3. Materials and Methods

3.1. Plant materials

Two types of plant materials were used in the present study. Different varieties of tea plants used as host plants and have been described in details in the following section (section 3.1.1). Some other plant materials have been used for extraction of botanicals used in the experiments to control the major pathogens of tea.

3.1.1. Host plant

Several varieties of tea plants are commercially available in the present study area. Most of the varieties are approved by Tocklai Experimental Station Jorhat, Assam. Some tea plants are raised from cuttings (commonly called as clonal varieties) and some others are raised from seeds (called as seed varieties). Four different tea varieties (two clonal varieties and two seed varieties) were selected for the experiments of the present study. Out of these, two varieties (TV-26 and Teenali) were clonal varieties and the other two (TS-463 and TS-520) were seed varieties (Fig. 3.1 & 3.2). Selected tea varieties were procured from Gayaganga Tea Estate situated near the city of Siliguri in the district of Darjeeling. All the four varieties were selected on the basis of their growing suitability in the tea gardens of the Darjeeling and neighboring districts. The procured varieties were planted in pots as well as in soil bed in the experimental garden of Department of Botany, University of North Bengal. All the four plants were recommended for commercial cultivation in the sub-Himalayan West Bengal and Assam by Bezbaruah and Singh (1988).

3.1.2. Maintenance of planted varieties

After plantation of the tea plants in the experimental garden (pots as well as in soil bed), the plants were maintained for several weeks under the normal daylight condition with mean monthly temperature between 30^0C

maximum to 10^0C minimum. The plants were watered as and when required using ordinary tap water.

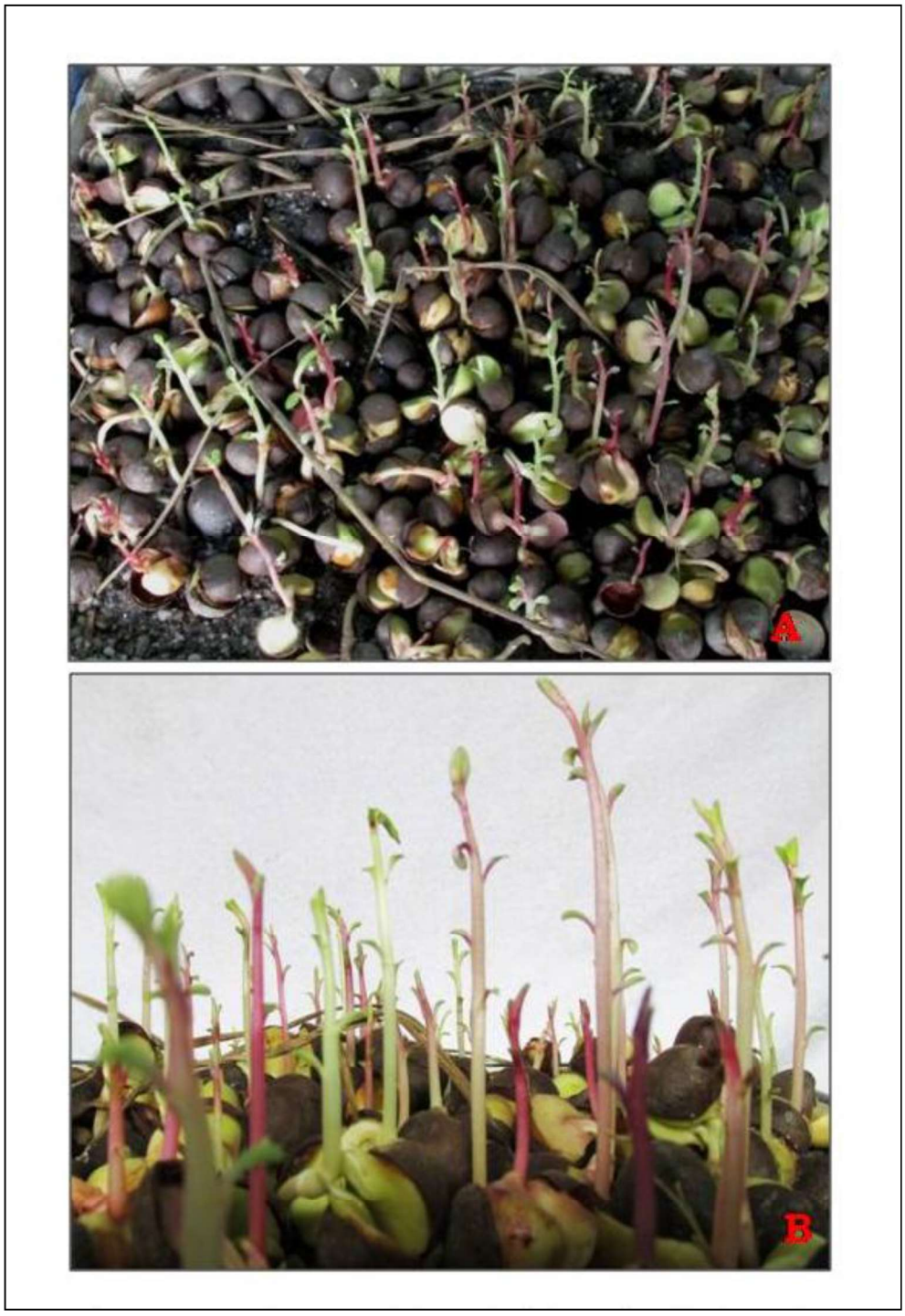

Fig. 3.1: A) Tea seeds TS-520 being raised in sand-soil mixture in a tray in the net house of the experimental garden of University of North Bengal after 30 days of sowing; B) Seedlings of TS-520 after 45 days of sowing.

