated by using Variation in fresh weight and RWC (degree in percent decrease) using following formula (Hameed *et al.* 2012).

$$\textbf{HSI = (\% reduction in seedling FW+ \% reduction in RWC)/ 2}$$

3.13. 3. Cell viability

Cell viability percentage was determined by MTT assay (Chen *et al.* 1982). 10 mm leaf disc were kept in glass vials with 1% MTT 3-[4,5-dimethyltiazol-2-yl] 2,5-

diphenyltetrazolium bromide] solution in dark for 12 h. Leaf samples were placed in 5% alcohol and kept for boiling till alcohol evaporated. Thereafter absorbance of the purple coloured extract was measured at 485 nm.

3.13.4. Leaf disc bioassay

Leaf disc bioassay was carried out following the method of Amano *et al.* (2012) with sligh modification. The healthy and fully expanded youngest leaves from the plants were briefly washed in deionized and 1 cm diameter leaf discs were finely cut and floated in 5 ml sterile distilled water and subjected to heat shock for 12 h at 40^0C. Leaf disc senescence bioassay reveals degree of susceptibility of eight wheat cultivars, represented in terms of degree of decoloration of leaf discs after 12 h of heat treatment at 40^0C in comparison to leaf discs kept in water in normal temperature.

3.13. 5. Cell ultrastructure observation by TEM

For TEM analysis, modified protocol of Campbell *et al.* (1990) was followed. For visualization of ultrastructural changes after heat treatment leaf samples were sliced into 2 x 2 mm and fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2% paraformaldehyde in for 12 h at 4^0C. After cleaning in phosphate buffer leaf pieces were fixed with 2% OsO_4 and then dried in ethyl alcohol and propylene oxide. Leaf slices were then implanted in LR white resin blocks and were cut with an ultra microtome (Leica UC6). Then ultra thin sections, stained with uranyl acetate and lead citrate were observed under transmission electron microscope (Technai).

3.13.6. Electrolyte leakage

Electrolyte leakage was measured as described by Lutts *et al.* (1996). Leaves of similar size were collected from four plants for each treatment and washed thoroughly with deionised water to remove surface-adhered electrolytes. The samples were placed in closed vials containing 10 ml of deionised water, incubated at 25°C on a rotary shaker for 24 h. Subsequently, the electrical conductivity of the solution (L_t) was determined conductivity meter. The samples were then autoclaved at 120°C for 20 min and cooled at 25°C before determining the final electrical conductivity (L_0). Electrolyte leakage was defined as follows:

$$\textbf{Electrolyte leakage (\%)} = (L_t / L_0) \times 100$$

3.13.7. Membrane lipid peroxidation

Membrane lipid peroxidation was measured following the method of Heath and Packer (1968). 0.5 g leaf sample was homogenized in 0.1 % (w/v) TCA and centrifuged for 10 min at 10,000 rpm. For estimation, 0.5 ml of the supernatant was mixed with 2 ml of 0.5 % (w/v) TBA in 20% TCA, followed by heating for 30 min at 95°C and cooling it on ice. The absorbance of the reaction mixture was determined at 532 and 600 nm. MDA content was then calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

3.13.8. Carbohydrates

3.13.8. 1. Extraction of total and reducing sugars

Total and reducing sugar was extracted following the method of Harborne (1973). 1 g of leaf and root tissues was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. The residue was reextracted and the process was repeated. The aqueous fraction was redissolved in distilled water and made up to 5 ml which was then centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for estimation.

3.13.8.2. Estimation of total soluble sugars

Estimation of total sugar was done by anthrone reagent following the method of Plummer (1978). To 1 ml of test solution, 4 ml of anthrone reagent was added (0.2% Anthrone in conc. H_2SO_4). The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10mins. Then the reaction mixture was cooled under running tap water and absorbance was measured in a colorimeter at a wavelength of 620 nm and quantified by using a standard curve of glucose.

3.13.8. 3. Estimation of reducing sugar

Reducing sugar was estimated by Nelson-Somogyi method as described by Plummer (1978). 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution (2 g $CuSO_4$, 12 g Na_2CO_3 anhydrous, 8 g Na^+-K^+ tartarate, 90 g Na_2SO_4 anhydrous in 500 ml of distilled water) and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's arseno molybdate reagent was added to the reaction mixture along with 2 ml of distilled water and mixed vigorously. A blue colour was developed, the absorbance of which was

the measured in a colorimeter at 515 nm and quantified using a standard curve of glucose.

3.13.8.4. Extraction and estimation starch

The extraction of starch from leaf and seed from the sample plant was done by homogenizing 1g of the plant tissue in hot 80% ethanol by the method described by Thimmaiah (1999) with some minor modifications. The homogenized mixture was filtered using Whatman no. 1 filter paper and extraction was done for 3–4 times from the residue by repeated washing using hot 80% ethanol till the washings did not give any colour with Anthrone reagent (0.2% anthrone in conc. H_2SO_4) and the residue was kept. The residue was then dried over a water bath at about 70°C. To the dried residue, 5mL of distilled water, 6.5mL of 52% perchloric acid was added which was then centrifuged for 20 minutes at about 5000 rpm. This process was repeated twice and the supernatant was pooled and kept separately. The final volume was made up to 25mL. Estimation of starch was done using the method as given by Thimmaiah (1999) in which 1mL of test solution was reacted with 4mL of Anthrone reagent (0.4% in conc. H_2SO_4) and mixed thoroughly. The mixture was then placed in a boiling water bath at 100°C for 8 minutes taking proper precautions and cooled under running tap water. The absorbance was measured at 630nm in a colorimeter against a proper blank. Starch content was then calculated from the standard curve of starch solution.

