Analysis of specific transcripts following induction of defense in tea against foliar fungal pathogens

Thesis submitted to the University of North Bengal
for the Award of Doctor of Philosophy in
Botany

Submitted by
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Declaration

*I, Shibu Das hereby declare that the work embodied in my thesis entitled "**Analysis of specific transcripts following induction of defense in tea against foliar fungal pathogens**" has been carried out by me under the supervision of **Dr. Aniruddha Saha**, Professor, Department of Botany, University of North Bengal and **Dr. Dipanwita Saha**, Assistant Professor, Department of Biotechnology, University of North Bengal for the award of the Degree of Doctor of Philosophy in Botany. I also declare that, this thesis or any part thereof has not been submitted for any other degree/ Diploma either to this or other university.*

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Dated: 02-11-2017

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Abstract

Tea (*Camellia sinensis* (L.) O. Kuntze.) as a beverage is an important plantation crop. Tea has stimulating and good health effects. Like many other plants tea also affected by a number of pathogens. Differential interaction of different varieties of tea against different pathogens has been observed by several scientists. The understanding of molecular events of the host pathogen interaction leading to induction of resistance in a susceptible variety is of great importance in the present day agriculture. Hence, the present study has been taken in to consideration. The title of the present work is "Analysis of specific transcripts following induction of defense in tea against foliar fungal pathogens". To fulfill the study the whole work has been divided into following objectives: 1) Control of disease by different abiotic inducers and disease assessment. 2) Induction of defence-related enzymes in tea by abiotic inducers. 3) Molecular identification of defense related genes of tea. 4) Analysis of selected gene specific transcript accumulation in induced tea plants and 5) *In vitro* control of pathogenic microorganisms by botanicals and biocontrol agents.

To fulfil the objectives, at the onset of the study, review of literature has been done, in the line of the present work, in a selective manner rather than in a comprehensive one.

Before initiation of the experimental work a survey was made for prevalence of the pathogens in the present study area (sub-Himalayan West Bengal). During the survey, four pathogens such as *Colletotrichum gloeosporioides, Curvularia eragrostidis, Pestalotiopsis theae* were found to attack tea plants and affect tea plants leading to loss of production. On the basis of Pathogenicity tests, two pathogens were selected for the study. Three susceptible tea varieties were also selected to study induction of resistance.

To understand the resistance induction by inducers at the time of host-pathogen interaction three different abiotic inducers (BTH, 3-ABA,

and GABA) were used. Detailed procedures of experiments and materials used for the experiments have been presented in the materials and methods section. Teenali variety was found to be most susceptible among the tested varieties.

BTH induced plants showed best induction of resistance, whereas, BABA and GABA showed moderate induction of resistance on the basis of disease index in comparison to control (untreated-inoculated) plants.

Three inducers (BTH, BABA and GABA) were further used to induce resistance in susceptible tea plants ('Teenali variety). Following induction up and down regulation of three different defense related enzymes were studied against challenge inoculation by *C. gloeosporioides,* the most virulent pathogen as found from results of pathogenicity tests. The enzymes studied were β-1, 3-glucanase, phenylalanine ammonia-lyase and peroxidase.

From the results it was found that BTH treated tea plants following challenge-inoculation by *C. gloeosporioides* showed maximum phenylalanine ammonia-lyase (PAL) activity after 4 days but highest activity of PAL was found only in BTH pre-treated plants after six days. In case of β-1, 3-glucanase, highest enzyme activity was found after six days of BTH treatment following challenge-inoculation with *C. gloeosporioides*. Marginally different results were found in case of peroxidase activity. GABA pretreated and *C. gloeosporioides* inoculated plants showed maximum peroxidise activity after six days.

The phylogenetic analysis of PAL gene showed three different clusters among three different *Camellia* species (*C. sinensis*, *C. talensis* and *C. flavida*), where the present isolates clusters together with *C. sinensis*. Phylogenetic tree of CHS gene showed five different clusters with different *Camellia* species but APX gene did not show any significant clusters like PAL and CHS gene. The results of BLASTn analysis of all the three genes (PAL, CHS and APX) showed more than 90% nucleotide sequence similarity with other *Camellia sinensis* gene submitted in the GenBank. From the sequence similarity matrix it was found that PAL gene of *Camellia sinensis*

showed above 90% nucleotide identity within the species and below 60% identity between *C. sinensis* and other species of *Camellia*. CHS gene showed above 95% nucleotide identity within the species and below 75% to 95% identity between *C. sinensis* and other species of *Camellia*. In case of APX gene, no significant group was observed as PAL and CHS gene through sequence similarity matrix.

Further analysis of sequence diversity among these three defense related genes (PAL, CHS and APX) relative synonymous codon usage (RSCU) pattern was also studied. From the results it was observed that highest %GC and GC3 along with overall pyrimidine content at third codon position were higher in CHS and PAL than APX.

To understand the pattern of codon usage and probable position of a particular gene an Nc plot were prepared. The results showed that codon usage bias was mainly influenced by mutational bias along with translational selection. To interpret this result a neutrality plot analysis was carried out to further understand the effect of GC content in different codon position to influence the major factor (mutational pressure or natural selection) in overall codon usage bias through a slope of regression line. The results indicated that natural selection pressure was much higher in APX gene followed by CHS and PAL gene respectively, whereas stronger correlation was found between GC12 and GC3 content in PAL gene which indicated equal forces of mutational pressure on each codon position worked on PAL gene.

From the results of codon usage bias of 59 sense codons on the basis of RSCU values it was found that number of frequently used codons as well as optimal codons are higher in PAL genes followed by CHS and APX respectively which indicated strong bias in PAL genes than CHS and APX genes. Correlation analysis indicated that A3 and L_aa affected codon usage in tea plants irrespective of different genes. But, correlation of other factors with Nc was more in PAL followed by CHS than APX.

Relative expression of phenylalanine ammonia-lyase (PAL) gene in tea was analyzed using quantitative real-time PCR (qRT-PCR) to compare the

effect of BTH on PAL transcript level against challenge inoculation with two foliar fungal pathogens *C. gloeospoiroides* (thought to be major pathogen) and *C. eragrostidis*. From the results it was observed that PAL transcript accumulation was elevated on 4th, 6th and 7th day in response to pre-treatment (by BTH) and challenge inoculation (by *C. gloeospoiroides*), whereas, in BTH treated and *C. eragrostidis*-inoculated plants, elevation in PAL gene expression was observed on 1st, 5th and 7th day post treatment. During enzyme estimation also, increased level of phenylalanine ammonia lyase was observed on 4th and 6th days after BTH pretreatment and challenge inoculation (by *C. gloeospoiroides).*

In vitro study of antagonistic activity of some common botanicals and four known biocontrol agents such as *Bacillus subtilis*, *B. pumilus*, *B. megaterium* and *Trichoderma harzianum* were tested to control mycelia growth of one of the most virulent pathogen *Colletotrichum gloeosporioides*. From the results it was found that 50% ethanolic extracts of *Datura metel* and *Clerodendrum viscosum* could inhibit more than 70% mycelia growth of the fungus. *Bacillus pumilus* was found to be the best antagonist and could inhibit 78% radial growth of the fungus tested. The other three bacterial antagonists also could inhibit the growth of the fungus *Colletotrichum gloeosporioides* upto a level of 60% and above.

This study also revealed certain new facts of fundamental importance. The significance of some defense related genes and their molecular characteristics have been demonstrated. Our investigations have provided an insight in to the mechanism of resistance induction in tea plants against some pathogens of tea. Differential expression of PAL gene have been studied by semi-quantitative (by RT PCR) and quantitative (by qRT PCR) methods following induction of resistance by known resistance inducer (BTH). The studies on expression of mRNA of PAL gene have extended our present knowledge of molecular expression of PAL gene in tea plants.

Preface

Tea is an evergreen perennial woody, shrub plants which is used worldwide as a non-alcoholic beverage. Green leaves of tea are used as vegetables in Burma and Thailand. Tea has some medicinal importance against blood pressure, coronary heart Diseases, and diabetes. Tea is characterized into three types green, black and oolong tea on the basis of its fermentation process of tender leaves. Now a day's the commercial production of tea is done in about 46 countries worldwide. North East India is one of the major tea producing zones of India. Several pathogens attack tea plants and damage the plants by reducing the production. In certain cases production is substantially reduced due to fungal attack on the leaves.

Management of diseases of tea plant by chemical pesticides have several drawbacks like harmful effects on human health and also has negative impact on the environment. The dependence on chemical pesticides and fungicides need to be reduced in future. For this one of the major practices in different crops *i.e.* induction of resistance by resistance inducers, also need to be introduced in tea following field trials etc.

Several scientists have shown that defense response may be initiated by exogenous application of some abiotic inducers. During the last twenty years several such inducers have been shown to induce defense (systemic acquired resistance) in a variety of plants. When a plant is infected by pathogens, a large number of genes that are involved in various metabolic activities, signal transduction, transcriptional regulation, and defense responses are activated. The regulation of defense-related genes is one of the important defense mechanism that is used by plants against pathogenic organisms.

To know the regulation of defense-related genes, study of transcripts by semiquantative and/or quantitative polymerase chain reaction (PCR) is essential. Hence, one of the important defense enzymes, Phenylalanine

ammonialyase (PAL), which is frequently increased in plants in response to pathogen invasion, has been studied in details in the present study. Another two defense related enzymes (β-1, 3 glucanase and peroxidase) have also been studied up to the level of enzyme induction. Molecular identification of defense related gene (PAL) of tea (*Camellia sinensis*) has also been done and the sequence of the gene has also been submitted to Genbank. Additionally, two other defense related genes such as Ascorbate peroxidase (APX) and Chalcone synthase (CHS) were also amplified from tea plants. Molecular characteristics the three genes have been studied in details.

Specific transcripts of PAL have been studied against one of the best known defense inducer of plants such as Benzothiodiazole (BTH). Before transcript studies the assay of enzymes have also been done by BTH along with two other abiotic inducers (BABA and GABA). As BTH was found to be best inducer of defense enzyme PAL, therefore PAL transcripts were thought to be studied initially by semi quantitative method. Following semi-quantitative method, quantitative method by real time PCR were also done to confirm the regulatory status of specific transcripts of PAL in a susceptible tea variety against two pathogens of tea. One pathogen is *Colletotrichum gloeosporioides* (highly virulent) and *Curvularia eragrostidis* (comperatively less Virulent). At the end of the study *in-vitro* control of one of the pathogen has been done by some botanicals and biocontrol agents.

The present work was initiated with major objective of study transcriptomes of defense related genes. That has been achieved through the transcriptome studies of PAL. Finally the work has been compiled in six major sections and several sub-sections and presented in the thesis.

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Abbreviations

%GC	Percentage of GC
µg	Microgram
µl	Microlitre
µm	Micrometer
^{0}C	Degree Celcious
A3	Adenine at 3rd codon position
A3	Adenine at 3rd codon position
aa	Amino acid
APX	Ascorbate peroxidise
Aromo	Aromaticity
ASM	Acibenzolar-S-methyl
ASM	Acibenzolar-S-methyl
BABA	3- Amino butyric acid
BLAST	Basic Local Alignment Search Tool
BN	Bios noir
bp	base pair
BTH	Benzothiadiazole
C3	Cytosine at 3rd codon position
CAI	Codon adaptation index
CAT	Catalase
CBI	Codon bias index
CD	Critical Difference
CHI	Chitinase
CHS	Chalcone synthase
CJ	Cis-jasmone
CTAB	Cetyl trimethyl ammonium bromide
d	Day(s)
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide tri phosphates
dpi	Day post inoculation
dT	Deoxythymine
EDTA	Ethylenediamine tetra acetic acid
Fop	Frequency of optimal codon
g	gram
G3	Guanine at 3rd codon position
GABA	4- Amino butyric acid
GC2	GC content at 2nd codon position
GC3	GC content at 3rd codon position
Gravy	Hydropathicity
h	hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric Acid
IAA	Indole Acetic Acid
IARI	Indian Agricultural Research Institute
INA	2, 6-dichloroisonicotinic acid
IPTG	Isopropyl-β-Dithiogalactopyran oside
ISR	Induced Systemic Resistance
ITCC	Indian Type Culture Collection
JA	Jasmonic Acid
K_2HPO_4	Dipotassium hydrogenphosphate
K_2PO_4	Dipotassium phosphate
kb	kilo bases
KCL	Potassium chloride
L	Litre
L_sym	Length of synonymous codon
LB	Luria Bertain
LOX	Lipoxygenase
lt	litre
M	Molar

m	Meter
M	Mole
M	Mole
mAmp	Miliampere
MEGA	Molecular evolutionary genetics analysis
MeJA	Methyl jasmonate
mg	Miligram
min	Minutes
ml	Milliliter
mM	Milimole
mm	Millimeter
M-MulV	Moloney Murine Leukemia Virus
MOPS	3-(N-Morpholio) propanesulphonic acid
mRNA	messenger RNA
N	Normal
Na_2PO_4	Dipotassium phosphate
NaCl	Sodium chloride
Nc	Effective number of codons
NCBI	National Centre for Biotechnology Information
nm	Nanometer
NO	Nitric oxide
nt	Nucleotide
OX	Over expression
PAL	Phenylalanine ammonialyase
PCR	Polymerase chain reaction
PDB	Potato Dextrose Broth
PGPR	Plant Groth Promoting Rhizobactera
POX	Preoxidase
PPO	Polyphenol oxidase
PR	Pathogenesis-related
PVP	Polyvinyl Pyrrolidone
qRT-PCR	quantitative real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotation per minute
RSCU	Relative synonymous codon usage
RT-PCR	Reverse transcription PCR
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SE	Standard Error
SIR	Systemic inducing resistance
ss	Single-stranded
T3	Thymine at 3rd codon position
TAE	Tris Acetate EDTA
TDL	N-(3-chloro-4-methylphenyl)-4-methyl-1, 2, 3 -thiadiazole-5-carboxamide
TV	Tocklai Variety.
UPASI	United Planters Association of South India
V	Volt
v/v	Volume by Volume
w/v	Weight by Volume
Wt	Weight
X-Gal	5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside

CONTENTS

1. Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze.) occupies an important position among the plantation crops due to its popularity. Among the beverages tea is one of the low caffeine containing plant. Tea has stimulating and good health effects. Flavan 3-ols of green tea has health-promoting effects (Chacko *et al*, 2010). Tea leaves contain four different compounds like (–)-epigallocatechin, (–)-epicatechin, (+)-gallocatechin, and (+)-catechin along with their derivatives which has positive effects on human health as reported by Jigisha *et al*. 2012; Macedo *et al*., 2012; Azzahra *et al*. 2012; Punyasiria *et al*. 2004).

Tea originated in southwest of China over 4000-5000 years ago (Mondal *et al*., 2004; Chen and Lin, 2015). In 2017, about 47 countries have been reported to grow tea for commercial production (https://ratetea.com/region.php). Tea plants are cultivated between 42º north to 35º south latitude. Tea plants thrive well in, humid climate, well drained acidic soil (pH- 4.5 to 5.5) well distributed rainfall and long sunshine hours. Three major tea growing areas of India are Northeast India, Northwest India and South India. Northeast Indian tea growing areas belong to states of Assam, West Bengal, Bihar, Tripura, Sikkim, Manipur, Nagaland, Meghalaya, Arunachal Pradesh, and Mizoram. Southern region include three states (Tamil Nadu, Kerala and Karnataka) and Northwest region include two states Himachal Pradesh and Uttarakhand (Sharma *et al*., 2010; Bhardwaj *et al*., 2014).

Plants generally initiate various defense reactions to protect it. Some of these are production of phytoalexines, antimicrobial proteins, reactive oxygen species etc. when they are infected by any of the pathogens. Successful defense reactions do not allow the infection to proceed. Successful defense depends upon several factors. If the reactions occur within a very short time the plants can protect themselves and the plants

are considered to be resistant plants. However, if the defense reactions do not occur or occur at a later stage, the infection process, proceed successfully and plants are said to be susceptible plants (Somssich and Hahlbrock, 1998; Sharma *et al.*, 2012; Leon and Montesano, 2013).

Fungal infection is one of most destructive of 'biotic stress' affecting tea plants. Like many other plants, tea plants are also susceptible to several fungal pathogens. Some of the diseases like blister blight, grey blight, brown blight and leaf spot are of serious nature and caused by fungal pathogens such as *Exobasidium vexans, Pestalotiopsis theae, Colletotrichum camelliae* and *Curvularia eragrostidis* respectively. Tea plants are also exposed to some other fungal pathogens like *Lasiodiplodia theobromae, Corticum theae, Fusarium oxysporum, Rhizoctonia bataticola, Fusarium solani, etc.* (Sarmah *et al.* 2016; Saha *et al.*, 2001; Sarmah, 1960; Naglot *et al.*, 2015; Liu *et al.*, 2017).

Several scientists have shown that defense response may be initiated by exogenous application of some biotic and abiotic inducers. During the last twenty years several such inducers have been shown to induce defense (systemic acquired resistance) in a variety of plants (Ghosh and Purkayastha, 2003; Kaur and Kolte, 2001; Justyna and Ewa, 2013; Ghosh, 2015). When a plant is infected by pathogens, a large number of genes that are involved in various metabolic activities, signal transduction, transcriptional regulation, and defense responses are activated (De Vos *et al.,* 2005). The regulation of defense-related genes is one of the important defense mechanism that is used by plants against biotic and abiotic agents (Edreva, 2005).

Although, the molecular interaction between fungus and plant is yet to be elucidated in details but only comprehensive approaches of transcriptome and proteome analysis have become available (Campo *et al.*, 2004; Tan *et al.*, 2015; Thatcher *et al.*, 2016). Differential expression of messenger RNAs has provided intriguing results in different host-pathogen interactions. High degree of variability has been detected between "*Fusarium verticillioides* and susceptible maize lines infection" and

"*Fusarium verticillioides* and resistant maize lines infection". Although similar functional categories of genes were involved in the response to infection in resistant and susceptible maize genotypes, in the susceptible line, the genes were qualitatively induced from a basal level and responded specifically to pathogen infection. In the resistant line, the defense-related genes assayed were transcribed at high level before infection and provided basic defense to the fungus (Lanubile *et al.*, 2010 & 2012).

Some of the major challenges of the present day cultivation of tea plants include susceptibility of the tea plants to harmful pests and fungal diseases. In the present study, from the fields it has been experienced that brown blight caused by *Colletotrichum camelliae* and leaf spot disease caused by *Curvularia eragrostidis* and *Lasiodiplodia theobroamae* are three important diseases to be taken into consideration for successful cultivation of tea in the sub-Himalayan West Bengal, the present study area (Figs. 1.1, 1.2 & 1.3).

A separate term 'induced systemic resistance' (ISR) was proposed by Kloepper *et al.*, in 1992. ISR differs mechanically from SAR (Systemic acquired resistance). Plant growth promoting rhizobacteria (PGPR) trigger plant-mediated resistance mechanism also called induced systemic resistance (ISR), which can suppress the disease by suppressing the causal foliar pathogen (Dube, 2001; Beneduzi *et al.*, 2012).

Plant enzymes are involved in defense reactions and in some cases successfully protect plants against plant pathogens. In cases where enzymes are produced at a later stage or the enzymes cannot be produced up to a threshold level required for resistance, results to susceptibility. Some of those enzymes are oxidative enzymes such as phenylalanine ammonia- lyase (PAL) and polyphenol oxodase (PPO), which catalyse the formation of lignin as well as other oxidative phenols which ultimately contribute to the formation of defense barriers making the plant cell structure inaccessible to the pathogen (Avdiushko *et al.*, 1993). In response to pathogen invasion in plants, Phenylalanine ammonialyase (PAL) frequently increase. Increased activity, in the phenylpropanoid pathway,

help in synthesis of defense-related compounds like lignin, flavonoid, phytoalexin, signalling molecules and salicylic acid (Mandal *et al.*, 2010; Duan *et al.*, 2014; Le Roy *et al.*, 2016; Boba *et al.*, 2017).

Pathogenesis related proteins (PR-proteins) are also assumed to play an important role in defense of plants. Two PR-proteins such as plant hydrolases, β-1,3-glucanase and chitinase are of special importance. Because many pathogenic fungi contain β-1,3-glucans and chitin as major structural cell wall components (Wessels and Sietsma, 1981). Several authors (Mauch *et al.*, 1988; Arlorio *et al.*, 1992; Bishop *et al.*, 2000) have demonstrated the activity of β-1,3-glucanase and chitinase to degrade fungal wall components *in vitro*, resulting in growth inhibition of fungi. Peroxidase is related with the defense reaction in plants. Peroxidase detoxifies the reactive oxygen species (Higa *et al.*, 2001). Its activity is changed under various environmental stresses such as temperature, salts and heavy metals and also by air pollution (Kiwan and Lee, 2003; Langebartels *et al.*, 2002; Das and Roychoudhury, 2014; Liu *et al.*, 2015).

Understanding the molecular responses associated with host defence mechanism in tea is very important for better management of the crop production. Since the whole tea genome sequence is not yet deciphered, very little information is known about the genes and genetic regulations associated with tea stress responses. The molecular basis of induction of some transcripts of the some defence-related enzymes has been thought to be studied for validation of the semi-quantitative estimation of the transcripts of some defence related enzymes. For performance of these following objectives were thought to be fulfilled in the present dissertation.

Objectives:

1. Control of disease by different abiotic inducers and disease assessment.
2. Induction of defence-related enzymes in tea by abiotic inducers.
3. Molecular identification of defense related genes of tea.

4. Analysis of selected gene specific transcript accumulation in induced tea plants.
5. *In vitro* control of pathogenic microorganisms by botanicals and biocontrol agents.

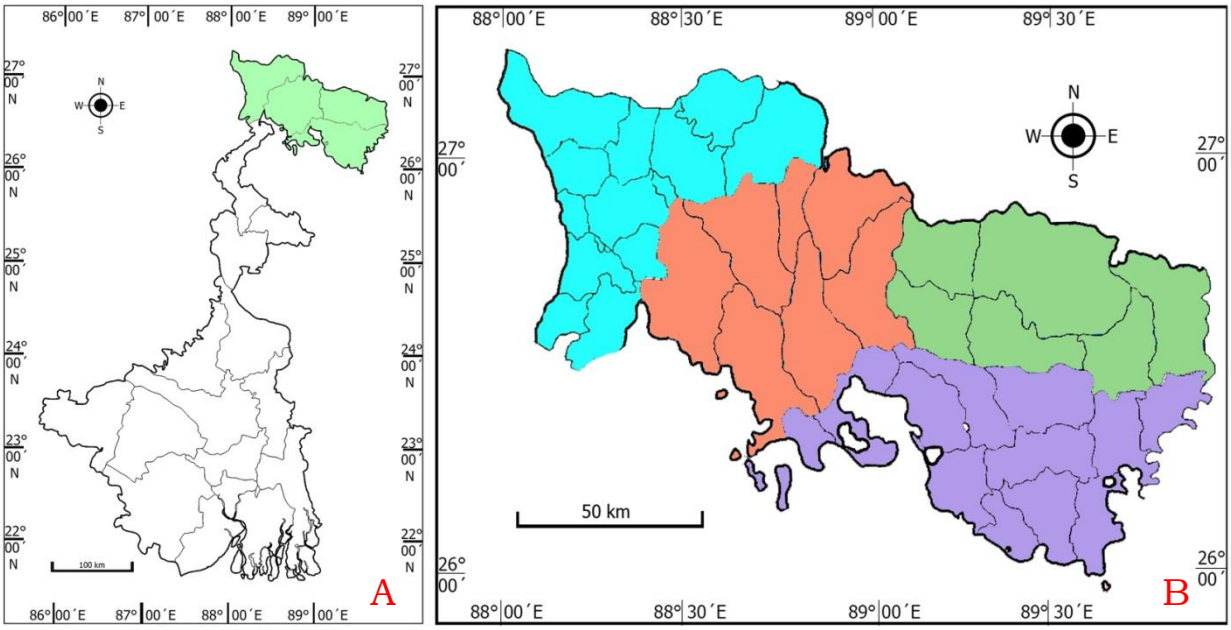

Fig. 1.1: (A) Map of West Bengal. (B) Four districts of northern part of West Bengal (The present study area) where tea is grown as an important cash crop.

Fig. 1.2: Different views of Gayaganga Tea State, Siliguri; (A) Ten years old tea plants. (B) Nursery for raising clonal tea plants. (C) Nursery for raising tea plants from seeds. (D) 25 years old tea plants Gayaganga Tea State, Siliguri.

Fig. 1.3: Naturally infected tea plants. (A) & (B) Brown blight of tea. (C) Leaf spot disease of tea.

2. Literature review

2.1. General aspects of Host-pathogen Interaction

Host pathogen interaction is one of the important areas of research. Although a variety of pathogen attack plants, some pathogens are considered to be of virulent in nature. Response of plants to fungal attack leads to either resistance or susceptibility depending upon interaction between plant and the pathogen. Pathogenesis and disease resistance are closely related. Compatible interactions lead to successful establishment of the pathogen which leads to susceptibility while resistance is related to incompatible ones. Application of exogenous inducers (may be biotic or abiotic) induce defense enzymes and its production at a threshold level leads to resistance of plants. Study of defense enzymes is one of the important areas which have been studied by several workers in the last three decades. Molecular basis of the defense genes particularly in tea have recently being initiated in different laboratories. In the present study defense related enzyme of tea has been proposed to be induced by abiotic inducers. Further, it has also been proposed to study the regulation (up and down) of selected defense related transcripts of selected defense related enzymes. Hence, it has been considered to review the works of the earlier workers. The studies, reports and observations of the previous workers in concord with the present line of investigation are being presented in the following paragraphs, in a selective rather than in a comprehensive manner. The present review, for convenience, has been grouped in to some aspects. The aspects of the review are as follows:

1. Diseases of tea
2. Induction of defense related enzymes and disease assessment
3. Molecular aspects of defense related genes (identification and expression analysis)

4. Management of pathogenic microorganisms by some botanicals and biocontrol agents.

2.2. Diseases of tea

Foliar fungal diseases of tea are main obstacle and also have adverse effect on quality and productivity of tea in all over the world. The fungus, *Colletotrichum sp.* and *Curvularia eragrotidis* are the main causal agent of brown blight disease and leaf spot disease of tea respectively in north-east India (Dasgupta *et al.* 2005).

Gray blight disease of tea caused by *Pestalotiopsis theae* is another important foliar disease of all tea growing countries of the world. The disease appears on mature foliage, bare stalks as well as in young shoots of the tea bushes. The disease symptoms are generally circular, with upper surface concentrically zoned with different colors (Chen and Chen, 1982; Baby and Sanjay, 2006; Premkumar *et al.*, 2012; Kumhar *et al.*, 2016; Pallavi *et al.*, 2012).

Alternaria alternata, is also a foliar fungal pathogen of tea in North Bengal, India. Disease symptoms first appear as grayish brown patches around tips and margins of young leaves which extend towards the midrib resulting to leaf curl, death and defoliation (Chakraborty *et al.*, 2006).

Black rot is an important disease of the mature leaf and stem caused by fungi *Corticium theae* and *Corticium invisum.* They attack the maintenance leaves, causing gradual deterioration in the health of the bush and consequent loss of crop. The infected leaves turn black as they rot during the wet weather. The dead leaves, which are detached from the bush but remain suspended on the bush and the mycelial chords come out from the infected leaves hold the bush. Then the fungal sclerotia embed themselves in the cracks and crevices of the stem and bark (Chen and Chen, 1982; Singh *et al.*, 2006).

2.3. Induction of defense related enzymes and disease assessment

Pajot *et al.* (2001) reported about two elicitors [(DL-*β*-amino butyric acid (BABA) and Phytogard (K_2HPO_3)] which induced systemic resistance in lettuce against *Bremia lactucae* causal organism of downy mildew disease and protected the plants. Among the two elicitors Phytogard reduced spore germination completely but BABA reduced partially. They also reported that BABA increased the activity of PR proteins like β-1,3-glucanase, peroxidase etc. but Phytogard did not induce this PR proteins.

Amzalek and Cohen (2007) studied the effect of SAR inducer like BABA, BTH, INA, NaSA, AABA and GABA to control sunflower rust caused by *Puccinia helianthi*. They applied all the compounds in sunflower plants as a foliar spray, root dip or leaf disc assay techniques. From the foliar spray results they found BABA was more effective and NaSA was least effective to induce resistance against rust but in leaf disc assay BTH, BABA and INA were fully protective. NaSA and AABA were less effective against rust but GABA did not have any potential effect. They also reported that BABA did not have any effect to defense compounds accumulation and on spore germination but suppressed mycelial colonization in the mesophyll.

Hassan and Abo-Elyousr (2013) pre-treated tomato plants with DL-3-aminobutyric acid (BABA) by soil drenching and studied the ability of BABA to protect tomato plants as well as the accumulation of total phenolic compounds, SA and the activity of PPO, CAT against bacterial wilt caused by *Ralstonia solanacearum*. They showed that, plants treated with BABA reduced disease incidence and also reduced leaf wilting index and vascular browning index. According to them BABA treated tomato plants showed increased activity of PPO, SA and total phenolic compound but decreased the activity of CAT.

Aleandri *et al.* (2010) studied the effect of three resistance inducers methyl jasmonate (MeJA), acybenzolar-S-methyl (BTH) and dipotassium hydrogenphosphate (K_2HPO_4) to control root rot and vine decline disease of melon caused by *Monosporascus cannonballus*. They applied the inducers either by foliar application or as seed soaking technique. Plants treated

with MeJA and BTH reduced disease severity in case of artificially inoculated soil or by naturally infected greenhouse soil by *M. cannonballus* but K_2HPO_4 did not reduced disease severity under similar condition. They also reported that Different PR-proteins and their isozymes were increased in root system treated with resistance inducers.

Hukkanen *et al.* (2007) reported that, benzothiadiazole (BTH) enhanced the accumulation of soluble and cell-wall-bound phenolics in strawberry leave and also improved the resistance to powdery mildew infection under greenhouse conditions. Most pronounced change was seen in the levels of ellagitannins, which increased up to 2- to 6-fold in 4 days post BTH application, but persisted only in the inoculated plants. The induction of phenolic metabolism by BTH was also reported in the fruits. Basal salicylic acid (SA) content was high in strawberry leaves, but increased in a similar fashion like other phenolics following treatments. The several new compounds have been identified by the authors for the first time in strawberry such as ellagic acid deoxyhexose, three agrimoniin-like ellagitannins, sanguiin H-10- and lambertianin C-like ellagitannins in the leaves, ellagic acid, p-coumaric acid, gallic acid, and kaempferol hexose in the cell-wall-bound fraction of the leaves, and kaempferol malonylglucoside in the fruits. According to them BTH enhanced the accumulation of phenolics in strawberry plants which might have been involved in the BTH-induced resistance against powdery mildew.

According to Romanazzi *et al.* (2009) studied phytoplasma caused diseases of grapevine. They evaluated the effectiveness of field treatments with resistance inducers (Chito Plant, Aliette, Kendal, Bion, and Olivis) to promote recovery in Bois noir (BN) infected grapevines. Treatments consisted of weekly sprays in spring-summer 2007 (seven applications) and 2008 (thirteen applications). All treatments increased the number of recovered plants but best results were obtained with Kendal, Olivis and Bion. Phytoplasma were reported to be absent in induced and recovered plants.

Percival (2010) used a detached leaf bioassay to evaluate systemic inducing resistance (SIR) agents, biostimulant products and one triazole fungicide (myclobutanil) on apple scab (*Venturia inaequalis*) development under laboratory conditions. They found that SIR agents (potassium phosphonate, potassium phosphite, harpin protein, salicylic acid, salicylic acid derivative) and myclobutanil inhibited germination and subsequent formation of appressoria of conidia and also found reduced leaf scab severity.

Tamm *et al*. (2011) focussed on the use of DL-β-aminobutyric acid (BABA) and an aqueous extract of *Penicillium chrysogenum* (Pen) as elicitors. In their studies, BABA as well as Pen could contribute to control diseases caused by *Rhizoctonia solani*.

According to Cohen *et al*. (2011) DL-3-amino-butyric acid (BABA) induced local and systemic resistance against disease in numerous plant species. In their study they showed that preventive application of BABA to lettuce (*Lactuca sativa*) plants induced resistance against downy mildew caused by the oomycete *Bremia lactucae* by callose encasement of the primary infection structures of the pathogen. They also showed that post-infection application of BABA to the foliage or the roots, even at progressive stages of disease development, is highly protective against *B. lactucae*. Resistance induced by BABA is manifested in multiple microscopic forms, depending on the time of its application. When applied at 1 day post inoculation (dpi) BABA induced HR in penetrated epidermal cells; at 2 dpi it caused massive encasement with callose of the primary haustoria; and, at 3 or 4 dpi it enhanced the accumulation of H_2O_2 in the developing mycelia runners and altered their colour to red. The pronounced change in the colour of the mycelium was visually apparent to the naked eye. In all cases the pathogen failed to sporulate on the treated plants.

Abdel-Kader *et al*. (2012) carried out an experiment to evaluate the efficacy of some plant resistance inducers against downy and powdery mildew of cucumber plants. They evaluated foliar spray treatment at a schedule of four times (at fifteen days intervals) starting at thirty days after

transplanting. They found two treatment mix resulted in the highest reduction in foliar Downy and Powdery mildew disease incidence reported increased production the under plastic houses conditions. Treatment mixture 1) was Calcium chloride (20mM) + *S. cerevisiae* 10x1010cfu/mL (10ml/L) + Chitosan (0.05mM) and Treatment mixture 2) was Potassium bicarbonate (20mM) +Thyme oil (5ml/L).

Gilardi *et al.* (2013) worked to control downy mildew of sweet basil (*Ocimum basilicum* L.), incited by *Peronospora belbahrii* Thines. According to them greatest reduction in disease incidence and severity was found with treatments that included metalaxyl-M+copper hydroxide, a mineral fertilizer 'Alexin', mandipropanid, and azoxystrobin. The glucohumates activator complex and acibenzolar-S-methyl also provided significant disease control ($P<0.05$). The mineral fertilizer Alexin, the glucohumates activator complex and acibenzolar-S significantly reduced disease incidence and severity after 20 days of the last treatment. They reported that effective control could be achived by either using a rotation of fungicides with compounds that can induce resistance, or by using rotation with different resistance-inducing compounds on their own.

El-Mougy *et al.* (2013) also evaluated foliar spray treatments in cucumber, pepper and tomato plants to control foliar diseases, powdery and downy mildews. Their treatment schedule was four times with fifteen days intervals starting at thirty days after transplanting. Among the different treatments, treatment mixture (1) contained Calcium chloride (20mM) + *S. cerevisiae* 10x1010cfu/mL (10ml/L) + Chitosan (0.05mM)] but Treatment mixture (2) contained Potassium bicarbonate (20mM) + Thyme oil (5ml/L) reduced diseases mentioned above as well as early and late blight diseases. The activity of Peroxidase, Polyphenol oxidase, Phenylalanine ammonia-lyase, chitinase and β-1,3-Glucanase enzymes significantly escalated defense response in cucumber, pepper and tomato plants resulting to reduced disease symptoms.

Gilardi *et al.* (2014) performed an experiment to control crown and root rot of tomato incited by *Phytophthora nicotianae*. Five different

treatments were made under greenhouse condition to test the efficacy of spray programmes. The disease reduction achieved with a single application of azoxystrobin and metalaxyl-M. Partial disease control was found by other four Treatments such as Phosetyl-Al and the biocontrol agents *Glomus* spp. + *Bacillus megaterium* + *Trichoderma*, *B. subtilis* QST713, *B. velezensis* IT45 and the mixture of *T. asperellum* ICC012 + *T. gamsii* ICC080.

Alkahtani *et al*. (2011) studied effect of six abiotic elicitors (Oxalic acid, Potassium oxalate, and salicylic acid, Bion, Fungastop and Photophor) for inducing reisitance in cucumber (*Cucumis sativus* L.) against powdery mildew (caused by *Sphaerotheca fuliginea*) disease. They studied disease severity to evaluate inducers efficiency and measured the biochemical changes in both PR-related protein and phytoalexins accumulation in treated plant and compaired with the control plants. All the elicitors they tested showed decreased powdery mildew disease. Among the six elicitors Bion was most effective and potassium oxalate showed lowest effectiveness in both single and booster spray. PR-proteins such as POX, PPO, CHI and ß-1, 3- glucanase was found to increase and accumulation of phytoalexin was also increased.

Altinok and Dikilitas (2014) reported Acibenzolar-S-methyl (ASM) as an abiotic plant activator. They applied the compound in eggplant seedlings and found increased resistance to *Fusarium oxysporum* f. sp. *melonis*, a virulent pathogen of brinjal wilt. ASM pretreated brinjal plants significantly reduce wilt disease. ASM pretreatment resulted hypersensitive reaction (HR) and callose formation and H_2O_2 synthesis was increased. ASM treated plants showed increased activity of catalase and polyphenol oxidase. They also applied a non-host isolate of the pathogen on eggplant as biotic inducer and got decreasd disease severity like the ASM application but abiotic inducers was found to better than the biotic inducer they used.

Amer *et al*. (2015) induced cucumber plants by three biotic inducers (*Trichoderma harazianum*, *Pseudomonas fluorescen* and *Ampelomyces*

quisqualis) against *Pseudoperonospora cubensis*, a downy mildew disease causing fungi. They reported that Peroxidase and β-1, 3-glucanase activities were increased by application of *Trichoderma harazianum* and downy mildew was controlled to a significant extent. Although *Pseudomonas fluorescen* also reduce the disease but it did not induced β-1, 3-glucanase significantly. When *Ampelomyces quisqualis* was applied as an inducers neither peroxidase nor β-1, 3-glucanase was increased but it gave a good results in SA signal pathway induction.

Sreedevi *et al*. (2011) reported that *Trichoderma harazianum* can induce systemic resistance in groundnut against *Macrophomina phaseolina*. Defense enzymes (POX and PPO) were induced by application of *T. harazianum*. Their observation let them too concluded that *T. harazianum* was capable of inducing systemic resistance against *Macrophomina phaseolinai* by triggering defense enzyme production.

Abhayashree *et al*. (2016) applied five abiotic elecitor such as L-isoleucine, L- leucine, L-methionine, L- phenylalanine and L- proline to induced resistance in chili against *Colletotrichum capsici*, an anthracnose disease causing fungi. They reported that 50 mM concentration of the elecitors performed well to induce defense and to control the disease significantly. The abiotic elicitors could induce activity of Phenylalanine ammonia lyase (PAL) and Peroxidase (POX) enzymes.

Acharya *et al*. (2011) used five abiotic elecitors (arachidonic acid, cupric chloride, chitosan, isonicotinic acid and salicylic acid) to induced systemic resistance in *Raphanus sativus* L. Significant increase of b-1,3 glucanase, peroxidase and polyphenoloxidase was reported alonghwith a remarkable increased of nitric oxide (NO). They suggested that NO might be a signaling molecule while inducing ISR in the host by abiotic elecitors.

Al-Sohaibani *et al*. (2011) used four organic acids (ascorbic acid, salicylic acid, oxalic acid and tannic acid); four different salts (KCL, K_2PO_4, NaCl and Na_2PO_4) and two growth regulators (Indole acetic acid and indole butyric acid) to control root rot disease of sweet basil caused by three different fungal pathogens such as *Macrophomina Phaseolina, Rhizoctonia*

solani and *Fusarium oxysporum* f. sp. *basilica*. Salicylic acid effectively decreased dumping off caused by *Macrophomina Phaseolina* and *Fusarium oxysporum* f. sp. *basilica*. While oxalic acid was best effective inducers against root rot disease caused by *Rhizoctonia solani*. They showed that inducers increased POX and CHI activity.

Baysal *et al*. (2003) applied three different defense activator BTH, ASM and Bion to induced resistance in tomato against *Clavibacter michiganensis* ssp. *michiganensis*, causal organism of bacterial canker of tomato. ASM pretreated plants showed reduction in disease severity upto 76.3%. In the resistance induced plants peroxidase and chitinase increased significantly.

Walters *et al*. (2011) reported that powdery mildew disease caused by *Blumeria graminis* f. sp. *hordei* and leaf scald disease caused by *Rhynchosporium secalis* of two spring barley varieties was controlled by the combined application of three resistance elicitors acibenzolar-S-methyl (ASM), β-aminobutyric acid (BABA) and cis-jasmone (CJ) in field conditions. They also showed the effect of combined application of those elicitors on greenhouse-grown barley leaves. The treated leaves activated the systemic acquired resistance (SAR) marker gene PR1-b and suppressed the jasmonic acid (JA) biosynthesis gene LOX2.

Romanazzi *et al*. (2009) studied the effect of five resistance inducers (Chito Plant, Aliette, Kendal, Bion, and Olivis) to reduce the Bois noir (BN) infected severe diseases of grapevine. Inducer treated plants decreased the disease severity in comparison to the control one. They sprayed the inducers by weekly manner in spring-summer 2007 (seven applications) and 2008 (thirteen applications). Among the inducers Kendal, Olivis and Bion showed the better results but on the other hand in the first year Aliette and Chito plant treatments showed better results than the second year.

Perez-de-Luque *et al*. (2004) studied the effect of foliar application of three SAR activator like, salicylic acid, glutathione and benzothiadiazole (BTH) to control the broomrape infected pea disease. They reported that the

broomrape infection was controlled under the growth chamber and greenhouse conditions by the application of BTH (0.6 to 1.0 mM), in the form of Bion 50 (50% a.i.).

Boro *et al.* (2011) studied the effects of abiotic inducer acibenzolar-S-methyl (ASM) and two *Xanthomonas* species extracted biotic agents, harpin protein and glycoproteins to control the bacterial leaf spot of yellow passion fruit. They applied all the inducers through seed immersion as well as by spraying and the seedlings were inoculated at four true leaves stage. They showed that ASM or harpin treated seed protect plants up to 90% and 47% and leaf treated with ASM or the glycoproteins from *Xanthomonas* spp protect plants up to 70% and 72% respectively against inoculation of *Xanthomonas axonopodis* pv. *Passiflorae*.