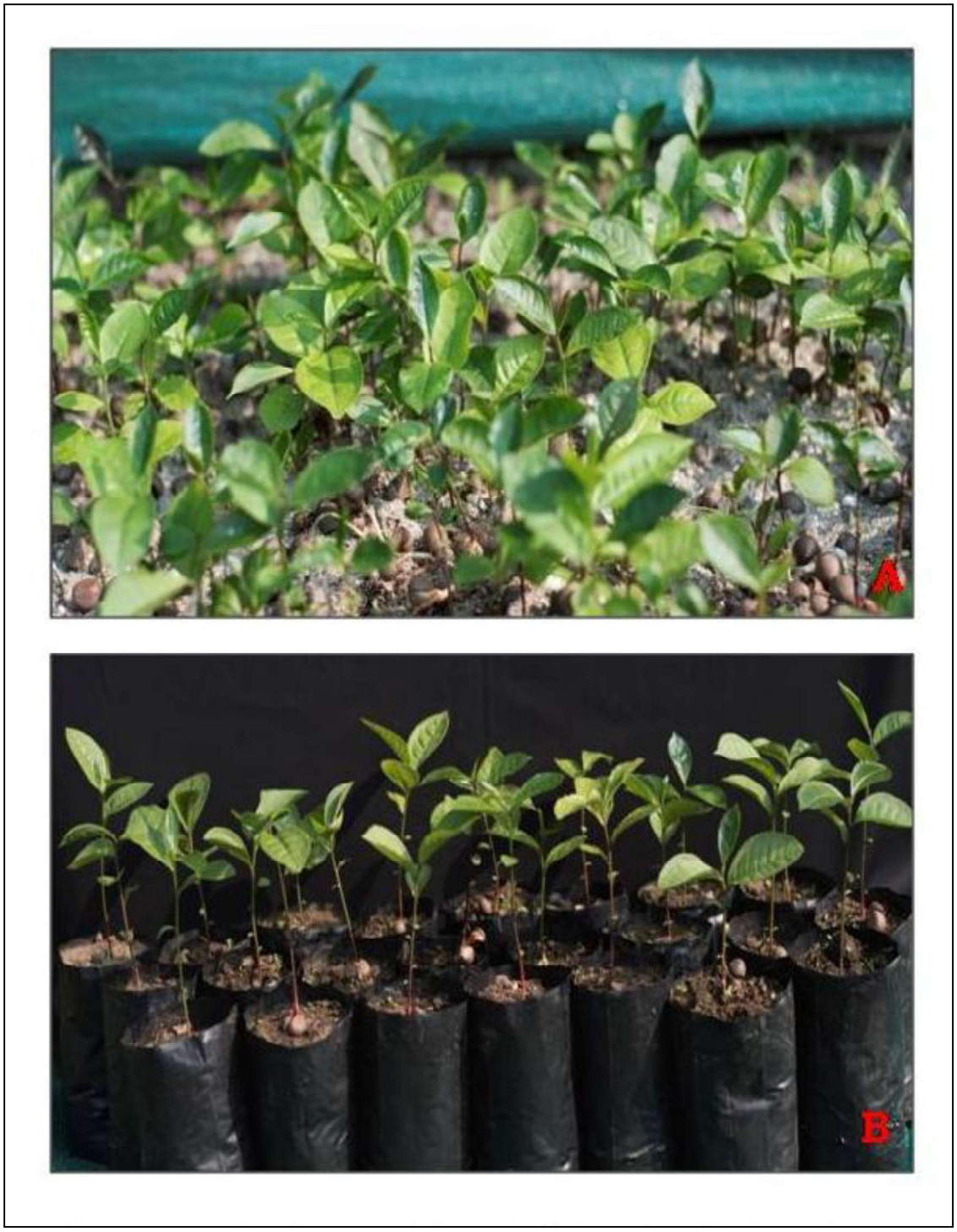

Fig. 3.2: A) Tea seeds of TS-463 being raised in sand-soil mixture in a tray in the net house of the experimental garden of University of North Bengal after 90 days of sowing; B) Seedlings of TS-463 after 120 days of sowing.

3.1.3. Collection of plants/plant materials for extraction of botanicals

Nine different plants were selected for extraction of botanicals to be used for *in vitro* control of the pathogens. The plants were selected on the basis of local availability as well as their previous reports of antifungal activity somewhere else. The list of plants have been provided in the following table (Table no. 3.1)

Table 3.1: List of plants used for extraction of botanicals

Name of the plants	Common name	Plant parts used	Reference for selection
Leucas indica (L.) Vatke	Thumbai	Leaf	Pranoothi *et al.* 2014
Clerodendrum viscosum Vent.	Bhant	Leaf	Oly *et al.* 2011
Lagerstroemia speciosa (L.) Pers.	Jarul	Leaf	Nasrin *et al.* 2012
Casuarina equisetifolia L.	Horsetail she oak	Leaf	Kumar and Panneerselvam 2015
Ricinus communis L.	Castor oil plant	Leaf	Khan and yadav 2011
Boerhavia diffusa L.	Punarnava	Leaf	Svecova *et al.* 2017
Azadirachta indica L.	Neem	Leaf	Shrivastava and swarnkar 2014
Datura metel L.	Dhutora	Leaf	Rinez *et al.* 2013
Polyalthia longifolia Sonn.	Debadaru	Leaf	Lalitha *et al.* 2011

3.1.3.1. Preparation of plant extract (from fresh plant parts)

Fresh leaves were collected and washed thoroughly with tap water and soaked by blotting paper at room temperature and allowed to dry. After drying the materials were weighed, ground and extracted separately with sterile distilled water and ethanol (0.5g/ml). Extraction was done following the method of Mahadevan and Sridhar (1982) with some modifications. The extracts were filtered through double-layered cheese cloth and then

centrifuged at 10,000 g for 15 minutes. The supernatants of the aqueous extracts were sterilized by passing through a Millipore filter (0.2µm). All extracts were stored as stock solution at 4^0C for screening of their antifungal activity within 7 days of preparation.

3.1.3.2. Bioassay of botanical extracts by poisoned food technique and assessment of antagonism of bio-control agents by dual culture method.

Poisoned food technique: Plant extracts were mixed with molten PDA medium to make 10% concentration of the extract. The medium was mixed well with the extracts and poured in a sterile petriplate (90 mm diameter) under aseptic condition and was allowed to solidify. In control sets sterile distilled water was added instead of plant extracts. Mycelium were cut out from the periphery of a 7-day old growing culture by sterile cork borer and one such disc was placed in the centre of each petriplate. Experimental and control plates were incubated at 28±1°C in an incubator and radial growth was measured after intervals of one day till radial mycelia growth of the control reached the periphery. Results after three days have been noted and percent reduction of radial growth have been tabulated.

Dual culture method: In case of fungal antagonist, agar disc (4 mm) were cut out from the periphery of a 7-day old growing culture of the pathogen and of a biocontrol agent by sterile cork borer. One such disc of pathogen and of biocontrol agent was placed at the periphery but opposite to each other of the same sterile PDA Petri plate (90 mm). In case of bacterial antagonist, 4 mm of mycelia disc of test pathogen were cut and placed at the centre of the petri plate. After that bacterial antagonists were streaked in a square pattern surrounding the test pathogen in a same pate. Both the control (where no biocontrol agent was placed) and experimental plates were incubated at 28±1 ^{0}C and radial growth was measured and percentage of growth inhibition (in comparison to control) was calculated.

3.2. Fungal culture

Three virulent fungal pathogens of tea have been taken in to consideration for the present study. One of the pathogen (*Colletotrichum gloeosporioides*) widely affects tea plants and cause brown blight of tea. The other pathogen (*Curvularia eragrostidis*) cause leaf spot disease in tea leaves. The third pathogen (*Lasiodiplodia theobromae*) causes diplodia disease in tea leaves, young tender stems and also in roots.

3.2.1. Source of fungal culture

Fungal cultures (*Colletotrichum gloeosporioides, Curvularia eragrostidis* and *Lasiodiplodia theobromae*) were obtained from Molecular Plant Pathology Laboratory; University of North Bengal; Siliguri. The cultures were originally isolated by Prof. Aniruddha Saha and the identifications were confirmed by IARI, New Delhi. The details of the source of the fungal cultures are given in the following table (Table 3.2).

Table 3.2: List of fungal cultures

Fungal culture	Source	Identification
Curvularia eragrostidis (P. Hennings) Meyer	Molecular Plant Pathology Laboratory, Department of Botany, University of North Bengal	Indian type culture collection, IARI, New Delhi (ITCC-4150.2K).
Colletotrichum gloeosporioides	Originally isolated from Mohurgong and Gulma Tea Estate, Siliguri, West Bengal	Identification done by 18s rRNA studies (GenBank Acc. No. MF661903)
Lasiodiplodia theobromae	Originally isolated from Bagdogra tea estate	Indian type culture collection, IARI, New Delhi (ITCC-4151.2K).