3.13.8.5. Scanning electron microscopy of starch grain and endosperm

For visualization and comparative analysis of ultrastructural changes of starch grains and endosperm in late planted control and PGPR primed plants after exposure to elevated temperature seeds were observed under scanning electron microscope.

For scanning electron microscopy 1 mm transverse sections of mature seeds were cut and transferred to 0.1 M phosphate buffer (pH 7.2). Subsequently transverse sections of seeds were fixed in primary fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (ph 7.2) for 8- 12 h at 4^0C. The samples were washed thrice after every 1 h with 0.1 M phosphate buffer (pH 7.2). Then seed transverse sections were dehydrated with graded ethyl alcohol series from 10-100%, followed by critical point drying. For SEM analysis samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold and examined under scanning electron microscope.

3.13.9. Phenols

3.13.9. 1. Extraction of total and ortho-dihydroxy phenols

The phenols were extracted by the method given by Mahadevan and Sridhar (1982). 1 g of leaf tissue was immersed in 5 ml of boiling absolute alcohol in dark for 10 mins. On cooling the sample was crushed using 80% alcohol and then filtered in a dark chamber. The residue was reextracted with 80% alcohol and then final volume was made up to 10 ml with 80% alcohol.

3.13.9. 2. Estimation of total phenols

Total phenols from leaf tissue were estimated by the method given by Bray and Thorpe (1954). To 1ml of extract, 1ml of 50% (1:1) diluted Folin ciocalteu's phenol reagent and 2ml of 20% Na_2CO_3 solution was added. The reaction mixture was boiled in a water bath for 1min and then cooled under running tap water followed by dilution with distilled water to make up the final volume to 25 ml. The absorbance was measured at 650 nm in a colorimeter and quantified using a standard curve of catechol.

3.13.9.3. Estimation of ortho-dihydroxy phenol

Estimation of ortho-dihydroxy phenols from leaf tissue was done by the method given by Arnows (1937). To 1 ml of the extract, 2 ml of 0.5(N) HCl, 1 ml of Arnow's reagent (10 g $NaNO_2$ and 10 g Na_2MoO_4 in 100 ml of distilled water) and 2 ml of 1(N) NaOH was added. A pink colour was developed and volume was made up to 10 ml with distilled water. After vigorous shaking the absorbance was measured at 515 nm in a colorimeter and quantified using a standard curve of caffeic acid.

3.13.10. Extraction and estimation of chlorophyll

Chlorophyll was estimated according to the method of Harborne (1973) by homogenizing 0.5 g of leaf tissue in 80% acetone and filtered through Whatman No.1 filter paper. 80% acetone was repeatedly added from the top till the residue became completely colourless. Then the filtrate was collected and total volume was measured. Estimation of chlorophyll was done by measuring the absorbance at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer against a blank of 80% acetone and calculated using the formula as given by Arnon (1949).

Total chlorophyll= $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} fresh weight

Chlorophyll a = (12.7 A_{663} – A_{645}) mg g^{-1} fresh weight

Chlorophyll b = (22.9 A_{645} – 4.68 A_{663}) mg g^{-1} fresh weight

3.13.11. Proteins

3.13.11. 1. Extraction

Soluble protein from the leaves and roots were extracted by following the method of Chakraborty *et al.* (1995). Fresh plant tissue was homogenized in a pre-chilled mortar and pestle with liquid nitrogen using 5 ml of 50 mM sodium phosphate buffer (pH 7.2) and PVP under ice cold condition and centrifuged at 10,000 rpm at -4^{0}C for 15 mins.The supernatant obtained was used as the crude extract for estimation.

3.13.11. 2. Estimation

Quantification of soluble protein was done following the metho of Bradford (1976). To 100 µl of crude extract of protein sample, 5 ml of Coomassie Brilliant Blue (CBB) G250 dye reagent (100 mg CBB G250 dye in 200 ml distilled H_2O containing 50 ml of 95% ethanol and 100 ml of conc. orthophosphoric acid) diluted in distilled H_2O in the ratio of 1:4 (v/v) was mixed. The absorbance of the change in colour of the dye from red to blue on binding to protein was measured in a UV-VIS spectrophotometer at 595 nm and protein was quantified using a standard curve of Bovine serum albumin.

3.13.12. SDS-PAGE analysis of proteins

SDS PAGE analysis for observing the protein pattern of the sample was done by the methodology given by Sambrook *et al.* (1989).

3.13.12. 1. Preparation of stock solutions

In order to prepare the gel for electrophoresis the following solutions were prepared:

(A) 30% Acrylamide solution:

29% Acrylamide and 1% N' N'-Methylene bis-acrylamide (7.25 g of acrylamide and 0.25 g of N' N'-Methylene bis-acrylamide) was mixed in 25 ml of warm distilled H_2O. Then the solution was filtered through Whatman No.1 filter paper, and kept in a dark bottle at 4^{0}C for further use.

(B) SDS (Sodium Dodecyl Sulphate):

10% stock solution of SDS (1 g SDS dissolved in 10 ml of distilled H_2O) was prepared in warm distilled water and stored at room temperature.