Chen *et al.* (1995) studied the role of salicylic acid (SA) in plant defense against pathogens. They found endogenous SA level increased in correlation with both resistance of tobacco to infection with tobacco mosaic virus and induction of defense-related genes [such as that encoding pathogenesis-related protein 1 (PR-1)]. Newly synthesized SA was conjugated to glucose to form SA β-glucoside. They also found a cell wall-associated S-glucosidase activity that releasesd SA from this glucoside. According to them SA β -glucoside served as an inactive storage form of SA. They purified a soluble SA-binding protein and isolated its encoding cDNA from tobacco. Finally they were able to further characterize the mechanism of SA signaling. The protein was a catalase, and binding of SA and its biologically active analogues inhibited catalase's ability to convert H_20_2 to 0_2 and H_20. Thus elevated levels of cellular H_20_2 appeared to induce PR-1 gene expression, perhaps by acting as a second messenger. Additionally, transgenic tobacco expressed an antisense copy of the catalase gene and exhibitied depressed levels of catalase also showed constitutive expression of PR-1 genes. They tested several abiotic inducers of PR gene expression and disease resistance for their ability to stimulate SA production. From their experiments it was found that levels of SA and its glucoside rose following application of all of the inducers except 2,6-dichloroisonicotinic

acid. 2, 6-Dichloroisonicotinic acid was found to bind catalase directly and inhibit its enzymatic activity.

Chen *et al.* (2000) reported that root and crown rot of cucumber caused by *Pythium aphanidermatum* could be suppressed by various plant growth-promoting rhizobacteria (PGPR). They treated cucumber roots with *Pseudomonas corrugata* 13 or *Pseudomonas aureofaciens* 63-28 which stimulated the activity of phenylalanine ammonia-lyase (PAL) in root tissues in 2 days and this activated accumulation lasted up to 16 days of bacterization. Peroxidase (POX) and polyphenol oxidase (PPO) activities were also increased in roots within 2-5 days. Isoperoxidase native PAGE (polyacrylamide gel electrophoresis) analysis indicated that the peroxidase isomer forms in cucumber roots induced by rhizobacteria were different from that in roots infected with *P. aphanidermatum*. Thus the activation mechanisms of PO by the rhizobacteria were thought to be different from those of pathogen infection.

Christ and mosinger (1989) reported increase of eleven acid soluble proteins (with apparent molecular masses ranging from 13-82 kD) in tomato (*Lycopersicon esculentum* Mill.) leaves following *Phytophthora infestans* or *Fulvia fulva* infection. Prominent changes in the protein pattern were also detected in the untreated leaves of infected plants which indicated systemic effects of the infection. Similar changes in the proteins were also induced either by moderate irradiations of the leaves with UV light (254 nm) or by injecting the leaves with chemical inducers [indole-3-acetic acid, 2-chloroethyl-phosphoric acid (ethephon), fusicoccin or an elicitor preparation from *Phytophthora megasperma* f.sp. *glvcinea*]. Acetylsalicylic acid (aspirin), kinetin, and abscisic acid did not induce detectable changes in protein pattern nor did they induce resistance.

Conrath *et al.* (1995) used 2,6-Dichloroisonicotinic acid (INA) and salicylic acid (SA) as potent inducers of plant defense responses including the synthesis of pathogenesis-related (PR) proteins and the development of enhanced disease resistance. They purified a SA receptor protein (a soluble SA-binding protein) from tobacco with an affinity and specificity of binding.

The protein has been reported to be a catalase whose enzymatic activity was inhibited by SA binding. They have proposed that increase in intracellular levels of reactive oxygen species plays a role in the induction of defense responses such as PR protein gene expression. The dose-response curves for inhibition of catalase by two compounds (INA and SA) are similar. Furthermore, the ability of both INA analogues and SA derivatives to bind and inhibit tobacco catalase correlates with their biological activity to induce PR-1 gene expression and which ultimately enhanced resistance to tobacco mosaic virus. Comparison of the structures of INA, SA, andtheir analogues revealed several common features that appeared to be important for biological activity.

Cortes-Barco *et al.* (2010) showed induced resistance in *Nicotiana benthamiana* against anthracnose caused by the hemibiotrophic fungus *Colletotrichum orbiculare.* The inducers were benzothiadiazole (BTH), (2R, 3R)-butanediol or PC1, an isoparaffin-based mixture. In disease assessment experiments, BTH, (2R, 3R)-butanediol and PC1 reduced the number of lesions per leaf area caused by *C. orbiculare* to a significant extent. They also reported that foliar application of BTH induced expression of genes for the acidic pathogenesis-related (PR) proteins, NbPR-1a, NbPR-3Q and acidic NbPR-5. In contrast, soil application of (2R, 3R)-butanediol or PC1 primed expression of genes for the basic PR proteins, NbPRb-1b, basic NbPR-2 and NbPR-5dB. These results are consistent with the activation of salicylic-acid-dependent systemic acquired resistance (SAR) by BTH and that of jasmonate/ethylene-dependent induced systemic resistance (ISR) by (2R, 3R)-butanediol or PC1, and show that (2R, 3R)-butanediol and PC1 can affect gene expression similarly to plant growth-promoting rhizobacteria. The effects of (2R, 3R)-butanediol and PC1 were not identical. In addition to priming, (2R, 3R)-butanediol induced expression of basic NbPR-2, whereas PC1 treatment induced expression of both NbPRb-1b and basic NbPR-2. Although a number of microbial products, such as (2R, 3R)- butanediol, have been shown to produce ISR, but the first demonstration of an isoparaffin-based mixture (not derived from a microorganism) could produce ISR.

In 2013, Dufour *et al.* reported that a salicylic acid analogue [Benzothiadiazole (BTH)] strengthens plant defence mechanisms against a pathogens diverse spectrum. They reported the role of BTH-pretreatment in enhancing resistance against infection with various isolates of *Plasmopara viticola* and *Erysiphe necator* causing downy and powdery mildews in grapevine leaves. The authors developed some tools for better assessment of the defence status of the plant. They reported that in compatible interactions more than 57.2% of differentiated transcripts from *P. viticola* infected-leaves (Pv-infected leaves) out of a set of 19 genes were down-regulated at 24 h post-inoculation (hpi). Under similar conditions, they also showed down regulation of about 90% of differentiated transcripts from from *E. necator* infected leaves (En-infected leaves), indicating a manipulation of host responses by the pathogens. Pathogen growth was reported to be inhibited by 61–98% (depending on the pathogen isolate) following BTH treatment that enhanced grapevine defences. Up-regulation of pathogenesis-related protein genes (PR-1, PR-2, PR-3, PR-8 and PR-10) were observed by the authors in BTH treated-Pv-infected leaves. Under similar conditions of treatment and En-infection the leaves showed up regulation of PR-3, PR-6 and PR-10 genes. They also showed that treatment with BTH led to regulation of indole pathway transcripts. According to them, particularly, anthranilate synthase was down-regulated at 24 hpi in all infected leaves but strongly up-regulated afterwards in relation to rate of pathogen development. Their quantitative studies (of polyphenols and stilbenes) showed that pterostilbene was specifically accumulated in pre-treated leaves and associated with biological efficacy.

Ghosh (2015) investigated a variety of enzymatic responses of ginger plants to *Pythium* infection after induction of SAR (systemic acquired resistance). They reported that *P. aphanidermatum* lnfected a susceptible ginger cultivar which showed increased disease intensity up to 28 days but Polyphenol oxidase (PPO), Lipoxygenase (LOX) and Phenyl alanine ammonia lyase (PAL) activities were found to be increased up to 14 days. Peroxidase (PO) activity reached their peaks on 21st day after inoculation and then decreased sharply as reported. To induce SAR, the authors

soaked rhizome seeds for 1 hour prior to sowing, separately, in salicylic acid (SA-5 mM) and *Acalypha* leaf extract (ALE – 10%). They observed significant disease reduction in both SA and ALE treated plants. SA and ALE treatment, enhanced activities of all four defence related enzymes (as studied by the authors) in ginger leaves. Untreated inoculated and treated non-inoculated plants in relation to their respective controls were tested by the authors. Treated inoculated plants exhibited maximum activity for all four enzymes they studied. SA stimulated PO and PAL more than that of ALE. According to them a correlation exists between reduction of disease intensity due to SAR induction and greater stimulation of specific enzymatic activities in ginger plants. They also suggested that all four enzymes were not equally responsive to a defence activator.

Chandra *et al*. (2007) studied phenylalanine ammonia lyase (PAL) activities leading to decline in disease formation caused by *Rhizoctonia solani* following application of salicylic acid (SA). They applied 1.4 mM SA (pH 6.5) twice. There after they inoculated the plants with *Rhizoctonia solani*. Finally, they observed quantitative change in polyphenol oxidase (PPO), peroxidase (POX) isoforms and increasing activity of PAL in Bundel-1, UPC-4200 and IFC- 902 cowpea genotypes. PAL activity was increased in *Rhizoctonia solani* inoculated UPC-4200, whereas total soluble protein were significantly increased in the same genotype after SA treatment and *Rhizoctonia* inoculation. In their isoform analysis (out of ten isoforms) isoforms 7 and 10 of polyphenol oxidase and isoform 4 of peroxidase showed increased activities when SA application were done. The disease symptoms measured by them, indicated less disease formation in SA sprayed Bundel-1 and UPC-4200 genotypes in compare to controls.

Azami-Sardooei *et al*. (2013) reported great economic losses, due to grey mould caused by *Botrytis cinerea*, in greenhouse-cultivated tomato, bean and cucumber. They also investigated the effects of foliar applications of different concentrations of BTH (a chemical analog of salicylic acid) on resistance to *B. cinerea.* According to their observation increased protection of tomato against *B. cinerea* were found in case of leaf treatments with 50 mg/l BTH in one spray. In case of bean and cucumber, only concentrations

of 250 mg/l and higher were reported to reduce susceptibility against B. cinerea. The authors also reported that BTH concentrations above 100 mg/l had a negative effect on plant height, flower and fruit numbers in bean and cucumber plants under pathogen-free conditions. But in tomato only the highest BTH dose (1000 mg/l) resulted in a significant negative effect on vegetative and generative growth.

Pye *et al.* (2013) used BTH (1,2,3-benzothiadiazole-7-thio carboxylicacid-S-methyl-ester) commercially known as 'Actigard' and TDL [N-(3-chloro-4-methylphenyl)-4-methyl-1, 2, 3 -thiadiazole-5-carboxamide,] commercially known as 'Tiadinil' for induction of defense in tomato plants. BTH and TDL were examined for their role on abscisic acid (ABA)-mediated, salt-induced disease predisposition in tomato seedlings. They showed that salt stress to roots significantly increased the severity of disease caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Phytophthora capsici* relative to non-stressed plants. According to their results, root treatment with TDL induced resistance to Pst in leaves and provided protection in both non-stressed and salt-stressed seedlings in wild-type and highly susceptible NahG plants. Non-stressed and salt-stressed ABA-deficient mutants were highly resistant to *Pst*. Neither TDL nor BTH induced resistance to root infection by *Phytophthora capsici*, nor did they moderate the salt-induced increment in disease severity. Root treatment with these plant activators increased the levels of ABA in roots and shoots similar to levels observed in salt-stressed plants. From their results they indicated that SAR activators could protect tomato plants from bacterial speck disease under predisposing salt stress. They were also of the opinion that some SA-mediated defense responses function sufficiently in plants with elevated levels of ABA.

2.4. Molecular aspects of defense related genes (identification and expression analysis)

In 1996, Lawton *et al.* reported that Benzothiadiazole (BTH) was a novel chemical activator of disease resistance in tobacco, wheat and other important agricultural plants. In their report, it was shown that BTH works

by activating SAR in *Arabidopsis thaliana.* They treated plants with BTH and showed that treated plant were resistant to infection by turnip crinkle virus, *Pseudomonas syringae* pv 'tomato' DC3000 and *Peronospora parasitica.* Thus, they reported the induction of resistance against viras, bacteria and fungus. According to them chemical treatment induce accumulation of mRNAs from the SAR-associated genes, PR-1, PR-2 and PR-5. They found that BTH induced both *PR-1* mRNA accumulation and resistance against *P. parasitica* in the ethylene response mutants, *etrl* and *ein2,* and in the methyl jasmonate-insensitive mutant, *jar1.* From their results, they suggested that BTH action was independent of plant hormones, whose mutants were taken in the tests. They also reported that BTH can induce both *PR-1* mRNA accumulation and *P. parasitica* resistance in transgenic *Arabidopsis* plants expressing the *nahG* gene, suggesting that BTH action does not require salicylic acid accumulation. They were also of the opinion that BTH activates the SAR signal transduction pathway because BTH-treatment failed to induce either *PR-1* mRNA accumulation or *P. parasitica* resistance in the noninducible immunity mutant, *niml.*

Xu *et al.* (2008) isolated a full-length cDNA and genomic DNA of phenylalanine ammonia-lyase gene from *Ginkgo biloba* (*GbPAL*). From their sequenced results, they found out that both the sequences of *GbPAL* were same having a gene coding region of about 2172 bp long. The deduced protein of the genes were consists of 724 amino acids with a predicted molecular mass of 79.1 kDa and a pI of 5.96. They reported that *Gb*PAL protein was highly identical to other plant PALs. According to the workers (on the basis of southern hybridization analysis of the genomic DNA), *GbPAL* belonged to a small multi-gene family. Real-time PCR analysis of tissues (tissue expression analysis) revealed that *GbPAL* constitutively expressed in all the tested tissues but high expression were reported in leaf and stem tissues. Induction of *GbPAL* has been reported by a variety of stresses including UV-B, wounding, cold and salicylic acid. On the basis of temporal expression profiling analyses, the tanscription levels of *GbPAL* were found to be significantly correlated with flavonoid accumulation.

Thus, the authors suggested that *GbPAL* might play a regulatory role in flavonoid biosynthesis in leaves of *G. biloba* at the transcriptional level.

Huang *et al.* (2010) worked with Phenylalanine ammonia-lyase (PAL) that catalyzes the first step of the phenylpropanoid pathway that produces precursors to a variety of important secondary metabolites. According to them the Arabidopsis (*Arabidopsis thaliana*) contains four PAL genes (PAL1–PAL4). They analysed combined mutations for the four Arabidopsis PAL genes. Contrary to others the workers found that three independent *pal1 pal2* double mutants were fertile and generated yellow seeds due to the lack of condensed tannin pigments in the seed coat. The *pal1 pal2* double mutants were also deficient in anthocyanin pigments in various plant tissues, which accumulate in wild-type plants under stress conditions. The authors are of opinion that, PAL1 and PAL2 have a redundant role in flavonoid biosynthesis. Furthermore, the *pal1 pal2* double mutants were more sensitive to ultraviolet-B light but more tolerant to drought than wild type plants. They also generated two independent *pal1 pal2 pal3 pal4* quadruple knockout mutants, which were stunted and sterile. Interestingly, from their study it was evident that quadruple knockout mutants contained about 10% of the wild-type PAL activity even after knocking out of the genes. Thus, they suggested about leaky PAL mutant genes or presence of one or more other unknown PAL genes. Further, substantially reduced levels of salicylic acid accumulation were found in case of the quadruple mutants that also showed increased susceptibility to a virulent strain of the bacterial pathogen (such as *Pseudomonas syringae).* Distinct and overlapping roles of the Arabidopsis PAL genes in plant growth, development, and responses to environmental stresses were, finally, stressed by the authors.

Ziaei *et al.* (2012) studied gene expression and activity of PAL in *Ocimum basilicum* L. at different stages of growth (such as seedling stage, beginning and middle of growth phase, budding stage and flowering). The level of gene expression was analysed by semi quantitative RT-PCR and by identification of phenylpropanoid compounds (by gas chromatography/ mass spectrometry). They indicated that the level of gene expression and

activity of enzyme (PAL) were altered during the plant development, where the highest expression and activity was achieved at budding stage. In their experiment, changes of methylchvicol content were found to be correlated to the transcription and activity of PAL enzyme.

Xu *et al.* (2012) for the first time isolated a full-length cDNA of PAL gene from *Juglans regia*, and reported as *JrPAL*. The *JrPAL* gene (full-length cDNA) contained a 1935bp open reading frame which encodes a 645-amino-acid protein with molecular weight of about 70.4 kD and isoelectric point (pI) of 6.7. The deduced *JrPAL* protein was highly identical with other plant PALs. Molecular model of *JrPAL* (3D model of *JrPAL*) showed similarity to that of PAL protein from *Petroselinum crispum* (*PcPAL*). They reported that *JrPAL* might have similar functions with *PcPAL* due to their similarity in 3D molecular model. On the basis of phylogenetic tree analysis it was reported by the authors that *JrPAL* shared the same evolutionary ancestor of other PALs and had a closer relationship with other angiosperm species. They also reported that *JrPAL* was expressed in all tested tissues including roots, stems, and leaves, but the highest transcription level was found in roots. Real-time PCR (expression profiling) analyses revealed that *JrPAL* expression was induced by a variety of abiotic and biotic stresses including UV-B, wounding, cold, abscisic acid and salicylic acid.

Three different PAL genes have been isolated from the *Epimedium sagittatum* (*EsPAL1*, *EsPAL2* and *EsPAL3*). Among these three gene isolates, the metabolic accumulation as well as expression profile was studied by the authors. They found that *EsPAL3* contain high levels of active components and highly expressed in flavonoid-rich leaves and tissues, whereas *EsPAL1* highly expressed in leaves and tissues containing high lignin content (Zeng *et al.*, 2013).

Alvarez *et al.* (2013) suggested Phenylalanine-ammonia-lyase (PAL) plays an important role in resistance against *Mycosphaerella fijiensis* causal organism of Black leaf streak disease of banana. They sequenced partial or complete PAL gene from four different cultivar of banana

('Calcutta 4', 'Grain Nain', 'Yangambi Km5' and 'Williams') and secondary structures analysis and 3D model of deduced PAL protein were also done. They reported that the PAL gene expression was dependent on the cultivar and they found highest expression of PAL in 'Calcutta 4' (resistant cultivar) in the early hours of infection in comparison to 'Williams' (susceptible cultivar).

Jin *et al.* (2013) cloned a phenylalanine ammonia-lyase (PAL) gene from *Dendrobium candidum* using homology cloning and RACE. They also found the full-length sequence and catalytic active sites that appear in PAL proteins of *Arabidopsis thaliana* and *Nicotiana tabacum.* PAL cDNA of *D. candidum* (designated as *DcPAL1*, GenBank accession No. JQ765748) contain 2,458 bps and also contains a complete open reading frame (ORF) of 2,142 bps, which encodes 713 amino acid residues. The reported amino acid sequence of *DcPAL1* showed more than 80% sequence similarity (as indicated by multiple alignments) with the PAL genes of other plants. The dominant sites and catalytic active sites, which were similar to that showing in PAL proteins of *Arabidopsis thaliana* and *Nicotiana tabacum*, were also found in *DcPAL1*.According to phylogenetic tree analysis studies *DcPAL* is more closely related to PALs present in plants of orchidaceae than to those of other plants. The differential expression patterns of PAL found in protocorm-like body, leaf, stem, and root, suggested that the PAL gene had multiple physiological functions in *Dendrobium candidum*.

Hashemitabar *et al.* (2014) cloned and characterized a full length cDNA of sugarcane (*Saccharum officinarum*) phenylalanine ammonia-lyase (*SoPAL*). They also studied the Differential tissue expression pattern of the *SoPAL* transcript as well as the enzyme activity in the tillering stage of growth. They cloned 2118 bp *SoPAL* cDNA through encoding technique which contained a protein with 706 amino acids. They found highest gene expression levels of *SoPAL* transcript in the root and stem in comparison with leaves and sheath respectively but the enzyme activity of *SoPAL* was highest in the leaves.

Kim and Hwang (2014) identified the pepper (*Capsicum annuum*) *PAL* (*CaPAL1*) gene and subsequently they induced *PAL* gene expression in

pepper leaves by avirulent strain [*Xanthomonas campestris* pv. *vesicatoria* (*Xcv*)] infection. When *CaPAL1 gene was* silenced, pepper plants exhibited increased susceptibility to virulent and avirulent *Xcv* infection. They reported that PAL activity was significantly compromised during *Xcv* infection in the *CaPAL1*-silenced pepper plants. It was also observed by the authors that SA accumulation was reduced as expression of the salicylic acid (SA)-dependent marker gene *CaPR1* was found to be reduced. Reactive oxygen species (ROS) and hypersensitive cell death was also reported to be much slower in the *CaPAL1*-silenced pepper plants. Increased resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Hyaloperonospora arabidopsidis* infection in *Arabidopsis was observed due to o*ver expression (OX) of *CaPAL1* gene. Restricted *Pst* growth, increased ROS burst and cell death, and induction of *PR1* expression and SA accumulation was observed in *CaPAL1*-OX leaves. The degree of PAL activity was higher in over expressed (*CaPAL1*-OX) plants (both healthy and *Pst*-infected) than in wild-type *Arabidopsis*. Combining the two observations, the authors suggested that CaPAL1 acts as a positive regulator of SA-dependent defence signalling to control pathogens through its enzymatic activity (in the phenylpropanoid pathway).

Wang *et al*. (2014) constructed a cDNA library to obtain detailed and general data from the flowers of *Camellia chekiangoleosa*. They explored the transcriptome of *C. chekiangoleosa* and investigated genes involved in anthocyanin biosynthesis. A 454 GS FLX Titanium platform was used to generate an EST dataset. They got about 46,279 sequences and about 24,593 (53.1%) were annotated. They used Blast search against AGRIS, and 1740 unigenes were found to be homologous to 599 Arabidopsis transcription factor genes. Based on their transcriptome dataset they found nine anthocyanin biosynthesis pathway genes (PAL, CHS1, CHS2, CHS3, CHI, F3H, DFR, ANS, and UFGT). The genes were cloned and analysed the spatio-temporal expression patterns using quantitative real-time polymerase chain reaction. Their studies provided valuable information concerning anthocyanin biosynthesis study and also enriched the gene resource for further studies.

Okorska *et al.* (2014) studied part of the *PSPAL1* gene (corresponding to the proximal promoter, exon 1 and intron) from eight pea varieties. They also compared their sequences with the published sequences of *PSPAL1* gene from Midoriusui cultivar (GenBank: D10002.1). Their sequences showed a very high level of similarity (96–99%), except in five varieties where a motif TTATTACAAAATATTA close to the Goldberg-Hogness (TATA) box was found. The motif was not detected in the other four varieties, including Midoriusui. From the pathogenicity test of plants of eight pea varieties with *Mycosphaerella pinodes* and from results, the disease index was determined by the authors that ranged from 5.2 to 42.3%. The *PSPAL1* gene motif was reported to be present in most cultivar Walor (resistant cultivar) but it was not found in cultivar Polar, the most susceptible cultivar as reported. They also reported that, the relationship was not clear in varieties with intermediate levels of resistance. According to the authors, a weak negative correlation with disease severity (R=-0.53) was observed following analysis of expression level of *PSPAL1* gene in four varieties (Walor, Ezop, Ramrod and Polar) after 1, 3, 6, 9, 12 and 15 h post inoculation. They also showed that the activation of *PSPAL1* gene occurred in infected pea leaves, stems and roots but degree of expression varied a lot (with the relative level of *PSPAL1* transcripts amounting to 0.15 in roots and 38.75 in leaves). Thus, they indicated some kind of signal transmission beyond the infected plant tissues.

Optimal growth with minimal effects of biotic and abiotic stress is essential for growth of Willow as it is an important biomass crop for the bioenergy industry (Jong *et al.* 2015). They reported that phenylpropanoid pathway is responsible for the biosynthesis lignin, flavonoids, condensed tannins, benzenoids and phenolic glycosides. All the above mentioned compounds have a role in protecting the plant against biotic and abiotic stress. It has also been reported that all products of the phenylpropanoid pathway are important for the healthy growth of short rotation cropping species such as willow. However, the phenylpropanoid pathway in willow remains largely uncharacterised (Jong *et al.* 2015). They identified and characterised five willow phenylalanine ammonia-lyase (PAL) genes such as

Willow PAL1, PAL2, PAL3, PAL4 and PAL5. Four genes (PAL1, PAL2, PAL3 and PAL4) were orthologous to the poplar genes. They did not found any orthologue of PAL5 gene. Two tandemly repeated PAL2 orthologues were identified in a single contig. Willow PALs showed similar sub-cellular localisation as in poplar genes. The gene expression and enzyme kinetics of the willow PAL genes differed slightly. Willow PAL2 has been shown to be more widely expressed than its poplar orthologues and that led the authors to suggest a wider role for PALs in the production of flavonoids, condensed tannins, benzenoids, and phenolic glycosides, in willow.

Miltiadis *et al.* (2015) observed increased phenolics in mature fresh walnut (*Juglans regia* L.) kernels under cold storage condition. Based on that observation, they investigated changes in individual soluble phenolic compounds under cold stress in relation to changes in total phenols. Specific activity of phenylalanine ammonia-lyase (PAL) was also studied with the association of particular compounds with phenylpropanoid pathway. Several experiments were performed by them such as treatments with inhibitors of phenylalanine ammonia-lyase (PAL), mRNA level studies, RNA and protein synthesis, activities of the enzymes polyphenol oxidase (PPO) and peroxidase (POX). On the basis of their results, increase in specific and total activity of PAL during cold storage was found. Among phenolics they identified 2,4-dihydroxybenzoic acid and protocatechuic acid ethyl ester by HPLC–DAD–ESI-MS. They also reported increase in acids such as 4-hydroxybenzoic, 2,4-dihydroxybenzoic, syringic and vanillic with increased PAL activity. According to their studies ellagic acid was largely independent on PAL. Increase in protocatechuic acid and decreases in protocatechuic acid ethyl ester also could not be directly related to PAL.

Plant hormones play important roles in biotic and abiotic stresses in rice throughout its entire growth period. Most of the interactions of stresses in rice are not completely understood (Zhang *et al.* 2015). Zhang *et al.* (2015) determined physiological performance of rice seedlings under a single stress and a sequential combination of various stresses (intercross stress). They found that superoxide dismutase, catalase, and peroxidase

activities and malondialdehyde were highly regulated by intercross stresses. The expression levels of pathogenesis-related genes and drought stress-related genes under various treatments were also analyzed by them. In case of drought-disease intercross stress, the expression of the *PR4*, *PAL*, and *Cht-1* genes were upregulated but in case of salt-disease intercross stress, the expression levels of the *PR1a*, *PBZ1*, *Gns1*, and *Cht-1* genes changed significantly. The expression of *LOX-RLL* was significantly found to be changed regardless of the type of intercross stress. They also showed that the expression of drought stress-related genes (*OsSKIPa*, *OsNADPH1*, *JRC0594*, and *OsGL1-2*) *to be* significantly regulated.

Liew *et al.* (1998) isolated cDNA clones encoding chalcone syntbase (CHS) (EC 2.3.1.74), a key enzyme involved in flavonoid and anthocyanin biosynthesis from flowers of the orchid, *Bromheadia* finlaysoniana (Lindl.). They determined complete nucleotide sequences of the 3 clones such as *OCHS3*, *OCHS4* and *OCHS8.* The lengths of *OCHS3*, *OCHS4* and *OCHS8* were 1445, 1382 and 1439 bp, respectively. All the cDNAs contained a single open reading frame of 1 185 bp, encoding a polypeptide of 394 amino acids with molecular weight of 42.9 kDa. A high degree of nucleotide sequence similarity (> 97 %) was observed within the three cDNAs. The deduced amino acid sequences showed 76-82 % homology, but the nucleotide sequence showed 59-68 % homology to CHS of other plants.

Pang *et al.* (2005) the genomic DNA sequence of chalcone synthase (CHS) gene was cloned from *Ginkgo biloba*. The *Gbchs* was 1295 bp long and composed of two exons and one intron, one of the typical features of chalcone synthase genes. The genomic Southern blot analysis indicated that *Gbchs* belonged to a multigene family. RT-PCR analyses revealed that *Gbchs* expressed differentially in the root, stem and leaf tissues of *G. biloba*, and the expression could be induced by UV-B and wounding treatments. The recombinant GbCHS protein was successfully expressed in Escherichia coli strain M15 [pREP4] with pQE30 vector and the result showed that the expressed GbCHS protein had molecular weight of about 42 kDa, a size matching with that of the predicted one by bioinformatic analysis.

Farzad *et al.* (2005) reported that chalcone synthase (CHS), the first committed enzyme in the flavonoid biosynthetic pathway, is commonly encoded by multi-gene families with select members of these families accounting for the majority of expression. They examined the CHS gene family in *Viola cornuta*, a plant whose flowers undergo ontogenetic color change. Using both RNA and RNA/DNA samples isolated from floral tissues at different pigment stages, they obtained 14 unique sequences from 60 total clones of a 288 bp fragment from the catalytic region of CHS. The *V. cornuta* sequences were monophyletic when compared to CHS orthologs from other taxa. According to them substitution models generally indicated unequal rates of transition and transversion. They also found significant rate variation among sites. With a Tamura-Nei correction, nucleotide divergence ranged from 0.3 to 10.6% with the vast majority as synonymous changes. The nucleotide divergence pattern suggested designation of three *V. cornuta* CHS clades. Based on divergence of CHS orthologs, the reported clades were consistent with three CHS orthologs in *V. cornuta*. Sequences from only a single clade were found to be expressed in all three floral pigment stages.

Tian *et al.* (2006) presented the expression of a full-length *chs* cDNA with 1225 bp from grape seedlings as well as they prepared antibody against the expressed protein. They introduced a full-length *chs* cDNA into an expressed plasmid pET-30a (+) vector at the *Eco*RI and *Sal*I restriction sites. pET-*chs* was found to be highly expressed in *Escherichia coli* BL21(DE3) pLysS cells with isopropyl-β-D-thiogalactopyranoside (IPTG) induction. A fusion protein with the His · tag label was purifed by Ni–NTA His · Bind Resin and then used as the antigen to immunize rabbit. The resulting antibody was purified to immuno-recognize the CHS of the crude protein extracts of different grape tissues with a molecular wt. of 43 kDa.

Since the early evolution of land plants from primitive green algae, flavonoids have played an important role as UV protective pigments in plants. Flavonoids occur in liverworts and mosses, and the first committed step in the flavonoid biosynthesis is catalyzed by chalcone synthase (CHS Jiang *et al.* (2006)). They cloned and characterized CHS from the

gametophores of *Physcomitrella patens*, a moss. *PpCHS* exhibited similar kinetic properties and substrate preference profile to those of higher plant CHS. p-Coumaroyl-CoA was the most preferred substrate, suggesting that *PpCHS* is a naringenin chalcone producing CHS. Consistent with the evolutionary position of the moss, phylogenetic analysis placed *PpCHS* at the base of the plant CHS clade, next to the microorganism CHS-like gene products. Thus, the authors were of opinion that *PpCHS* was one of the oldest CHSs that appeared on earth.

Dao *et al.* (2011) reported that CHS gene expression could be induced in plants by stress conditions such as UV light, bacterial or fungal infection. According to them CHS expression accumulates flavonoid and isoflavonoid phytoalexins and also involve in the salicylic acid defense pathway.

Roslan *et al.* (2013) isolated a cDNA encoding a chalcone synthase from the leaves of *Polygonum minus* by rapid amplification of cDNA ends (RACE) and designated as *pmCHS* (GenBank accession no. JQ801338). The full-length cDNA of *P. minus pmCHS* was 1472 bp with an 1179 bp open reading frame (ORF) that corresponded to a predicted protein of 392 amino acid deduced protein. *In silico* analysis showed that the calculated molecular weight and theoretical isoelectric point (pI) of *pmCHS* were 43.1 kDa and 5.78, respectively. Several important motifs, such as the product binding site, active site and dimer interface, were also successfully identified from the deduced amino acid sequence. Multiple sequence alignment indicated that the *pmCHS* sequence was highly conserved and shared high sequence identity (>90%) with chalcone synthases from other plants. Gene expression analysis via qRT-PCR showed that *pmCHS* was most highly expressed in the roots, showing a 10-fold increase compared to leaves and a 15-fold increase compared to stems.

2.5. Management of pathogenic microorganisms by botanicals and biocontrol agents

Ravikumar and Garampalli (2013) evaluated antifungal property of 39 plant extracts against *Alternaria solani* a pathogen of early blight of

tomato by poison food technique in Potato Dextrose Agar medium. From their results, they found that out of 39 plant extracts 13 plants extracts significantly reduced the mycelial growth of the pathogen they tested. Seven plant extracts *Crotalaria trichotoma*, *Citrus aurantifolia*, *Azadirachta indica*, *Polyalthia longifolia*, *Datura metel*, *Muntingia calabura*, and *Oxalis latifolia* showed maximum inhibition (above 20%) of the disease at 4% concentration and six extracts *Crotalaria trichotoma, Azadirachta indica, Polyalthia longifolia*, *Datura metel*, *Capsicum annum* and *Citrus aurantifolia* showed significant growth inhibition at 2% concentration.

Falade (2017) tested the *in vitro* effect of 30, 50 and 65% concentrations of six plant extracts. Plants they selected were *Tridax procumbens, Jatropha gossypifolia, Sida acuta, Blighia sapida, Ricinus communis* and *Datura stramonium* on growth, conidial germination and sporulation of *Colletotrichum lindemuthianum*. Their results showed that the extracts of all six plants did not have any inhibitory effect on conidial germination and sporulation but significantly reduced the growth rates of fungus in comparison to the control. The maximum growth inhibition rate was to be found at 65% concentrations. Out of six extracts *Datura stramonium, Ricinus communis* and *Jatropha gossypifolia* showed significant effect and reduced the growth while *Blighia sapida* caused the least inhibition of growth. The growth inhibition rate of *Datura stramonium* at 30, 50 and 65% concentrations were 10, 16 and 33% respectively whereas *Blighia sapida* showed 2, 8 and 10% respectively.

Pawar (2011) examined antifungal activity of 18 plant leaf extracts against 5 seed-borne pathogenic fungi *viz. Alternaria alternata, Aspergillus niger, Curvularia lunata, Fusarium moniliforme* and *Trichoderma viride* by using cup-plate method. From their results it was observed that nine plant leaf extracts showed antifungal activity. *Azadirachta indica* leaf extracts showed maximum activity; while *Holoptelia integrifolia* leaf extracts showed minimum activity against the pathogenic fungi they tested.

Parimala and Sangeetha (2016) reported the antifungal activity of *Ricinus communis* leaf extracts against fungal isolates *Aspergillus niger*,

Aspergillus flavus, *Aspergillus fumigatus*, *Aspergillus terreus*, *Penicillium chrysogenum, Rhizopus stolonifer, Fusarium oxysporum*, *Candida albicans, Candida krusei, Candida tropicalis* and *Candida glabrata*. Methanol extracts showed highest inhibition zone; while minimum inhibition zone was observed in aqueous extracts against the fungal isolates.

In 2005, Peraza-Sanchez *et al*. screened 7 Yucatecan plant extracts and tested for fungicidal activity against *C. gloeosporioides*. They purified root extract of one of the most active plants, *Acacia pennatula,* following bioassay. They isolated new compound 15,16-dihydroxypimar-8(14)-en-3-one. Inhibitory activity on growth, sporulation, and germination of the fungus was observed by the isolated compound in "agar dilution" bioassay *in vitro*.

Deepak *et al*. (2005) screened antisporulant activity of forty commonly growing plant species of India. They used methanolic extracts against *Sclerospora graminicola*, the causative organism of pearl millet downy mildew. Out of the plant extracts tested, they showed that the extracts of 11 species (*Agave americana, Artemisia pallens, Citrus sinensis, Dalbergia latifolia, Helianthus annus, Murraya koenigii, Ocimum basilicum, Parthenium hysterophorus, Tagetes erecta, Thuja occidentalis* and *Zingiber officianale*) exhibited antisporulant effect even after 10-fold dilution of the crude extracts.

Bioactive compounds from lipophilic leaf extracts of medicinal plants (used by Himalayan people), were screened for antifungal properties by Guleria and Kumar (2006) by direct bioautography. Two fungi (*Alternaria alternata* and *Curvularia lunata*) were used as test organisms in bioautography. They evaluated fungal growth by measurement of radial growth. Out of 12 plants tested, they showed five plant species showed antifungal activity. They used $CHCl_3$: CH_3OH (1:9, v/v) as a solvent to develop silica gel TLC plates. Lipophilic extracts of *Vitex negundo*, *Zantoxylum alatum*, *Ipomea carnea*, *Thuja orientalis* and *Cinnamomum camphora* showed clear inhibition zones on TLC plates. According to them *T. orientalis showed* best antifungal activity.

Thirty aquous plant extracts were screened *in vitro* against *Sclerotium rolfsii* by Kiran *et al.* (2006) to examine the inhibitory effect on mycelial growth and sclerotial production. Plant extracts of *Prosopis juliflora* (10% concentration) inhibited (74%) of mycelial growth. Two other plant extracts (*Agave Americana* and *Nerium indicum*) also showed antifungal activity by inhibition of growth. But, best inhibition of sclerotial production was shown by *Agave americana* and *Clerodendron inerme.* Leaf and fruit extract of *Riccinus communis* could also inhibit sclerotial production.

Antifungal efficacy of cloves against *Aspergillus* spp was reported by Reddy *et al.* (2007). They isolated, characterized and tested the components of cloves. They identified eugenol as a major component on TLC plate as dark coloured spot with R_f 0.5 along with standard. In TLC plate bioautography test, TLC plates were spray-inoculated with four species of *Aspergillus* (*A. flavus*, *A. paraciticus*, *A.niger*, *A. ochraceus*) and they reported that eugenol on TLC plates inhibited mycelia growth of all four species of *Aspergillus*.

Antibacterial activity of seven semi purified plant extracts made from flowers, leaves, fruits, stems, pods and seeds of some plants and four antimicrobial chamicals were evaluated Meena *et al.*(2007). The bacterial plant pathogens used for the purpose were *Pseudomonas solanacearum*, *Xanthomonas campestris* pv. *Campestris*, *Xaxonopodis* and *Xanthomonas* pv. *Citri.* They followed disc diffusion method to test the antibacterial activity. Product componantes from mahua flowers and Satyanashi leaves were found effective, at 1000 ppm, against *Pseudomonas solanacearum*.

Mewari *et al.* (2007) tested two mosses viz. *Entodon plicatus* C. Muell and *Rhynchostegium vagans* jaeg for their antimicrobial activity against *Bipolaris sorokiniana, Fusarium solani, Pseudomonas sclanacearum*, and *Xanthomonas oryzae.* Aquous extracts of the two mosses were ineffective. Ethanolic extracts of E. plicatus and petroleum ether extract of *R. vagans* showed inhibitory effects against *B. Sporokiniana.* Extract of *R. vagans* were more effective inhibitors of *F. solani* than those of *E. plicatus*.

Antifungal activities of leaf extracts of four plant species (*Acacia pennata*, *Anaphylis wightiana*, *Capparis pepiaria* and *Catunaregum spinosa*) have been studied Malabadi and Vijay kumar (2007). They evaluated the of dichloromethen, aceton, hexane, and methanol extracts of leaves against four pathogens viz. *Candida albicans*, *Kluyeromyces polysporus*, *Aspergillus niger*, *Aspergillus fumigantus*. On the basis of MIC values of methanolic extract of *Anaphylis wightiana it was reported that the plant extract was highly antifungal* against particularly *C. albicans* and *K. polysporus.*

Broad spectrum of antimicrobial activity on human pathogenic microorganisms of six bacteria and two fungal strains by ether and ethyl acetate extracts *Crotalaria madurensis* was studied by Bhakshu *et al.* (2008). The plant is an endemic medicinal plant found in the forest of Nallamallias of Eastern-ghat of India.

Antifungal activity of essential oils of some medicinal plants was studied by Bansod and Rai (2008). They screened the activity against *A. fumigatus* and *A. niger* by determination of MIC. The oil of plants they found to be antifungal were *Cymbopogon martini, Eucalyptus globules, Cinnamomum jeylenicum*, *Cymbopogon citrates*. Antifungal activity of some plants oils was similar to control by Miconazole nitrate. The oils of *Mentha spicata, Azadirachta indica, Eugenia caryophyllata, Withania somnifera* and *Zingiber officinale* exhibited moderate activity. The oils *Cuminum cyminum, Allium sativum, Ocimum sanctum, Trachyspermum copticum, Foeniculum vulgare* and *Elettaria cardamomum* showed comparatively low activity against the two pathogens.

Salar and Suchitra (2009) evaluated antimicrobial activity of different parts (roots, stems, leaves and fruits) of *Solanum xanthocarpum* against bacteria and fungus. They extracted the antimicrobial properties of the plant parts in aqueous and organic solvents viz. ethanol, benzene, acetone and methanol. They studied the activity (antimicrobial) against Gram-positive (*Staphylococcus aureus*, *S. epidermidis*), Gram-negative

(*Escherichia coli, Pseudomonas aeruginosa*) bacteria and the fungus *Aspergillus niger*.

Thembo *et al.* (2010) screened the antifungal activity of aqueous, hexane, dichloromethane and methanol extracts of four weeds (*Tagetes minuta*, *Lippia javanica*, *Amaranthus spinosus* and *Vigna unguiculata*) against four fungi (*Fusarium verticillioides*, *F. proliferatum, Aspergillus flavus* and *A. parasiticus*). All extracts except water extracts of *Vigna unguiculata* and *Amaranthus* spinosus showed antifungal activity against *Fusarium* spp.