3.2.2. Maintenance of stock cultures

All the cultures were maintained in freshly prepared sterile PDA slants. For experimental works cultures were transferred to fresh PDA slants at regular intervals and were maintained at room temperature. For preservation, two weeks old fungal cultures in PDA medium were stored at low temperature in refrigerator (6^0 C). Apart from weekly transfer for

experimental purpose, the cultures were also examined at regular intervals to test their pathogenicity.

3.2.3. Microbial antagonists used during the study

Four well known microbial antagonists were used during the study. Among the four three were bacterial antagonists and other one was fungal antagonist. The sources of the cultures have been given in the following table (Table no. 3.3).

Table 3.3: List of microbial cultures

Name of the fungal antagonists	Source and Number
Bacillus subtilis *Bacillus megaterium* *Bacillus pumilus*	Dr. A. Saha, Molecular plant pathology laboratory, Dept. of Botany, NBU
Tricoderma harzianum	IARI, New Delhi; ITCC No. 4572

3.3. Morphological characterization and microscopy

The morphology of the fungal pathogens was studied in the following steps. First, the fungi were inoculated separately on PDA plates and were incubated up to 7 days for the culture to develop. At regular intervals the plates were observed for formation of fungal growth, sporulation, color of mycelial mat and growth pattern. In the second step, microscopic observations were made and for doing this, a bit of mycelia were taken from pure culture and were placed on glass slide (clean and grease free) for microscopy. One drop of cotton-blue (mixed in lactophenol) was placed on the clump of mycelia. The clump of hyphae was separated carefully using sterile needles. There after the slides were mounted with cover glass, excess stain were soaked, edge of the cover glass were sealed and finally the slides were observed under compound microscope at 10 x 40 magnification.

3.4. Preparation of conidial suspension (spore suspension)

Fourteen days old sporulated fungal culture in PDA slants were taken. Approximately, 5 ml sterile distilled water was poured in the

cultured slants aseptically in a laminar air flow hood. Gentle scrapping by an inoculating needle was done on the surface of the mycelium grown on the agar medium. Then the tube was shaken well to suspend the spores from the mycelium and to mix with the sterile distilled water. The resultant mixture was strained through sterile muslin cloth. The filtrate was taken as spore suspension. The desired concentration of the spores in the suspension was adjusted by adding sterile distilled water following haemocytometer count.

3.5. Verification of Koch's Postulates

At the onset of present study, both the fungal cultures were tested for their pathogenicity and verification of the Koch's postulations. Fresh young tea leaves of nursery tea plants were collected from experimental garden, Dept. of Botany, University of North Bengal, Siliguri. At first, the leaves were inoculated with conidial suspensions of *Colletotrichum gloeosporioides*, *Curvularia eragrostidis* and *Lasiodiplodia theobromae* separately following detached leaf inoculation technique (Dickens and Cook, 1989) and after 72 hours of inoculation, the pathogen in each case was re-isolated from infected tea leaves. Before isolation, the infected portions were cut into small pieces, washed thoroughly with sterile distilled water; surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 1 minute, washed two times with sterile distilled water and finally transferred aseptically into sterile Potato Dextrose Agar (PDA) slants. These isolates were examined after one week of inoculation and the identity of the organisms were confirmed after comparing them with the respective stock cultures.

3.6. Studies on germination spores

Spore suspension drops of 20 µl each of test pathogen were placed on clean, grease free glass slides. The slides were incubated in a glass humid chamber. The chamber was placed within an incubator at 28±1 ^{0}C. The incubator was also fitted with fluorescent tube which was automatically operative to create 12h dark and 12h light period. After 24

hours of incubation period, the slides were stained with cotton blue mixed with lactophenol and observed under microscope. Finally, the percentage of spore germination was calculated.

3.7. Inoculation technique and disease assessment

3.7.1. Detached leaf inoculation technique

Artificial inoculation of detached leaves with test pathogen was performed following the detached leaf inoculation technique of Dickens and Cook (1989) with certain modifications. To perform the experiment fresh young fully expanded and detached tea leaves were placed on trays lined with moist blotting papers. The leaves in the trays were inoculated with spore suspension of the pathogen. Initially two or four wounds (light scratch of 2 mm length) were made on the adaxial surface of each leaf with the help of a sterile, sharp needle. 20 μl drop of spore suspension (bearing about $1X10^6$ conidia/ml) of test pathogen (prepared from 14 d old cultures) were placed on the wounds of each leaf with a micropipette. In control sets, drops of sterile distilled water were placed on the leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to maintain the required moistures inside the trays during incubation.

3.7.2. Cut-shoot inoculation technique

As tea plants are perennially grown plants and the bush is large enough, hence, cut-shoot inoculation has been preferred by several scientists. The inoculation technique described by Yanase and Takeda (1987) was followed for cut shoot inoculation in the present study. Twigs with 3-4 leaves of nursery tea plants grown in the experimental tea garden (Department of Botany, University of North Bengal) were cut with a sharp blade and immediately introduced into glass vials containing sterile tap water to avoid damage of vascular bundle and were transferred to the laboratory. Leaves were inoculated with spore suspensions as well as the mycelial plugs (4mm diameter) bearing conidia of test pathogens as used by Dickens and Cook (1989). The mycelial plugs were covered with moistened cotton wool and sterile PDA plugs were used as controls. For

each treatment sixteen cut shoots of a variety were placed into the holes of a floating board, and the board was floated on the modified Hoagland and Knop's solution in a glass chamber. Top of the chambers were covered with perforated transparent cellophane papers so that light and air could pass through. The glass chambers were incubated at the room temperature of 28±2°C.

3.7.3. Treatment of whole tea plants by different resistance inducers and post treatment inoculation

Following the whole plant inoculation method of Dickens and Cook (1989), six month old tea plants (raised through seed germination and clonal cuttings) were inoculated with the test pathogen. Inoculation was done by spraying conidial suspensions ($1X10^6$ conidia/ml) prepared from 14d old cultures of test pathogen grown on sterile PDA slants. In control sets, plants were sprayed with sterile distilled water. In treated-control sets, plants were sprayed with the chemicals (resistance inducers). In treated inoculated sets inoculation was done after spraying of resistance inducing chemical. All plants were kept for 48 hours in transparent polythene chamber to maintain high humidity. There after the plants were kept in natural air and light. Concentration of all the chemicals used for treatment was 1mM.

3.8. Leaf disease assessment

3.8.1. Assessment of disease in detached leaves inoculated by pathogen

Disease assessment was done after 24, 48 and 72h of inoculation. Percentage of lesions was calculated as follows:

(Total no. of lesions formed/Total no. of inoculation drops) X 100.

In addition, mean diameter of lesions in millimeter were also recorded after 24, 48 and 72h of inoculation.

3.8.2. Assessment of disease in cut-shoots and whole plants inoculated by pathogen

Leaf disease was assessed following the method of Sinha and Das (1972). On the basis visual observations number of lesions developed on

the leaves after desired hours or days of inoculation, were noted and diameters of each lesion were measured. The diameters were categorized into four groups and a value was given to each group as follows:

Group 1 = Very small-restricted lesions of 1-2 mm diameter: 0.1

Group 2 = Lesions with sharply defined margins of 2-4 mm diameter: 0.25

Group 3 = Slow spreading lesions of 4-6 mm diameter: 0.5

Group 4 = Spreading lesions of variable size (beyond 6 mm in diameter) with diffused margin: 1.0

Finally disease index was calculated in the following way

Disease Index = (No. of lesion of Group 1) X 0.1 + (No. of lesion of Group 2) X 0.25 + (No. of lesion of Group 3) X 0.5 + (No. of lesion of Group 4) X 1.0.