(C) Tris buffer:

(i) 1.5 M Tris buffer for resolving gel was prepared by dissolving 4.542 g of Tris base in 15 ml of distilled H_2O and the pH of the solution was adjusted to 8.8 using conc. HCl. The final volume of the solution was made up to 25 ml with distilled water and stored at -4°C.

(ii) 1.0 M Tris buffer for stacking gel was prepared by dissolving 3.029 g Tris base in 15 ml of distilled H_2O and the pH of the solution was adjusted to 6.8 using conc. HCl. The final volume of the solution was made up to 25 ml with distilled water and stored at -4°C.

(D) Ammonium Persulphate (APS) solution:

Ammonium per sulphate solution was freshly with 10% APS dissolved in distilled H_2O.

(E) Tris-Glycine electrophoresis running buffer:

Tris running buffer was freshly prepared by dissolving 25 mM of Tris base and 250 mM of Glycine in distilled water, and after mixing it thoroughly 0.1% of SDS was also mixed with the solution.

(F) SDS gel loading buffer:

This buffer was made by mixing 50 mM Tris-Hcl (pH 6.8), 10 mM β-mercaptoethanol, 10% SDS, 0.1% Bromophenol blue and 10% Glycerol. Therefore a 1X solution in 10ml contained 1ml of 50 mM Tris-HCl, 0.5 ml of ß-mercaptoethanol, 2ml of 10% SDS, 10 mg of bromophenol blue, 1 ml of glycerol, and the volume was made up to 10 ml using distilled H_2O and stored at -4°C.

(G) Fixing solution:

The fixing solution was prepared by mixing glacial acetic acid, methanol and distilled water in the ratio of 10:20:10 and was stored at normal room temperature.

(H) Staining solution:

The staining solution was prepared by dissolving 250 mg of Coomasie brilliant blue (Sigma R-250) in 45 ml of methanol. After which 45 ml of distilled water along with 10 ml of glacial acetic acid was added to it. The prepared stain was filtered through Whatman No. 1 filter paper and stored at room temperature.

(I) Destaining solution:

The destaining solution was prepared by mixing methanol, distilled water and acetic acid in the ratio of 45:45:10 and stored at room temperature.

3.13.12.2. Preparation of slab gel and electrophoresis

For preparation of slab gel, 2 glass plates (8 cm X 8 cm) were washed with dehydrated alcohol and dried. Then 1.5 mm thick spacers were placed in between the glass plates at the bottom and two sides of the glass plates and then sealed with petroleum jelly.
The resolving gel solution was cast very slowly up to a height of 5cm. The gel was over layered with water and kept for 2-3 h for polymerization. 10% resolving gel solution was prepared as follows:

Distilled H_2O	2.85 ml
30% Acrylamide	2.55 ml
1.5M Tris (pH 8.8)	1.95 ml
10% APS	0.075 ml
10% SDS	0.075 ml
TEMED	0.003 ml

After polymerization of the resolving gel, water overlay was decanted off. Stacking gel solution was poured carefully up to a height of 1 cm over the resolving gel and a 1 mm thick comb was placed. Finally the gel was kept for 60
mins for polymerization. 5% stacking gel solution was then prepared as follows:

Distilled H_2O	2.10 ml
30% Acrylamide	0.50 ml
1.0M Tris (pH 6.8)	0.38 ml
10% APS	0.03 ml
10% S.D.S	0.03 ml
TEMED	0.003 ml

3.13.12.3. Preparation of sample

Sample was prepared by mixing the sample protein with SDS gel loading buffer in an Eppendorf tube. Sample volume containing 20 µg protein was taken along with 10-15 µl of the gel loading buffer and then boiled in a water bath for 3mins.

3.13.12.4. Electrophoresis

After polymerization of the stacking gel the comb was removed carefully and the wells were cleaned thoroughly with distilled water. After removing the lower spacer, the gel plate was mounted in the electrophoresis apparatus. The Tris-Glycine running buffer that was prepared was then poured in the apparatus and was added sufficiently in both the upper and lower reservoir. Any bubbles trapped at the bottom of the gel were removed very carefully with a bent syringe. Then the electrodes were adjusted in their respective places. 40 µl of the dye mixed sample was loaded in to the bottom of the well of respective lanes with a micropipette and separated at 18mA current till the dye front reached the bottom of the gel.

3.13.12.5. Fixing, staining and destaining

After electrophoresis was complete the gel plate was taken out from the apparatus and then the gel was carefully separated from the glass plate. The stacking layer was discarded and the remaining resolving gel portion was carefully transferred to a gel box of appropriate size and left overnight in 40 ml of fixing solution. Then the next day, the fixing solution was properly decanted from the gel box after which 40ml of staining solution was immediately poured on top of the gel and kept for about 4-6 hours on a shaker with a constant speed of 30 r.p.m for proper staining. After the staining of the gel was done, the staining solution was decanted out from the gel box and destaining solution was added to it and kept on a shaker with 30 rpm until destaining was achieved. The destaining solution in the gel was repeatedly replaced with fresh destaining solution till the time the protein patterns were distinct. Then the gel was stored in 7% acetic acid solution for analysis. Gels were analysis using BIORAD gel imagining system.