Johnny *et al.* (2011) studied antifungal activities of 15 selected medicinal plants such as *Alpinia galanga* (L.) Willd.,*Alstonia spatulata* Blume., *Annona muricata* L., *Blechnum orientale* L., *Blumea balsamifera* L., *Centella asiatica* L., *Dicranopteris linearis* (Burm. f.) Underw., *Dillenia suffruticosa* (Griff ex Hook.f. and Thomson) Martelli, *Litsea garciae* Vidal., *Melastoma malabathricum* L., *Momordica charantia* L., *Nephrolepis biserrata* (Sw.), *Pangium edule* Reinw., *Piper betle* L. and *Polygonum minus* Huds., against pathogenic fungus, *Colletotrichum capsici*. They used methanol, chloroform, acetone and Kocide 101 leaf extracts. *Piper betle* extracts in all the solvents have shown antifungal activities against *C. capsici*.

Aye and Matsumoto (2011) selected sixteen naturally available phytoextracts and tested *in-vitro* for their potential to control phytopathogens of rice, such as *Rhizoctonia solani, Rhizoctonia oryzae, Rhizoctonia oryzae-sativae* and *Sclerotium hydrophilum*. Four plant extracts (Clove, Neem, rosemary and pelargonium) showed significant antifungal activity against the rice pathogens mentioned above.

Naz and Bano (2012) investigated the antimicrobial activity of methanol, ethanol and aqueous leaf extracts of *Ricinus communis* against gram positive bacteria like, *Bacillus subtilis* and *Staphylococcus aureus*; gram negative bacteria like, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* as well as against some fungal strain such as, *Aspergillus fumigates* and *Aspergillus flavus*. Methanol leaf extracts showed potential

activity to reduce the growth of pathogenic bacteria and fungal strain better than the ethanol and aqueous leaf extracts.

Ishnava *et al.* (2012) *In vitro* screened the antifungal activity of leaf extracts against nine different fungi by agar diffusion test and minimum inhibitory concentration (MIC). Out of 17 plants leaf extracts *Ocimum sanctum*, *Datura metel, Adhatoda vasica, Holoptelea integrifolia* and *Aegle marmelos* showed maximum antifungal activity against *Alternaria* sp., *Aspergillus parasi*, *Aspergillus nidulans*, *Aspergillus flavus* and *Trichoderma harzianum*.

Pandey *et al.* (2012) reviewed the pathogenicity of *Colletotrichum gloeosporioides* a major causal organism of mango anthracnose disease and its botanical management. They showed that phytoextracts of *Azadirachta indica* was most effective botanical to reduce the radial growth of the fungas. The activity of the plant extract was shown to be similar with that of *Trichoderma* spp (a biocontrol agent).

Crude extracts of different plants such as Neem, Tulsi, Onion, Garlic, Basak, Leucus, Ginger, Turmeric, Ashwagandha, etc. have been used as effective phytoextracts *i.e.* botanicals, against many pathogenic fungi and bacteria (Baljeet *et al.*, 2015; Jain *et al.*, 2015; Ambareen *et al.*, 2015). Other than botanicals many microorganisms like *Bacillus*, *Pseudomonus, Tricoderma* etc. have been used as biocontrol agents to control several plant pathogens. They suppress the pathogen either by producing a specific toxin or by preventing establishment of other microorganisms through competition or other modes of action (Arunachalam and Sharma 2012; Shaikh and Sahera, 2016).

Padder *et al.* (2010) evaluated the efficacy of three bioagents (*Trichoderma viride*, *T. harzianum* and *Gliocladium virens*) and five biopesticides (Achook, Neemgold, Wannis, Spictaf and Neemazal) against *Colletotrichum lindemuthianum.* All the three antagonistic fungi significantly could inhibit mycelial growth. Among the tested bioagents and biopesticides, *T. viride* and Wanis (1000 µl/ml) were reported to be most effective to reduce the seed borne infection. They also found that disease

could effectively be managed by seed-dressing either with Bavistin or biopesticide followed by foliar treatment of fungicide or biopesticide.

Pallavi *et al.* (2012) isolated three *Bacillus* strains (MB1, MB2, and MB3), and three *Pseudomonus* strains (MP1, MP2, and MP3) from various soil sample and evaluated their efficacy as biocontrol agents against *Pestalotiopsis theae*, a causal organism of grey blight disease of tea. They found that selected six strains showed highest antagonistic effect against the pathogen.

Fitsum *et al.* (2014) evaluated the efficacy of three biocontrol agents (*Trichoderma viridae, T. harzianum,* and *Pseudomonus fluoroscens*) by dual culture and double dilution microtiter method against *Colletotrichum lindemuthianum*. Anthracnose disease of *phaseolus vulgaris is* caused by *Colletotrichum lindemuthianum*. They found that *T. viride* showed highest effect to inhibit mycelia growth followed by *T. harzianum* and *P. fluorescens.*

Koley *et al.* (2015) studied the efficacy of six bio-control agents (BCAs) and 12 botanicals against fungus *Alternaria solani* causing early leaf blight of tomato by *in-vitro* growth inhibition technique. Among the BCAs *Bacillus subtilis* and among the botanicals *Datura stramonium* showed significant control of the pathogen in in vitro studies.

Mardanova *et al.* (2017) isolated two *Bacillus* strains (GM5 and GM2) from the rhizosphere soil of potato roots and evaluated their antagonistic activity against phytopathogenic fungi *Fusarium solani* and F. *oxysporium*. According to them GM5 strain was more effective than the GM2 strain to inhibit the growth of fungus *in vitro*. Thus they reported the efficacy of the two strains as biocontrol agents.

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.

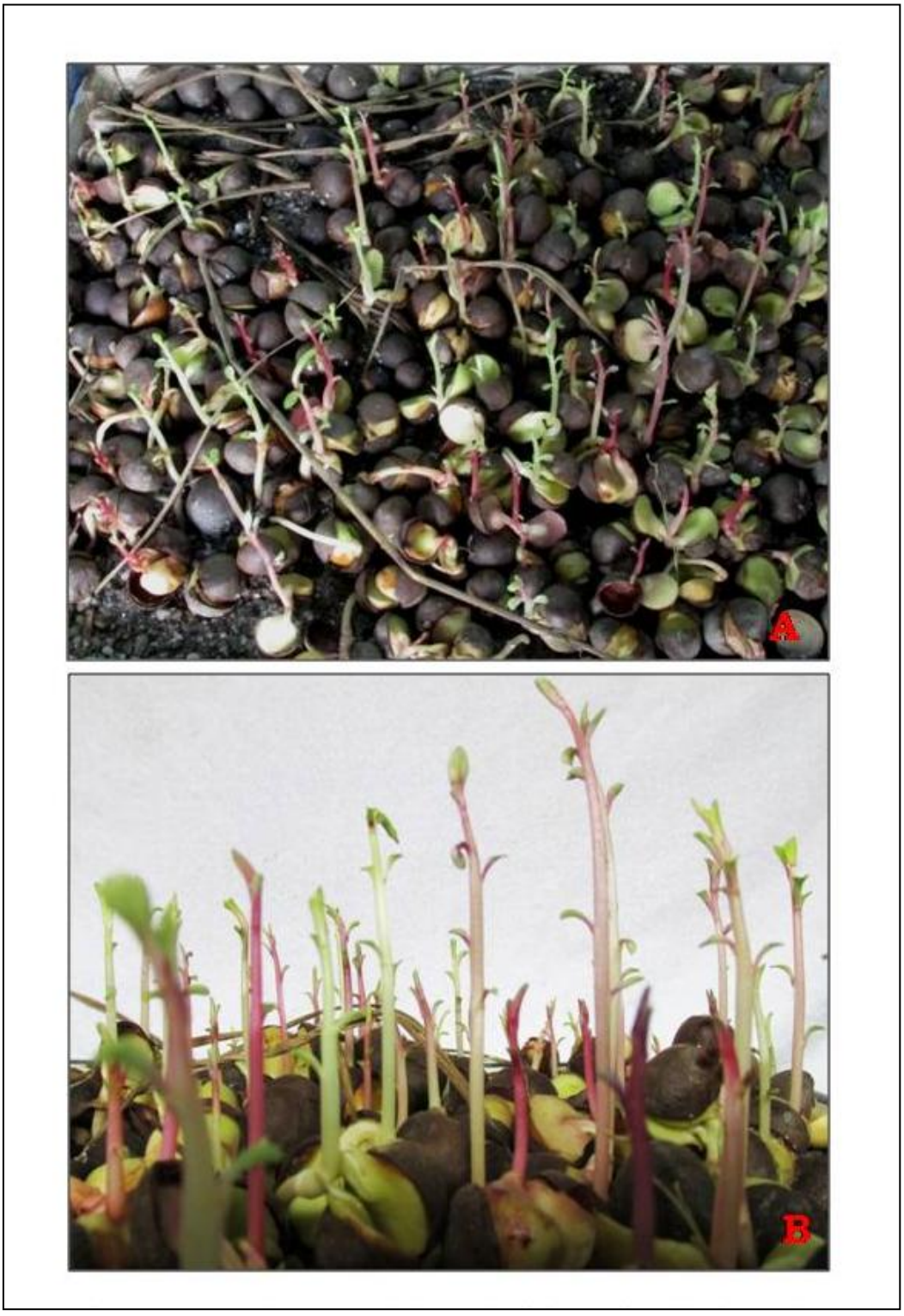

Fig. 3.1: A) Tea seeds TS-520 being raised in sand-soil mixture in a tray in the net house of the experimental garden of University of North Bengal after 30 days of sowing; B) Seedlings of TS-520 after 45 days of sowing.

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 37°C for 2 hrs with shaking at 200 rpm.
v. Centrifugation was done at 6000 rpm for 6 minutes at 4°C.
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 37°C.
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 - 60ºC. Optimum temperature was confirmed as 60ºC 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 50-55°C. 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.

4. Results

4.1: Selection of pathogens and survey of the study area

Several pathogens were isolated from the present study area by the previous workers of the present laboratory. Some of them are *Colletotrichum gloeosporioides* (producing brown blight disease symptoms in tea), *Curvularia eragrostidis* (producing leaf spot disease symptom in tea) and *Lasiodiplodia theobromae* (*which* attack young tender leaves as well as tender stem and root of tea plants). Study of the transcripts following induction (by resistance inducers) and inoculation by pathogens were the thrust area of the present study. Hence, at least one host and one pathogen need to be studied in details. In the present study detailed pathogenicity of three pathogens were determined in four different varieties of tea plants. Finally from that one host and pathogen were selected for the transcriptome analysis towards resistance induction.

However, before initiation of the present work it was considered worthwhile to survey the present status of four diseases prevalent in the present study area. This was done on the basis of visual observation of symptoms in four major tea gardens of the area. During survey, it was found that *Colletotrichum gloeosporioides, Curvularia eragrostidis* and *Lasiodiplodia theobromae* were present in the study area (Table 4.1). Out of these three two pathogens (*C. gloeosporioides* and *C. eragrostidis)* were found to attack leaves only. *L. theobromae* attacked leaf, root and stem in case of young plants. Another pathogen such as *Pestalotiopsis theae* although attack severely in the different tea plants of different tea estates surveyed (Table 4.1), but they generally attack mature leaves. Considering the damage created by the pathogens in the young leaves, three pathogens were initially selected among the disease causing pathogens for further studies.

To understand intriguing mechanisms of host pathogen interaction and resistance induction by inducers several experiments have been

performed. However, before going to the mechanisms, it was considered to evaluate the pathogens' pathogenicity at least in some seed and clonal varieties of tea. The fungal isolates used in the present study were collected from the molecular Plant pathology laboratory and were verified by Koch's postulations.

Table 4.1: Different tea diseases found in the tea gardens

Tea gardens visited	Disease symptoms visualized (%) on leaves			
	Brown blight (Caused by *C. gloeosporioides*)	**Gray blight (Caused by *P. theae*)**	**Leaf spot* (Caused by *C. eragrostidis*)**	**Dark brown necrotic spot (Caused by *L. theobromae*)**
Gaya ganga tea estate	76	74	60	54
Bagdogra tea estate	77	75	58	54
Matigara tea estate	74	75	60	55
Kharibari tea estate	72	70	59	52
Average of the four tea gardens	74.75±1.11	73.5±1.19	59.25±0.48	53.75±0.63

.*Leaf spot with restricted margin; Data after ± represent standard error values

4.2: Pathogenicity test of pathogenic fungus in different tea varieties

Pathogenicity of three fungal pathogens (*Colletotrichum gloeosporioides*, *Curvularia eragrostidis* and *Lasiodiplodia theobromae*) were tested by detached leaf inoculation technique and cut shoot inoculation technique. The inoculation techniques of detached leaf and cut shoot have been discussed in details in the materials and methods (Section 3.7). Four different tea varieties were used for the experiment. The disease assessment procedures and details of incubation periods have been mentioned in the materials and methods (Section 3.8).

4.2.1: Pathogenicity of three pathogens in tea varieties following detached leaf inoculation technique

Pathogenicity of *C. gloeosporioides, C. eragrostidis* and *Lasiodiplodia theobromae* were performed on detached leaves of 4 different varieties (viz.,

TS-463, TS-520, Teenali and TV-26) of tea. The results presented in the Table 4.2, 4.3 and 4.4 represents percentage of lesion formed and mean diameter of lesions after 24, 48 and 72 hours of incubation. Details of the inoculation technique and disease assessment have been discussed in the materials and methods section 3.7.1 and 3.8.1 respectively. The leaves were mounted with spore suspension droplets and the brown necrotic infected sites were termed as lesions (Fig. 4.1). The lesions were counted and diameters of the lesion were measured. The percentages of lesions formed in three separate experiments (i.e., replicates) were taken. The mean values of the three experiments were tabulated.

On the basis of the data presented in the Table 4.2, it is evident that the three varieties (Teenali, TS-520 and TS-463) were susceptible but TV-26 showed resistance against the pathogen *C. gloeosporioides. C. gloeosporioides* inoculated Teenali, TS-520 and TS-463 leaves produced 82.60%, 75.33% and 68.83% lesions respectively but TV-26 produced 27.83% lesion. In the present study we considered a variety as highly susceptible when it produced more than 70% lesion and resistant when the lesion production was less than 30% after 72 h of incubation. The varieties were considered as moderately susceptible when 40% to 60% lesion formation was observed. From the results it was found that Teenali and TS-520 are highly susceptible but TS-463 was moderately susceptible and TV-26 was resistant against *C. gloeosporioides*. The mean diameter of lesions produced after 72 h of inoculation by *C. gloeosporioides* were 10.16 mm, 9.0 mm and 8.56 mm in case of tea varieties Teenali, TS-520 and TS-463 respectively. In resistant variety TV-26, the mean diameter of lesions was 1.86 mm after 72 h of inoculation.

From the results (Table 4.3), it was found that 73.16%, 70.20%, 65.00% and 27.00% lesions were produced respectively in Teenali, TS-520, TS-463 and TV-26 by *C. eragrostidis* inoculation and after incubation of 72h. From the results, Teenali and TS-520 were considered as highly susceptible varieties but TS-463 was moderately susceptible. The variety TV-26 was also found to be resistant against *C. eragrostidis* inoculation.

The susceptible varieties Teenali, TS-520, TS-463 showed the mean lesion diameter of 9.20 mm, 8.50 mm and 7.25 mm respectively, where as the resistant variety TV-26 showed lesion diameter 1.60 mm after 72h of inoculation under similar conditions.

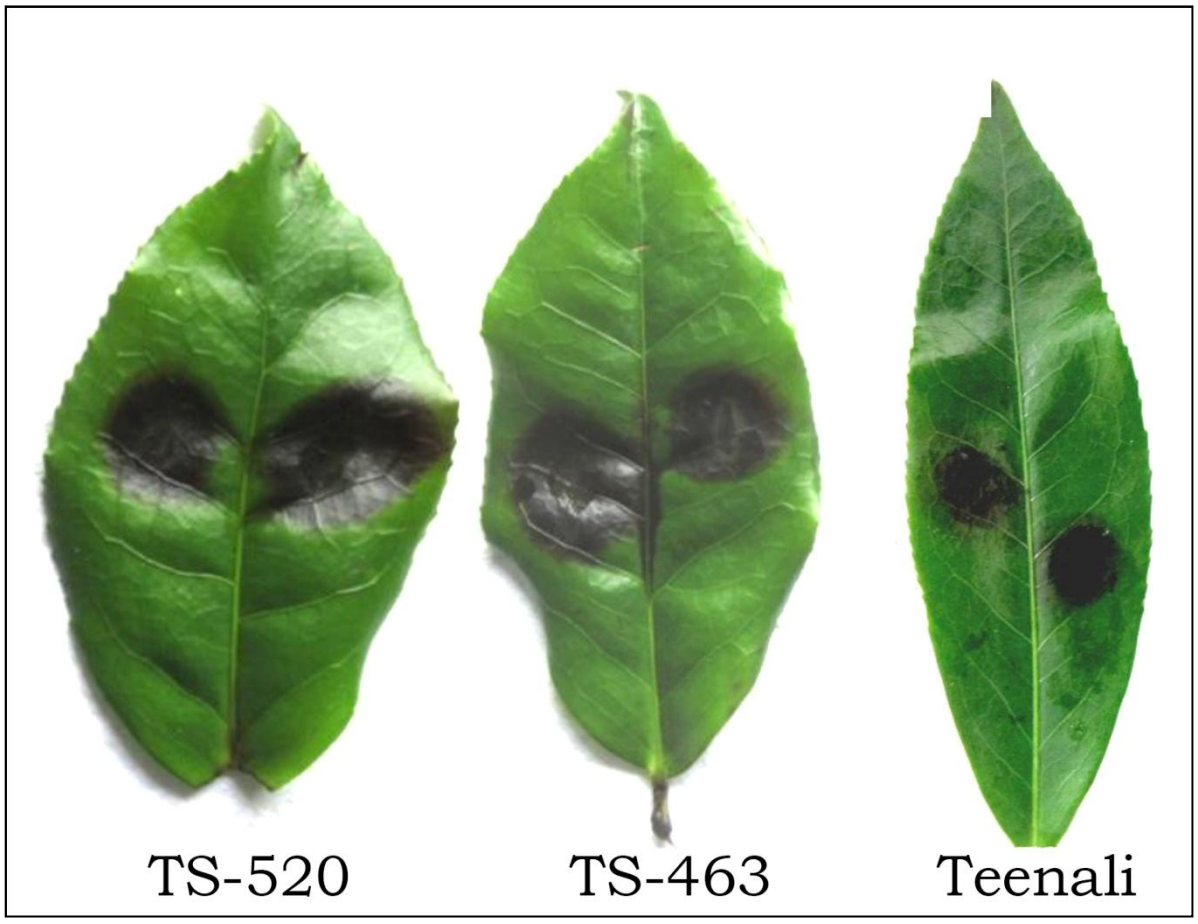

Fig. 4.1: Pathogenicity test by detached leaf inoculation showing black lesions.

On the basis of the data presented in the Table 4.4, it was evident that Teenali, TS-520 and TS-463 are moderately susceptible against *L. theobromae* because they produced 64.66%, 61.75% and 61.66% lesions respectively. TV-26 was considered as highly resistant variety because it produced 19.50% lesion against *L. theobromae* after 72h of inoculation. The mean lesion diameters after 72 h of inoculation by *L. theobromae* were 6.10 mm, 5.26 mm and 5.30 mm in case of Teenali, TS-520 and TS-463 respectively. The variety TV-26 showed the lesion diameter of 2 mm after 72h of inoculation with *L. theobromae*.

Table 4.2: Pathogenicity test of *Colletotrichum gloeosporioides* on detached leaves of selected tea varieties

Tea Varieties	[a] Percentage of lesion formed			[b] Mean diameter of lesion (mm)		
	Incubation periods (Hours)			Incubation periods (Hours)		
	24 h	48 h	72 h	24 h	48 h	72 h
TV-26	0	0	27.83±0.60	0	0	1.86±0.17
Teenali	42.25±0.52	62.91±0.46	82.60±0.95	1.70±0.20	4.0±0.11	10.16±0.72
TS-520	31.66±0.70	50.0±0.58	75.33±0.90	1.20±0.12	3.70±0.15	9.0±0.21
TS-463	30.66±0.33	45.0±0.57	68.83±0.60	1.20±0.10	3.83±0.12	8.56±0.23
CD (5%)	1.13	1.73	2.85	0.44	0.40	1.11

[a] Mean of 3 replications.
[b] Mean of 30 lesions.
Data after ± represent standard error values.

Table 4.3: Pathogenicity test of *Curvularia eragrostidis* on detached leaves of different tea varieties

Tea Varieties	[a] Percentage of lesion formed			[b] Mean diameter of lesion (mm)		
	Incubation periods (Hours)			Incubation periods (Hours)		
	24 h	48 h	72 h	24 h	48 h	72 h
TV-26	0	0	27.0±0.35	0	0	1.60±0.07
Teenali	34.66±0.82	56.25±0.81	73.16±0.54	1.87±0.04	4.06±0.08	9.20±0.20
TS-520	33.50±0.50	58.00±0.73	70.20±0.76	1.30±0.03	4.00±0.14	8.50±0.28
TS-463	35.00±1.15	55.00±1.73	65.00±0.57	1.25±0.12	3.50±0.17	7.25±0.22
CD (5%)	2.59	3.57	1.95	0.23	0.44	0.71

[a] Mean of 3 replications.
[b] Mean of 30 lesions.
Data after ± represent standard error values.

Table 4.4: Pathogenicity test of *Lasiodiplodia theobromae* on detached leaves of different tea varieties

Tea Varieties	[a] Percentage of lesion formed			[b] Mean diameter of lesion (mm)		
	Incubation periods (Hours)			Incubation periods (Hours)		
	24 h	48 h	72 h	24 h	48 h	72 h
TV-26	0	0	19.50±0.76	0	0	2.00±0.10
Teenali	28.00±1.00	43.66±0.73	64.66±1.10	1.30±0.11	3.53±0.14	6.10±0.15
TS-520	30.50±0.29	42.16±0.60	61.75±0.95	1.26±0.09	3.16±0.12	5.26±0.32
TS-463	31.50±0.86	41.00±1.15	61.66±1.20	1.20±0.10	3.20±0.11	5.30±0.30
CD (5%)	2.56	2.70	3.12	0.25	0.39	0.67

[a] Mean of 3 replications.
[b] Mean of 30 lesions.
Data after ± represent standard error values.

4.2.2: Pathogenicity of three pathogens in tea varieties following Cut shoots inoculation technique

Twigs containing three to four leaves were selected for pathogenicity test of *C. gloeosporioides, C. eragrostidis* and *L. theobromae*. Details of the experimental set up and diseasement have been discussed in the materials and methods (sections 3.7.2 and 3.8.2). The results were computed following the method of Sinha and Das (1972) and presented in the tables 4.5, 4.6 and 4.7 respectively. The tea varieties, which showed the mean disease index/shoot values between 1.00 and 1.50 after 72h of inoculation, were considered as moderately resistant tea varieties. When the value of mean disease index/shoot was less than 1.00 those tea varieties were considered as resistant varieties. Tea varieties were considered as susceptible when mean disease index/shoot values was more than 1.50.

From the result (Table 4.5) of mean disease index/shoot value, it was evident that Teenali was most susceptible variety and TV-26 was the most resistant variety against *C. gloeosporioides* after 72 h of inoculation. The mean disease index/shoot values of Teenali, TS-520, TS-463 and TV-26 varieties were 1.70, 1.50, 1.55 and 0.8 after 72 h of inoculation. From the disease index data it was found that the mean disease index was maximum in Teenali plants than that of TV-26 plants, so Teenali was considered as most susceptible and TV-26 was considered as most resistant variety against *C. gloeosporioides* after 72 h inoculation, while another susceptible variety TS-463 showed mean disease index value of 1.55. TS-520 was also considered as marginally moderately susceptible tea variety against *C. gloeosporioides* as it showed mean disease index/shoot value of 1.50 after 72 h of inoculation.

From the table 4.6 it was found that in case of Teenali the mean disease index/shoot value was 1.60 while TV-26 showed 0.75 mean disease index/shoot value after 72 h of post inoculation by *C. eragrostidis*. Hence, on the basis of mean disease index/shoot, Teenali was considered as most susceptible and TV-26 was a most resistant tea variety against *C. eragrostidis*. Other tea varieties like TS-520 and TS-463 showed the mean

disease index/shoot values of 1.45 and 1.50 respectively after 72 h of inoculation. From the disease value both the tea varieties (TS-520 and TS-463) were considered as moderately susceptible varieties against *C. eragrostidis*.

Table 4.5: Pathogenicity test of *Colletotrichum gloeosporioides* on cut shoots of different tea varieties

Tea Varieties	Mean foliar disease index/shoot*		
	Incubation periods (Hours)		
	24 h	48 h	72 h
TV-26	0	0	0.86±0.09
Teenali	0.18±0.01	0.57±0.01	1.73±0.08
TS-520	0.15±0.01	0.47±0.01	1.50±0.06
TS-463	0.16±0.01	0.50±0.05	1.55±0.03
CD (5%)	0.04	0.11	0.24

* Mean of 3 replications, Data after ± represent standard error values.
**Data presented on the basis of 10 cut shoots per treatment.

From the pathogenicity results of *L. theobromae* (Table 4.7), it was found that the tea varieties like Teenali, TS-520, TS-463 and TV-26 showed mean foliar disease index/shoot values of 1.30, 1.15, 1.20 and 0.70 respectively after 72h of inoculation. So, from the results it was evident that after 72h of inoculation TV-26 was most resistant and all the other three varieties like Teenali, TS-520 and TS-463 were moderately susceptible. No variety was found most susceptible on the basis of mean disease index value when inoculated with *L. theobromae*.

Table 4.6: Pathogenicity test of *Curvularia eragrostidis* on cut shoots of different tea varieties

Tea Varieties	Mean foliar disease index/shoot*		
	Incubation periods (Hours)		
	24 h	48 h	72 h
TV-26	0.10±0.01	0.25±0.01	0.75±0.02
Teenali	0.18 ±0.10	0.45±0.02	1.60±0.03
TS-520	0.15±0.00	0.43±0.02	1.45±0.03
TS-463	0.16±0.01	0.42±0.04	1.50±0.04
CD (5%)	0.07	0.05	0.34

* Mean of 3 replications, Data after ± represent standard error values.
**Data presented on the basis of 10 cut shoots per treatment.

Table 4.7: Pathogenicity test of *Lasiodiplodia theobromae* on cut shoots of different tea varieties

	Mean foliar disease index/shoot*		
	Incubation periods (Hours)		
Tea Varieties	**24 h**	**48 h**	**72 h**
TV-26	0.15±0.04	0.45±0.08	0.70±1.06
Teenali	0.25±0.02	0.81±0.03	1.30±0.08
TS-520	0.20±0.01	0.44±0.03	1.15±0.07
TS-463	0.21±0.01	0.50±0.02	1.20±0.09
CD (5%)	0.04	0.08	0.41

* Mean of 3 replications, Data after ± represent standard error values.

**Data presented on the basis of 10 cut shoots per treatment.

4.3. Induction of defense by abiotic inducers and disease assessment

On the basis of pathogenicity tests it was found that two pathogens *Colletotrichum gloeosporioides* and *Curvularia eragrostidis* were most destructive in comparison to *Lasiodiplodia theobromae* in case of young pluckable leaves. Hence, two fungi (*Colletotrichum camelliae* and *Curvularia eragrostidis)* were considered for further studies related to induction of defense in susceptible tea plants. Three different abiotic inducers (BTH, 3-ABA, and GABA) were used to study the induction of resistance in three susceptible tea plants by visual observation-based disease evaluation (by mean foliar disease index). Details of the treatment and disease index computation process have been discussed in the materials and methods (sections 3.7.3 and 3.8.2). Concentration of the chemicals used was 1mM.

4.3.1. Induction of defense in susceptible tea plants by abiotic inducers and disease assessment against *Colletotrichum gloeosporioides*

From the results presented in table 4.8 we find that disease index was reduced significantly to 3.1 from 6.0 (against *Colletotrichum gloeosporioides*) which were found in control plants where BTH induction was not done. BABA and GABA also could reduce disease incidence close to BTH induction in susceptible tea plants of variety TS-463

Similar experiments in another susceptible variety (TS 520) were also performed and results have been presented in table 4.9. From the results we find disease index reduced to 3.0 from 6.2 (found in control plants) in case of BTH treated plants after 7 days of treatment and inoculation. BABA and GABA also could reduce disease occurrence but BTH was best among the three abiotic inducers tested.

BTH induced plants of Teenali variety showed best induction of resistance as we find from the results presented in table 4.10. BTH induced plants of the Teenali variety showed reduction of disease index from 6.72 (found in control) to 2.80 (BTH treated plants). In this variety also BTH was found to be best inducer of resistance than the BABA and GABA.

4.3.2. Induction of defense in susceptible tea plants by abiotic inducers and disease assessment against *Curvularia eragrostiddis*

When induction of resistance by three abiotic inducers were studied against *Curvularia eragrostiddis,* we found that after seven days disease index was reduced significantly to 3.0 from 5.5 by BTH in susceptible tea plants of variety Teenali (Table 4.11). From the results of disease index presented in table 4.11 we also find that BABA and GABA also could reduce disease incidence almost similar to BTH induction.

Table 4.8: Disease incidence following application of abiotic inducers in susceptible tea plants (TS-463) against *Colletotrichum gloeosporioides*

Treatments	Mean foliar index disease /plant*			
	Incubation periods (Days)			
	1d	3d	5d	7d
Untreated-inoculated (Control)	0.18±0.02	1.55±0.17	4.10±0.26	6.00±0.41
Treated with BTH and *C. gloeosporioides* inoculated	0.16±0.01	1.15±0.07	2.90±0.52	3.10±0.29
Treated with BABA and *C. gloeosporioides* inoculated	0.17±0.01	1.20±0.11	3.00±0.57	3.20±0.39
Treated with GABA and *C. gloeosporioides* inoculated	0.17±0.02	1.25±0.14	3.10±0.57	3.26±0.49
CD (5%)	0.06	0.22	0.58	0.87

* Mean of 3 replications, Data after ± represent standard error values.

Table 4.9: Disease incidence following application of abiotic inducers in susceptible tea plants (TS-520) against *Colletotrichum gloeosporioides*

Treatments	Mean foliar index disease /plant*			
	Incubation periods (Days)			
	1d	3d	5d	7d
Untreated-inoculated (Control)	0.18±0.01	1.75±0.27	4.0±0.21	6.2±0.34
Treated with BTH and *C. gloeosporioides* inoculated	0.16±0.01	1.10±0.07	2.9±0.57	3.0±0.50
Treated with BABA *C. gloeosporioides* inoculated	0.17±0.01	1.20±0.11	3.0±0.55	3.2±0.41
Treated with GABA and *C. gloeosporioides* inoculated	0.17±0.01	1.25±0.19	3.1±0.55	3.3±0.49
CD (5%)	0.03	0.46	0.57	0.61

* Mean of 3 replications, Data after ± represent standard error values.

Table 4.10: Disease incidence following application of abiotic inducers in susceptible tea plants (Teenali) against *Colletotrichum gloeosporioides*

Treatments	Mean foliar index disease /plant*			
	Incubation periods (Days)			
	1d	3d	5d	7d
Untreated-inoculated (Control)	0.21±0.06	2.00±0.30	4.30±0.14	6.72±0.43
Treated with BTH and *C. gloeosporioides* inoculated	0.20±0.03	1.00±0.34	2.70±0.18	2.80±0.46
Treated with BABA *C. gloeosporioides* inoculated	0.21±0.02	1.10±0.46	2.80±0.29	3.2±0.39
Treated with GABA and *C. gloeosporioides* inoculated	0.21±0.02	1.20±0.30	2.90±0.46	3.0±0.46
CD (5%)	0.05	0.27	0.53	1.03

* Mean of 3 replications, Data after ± represent standard error values.

The same three inducers were also applied in another susceptible variety TS 520 (Table 4.12). In this case disease index was drastically reduced to 2.8 by BTH treatment in comparison to control where disease index was 5.0 after five days of treatment and inoculation. BABA and GABA also could significantly reduce disease occurrence in the variety TS 520.

When similar treatments were done on variety TS-463 (Table 4.13), we found reduction of disease index from 5.0 (in control set, where plants

were inoculated by *C. eragrostidis* only) to 3.0 after seven days of treatment by BTH followed by inoculation by the pathogen. Almost similar results were observed in case of induction treatments by BABA and GABA along with inoculation after treatment.

Table 4.11: Disease incidence following application of abiotic inducers in susceptible tea plants (Teenali) against *Curvularia eragrostidis*

Treatments	Mean foliar index disease /plant*			
	Incubation periods (Days)			
	1d	3d	5d	7d
Untreated-inoculated (Control)	0.19±0.03	1.2±0.15	4.25±0.30	5.50±0.29
Treated with BTH and *C. eragrostidis* inoculated	0.17±0.03	0.95±0.16	2.9±0.45	3.0±0.57
Treated with BABA and C. *eragrostidis* inoculated	0.18±0.06	1.0±0.23	3.0±0.51	3.1±0.47
Treated with GABA and *C. eragrostidis* inoculated	0.18±0.05	1.0±0.17	3.1±0.45	3.2±0.41
CD (5%)	0.03	0.55	0.54	0.89

* Mean of 3 replications, Data after ± represent standard error values.

Table 4.12: Disease incidence following application of abiotic inducers in susceptible tea plants (variety TS-520) against *Curvularia eragrostidis*

Treatments	Mean foliar index disease /plant*			
	Incubation periods (Days)			
	1d	3d	5d	7d
Untreated-inoculated (Control)	0.17±0.01	1.5±0.21	3.75±0.14	5.0±0.29
Treated with BTH and *C. eragrostidis* inoculated	0.16±0.01	1.10±0.26	2.70±0.29	2.8±0.58
Treated with BABA and C. *eragrostidis* inoculated	0.16±0.01	1.15±0.07	2.80±0.20	3.0±0.24
Treated with GABA and *C. eragrostidis* inoculated	0.17±0.02	1.17±0.11	2.85±0.42	3.1±0.26
CD (5%)	0.04	0.68	0.69	0.78

* Mean of 3 replications, Data after ± represent standard error values.

Table 4.13: Disease incidence following application of abiotic inducers in susceptible tea plants (variety TS-463) against *Curvularia eragrostidis*

Treatments	Mean foliar index disease /plant*			
	Incubation periods (Days)			
	1d	3d	5d	7d
Untreated-inoculated (Control)	0.15±0.02	1.30±0.21	3.80±0.29	5.00±0.58
Treated with BTH and *C. eragrostidis* inoculated	0.15±0.02	0.95±0.20	2.90±0.10	3.00±0.29
Treated with BABA and *C. eragrostidis* inoculated	0.15±0.02	1.00±0.11	2.80±0.34	3.06±0.29
Treated with GABA and *C. eragrostidis* inoculated	0.16±0.01	1.00±0.11	2.80±0.34	3.10±0.21
CD (5%)	0.03	0.61	0.42	0.49

* Mean of 3 replications, Data after ± represent standard error values.

4.4. Induction of defense-related enzymes in susceptible variety of tea by abiotic inducers and studies on some defense related enzymes.

Three different defense related enzymes were studied following induction of susceptible tea plants by three known abiotic inducers. Phenylalanine ammonia lyase (PAL) is a key player in phenyl-propanoid pathway. This pathway converts L-Phenylalanine to trans-cinnamic acid first and then tras- cinnamic acid lead to the synthesis of defense related compounds like lignin, phytoalexins such as isoflavonoids and caumarins (Dixon and Lamb, 1990; Mahadevan and Sridhar, 1996). PAL activity has been reported to increase by application of BABA (Newton *et al.* 1997) and BTH (Gorlach *et al.* 1996).

Pathogenesis related protein β-1,3 glucanase has an important role in hydrolyzing β-1,3 glucans present in chitin, one of the major cell wall component embedded in matrix (Lawrence *et al.* 1996). Thus β-1,3 glucanase plays an key role in plant defense against invading plant pathogenic fungi. Kini *et al.* (2000) has reported higher β-1,3 glucanase activity in some resistant plants and low in the susceptible plants. Thus induction of the enzyme by exogenous application of some abiotic inducers in susceptible varities of tea was taken into consideration.

Another defense enzyme, peroxidase, is related to stress and has been reported to be induced in plants by various environmental changes such as salts, temperature, heavy metals (Kiwan and Lee, 2003), Air pollution, (Lee *et al.* 2000). As peroxidase also treated as pathogenesis related proteins (PR 9) and peroxidase induction in plants has been reported by some scientists (Kiwan and Lee, 2003; Lee e*t al.*, 2000), its induction by abiotic inducers in susceptible varities of tea was also been taken into consideration.

4.4.1. Activity of PAL with abiotic inducers

In the present study, enzymatic response of PAL in susceptible tea variety was studied by the exogenous application of different abiotic inducers such as BTH, BABA and GABA. Six month old tea plants were used for induction of PAL. Plants are divided into eight sets on the basis of treatment and each set contained ten plants. The detailed procedure of application of abiotic inducers and challenge-inoculation with pathogens has been discussed in materials and methods (Section-3.7.3) and the procedures of enzyme assay has been discussed in Section 3.9

From the results (Table 4.14 & Fig. 4.2) it has been found that the activity of PAL was increased in BTH treated plants about two fold after 4days and then declined but further increased after 6 days after treatment. BABA and GABA treated plants showed minor increase in PAL activity. Plants inoculated with *Colletotrichum gloeosporioides* after treatment showed highest PAL activity. About three fold increase in PAL activity was observed in BTH treated-inoculated plants after six days. Untreated plants inoculated with *C. gloeosporioides* showed slight increase in enzyme activity after 6 d of inoculation. In control plant sets (untreated-uninoculated) PAL activity remained unchanged.

4.4.2. Activity of β-1, 3-glucanase with abiotic inducers

In the present study, β-1,3-glucanase activity, in a susceptible tea variety, was determined by three abiotic inducers (BTH, BABA and GABA). The detailed procedures of treatment of abiotic inducers and challenge-

inoculation with *Colletotrichum gloeosporioides* has been discussed in materials and methods (Section 3.7.3). The procedure for enzyme assay has been discussed in materials and methods (Section-3.9).

Table 4.14: Activity of phenylalanine ammonia-lyase in tea plants pretreated with three chemical inducers followed by *Colletotrichum gloeosporioides* inoculation

Treatments	PAL activity (µmol $min^{-1}g^{-1}$ fresh weight tissue)						
	Days after inoculation						
	0 d	1d	2d	3d	4d	5d	6d
Control	2.45 ±0.03	2.41 ±0.06	2.53 ±0.03	2.52 ±0.38	2.54 ±0.30	2.50 ±0.29	2.52 ±0.16
Colletotrichum gloeosporioides	2.50 ±0.11	3.00 ±0.05	3.81 ±0.04	4.10 ±0.02	5.50 ±0.57	4.75 ±0.08	5.25 ±0.07
BABA	2.45 ±0.20	2.50 ±0.25	2.95 ±0.33	4.00 ±0.34	4.26 ±0.17	4.10 ±0.38	4.53 ±0.32
GABA	2.40 ±0.30	2.55 ±0.25	3.00 ±0.23	3.95 ±0.25	4.10 ±0.38	4.10 ±0.17	4.66 ±0.40
BTH	2.45 ±0.10	2.83 ±0.10	3.50 ±0.30	4.20 ±0.20	5.60 ±0.40	5.00 ±0.57	7.10 ±0.20
BABA + *C. gloeosporioides*	2.50 ±0.18	2.60 ±0.38	3.20 ±0.34	4.10 ±0.29	4.63 ±0.44	4.40 ±0.09	5.00 ±0.58
GABA + *C. gloeosporioides*	2.55 ±0.03	2.70 ±0.21	3.20 ±0.20	4.00 ±0.29	4.50 ±0.36	4.60 ±0.46	4.85 ±0.41
BTH + *C. gloeosporioides*	2.60 ±0.10	2.70 ±0.20	3.40 ±0.30	4.30 ±0.20	5.90 ±0.30	5.20 ±0.06	5.50 ±0.30
CD (5%)	0.21	0.27	0.37	0.52	0.68	0.63	0.61

*Data are mean of three replications; Data after ± indicate standard error values.