3.9. Assay of some defense enzymes (extraction and estimation)

3.9.1. Peroxidase: Extraction and Estimation:

Hammerschmidt *et al.* (1982) described extraction and estimation procedure of plant Peroxidase activity. In the present study, peroxidase activities of differently treated plants have been determined according to the procedure given by Hammerschmidt *et al.* (1982) with some modifications. Fresh tea leaves (1 g) either treated or inoculated etc. were dipped in liquid nitrogen for freezing. After 10 min the frozen leaves were crushed to powder presence of in 0.1M sodium phosphate buffer (pH 6.5) at cold room (4°C). The homogenate was then subjected to filter through four-layered muslin cloth. The filtrate was then centrifuged at 6000 g at 4^0 C in a cooling centrifuge (Remi, India) for 15 min. Thereafter, supernatant was obtained and considered as crude enzyme for estimation.

For estimation of the enzyme activity following steps was taken. Guaiacol (0.05M)-1.5 ml was added to 200 µl of extracted crude enzyme in a cuvette. The cuvette was then placed in the slot of a UV-VIS Spectrophotometer (Systronics, Model no.118, India) and the initial reading was adjusted to zero at 420 nm. Then 100 µl of H_2O_2 (1% v/v) was added to the cuvette and the changes in absorbance values were recorded up to 5 min at 1 min intervals. The change in absorbance [ΔA420 min^{-1} g^{-1} fresh weight tissue], was expressed as enzyme activity.

3.9.2. β-1, 3-glucanase: Extraction and Estimation:

Pan *et al.*, (1991) used laminarin-dinitrosalicylate method for extraction and estimation of β-1, 3-glucanase. The process of Pan *et al* (1991) was followed with necessary modifications needed for the study of the present host-parasite interaction related changes in β-1, 3-glucanase activity in differentially treated plants. Fresh tea leaves (1 g) either treated or inoculated etc. (1g), were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were pulverized in 5 ml of 0.05 M Sodium acetate buffer (pH 5.0) in a pre chilled mortar and pestle in a cold room at 4^0C. The homogenized material was filtered through four-layered muslin cloth. The resultant filtrate was then centrifuged (at 10000 g at 4^0 C for 15 min). The supernatant thus obtained was used as crude enzyme.

To estimate the enzyme activity, laminarin was used as substrate. Crude enzyme extract (15.6 µl) was added to 15.6 µl of Laminarin (4% solution) (Sigma, USA) and the resultant mixture was incubated at 40^0C for 10 min. To stop the reaction 94 µl of dinitrosalicylic acid reagent was added. The mixture was heated for 5 minutes on a boiling water bath. A colored solution was obtained and the final colored solution was diluted with 1 ml distilled water. The resultant mixture was subjected to be placed in a cuvette and absorbance values were recorded at 500 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Expression of enzyme activity was computed as mg glucose g^{-1} fresh weight tissue min^{-1}.

3.9.3. Phenylalanine ammonia lyase (PAL): Extraction and Estimation:

PAL activity was determined following the method of Sadasivan and Manickam (1996). The rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm was considered as PAL activity. Fresh tea leaves (1 g) either treated or inoculated etc. were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.2 M borate buffer (pH 8.7) in a mortar and pestle at 4^0C. The homogenate was then filtered through four-layered muslin cloth and centrifuged at 12000 g at 4^0C for 15 min. Supernatant (The yellowish green filtrate) was used as crude enzyme extract.

To estimate the enzyme activity, 0.5 ml borate buffer, 0.2 ml crude enzyme, 1.5 ml distilled water and 1 ml of 0.1 M L-phenylalanine were mixed and was then incubated for 30 min at 30^0 C in a water bath. The reaction was stopped by adding 0.5 ml of Trichloroacetic acid (1M) in the mixture. The absorbance values were noted at 290 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was computed as μMol min $^{-1}$ g $^{-1}$ fresh weight tissue. The final results were obtained by using trans-cinnamic acid as standard.

3.10. Molecular detection of defense related gene

3.10.1. Extraction of total DNA

Leaves of 1 year old tea plants were selected for the isolation of total DNA. Leaf samples were collected and thoroughly washed in tap water. There after total DNA was extracted by the modified Cetyl trimethyl ammonium bromide (CTAB) method of Sharma *et al.* (2003). CTAB-DNA extraction buffer was prepared by mixing Tris-100mM [pH-8], 20mM EDTA [pH-8], 1.4M NaCl, 2% CTAB and 2μl/ml β-mercaptoethanol. Detailed step of CTAB method are as follows:

i. One gm leaf tissue was ground to powder in Liquid Nitrogen using a mortar and pestle.
ii. The tissue was then homogenized with 5ml pre-warmed (at 60˚C) 2% CTAB-DNA extraction buffer.
iii. The resultant homogenate was transfer in a 1.5ml micro centrifuge tube and incubated at 60˚C for 1h, in a dry bath and the homogenate was mixed occasionally by gentle swirling.
iv. After incubation, homogenate was removed from the dry bath and 0.6 volume of chloroform-isoamylalcohol (24:1) was added.
v. The homogenate was mixed thoroughly by inversion of the up side down and again down side up.
vi. After 15 minutes the mixture was centrifuged at 10,000 rpm for 10 min. and aqueous phase was transferred to another 1.5ml micro centrifuge tube.

vii. 0.6 volume of isopropanol was added to the sample to precipitate the DNA.

viii. DNA was pelleted by centrifugng at 10,000 rpm in 1.5 ml centrifuge tubes for 15 min at 4˚C.

ix. DNA was washed with 70% ethyl alcohol and dried overnight.

x. Dried DNA was dissolved in $T_{10}E_1$ buffer [pH-8].

xi. 2.5 µl RNAse was added to 0.5 µl of crude DNA and mixed gently and incubated at 37˚C for 1h.

xii. After this 0.3 ml of chloroform-isoamylalcohol (24:1) was added and was mixed thoroughly. Finally the resultant mixture was centrifuged for 15 min at 10,000 rpm.

xiii. Supernatant was removed except the white interface layer and DNA was reprecipitated from the supernatant by mixing absolute alcohol (supernatant: Alcohol:: 1:2).

xiv. DNA was pelleted by centrifugation at 10,000 rpm for 15 min and then washed with 70% alcohol.

xv. Dried DNA was redissolved in $T_{10}E_1$ buffer [pH-8] and stored at -20˚C for further use.

3.10.2. Agarose gel electrophoresis of extracted DNA

The frame of the gel-casting unit was cleaned and sealed with a tape to form a mould. The frame was placed on a flat platform to ensure a flat and levelled base; the comb was then positioned parallel to the open edge of the frame about 2 mm above the surface. Agarose powder was added to TAE buffer (1X) and was dissolved by melting at 100˚C, the solution was cooled to 50˚C; about 1.2 µl of the ethidium bromide (0.5 mg/ml final concentration) was added to the gel and then poured into the gel frame and allowed to set. After setting the gel, it was transferred to the gel tank such that the wells were towards the negative pole. The gel tank was filled with TAE buffer just enough to cover the surface of the gel. 5µl of the PCR product was mixed with 0.8µl of (6X) loading dye and loaded to the wells of submerged gel along with marker DNA using a micropipette. The electrophoresis apparatus connected to the power supply and electrophoresis was carried out at 70 V for one hours or upto deep blue dye

migrated to the end of the gel. It was then visualized under UV-transilluminator.