3.13.13. Extraction and Estimation of antioxidative enzymes

3.13.13.1. Extraction

For extraction of enzymes, leaf samples were initially ground to powder in liquid nitrogen and then extracted with 50 mM sodium phosphate buffer [for peroxidase and

catalase (pH 6.8); and ascorbate peroxidase (pH 7.2) and 100 mM potassium phosphate buffer pH 7.6 (For glutathione reductase and superoxide dismutase) using PVP under ice cold conditions. The homogenates were then centrifuged at 10,000 rpm for 10 min at 4^{o}C. Supernatants were used as crude enzyme extracts and estimation of protein content was done in each case following the method of Bradford (1976).

3.13.13. 2. Estimation

3.13.13.2.1. Superoxide dismutase (SOD EC 1.15.1.1)

SOD activity was determined following the method of Dhindsa *et al.* (1981) by following the inhibition of the photochemical reduction of NBT at 560 nm. The assay mixture contained 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.6), 0.8 ml of distilled H_2O, 0.1 ml of 1.5 M Na_2CO_3, 0.1 ml of freshly prepared 3 mM EDTA, 0.1 ml of 2.25 mM NBT, 0.2 ml of 0.2 M freshly prepared methionine, 0.1 ml of 60 µM riboflavin and 0.1 ml of enzyme extract was added just before use. The reaction tubes containing enzyme samples were illuminated with 15 W fluorescent lamp for 10min. Another set of tubes lacking enzymes were illuminated and served as control. A non-irradiated complete reaction mixture served blank. The absorbance of the samples was measured at 560 nm and 1 Unit of enzyme activity (EU) was defined as the amount of enzyme required to inhibit 50 % of the NBT reduction rate as compared with controls containing no enzymes.

3.13.13.2.2. Gluathione reductase (GR, EC 1.6.4.2)

GR activity was determined by measuring the oxidation of NADPH at 340 nm (€ = 6.2 $mmol^{-1}$ cm^{-1}) as described by Lee and Lee (2000). The reaction mixture consisted of 1 ml of 0.1 M potassium buffer (pH 7.6), 0.2 ml of 0.1 M EDTA, 0.1 ml of freshly prepared 6 mM glutathione, 0.2 ml of 0.1 mM NADPH and 0.2 ml of enzyme extract. Change in absorbance was measured immediately at specific intervals up to 3 mins in a UV-VIS spectrophotometer. Enzyme activity was expressed as µmol NADPH oxidized mg $protein^{-1}$ min^{-1}.

3.13.13.2.3. Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity was assayed as decrease in absorbance at 290 nm resulting from oxidation of ascorbate (€ = 2.8 $mmol^{-1}$ cm^{-1}) following the method of Asada and Takahashi (1987). The reaction mixture consisted of 0.01 ml of freshly prepared ascorbic acid (10

mg ascorbic acid in 10 ml of 0.05 M sodium phosphate buffer (pH 7.2), 0.01 ml of H_2O_2, 2.97 ml of 0.05 M of sodium phosphate buffer (pH 7.2) and 0.01ml of enzyme extract was added just before assay and the change in absorbance of the reaction mixture was measured immediately at specific intervals up to 3 mins in a UV-VIS spectrophotometer. Enzyme activity was expressed as mmol ascorbate mg protein^{-1} min^{-1}.

3.13.13.2.4. Peroxidase (POX, EC 1.11.17)

POX activity activity was assayed by monitoring the oxidation of o-dianisidine (€ = 11.3 mmol^{-1} cm^{-1}) in the presence of H_2O_2 at 460 nm following the method of Chakraborty *et al.* (1993). The reaction mixture consisted of 1.7 ml of distilled H_2O, 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 0.1 ml of H_2O_2, 0.1 ml of freshly prepared o-dianisidine (5 mg/ml o-dianisidine in methanol) and 0.1ml of enzyme extract. The change in absorbance was noted down immediately at specific intervals up to 3 mins in a UV-VIS spectrophotometer. The POX activity was expressed as mmol o-dianisidine mg protein^{-1} min^{-1}.

3.13.13.2.5. Catalase (CAT, EC 1.11.1.6)

CAT activity was assayed following the method of Chance and Machly (1955) by estimating the decomposition of H_2O_2 (€ = 43.6 mol^{-1} cm^{-1}) at 240 nm. 40 µl of the enzyme extract was added to 3 ml of H_2O_2 buffer (0.1 ml of H_2O_2 in 25 ml of $NaPO_4$ buffer of pH 6.8) and mixed well. . The change in absorbance was noted down immediately at specific intervals up to 3 mins in a UV-VIS spectrophotometer. The enzyme activity was expressed as mol H_2O_2 mg protein^{-1} min^{-1}.

3.13.14. Native PAGE analysis of peroxidise and catalase isozymes

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analyses of peroxidase and catalse enzymes by following the method given by Davis (1964).

3.13.14. 1. Preparation of stock solution

Solution A: Acrylamide stock solution for resolving gel

28g of acrylamide and 0.76g of N' N' Methylene bis-acrylamide was dissolved in 50 ml of warm distilled water and then final volume was made up to 100 ml and filtered through Whatman No.1 filter paper and stored at -4^{0}C in a dark bottle for further use.

Solution B: Acrylamide stock solution for stacking gel

10g of acrylamide and 2.5g of N' N' Methylene bis-acrylamide was dissolved in 50 ml of warm distilled water and then the volume was made up to 100 ml and filtered through Whatman No.1 filter paper and stored at - 4^0C in a dark bottle for further use.

Solution C: Tris-HCl (pH 8.9) for resolving gel

9.15 g of Tris was dissolved in 15 ml of distilled water and the desired pH was maintained by adding 1(M) HCl to the solution. After the pH was adjusted, the volume of the solution was made up to 25mL with distilled water and then stored at - 4^0C for further use.