The activity of β–1,3-glucanase was increased in case of 'treated', 'inoculated' and 'treated-inoculated' plants but in case of control plants (untreated-uninoculated) no significant changes in β–1,3-glucanase activity was observed. From the results (Table 4.15 & Fig. 4.3) it was evident that all the inducer treated plants showed similar type of β–1, 3-glucanase activity. BTH treated plants showed highest activity after 4 days

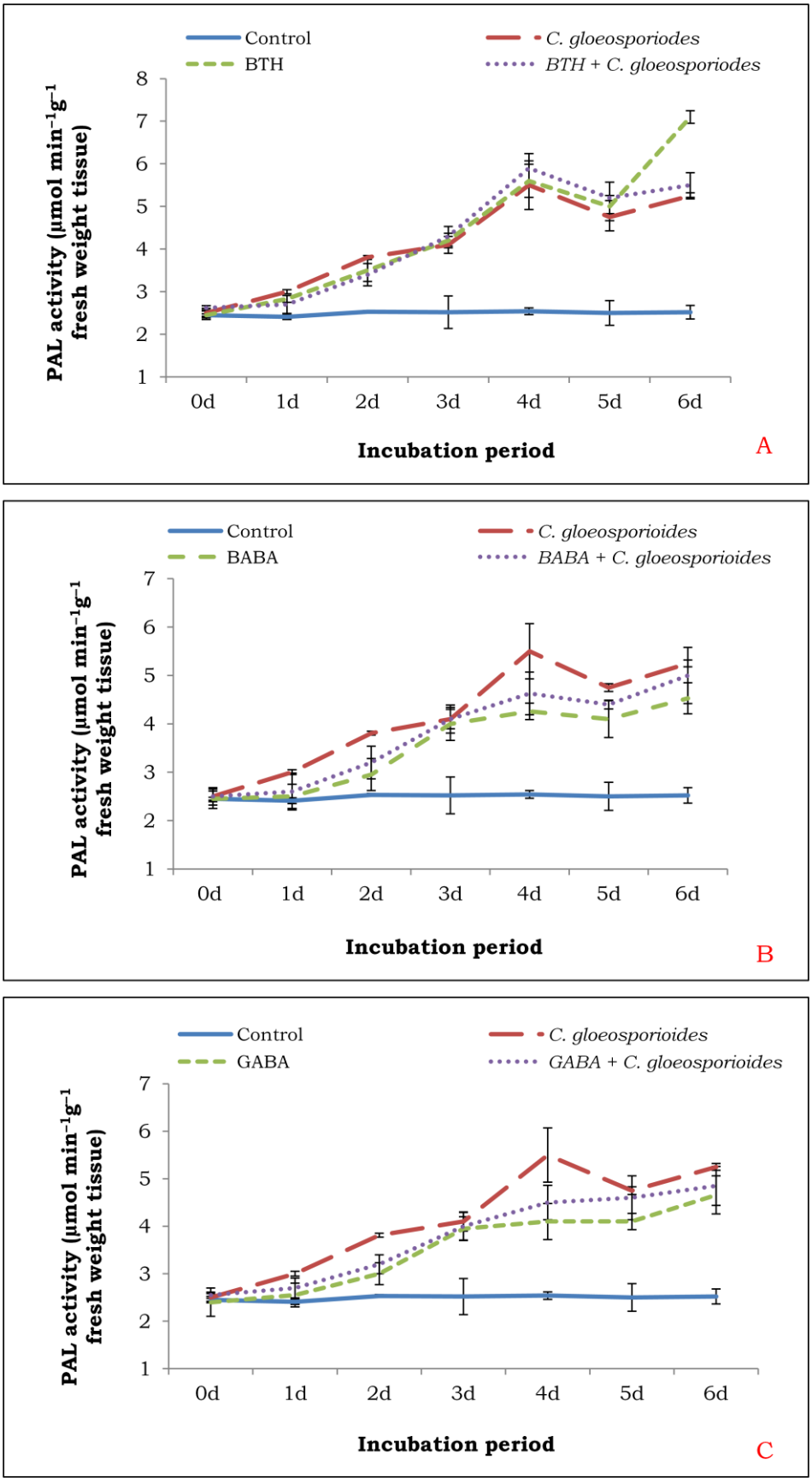

Fig. 4.2: Activity of phenylalanine ammonia-lyase in tea inoculated with *C. gloeosporioides* and pretreated with different abiotic inducers (A) BTH; (B) BABA; (C) GABA.

Table 4.15: β-1, 3-glucanase activity in young tea plants of Teenali variety pretreated with three chemical inducers and challenge inoculated with *C. gloeosporioides*

Treatments	β-1,3-glucanase activity [mg glucose g-1 fresh weight tissue min-1]*						
	Incubation period [days]						
	0d	1d	2d	3d	4d	5d	6d
Control	12.00 ±0.58	12.00 ±1.53	12.26 ±1.07	12.33 ±0.33	13.15 ±0.93	13.33 ±1.20	13.0 ±0.56
Colletotrichum gloeosporioides	14.20 ±0.46	19.33 ±1.10	22.66 ±0.88	26.86 ±0.32	30.65 ±0.36	23.48 ±0.77	31.68 ±0.45
BABA	14.00 ±0.81	18.33 ±0.88	21.45 ±0.99	23.66 ±1.20	24.5 ±0.77	23.25 ±0.72	25.00 ±1.73
BTH	14.33 ±0.66	18.66 ±0.88	23.5 ±0.76	25.54 ±1.15	27.86 ±1.29	25.00 ±1.15	26.5 ±1.75
GABA	14.33 ±1.20	18.33 ±0.88	21.50 ±0.29	22.00 ±0.58	23.00 ±1.73	21.00 ±1.0	22.00 ±1.52
BABA + *C. gloeosporioides*	16.31 ±0.63	22.76 ±1.29	25.63 ±1.91	26.65 ±1.79	28.90 ±1.45	29.00 ±1.53	30.55 ±0.75
GABA + *C. gloeosporioides*	15.0 ±1.73	18.25 ±0.90	20.56 ±1.65	21.65 ±2.25	25.66 ±1.76	20.46 ±1.35	28.5 ±1.44
BTH + C. gloeosporioides	15.25 ±1.28	21.53 ±1.28	24.30 ±0.66	26.45 ±1.17	28.5 ±1.04	26.5 ±0.76	31.00 ±1.52
CD (5%)	1.69	2.04	1.95	2.36	1.96	1.99	1.42

*Data are mean of three replications; Data after ± indicate standard error values.

of treatment and then activity declined but in case of BABA treated plants highest activity (25.00 mg glucose g-1 fresh weight tissue min-1) was found after 6 days of treatment in comparison to control plants (untreated-uninoculated). Plants inoculated with pathogen showed further increased activity of β–1, 3-glucanase about 30.65±0.36 and 31.68±0.45 after 4 days and 6 days of inoculation respectively in comparison to control plants. Similar type of induction of β-1, 3-glucanase was also found in treated-inoculated plant sets.

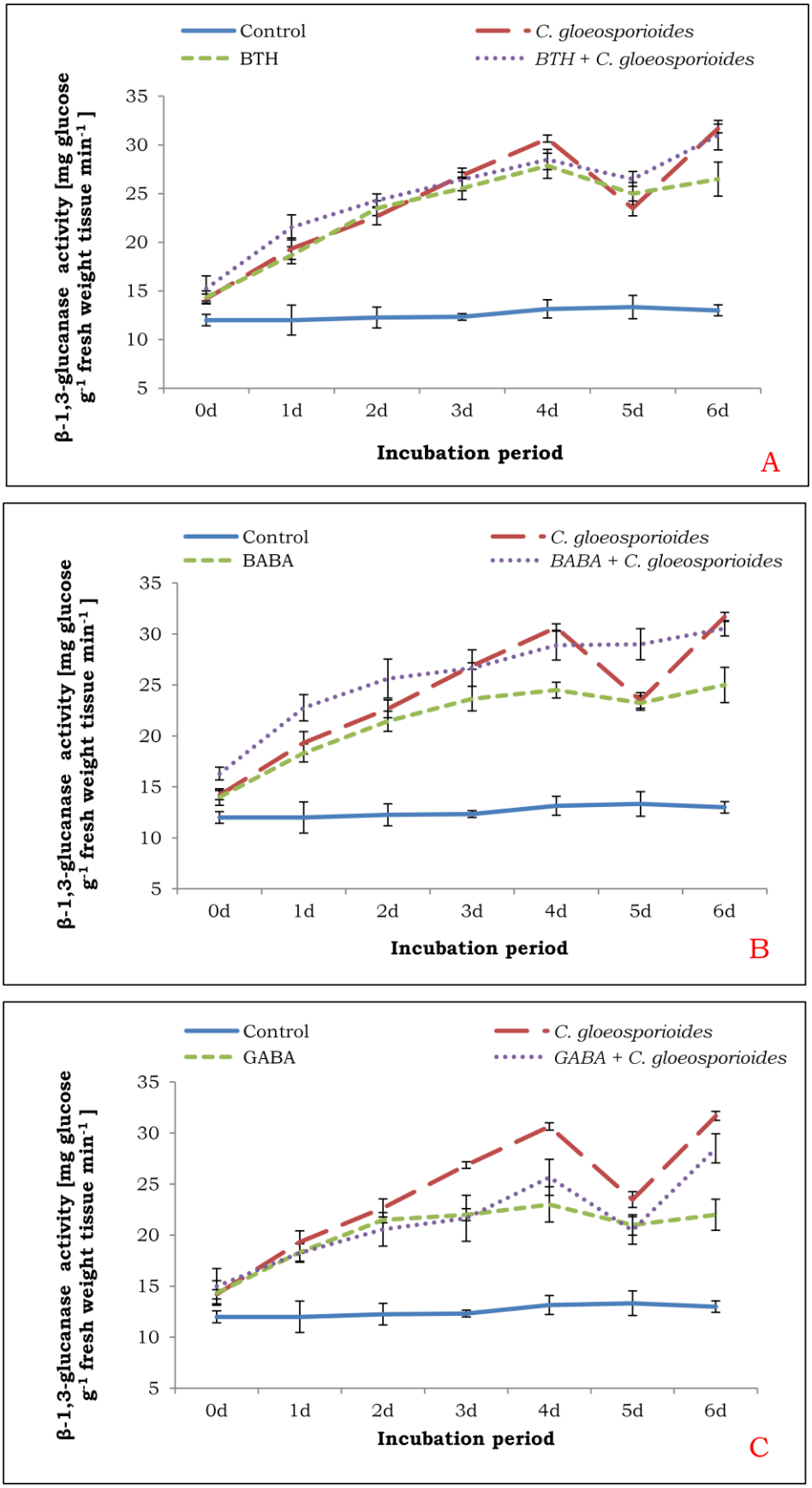

Fig. 4.3: Activity of ß-1,3-glucanase in tea inoculated with *C. gloeosporioides* and pretreated with different abiotic inducers (A) BTH; (B) BABA; (C) GABA.

4.4.3. **Activity of Peroxidase with abiotic inducers**

Peroxidase activity levels in susceptible tea plants, by treatment of different abiotic inducers, such as BTH, BABA and GABA individually or inoculated with *Colletotrichum gloeosporioides* were determined. To compare the induction level of peroxidase activity, one set of plants were inoculated with pathogen followed by the inducer treatment, whereas another two plant sets, such as control (untreated-uninoculated) and untreated-inoculated were also prepared. The detailed procedure of application of different inducers has been discussed in materials and methods (Section-3.7.3) and the detailed procedures of enzyme extraction and assay have been discussed in materials and methods (Section-3.9).

Peroxidase induction was measured up to six day in inoculated, treated and treated inoculated plants. Treatment was done with three chemical inducers and half of the pretreated plants were challenge inoculated by one of two test pathogens of tea plants and the results were noted in table 4.16 & Fig. 4.4. Among the inducers, GABA and BTH showed maximum peroxidase induction where enzyme activity increased from 71 in 0 days to 210 after 6th days of GABA treatment whereas BTH treated plants showed the increasing activity from 72.00 in 0 days to 200 in 6th days after treatments. Similar type of induction was also found in challenge-inoculated plants pretreated with BTH and GABA treatments. BTH pretreated-inoculated plants showed maximum peroxidase activity about 270 and GABA pretreated-inoculated plants showed maximum activity about 280 after 6^{th} days. Control sets did not show any significant increase in peroxidase levels.

Table 4.16: Peroxidase activity in young tea plants of Teenali pretreated with three chemical inducers and challenge inoculated with *C. gloeosporioides*

Treatments	Peroxidase activity (ΔA_{420} min $^{-1}$ g $^{-1}$ fresh weight tissue)*						
	1 unit = 0.001 Absorbance						
	Incubation period [days]						
	0 d	1d	2d	3d	4d	5d	6d
Control	70.00 ±1.15	71.00 ±0.58	71.00 ±1.00	71.00 ±0.58	73.00 ±0.58	73.00 ±1.53	74.00 ±0.58
Colletotrichum gloeosporioides	72.00 ±0.58	78.00 ±1.15	82.00 ±1.15	88.00 ±1.15	91.00 ±0.58	101.0 ±1.53	112.0 ±1.15
BABA	72.00 ±0.58	82.00 ±1.15	89.00 ±1.73	95.00 ±0.58	114.0 ±1.53	125.0 ±1.00	140.0 ±1.15
GABA	71.00 ±0.58	85.00 ±1.73	100.0 ±1.15	120.0 ±1.15	150.0 ±1.53	152.0 ±1.53	210.0 ±2.89
BTH	72.00 ±0.58	82.00 ±1.15	90.00 ±0.58	110.0 ±1.73	145.0 ±1.73	130.0 ±1.15	200.0 ±1.53
BABA + *C. gloeosporioides*	71.10 ±0.56	88.33 ±0.88	103.0 ±0.58	125.0 ±1.00	140.0 ±1.15	141.33 ±0.88	180.0 ±1.73
GABA + *C. gloeosporioides*	73.00 ±0.58	95.00 ±1.53	115.0 ±1.73	135.0 ±1.73	210.0 ±1.53	205.0 ±2.89	280.0 ±1.15
BTH + *C. gloeosporioides*	72.17 ±1.17	90.67 ±0.88	105.0 ±1.00	132.0 ±1.53	200.0 ±1.73	205.0 ±2.89	270.0 ±1.15
CD (5%)	1.25	2.11	2.26	2.14	2.30	2.78	2.64

*Data are mean of three replications; Data after ± indicate standard error values.

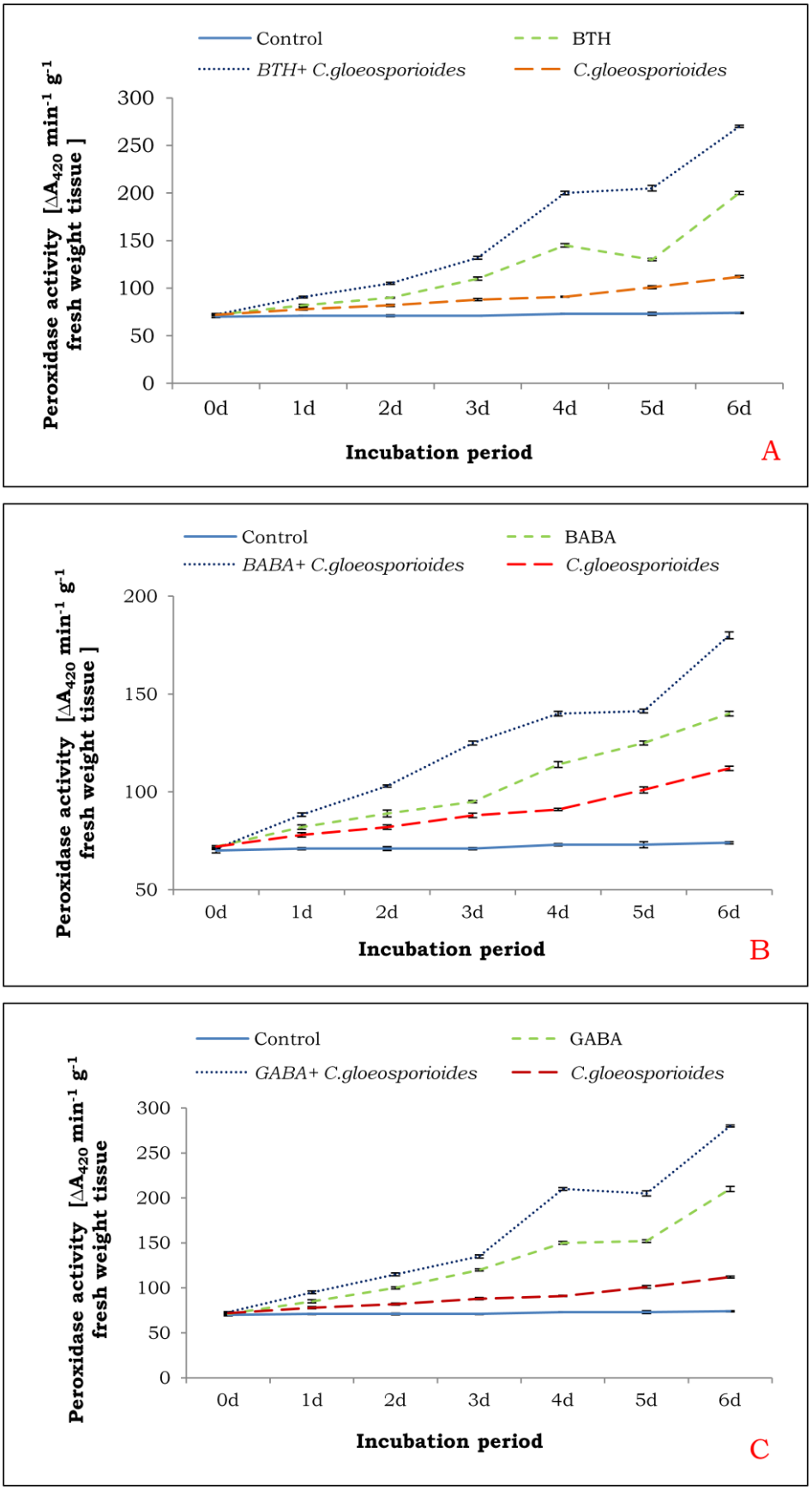

Fig. 4.4: Activity of peroxidase in tea inoculated with *C. gloeosporioides* and pretreated with different abiotic inducers (A) BTH; (B) BABA; (C) GABA.

4.5. Isolation and molecular characterisation of three defense related genes in tea

4.5.1. Detection and analysis of defense related gene in tea

Three different defense related genes such as Phenylalanine ammonia-lyase (PAL), Chalcone synthase (CHS) and Ascorbate peroxidase (APX) were amplified from tea leaves through RT-PCR under optimal RT-PCR conditions with the help of gene specific primers (Table. 3.4), where total RNA was used as a template. Expected amplicons of different genes such as APX, CHS and PAL respectively were isolated (Fig. 4.5). The amplicons were cloned and sequenced.

4.5.1.1. Analysis of Phenylalanine ammonia-lyase (PAL) gene isolated from tea

After sequencing of the PAL gene amplicon, a 333 nt long sequence was obtained. The PAL gene sequence was analysed through BLASTn programme. The BLASTn analysis showed high nucleotide (nt) identity with *Camellia chekiangoleosa* (99%) and *Camellia sinensis* (91%) and the nt sequences were submitted to GenBank (Acc. no. KF303590). The PAL sequence was aligned with other PAL gene sequences of *C. sinensis* and other species of *Camellia* submitted in the GenBank. Nt sequence identity matrix of the isolated PAL genes, isolated from different *Camellia* spp., were created in SDT v1.2 and presented in Fig. 4.6. From the sequence identity matrix it was observed that the PAL gene of *C. sinensis* showed above 90% nt identity within the species, whereas, below 60% nt identity was observed between *C. sinensis* and other species of *Camellia*. Phylogenetic tree was also created to study the evolutionary relationship among the above mentioned sequences (Fig. 4.7). In the phylogeny, *C. sinensis*, *C. talensis* and *C. flavida* formed three different clusters where our sequence clustered together with *C. sinensis*. Among the clusters *C. talensis* and *C. flavida* were more closely related than *C. sinensis*.

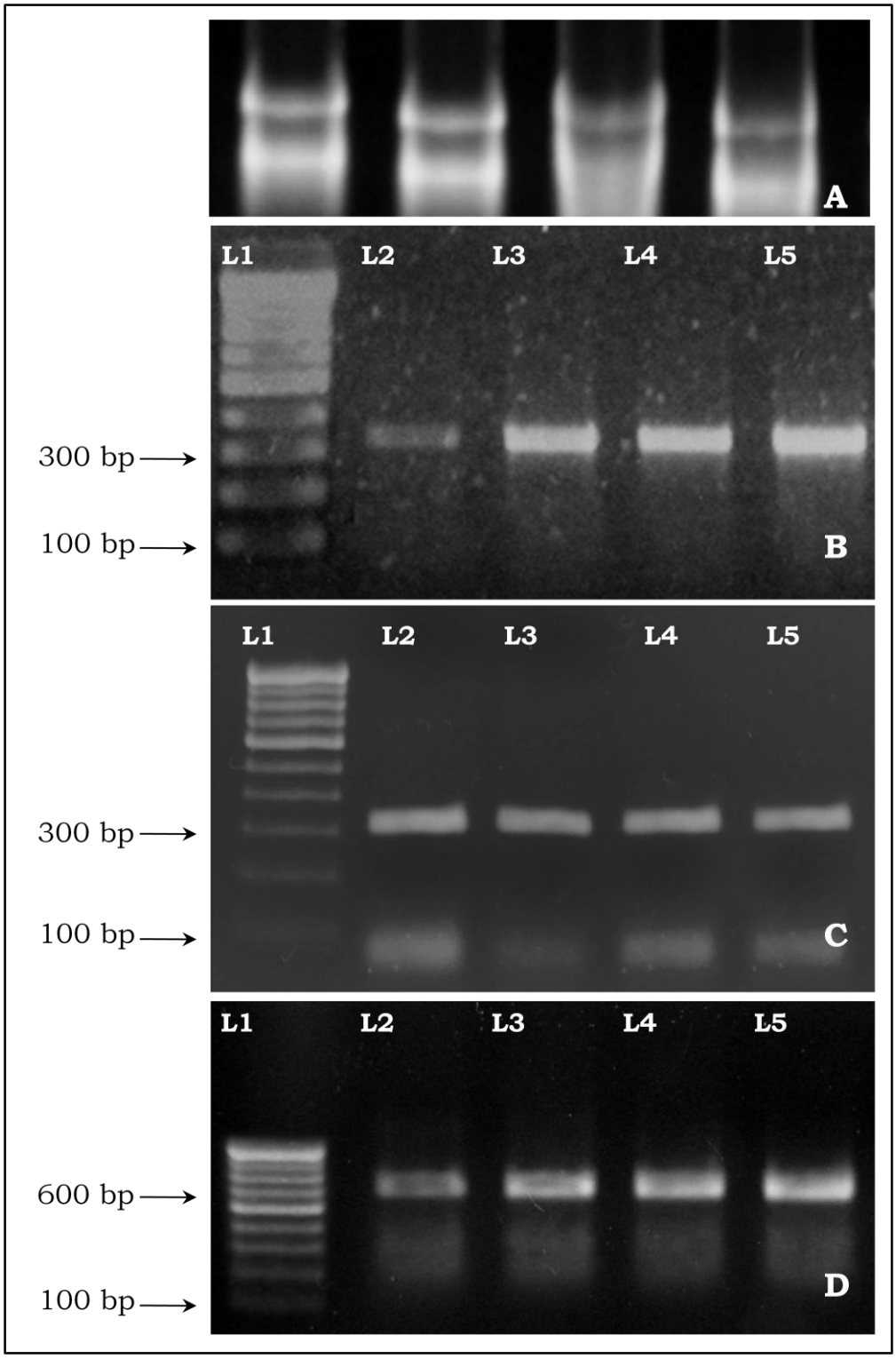

Fig. 4.5: A) Extracted total RNA on 1% agarose gel. B-D) Amplified RT-PCR product of PAL gene (B); CHS gene (C) and APX gene (D) on 1% agarose gel [L1: 100 bp DNA ladder, L2-L5: amplified gene product].

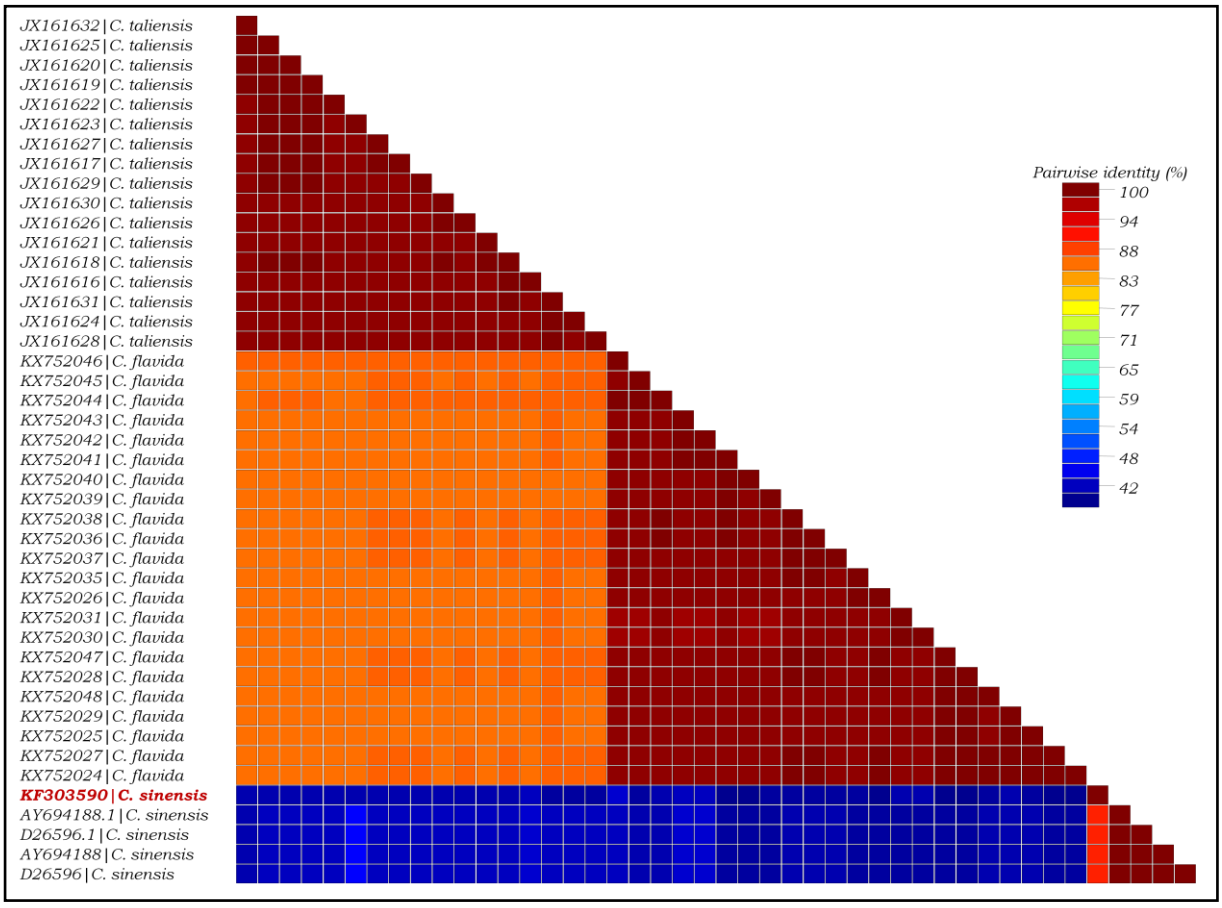

Fig. 4.6: Sequence identity matrix of isolated PAL gens with other PAL gene sequences of *Camellia* sp. Identity percentage corresponding to the colour matrix is indicated in the right-hand side of the figure.

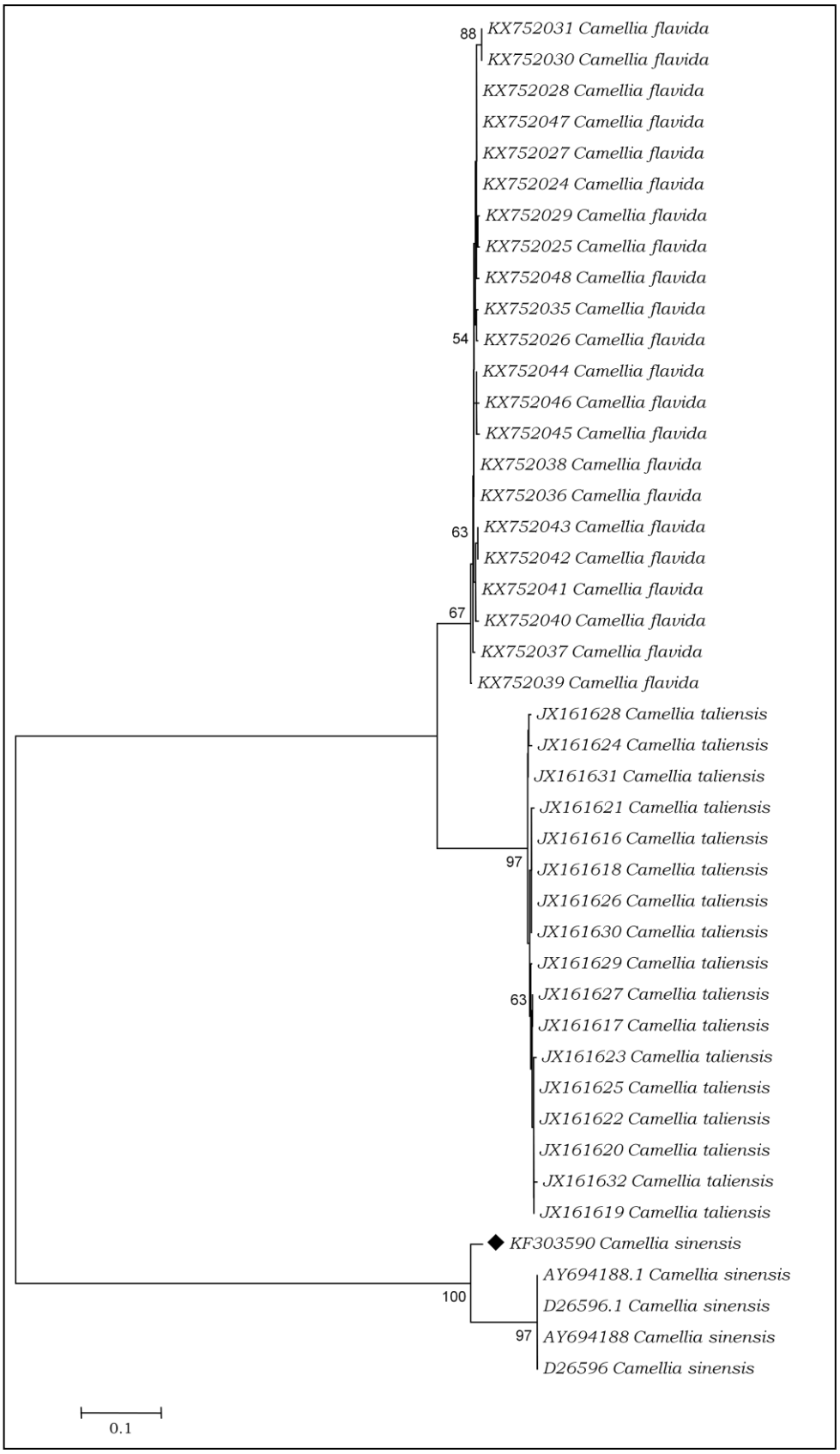

Fig. 4.7: Phylogenetic relationship of present isolated PAL gene with the other PAL gene sequences of *Camellia* sp. published in the GenBank using the neighbor joining method. Numbers at the nodes indicate the bootstrap percentage scores out of 1000 replicates.

4.5.1.2. Analysis of Chalcone synthase (CHS) gene isolated from tea

CHS gene amplicon was subjected to sequencing. At first, sequence of 345bp CHS gene (Acc. no. KJ866952) was analysed using BLASTn programme of NCBI database. The sequence analysis showed 99% nt sequence similarity with CHS gene of *C. sinensis* submitted in the GenBank. The sequence of isolated CHS gene was aligned with other CHS gene sequences of *C. sinensis* and other species of *Camellia* submitted in the GenBank. Percentage of nt sequence similarity of the isolated CHS genes with the other CHS genes of different *Camellia* species has been presented in Fig. 4.8. Sequenced similarity matrix showed that the CHS gene of *C. sinensis* showed above 95% nt identity within the species, whereas 75% to 95% nt identity was observed between *C. sinensis* and other species of *Camellia*. Evolutionary relationship of CHS gene of different *Camellia* species was studied using neighbor-joining method with Kimura-2 parameter model. The sequence identity matrix of CHS genes of different *Camellia* species formed five different groups which is evident from the phylogenetic tree (Fig. 4.9).

4.5.1.3. Analysis of Ascorbate peroxidase (APX) gene isolated from tea

Sequencing of the isolated APX gene gave a 663 nt long sequence (Acc. No. KR296654), which showed 98% nt identity with APX gene of *C. sinensis* after BLASTn analysis. The sequence identity matrix of the APX gene (Fig. 4.10A) of different *Camellia* species showed no significant group as observed in the previously mentioned gene sequences (viz., PAL and CHS). The phylogenetic tree also revealed similar type of result indicating that the APX gene did not evolutionarily segregated from species to species (Fig. 4.10B). Rather, their diversity within the *C. sinensis* species was more prominent. However, only nine sequences of APX gene of *Camellia* spp. have been found so far. Nevertheless, more sequences from different species of *Camellia* might give better insight towards understanding of their evolutionary trend.

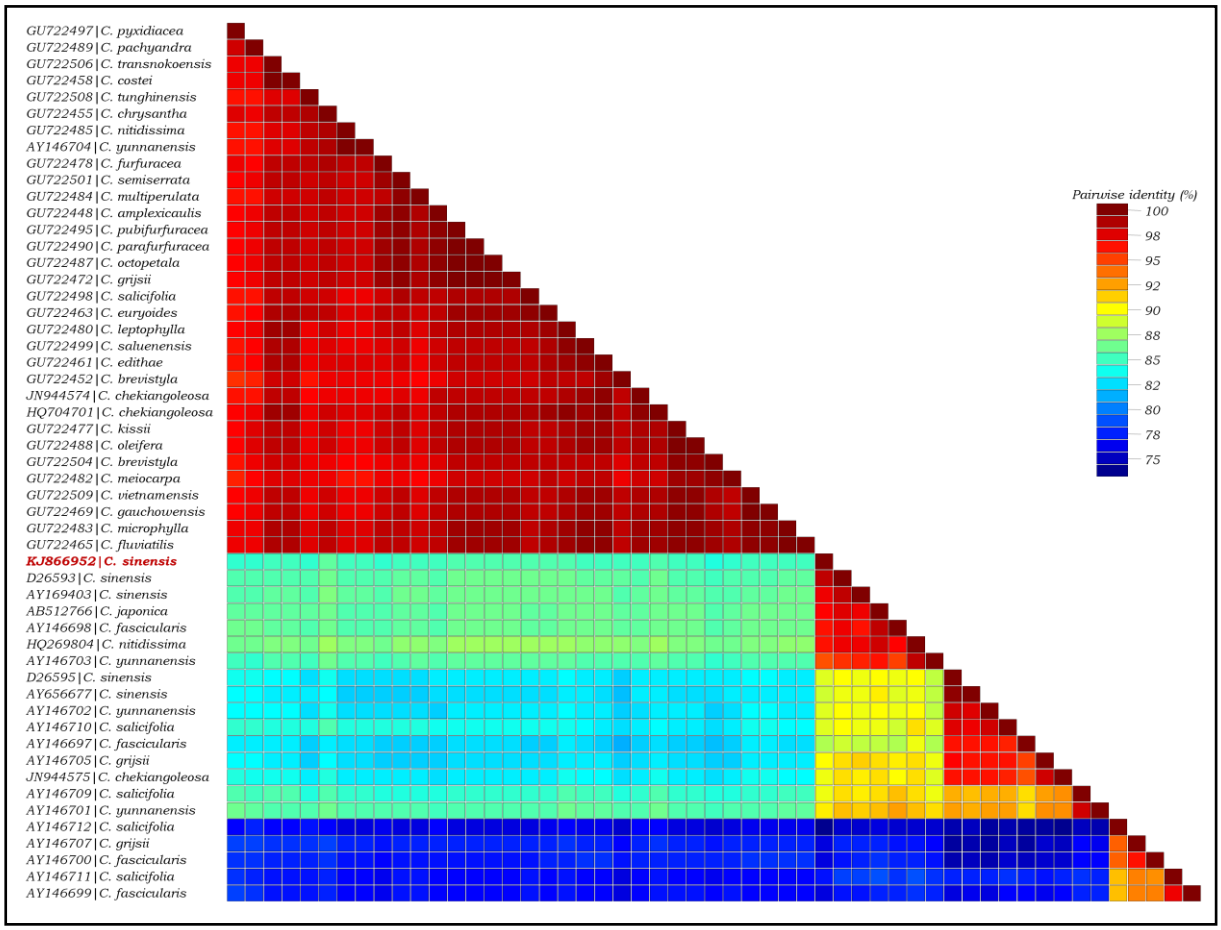

Fig. 4.8: Sequence identity matrix of isolated CHS gens with other PAL gene sequences of *Camellia* sp. Identity percentage corresponding to the colour matrix is indicated in the right-hand side of the figure.

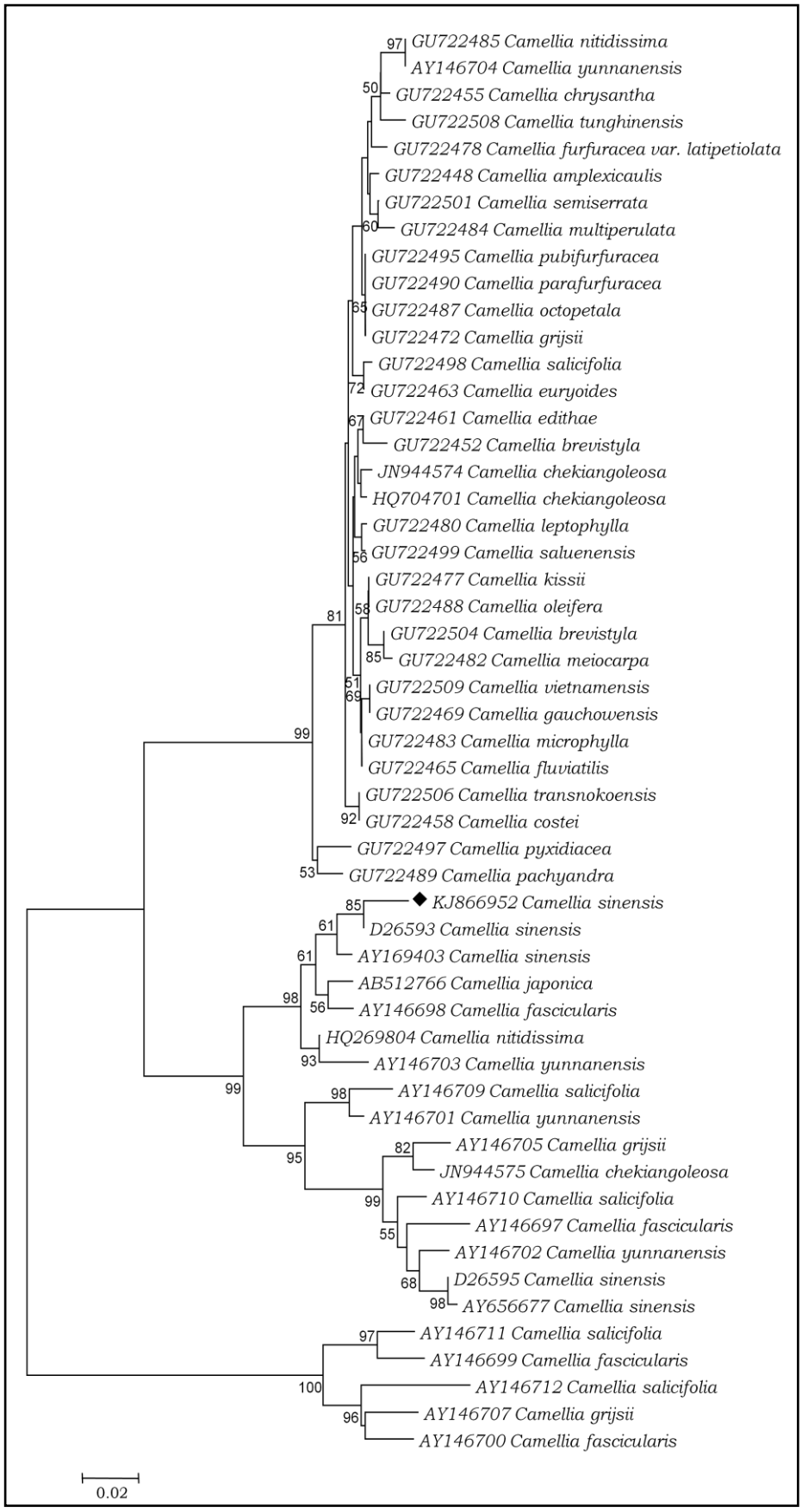

Fig. 4.9: Phylogenetic relationship of present isolated CHS gene with the other CHS gene sequences of *Camellia* sp. publish in the GenBank using the neighbor joining method. Numbers at the nodes indicate the bootstrap percentage scores out of 1000 replicates.

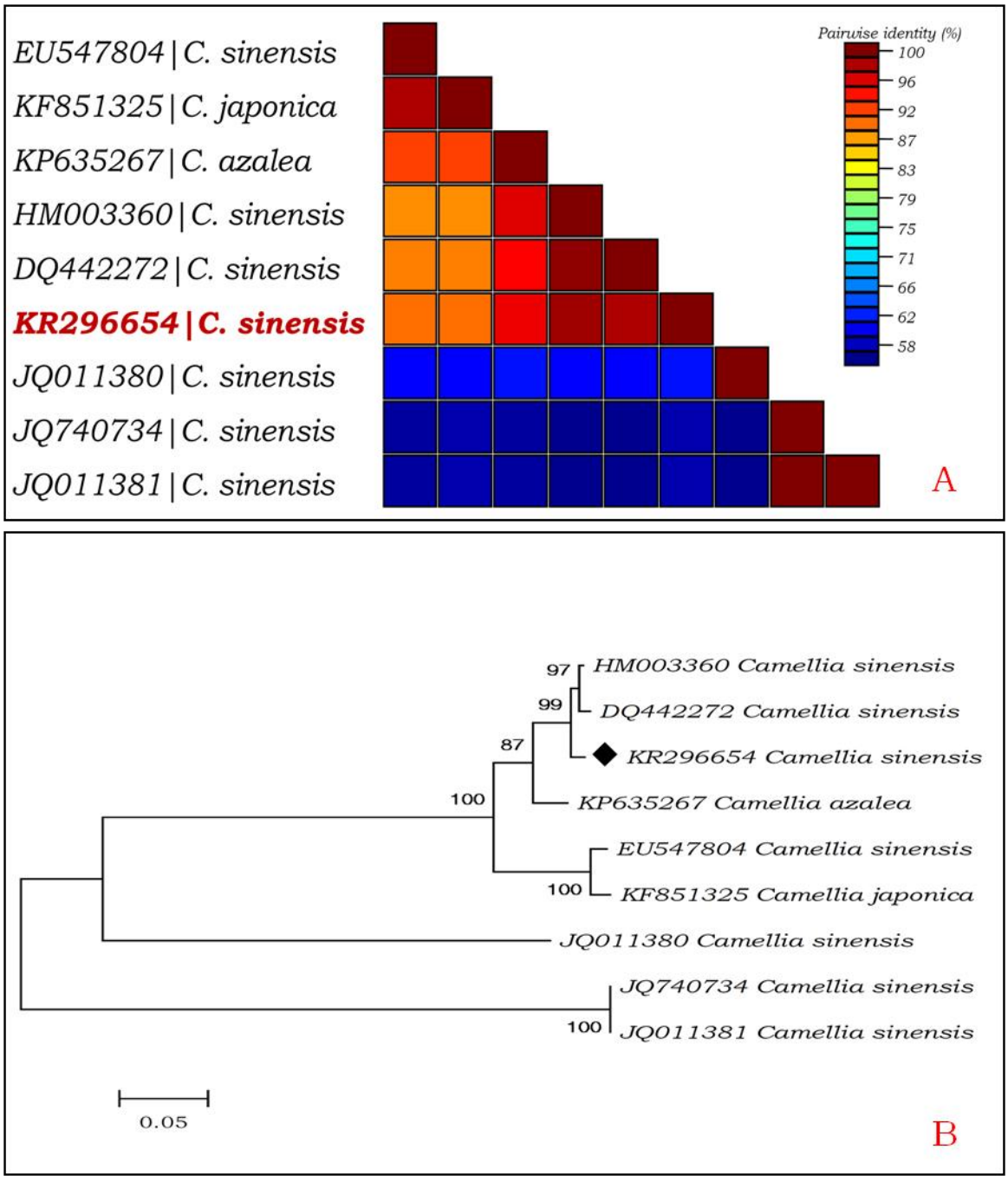

Fig. 4.10: A) Sequence identity matrix; B) Phylogenetic tree of present isolated APX gens with other APX gene sequences of *Camellia* sp. Identity percentage corresponding to the colour matrix is indicated in the right-hand side of the figure.