3.10.3. Extraction of total RNA

Total RNA were extracted from the tea leaves following the method of Ghawana *et al.* (2011). All the plastic wares and glass wares used for this experiment are made RNase free by treating with DEPC for overnight and autoclaving until the traces of DEPC removed. These were then dried in Hot Air Oven at 90^{o}C before use. Detailed procedures of RNA extraction method are as follows:

i. 100mg leaf tissue was ground to powder in Liquid N_2 using a sterile mortar and pestle.
ii. Add 1ml of extraction buffer and grind further to make a homogenous mixture.

RNA extraction buffer: Tris saturated phenol
0.1% SDS
0.5M EDTA (pH-8.0)
1.6M sodium acetate

iii. 400µl of DEPC treated RNase free water was added and mixed it by grinding.
iv. The mixture was transferred to 2ml micro centrifuge tube and left for 5 minutes at room temperature.
v. 200µl of chloroform was added to each micro centrifuge tube and vortexes vigorously for 5-10 sec and left for 10 minutes at room temperature.
vi. The sample was centrifuged at 12,000 rpm for 10 minutes at 4^{0}C.
vii. The upper aqueous phase was transferred to a fresh 1.5ml micro centrifuge tube.
viii. 0.6 volumes of isopropanol were added to the sample to precipitate the RNA and vortexed vigorously for 5-10 sec and left for 10 minutes at room temperature.

ix. RNA was pelleted by centrifugng at 12,000 rpm for 10 min at 4˚C in 1.5 ml centrifuge tubes and the supernatant was discarded.
x. The pellet was washed with 70% ethyl alcohol and air-dried.
xi. Dried samples were dissolved in DEPC treated RNase free water and stored at -20ºC for further use.

3.10.4. Agarose gel electrophoresis of extracted RNA

Agarose gel electrophoresis was done using the Genei RNA gel electrophoresis kit (Genei, Bangalore) for visualization of extracted RNA. 4-5 µl extracted RNA was run on agarose gel in 1X MOPS buffer. 2 µl of 10mg/ml ethidium bromide was added to the RNA along with 14 µl RNA sample buffer. The gel was run at 50V till the dye front bromophenol blue reaches ¾ of the gel.

3.10.5. Polymerase chain reaction (PCR) from genomic DNA

Polymerase chain reactions (PCR) were performed with 2 µl of genomic DNA as templates in 25µl reaction mixture. The amplification was carried out using a MJ Mini Personal Thermal Cycler (Bio-Rad) with the help of gene specific primer (Table 3.4). The reaction mixtures containing the following components:

Reagents	Amount
Taq buffer (5X)	5.0 µl
Taq DNA polymerase(5u/µl)	0.13 µl
dNTP mix (10mM each)	1.0 µl
$MgCl_2$ (25mM)	1.5 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Template (DNA)	2.0 µl
Sterile water	13.37µl

3.10.6. RT-PCR (Reverse Transcriptase-PCR) from total RNA

First step in the RT-PCR, first strand cDNA was synthesised from the isolated RNA using the M-MuLV RT-PCR kit (Genei, Bangalore) following

manufacturer protocol. Reverse transcription reaction was done by M-MuLV reverse transcriptase with oligo-(dT) primers, 10X RT-PCR buffer (2µL), RNase inhibitor (1 µL). The mixture was incubated at 45ºC for 1 h after that the reaction was terminated by heating at 95ºC for 5 min and the product was stored at -20ºC up to 2hr or directly used as a template for PCR amplification. Polymerase chain reactions (PCR) were performed using a MJ Mini Personal Thermal Cycler (Bio-Rad) with the help of gene specific primer (Table 3.4) which containing 2µl cDNA as templates in 25µl reaction mixture. Others components of the reaction mixtures are as follows:

Reagents	Amount
RT-PCR buffer (10X)	2.5 µl
Taq DNA polymerase(3u/µl)	0.5 µl
dNTP mix (10mM each)	1.0 µl
$MgCl_2$ (25mM)	1.5 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Template (cDNA)	2.0 µl
Sterile water	15.5µl

3.10.7. Agarose gel electrophoresis of PCR products

After successful completion of PCR, amplification product were electrophoresed at 1% agarose gel in 1X TAE (Tris Acetic acid EDTA) buffer and gel was visualized under UV- transilluminator following ethidium (10mg/ml) bromide staining. Expected amplicon size was measured by using standard molecular weight markers of DNA.

Table 3.4: List of primer used for PCR amplification

Primer name	Primer sequence
PAL	F: 5'-ACAACAATGGGTTGCCATCGAATC-3'
	R: 5'-ACTTGGCTAACACTGTTCTTGACA-3'
CHS	F: 5'-AACAAGGTTGCTTTGCCGGTGGCA-3'
	R: 5'-GATGAGCCCAGGAACATCCTTGAG-3'
APX	F: 5'-AAGAAGGCTAATGACAAAGCCAAG-3'
	R: 5'-GCTTCATGTGGGCTTCTGCATA-3'

F-Forward primer; R- Reverse primer

3.10.8. Purification of PCR products

After electrophoresis, the purification process was done using Wizard SV Gel and PCR Clean-Up System (Promega) following manufacturer's protocol. The detailed processes are as follows:

i. The expected DNA fragments were excised from the gel using a sharp blade under the UV-transilluminator and placed in a 1.5ml micro centrifuge tube.
ii. Then 10µL Membrane Binding Solution was added per 10mg of gel slice and vortexed.
iii. After that the tube was incubated at 55-65°C until the gel slice was completely dissolved and adds an equal volume of Membrane Binding Solution.
iv. Then the dissolved gel mixture was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute.
v. The mixture was centrifuged at 16,000×g for 1 minute and the flow-through was discarded.
vi. The Minicolumn was reinserted into the collection tube and 700µL of Membrane Wash Solution was added.
vii. The tube (with column) was centrifuged at 16,000×g for 1 minute and the flow-through was discarded
viii. After that 500µL Membrane Wash Solution was added in the column and centrifuged at 16,000×g for 5 minutes.
ix. After centrifugation the collection tube was emptied and recentrifuged the Minicolumn (with empty collection tube) for 1 minute with the lid open to allow evaporation of any residual ethanol.
x. Then the Minicolumn was transferred to a clean 1.5ml microcentrifuge tube and added 50µL nuclease-free water to the Minicolumn and incubated at room temperature for 1 minute.
xi. Finally the tube was centrifuged at 16,000×g for 1 minute and the Minicolumn was discarded and purified DNA was stored at -20°C.