Solution D: Tris-Hcl (pH-6.7) for stacking gel

1.495g of Tris was dissolved in 15ml of distilled water and the desired pH was maintained by adding 1(M) HCl to the solution. After the pH was adjusted, the volume of the solution was made up to 25ml with distilled water and then stored at -4^0C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of Ammonium persulphate was prepared by dissolving 0.15g of APS in 100 ml of distilled water.

Solution F: Riboflavin solution

4 mg of riboflavin was dissolved in 10 ml of distilled water and stored in dark brown bottles at -4°C and stored for further use.

Gel loading dye

0.1 g of Bromophenol blue and 4 g of sucrose was mixed in 10 ml of distilled water and filtered and stored at -4°C for further use.

Electrophoresis buffer

Freshly prepared on the day of experiment by dissolving 0.18 g of Tris and 0.87 g of Glycine in 100 ml of distilled water and the final volume was made up to 300 ml and stored at -4°C.

3.13.14.2. Preparation of slab gel

Gel was prepared by taking two mini slab glass gel plates (8 cm × 8 cm) which was thoroughly cleaned in order to remove any traces of grease and then dried. 1 mm thick spacers were placed between two glass plates (both sides and below) and sealed with vacuum grease and clipped from all sides in order to prevent leakage of the gel solution leaving the top open.

Resolving gel solution was prepared by mixing solution A, C, E and distilled H_2O in the ratio of 1:1:4:1. 5 µL of TEMED (N,N,N',N'-Tetramethyl ethylenediamine) was added to it and gently mixed, then immediately it is poured in to the prepared gel plate leaving sufficient space for the stacking gel on top. Then the resolving gel was immediately over layered with water and kept for polymerization for 2 hrs. After 2 hrs the over layered water was poured off and washed with distilled water to remove any unpolymerized acrylamide.

Stacking gel solution was prepared by mixing solution B, D, F and distilled H_2O in the ratio of 2:1:1:4. 4 µL of TEMED (N,N,N',N'-Tetramethyl ethylenediamine) was added to it and gently mixed, then poured on top of the resolving gel, immediately after which the comb was inserted, leaving a sufficient space between the resolving and stacking gel. Then again it is over layered with distilled water. The gel was then kept for polymerization for 1-2 hrs in strong sunlight. After polymerization of the stacking gel, the comb was removed carefully and the wells were cleaned thoroughly with distilled water. After removing the clips and the lower spacer the gel plate was mounted in the electrophoresis apparatus.

3.13.14. 3. Sample preparation

1 g of plant leaf tissue was crushed in 5 mL of sodium phosphate buffer (pH 7.2) in liquid nitrogen in a pre-chilled mortar and pestle, centrifuged at 10,000 rpm in a cooling centrifuge at -4°C for 20 min. Sample volume containing 20 µg protein was taken along with 10-15 µl of the gel loading dye and kept for further use.

3.13.14. 4. Electrophoresis

The Tris-Glycine running buffer that was prepared was then poured in the gel apparatus and was added sufficiently in both the upper and lower reservoir. Any bubbles trapped at the bottom of the gel were removed very carefully with a bent syringe and the

electrodes were adjusted in their respective places. The gel was pre-run for about 10 mins before loading the samples. Then the mixed sample was loaded in respective lanes of the gel and separated for about 2-3 hrs at 10 mA current in ice-cold condition at 4°C inside a refrigerator till the dye front reached the bottom of the gel.

3.13.14.5. Staining

3.13.14.5.1. Superoxide dismutase (SOD EC 1.15.1.1)

SOD gels were stained following the method of Beauchamp and Fridovich (1971). After the run, the gel was immersed in 5 mM H_2O_2 for 30 min. Then, the gel was soaked in 50 mM sodium phosphate buffer containing 0.24 mM NBT and 28 µM riboflavin for 20 min in dark. Gel was then transferred the in 28 mM TEMED solution and exposed to light source (box with 40 W fluorescent bulbs) at room temperature for 20 min. Stained native gels have purple background and clear achromatic bands representing the SOD isozymes.

3.13.14.5.2. Ascorbate peroxidase (APX, EC 1.11.1.11)

APX gels were stained following the method of Mittler and Zilinskas (1993). After the run, the gel was immersed in ice cold sodium phosphate buffer (50 mM, pH 7.0) containing 2 mM ascorbate for 30 min. Then the gel wastransferred to a solution of sodium phosphate buffer (50 mM, pH 7.0) containing 2 mM H_2O_2 and 4 mM ascorbate for 20 min. The gel was then with sodium phosphate buffer (50 mM, pH 7.0) for 1 min. The gel wasvisualized by submerging the gel in solution of sodium phosphate buffer (50 mM, pH 7.0) containing 28 mM TEMED and 2.45 mM NBT for 5 min and the reaction was stoped by washing the gel with distilled water. Stained native gels have purple background and clear achromatic bands representing the APX isozymes.

3.13.14.5.3. Gluathione reductase (GR, EC 1.6.4.2)

GR gels were stained following the method of Anderson *et al.* (1990). The gel was incubated in 50 mM Tris-Cl (pH 7.0) containing 0.24 mM of (3-[4, 5-Dimethyl thiazol –2, yl] -2, 5 –diphenyl –tetrazolium bromide (MTT), 0.34 mM of 2, 6 –dichlorophenolindophenol, 3.6 mM GSSG and 0.4 mM NADPH for 1 h. Then the gel wass de-stained with distilled water. Stained native gels have beige colored background and bark brown bands representing the GR isozymes.