4.5.2. Codon bias of defense related genes

4.5.2.1. Codon usage pattern

The neucletide composition at 3rd position (A3, T3, C3 and G3), average GC content, GC content at 1st, 2nd and 3rd position of different synonymous codon, Nc, Fop, Gravy, Aroma of the three defense genes have been shown in Table 4.18.

From the results (Table 4.17) it has been found that among the three genes, highest average GC percent (0.529) was found in CHS gene whereas PAL and APX gene contained average GC percentage (0.489 and 0.487 respectively). The highest GC content at first synonymous codon position was observed in CHS gene (0.640) followed by PAL gene (0.571) and APX gene (0.464). Similar type of observation was also found in case of average GC3 contents. The average GC3 contents in CHS, PAL and APX genes were 0.532, 0.447 and 0.482 respectively. However, no significant variation in GC2 content has been found among the three genes. The GC2 content in APX, PAL and CHS are 0.479, 0.432 and 0.402 respectively.

The overall pyrimidine content at 3rd codon position (T3 and C3) was higher than the overall purine content at 3rd codon position (A3 and G3) in all the three genes (Table 4.17). However, the highest T3 and C3 contents (0.36 and 0.38) have been found in CHS followed by PAL and APX. PAL showed highest A3 content (0.34) whereas APX showed highest G3 content (0.286). So, the nucleotide composition at third position showed that T3, C3 and A3 contents were higher than the G3 contents in PAL and APX. In case of CHS, T3 and C3 contents higher than the A3 and G3 contents. In case of Nc value, it was found that CHS showed maximum Nc value (56.014) followed by Nc values of APX (52.980) and PAL (51.393).

To consider the pattern of codon usage variation among the three genes, an Nc plot were prepared. The variation of GC3 content determined the codon usage pattern of a gene and the Nc plot showed probable position of that gene. The Nc plots of three defense related genes (APX, CHS and PAL) have been presented in Fig. 4.11A. The results showed that the positions of most of the genes are below the standard curve which

indicated that mutational bias along with translational selection may influence the codon usage bias of these three genes.

Table 4.17: Nucleotide and amino acid composition of the three defense related genes

	APX	CHS	PAL
T3s	0.317±0.031	0.360±0.002	0.325±0.003
C3s	0.306±0.016	0.380±0.002	0.375±0.002
A3s	0.311±0.019	0.196±0.002	0.340±0.001
G3s	0.286±0.027	0.270±0.001	0.175±0.002
CBI	0.020±0.009	-0.059±0.002	-0.004±0.004
Fop	0.423±0.009	0.363±0.001	0.409±0.002
Nc	52.980±1.275	56.041±0.159	51.393±0.274
GC1s	0.464±0.033	0.640±0.002	0.571±0.003
GC2s	0.479±0.024	0.402±0.001	0.432±0.005
GC3s	0.482±0.021	0.532±0.002	0.447±0.003
GC	0.487±0.009	0.529±0.001	0.489±0.001
L_sym	321.444±29.363	188.839±0.811	207.536±1.186
L_aa	336.889±31.079	194.118±0.860	212.836±1.166
Gravy	-0.241±0.081	0.126±0.005	-0.143±0.024
Aromo	0.090±0.008	0.056±0.000	0.069±0.002

Values after '±' represent the standard error

4.5.2.2. Analysis of neutral evolution

Neutrality plot analysis was carried out to understand the major factor (mutational bias or natural selection) responsible for shaping codon usage bias; where the GC content in different codon position played a major role in evaluating the selection process. Slope of regression closer to 1 indicated strong effect of mutational pressure on synonymous codon usage bias. From the Fig. 4.11B it is evident that the slope of regression line of APX, CHS and PAL were 0.069, 0.194 and 0.478 indicating that contribution of mutational pressure on APX, CHS and PAL synonymous codon usage were 6.9%, 19.4% and 47.8%. This data indicated the strong involvement of natural selection on these three genes where natural selection pressure was calculated much higher in APX (93.1%) and CHS (80.6%) gene than PAL (52.2%) gene. Significant correlation (R^2 close to 1) between GC12 and GC3 implied effect of similar mutational pressure on each codon position i.e., GC1, GC2 and GC3. CHS and APX genes showed lower correlation (R^2=0.342 and 0.013 respectively) between GC12 and GC3. Whereas, strong correlation was found in PAL gene (R^2=0.71). This indicated equal forces of mutational pressure on each codon position worked on PAL gene, but in case of CHS and APX different forces of mutational pressure acted on three different codon positions.

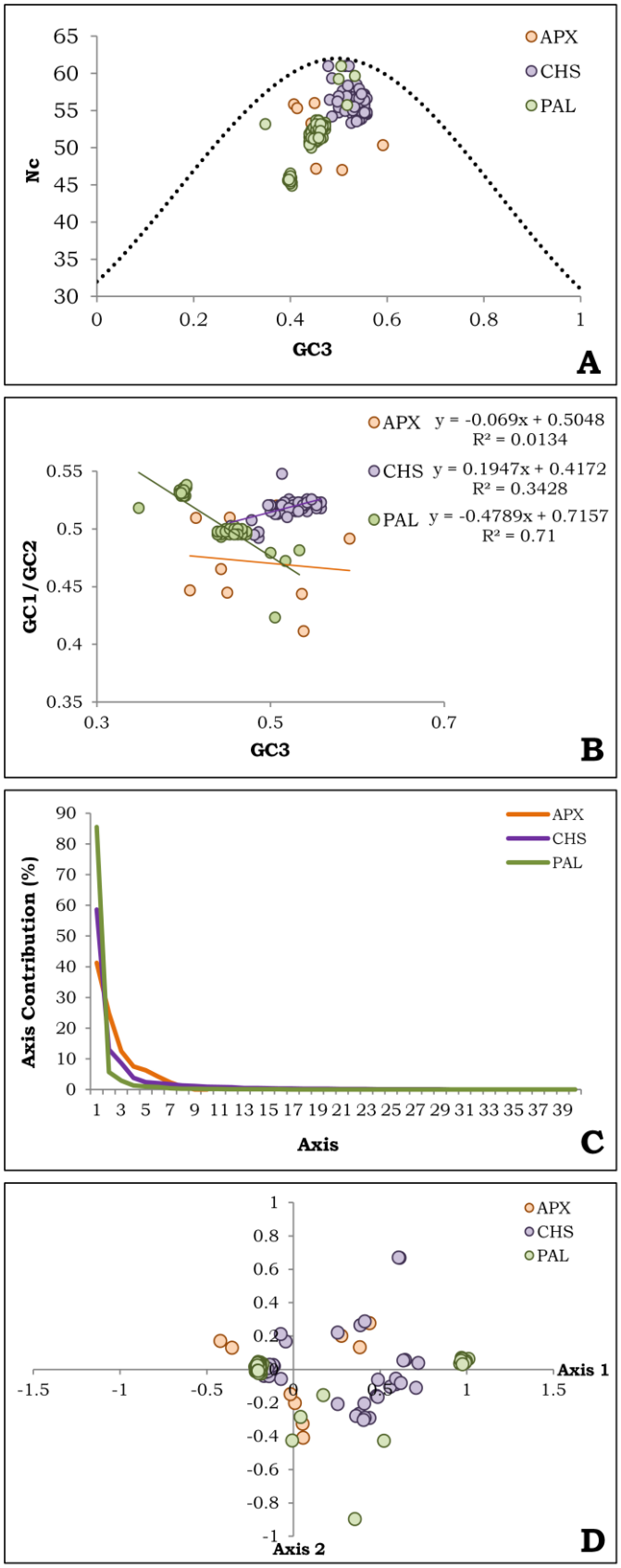

Fig. 4.11: (A) Relationship between effective number of codons (Nc) and GC3 contents; (B) Neutrality plot analysis of GC1/GC2 contents and GC3 contents; (C) Contribution of two major axis (Axis1 and Axis2); (D) Correspondence analysis of codon usage pattern.

4.5.2.3. Correlation of nucleotide and amino acid composition

To determine the effect of different factors in codon usage bias of a gene, the COA was performed on the basis of relative synonymous codon usage (RSCU) values. Through COA analysis different sets of indices [nucleotide composition at the third position of the synonymous codons (i.e., A3, T3, G3, C3, GC1, GC2 and GC3); overall frequency of GC content (%GC); length of synonymous codon (L_sym); length of amino acids (L_aa); average of hydropathy (GRAVY); frequency of aromatic amino acids (Aromo); codon bias index (CBI) and frequency of optimal codon (Fop)] were compared with the effective number of codons (Nc).

From the correlation analysis of APX gene (Table 4.18 & Fig. 4.12) it was found that, the Nc was positively correlated with A3 ($r=0.785$, $p<0.01$), L_aa ($r=0.782$, $p<0.01$), Axis1 ($r=0.695$, $p<0.05$) and negatively correlated with GC1 ($r=-0.835$, $p<0.01$). In case of CHS gene (Table 4.19 & Fig. 4.13), Nc was positively correlated with A3 ($r=0.443$, $p<0.01$), GC2 ($r=0.276$, $p<0.01$), Gravy ($r=0.306$, $p<0.01$) and negatively correlated with L_sym ($r=-0.347$, $p<0.01$), L_aa ($r=-0.336$, $p<0.01$), Axis4 ($r=-0.313$, $p<0.01$). But in case of PAL gene (Table 4.20 & Fig. 4.14, 4.15), all the factors were either positively or negatively correlated with Nc. Here, C3 ($r=0.675$, $p<0.01$), G3 ($r=0.866$, $p<0.01$), CBI ($r=0.529$, $p<0.01$), Fop ($r=0.437$, $p<0.01$), GC1 ($r=0.600$, $p<0.01$), GC3 ($r=0.861$, $p<0.01$), L_sym ($r=0.286$, $p<0.01$), L_aa ($r=0.235$, $p<0.05$) and Gravy ($r=0.780$, $p<0.01$) were positively correlated. Whereas, T3 ($r=-0.862$, $p<0.01$), A3 ($r=-0.557$, $p<0.01$), GC2 ($r=-0.855$, $p<0.01$), GC ($r=+0.645$, $p<0.01$), Aromo ($r=-0.804$, $p<0.01$) and Axis1 ($r=-0.749$, $p<0.01$) were negatively correlated. However, A3 and L_aa were either positively or negatively correlated with Nc in all the three genes. This indicated that these two factors affected the overall codon usage in tea irrespective of different genes, whereas other factors played specific roles in shaping codon bias in three different genes.

Table 4.18: Correlation coefficients of nucleotide constraints along four axes with indices of APX genes that influence codon usage bias

	T3s	C3s	A3s	G3s	CBI	Fop	Nc	GC1s	GC2s	GC3s	GC	L_sym	L_aa	Gravy	Aromo	Axis1	Axis2	Axis3
C3s	.329																	
A3s	-.262	.169																
G3s	-.549	-.675*	-.574															
CBI	.177	.387	-.063	-.109														
Fop	.639*	.715*	.169	-.706*	.740*													
Nc	-.371	-.382	**.785****	-.109	-.200	-.177												
GC1s	.742*	.210	-.805**	-.011	.159	.302	**-.835****											
GC2s	-.607	.078	.632*	-.183	-.545	-.418	.388	-.684*										
GC3s	-.727*	-.293	-.456	.865**	-.028	-.615	-.258	-.104	.098									
GC	-.152	.118	-.616	.359	-.315	-.406	-.729*	.444	.177	.578								
L_sym	-.063	.254	.900**	-.582	.206	.419	.764*	-.669*	.280	-.536	-.819**							
L_aa	-.101	.232	.905**	-.553	.202	.391	**.782****	-.698*	.297	-.506	-.815**	.999**						
Gravy	-.676*	-.757*	-.107	.806**	-.191	-.709*	.320	-.443	.041	.641*	-.080	-.142	-.112					
Aromo	.495	.649*	.527	-.822**	.486	.865**	.213	-.069	-.124	-.747*	-.636*	.737*	.720*	-.601				
Axis1	-.890**	-.510	.520	.379	-.231	-.584	**.695***	-.899**	.599	.407	-.229	.327	.361	.711*	-.334			
Axis2	-.254	-.488	-.837**	.882**	-.144	-.603	-.514	.382	-.294	.785**	.644*	-.870**	-.854**	.543	-.849**	.000		
Axis3	-.321	.543	-.061	.073	.390	.201	-.266	-.034	.118	.446	.340	-.014	-.008	-.179	.069	.000	.000	
Axis4	-.189	.420	.145	-.174	-.066	-.014	-.211	-.139	.431	.125	.306	.024	.035	-.122	.080	.000	.000	.000

**Correlation is significant at the 0.01 level

*Correlation is significant at the 0.05 level

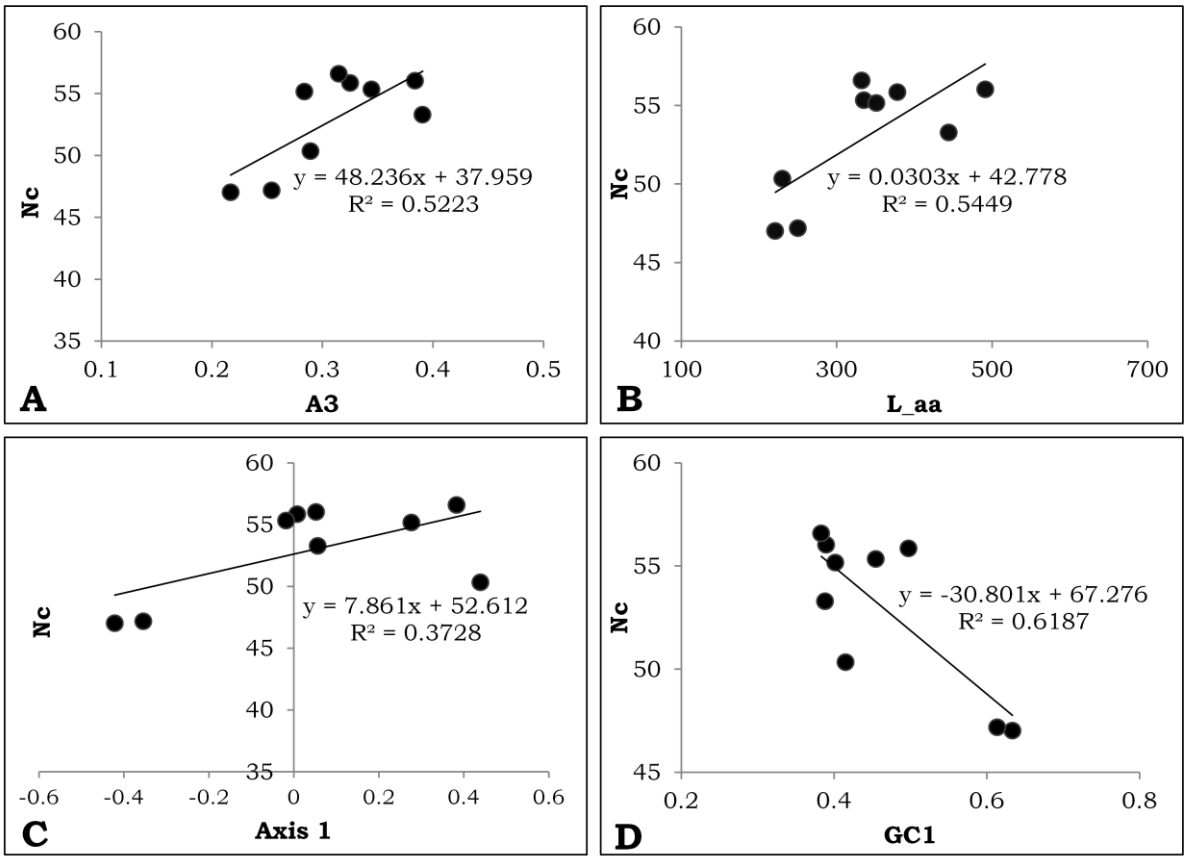

Fig. 4.12: Correlation between effective number of codons (Nc) of APX gene with A) adenine at 3rd codon position (A3), B) length of amino acid (L_aa), C) Axis1 and D) GC content at 1st codon position (GC1).

Table 4.19: Correlation coefficients of nucleotide constraints along four axes with indices of CHS genes that influence codon usage bias

	T3s	C3s	A3s	G3s	CBI	Fop	Nc	GC1s	GC2s	GC3s	GC	L_sym	L_aa	Gravy	Aromo	Axis1	Axis2	Axis3
C3s	-.324**																	
A3s	-.380**	-.644**																
G3s	-.599**	.285**	-.185															
CBI	-.359**	-.016	.344**	-.051														
Fop	-.334**	-.077	.394**	-.087	.989**													
Nc	-.187	-.164	**.443****	-.210*	.211*	.245*												
GC1s	-.161	.792**	-.552**	.154	-.192	-.254*	-.113											
GC2s	-.197	-.544**	.657**	-.173	.410**	.464**	**.276****	-.515**										
GC3s	-.558**	.880**	-.553**	.700**	-.001	-.067	-.222*	.657**	-.444**									
GC	-.474**	.910**	-.574**	.537**	-.021	-.088	-.184	.844**	-.405**	.945**								
L_sym	.108	.067	-.308**	.353**	-.290**	-.347**	**-.347****	-.143	-.384**	.194	.028							
L_aa	.168	-.034	-.261*	.301**	-.258*	-.305**	**-.336****	-.266**	-.329**	.095	-.087	.990**						
Gravy	.207*	-.607**	.449**	-.353**	.253*	.331**	**.306****	-.658**	.360**	-.613**	-.677**	-.462**	-.344**					
Aromo	.461**	-.369**	-.041	-.168	.005	.039	-.214*	-.594**	-.029	-.371**	-.508**	.504**	.596**	.258*				
Axis1	-.300**	-.712**	.866**	-.053	.363**	.389**	.295**	-.740**	.646**	-.528**	-.635**	-.162	-.089	.560**	.081			
Axis2	.761**	-.385**	-.232*	-.404**	-.123	-.089	-.060	-.468**	-.108	-.494**	-.556**	.053	.155	.549**	.526**	.001		
Axis3	.015	.135	-.244*	.141	-.279**	-.382**	-.194	.252*	-.258*	.183	.199	.165	.123	-.259*	-.353**	-.002	-.002	
Axis4	-.158	-.039	-.004	.333**	.097	.127	**-.313****	.006	.044	.137	.106	-.235*	-.227*	.169	-.113	.000	-.002	.000

**Correlation is significant at the 0.01 level

*Correlation is significant at the 0.05 level

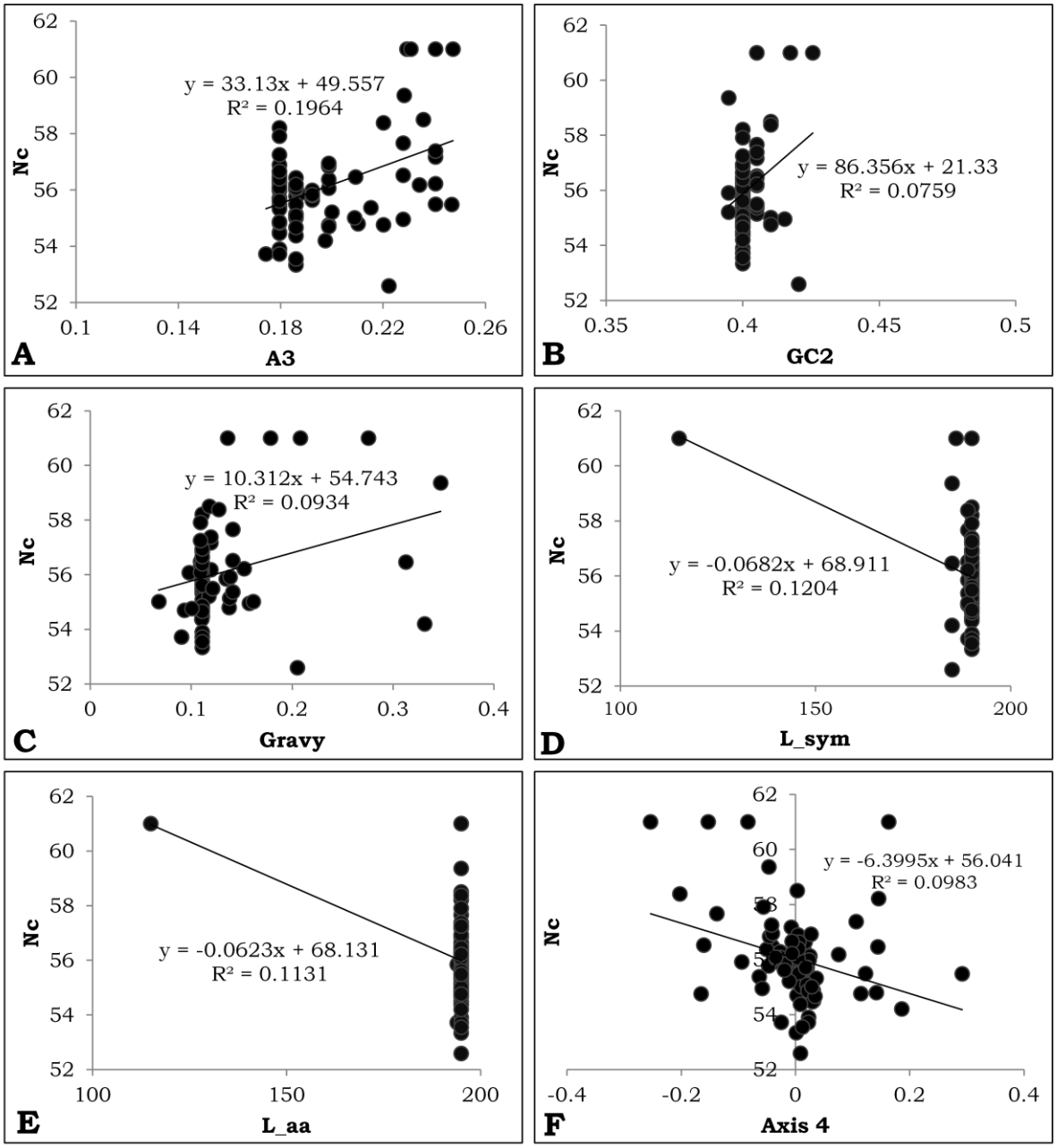

Fig. 4.13: Correlation between effective number of codons (Nc) of CHS gene with A) adenine at 3rd codon position (A3), B) GC content at 2nd codon position (GC2), C) hydropathicity (Gravy), D) length of synonymous codons (L_sym), E) length of amino acid (L_aa), and F) Axis4.

Table 4.20: Correlation coefficients of nucleotide constraints along four axes with indices of PAL genes that influence codon usage bias

	T3s	C3s	A3s	G3s	CBI	Fop	Nc	GC1s	GC2s	GC3s	GC	L_sym	L_aa	Gravy	Aromo	Axis1	Axis2	Axis3
C3s	-.936**																	
A3s	.616**	-.653**																
G3s	-.680**	.465**	-.706**															
CBI	-.793**	.869**	-.383**	.205*														
Fop	-.718**	.832**	-.379**	.123	.988**													
Nc	**-.862****	**.675****	**-.557****	**.866****	**.529****	**.437****												
GC1s	-.821**	.825**	-.236*	.235*	.899**	.832**	**.600****											
GC2s	.944**	-.832**	.454**	-.630**	-.778**	-.680**	**-.855****	-.878**										
GC3s	-.941**	.885**	-.830**	.817**	.652**	.593**	**.861****	.620**	-.831**									
GC	.531**	-.281**	.026	-.525**	-.305**	-.182	**-.645****	-.496**	.744**	-.399**								
L_sym	-.566**	.672**	-.042	-.106	.844**	.828**	**.286****	.847**	-.568**	.352**	-.124							
L_aa	-.520**	.640**	-.015	-.156	.821**	.812**	.235*	.814**	-.516**	.307**	-.072	.998**						
Gravy	-.939**	.871**	-.436**	.527**	.847**	.759**	**.780****	.925**	-.986**	.804**	-.678**	.665**	.618**					
Aromo	.930**	-.840**	.422**	-.561**	-.812**	-.719**	**-.804****	-.915**	.990**	-.799**	.708**	-.621**	-.571**	-.988**				
Axis1	.901**	-.830**	.335**	-.457**	-.856**	-.768**	**-.749****	-.950**	.973**	-.736**	.691**	-.701**	-.656**	-.987**	.982**			
Axis2	.173	.110	.282**	-.787**	.295**	.359**	-.550**	.263**	.177	-.330**	.437**	.520**	.552**	-.036	.087	.000		
Axis3	.329**	-.448**	.713**	-.339**	-.180	-.210*	-.206*	-.020	.106	-.510**	-.317**	-.110	-.113	-.136	.085	.000	.000	
Axis4	-.029	.054	-.303**	.111	-.108	-.115	-.075	-.092	-.061	.117	-.167	-.459**	-.483**	.044	-.064	.000	.000	.000

**Correlation is significant at the 0.01 level

*Correlation is significant at the 0.05 level

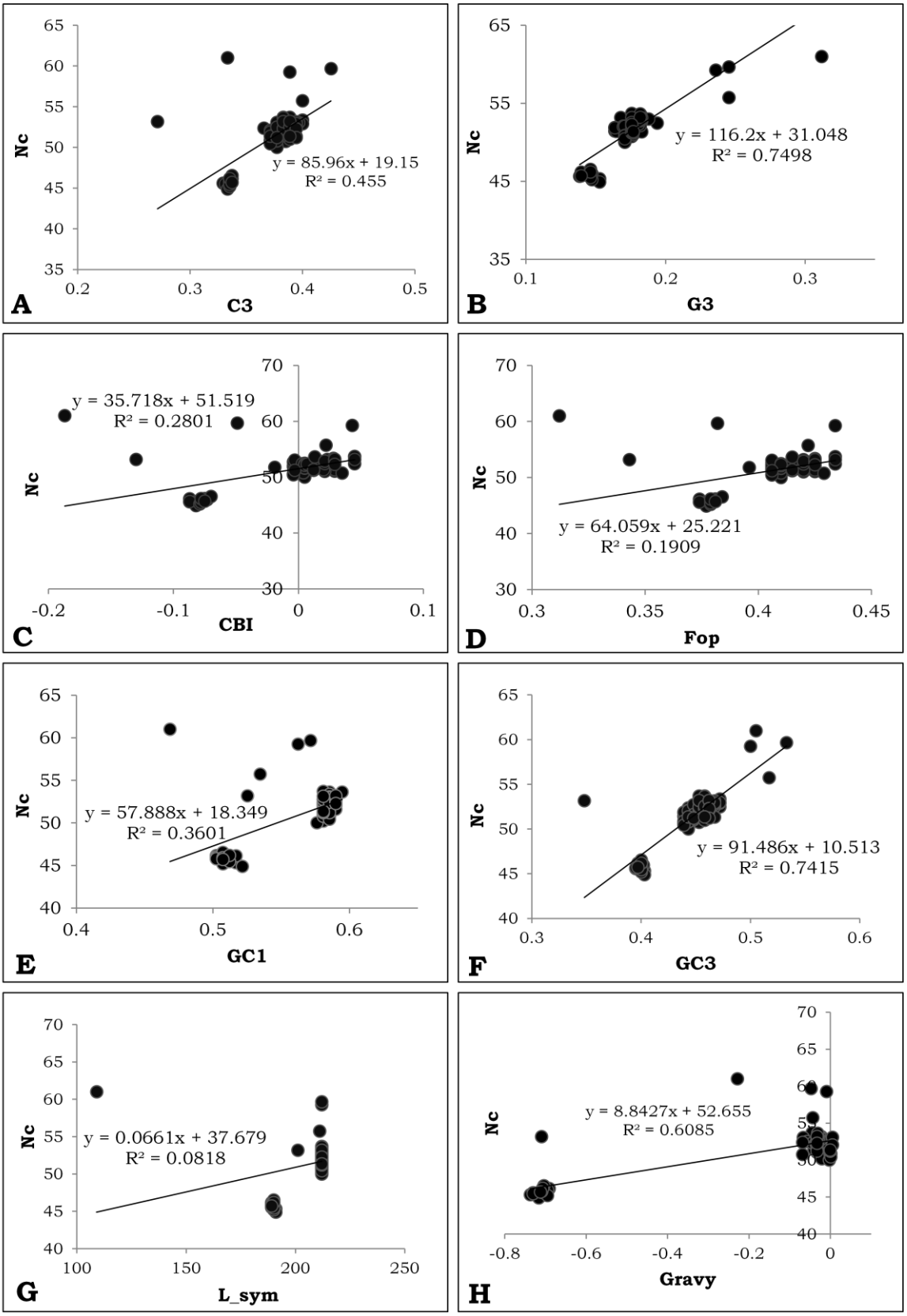

Fig. 4.14: Correlation between effective number of codons (Nc) of PAL gene with A-B) cytosine and guanine at 3rd codon position (C3 and G3), C) codon bias index (CBI), D) frequency of optimal codon (Fop), E-F) GC content at 1st and 3rd codon position (GC1 and GC3), G) length of synonymous codons (L_sym) and H) hydropathicity (Gravy).

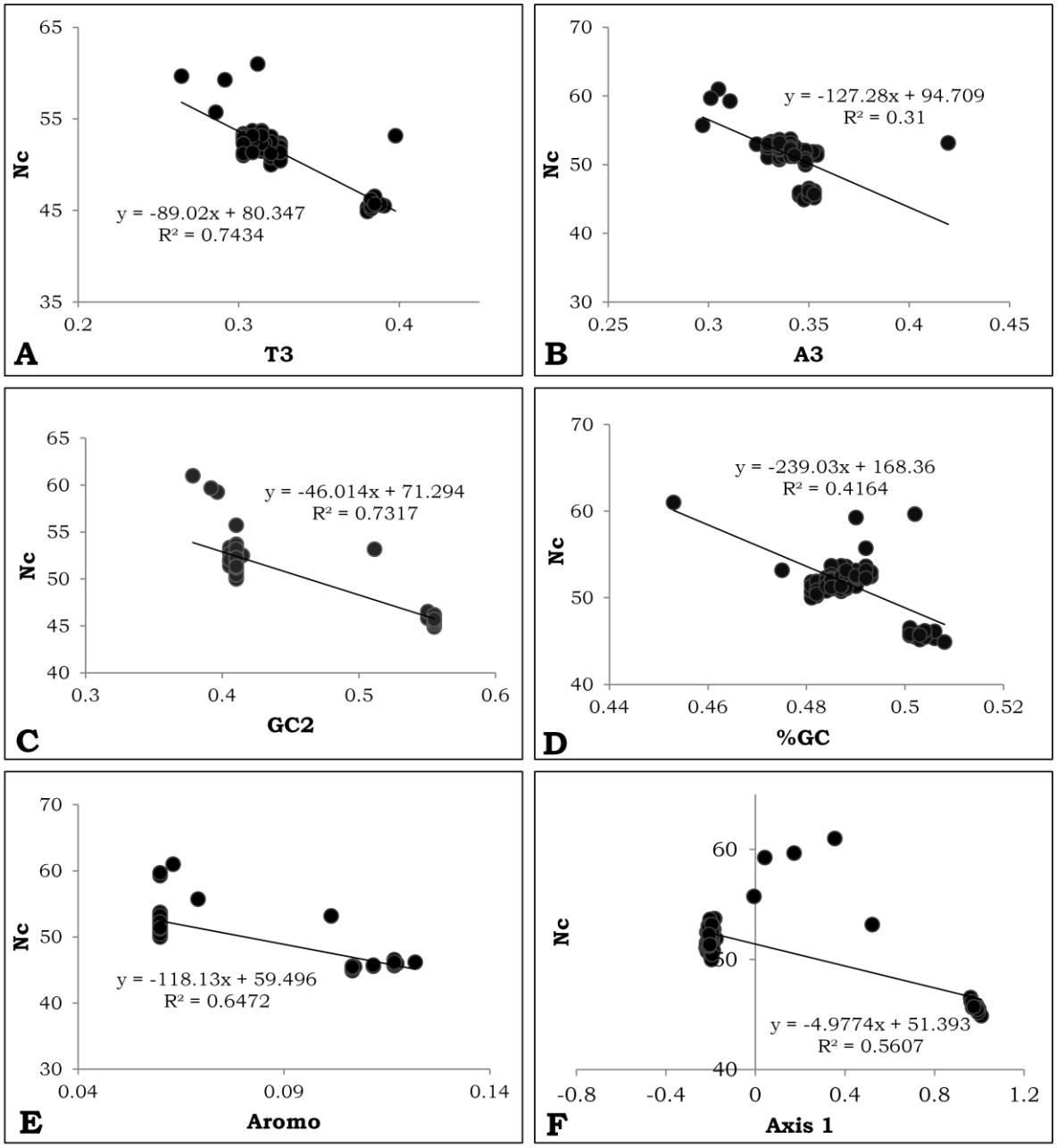

Fig. 4.15: Correlation between effective number of codons (Nc) of PAL gene with A-B) thymine and adenine at 3rd codon position (T3 and A3), C) GC content at 2nd codon position (GC2), D) percentage of GC (%GC), E) aromaticity (Aromo) and F) Axis1.

Another factor that played important role in codon usage bias was the contribution of axes. The results showed that (Fig. 4.11C) in APX and CHS gene, the first axis contributed for 41.26% and 58.61% respectively and the contribution of second axis for these two genes were 24.95% and 13.06% respectively, whereas, in case of PAL gene major contribution was observed by the first axis which contributed for 85.52%. Here the second axis accounted for only 5.69%. From the Fig. 4.11D it was evident that CHS and PAL genes were moderately clustered along Axis1, whereas, APX genes are scattered along both the axes i.e., Axis1 and Axis2. However, the clustering of PAL genes were more prominent than CHS, which was also reflected in the phylogenetic analysis where PAL genes formed three prominent clusters and CHS genes were diversified in several small clusters but no significant clusters were found in APX genes. This indicated that PAL genes showed less codon usage variation among themselves than that of CHS.

4.5.2.4. Codon usage bias in three different genes

On the basis of RSCU values the codon usage bias of 59 sense codons (except Met, Trp and three termination codon) of the three defense related genes (PAL, CHS and APX) were calculated (Table 4.21). From the table it was evident that APX genes did not showe any optimal codon, whereas, CHS and PAL genes showed 12 (UGU, UUC, UAC, UCG, CAU, CCC, CUA, CAA, CCG, AUA, AGA, GCU) and 17 (UCC, UUA, CCU, CUC, CAA, CCG, CGG, AAU, AGU, AUC, AGC, AUA, AAA, GUU, GAU, GCA, GGA) optimal codons respectively. Out of these optimal codons both CHS and PAL genes showed more A/U ending codons than G/C ending codons. Optimal codons of CHS genes showed 7 A/U ending and 5 G/C ending codons respectively, whereas, PAL genes showed and 11 A/U ending and 6 G/C ending optimal codons respectively. From the RSCU of the 59 sense codons APX contained 24 frequently used codons (RSCU>1) out of which 9 were more frequently used (RSCU>1.5). But, CHS and PAL contained 31 and 32 frequently used codons (RSCU>1) out of which 10 and 11 were more frequently used (RSCU>1.5) respectively. This indicated PAL contained more numbers of codons with strong bias followed by CHS and APX (Table 4.21).

Table 4.21: Relative synonymous codon usage of APX, CHS and PAL genes

Amino acid	Codon	APX		CHS		PAL	
		Frequency	RSCU	Frequency	RSCU	Frequency	RSCU
Phe	UUU	7.20	1.14	3.60	1.02	7.70	1.13
	UUC	5.40	0.86	**3.50**	**0.98**	5.90	0.87
Leu	UUA	4.10	0.86	0.80	0.16	**2.00**	**1.05**
	UUG	4.10	0.86	6.00	1.25	1.00	0.54
	CUU	6.00	1.26	7.80	1.62	0.30	0.14
	CUC	7.70	1.60	8.50	1.77	**5.70**	**3.04**
	CUA	3.10	0.65	**5.40**	**1.12**	1.50	0.79
	CUG	3.70	0.77	0.40	0.07	0.80	0.44
Ile	AUU	6.00	1.34	4.80	1.33	5.40	1.52
	AUC	4.10	0.92	3.50	0.96	**5.10**	**1.43**
	AUA	3.30	0.74	**2.60**	**0.71**	**0.20**	**0.05**
Met	AUG	4.10	1.00	3.20	1.00	1.00	1.00
Val	GUU	5.70	1.91	4.20	1.20	**3.90**	**1.91**
	GUC	1.30	0.45	4.00	1.12	3.40	1.67
	GUA	2.30	0.79	0.40	0.12	0.80	0.41
	GUG	2.60	0.86	5.50	1.56	0.00	0.01
Ser	UCU	11.60	2.02	1.50	0.87	4.50	1.64
	UCC	9.00	1.57	1.90	1.07	**3.00**	**1.11**
	UCA	4.10	0.72	2.10	1.20	0.20	0.09
	UCG	2.40	0.43	**0.80**	**0.47**	0.80	0.30
	AGU	2.80	0.49	2.40	1.35	**3.90**	**1.43**
	AGC	4.40	0.78	1.80	1.04	**3.90**	**1.42**
Pro	CCU	5.90	1.39	4.20	1.46	**2.30**	**0.68**
	CCC	3.30	0.78	**4.10**	**1.43**	3.10	0.92
	CCA	4.90	1.15	2.00	0.69	6.00	1.80
	CCG	2.90	0.68	**1.20**	**0.42**	**2.00**	**0.60**
Thr	ACU	6.90	1.89	1.90	0.94	4.70	1.22
	ACC	2.40	0.67	3.60	1.81	5.20	1.35
	ACA	4.10	1.13	1.50	0.73	5.60	1.43
	ACG	1.10	0.31	1.10	0.53	0.00	0.00
Ala	GCU	6.20	1.61	**5.30**	**1.15**	2.20	0.91
	GCC	3.60	0.92	8.80	1.91	4.30	1.77
	GCA	4.10	1.06	3.00	0.66	**3.20**	**1.31**
	GCG	1.60	0.40	1.30	0.29	0.00	0.01
Tyr	UAU	3.80	1.01	0.10	0.12	3.10	1.21
	UAC	3.70	0.99	**1.80**	**1.88**	2.00	0.79
TER	UAA	6.00	0.72	0.00	0.00	1.00	0.26
	UAG	2.00	0.24	0.00	0.00	1.20	0.30
	UGA	16.90	2.04	0.00	0.00	9.70	2.44
His	CAU	5.40	1.15	**3.60**	**1.44**	3.10	0.90
	CAC	4.00	0.85	1.40	0.56	3.80	1.10
Gln	CAA	13.30	1.45	**5.60**	**1.61**	**8.70**	**1.79**
	CAG	5.00	0.55	1.40	0.39	1.00	0.21
Asn	AAU	4.40	1.36	1.90	0.68	**3.30**	**1.00**
	AAC	2.10	0.64	3.70	1.32	3.30	1.00
Lys	AAA	9.60	1.38	2.20	0.42	**6.90**	**1.62**
	AAG	4.30	0.62	8.50	1.58	1.60	0.38
Asp	GAU	3.90	0.88	5.90	1.01	**3.80**	**1.02**
	GAC	5.00	1.13	5.80	0.99	3.60	0.98
Glu	GAA	9.80	1.43	3.40	0.68	4.00	1.86
	GAG	3.90	0.57	6.70	1.32	0.30	0.14
Cys	UGU	9.90	0.82	**0.80**	**0.81**	4.10	0.92
	UGC	14.10	1.18	1.20	1.19	4.90	1.08
Trp	UGG	11.90	1.00	2.00	1.00	4.10	1.00
Arg	CGU	1.70	0.34	2.80	2.13	1.50	0.39
	CGC	3.70	0.74	1.90	1.40	5.70	1.44
	CGA	4.30	0.88	0.10	0.10	3.00	0.75
	CGG	3.10	0.63	1.90	1.43	**4.10**	**1.03**
	AGA	8.90	1.80	**0.80**	**0.57**	4.50	1.13
	AGG	7.90	1.60	0.50	0.37	5.00	1.26
Gly	GGU	2.20	0.34	7.00	1.63	2.10	0.57
	GGC	4.70	0.72	5.80	1.34	6.00	1.67
	GGA	12.70	1.97	0.50	0.12	**4.40**	**1.23**
	GGG	6.20	0.97	4.00	0.92	1.90	0.52

Frequency and RSCU values of the optimal codons are marked as bold

4.6. Relative gene expression studies of PAL gene by quantitative real-time PCR (qRT-PCR)

Relative expression of PAL gene was analyzed using quantitative real-time PCR (qRT-PCR) to compare the effect of BTH in inducing resistance in tea against two different foliar fungal pathogens such as *Colletotrichum gloeospoiroides* and *Curvularia eragrostidis*. The relative expression of PAL gene was quantified in unit 'fold change' (i.e., how many fold increased or decreased) of transcript level in the 'treated', 'inoculated' and 'treated-inoculated' plants in comparison to control (Table 4.22). For expression analysis PAL gene specific primers were used where actin used as endogenous control (Fig. 4.16). The transcript accumulation (fold change) in tea leaves were noted and analyzed in three different cases such as plants inoculated with pathogens (*C. gloeospoiroides* and *C. eragrostidis*), plants treated with BTH and plants treated with BTH followed by challenge inoculation with pathogens (*C. gloeospoiroides* and *C. eragrostidis*). Results of transcript accumulation were observed after one day intervals up to 7 days.