3.10.9. Cloning of PCR product

The PCR product with expected size were cloned into the pGME®-T Easy Vector using pGME®-T easy cloning kit (Promega, USA) following manufactures protocol. The details cloning procedure are as follows:

3.10.9.1. Preparation of competent cells

Competent cells of *E. coli* (JM109, Promega) for transformation were prepared following the method of Sambrook and Russel (2001) with some modification. Detailed method of component cells preparation is carrying out with following steps:

i. A single colony of JM109 cells were grown overnight at 37°C on 10ml LB (Luria-Bertani) broth medium.
ii. 100µl of overnight growing culture was inoculated into 5ml of fresh LB broth.
iii. After that the culture was grown 2 to 4 hrs. (till O.D. reached 0.3 to 0.4 at 600nm) at 37°C on a rotary shaker at 100 rpm.
iv. The growing culture was transferred to 1.5ml micro centrifuge tube and was pelleted by centrifugation at 6,000 rpm for 10 min at 4˚C and the supernatant was discarded.
v. 750µl of solution-I (mixture of $MgCl_2$ and $CaCl_2$) was mixed with pelleted cells and the cells were resuspended by gently mixing.
vi. The mixture was centrifuged at 5000 rpm for 7 minutes at 4^0C and the supernatant was discarded.
vii. The pellet was resuspended in 750µl of 100mM $CaCl_2$ solution by gently mixing and incubated on ice for 45 minutes.
viii. The cells pellet was again recovered from the mixture by centrifugation at 5000 rpm for 5 minutes at 4^0C.
ix. The supernatant was discarded and the pellet was resuspended in 500µl of 100mM $CaCl_2$ and stored at -20°C for overnight.

3.10.9.2. Preparation of ligation mixture

The ligation mixture was prepared by using pGME®-T Easy Vector, purified PCR product and 2X rapid ligation buffer in a 0.5 ml PCR tubes.

Tubes containing ligation mixture were incubated overnight at 4^0C. The ligation mixtures containing the following components:

2X rapid ligation buffer	5 µl
pGME®-T Easy Vector	1 µl
T_4 DNA Ligase	1 µl
DNA (PCR product)	2 µl
Deionized H_2O	1 µl

3.10.9.3. Transformation

Previously prepared competent cells (section 3.10.9.1.) were used for the transformation purpose. The detailed transformation procedures are as follows:

i. 2µl of ligation mixture was added to the 100µl competent cells and incubated in ice for 45 minutes.
ii. After ice incubation heat shock was given at 42^0C for 90sec.
iii. Then the tube was transferred directly to ice and incubated for 10 minutes.
iv. After ice incubation 300µl LB broth was added to the tube and incubated at 37ºC for 2 hrs with shaking at 200 rpm.
v. Centrifugation was done at 6000 rpm for 6 minutes at 4ºC.
vi. After centrifugation 300 µl supernatant was discarded and the remaining one was gently mixed with the pellet.
vii. Then 100µl transformed culture were spread onto LB agar plates containing ampicillin (50mg/ml), IPTG (20%) and X-gal (4%).
viii. The plates were incubated overnight at 37ºC.
ix. Positive transformed cells were selected by blue-white screening.

3.10.9.4. Detection of positive clone by colony PCR

After successful transformation, the positive transformed cells were further tested through boiling lysis followed by PCR. Each of the colonies was mixed with 200µl sterile water in a 0.5 ml PCR tubes. The mixture was then boiled at 100˚C for 10 minutes in water bath. After boiling the mixture was rapidly cooled down and centrifuged at 7000 rpm for 5 minutes at 4˚C.

The supernatant was used as template for PCR reaction. PCR reaction was carried out following the process as described in the section 3.10.5.

3.10.10. Sequence analysis of defense related genes

3.10.10.1. Sequencing and phylohenetic analysis of cloned product

The positive cloned products were sent for sequencing to Chromous Biotech Pvt. Ltd., Bangalore, India. The nucleotide (nt) sequence was aligned using ClustalW 1.6 (Thompson *et al.*, 1994) and submitted in the GenBank after BLASTn analysis (Altschul *et al.*, 1997). Nucleotide identity was calculated using SDT v1.2 (Muhire *et al.*, 2014). The phylogenetic tree of the three defense related genes viz., Ascorbate peroxidase (APX), Chalcone synthase (CHS) and Phenylalanine ammonia lyase (PAL) were generated through neighbour-joining method with Kimura-2 parameter using MEGA 6.0 (Tamura *et al.*, 2013).

3.10.10.2. Codon usage analysis

CodonW version 1.4.2 software was used to analyze the codon usage pattern of the above mentioned three defense related genes (Peden 1991). GC1 and GC2 contents of the same were calculated using Codon O (Angellotti *et al.*, 2007). Correlation among nucleotide composition and synonymous codon usage patterns were analyzed in SPSS Statistics 20.

3.10.10.2.1. Nucleotide and amino acid composition

Four nitrogenous bases (A, T, G and C) are the basic composition of nucleotides of any gene. The nucleotide composition at the third position of the synonymous codons (i.e., A3, T3, G3 and C3) of PAL, APX and CHS coding genes were calculated. The overall frequency of GC content and the frequency of GC at the first, second and third position of synonymous codon (GC1, GC2 and GC3) were also calculated (excluding the codons AUG and UGG that codes for Met and Trp respectively along with the three termination codons UAA, UAG and UGA). Number of synonymous codons and translatable codons were assessed by length of synonymous codon (L_sym) and length of amino acids (L_aa) respectively. Chemical property of amino acids for hypothetical translated gene products were analyzed by

using two different index- (a) Grand average of hydropathy (GRAVY) which indicated average hydropathicity and (b) Aromo that indicated aromaticity i.e. frequency of aromatic amino acids (Xu *et al.*, 2008; Wei *et al.*, 2014).

3.10.10.2.2. Effective number of codon (Nc)

Effective number of codons (Nc) is one of the useful index to study the effect of mutation on overall codon bias. Effective number of codons (Nc) measure the factors affecting codon bias among absolute synonymous codons for the corresponding amino acids in a gene. The Nc values ranges from 20 to 61. To measure the synonymous codon bias Nc values were plotted in a graph in correspondance to GC3 values. The Nc values located on or just below the standard curve indicated the strong effect of mutational pressure on codon usage bias whereas, the values far below from the standard curve indicated involvement of other factors like translational selection in shaping codon usage bias apart from mutational pressure. Nc values close to 20, points to highly biased genes where only one codon was used for each amino acid. Nevertheless, the unbiased genes show Nc values closer to 61, where all the synonymous codons were used uniformly for every amino acid.

3.10.10.2.3. Analysis of neutrality plot

Neutrality plot analysis was used to estimate the influence of mutational pressure and natural selection in overall codon usage bias. In the neutrality plot analysis, GC12 contents were plotted in Y axis against the GC3 contents in X axis and a regression line was calculated.

3.10.10.2.4. Correspondence analysis (COA)

Correspondence analysis was used to analyze the effect of different factors that influenced the synonymous codon usage bias of a gene. COA is a mathematical calculation that was used to measure the major variation trends in codon usage pattern of a gene (i.e. major variation in gene order according to their positions along the major axes) by using the RSCU values (Wright, 1990). In COA analysis, each coding sequence of a gene was represented as a 59 dimensional vector according to their 59 sense

codons and each dimension corresponded to the RSCU value of each sense codon.