3.13.14.5.4. Peroxidase (POX, EC 1.11.17)

Peroxidase isozyme was visualized by following the staining method of Reddy and Garber (1971) in ice-cold condition. For the staining purpose benzidine (4,4'-diaminobiphenyl) dye was prepared by mixing 0.52 g benzidine in 4.5 ml of glacial acetic acid. 20 ml of distilled water was added to it after that and finally filtered through Whatman no. 1 filter paper. 3% H_2O_2 was prepared separately in 25ml of chilled distilled water. The two solutions were mixed just before staining and immediately poured on top of the resolving gel in a gel staining box. Dark blue coloured bands were observed after staining. The reaction was arrested by immersing the gel in 7% acetic acid solution.

3.13.14.5.5. Catalase (CAT, EC 1.11.1.6)

Catalase isozyme staining was performed according to Woodbury *et al.* (1971) with modifications. The gel was washed thrice in distilled water for about 6 min to remove the buffer from the gel surface where staining occurred. The gel was then soaked in 3.3mM H_2O_2 for 15 min at 20 °C in the dark with light agitation, and then in the stain mixture containing 1% (w/v) $FeCl_3$ and 1% (w/v) $K_3Fe(CN)_6$ in distilled water under 20 °C for 10 min or until the light yellow bands were visible.

3.13.15. Extraction and assay of defense enzymes

3.13.15.1. β-1, 3-glucanase (β-GLU, EC 3.2.1.38)

β- 1, 3-glucanase was extracted from leaf samples following the method of Pan *et al.* (1991). 1g of leaf sample was crushed and extracted using 5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0). Finally the extract was centrifuged at 10,000 r.p.m for 15 min at 4°C and the supernatant was used as crude enzyme extract. Estimation of β-1, 3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.* (1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was discontinued by mixing 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was dissolved in 4.5 ml of water, mixed and absorbance was recorded at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissue.

3.13.15.2. Chitinase (CHT, EC 3.2.1.14)

Extraction and estimation of chitinase was done by following the method described by Boller and Mauch (1988) with modifications. 1g of leaf sample from the jute plants were crushed and extracted using 5 ml of chilled 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 12,000 r.p.m. for 10 min at 4°C, and precipitate was discarded. The supernatant was used as enzyme source. Enzyme assay mixture consisted of 10µl of 1M Na-acetate buffer (pH 4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m for 3 min. 0.3 ml supernatant, 30µl of 1M KPO_4 buffer (pH 7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH 9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C.The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue.

3.13.15. 3. Phenyl alanine ammonia Lyase (PAL EC 4.3.1.5)

Extraction of PAL (E.C. 4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1 gm leaf sample was crushed in 5ml of 0.1M sodium borate buffer (pH 8.8) with 2mM of ß mercaptoethanol in ice cold temperature. The slurry was centrifuge in 15,000 rpm for 20 min at 4ºC. Supernatant was collected and after recording its volume, was immediately used for assay or stored at -20ºC. Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 30 µM L- phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1 h at 40 ºC the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissues.

3.13.16. Extraction and Estimation of non-enzymatic antioxidants

3.13.16. 1. Ascorbate

Extraction and quantification of ascorbic acid from the leaves was done following the protocol of Mukherjee and Choudhuri (1983). 1 g leaf tissue was homogenised in pre-chilled motor and pestle on ice using 6% Trichloroacetic acid, filtered and the final volume was made up to 10 ml. For estimation, 4 ml of the extract was mixed with 2 ml of 2% Dinitro phenyl hydrazine and 1 drop of 10% Thiourea (in 70% ethanol). The reaction mixture was thoroughly shaken, kept in boiling water bath for 15 mins and cooled at room temperature. 5 ml of 80% (v/v) H_2SO_4 was finally added to the reaction mixture at 0^0C. The absorbance was then taken at 530 nm against a blank in UV-VIS spectrophotometer. The amount of ascorbic acid was quantified using a standard curve of ascorbic acid.

3.13.16. 2. Carotenoid

The extraction and quantification of carotenoids was done by following the method of Litchtenthaler (1987). For extraction, 0.5 g of leaf tissue was homogenized in 100% methanol in dark, filtered in Whatman no.1 filter paper and the final volume was made up to 10 ml. The absorbance was then measured at 480 nm, 645 nm and 663 nm against a blank in UV-VIS spectrophotometer and the carotenoid content was calculated using standard formula:

$$\mathbf{A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})\ \mu g\ g^{-1}\ fresh\ weight}$$

3.13.16. 3. Total glutathione

Total glutathione was measured according to the methods of Griffith, 1985 and Smith, 1985. 1 g leaf tissue was homogenized in 10 ml of ice cold 5% metaphosphoric acid. The homogenate is centrifuged at 10, 000 r.p.m for 15 min at 4°C. The supernatant is used for the glutathione pool. For GSSG (glutathione disulphide) assay, the GSH (glutathione in reduced form) was neutralized by adding 20 µl of 2- vinylpyridine to the supernatant and 20 µl of water was added in the aliquots used for the total glutathione (GSH + GSSG) assay. Tubes were mixed until an emulsion was formed. Glutathione content in 1 ml of reaction mixture was assessed by adding up 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5′ dithiobis (2- nitrobenzoic acid), and 0.1 ml of sample acquired as described above. The reaction

was initiated with addition 3 units of GR and was observed by measuring the change in absorbance at 412 nm for 1 min. GSH was estimated as the difference between the amount of total glutathione. A standard curve for GSH in the range of 0–30 μmol ml^{-1} was prepared.