From the results (Table 4.22 & Fig. 4.17) it was evident that in BTH treated plants the PAL gene expression was elevated (from approximately 1 fold to 4 folds) from 4 days up to 7 days post treatment. But, in case of *C. gloeospoiroides* inoculated plants the expression was down-regulated up to 7 days post inoculation except 4th and 6th days, where the plants showed increased transcript accumulation of about 1 fold and 2 folds respectively. However, in case of BTH-treated and *C. gloeospoiroides* inoculated plants initial increase in PAL gene expresasion was observed on the day of treatment and inoculation which was down-regulated upto 3rd day. On the 4th day approximately 1 fold transcript accumulation was observed in the treated-inoculated one which was again decreased on the next day. Nevertheless, from 6th day onward the PAL gene expression was elevated from about 2 to 6 folds.

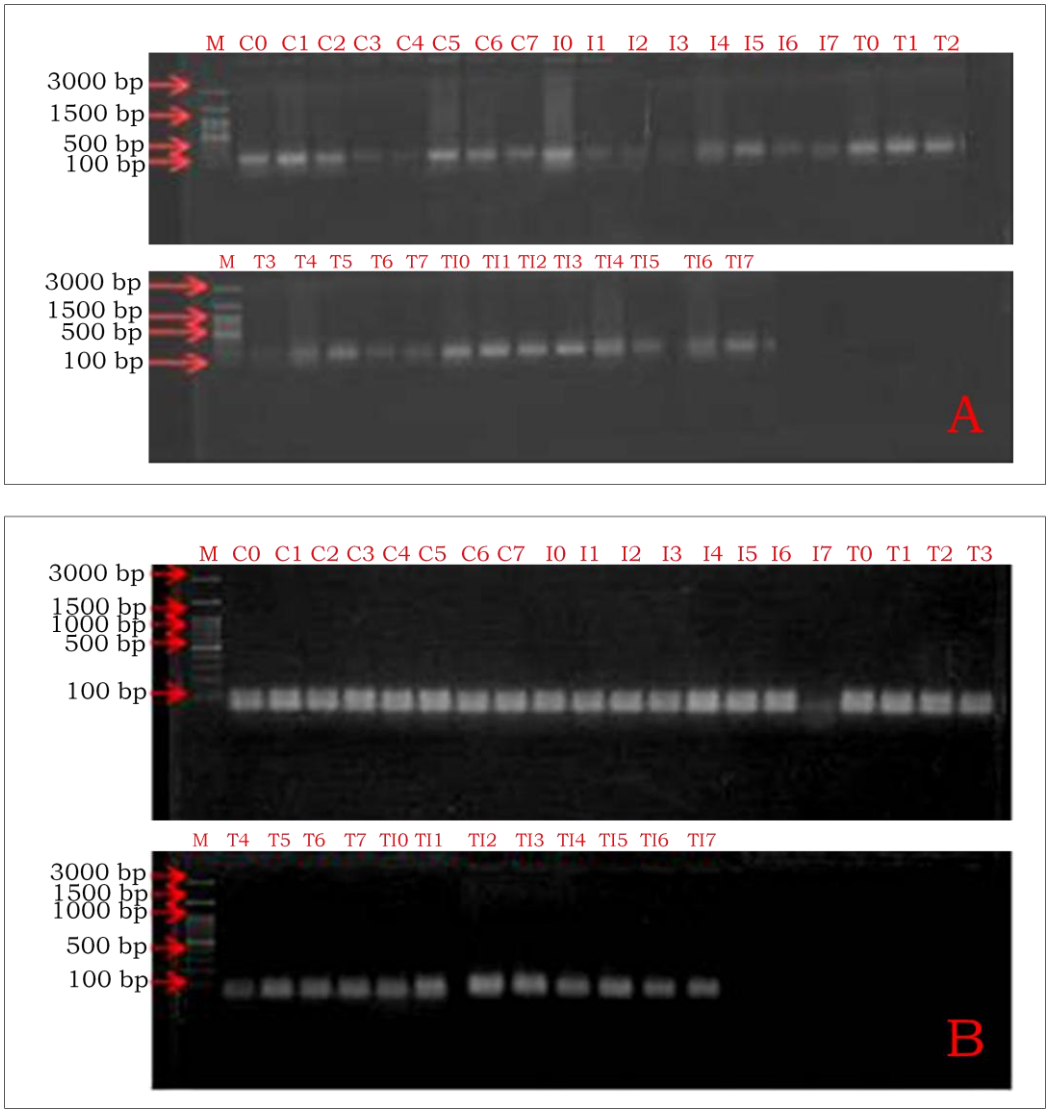

Fig. 4.16: Agarose gel electrophoresis of amplified RT-PCR product of A) PAL gene and B) plant actin gene [M: molecular marker, C0-C7: control after every one day intervals from 0 day to 7 days, I0-I7: pathogen inoculated after every one day intervals from 0 day to 7 days, T0-T7: BTH treated after every one day intervals from 0 day to 7 days, TI0-TI7: treated-inoculated after every one day intervals from 0 day to 7 days].

Table 4.22: PAL transcript accumulation (fold change) in BTH pre-treated tea plants following challenge inoculation with *C. gloeospoiroides* and *C. eragrostidis*

Days	T	*Cg*	T+Cg	*Ce*	T+*Ce*
0d	0.454398	0.937309	1.019716	0.336438	0.671845
1d	0.448165	0.993375	0.75867	11.93824	2.721812
2d	0.519373	0.833203	0.044279	0.041366	0.004905
3d	0.403849	0.700007	0.401645	1.693297	0.365938
4d	1.258599	1.324091	1.814871	0.583592	0.344213
5d	1.008413	0.69493	0.523037	0.004053	4.56797
6d	3.448775	2.189918	2.685562	0.091216	0.039395
7d	4.408507	0.384771	6.685562	0.134234	14.85545

T= BTH treated; ***Cg***= *Colletotrichum gloeospoiroides* inoculated; **T+*Cg*=** BTH treated and *Colletotrichum gloeospoiroides* inoculated; ***Ce***= *Curvularia eragrostidis* inoculated; **T+*Ce***= BTH treated and *Curvularia eragrostidis* inoculated

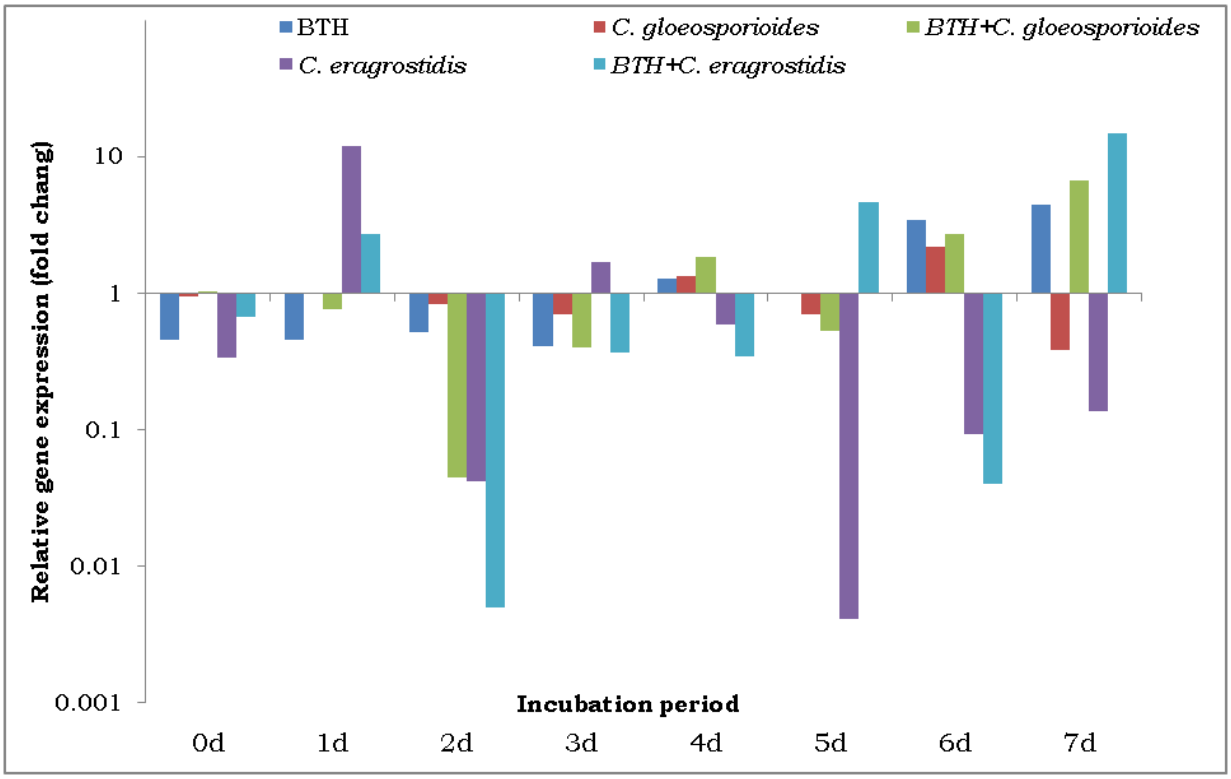

Fig. 4.17: Expression analysis of PAL transcripts levels in tea plants by Real-time PCR after BTH treated, inoculated (with *C. gloeosporioides* and *C. eragrostidis*) and BTH treated-inoculated.

C. eragrostidis inoculated plants showed slightly different PAL gene expression pattern than *C. gloeospoiroides* inoculated one. When the plants were inoculated with *C. eragrostidis* only, transcript level of PAL gene was increased on the 1st and 3rd days post inoculation. However, on the other days it remained down-regulated. Whereas, in case of BTH-treated and *C. eragrostidis* inoculated plants elevated PAL gene expression was observed as follows: 2 fold increase was observed on the 1st day, followed by 4 fold increase on the 5th day and finally 14 fold increase was observed on the 7th day.

4.7. Control of *Colletotrichum gloeosporio*ides by botanicals, and biocontrol agents.

4.7.1. Control of *Colletotrichum gloeosporio*ides by botanicals

Ten plants were collected from different parts of North Bengal University campus and adjoining areas of Siliguri in the district of Darjeeling to evaluate antifungal activity of the plant extracts against *Colletotrichum gloeosporio*ides. This was done with an objective to control brown blight disease caused by *C. gloeosporioides* by botanicals available around the tea gardens of north Bengal, the present study area. Aqueous and 50% ethanolic extracts were prepared and tested for their antifungal properties *in vitro,* against *C. gloeosporioides*. From the results (Table 4.23; Fig. 4.18 & 4.19) it was found that 50% ethanolic extract of *Datura metel* and *Clerodendrum viscosum* could inhibit 72.12% and 71.52% radial mycelial growth respectively in comparison to control. Least growth inhibition was observed in aqueous and 50% ethanolic leaf extracts of *Lantana camara*. Both aqueous and 50% ethanolic leaf extracts of *Leucas indica, Richinus communis* and *Lagestroemia speciosa* showed moderate mycelia-growth inhibition in petriplates. Aqueous extracts of *Clerodendrum viscosum* and *Datura metel* and ethanolic extracts of *Casuarina equisetifolia,* and *Polyalthia longifolia* also showed moderate (about 50% to 70%) inhibition of mycelia growth against *C. gloeosporioides*.

Table 4.23: Aantifungal efficacy of plant extracts on the radial growth of *Colletrotrichum gloeosporioides* (following poisoned food technique)

Plant extracts	Leaf extracted in aqueous		Leaf extracted in 50% ethanol	
	Radial Growth of fungus (mm)	Growth Inhibition (%)	Radial Growth of fungus (mm)	Growth Inhibition (%)
Leucas indica	22.67 ±0.88	58.79 ±0.94	18.33 ±0.33	66.67 ±1.24
Ricinus communis	27.00 ±0.58	50.91 ±1.91	25.33 ±0.33	53.94 ±0.75
Lagerstroemia speciosa	26.33 ±0.33	52.12 ±0.77	24.33 ±0.67	55.76 ±0.42
Azadirachta indica	33.00 ±0.58	40.00 ±0.55	32.67 ±0.33	40.61 ±0.48
Clerodendrum viscosum	22.00 ±0.58	60.00 ±0.55	15.67 ±0.33	71.52 ±0.98
Casuarina equisetifolia	24.33 ±0.67	55.76 ±0.42	22.00 ±0.58	60.00 ±0.55
Polyalthia longifolia	27.00 ±0.58	50.91 ±1.91	26.33 ±0.33	52.12 ±1.50
Datura metel	19.00 ±0.58	65.45 ±1.21	15.33 ±0.33	72.12 ±0.56
Boerhavia diffusa	28.00 ±0.58	49.09 ±0.52	26.00 ±0.58	52.73 ±1.35
Control	55.00 ±1.00	-	55.00 ±1.00	-
CD (5%)	0.93	1.61	0.77	1.10

Data after ± Data after ± represent standard error values

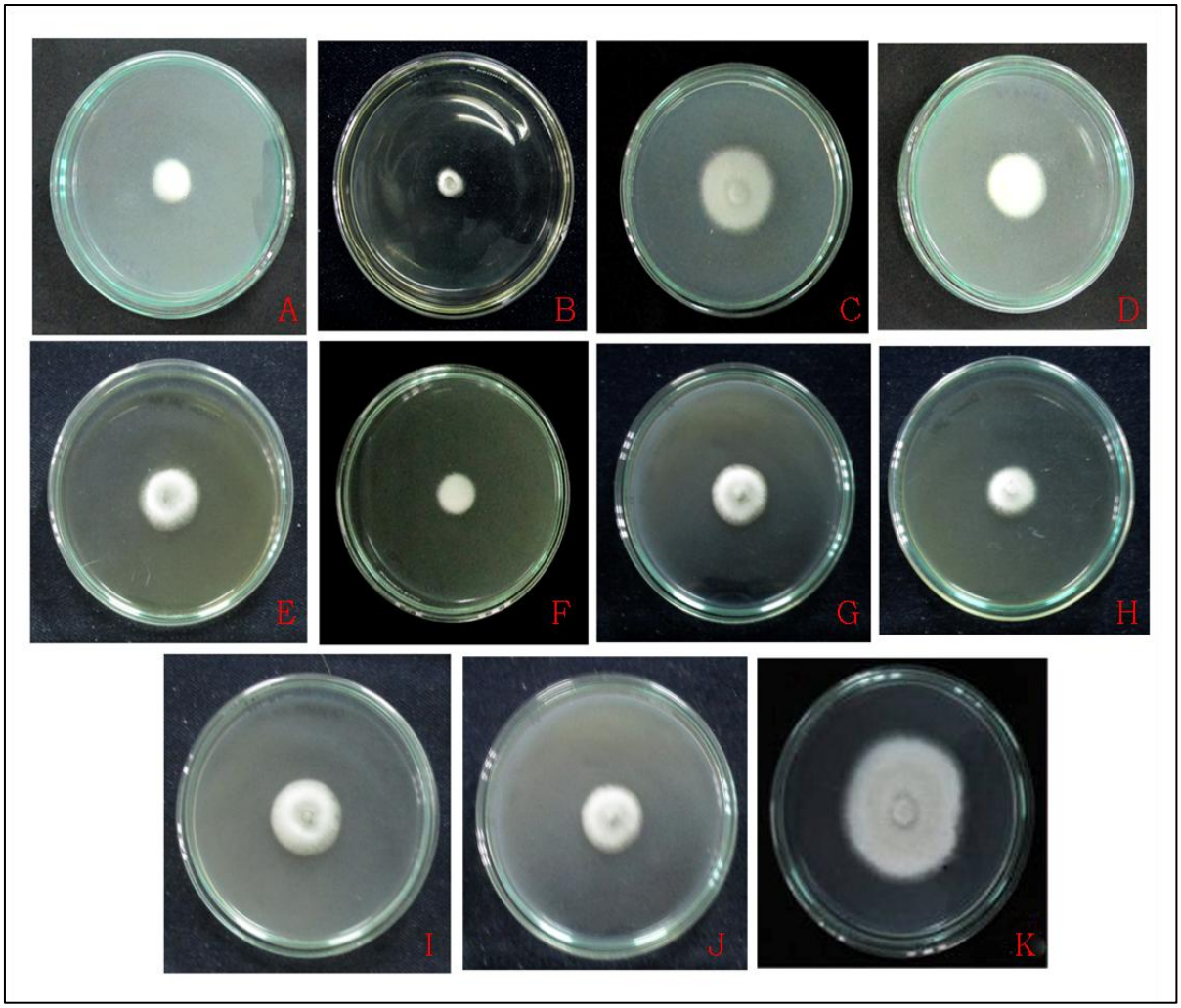

Fig. 4.18: Radial growth inhibition of *C. gloeosporioides* by aqueous solution of A) *Datura metel,* C) *Azadirachta indica,* E) *Leucas indica,* G) *Boerhavia diffusa,* I) *Lagerstroemia speciosa;* 50% ethanolic solution of B) *Datura metel,* D) *Azadirachta indica,* F) *Leucas indica,* H) *Boerhavia diffusa,* J) *Lagerstroemia speciosa* and K) Radial growth of *C. gloeosporioides* (grown in PDA not amended with leaf extract).

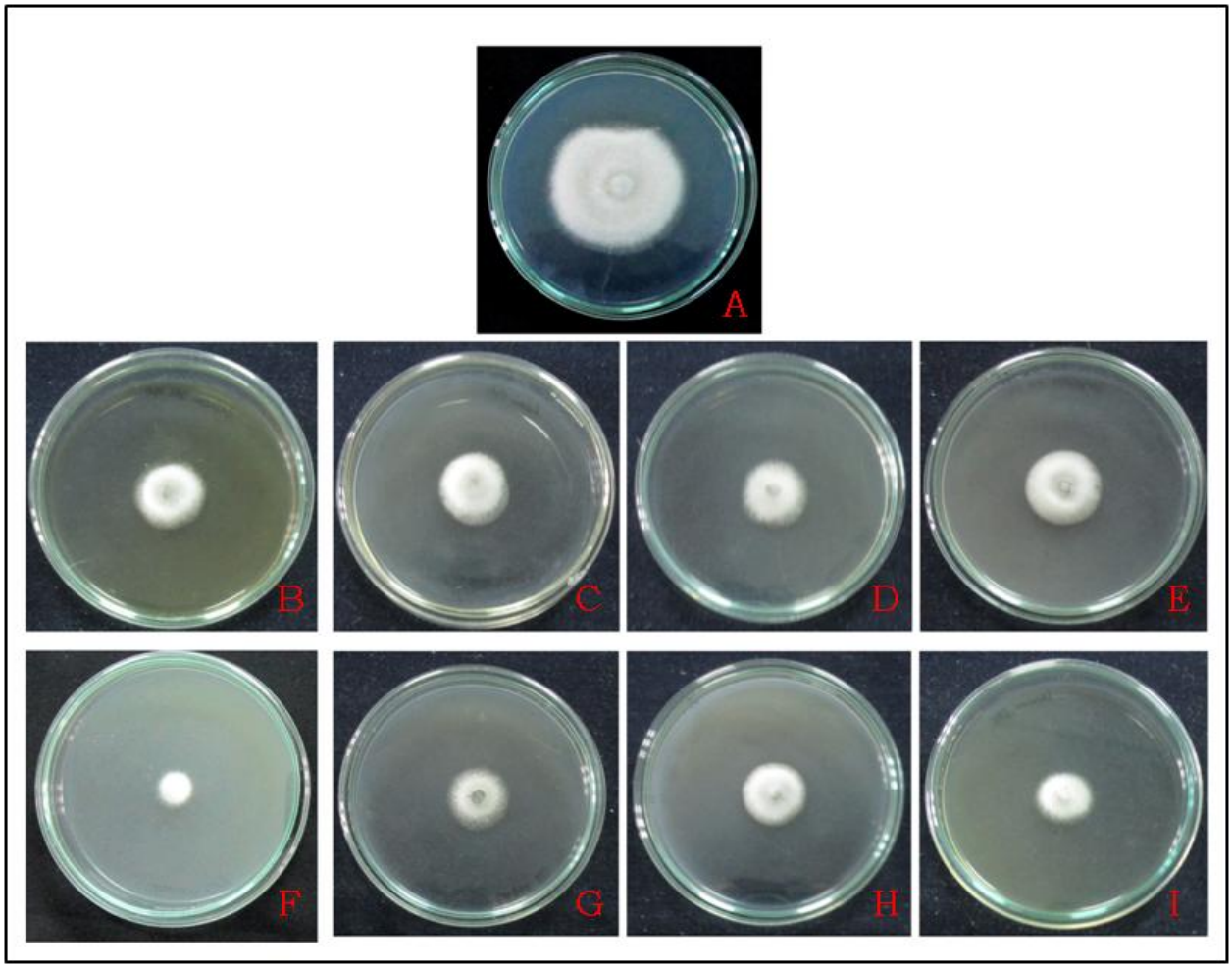

Fig. 4.19: Radial growth inhibition of *C. gloeosporioides* by aqueous solution of B) *Clerodendrum viscosum,* C) *Ricinus communis,* E) *Polyalthia longifolia, G*) *Casuarina equisetifolia;* 50% ethanolic solution of F) *Clerodendrum viscosum,* D) *Ricinus communis,* H) *Polyalthia longifolia, I*) *Casuarina equisetifolia,* and A) Radial growth of *C. gloeosporioides* (grown in PDA not amended with leaf extract).

4.7.2. Control of *Colletotrichum gloeosporio*ides by biocontrol agents

Four known biocontrol agents such as *Bacillus subtilis*, *B. pumilus*, *B. megaterium* and *Trichoderma harzianum* were tested to control *Colletotrichum gloeosporioides* one of the most virulent pathogen of tea plants. The antagonistic efficacy of the four antagonists against the fungus has been presented in the table 4.24 and in Fig. 4.20. From the results (Table 4.25) it was found that *Bacillus pumilus* showed maximum inhibitory effect against the fungus *C. gloeosporioides* followed by *Bacillus megaterium* and *Bacillus subtilis* respectively. *B. pumilus*, *B. megaterium* and *Bacillus subtilis* showed 78.17%, 63.61% and 61.79% inhibition of radial growth respectively in comparison to control. *Trichoderma harzianum* although showed least growth inhibition (i.e. about 59.97% inhibition of growth) in dualculture but within 4 days it showed over and fully covered the petriplate.

Table: 4.24: Control of *Colletotrichum gloeosporioides* by known biocontrol agents (*in-vitro*) after 4 days of growth.

Biocontrol agents	Radial Growth Of fungus (mm)	Growth Inhibition (%)
Bacillus pumilas	12.00±0.58	78.17±1.11
Bacillus megaterram	20.00±0.58	63.61±1.23
Bacillus subtilis	21.00±0.58	61.79±1.25
Trichoderma harzianum	22.00±0.58	59.97±1.27
Control	55.00±1.00	-
CD (5%)	2.00	2.99

Data after ± represent standard error values

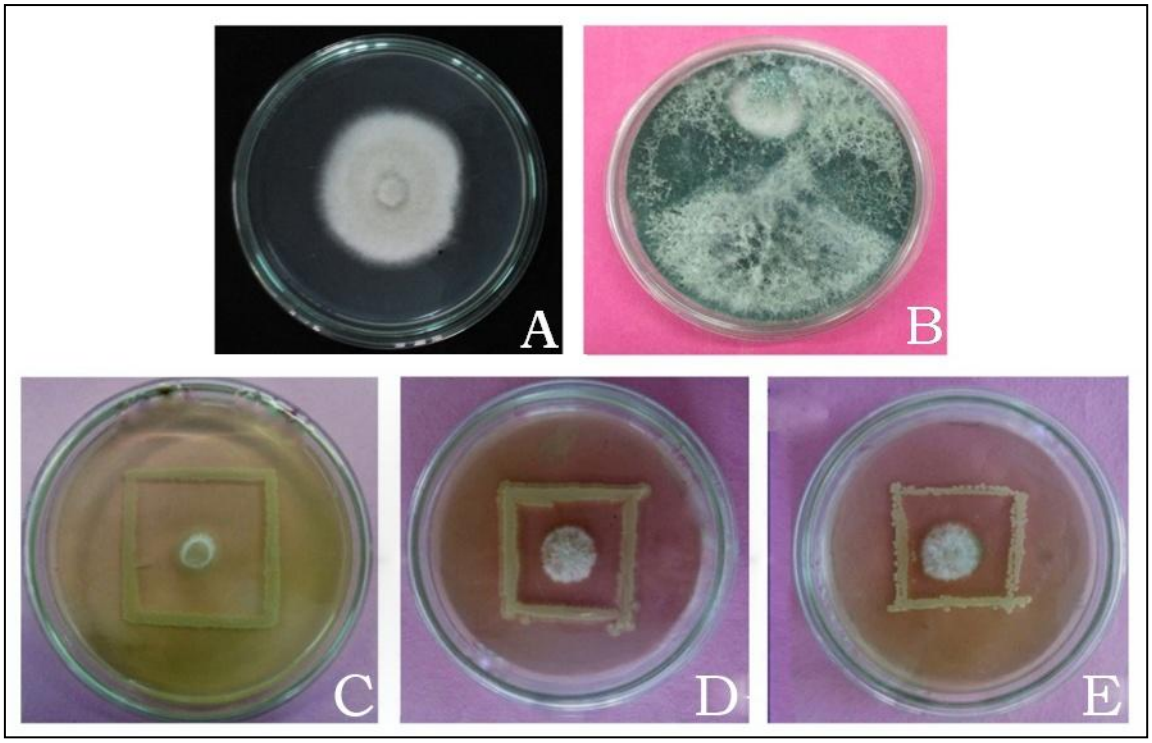

Fig. 4.20: A) Radial growth of *C. gloeosporioides* in PDA (control); growth of *C. gloeosporioides* co-cultured with B)*Trichoderma harzianum*; C) *Bacillus pumilus;* D) *Bacillus megaterium;* E) *Bacillus subtilis* .

6. Discussion

Tea a non-alcoholic beverage, occupies an important position among the people worldwide. Tea [*Camellia sinensis* (L.) O. Kuntze.], is a plantation crop and in view of its popularity it is grown as a cash crop. Commercially, Tea plants are cultivated in 46 countries. Tea plants grow between 42º north to 35º south latitude and from sea level to 2300m above mean sea level with environmental conditions like, humid climate, acidic soil (pH- 4.5 to 5.5), well distributed rainfall and long sunshine hours. On the basis of productivity and consumers, India is the second largest tea producer after china. It is thought that tea originated in southwest of China over 4000-5000 years ago (Chen and Lin, 2015; Yamanishi, 1991). In India major tea producing areas are geographically separated into three distinct regions. These regions are northeast India (Assam, West Bengal, Bihar, Tripura, Sikkim, Manipur, Nagaland, Meghalaya, Arunachal Pradesh, and Mizoram), South India (Kerala, Karnataka, and Tamil Nadu), and north India (the hills of Himachal Pradesh and Uttarakhand) (Sharma *et al.*, 2010; Bhardwaj *et al.*, 2014; Meegahakumbura *et al.*, 2016).

Due to high commercial value, tea plantations have been extended in several new places, remote from their place of origin. Moreover, for high production several new varieties have also been introduced in the plantations. All these changes have made the plants prone to attack by a number of fungal pathogens. Thus, tea plants are exposed to a number of fungal pathogens (*Pestalotiopsis theae, Colletotrichum gloeosporioides, Exobasidium vexans, Lasiodiplodia theobromae, Corticum theae, Fusarium oxysporum, Rhizoctonia bataticola and Curvularia erragrostidis*), some of them are of serious nature. The most serious disease (blister blight, grey blight, brown blight, black rot) causing fungi and also some other leaf disease-causing fungi frequently encounter tea plants in the present study area *i,e.* Sub-Himalayan West Bengal (Guo *et al.* 2014; Karakaya and Bayraktar, 2010). The pathogens attack tea plants and damage the plants.

This ultimately results to reduced production of tea. In certain cases production is substantially reduced due to fungal attack on the leaves.

Understanding the molecular responses associated with host defence mechanism in tea is thus very important for better management of the crop production. Since the whole tea genome sequence has not yet been deciphered, very little information is known about the genes and genetic regulations associated with tea stress responses. The molecular interaction between the fungus and the plant is not well known and only some comprehensive approaches of transcriptome and proteome analysis have become available (Campo *et al.*, 2004). The regulation of defense-related genes is one of the key elements of the defense mechanism that is used by plants against biotic and abiotic agents (Rejeb *et al.* 2014; Edreva, 2005). Differential expression of messenger RNAs has provided intriguing results. A high level of variability was detected in response to *Fusarium verticillioides* infection between susceptible and resistant maize lines. Although similar functional categories of genes were involved in the response to infection in resistant and susceptible maize genotypes, in the susceptible line, the genes were qualitatively induced from a basal level and responded specifically to pathogen infection. In the resistant line, the defense-related genes assayed were transcribed at high level before infection and provided basic defense to the fungus (Lanubile *et al.*, 2010 and 2012; Huang *et al.*, 2016).

In order to provide protection against pathogens, management of diseases has been done by exogenous application of a variety of biotic and abiotic inducers (Ryals *et al.*, 1996; Meena *et al.*, 2001; Chitra *et al.*, 2008; Anand *et al.*, 2007; Narayanasamy, 2013; Oliveira *et al.*, 2016; Llorens *et al.*, 2017).

Hence, in the present study, it was thought to assess the role of some known abiotic inducers against some pathogens of tea leaf. Before initiation of the experiments on induction, it was thought worthwhile to study the pathogenicity of the selected pathogens (*Colletotrichum gloeosporioides, Curvularia eragrostidis, Lasiodiplodia theobromae*). Hence,

pathogenicity of the selected pathogens (*C. gloeosporioides* (producing brown blight disease symptoms in tea), *C. eragrostidis* (producing leaf spot disease symptom in tea) and *L. theobromae* (*which* attack young tender leaves as well as tender stem and root of tea plants) were determined. The selected pathogens were originally isolated from the present study area by the previous workers of the present laboratory. Pathogenicity tests were done to select most susceptible plant against a pathogen. Finally, one of the selected susceptible plants (Variety Teenali) was taken in to consideration for studies of regulation (up or down regulation) of a selected defense related enzyme [Phenyl alanine ammonia lyase (PAL)]. For this, study, one abiotic inducer benzothiodiazole (BTH) was taken into consideration for induction of PAL. However, before selection of the enzyme of study (i,e. PAL) for up or down regulation, two other enzymes β-1,3 glucanase and peroxidase were also studied.

Before initiation of the study of PAL transcriptomes induced by BTH, it was also considered to study characteristics of some of the defense related genes of tea plants. Three different defense related genes [PAL, Chalcone synthase (CHS) and Ascorbate peroxidase (APX)] were studied in details to find out the molecular characteristics of the genes sequenced from tea plants. More specifically, the characters that were studied are as follows: the neucletide composition at 3rd position (A3, T3, C3 and G3), average GC content, GC content at 1st, 2nd and 3rd position of different synonymous codon, Nc, Fop, Gravy and Aroma of the three defense genes as mentioned above.

Study of the transcripts following induction (by resistance inducers) and inoculation by pathogens were the thrust area of the present study. Hence, detailed pathogenicity of three pathogens was determined in four different varieties of tea plants. Finally from that, one variety (Teenali) and two pathogens (*Colletotrichum gloeosporioides* and *Curvularia eragrostidis*) were selected for the PAL transcriptome analysis towards resistance induction.

Additionally, in the last part of the present works some botanicals and bio-control agents have also been tested *in vitro* to know their efficacy to control the pathogens of the present study.

During survey of the present study area, predominantly four fungi were found. Considering the damage created by the pathogens in the young leaves, three pathogens were selected for the present study. During survey, on the basis of visual observation relative presence of the fungi was estimated. From that we have got *Colletotrichum gloeosporioides, Curvularia eragrostidis, Lasiodiplodia theobromae* and *Pestalotiopsis theae* as major pathogens of tea leaves in the sub-Himalayan west Bengal, the present study area. The report of *C. gloeosporioides, L. theobromae* and *P. theae* is in conformity with that of Sarmah (1960). Sarmah (1960) reported that *C. gloeosporioides* and *P. theae* were associated with mature leaves. They also reported that *L. theobromae* was associated with the tea plants as pathogen of leaves, tender stems and roots. *C. eragrostidis*, has been reported by Saha *et al.* (2001) from the young tea plants of North Bengal. Thus our selection of fungi for the present study was significant in view of controlling the diseases they cause by inducing resistance in the susceptible tea plants.

The fungal isolates used in the present study were collected from the molecular Plant pathology laboratory and were verified by Koch's postulations. However, before going to the mechanisms, it was considered to evaluate the pathogens' pathogenicity at least in some seed and clonal varieties of tea. Hence, all the fungal pathogens were subjected to pathogenicity test in four different tea varieties, as differential pathogenicity of a fungus differentiates degree of susceptibility or resistance of a particular variety of plant or pathogenicity of different fungi to a particular variety gives the information of infecting capacity (i.e. degree of virulence or avirulence). In the present study, pathogenicity test have been done in two different techniques viz. 'Detached leaf' and 'Cut-shoot' inoculation technique. Results obtained from the two different techniques were in agreement with each other. Dickens and Cook (1989) could detect resistance and susceptibility of *Camellia* plants against *Glomerella*

cingulata. Brennan *et al.* (2003) tested the pathogenicity of five different species of *Fusarium* in wheat seedlings. In1987, Yanase and Takada used cut shoot method for determining resistance of tea plants to grey blight disease-causing fungi *Pestalotiopsis longiseta*.

From the results of pathogenicity tests following detached leaf inoculation technique and cut shoot inoculation technique three varieties (Teenali, TS-520 and TS-463) were found to be susceptible and one variety (TV-26) was found to be resistant against all the three pathogens tested. Saha (1992) observed pathogenicity of *Bipolaris carbonum* in several varities of tea including TV-26. He also found that TV-26 was resistant variety against *Bipolaris carbonum*. Thus, present experiment is also in agreement with that of Saha (1992) to detect resistant variety against a pathogen although his pathogen was different. In 1995, Chakraborty *et al*. reported that in the tested tea varities TV 18 was susceptible and TV 9 was moderately resistant against *Colletotrichum* camelliae and *Pestalotiopsis theae*. Hu-Shu Xia (1996) in china also detected two cultivars as highly resistant against *P. theae* following pathogenicity test. Thus our studies were found in the same line with that of some previous workers mentioned above. Results of both disease assessment techniques were in good agreement with each other.

To understand intriguing mechanisms of host pathogen interaction and resistance induction by inducers, several experiments have been performed. In the present study, the role of BTH (Benzothiadiazole, a chemical analogue of salicylic acid), in inducing defense and also in reducing disease in tea plants against foliar fungal pathogens was studied. Two more inducers such as BABA and GABA were also studied to compare the role of BTH as inducer of resistance. Results indicated that BTH induced resistance in most susceptible tea variety (Teenali) against *Colletotrichum gloeosporioides*. Induction of resistance was evident (Table 4.10.), due to appearance of less-severe disease symptoms (disease index 2.80) in BTH treated plants in comparison to symptoms appeared in control plants (disease index 6.72). Similar results were also found when BABA and GABA were used as inducers but treated plants showed disease

index of 3.2 and 3.0 respectively. Induction of resistance by BABA and GABA was much lower than that of BTH. Thus, BTH was the best resistance inducer in susceptible tea variety 'Teenali' against *Colletotrichum gloeosporioides*. In plants BTH play an important role as a potential SAR activator which increases disease resistance capacity by activation of SAR signaling transduction pathway (Thakur and Sohal, 2013). BTH pre-treated pepper plants have been found to show less severe symptoms and also reduced infection percentage (Trejo-Saavedra *et al.,* 2013). Sood *et al*. (2013) showed BTH was more effective than SA in inducing resistance in rice plants against sheath blight. Time course analysis done by them showed peak accumulation of defense related enzymes and phenols in the rice leaves treated with BTH and SA. According to them accumulation was highest at the flowering stage. Higher enzymatic activity was reported in elicitor treated plants inoculated with *R. solani*. Thus, their results supports our studies where we also observed higher enzymatic activity in elicitor (BTH, BABA and GABA) treated tea plants inoculated with pathogen. PAL, is an important enzyme in the biosynthesis of phenyl propane unit and Phenyl propane unit is a component of flavonoids, phenolic acids and lignins.

PAL has been reported to be induced in many cases of disease resistance following treatment with various abiotic elicitors or inducers of defense reaction. Benzothiadiazole-mediated induced resistance of banana plants to *Colletotrichum musae* was reported by Zhu *et al*. (2016). They also reported BTH effectively inhibited the invasion and development of pathogenic organism and controlled the occurrence of disease. BTH treatment enhanced the activities of defense-related enzymes, including chitinase, phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase. Raju *et al*, (2008) reported increased PAL activity level in response to *Fusarium oxysporum* inoculation and elicitor (SA) application in *Cicer arietinum*. This supports our study in tea plants where we found increased PAL activity after elicitors (BTH, BABA & GABA) treatment followed by challenge inoculation either with *C. gloeosporioides* or with *C. eragrostidis.*

BABA induced systemic resistance in lettuce against *Bremia lactucae* causal organism of downy mildew disease and protected the plants. They also reported that BABA increased the activity of PR proteins like β-1,3-glucanase, peroxidase etc. (Pajot *et al.*, 2001). In our experiments also BABA induced PR proteins in tea plants almost like that of BTH. Amzalek and Cohen (2007) also studied the effect of six SAR inducers including BABA, BTH and GABA to control sunflower rust caused by *Puccinia helianthi* and could show the effect of the inducers. Thus, our inclusion of the three resistance inducers such as BABA, BTH and GABA was justified. And we found all three tested inducers could induce resistance in susceptible tea plant (Variety 'Teenali'). From the foliar spray results Amzalek and Cohen (2007) found BABA was more effective to induce resistance against rust but in leaf disc assay BTH and BABA could protect fully but GABA did not have any potential effect.

Similarly, a large number of workers have worked with BTH as inducer of disease resistance and could reduce diseases in many host-pathogen interactions (Oumar *et al.*, 2015; Azami-Sardooei *et al.*, 2013; Kogel *et al.*, 2005). In our studies we have found BTH as a best inducer of resistance in tested tea plants against *C. gloeosporioides* and *C. eragrostidis*. Thus like many other workers we also found BTH as good resistance inducer-chemical for tea plants susceptible to foliar fungal pathogens tested in this study.

Several workers have also used BABA as inducer of PR proteins in different plant host-pathogen interactions and could reduce disease incidences (Navarova *et al.*, 2012; Walters *et al.*, 2011; Slaughter *et al.*, 2012; Conrath, 2009; Beckers and Conrath, 2007; Ton and Mauch-Mani, 2004). In the present study also disease incidences could be reduced in tea plants against two pathogens. Hence, our results are in good agreement with them also.

Plants evolved several metabolic pathways to cope up with different biotic and abiotic stress. Polyphenols such as flavonoids, isoflavons, anthocyanins, phytoalexins and lignin play important role in combating

different pathogens. These polyphenols are synthesized in plant through phenylpropanoid pathway Phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase are the key enzymes of phenylpropanoid pathway (Dixon and Paiva, 1995; La Camera *et al.*, 2004; Jones and Dangl, 2006; Ferrer *et al.*, 2008; Xu *et al.*, 2010; Payyavula *et al.*, 2012). Reactive Oxygen Species (ROS) like hydrogen peroxide (H_2O_2) may accumulate in plant cells during heat stress leading to the damage of plant tissue. Ascorbate peroxidase (APX), superoxide dismutase (SOD), monodehydroascorbate reductase and glutathione reductase are the key enzymes that nullify the effect of H_2O_2. APX is the key enzyme in of ascorbate-glutathione cycle that leads to the production of these oxidoreductases (Asada, 1992; Padaria *et al.*, 2014).

As in the present study, BTH has shown its ability to induce several defense related enzymes including PAL, therefore it was considered worthwhile to study the molecular phylogeny of PAL gene in different *Camellia* species. In the molecular phylogenetic study two more defense related genes were also considered, one from the same phenylpropanoid pathway (CHS) and another from different biochemical pathway (APX), to see wheather the genes encoding the enzymes that catalyzes phenylpropanoid intermediates are conserved or not. In our study we found that, PAL genes were conserved in different species of *Camellia* and were clustering according to different species, whereas, in case of CHS several small clusters were observed. But, APX gene of different *Camellia* species showed no significant group, rather, their diversity within the *C. sinensis* species was more prominent. This indicated that PAL and CHS genes were more conserved among different species of *Camellia* than APX.