3.10.10.2.5. Relative synonymous codon usage (RSCU)

The RSCU value refers to the ratio of the observed frequency of a specific codon and the expected frequency of synonymous codon for an amino acid sequence. If the RSCU value of a codon was 1.0 to 1.5 meant codon was used frequently. The RSCU values less than 1.0 denoted that codon was used less frequently and the RSCU values greater than 1.5 denoted that codon was used more frequently in a particular gene (Sharp and Li, 1986; Zhao *et al.*, 2016).

3.11. Gene expression analysis by qRT-PCR

Real time PCR is a method that quantifies expression levels of mRNA. This method quantifies the amount of final products at the end-time. This method is more specific and sensitive than other PCR methods. This method is reproducible too. The different steps for conducting real time PCR have been discussed in the next sub-sections.

3.11.1. Preparation of plant samples for gene expression analysis

Six month old young tea plants were divided into four sets: control (untreated-uninoculated, where only sterile distilled water was used), uninoculated-BTH treated, inoculated-untreated and inoculated-BTH treated. Each set contained three biological replicates. All the chemicals were supplemented with 0.5% Tween-20 as adherent. All the test plants were observed after every 24 hours of inoculation up to 4 days and 1 gm leaf sample from each replica was collected and dipped in liquid nitrogen and total RNA was isolated immediately for expression analysis or stored at -20^{o}C.

3.11.2. Extraction of RNA for gene expression analysis

All the plasticwares and glasswares 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. Detailed methods are as follows:

i. One gram of leaf sample each, stored at -20˚C were pulverized to powder in liquid N_2 using sterile mortar and pestle.
ii. After that 10ml of extraction buffer (8M Guanidium hydrochloride, 20mM EDTA and 20mM HEPES) was added to the powdered sample with fresh addition of 20mM Beta Mercaptoethanol and finally vortexes vigorously for 5 minutes.
iii. Then the mixture was centrifuged at 12,000 rpm for 10 minutes at 4^{o}C and the supernatant was transferred to a fresh vial.
iv. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added with the supernatant and vortexes vigorously.
v. The sample was centrifuged for 20 minutes at 12000 rpm.
vi. The upper aqueous phase was transferred to a fresh vial and 2ml of 1M glacial acetic acid was added to it along with 7ml of 100% chilled ethanol.
vii. Then the mixture was incubated at -20˚C for overnight.
viii. After that incubation mixture was centrifuged at 12000 rpm for 20 minutes at 4˚C to pellet the RNA.
ix. The pellet was resuspended in 5 ml of sodium acetate by vortexing vigorously for 1 minute.
x. Then the mixture was centrifuged at 12000 rpm for 5 minutes at 4˚C and was repeated twice.
xi. After that the pellet was washed with 5ml of 70% ethanol and centrifuged at 12000rpm for 5 minutes at 4˚C.
xii. This ethanol washing was repeated thrice and the pellet was dried at room temperature and was suspended in RNase free water.

3.11.3. DNase treatment

The extracted RNA was treated with DNase enzyme to remove any traces of DNA contamination. One micro liter of DNase was added to above RNA and incubated for 1hr at 37˚C and after the temperature was raised to

70˚C for 5 minutes to inactivate the enzyme. The RNA was stored at -20˚C for future use.

3.11.4. RNA Quanitifcation

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 was deemed indicative of pure RNA.

3.11.5. cDNA synthesis

After quantification RNA was reverse transcribed using oligo dT (Sigma Aldrich). Hundred nano gram of RNA was aliquoted to a fresh sterile microfuge tube and 2µl of oligo dT was added and incubated at 70˚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˚C for 90 minutes and reaction was terminated by incubating at 70˚C for 15 minutes.

3.11.6. Primer design and synthesis

The primers for Relative quantification analysis were designed using Primer Express software of Applied Biosystems. The Tm was calculated and adjusted and the synthesized primers were purified by HPLC. Following primer sets are used for experimental purposes:

Primer name	Primer sequence
PAL1	FP 5' GTGTTCTTTGCCTCGTCAGA 3'
	RP 5' ATCCCTGGAGCAAGGTATTG 3'
PAL2	FP 5' TCCGATCATCGACAAAATCA 3'
	RP 5' AGCTCAGAGAATTGGGCAAA 3'
Plant Actin	FP 5' CAAGCAGCATGAAGATCAAGGT 3'
	RP 5' CACATCTGTTGGAAAGTGTGAG 3'

FP- Forward primer; RP- Reverse primer

3.11.7. PCR standardization

A gradient PCR was performed to standardize the optimum annealing temperature of the designed primer using 30 ng of synthesized cDNA

keeping the temperature range between 50 - 60°C. Optimum temperature was confirmed as 60°C for all the primers.

3.11.8. Relative Quantification using Real Time PCR

The quantification was done in Applied Biosystems StepOne Real Time PCR using the SYBR Green Chemistry. The relative changes in gene expression were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The reaction was carried out in a 10µl reaction volume with the components as given below:

First strand cDNA	0.5 µL
SYBR Green Master Mix (2X)	5.0 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Nuclease-free water	3.5 µL

qRT PCR conditions

Conditions	Temperature	Time	Cycles
Initial denaturation	95˚C	20 sec	} 40 cycles
Denaturation	95˚C	15 sec	
Annealing & Extension	60˚C	30 sec	-

3.12. List of major chemicals used

In addition to the common laboratory reagents, following chemicals were used during the work:

Chemicals	Company
10x Taq DNA buffer B	Genie, Bangalore
6x loading dye	Genie, Bangalore
Acetic acid	SRL Pvt. Ltd., Mumbai, India
Agar powder	SRL Pvt. Ltd., Mumbai, India
Agarose	SRL Pvt. Ltd., Mumbai, India
Ampicilin	HiMedia, Mumbai, India
2, 1, 3-Benzothiadiazole	Fluka, Switzerland
3- Amino butyric acid	Fluka, Switzerland
4- Amino butyric acid	Fluka, Switzerland
Calcium chloride	HiMedia, Mumbai, India
Chloroform	E. Merck, Mumbai, India
CTAB	CDH Pvt. Ltd., New Delhi, India
3, 5- Dinitrosalicylic acid	Hi Media Laboratories, Mumbai, India
DEPC-treated water	Chromus Biotech, Bangalore, India
dNTP mix	Genie, Bangalore
EDTA	SRL Pvt. Ltd., Mumbai, India
Ethydium bromide	SRL Pvt. Ltd., Mumbai, India
Ethyl alcohol	JHI Co. Ltd., China
Formaldehyde	SRL Pvt. Ltd., Mumbai, India
Glacial acetic acid	SRL Pvt. Ltd., Mumbai, India
Glycerol	SRL Pvt. Ltd., Mumbai, India
Guiacol	SRL Pvt. Ltd., Mumbai, India
Hydrochloric acid	E. Merck, Mumbai, India
Hydrogen peroxide	E. Merck, Mumbai, India
IPTG	Promega Corporation, USA
Isoamyl alcohol	E. Merck, Mumbai, India
Isopropanol	SRL Pvt. Ltd., Mumbai, India
JM 109	Promega Corporation, USA

Table 3. 12. contd...