3.13.17. Non compatible osmolytes

3.13.17. 1. Extraction and estimation of proline

Extraction of proline from the leaves and roots was done by the method of Bates *et al.* (1973). 1 g of plant tissue was homogenized in 10 ml of 3% Sulfosalicylic acid and filtered through a Whatman No. 1 filter paper. The supernatant was collected for estimation.To 1 ml of extract, 3 ml of distilled water and 1 ml of Ninhydrin solution (1g Ninhydrin in 10 ml acetone and 15 ml distilled water) was added. The reaction mixture was kept on a boiling water bath for 30 mins and then cooled at room temperature. The reaction mixture was transferred in separating funnel and 5 ml of Toluene was added and mixed vigorously. The lower coloured layer was taken and the absorbance values were measured at 520 nm in a colorimeter against a blank and quantified from a standard curve of proline.

3.13.17. 2. Extraction and estimation of glycine betaine

Glycine betaine extraction and estimation was performed following the method of Yildiztugay *et al*. (2014). Leaf tissues crushed in distilled water were mixed with 2 N HCl. Half volume of acidified prechilled solution was taken and blended with 0.2 ml of potassium tri-iodide solution and was then allowed to cool in an ice 90 min with. Then 20 ml 1, 2 dichloromethane (cooled at −10 °C) were put in the solution. Then absorbance of lower organic level was observed at 365 nm and quantity of glycine betain (GB) was worked out using standard curve.

3.13.18. Hydrogen peroxide (H_2O_2) content

3.13.18.1. Extraction

The extraction of hydrogen peroxide was done by the method given by Jena and Choudhuri (1981) with slight modification. 0.5 g leaf extract was homogenized in 10 ml of phosphate buffer (50 mM, pH 6.5) in a dark chamber and centrifuged at 6000 rpm for 25 mins.

3.13.18. 2. Estimation

The estimation of hydrogen peroxide was done by the method given by Jena and Choudhuri (1981). The supernatant was used for estimation, where 3 ml of extract was mixed with 1 ml of 0.1% Titanium sulphate in 20% (v/v) H_2SO_4 along with. Both the blank and the reaction mixture was centrifuged at 6000 rpm for 15 mins and the intensity of yellow colour obtained was measured at 410 nm in a UV-VIS spectrophotometer. H_2O_2 content was quantified using the extinction coefficient value of $\epsilon = 0.28\ \mu mol^{-1}\ cm^{-1}$.

3.13.19. *In situ* localization of H_2O_2 and O_2^-

The detection of hydrogen peroxide in the leaf tissue was done according to the method given by Thordal-Christensen *et al.* (1997) with minor modifications. The leaf tissues were washed thoroughly with doubled distilled water and cut into leaf discs. Then these leaf discs were incubated in dark for 24 h in 1 mg/ml of 3,3'-Diaminobenzidine (DAB) having pH 3.8. After 24 h the leaf disc was transferred to a beaker containing ethanol/lactic acid/glycerol in the ratio 4:1:1 and was heated at a temperature of 70^0C until all the chlorophyll was removed. Hydrogen peroxide was visualized as reddish-brown colour at the site of Diaminobenzidine polymerization.

Superoxide (O_2^-) production was examined in leaf tissue using a modification of the Nitro blue tetrazolium (NBT) staining technique described by Romero-Puertas *et al.* (2004). Leaf segments were incubated in 0.05% (w/v) NBT in 10-mM phosphate buffer (pH 7.5) at room temperature for 30 min. Bleaching was performed by the immersion of stained segments in boiling ethanol/lactic acid/glycerol (4:1:1) for 5 min. The formation of a blue formazan precipitate indicates the reduction of NBT by superoxide (O_2^-).

3.14. Real time PCR analysis

3.14. 1. RNA extraction

All the plastic wares and glass wares used for this experiment were made RNase free by treating with DEPC for overnight and autoclaving twice at 121°C until the traces of DEPC removed. These were then dried in Hot Air Oven at 90°C before use. The samples stored at 80°C were ground to powder in liquid N2 using sterile mortar and pestle. To the powdered sample 1ml of Trizol Reagent (Takara Bio Inc, Japan) was added mixed by grinding, transferred to a 2ml microfuge tube and incubated at room

temperature for 15 – 20 mins. It was then centrifuged at 10,000 rpm (Sigma) for 5 min and the supernatant was transferred to a fresh sterile microfuge tube. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed gently and incubated in ice for 2 minutes. After centrifuging the above at 12,000 rpm for 5 minutes the supernatant was transferred to a fresh vial and then incubated in ice for 2 minutes after adding 500 µl of isopropanol. The RNA was pelleted down by centrifuging the above for 10 minutes at 10,000 rpm and the pellet was vortexed gently with 300 µl of 70% of ethanol. Centrifugation at 10,000 rpm was repeated for 10 minutes and the pellet was air dried. 20µl of RNase free water was added to the pellet and heated gently (60^{o}C) to dissolve the pellet in water. The extracted RNA was treated with DNase enzyme to remove any traces of DNA contamination. One micro litter of DNase was added to above isolated RNA and incubated for 1hr at 37^{o}C and after the temperature was raised to 70^{o}C for 5 minutes to inactivate the enzyme. The RNA was stored at -20^{o}C for later use. The concentration and purity of RNA was assessed using a spectrophotometer (Sartorius). A 1µL aliquot of RNA was pipetted onto the apparatus pedestal. RNA with an absorbance ratio at 260 and 280 nm (A260/A280) between 1.8 and 2.2 will be deemed indicative of pure RNA.