In a study on willow Jong *et al.* (2015) characterised five PAL gene isoforms, where PAL1, PAL2, PAL3 and PAL4 genes were orthologous to the poplar PAL genes, but PAL5 orthologue was absent in willow. Phylogenetic analysis of the corresponding amino acid sequences showed that the PAL genes isolated from willow and poplar were more closely related than Arabidopsis and tobacco. In other studies it was observed that PAL genes isolated from bacteria, fungi, bryophytes, gymnosperms, monocot and dicot

plants formed separate groups, where several small sub-groups were observed in monocot and dicot PALs (Paolis *et al.*, 2008; Xu *et al.*, 2012; Hashemitabar *et al.*, 2014; Zhu *et al.*, 2015). This suggested that PAL genes are conserved among the different families and genera. Jin *et al.* (2013) also suggested that PAL gene isolated from *Dendrobium candidum* was conserved in the orchidaceae family. Kumar and Ellis (2001) reported that two PAL genes of raspberry viz., RiPAL1 and RiPAL2 showed diverged phylogeny, where both the genes were diverged temporally i.e., one was associated with early fruit ripening events and the other was associated with flower and fruit development events at later stage of growth. This indicated that PAL genes are conserved not only in different plant families but it also diverged with different mode of function. Lo *et al.* (2002) isolated seven CHS isoforms (CHS1-7) and one CHS-like gene (CHS8) from Sorghum, where they found that all the monocot CHS (CHS1-7) grouped together and the CHS-like gene was clustered separately. Pitakdantham *et al.* (2010) also reported that CHS genes are conserved among the *Dendrobium* genus. Zhou *et al.* (2011) reported that, CHS genes of eudicot plant families (salicaceae, malvaceae and rosaceae) were more closely related than that of monocot and dicot families. Farzad *et al.* (2005) also reported that *Viola cornuta* was of monophyletic origin, where all the CHS from eudicots grouped together and CHS of *Zea mays* (monocot) was placed as an out-group. Phylogenetic study of APX from *Eleusine coracana* revealed that sequence homology among the APX family varied from about 74% to 97% (Bhatt *et al.*, 2013). Similar wide range of diversity (83-98%) in APX gene sequences was also observed in *Hordeum vulgare*, *Aegilops tauschii*, *Puccinellia tenuiflora*, *Oryza sativa* and *Brachypodium distachyon* (Padaria *et al.*, 2014). Genome-wide identification and phylogenetic and syntenic comparison of PAL and peroxidase (POX) genes suggested that PAL genes were highly conserved among monocots or dicots, whereas, POX genes that are present at the subtelomeric region of chromosomes are more diversified due to higher evolutionary rate than PAL resulting in the evolution of several subgroups (Rawal *et al.*, 2013).

For further analysis of sequence diversity among these three defense related genes (PAL, CHS and APX) relative synonymous codon usage (RSCU) pattern was also studied. From the results it was observed that highest %GC and GC3 along with overall pyrimidine content at third codon position were higher in CHS and PAL than APX. According to several scientists codon usage pattern is influenced by several factors like mutational bias, translational selection, t-RNA abundance (Kanaya *et al.*, 2001; Sharp and Li, 1986). From the Nc plot it was evident that in all the three genes codon usage was influenced by mutation and translational selection as all the values of Nc plot were placed far lower from the standard curve (Wright, 1990; Xu *et al.*, 2008; Zhang *et al.*, 2011; Su *et al.*, 2017). According to several studies (Xu *et al.*, 2008; Wei *et al.*, 2014; Zhao *et al.*, 2016) slope of regression closer to 1 indicated strong effect of mutational pressure on synonymous codon usage bias. However from the neutrality plot analysis it was found that maximum effect of natural selection was observed in APX (93.1%) followed by CHS (80.6%) and PAL (52.2%). Significant correlation between GC content at first, second and third codon position was also higher in PAL (R^2=0.71) and CHS (R^2=0.342) than APX (R^2=0.013). Significant correlation (R^2 close to 1) between GC12 and GC3 implied effect of similar mutational pressure on each codon position i.e., GC1, GC2 and GC3 (Zhao *et al.*, 2016; Su *et al.*, 2017). From the correlation analysis it was found that A3 and L_aa affected codon usage in tea plants irrespective of different genes. But, correlation of other factors with Nc was more in PAL followed by CHS than APX. On the basis of RSCU values the codon usage bias of 59 sense codons (except Met, Trp and three termination codon) of all the three defense related genes were also calculated (Table 4.22). If RSCU value of a codon falls within the range of 1.0 to 1.5, then it indicates that the codon was used frequently. If RSCU value is less than 1.0 then it denotes that codon was used less frequently and if the RSCU value is greater than 1.5 then it denotes that codon was used more frequently in a particular gene (Sharp and Li, 1986; Zhao *et al.*, 2016). From the RSCU of the 59 sense codons APX contained 24 frequently used codons (RSCU>1) out of which 9 were more frequently used (RSCU>1.5). But, CHS and PAL contained 31 and 32 frequently used

codons (RSCU>1) out of which 10 and 11 were more frequently used (RSCU>1.5) respectively. This indicated that PAL genes contained more numbers of optimal codons with strong bias than CHS and APX.

According to several workers codon usage pattern affects translational efficiency in several organisms (Xu *et al.*, 2008; Zhang *et al.*, 2011; Zhao *et al.*, 2016). But in recent years it was found that preferred codons were found frequently in highly expressed genes. In a study on *Neurospora* it was observed that codon usage significantly affected both translational (protein expression) and transcriptional (mRNA up or down regulation) levels of gene expression especially in regulation of transcription. It was also found that biased codons increased gene expression levels along with specification in protein sequences (Zhou *et al.*, 2016). Boel *et al.* (2016) also reported that codon usage pattern mainly affected mRNA folding rather than influencing protein expression. In the present study it was observed that PAL genes showed relatively higher codon bias than CHS and APX. In the phylogenetic study also it was found that, PAL genes were more conserved in different families of plants.

The present study was further progressed with the expression of PAL gene in tea plants in response to pathogen attack and/or induction by abiotic inducer BTH. Exogenous application of BTH has proved to be effective in developing SAR through SA-signaling pathway and production of polyphenols through induction of several compounds of phenylpropanoid pathway (Friedrich *et al.*, 1996; Lawton *et al.*, 1983; Sticher *et al.*, 1997; Brisset *et al.*, 2000; Bressan and Purcell, 2005; Hukkanen *et al.*, 2007; Polesani *et al.*, 2008). Study of expression of 11 PAL isoforms (PAL01 through PAL11) in blast-resistant rice variety showed that 2 isoforms viz., PAL04 and PAL07 were up regulated after 48 hours post inoculation in resistant rice variety in response to *Magnaporthe oryzae*-cell wall hydrolysates, suggesting the involvement of the two isoforms in regulating resistance against the fungal pathogen (Giberti *et al.*, 2012).

In the present study, change in relative expression of phenylalanine ammonia-lyase (PAL) gene in tea after induction with BTH and challenge

inoculation with two foliar fungal pathogens (*Colletotrichum gloeospoiroides* and *Curvularia eragrostidis*) was analyzed using quantitative real-time PCR (qRT-PCR) to compare the effect of BTH on PAL transcript level against both *C. gloeospoiroides* (thought to be major pathogen) and *C. eragrostidis*. As discussed in the section 4.6. all the plants were divided into six sets i.e., untreated-uninoculated control, untreated-inoculated (2 sets for two pathogens), treated-uninoculated and treated-inoculated (2 sets for two pathogens) and the results were recorded post treatment up to 7 days with 1 day interval. From the results it was observed that PAL transcript accumulation was elevated on 4th, 6th and 7th day post treatment in response to BTH treatment and *C. gloeospoiroides* inoculation, whereas, in BTH treated and *C. eragrostidis*-inoculated plants, elevation in PAL gene expression was observed on 1st, 5th and 7th day post treatment. However, in both the cases simultaneous reduction in disease index was observed up to 7 days. During enzyme estimation also, increased level of phenylalanine ammonia lyase was observed on 4th and 6th days post treatment with BTH and challenge inoculation with *C. gloeospoiroides*.

Dufour *et al*. (2013) stated that, treatment with BTH induced up regulation of PR-1, PR-2, PR-3, PR-6, PR-8, PR-10, anthranilate synthase (enzyme that catalyses intermediate product of indole pathway), PAL and some other defense related gene transcripts in grapevine after 48 hour post treatment and gave enhanced resistance to *Plasmopara vaticola* and *Erysiphe necator*. Duan *et al*. (2014) compared the expression of SA synthesis pathway related genes [phenylalanine ammonia lyase (PAL), EDS1 (enhanced disease susceptibility 1) and PAD4 (phytoalexin deficient 4)] in response to small brown planthopper (*Laodel phaxstriatellus*) infestation in resistant variety Kasalath and susceptible variety Wuyujing 3. They found that in resistant rice variety Kasalath PAL expression after 48 and 72 hours post infestation was higher than the susceptible variety Wuyujing 3, whereas, peroxidase and polyphenol oxidase gene expression were at elevated level after 24 hours post infestation. So, they suggested that PAL gene played a significant role in developing resistance through induction of SA signaling pathway in Kasalath variety. Ejtahed *et al*. (2015)

studied PAL gene expression and rosmarinic acid (RA) accumulation in two species of *Salvia* in response to SA using semi-quantitative reverse transcriptase PCR (RT-PCR) where up-regulation of PAL gene expression were observed. However, they also sensed the involvement of some other unknown factors in elevation of phenolics in the above mentioned plant species. Kim and Hwang (2014) stated that increased PAL activity via phenylpropanoid pathway showed resistance to *Pseudomonas syringae* pv. *tomato* in pepper plant up to 48 hours. In 2014, Landi *et al.* studied the expression of 12 defense related genes including PAL in strawberry fruit in response to three different elicitors, viz., chitosan, BTH and calcium and organic acids (COA) using real time PCR (qRT-PCR). They observed that expression of calcium-dependent protein kinase (CDPK), K+ channels, glutathione S-transferase (GST), ascorbate peroxidase (APX), chalcone synthase (CHS), chalcone isomerase (CHI), flavanon3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), flavonoid 3-O-glucosyltransferase (UFGT) were increased up to 48 hours post BTH treatment. Although, PAL, flavonol synthase (FLS), and anthocyanidin synthase (ANS) expression was not elevated much higher up to 48 hours after BTH treatment. Nevertheless, in all these cases PAL gene expression was observed up to 3 days post treatment or inoculation. In our study also PAL gene expression was not up regulated up to 3 days (i.e., 72 hours) in both cases (BTH treated and 'BTH treated-*C. gloeosporioides inoculated*) plants. However, in our study induction in PAL transcript level was observed after 4 days post treatment or inoculation. From the above mentioned experiments it can be concluded that, BTH might be an effective inducer of PAL gene (defense related gene) in tea for combating with *C. gloeosporioides* and *C. eragrostidis*.

Several scientists (Parimala and Sangeetha, 2016; Pawar, 2011; Falade, 2017; Ravikumar and Garampalli, 2013; Thembo *et al.*, 2010; Johnny *et al.*, 2011; Naz and Bano, 2012; Ishnava *et al.*, 2012) have shown that botanicals from common plants may be used for control of different pathogens causing severe disease symptoms in a variety of crops. In the present study also mycelia growth of one of the most virulent pathogen (*C.*

gloeosporioides) was controlled *in vitro* by some common botanicals. From the results it was evident that 50% ethanolic extracts of *Datura metel* and *Clerodendrum viscosum* could inhibit mycelia growth of the fungus. Inhibition was more than 70%. Least growth inhibition was observed when leaf extracts of *Lantana camara* was tested *in vitro*. All other extracts tested showed moderate growth reduction of the fungus.

Ravikumar and Garampalli (2013) evaluated antifungal property of *Datura metel* leaf extracts against *Alternaria solani* a pathogen of early blight of tomato by poison food technique in Potato Dextrose Agar medium. They found that 2% concentration of the extract could significantly inhibited the growth of the fungus. Thus antifungal activity of *Datura metel* has been confirmed by them. Falade (2017) tested the *in vitro* effect of *Datura stramonium* on growth, conidial germination and sporulation of *Colletotrichum lindemuthianum*. They found maximum growth inhibition of 65%. Thus their result also showed *Datura* leaf extract had potential antifungal property against *Colletotrichum* sp. We also got similar results but in some other species.

Antifungal property of *Clerodendrum viscosum* has recently been documented by Oly *et al*. (2011). They showed that alcoholic extract of the plant leaves could inhibit growth of fifteen bacteria and seven fungi including *Fusarium oxysporum*. Thus they also confirmed the antifungal efficacy of the plant extract. Thus our study of antifungal efficacy of the same plant extract against *Colletotrichum gloeosporioides* are in good agreement with that of Oly *et al*. (2011). Parimelazhagan and Francis (1999) showed that growth of Seed pathogen *Curvularia lunata* could be checked *in vitro* by the leaf extract of *Clerodendrum viscosum*. Their works also supports our finding of antifungal properties of *Clerodendrum viscosum* against *C. gloeosporioides*. Choudhury *et al*. (2009), isolated antibacterial properties of *Clerodendrum viscosum* as 'Viscosene'. Das *et al* 2011 also showed antihelminthic property of *C. viscosum*. Siju *et al*. (2011) reported antibacterial and antifungal activities of leaf extracts of *C. viscosum*. They also reported about broad spectrum antimicrobial activity of the plant leaf extract.

Use of antagonistic microorganisms in the practice of the present day agriculture is of great importance. Tea is one of the important export oriented beverage product of India and more specifically of north east India. Residual chemicals present in tea often become hurdle for qualifying the criteria of minimum residual presence of different chemicals in made tea. Hence, biocontrol of fungal pathogens in tea plants is of great importance, in view of its' eco-friendly nature. Four known biocontrol agents such as *Bacillus subtilis*, *B. pumilus*, *B. megaterium* and *Trichoderma harzianum* were tested to control *Colletotrichum gloeosporioides*. It is evident from the results that *Bacillus pumilus* could inhibit the growth of the fungus up to a level of 78% *in vitro*. About 60% growth inhibitions were observed in case of other two *Bacillus* species in dual culture. *Bacillus pumilus* was found to be the best antagonist but the other three also could inhibit the growth of the fungus *Colletotrichum gloeosporioides* upto a level of 60% and above.

Nielsen and Sorensen (1997) showed antifungal activity of *Bacillus pumilus* against a large number of plantpathogenic microfungi. They also stated that the bacteria released cell wall degrading enzymes to inhibit the growth of the tested fungi. Abdel Kader and El-Mougy (2013) showed *Trichoderma harzianum* and B. subtilis along with some resistance inducers could inhibit fungal diseases like Powdery, Downy mildews of Cucumber, Cantaloupe and Pepper as well as Early, Late blights of Tomato.

Gajera *et al*. (2013) explained the antagonistic properties of *Trichoderma* strains act as bio-control agents against fungal phytopathogens either indirectly or directly. Indirect mechanism comprises competition for nutrients and space, modification of the environmental conditions, antibiosis and induction of plant defensive mechanisms. In the present study we observed overgrowth of *Trichoderma harzianum* on *Colletotrichum gloeosporioides*. This is probably due to mycoparasitism as suggested by Gajera *et al*. (2013). They also reported the antifungal properties of *Bacillus subtilis*. In our studies also *Bacillus subtilis* has shown substantial antifungal efficacy. Thus our results of antagonism of

biocontrol agents are in good agreement with that of others as reported. Leclere *et al.* (2005) reported about strain (BBG100) of *Bacillus subtilis* for over production of antagonistic property in comparison to a wild strain which could not protect pathogenic fungi.

Thus our results of antagonism of *Bacillus pumilus, Bacillus subtilis* and of *Trichoderma harzianum* are in the line of several previous scientists. We have observed antagonism of *Bacillus megatorium* against *Colletotrichum gloeosporioides in vitro* but *in vitro* antagonisms of the bacteria against fungal pathogens are scanty, although several *in vivo* reports are there (Chakraborty *et al.*, 2006; Kildea *et al.*, 2007).

The present study has supported and also elaborated some findings of previous workers. This study also reveals certain new facts of fundamental importance. The significance of some defense related genes and their molecular characteristics have been demonstrated. Induction of defense related enzymes by inducing chemicals have been observed. Differential expression of PAL gene have been studied by semi-quantitative (by RT PCR) and quantitative (by qRT PCR) methods following induction of resistance by known resistance inducers. The diseases of susceptible plants could be reduced significantly by BTH. Our investigations have provided an insight in to the mechanism of resistance induction in tea plants against some pathogens of tea. More works need to be done for formulating some defense inducers applicable to tea plants. Suitable control measures may be designed from the present study at least for controlling tea diseases caused by *C. gloeosporioides* and *Curvularia eragrostidis*.

Bibliography

Abdel-Kader, M. M., El-Mougy, N. S. and Embaby, E. I. (2012) Resistance inducers treatments against downy and powdery mildews of cucumber under commercial plastic houses conditions. *Australian Journal of Basic and Applied Sciences* **6:** 249-259.

Abhayashree, M. S., Murali, M. and Amruthesh, K. N. (2016) Abiotic elicitors mediated resistance and enhanced defense related enzymes in *Capsicum annuum* L. against anthracnose disease. *Scientia Horticulturae* **204:** 172–178.

Acharya, K., Chakraborty, N., Dutta, A. K., Sarkar, S. and Acharya, R. (2011) Signaling role of nitric oxide in the induction of plant defense by exogenous application of abiotic inducers. *Archives of Phytopathology and Plant Protection* **44:**1501-1511.

Aleandri, M. P., Reda, R., Tagliavento, V., Magro, P. and Chilosi, G. (2010) Effect of chemical resistance inducers on the control of *Monosporascus* root rot and vine decline of melon. *Phytopathologia Mediterranea* **49:** 18–26.

Alkahtani, M., Omer, S. A., El-Naggar, M. A., Abdel-Kareem, E. M. and Mahmoud, M. A. (2011) Pathogenesis-related protein and phytoalexin induction against cucumber powdery mildew by elicitors. *International Journal of Plant Pathology* **2:** 63-71.

Al-Sohaibani, S. A., Mahmoud, M. A., Al-Othman, M. R., Ragab, M. M. M., Saber, M. M. and Abd El- Aziz, A. R. M. (2011) Influence of some biotic and abiotic inducers on root rot disease incidence of sweet basil. *African Journal of Microbiology Research* **22:** 3628-3639.

Altinok, H. H. and Dikilitas, M. (2014) Antioxydant response to biotic and abiotic inducers for the resistance against fusarium wilt disease in eggplant (*Solanum melongena* L.) *Acta Botanica Croatica* **73:** 79–92.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25:**3389-3402.

Alvarez, J. C., Rodriguez, H. A., Rodriguez-Arango, E., Monsalve, Z. I. and Arango, R. E. (2013) Characterization of a differentially expressed phenylalanine ammonia-lyase gene from banana induced during Mycosphaerella fijiensis infection. *Journal of Plant Studies* **2:** 35.

Ambareen, Z., Konde, S., Raj, S. N. and Kumar, N. C. (2015) Antimicrobial efficacy of herbal extracts. *International Journal of Oral Health Dentistry* **1:**108-113.

Amer, M. A., El-Abd, S. M., Deraz, S. F. and Zaid, N. A. (2015) Systemic acquired resistance induced by some biotic agents against downy mildew of cucumber disease. *International Conference on Plant, Marine and Environmental Sciences (PMES-2015) Jan. 1-2, 2015 Kuala Lumpur (Malaysia) pp* 76-83.

Amzalek, E., and Cohen, Y. (2007) Comparative efficacy of systemic acquired resistance-inducing compounds against rust infection in sunflower plants. *Phytopathology* **97:** 179-186.

Anand, T., Raguchander, T., Karthikeyan, G., Gopalakrishnan, C., Bhaskaran, R. and Ganeshamoorthi, P. (2007) Induction of systemic resistance by plant growth promoting rhizobacteria in chilli plants against fruit rot disease. *World Journal of Agricultural Sciences* **3:** 815-824.

Angellotti, M. C., Bhuiyan, S. B., Chen, G. and Wan, X. F. (2007) CodonO: codon usage bias analysis within and across genomes. *Nucleic Acids Research* **35:** W132–W136.

Arlorio, M., Ludwig, A., Boller, T. and Bonfante, P. (1992) Inhibition of fungal growth by plant chitinases and β-1,3-glucanases: A morphological study. *Protoplasma* **171**: 34-43.

Arunachalam, M. K. and Sharma, P. (2012). Confrontation assay for *Trichoderma* as a potential biocontrol agent against *Pythium aphanidermatum* and *Sclerotinia sclerotiorum. Pest Management in Horticultural Ecosystems* **18:** 74-77.

Asada, K. (1992) Ascorbate peroxidase- a hydrogen peroxide-scavenging enzyme in plants. *Physiologia Plantarum* **86:** 235-241.

Avdiushko, S. A., Ye, X. S. and Kuc, J. (1993) Detection of several enzymatic activities in leaf prints cucumber plant. *Physiological and Molecular Plant Pathology* **42**: 441-454.

Aye, S. S. and Matsumoto, M. (2011) Effect of some plant extracts on *Rhizoctonia* spp. and *Sclerotium hydrophilum*. *Journal of Medicinal Plants Research* **5:** 3751-3757.

Azami-Sardooei, Z., Seifi, H. S., De Vleesschauwer, D. and Hofte, M. (2013) Benzothiadiazole (BTH)-induced resistance against Botrytis cinerea is inversely correlated with vegetative and generative growth in bean and cucumber, but not in tomato. *Australasian Plant Pathology* **42:** 485-490.

Azzahra, L. F., Fouzia, H., Mohammed, L. and Noureddine, B. (2012) Antioxidant response of *Camellia sinensis* and *Rosmarinus officinalis* aqueous extracts toward H_2O_2 stressed mice. *Journal of Applied Pharmaceutical Science* **2:** 70-76.

Baby, U. I. and Sanjay, R. (2006) Seasonal incidence and economic importance of grey blight disease of tea. *Journal of plantation Crops* **34**: 66-67.

Baljeet, S. Y., Simmy, G., Ritika, Y. and Roshanlal, Y. (2015) Antimicrobial activity of individual and combined extracts of selected spices against some pathogenic and food spoilage microorganisms. *International Food Research Journal* **22:** 2594-2600.

Bansod, S. and Rai, M. (2008) Antifungal activity of essential oils from Indian medicinal plants against human pathogenic *Aspergillus fumigatus* and *A. niger*. *World Journal of Medical Sciences* **3:** 81-88.

Baysal, O., Soylu, E. M. and Soylu, S. (2003) Induction of defence-related enzymes and resistance by the plant activator acibenzolar-S-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* ssp. *Michiganensis*. *Plant Pathology* **52:** 747–753.

Beckers, G. J. and Conrath, U. (2007) Priming for stress resistance: from the lab to the field. *Current Opinion in Plant Biolo*gy **10:** 425–431.

Beneduzi, A., Ambrosini, A. and Passaglia, L. M. P. (2012) Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology* **35:** 1044-1051.

Bhakshu, L. M., Ratnam, K. V. and Venkataraju, R. R. (2008) Medicinal properties and antimicrobial activity of *Crotalaria madurensis* var. *kurnoolica*. *Ethnobotanical Leaflets* **1:** 104.

Bhardwaj, P., Sharma, R. K., Kumar, R., Sharma, H. and Ahuja, P. S. (2014) SSR marker based DNA fingerprinting and diversity assessment in superior tea germplasm cultivated in western Himalaya. *Proceedings of the Indian National Science Academy* **80:** 157-162.

Bhatt, D., Saxena, S. C., Jain, S., Dobriyal, A. K., Majee, M. and Arora, S. (2013) Cloning, expression and functional validation of drought inducible ascorbate peroxidase (Ec-apx1) from *Eleusine coracana*. *Molecular Biology Reports* **40:** 1155–1165.

Bishop, J. P., Dean, A. M. and Olds, T. M. (2000) Rapid evolution in plant chitinases: Molecular targets of selection in plant pathogen coevolution. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 5322-5327.

Boba, A., Kostyn, K., Kostyn, A., Wojtasik, W., Dziadas, M., Preisner, M., Szopa, J. and Kulma, A. (2017) Methyl salicylate level increase in flax

after *Fusarium oxysporum* infection is associated with phenylpropanoid pathway activation. *Frontiers in Plant Science* **7:** 1951.

Boel, G., Letso, R., Neely, H., Price, N. W., Wong, K., Su, M., Luff, J. D., Valecha, M., Everett, J. K., Acton, T. B., Xiao, R., Montelione, G. T., Aalberts D. P. and Hunt, J. F. (2016) Codon influence on protein expression in *E. coli* correlates with mRNA levels. *Nature* **529:** 358-363.

Boro, M. C., Beriam, L. O. S. and Guzzo, S. D. (2011) Induced resistance against *Xanthomonas axonopodis* pv. *passiflorae* in passion fruit plants. *Tropical Plant Pathology* **36:** 074-080.

Brennan, J.M., Fagan, B., van Maanen, A., Cooke, B. M. and Doohan, F. M. (2003) Studies on in vitro growth and pathogenicity of European *Fusarium* fungi. *European Journal of Plant Pathology* **109:** 577–587.

Bressan, A. and Purcell, A. H. (2005) Effect of benzothiadiazole on transmission of X-disease phytoplasma by the vector Colladonus montanus to *Arabidopsis thaliana*, a new experimental host plant. *Plant Disease* **89:** 1121-1124.

Brisset, M-N., Cesbron, S., Thomson, S. V. and Paulin, J-P. (2000) Acibenzolar-S-methyl induces the accumulation of defense-related enzymes in apple and protects from fire blight. *European Journal of Plant Pathology* **106:** 529-536.

Campo, S., Carrascal, M., Coca, M., Abian, J. and San, S. B. (2004) The defense response of germinating maize embryos against fungal infection: a proteomics approach. *Proteomics* **4:** 383–396.

Chacko, S. M., Thambi, P. T., Kuttan, R. and Nishigaki, I. (2010) Beneficial effects of green tea: A literature review. *Chinese Medicine* **5:**13.

Chakraborty B. N., Basu, P., Das, R., Saha, A. and Chakraborty, U. (1995) Detection of cross reactive antigens between *Pestalotiopsis thae* and

tea leaves and their cellular location. *Annals of Applied Biology* **127:** 11-21.

Chakraborty, B. N., Das-Biswas, R. and Sharma, M. (2006) *Alternaria alternata*–a new foliar fungal pathogen of tea in North Bengal, India. *Plant pathology* **55:** 303-303.

Chakraborty, U., Chakraborty, B. and Basnet, M. (2006) Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. *Journal of Basic Microbiology* **46:** 186-195.

Chandra, A., Saxena, R., Dubey, A. and Saxena, P. (2007) Change in phenylalanine ammonia lyase activity and isozymevpatterns of polyphenol oxidase and peroxidase by salicylic acidvleading to enhance resistance in cowpea against *Rhizoctonia solani*. *Acta Physiologiae Plantarum* **29:** 361–367.

Chen, C., Belanger R. R., Benhamou, N. and Paulitz, T. C. (2000) Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiological and Molecular Plant Pathology* **56:** 13-23.

Chen, T. M. and Chen, S. F. (1982) Diseases of tea and their control in the people's republic of China. *Plant Disease* **66**: 961-965.

Chen, Z. and Lin, Z. (2015) Tea and human health: biomedical functions of tea active components and current issues. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)* **16:** 87-102.

Chen, Z., Malamy, J., Henning, J., Conrath, U., Sanchez-Casas, P., Silva, H., Riciglianoi, J., and Klessigii, D. F. (1995) Induction, modification, and transduction of the salicylic acid signal in plant defense responses. *Proceedings of the National Academy of Sciences* **92:** 4134-4137.

Chitra, K., Ragupathi, N., Dhanalakshmi, K., Mareeshwari, P., Indra, N., Kamalakannan, A., Sankaralingam, A. and Rabindran, R. (2008) Salicylic acid induced systemic resistant on peanut against

Alternaria alternate. *Archives of Phytopathology and Plant Protection* **41:** 50-56.

Choudhury, M. D., Paul, S. B., Choudhury, S., Choudhury, S. and Choudhury, P. P. N. (2009) Isolation, characterization and bio-activity screening of compound from *Clerodendrum viscosum* Vent. *Assam University Journal of Science and Technology: Biological Sciences* **4:** 29-34.

Christ, U. and Mosinger, E. (1989) Pathogenesis-related proteins of tomato: I . Induction by *Phytophthora infestans* and other biotic and abiotic inducers and correlations with resistance. *Physiological and Molecular Plant Pathology* **35:** 53-65.

Cohen, Y., Rubin, A. E. and Vaknin, M. (2011) Post infection application of DL-3-amino-butyric acid (BABA) induces multiple forms of resistance against *Bremia lactucae* in lettuce. *European Journal of Plant Pathology* **130:** 13-27.

Conrath, U. (2009) "Priming of induced plant defense responses." *In:* Advances in Botanical Research Vol. 51 ed. VanLoon L. C., editor. (London: Elsevier), pp. 361-395.

Conrath, U., Chen, Z., Ricigliano, J. R. and Klessig, D. F. (1995) Two inducers of plant defense responses, 2,6-dichloroisonicotinic acid and salicylic acid, inhibit catalase activity in tobacco. *Proceedings of the National Academy of Sciences* **92:** 7143-7147.

Cortes-Barco, A. M., Goodwin, P. H. and Hsiang, T. (2010) Comparison of induced resistance activated by benzothiadiazole, (2R, 3R)-butanediol and an isoparaffin mixture against anthracnose of *Nicotiana benthamiana*. *Plant Pathology* **59:** 643–653.

Dao, T. T. H., Linthorst, H. J. M. and Verpoorte, R. (2011) Chalcone synthase and its functions in plant resistance. *Phytochemistry Reviews* **10:** 397-412.

Das, K. and Roychoudhury, A. (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* **2:** 53.

Das, K. and Roychoudhury, A. (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* **2:** 53.

Dasgupta, S., Saha, D. and Saha, A. (2005) Levels of common antigens in determining pathogenicity of *Curvularia eragrostidis* in different tea varieties. *Journal of Applied Microbiology* **98**:1084-1092.

De Vos, M., Van Oosten, V. R., Van Poecke, R. M. P., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., Buchala, A. J., Metraux, J. P., Van Loon, L. C., Dicke, M. and Pieterse, C. M. J. (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions* **18:** 923-937.

Deepak, S. A., Oros, G., Sathyanarayana, S. G., Shetty, N. P., Shetty, H. S. and Sashikanth, S. (2005) Antisporulant activity of leaf extracts of Indian plants against Sclerospora graminicola causing downy mildew disease of pearl millet. *Archives of Phytopathology and Plant Protection* **38:** 31-39.

Dickens, J. S. W. and Cook, R. T. A. (1989) *Glomerella cingulata* on *Camellia. Plant pathology* **38:** 75-85.

Dixon, R. A. and Paiva, N. (1995) L. Stress-induced phenylpropanoid metabolism. *The Plant Cell* **7:**1085-1097.

Duan, C., Yu, J., Bai, J., Zhu, Z. and Wang, X. (2014) Induced defense responses in rice plants against small brown plant hopper infestation. *The crop journal* **2:** 55-62.

Duan, C., Yu, J., Bai, J., Zhu, Z. and Wang, X. (2014) Induced defense responses in rice plants against small brown planthopper infestation. *The Crop Journal* **2:** 55-62.

Duan, C., Yu, J., Bai, J., Zhu, Z. and Wang, X. (2014) nduced defense responses in rice plants against small brown planthopper infestation. *The Crop Journal* **2:** 55-62.

Dube, H. C. (2001) Rhizobacteria in biological control and plant growth promotion. *Journal of Mycology and Plant Pathology* **31**: 9-21.

Dufoura, M. C., Lambertb, C., Bouscauta, J., Merillonb, J. M. and Corio-Costet, M. F. (2013) Benzothiadiazole-primed defence responses and enhanced differential expression of defence genes in *Vitis vinifera* infected with biotrophic pathogens *Erysiphe necator* and *Plasmopara viticola. Plant Pathology* **62:** 370-382.

Edreva, A. (2005) Pathogenesis-related proteins: research progress in the last 15 years. *General and Applied Plant Physiology* **31:** 105-124.

Ejtahed, R. S., Radjabian, T. and Tafreshi, S. A. H. (2015) Expression analysis of phenylalanine ammonia lyase gene and rosmarinic acid production in *Salvia officinalis* and *Salvia virgata* shoots under salicylic acid elicitation. *Applied Biochemistry and Biotechnology* **176:** 1846-1858.

El-Mougy, N. S., Abdel-Kader, M. M., Lashin, S. M. and Megahed, A. A. (2013) Fungicides alternatives as plant resistance inducers against foliar diseases incidence of some vegetables grown under plastic houses conditions. *International Journal of Engineering and Innovative Technology* **3:** 71-81.

Falade M. J. (2017) *In vitro* evaluation of antifungal activities of six plant extracts against *Colletotrichum lindemuthianum sensu-lato. American Journal of Plant Biology* **2:** 61-65.

Farzad, M., Soria-Hernanz, D. F., Altura, M., Hamilton, M. B., Weiss, M. R. Elmendorf, H. G. (2005) Molecular evolution of the chalcone synthase gene family and identification of the expressed copy in flower petal tissue of *Viola cornuta. Plant Science* **168:** 1127-1134.

Ferrer, J. L., Austin, M. B., Stewart, C. Jr., Noel J. P. (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiology and Biochemistry* **46:** 356–370.

Fitsum, S., Amin, M., Selvaraj, T. and Alemayehu, A. (2014) *In vitro* evaluation of some fungicides and bioagents against common bean anthracnose (*Colletoteichum lindemuthianum* Sacc. Magnus) Briosi and Cavara. *African journal of microbiology research* **8:** 2000-2005.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G. Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J. P., Kessmann, H. and Ryals, J. (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *The Plant Journal* **10:** 61-70.

Gajera, H., Domadiya, R., Patel, S., Kapopara, M. and Golakiya, B. (2013) Molecular mechanism of Trichoderma as bio-control agents against phytopathogen system - a review. *Current Research in Microbiology and Biotechnology* **1:** 133-142.

Ghawana S., Paul A., Kumar H., Kumar A., Singh H. and Bhardwaj P.K. (2011) An RNA isolation system for plant tissues rich in secondary metabolites. *BMC Research Notes* **4:** 85-89.

Ghosh, R. (2015) Enzymatic responses of ginger plants to *Pyithium* infection after SAR induction. *Journal of Plant Pathology & Microbiology* **6:** 283.

Ghosh, R. (2015) Enzymatic responses of ginger plants to *Pythium* infection after SAR induction. *Journal of Plant Pathology & Microbiology* **6:** 283.

Ghosh, R. and Purkayastha, R. P. (2003) Molecular diagnosis and induced systemic protection against rhizome rot disease of ginger caused by *Pythium aphanidermatum*. *Current Science* **85:** 1782-1787.

Giberti, S., Bertea, C. M., Narayana, R., Maffei, M. E. and Forlani, G. (2012) Two phenylalanine ammonia lyase isoforms are involved in

the elicitor-induced response of rice to the fungal pathogen *Magnaporthe oryzae. Journal of Plant Physiology* **169:** 249-254.

Gilardi, G., Demarchi, S., Garibaldi, A. and Gullino, M. L. (2013) Management of downy mildew of sweet basil (*Ocimum basilicum*) caused by *Peronospora belbahrii* by means of resistance inducers, fungicides, biocontrol agents and natural products. *Phytoparasitica* **41:** 59-72.

Gilardi, G., Demarchi, S., Gullino, M. L. and Garibaldi, A. (2014) Managing Phytophthora crown and root rot on tomato by pre-plant treatments with biocontrol agents, resistance inducers, organic and mineral fertilizers under nursery conditions. *Phytopathologia Mediterranea* **53:** 205–215.

Guleria, S. and Kumar, A. (2006) Antifungal activity of some Himalayan medicinal plants using direct bioautography. *Journal of Cell and Molecular Biology* **5:** 95-98.

Guo, X., Park, Y., Freedman, N. D., Sinha, R., Hollenbeck, A. R., Blair, A., Chen, H. (2014). Sweetened beverages, coffee, and tea and depression risk among older US adults. *PLoS ONE* **9:** e94715.

Hammerschmidt, R., Nuckles, E. and Kuc, J. (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium. Physiological Plant Pathology* **20:** 73-82.

Hashemitabar, M., Kolahi, M., Tabandeh, M. R., Jonoubi, P. and Majd, A. (2014) cDNA cloning, phylogenic analysis and gene expression pattern of phenylalanine ammonia-lyase in sugarcane (*Saccharum officinarum* L.). *Brazilian Archives of Biology and Technology* **57:** 456-465.

Hassan, M. A. E. and Abo-Elyousr, K. A. M. (2013) Activation of tomato plant defence responses against bacterial wilt caused by *Ralstonia*

solanacearum using DL-3-aminobutyric acid (BABA). *European Journal of Plant Pathology* **136:**145-157.

Higa, A., Hidaka, T., Minai, Y., Matsuaka, Y. and Higa, M. (2001) Active oxygen radicals induce peroxidase activity in rice blade tissue. *Bioscience, Biotechnology, and Biochemistry* **65**: 1852-1855.

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y. H., Yu, J. Q. and Chen, Z. (2010) Functional analysis of the Arabidopsis pal gene family in plant growth, development, and response to environmental stress. *Plant Physiology* **153:** 1526–1538.

Huang, Y., Li, L., Smith, K. P. and Muehlbauer, G. J. (2016) Differential transcriptomic responses to *Fusarium graminearum* infection in two barley quantitative trait loci associated with *Fusarium* head blight resistance. *BMC Genomics* **17:**387.

Hukkanen, A. T., Kokko, H. I., Buchala, A. J., Mcdougall, G. J., Stewart, D., Renlampi, S. O. K. and Karjalainen, R. O. (2007) Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. *Journal of Agricultural and Food Cheistry* **55:** 1862-1870.

Hu-Shu Xia. (1996) A preliminary evaluation of tea germplasm resource resistance to tea grey blight disease in Anhui Province, China. *Crop Genetic Resources* **2**: 41-42.

Ishnava, K. B., Chauhan, K. H. and Bhatt, C. A. (2012) Screening of antifungal activity of various plant leaves extracts from Indian plants. *Archives of Phytopathology and Plant Protection* **45:** 152-160.

Jain, I., Jain, P., Bisht, D., Sharma, A., Srivastava, B. and Gupta, N. (2015) Comparative evaluation of antibacterial efficacy of six Indian plant extracts against *Streptococcus* m*utans*. *Journal of Clinical and Diagnostic Research* **9:** ZC50-ZC53.

Jiang, C., Schommer, C. K., Kim, S. Y. and Suh D. Y. (2006) Cloning and characterization of chalcone synthase from the moss, *Physcomitrella patens. Phytochemistry* **67:** 2531–2540.

Jigisha, A., Nishant, R., Navin, K. and Pankaj, G. (2012) Green tea: a magical herb with miraculous outcomes. *International Research Journal of Pharm*acy **3**:139-148.

Jin, Q., Yao, Y., Cai, Y. and Lin, Y. (2013) Molecular cloning and sequence analysis of a phenylalanine ammonia-lyase gene from *Dendrobium*. *PLoS ONE* **8:** e62352.

Johnny, L., Yusuf, U. K. and Nulit, R. (2011) Antifungal activity of selected plant leaves crude extracts against a pepper anthracnose fungus, *Colletotrichum capsici* (Sydow) butler and bisby (Ascomycota: Phyllachorales). *African Journal of Biotechnology* **10:** 4157-4165.

Jones, J. D. G. and Dangl, J. L. (2006). The plant immune system. *Nature* **444:** 323-329.

Jong, F. D., Hanley, S. J., Beale, M. H. and Karp, A. (2015) Characterisation of the willow phenylalanine ammonia-lyase (PAL) gene family reveals expression differences compared with poplar. *Phytochemistry* **117:** 90–97.

Justyna, P-G. and Ewa, K. (2013) Induction of resistance against pathogens by β-aminobutyric acid. *Acta Physiologiae Plantarum* **35:**1735-1748.

Kanaya, S., Yamada, Y., Kinouchi, M., Kudo, Y. and Ikemura, T. (2001) Codon usage and tRNA genes in eukaryotes: correlation of codon usage diversity with translation efficiency and with CG-dinucleotide usage as assessed by multivariate analysis. *Journal of Molecular Evolution* **53:** 290-298.

Karakaya, A. and Bayraktar, H. (2010) Botrytis Disease of Tea in Turkey. *J Phytopathol* **158:** 705-707.

Kaur, A. and Kolte, S. J. (2001) Protection of mustard plants against staghead phase of white rust by Foliave treatment with benzothiadiazole. An activator of plant defense system. *Journal of Mycology and Plant Pathology* **31:** 133-138.

Khan, J. A. and Yadav, K. P. (2011) Assessment of antifungal properties of *Ricinus communis*. *Journal of pharmaceutical and biomedical sciences* **11:** 1-3.

Kildea, S., Ransbotyn, V., Khan, M. R., Fagan, B., Leonard, G., Mullins, E., and Doohan, F. M. (2007) *Bacillus megaterium* shows potential for the biocontrol of *Septoria tritici* blotch of wheat. *Biological Control* **47:** 37-45.

Kim, D. S. and Hwang, B. K. (2014) An important role of the pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *Journal of Experimental Botany* **65:** 2295–2306.

Kiran, K., Linguraju, S. and Adiver, S. (2006) Effect of plant extract on Sclerotium rolfsii, the incitant of stem rot of ground nut. *Journal of Mycology and Plant Pathology* **36:** 77-79.

Kiwan, Y. and Lee, M. Y. (2003) Environmental stress-induced extracellular isoperoxidase RC3 from rice. *Journal of Environmental Biology* **24**:17-22.