Laminarin	Sigma Aldrich, USA
L-Phenylalanine	SRL Pvt. Ltd., Mumbai, India
Luria Bertani Broth	SRL Pvt. Ltd., Mumbai, India
$MgCl_2$	Genie, Bangalore
M-MuLV reverse transcriptase	Genie, Bangalore
M-MuLV RT-PCR kit	Genie, Bangalore
NaCl	E. Merck, Mumbai, India
Oligo $(dT)_{18}$ primer	Promega Corporation, USA
pGEM- T easy cloning kit	Promega Corporation, USA
pGEM-T vector	Promega Corporation, USA
RNA gel electrophoresis kit	Genie, Bangalore
RNase	Promega Corporation, USA
Sodium acetate	SRL Pvt. Ltd., Mumbai, India
Sodium dodecyl sulphate (SDS)	E. Merck, Mumbai, India
Sodium Sulphite	E. Merck, Mumbai, India
T_4 ligase	Promega Corporation, USA
Taq DNA polymerase	Genie, Bangalore
Total RNA extraction kit	Genie, Bangalore
Trichloroacetic acid (TCA)	Universal laboratories Pvt. Ltd, Mumbai, India
Tris	SRL Pvt. Ltd., Mumbai, India
Tris saturated phenol	SRL Pvt. Ltd., Mumbai, India
X- Gal	Promega Corporation, USA
β-marcaptoethanol	SRL Pvt. Ltd., Mumbai, India

3.13: Composition of Buffers, solutions and media used

Several buffers, solutions and media were used in the the present study. Composition and procedure of preparation of those have been given in the following paragraphs/sub-sections.

3.13.1. POTATO DEXTROSE BROTH (PDB)

Peeled potato	40 g
Dextrose	2 g
Distilled water	100 ml

(Required amount of peeled potato was boiled in distilled water. The potato broth was taken by straining through cheesecloth and required amount of dextrose was added. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes).

3.13.2. POTATO DEXTROSE AGAR (PDA)

Potato dextrose agar was prepared by adding 2% agar powder to the final potato dextrose broth solution. The agar was melted by heating the media before sterilization.

3.13.3. OAT MEAL AGAR (OMA)

Oat meal	40 g
Agar agar	15 g
Distilled water	1000 ml

(Powdered oat was boiled in distilled water in a water bath, occasionally stirred and strained through cheese cloth. Then required amount of agar powder was added to it and boiled with constant shaking till the agar was dissolved. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes).

3.13.4. Sodium phosphate buffer 0.1M (pH 6.5)

Stock solution A:

Na_2HPO_4, $7H_2O$	3.5g
Distilled water	100ml

Stock solution B:

NaH_2PO_4, $2H_2O$	3.1g
Distilled water	100ml

32ml of stock solution A was added to 68ml of stock solution B to obtain a pH of 6.5.

3.13.5. 0.05 M Sodium acetate buffer, pH 5.0

Stock solution A:

Glacial acetic acid (0.1M)	5.77 ml
Distilled water	1000 ml

Stock solution B:

Sodium acetate ($C_2H_3O_2Na.3H_2O$)	13.61g
Distilled water	1000 ml

(6.4 ml of solution A and 3.6 ml of solution B were mixed and pH was adjusted to 5.0).

3.13.6. 0.2 M borate buffer, pH 8.7

Stock solution A:	
Boric acid	1.24 g
Distilled water	100 ml
Stock solution B:	
Borax	1.90g
Distilled water	100 ml

(50 ml of solution A and 22.5 ml of solution B were mixed and pH was adjusted to 8.7).

3.13.7. Potassium phosphate buffer [0.1M; pH- 7.0]

Stock solution A:	
KH_2PO_4	136.09g
Distilled water	1000 ml
Stock solution B:	
K_2HPO_4	174.18g
Distilled water	1000 ml

38.5 ml of stock solution A was added to 61.5 ml of stock solution B and distilled water was added to obtain a final volume of 1000 ml (pH 7.0).

3.13.8. 2X CTAB DNA extraction buffer (for per gram of leaf tissue):

Tris (1M)	500µl (pH 8.0)
NaCl (5M)	1.4ml
EDTA (0.5M)	200µl (pH 8.0)
β-marcaptoethanol	10µl
Sterile water	2.89ml
CTAB	100mg

3.13.9. TE buffer:

Tris-HCl	10mM
EDTA	1mM
Final pH	8.0

3.13.10. TAE buffer (composition of 50X TAE):

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml
Distilled water (final volume made up to)	1000 ml
Final pH	8.0

To make 1X TAE buffer, 1 ml 50X stock buffer was diluted in 49 ml distilled water to make final volume 50 ml.

3.13.11. Ethidium bromide stock solution (1 ml):

0.5mg ethidium bromide was dissolved in 1 ml distilled water; Stored in dark bottle at 4˚C.

3.13.12. Hogland and Knop solution

KNO_3	0.61 g
$Ca(NO_3)_2, 4H_2O$	0.95 g
$MgSO_4, 7H_2O$	0.49 g
$NH_4(H_2PO_4)$	0.12 g
$MnSO_4, 4H_2O$	3.00 g
$ZnSO_4, 7H_2O$	0.5 mg
H_3PO_3	0.5 ml
$CuSO_4, 5H_2O$	0.025 mg
$Na_2MoO_4, 2H_2O$	0.025 mg
H_2SO_4	0.5 µl
$FeC_6O_5H_7, 5H_2O$	0.2 g
Distilled water	1000 ml

Required amount of all the constituents were taken and mixed thoroughly in distilled water.

3.13.13. 1x MOPS buffer

10x MOPS Buffer Composition:

200 mM MOPS, pH 7.0	41.9 g
80 mM Sodium Acetate	4.1 g
10 mM EDTA, pH 8.0	3.7 g

Then the final volume was made up to 1.0 liter with distilled water. Then 100 ml of 10X MOPS buffer and 20 ml 37% Formaldehyde was mixed with 880 ml distilled water to make 1X MOPS buffer.

3.13.14. Luria-Bertani (LB) Agar

To make Luria-Bertani (LB) Agar 25.0 g of powder was mixed with distilled water. Then agar powder was added at the rate of 1 % i.e., 10 g and the final volume was made up to 1.0 liter with distilled water. Then it was autoclaved at 15 psi pressure at 121° C for 15 minutes.

3.13.15. Luria-Bertani (LB) Broth, Miller

To make Luria-Bertani (LB) Broth 25.0 g of powder was mixed with distilled water to make the final volume of 1.0 liter. Then it was autoclaved at 15 psi pressure at 121° C for 15 minutes.

3.13.16. Ampicillin stock solution (50 mg/ml)

One gram of ampicillin was dissolved in 10 ml of sterile distilled water. Solution was filter sterilized using Whatman poly ethersulfone membrane (0.2 µm pore size), stored in aliquots at -20°C.

3.13.17. X-Gal stock solution (4%)

400 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was dissolved in 10 ml of N, N-dimethylformamide. The solution was stored at -20˚C in a dark bottle.

3.13.18. IPTG stock solution (20%)

200 mg of IPTG (Isopropyl-β-D-thiogalactopyranoside) was dissolved in 1 ml of sterile distilled water. The solution was filter sterilized and stored in aliquots at 4˚C.

3.13.19. LB +Ampicillin+X-gal+IPTG plates

Autoclaved LB agar medium (100 ml) was allowed to cool to 50-55ºC. The medium was gently mixed with 100 µl of ampicillin stock solution (50 mg/ml), 100 µl of X-Gal stock solution (4%) and 20 µl of 20% IPTG stock solution and poured on 90-mm size petri plates. The plates were allowed to solidify and dried open under laminar air flow for 30 min.