3.14. 2. cDNA synthesis

After quantification, RNA was reverse transcribed using oligo dT (Sigma Aldrich). Hundred nano gram of RNA and 2µl of oligo dT were taken to a fresh sterile microfuge tube and incubated at 70^{o}C for 5 minutes and immediately transferred to ice. To this 2µl of dNTPs, 1µl of Reverse Transcriptase enzyme (Biolabs, New England) and 2 µl of 10x Reverse transcriptase buffer was added and made up the volume to 25µl using RNase free water. This mixture was incubated at 42^{o}C for 90 minutes and reaction was terminated by incubating at 70^{o}C for 15 minutes.

3.14. 3. Primer synthesis and validation

Gene specicific primers for Real Time PCR were designed using Perkin Elmer Primer Express® software on the sequences obtained for both the genes. All primers were designed to be intron-spanning to preclude amplification of genomic DNA. A gradient PCR was performed to standardize the optimum annealing temperature of the designed primer using 50 ng of synthesized cDNA keeping the temperature range of 50 – 60^{o}C

and for 35 cycles, and the best annealing temperature was opted to be at 60^0C for all the genes. The quality of PCR products was visually inspected by electrophoresis, the generation of single band of the expected size was taken as a criterion for specificity. To normalize relative levels of expression, plant Actin was used as an internal reference control.

Primers details

Genes	Length of Primer	Sequence of selected primer pair (5'-3' direction)	GC Content (%)	Predicted T_m of Primer	Measured T_m of Primer
P5CS					
FP	22	TCGGTGCTGAGGTTGGCATAAG	55	56.7	60
RP	23	TTGTCACCATTCACCACTTGCCC	52	57.1	60
P5CR					
FP	21	CGGGTAAACATCCAGGGCAGC	62	58.3	60
RP	21	TCGGCATCTTGTTGTGGCAGC	57	56.3	60
PDH					
FP	22	CGGGATCCTCGACTACGGCATC	64	60.4	60
RP	24	TGATCTTGATACACACGCTCGCCG	54	59.1	60
ADC1					
FP	20	CACCAAGATACCAGGCCACT	55	53.8	60
RP	20	GTGGAAGTGCAGCAACTTGA	50	51.8	60
ADC2					
FP	20	AGGAGGAGGAGCTCGACATT	55	53.8	60
RP	18	GCCGAACTTGCCCTTCTC	61	52.6	60
SAMDC2					
FP	20	GCGTCCTCATCTACCAGAGC	60	55.9	60
RP	22	CTTGCCTTCCTTGACCAGAG	55	53.8	60
TdHSP101C					
FP	20	GTTGGACAGTATGAGGCCGT	55	53.8	60
RP	22	CATTTCACCCCCAATTCAACAG	45	53	60
HSP90					
FP	17	TCCCGCACGCTTCTCCT	65	51.9	60
RP	21	AACTGTTCCACGAGTACCACA	48	52.4	60
HSP70					
FP	21	CATGGCCCGTCTGCTTGCTCT	62	56.3	60
RP	21	AGCACGCCGTTCTTCATCTCG	57	45.9	60
HSP26.3					
FP	16	AAGTACAACCGCCGCA	56	45.9	60
RP	17	GAAGACGTCCTTGCGCT	59	49.5	60
HSP 23.5					
FP	23	TCTTCTCCCCAAGTACTTCAAGC	48	55.3	60
RP	21	TTCCCCTGAGTACTTGCATCC	52	54.4	60
HsfA3					
FP	23	CCCAGCGCCAGGCCACTAAGGAC	70	64.2	60
RP	23	CAAAGCGAGCCCGTGTGATGGTA	57	58.8	60

3.14. 4. Relative Quantification using Real Time PCR

The quantification was done in Applied Biosystems StepOne Real Time PCR using the SYBR Green Chemistry. The reaction was carried out in a 10µl reaction volume. Reagents and temperature profile for Real time PCR are Listed below

Real time PCR components	Volume (µl)
SYBR Green Master mix	5.00
Primer FP (10picomoles/µl)	1.00
Primer RP (10picomoles/µl)	1.00
Template DNA	25ng/µl
PCR grade water	Volume make up
Total volume	10.00

Temperature profile for Real time PCR are presented below

Initial denaturation	94°C for 1min	
Denaturation	94°C for 15sec	35 cycles
Annealing & Extension	60°C for 30 sec	

To accurately and reliably determine gene expression values, raw fluorescence data (Ct values) generated by the real-time PCR instrument was exported to qBase plus software 13, the purpose of which was to scale raw data to an internally defined calibrator and an endogenous control gene (Actin). The ΔΔCt method was finally used to calculate the relative expression of the genes for individual treatments.

3.15. Statistical analysis

Statistical analysis was executed using in Microsoft excel and IBM SPSS version 21 and Fischer's LSD test was performed to determine significant mean differences at $P \leq 0.05$.