Kloepper, J. W., Tuzun, S. and Kud, J. A. (1992) Proposed definitions related to induced disease resistance. *Biocontrol Science and Technology* **2:** 349-351.

Kogel, K. H. and Langen, G. (2005) Induced disease resistance and gene expression in cereals. *Cellular Microbiology* **7:** 1555–1564.

Koley, S., Mahapatra, S. S. and Kole, P. C. (2015) In vitro efficacy of bio-control agents and botanicals on the growth inhibition of *Alternaria solani* causing early leaf blight of tomato. *International journal of bio-resource, environment and agricultural* sciences **1:** 114-118.

Kumar, A. and Ellis, B. E. (2001) The phenylalanine ammonia-lyase gene family in Raspberry. Structure, expression, and evolution. *Plant Physiology* **127:** 230-239.

Kumar, U. M. N. and Panneerselvan T. (2015) Efficacy of aqueous and ethanol extracts *Casuarina equisetifolia* for potential antimicrobial activity. *World Journal of Pharmacy and Pharmaceutical Sciences* **4:** 1877-1882.

Kumhar, K. C., Babu, A., Bordoloi, M., Benarjee, P. and Rajbongshi, H. (2016) Comparative bioefficacy of fungicides and *Trichoderma* spp. against *Pestalotiopsis theae*, causing grey blight in tea (*Camellia* sp.): An *In vitro* study. *International Journal of Current Research in Biosciences and Plant Biology* **3:** 20-27.

La Camera, S., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M. and Heitz, T. (2004) Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. *Immunological Reviews* **198:** 267-284.

Lalitha. V., Kiran. B. and Raveesha. K. A. (2011) Antifungal activity of *Polyalthia longifolia* (Sonn.) Thw. against seed borne fungi of paddy (*Oryza sativa*. L). *Journal of Phytology* **3:** 4-8.

Landi, L., Feliziani, E. and Romanazzi, G. (2014) Expression of defense genes in strawberry fruits treated with different resistance inducers. *Journal of Agricultural and Food Chemistry* **62:** 3047-3056.

Langebartels, C., Wohlgemuth, H., Kschieschan, S., Grun, S. and Sandermann, H. (2002) Oxidative burst and cell death in ozone-exposed plants. *Plant Physiol Biochem* **40:** 567-575.

Langebartels, C., Wohlgemuth, H., Kschieschan, S., Grun, S. and Sandermann, H. (2002) Oxidative burst and cell death in ozone-exposed plants. *Plant Physiology and Biochemistry* **40:** 567–575.

Lanubile, A., Bernardi, J., Battilani, P., Logrieco, A. and Marocco, A. (2012) Resistant and susceptible maize genotypes activate different transcriptional responses against *Fusarium verticillioides*. *Physiological and Molecular Plant Pathology* **77:** 52-59.

Lanubile, A., Pasini, L. and Marocco, A. (2010) Differential gene expression in kernels and silks of maize lines with contrasting levels of ear rot resistance after Fusarium verticillioides infection. *Journal of Plant Physiology* **167:** 1398-1406.

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *The Plant Journal* **10:** 71-82.

Lawton, M. A., Dixon, R. A., Hahlbrock, K. and Lamb, C. (1983) Rapid induction of the synthesis of phenylalanine ammonia-lyase and of chalcone synthase in elicitor-treated plant cells. *European Journal of Biochemistry* **129:** 593-601.

Le Roy, J., Huss, B., Creach, A., Hawkins, S. and Neutelings, G. (2016) Glycosylation is a major regulator of phenylpropanoid availability and biological activity in plants. *Frontiers in Plant Science* **7:** 735.

Le Roy. J., Huss, B., Creach, A., Hawkins, S. and Neutelings, G. (2016) Glycosylation is a major regulator of phenylpropanoid availability and biological activity in plants. *Frontiers in Plant Science* 7:735.

Leclere, V., Bechet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M. and Jacques, P. (2005) Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Applied and Environmental Microbiology* **71:** 4577-4584.

Leon, I. P. and Montesano, M. (2013) Activation of defense mechanisms against pathogens in mosses and flowering plants. *International Journal of Molecular Sciences* **14:** 3178-3200.

Liew, C. F., Loh, C. S., Goh, C. J. and Lim, S. H. (1998) The isolation, molecular characterization and expression of dihydroflavonol 4-reductase cDNA in the orchid, Bromheadia finlaysoniana. *Plant Science* **135:** 161-169

Liu, F., Hou, L., Raza, M. and Cai L. (2017) *Pestalotiopsis* and allied genera from *Camellia*, with description of 11 new species from China. *SCIENTIFIC REPORTS* **7:** 866.

Liu, X., Hou, F., Li, G. and Sang, N. (2015) Effects of nitrogen dioxide and its acid mist on reactive oxygen species production and antioxidant enzyme activity in Arabidopsis plants. *Journal of Environmental Sciences* **34:** 93-99.

Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**: 402-408.

Llorens, E., García-Agustín, P. and Lapena, L. (2017) Advances in induced resistance by natural compounds: towards new options for woody crop protection. *Scientia Agricola* **74:** 90-100.

Lo, C., Coolbaugh, R. C. and Nicholson, R. L. (2002) Molecular characterization and in silico expression analysis of a chalcone synthase gene family in *Sorghum bicolor. Physiological and Molecular Plant Pathology* **61:** 179-188.

Macedo, J. A., Ferreira, L. R., Camara, L. E., Santos, J. C., Gambero, A., Macedo, G. A. and Ribeiro, M. L. (2012) Chemopreventive potential of the tannase-mediated biotransformation of green tea. *Food chemistry* **133:** 358-365.

Mahadevan, A. and Sridhar, R. (1982) Methods in Physiological Plant Pathology 2nd *edition. Sivakami Publications*, Madras.

Malabadi, R. B. and Vijay Kumar, S. (2007) Assessment of antifungal activity of some medicinal plants. *International Journal of Pharmacology* **3:** 499-504.

Mandal, S. M., Chakraborty, D. and Dey S. (2010) Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signaling and Behavior* **5:** 359-368.

Mandal, S. M., Chakraborty, D. and Dey, S. (2010) Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signaling & Behavior* **5:** 359-368.

Mardanova, A. M., Hadieva, G. F., Lutfullin, M. T., Khilyas, I. V., Minnullina, L. F., Gilyazeva, A. G., Bogomolnaya, L. M. and Sharipova, M. R. (2017) *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi. *Agricultural Sciences* **8:** 1-20.

Mauch, F., Mauch-Mani, B. and Boller, T. (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase and ß-1,3- glucanase. *Plant Physiology* **88**: 936-942.

Meegahakumbura, M. K., Wambulwa, M. C., Thapa, K. K., Li, M. M., Moller, M., Xu, J. C., Yang, J. B., Liu, B. Y., Ranjitkar, S., Liu, J., Li, D. Z. and Gao, L. M. (2016) Indications for three independent domestication events for the tea plant (*Camellia sinensis* (L.) O. Kuntze) and new insights into the origin of tea germplasm in china and india revealed by nuclear microsatellites. PLoS ONE **11:** e0155369.

Meena, A. K., Mali, B. L. and Chaudhary, S. L. (2007) Evaluation of partially purified plant products and antimicrobial chemicals preparation against bacterial pathogens. *Journal of Mycology and Plant Pathology* **37:** 365-368.

Meena, B., Marimuthu, T. and Velazhahan, R. (2001) Salicylic acid induces systemic resistance in groundnut against late leaf spot caused by

Cercosporidium personatum. *Journal of Mycology and Plant Pathology* **31:** 139-145.

Mewari, N., Chaturvedi, P., Kumar, P. and Rao, P. B. (2007) Antimicrobial activity of moss extracts plant pathogen. *Journal of Mycology and Plant* Pathology **37:** 359-360.

Miltiadis, V., Christopoulos, Tsantili, E. (2015) Participation of phenylalanine ammonia-lyase (PAL) in increased phenolic compounds in fresh cold stressed walnut (*Juglans regia* L.) kernels. *Postharvest Biology and Technology* **104:** 17-25.

Mondal, T. K., Bhattacharya, A., Laxmikumaran, M. and Ahuja, P. S. (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell, Tissue and Organ Culture* **76:** 195–254.

Muhire, B. M., Varsani, A., Martin, D. P. (2014) SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS ONE* 9:e108277.

Naglot, A., Goswami, S., Rahman, I., Shrimali, D. D., Yadav, K. K., Gupta, V. K., Rabha, A. J., Gogoi H. K. and Veer V. (2015) Antagonistic potential of native *Trichoderma viride* strain against potent tea fungal pathogens in north east India. *The Plant Pathology Journal* **31:** 278-289.

Narayanasamy P. (2013) Abiotic Biological Control Agents for Crop Disease Management. *In:* Biological Management of Diseases of Crops. Progress in Biological Control, vol 15. Springer, Dordrecht, pp. 511-632.

Nasrin, F., Ahmad, S. and Kamrunnahar. (2012) Evaluation of antimicrobial, antioxidant and cytotoxic activities of methanolic extracts of *Lagerstroemia speciosa* leaves and barks. *Journal of Applied Pharmaceutical Science* **2:** 142-147.

Navarova, H., Bernsdorff, F., Doring, A-C. and Zeiera, J. (2012) Pipecolic Acid, an endogenous mediator of defense amplification and priming,

is a critical regulator of inducible plant immunity. *The Plant Cell* **24:** 5123-5141.

Naz, R. and Bano, A. (2012) Antimicrobial potential of *Ricinus communis* leaf extracts in different solvents against pathogenic bacterial and fungal strains. *Asian Pacific Journal of Tropical Biomedicine* **2:** 944-947.

Nielsen, P. and Sørensen, J. (1997) Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. *FEMS Microboilogy Ecology* **22:** 183-192.

Okorska, S., Michalczyk, D., Okorski, A., Piotrowicz-Cieślak, A., Pupel, P., Głowacka, K., Jagielska, T. and Górecki, R. (2014) Variability of *PSPAL1* (phenylalanine ammonia-lyase gene-1) proximal promoter sequence and expression in pea challenged with *Mycosphaerella pinodes*. *Czech Journal of Genetics and Plant Breeding* **50**: 163-170.

Oliveira, M. D. M., Varanda, C. M. R. and Felix, M. R. F. (2016) Induced resistance during the interaction pathogen x plant and the use of resistance inducers. *Phytochemistry Letters* **15:** 152-158.

Oly, W.T., Islam, W., Hasan, P. and Parween, S. (2011) Antimicrobial activity of *Clerodendrum viscosum* vent. (Verbenaceae). *International Journal of Agriculture and Biology* **13:** 222-226.

Oumar, D., Akumah, B. E. and Tchinda, N. D. (2015) Induction of resistance in cocoyam (*Xanthosoma sagittifolium*) to *Pythium myriotylum* by corm treatments with Benzothiadiazole and its effect on vegetative growth. *American Journal of Experimental Agriculture* **5:** 164-171.

Padaria, J. C., Vishwakarma, H., Biswas, K., Jasrotia, R. S. and Singh, G. P. (2014) Molecular cloning and in-silico characterization of high temperature stress responsive pAPX gene isolated from heat tolerant Indian wheat cv. Raj 3765. *BMC Research Notes* **7:** 713.

Padder, B. A., Sharma, P. N., Kapil, R., Pathania, A. and Sharma, O. P. (2010) Evaluation of bioagents and biopesticides against *Colletotrichum lindemuthianum* and its integrated management in common bean. *Notulae Scientia Biologicae* **3:** 72-76.

Pajot, E., Corre, D. L. and Silue, D. (2001) Phytogard and DL-*β*-amino butyric acid (BABA) induce resistance to downy mildew (*Bremia lactucae*) in lettuce (*Lactuca sativa* L). *European Journal of Plant Pathology* **107:** 861-869.

Pallavi, R. V., Nepolean, P., Balamurugan, A., Jayanthi, R., Beulah, T. and Premkumar, R. (2012) *In vitro* studies of biocontrol agents and fungicides tolerance against grey blight disease in tea. *Asian Pacific Journal of Tropical Biomedicine* **2012:** S435-S438.

Pan, S.Q., Ye, X. S., and Kuc, J. (1991) A technique for detection of chitinase, β-1,3-glucanases and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocussing. *Phytopathology journal* **81**: 970-974.

Pandey, A., Yadava, L. P., Mishra, R. K., Pandey, B. K., Muthukumar M. and Chauhan, U. K. (2012) Studies on the incident and pathogenesis of *Colletotrichum gloeosporioides* penz. causes anthracnose of mango. *International Journal of Security and Networks* **3:** 220-232.

Pang, Y., Shen, G., Wu, W., Liu, X., Lin, J., Tan, F., Sun, X. and Tang, K. (2005) Characterization and expression of chalcone synthase gene from *Ginkgo biloba*. *Plant Science* **168:** 1525-1531.

Paolisa, A. D., Pignoneb, D., Morgeseb, A. and Sonnante, G. (2008) Characterization and differential expression analysis of artichoke phenylalanine ammonia-lyase-coding sequences. *Physiologia Plantarum* **132:** 33-43.

Parimala, S. and Sangeetha, D. (2016). Antifungal activity of *Ricinus communis* L. solvent extracts against pathogenic fungi. *Life Science Archives* **2:** 741-746

Parimelazhagan, T. and Francis, K. (1999) Antifungal activity of *Clerodendrum viscosum* against *Curvularia lunata* in rice seeds. *J. Mycology and Plant Pathology* **29:** 139-141.

Pawar, B. T. (2011) Antifungal activity of some leaf extracts against seed-borne pathogenic fungi. *International Multidisciplinary Research Journal* **4:**11-13.

Payyavula, R. S., Navarre, D. A., Kuhl, J. C., Pantoja, A, and Pillai, S. S. (2012) Differential effects of environment on potato phenylpropanoid and carotenoid expression. *BMC Plant Biology* **12:** 39

Peden, J. F. (1991). Analysis of Codon Usage. Ph. D. thesis, University of Nottingham, Nottingham, UK.

Peraza-Sánchez, S. R., Chan-Che, E. O. and Ruiz-Sánchez, E. (2005) Screening of Yucatecan plant extracts to control *Colletotrichum gloeosporioides* and isolation of a new pimarene from *Acacia pennatula*. *Journal of agricultural and food chemistry* **53:** 2429-2432.

Percival, G. C. (2010) Effect of Systemic Inducing Resistance and Biostimulant Materials on Apple Scab Using a Detached Leaf Bioassay. *Arboriculture and Urban Forestry* **36:** 41-46.

Perez-de-Luque, A., Jorrfn, J. V., and Rubiales, D. (2004) Crenate broomrape control in pea by foliar application of benzothiadiazole (BTH). *Phytoparasitica* **32:** 21-29.

Pitakdantham, W., Sutaburtra, T., Chiemsombat, P. and Pitaksutheepong, C. (2010) Isolation and characterization of chalkone synthase gene isolated from *Dendrobium* Sonia Earsakul. *Pakistan Journal of Biological Sciences* **13:** 1000-1005.

Pitsch, N. T., Witsch, B. and Baier, M. (2010) Comparison of the chloroplast peroxidase system in the chlorophyte *Chlamydomonas reinhardtii*, the bryophyte *Physcomitrella patens*, the lycophyte *Selaginella moellendorffii* and the seed plant *Arabidopsis thaliana*. *BMC Plant Biology* **10**: 133.

Polesani, M., Desario, F., Ferrarini, A., Zamboni, A., Pezzotti, M., Kortekamp, A. and Polverari, A. (2008) cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected by Plasmopara viticola. *BMC Genomics* **9:** 142-156.

Pranoothi, E. K., Narendra, K., Joshi, D.S.D.S., Swathi, J., Sowjanya, K.M., Rathnakarreddi, K.V.N., Emmanuel S., Padmavathi, C. and Satya, A. K. (2014) Studies on qualitative, quantitative, phytochemical analysis and screening of *in vitro* biological activities of *Leucas indica* (L) var. Nagalapuramiana. *International Journal of Herbal Medicine* **2:** 30-36.

Premkumar, R., Nepolean, P., Pallavi, V. R., Balamurugan, A. and Jayanthi, R. (2012) Integrated disease management of grey blight in tea. *Two and a Bud* **59:** 27-30.

Punyasiria, P. A. N., Abeysinghea, I. S. B., Kumarb, V., Treutterc, D., Duyd, D., Goschd, C., Martense, S., Forkmannd, G. and Fischer T. C. (2004) Flavonoid biosynthesis in the tea plant *Camellia sinensis*: properties of enzymes of the prominent epicatechin and catechin pathways. *Archives of Biochemistry and Biophysics* **431:** 22–30.

Pye, M. F., Hakuno, F., MacDonald, J. D. and Bostock, R. M. (2013) Induced resistance in tomato by SAR activators during predisposing salinity stress. *Frontiers in plant science* **4:** 116.

Raju, S., Jayalakshmi, S. K., and Sreeramulu, K. (2008) Comparative study on the induction of defense related enzymes in two different cultivars of chickpea (*Cicer arietinum* L) genotypes by salicylic acid, spermine and *Fusarium oxysporum* f. sp. *ciceri. Australian Journal of Crop Science* **2:**121-140.

Ravikumar M.C. and Garampalli R. H. (2013) Antifungal activity of plants extracts against *Alternaria solani*, the causal agent of early blight of tomato. *Archives of Phytopathology and Plant Protection* **46:** 1897-1903.

Rawal, H. C., Singh, N. K. and Sharma, T. R. (2013) Conservation, Divergence, and Genome-Wide Distribution of *PAL* and *POX A* Gene Families in Plants. *International Journal of Genomics* **2013:** Article ID 678969, 10 pages.

Reddy, L., Nagaraju, C. N., Prasanna, Kumar, M. K. and Venkataravanappa, V. (2007) Transmission and host range of the *Papaya ringspot virus* (PRSV). *Journal of Plant Diseases and Protection* **2**: 9-13.

Rejeb, I. B., Pastor, V. and Mauch-Mani, B. (2014) Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. *Plants (Basel)* **3:** 458-475.

Rinez, A., Remadi, M. D., Ladhari, A., Omezzine, F., Rinez, I. and Haouala, R. (2013) Antifungal activity of *Datura metel* L. organic and aqueous extracts on some pathogenic and antagonistic fungi. *African Journal of Microbiology Research* **7:** 1605-1612.

Romanazzi, G., D'Ascenzo, D. and Murolo, S. (2009) Field treatment with resistance inducers for the control of grapevine bois noir. *Journal of Plant Pathology* **91:** 677-682.

Roslan, N. D., Tan, C. S., Ismail, I. and Zainal, Z. (2013) cDNA cloning and expression analysis of the chalcone synthase gene (CHS) from'polygonum minus. *Australian Journal of Crop Science* **7:** 777.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y. (1996) Systemic acquired resistance. *Plant Cell* **8:** 1809-1819.

Sadasivam, S. and Manickam, A. (1996) Biochemical Methods (2nd Edition), new Age International Private Limited. pp. 110-112.

Saha, A. and Chakraborty, B. N. (1992) Phytotoxic effect of metabolic byproducts in the culture filtrate of *Bipolaris carbonum* and *Camellia sinensis. Geobios* **19:** 15-17.

Saha, A., Dasgupta, S. and Saha, D. (2001) Discovery of *Curvularia eragrostidis* on Tea (*Camellia sinensis* (L.) O. Ktze.) leaves from

clonal-cutting nurseries in North Bengal. *Environment and Ecology* **19**: 846–848.

Salar R.K. and Suchitra,. (2009) Evaluation of antimicrobial potential of different extracts of *Solanum xanthocarpum* Schrad. and Wendl. *African Journal of Microbiology Research* **3:** 97-100.

Sambrook J. and Russel D. W. (2001) *A laboratory manual*, Second edition, Cold Spring Harbour, Laboratory Press, USA.

Sarmah, K. C. (1960) Disease of tea and associated crops in North East. Memorandum No.26. Indian Tea Association, Tocklai Experimental Station.

Sarmah, S. R., Dutta, P., Bhattacharyya, P. N., Payeng, B. and Tanti, A. J. (2016) Growth habit of tea pathogens (*Cephaleuros* spp. and *Fusarium solani*) and evaluation of relative susceptibility of selected Tea cultivars. *International Research Journal of Biological Sciences* **5:** 1-9.

Shadlea, G. L., Wesleya, S. V. Kortha, K. L., Chena, F., Lambb, C. and Dixon, R. A. (2003) Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of l-phenylalanine ammonia lyase. *Phytochemistry* **64:** 153-161.

Shaikh, F. T. and Sahera, N. (2016) Antifungal activity of *Pseudomonas aeuroginosa* and *Bacillus subtilis* against pathogens of cucurbitaceous fruits. *IJIRSET* **5:** 3320-3324.

Sharma, P., Jha, A. B., Dubey, R. S. and Pessarakli, M. (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany* **2012:** Article ID 217037, 26 pages.

Sharma, R. K., Negi, M. S., Sharma, S., Bhardwaj, P., Kumar, R., Bhattachrya, E., Tripathi, S. B., Vijayan, D., Baruah, A. R., Das, S. C., Bera, B., Rajkumar, R., Thomas, J., Sud, R. K., Muraleedharan, N., Hazarika, M., Lakshmikumaran, M., Raina, S. N. and Ahuja, P. S.

(2010) AFLP-based genetic diversity assessment of commercially important tea germplasm in India. *Biochemical Genetics* **48:** 549-564

Sharma, R., Mahila, H. R., Mohapatra, T., Bhargava, S. C. and Sharma M. M. (2003) Isolating plant genomic DNA without liquid nitrogen. *Plant molecular biology Reporter* **21:** 43-50.

Sharp, P. M. and Li, W. H. (1986) An evolutionary perspective on synonymous codon usage in unicellular organisms. *Journal of Molecular Evolution* **24:** 28-38.

Sharp, P. M. and Li, W. H. (1987) The codon adaptation index- a measure of directional synonymous codon usage biase, and its potential application. *Nucleic Acid Research* **15:** 1281-1295.

Shrivastava, D. K.and Swarnkar K. (2014) Antifungal activity of leaf extract of neem (*Azadirachta Indica* Linn). *International Journal of Current Microbiology and Applied Sciences* **3:** 305-308.

Shrivastava, D.K. and Swarnkar, K. (2014) Antifungal Activity of leaf extract of Neem (*Azadirachta Indica* Linn). *International Journal of Current Microbiology and Applied Sciences* **3:** 305-308.

Siju, E. N., Rajalakshmi, G. R., Sreejith, K. R., Hariraj, N., Venugopal, V. P., Sneha, V. N. (2011) Anthelmintic activity of leaves of *Clerodendrum viscosum* Vent. *International Journal of Pharma and Bio Sciences* **5:** 25-28.

Singh, A., Sharma, O. P. and Garg, D. K. (2006) Integrated pest management, principle and applications, Vol. 2: applications. *CBS Publishers and distributor, New Delhi.*

Sinha, A. K. and Das, N. C. (1972) Induced resistance in rice plants to *Helminthosporium oryzae*. *Physiological Plant Pathology* **2**:401-410.

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B. and Mauch, M. B. (2012) Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plant Physiology* **158:** 835-843.

Somssich, I. E., and Hahlbrock, K. (1998) Pathogen defence in plants—a paradigm of biological complexity. *Trends in Plant Science* **3:** 86-90.

Sood, N., Sohal, B. S. and Lore, J. S. (2013) Foliar application of benzothiadiazole and salicylic acid to combat sheath blight disease of rice. *Rice Science* **20:** 349-355.

Sreedevi, B., Devi, M. C. and Saigopal, D. V. R. (2011) Induction of defense enzymes in *Trichoderma harzianum* treated groundnut plants against *Macrophomina phaseolina. Journal of Biological Control* **25:** 33–39.

Sticher. L, Mauch-Mani, B. and Métraux, J. P. (1997) Systemic acquired resistance. *Annual Review of Phytopathology* **35:** 235-270.

Su, W., Li, X., Chen, M., Dai, W., Sun, S., Wang, S., Sheng, X., Sun, S., Gao, C., Hou, A., Zhou, Y., Sun, B., Gao, F., Xiao, J., Zhang, Z. and Jiang, C. (2017) Synonymous codon usage analysis of hand, foot and mouth disease viruses: A comparative study on coxsackievirus A6, A10, A16, and enterovirus 71 from 2008 to 2015. *Infection, Genetics and Evolution* **53:** 212–217

Svecova, E., Colla, G. and Crino, P. (2017) Antifungal activity of *Boerhavia diffusa* L. extract against *Phytophthora* spp. in tomato and pepper. *European Journal of Plant Pathology* **148:** 27-34.

Tamm, L., Thüriga, B., Fliessbacha, A., Goltliebb, A. E., Karavanib, S. and Cohen, Y. (2011) Elicitors and soil management to induce resistance against fungal plant diseases. *NJAS - Wageningen Journal of Life Sciences* **58:** 131-137.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30:** 2725-2729.

Tan, G., Liu, K., Kang, J., Xu, K., Zhang, Y., Hu, L., Zhang, J. and Li, C. (2015) Transcriptome analysis of the compatible interaction of tomato with *Verticillium dahliae* using RNA-sequencing. Frontiers in Plant Science **6:** 428.

Thakur, M. and Sohal, B. S. (2013) Role of elicitors in inducing resistance in plants against pathogen infection: A review. *International Scholarly Research Notices Biochemistry* **2013:** Article ID 762412, 10 pages.

Thatcher, L. F., Williams, A. H., Garg, G., Buck, S-A. G. and Singh, K. B. (2016) Transcriptome analysis of the fungal pathogen *Fusarium oxysporum* f. sp. *medicaginis* during colonisation of resistant and susceptible *Medicago truncatula* hosts identifies differential pathogenicity profiles and novel candidate effectors. *BMC Genomics* **17:** 860.

Thembo, K. M., Vismer, H. F., Nyazema, N. Z., Gelderblom, W. C. A. and Katerere, D. R. (2010) Antifungal activity of four weedy plant extracts against selected mycotoxigenic fungi. *Journal of Applied Microbiology* **109:** 1479-1486.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673-4680.

Tian, L., Kong, W. F., Pan, Q. H., Zhan, J. C., Wen, P. F., Chen, J. Y., Wan, S. B. and Huang, W. D. (2006). Expression of the chalcone synthase gene from grape and preparation of an anti-CHS antibody. *Protein Expression and Purification* **50:** 223-228.

Ton, J. and Mauch-Mani, B. (2004) β-amino-butyric acid-induced resistance against necrotrophic pathogensis based on ABA-dependent priming for callose. *The Plant Journal* **38:** 119-130.

Trejo-Saavedra, D. L., García-Neria, M. A. and Rivera-Bustamante, R. F. (2013) Benzothiadiazole (BTH) induces resistance to Pepper golden mosaic virus (PepGMV) in pepper (*Capsicum annuum* L.). *Biological Research* **46:** 333-340.

Walters, D. R., Havis, N. D., Sablou, C. and Walsh, D. J. (2011) Possible trade-off associated with the use of a combination of resistance elicitors. *Physiological and Molecular Plant Pathology* **75:** 188-192.

Wang, Z. W., Jiang, C., Wen, Q., Wang, N., Tao, Y. Y. and Xu, L. A. (2014) Deep sequencing of the *Camellia chekiangoleosa* transcriptome revealed candidate genes for anthocyanin biosynthesis. *Gene* **538:** 1-7.

Wei, L., He, J., Jia, X., Qi, Q., Liang, Z., Zheng, H., Ping, Y., Liu, S. and Sun, J. (2014) Analysis of codon usage bias of mitochondrial genome in *Bombyx mori* and its relation to evolution. *BMC Evolutionary Biology* **14:** 262.

Wessels, J. G. H. and Sietsma, J. H. (1981) Fungal Cell Wells: a survey in plant carbohydrates II. extracellular carbohydrates. *In*: Encyclopedia of Plant Physiology, *eds*. Tanner, W. and Loewuse, F. A. *Springer Verlag*. Barlin, Germany. pp. 352-394.

Wright, F. (1990) The 'effective no of codons' used in a gene. *Gene* **87:** 23-29.

Xu, F., Cai, R., Cheng, S., Du, H., Wang, Y. and Cheng, S. (2008) Molecular cloning, characterization and expression of phenylalanine ammonia-lyase gene from *Ginkgo biloba. African Journal of Biotechnology* **7:** 721-729.

Xu, H., Park, N., Li, X., Kim, Y. K., Lee, S. Y. and Park, S. U. (2010) Molecular cloning and characterization of phenylalanine ammonia-lyase, cinnamate 4-hydroxylase and genes involved in flavone biosynthesis in *Scutellaria baicalensis*. *Bioresource Technology* **101:** 9715-9722.

Xu, X. Z., Liu, Q. P., Fan, L. J., Cui, X. F. and Zhou X. P. (2008) Analysis of synonymous codon usage and evolution of begomoviruses. *Journal of Zhejiang University SCIENCE B* **9:** 667-674.

Yamanishi, T. (Ed.) (1991) Proceedings, International Symposium on Tea Science. Organizing Committee, ISTS, NIVOT, Shizuoka, Japan.

Yanase, Y. and Takeda, Y. (1987) Method for testing the resistance to tea grey blight caused by *Pestalotia longiseta*. Spegazzini in tea breeding. *Bulletin of the National Research Institute of Vegetables, Ornamental plants and tea*, B (Kenya) **1**:1-4.

Zeng, S., Liu, Y., Zou, C., Huang, W. and Wang, Y. (2013) Cloning and characterization of phenylalanine ammonia-lyase in medicinal *Epimedium* species. *Plant Cell, Tissue and Organ Culture* **113:** 257-267.

Zhang, J., Wang, M., Liu, W. Q., Zhou, J. H., Chen, H. T., Ma, L. N., Ding, Y. Z., Gu, Y. X. and Liu, Y. S. (2011) Analysis of codon usage and nucleotide composition bias in polioviruses. *Virology Journal* **8:** 146.

Zhang, Y. P., E, Z. G., Jiang, H., Wang, L., Zhou, J. and Zhu, D.F. (2015) A comparative study of stress-related gene expression under single stress and intercross stress in rice. *Genetics and Molecular Research* **14:** 3702-3717.

Zhao, Y., Zheng, H, Xu, A., Yan, D., Jiang, Z., Qi, Q. and Sun, J. (2016) Analysis of codon usage biase of envelope glycoprotein genes in nuclear polyhedrosis virus (NPV) and its relation to evolution. *BMC Genomics* **17:** 677.

Zhou, L., Wang, Y. and Peng, Z. (2011) Molecular characterization and expression analysis of chalcone synthase gene during flower development in tree peony (*Paeonia suffruticosa*). *African Journal of Biotechnology* **10:** 1275-1284.

Zhou, Z., Dang, Y., Zhou, M., Li, L., Yu, C. H., Fu, J., Chen, S. and Liu, Y. (2016) Codon usage is an important determinant of gene expression levels largely through its effects on transcription. *Proceedings of the National Academy of Sciences of the United States of America* **113:** E6117-E6125.

Zhu, Q., Xie, X., Lin, H., Sui, S., Shen, R., Yang, Z., Lu, K., Li, M. and Liu, Y. (2015) Isolation and functional characterization of a phenylalanine ammonia-lyase gene (*SsPAL1*) from *Coleus* (*Solenostemon scutellarioides* (L.) Codd). *Molecules* **20:** 16833-16851.

Zhu, X., Lin, H., Si, Z., Xia, Y., Chen, W. and Li, X. (2016) Benzothiadiazole-mediated induced resistance to *Colletotrichum musae* and delayed ripening of harvested banana fruit. *J. Agric. Food Chem*. **64:** 1494-1502.

Ziaei, M., Sharifi, M., Behmanesh, M. and Razavi, K. (2012) Gene expression and activity of phenyl alanine amonialyase and essential oil composition of *Ocimum basilicum* L. at different growth stages. *Iranian Journal of Biotechnology* **10:** 32-39.

APPENDIX I

Nucleotide sequences of isolated defense related genes submitted in the GenBank

1. *Camellia sinensis* chalcone synthase (PAL) mRNA, partial cds (Acc. No.: KF303590)

TTCTACAACAATGGGTTGCCATCGAATCTTACAGGAGGGCGCAATCCGAGCTTGGATTATG
GTTTCAAGGGAGCAGAGATTGCCATGGCCTCGTATTGCTCAGAACTCCAGTTCCTTGCCAA
TCCTGTAACCAACCATGTCCAAAGCGCCGAGCAACATAACCAAGATGTGAACTCCTTAGGC
CTAATCTCTTCAAGAAAAACCGCTGAAGCTATTGATATCTTGAAGCTCATGTCCTCTACAT
ATCTAGTGGCACTATGTCAAGCCATAGATTTGAGGCATTTGGAGGAGAATTTGAGGAACAC
TGTCAAGAACAGTGTTAGCCAAGTAACG

2. *Camellia sinensis* chalcone synthase (CHS) mRNA, partial cds (Acc. No.: KJ866952)

CAAGGTTGCTTTGCCGGTGGCACAGTACTTCGCCTAGCCAAGGACTTGGCCGAGAACAACA
AAGGAGCCCGGGTTTTAGTCGTGTGCTCTGAGATAACTGCGGTCACATTCCGTGGGCCTAA
TGATACCCATCTCGACAGCCTTGTGGGTCAAGCCCTATTTGGCGATGGCGCGGCTGCTATT
ATAGTTGGGTCAGACCCAATTCCAGAAGTTGAGAAACCGTTGTTCGAGTTGGTCTCAGCGG
CCCAAACCATTCTCCCCGATAGTGACGGTGCCATTGACGGACACCTCCGTGAAGTGGGCCT
TACATTTCACCTCCTCAAGGATGTTCCTGGGCTCATCAAA

3. *Camellia sinensis* chalcone synthase (APX) mRNA, partial cds (Acc. No.: KR296654)

GACAAAGCCAAGAGGAAGCTCAGAGGCCTCATCGCTGAGAAGAACTGTGCTCCGATTATGC
TCCGTCTCGCATGGCACTCTGCTGGTACTTACGATGTGACGACGAAGACCGGAGGTCCGTT
CGGAACAATGAGGCACAAGCTTGAGCAAGGTCACGAGGCCAACAACGGCCTTGAGATCGCC
GTCAGGCTACTAGAGCCTATCAAGGAGCAGTTCCCGATGATCTCTTATGCTGACTTCTATC
AGTTGGCTGGAGTTGTTGCCGTTGAAATTACTGGGGGACCTGATGTTCCATTCCATCCAGG
AAGGGAGGATAAGCCTGAGCCACCTGTCGAAGGCCGCCTTCCTGATGCTACCAAGGGAACG
GACCATCTGAGAGATGTGTTTGTTAAACACATGGGCCTCACTGACAAGGACATTGTTGCTC
TATCTGGTGGCCACACCCTGGGAAGGTGCCACAAGGAGCGTTCTGGATTTGAAGGGCCCTG
GACTGCCAATCCACTCATCTTTGATAACTCCTACTTCACGGAACTCCTGACTGGAGAGAAG
GAAGGGCTTCTACAACTGCCATCTGACAAGGCTCTCCTCAATGATCCTGTCTTCCGCCCTC
TTGTTGAGAAATATGCTGCGGATGAGGATGCATTCTTTGCAGATTATGCAGAA

APPENDIX II

List of Publications

1. **Das S**, Chakraborty P, Mandal P, Saha D and Saha A. (2017) Phenylalanine ammonia-lyase gene induction with benzothiadiazole elevates defence against *Lasiodiplodia theobromae* in tea in India. *J Phytopathol.*; 00:1–7. https:// doi.org/ 10.1111/jph.12615.

2. Saha A., **Das S.,** Chakraborty P., Saha B., Saha D. and Saha A. (2016) Two new bottle gourd fruit rot causing pathogens from sub-Himalayan West Bengal. *International Journal of Agricultural Technology* **12(2):** 321-332.

3. Chakraborty P., **Das S.,** Saha D. and Saha A. (2016) First report of Soybean mosaic virus infecting bottle gourd plant in India. *Plant Disease* **100(7):** 1509.

4. Chakraborty P., **Das S.,** Saha B., Karmakar A., Saha D. and Saha A. (2017) *Rose rosette virus*: an emerging pathogen of garden roses in India. *Australasian Plant Pathology* **46(3):** 223–226.

5. Chakraborty P., **Das S.,** Saha B., Sarkar P., Karmakar A., Saha A., Saha D. and Saha A. (2015) Phylogeny and synonymous codon usage pattern of *Papaya ringspot virus* coat protein gene in sub-Himalayan region of north-east India. *Canadian Journal of Microbiology* **61(8):** 555-564.

6. Saha A., Saha B., Chakraborty P., Sarkar P., **Das S.,** and Saha D. (2014) Molecular detection and diversity analysis of some potyviruses associated with mosaic diseases of papaya, common bean and potato growing in sub-Himalayan West Bengal. *VEGETOS* **27(2):** 338-346.

7. Mandal H., Chakraborty P., **Das S.,** Saha A., Sarkar T., Saha D. and Saha A. (2017) Biocontrol of virulent *Ralstonia solanacearum* isolates by an indigenous *Bacillus cereus*. *International Journal of Agricultural Technology* **13(1):** 19-30.

8. Saha A., Saha B., Chakraborty P**.**, Sarkar P., **Das S**., and Saha D. (2014) Molecular detection and diversity analysis of some potyviruses associated with mosaic diseases of papaya, common bean and potato growing in sub-Himalayan West Bengal. *VEGETOS* **27(2):** 338-346.

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ORIGINAL ARTICLE

WILEY Journal of Phytopathology

Phenylalanine ammonia-lyase gene induction with benzothiadiazole elevates defence against *Lasiodiplodia theobromae* in tea in India

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UGC

Abstract

Tea is widely used as an aromatic beverage cultivated in more than 35 countries from different continents. The global production of tea during the year 2015–2016 was 5,303 million kg where India stands 2nd contributing 23% of the world production. Diplodia disease is one of the most common diseases of tea in sub-Himalayan West Bengal, India. Phenylpropanoids act as a precursor of several secondary metabolites that are synthesized at the time of biotic and abiotic stress conditions. During the induction of systemic acquired resistance, salicylic acid production is regulated by phenylalanine ammonia-lyase (PAL) gene expression. In this communication, twelve-month-old tea seedlings (TV-26) were treated with benzothiadiazole (BTH) and inoculated with *Lasiodiplodia theobromae*. Transcript accumulation in different treatments, *that is* treated-uninoculated, untreated-inoculated and treated-inoculated were measured in comparison with untreated-uninoculated control to study the effect of BTH in defence induction. From the results, it was evident that BTH-treated and *L. theobromae*-inoculated plants showed higher transcript accumulation (3.81 μg/μl) and reduced disease index (3.8 ± 0.02) in comparison with untreated control where transcript accumulation was 3.26 μg/μl and disease index reduced to 14.6 ± 0.05. After sequencing, nucleotide and deduced protein sequences were compared, and 3D structure was established where three lyase superfamily (cl26059) motifs were found. The increase in PAL activity was also observed in treated-inoculated plants (from 2.5 to 4.4 μmol min^{-1} g^{-1}) in comparison with healthy control (from 2.5 to 2.7 μmol min^{-1} g^{-1}) after 4th day of treatment. Nonetheless, BTH might be one of the significant inducers of PAL which can be used to induce of disease resistance in tea plants in India.

KEYWORDS
benzothiadiazole, diplodia disease, phenylpropanoids, transcript accumulation

1 | INTRODUCTION

Tea (*Camellia sinensis* (L.) O. Kuntze.) is a tree member of the family Theaceae. Processed tea leaves are widely used as an aromatic beverage cultivated in more than 35 countries from different continents. It is a rich source of antioxidant, epigallocatechin, epicatechin, gallocatechin and catechin and several other phenolic compounds that play important roles in improving human health and also providing resistance against a wide range of pathogens. Green leaves of tea are also used as vegetable in Burma, Thailand and also have some medicinal importance against blood pressure, coronary heart diseases and diabetes (Azzahra, Fouzia, Mohammed, & Noureddine, 2012; Chen, 1999;

Macedo & Oliveira, 2012; Mondal, Bhattacharya, Laxmikumaran, & Ahuja, 2004; Punyasiria et al., 2004). The global production of tea during the year 2015–2016 was 5,303 million kg. India contributes 23% of the world production (Tea Board India, http://www.teaboard.gov.in/pdf/Tea_Board_Annual_Report_2015_16_pdf3913.pdf). Tea plants are exposed to a number of pathogens, mostly fungi reducing the quality and production substantially. Blister blight, grey blight, brown blight, black rot and diplodia disease caused by *Exobasidium vexans*, *Pestalotiopsis theae*, *Colletotrichum camelliae*, *Corticium invisum* and *L. theobromae* (Pat.) Griffon and Mauble (synonym: *Botryodiplodia theobromae*), respectively, are the major limitations to tea production worldwide. Diplodia disease is one of the most common diseases of tea in sub-Himalayan West Bengal as it can damage any part of tea plant at every stage of development (Sarmah, 1960).

Different defensive mechanisms have evolved in plant systems to combat against biotic and abiotic stresses (Jones and Dangl, 2006). Differential expression of secondary metabolites at an alleviated level promotes host stress response in a controlled manner and co-evolved with invading micro-organisms. Phenylpropanoids act as a precursor of several secondary metabolites through, anthocyanin molecules, lignin, plant hormones, phytoalexins, flavonoids and isoflavonoids that are synthesized at the time of wounding, UV irradiation, nutrient deficiency, herbivory, predation, pathogen infection and abiotic stress conditions (Edwards et al., 1985; Hahlbrock & Scheel, 1989; Dixon & Paiva, 1995; MacDonald & D'Cunha, 2007; Ferrera, Austinb, Stewart, & Noelb, 2008; Huang et al., 2010; Vogt 2010; Payyavula, Navarre, Kuhl, Pantoja, & Pillai, 2012). Trans-cinnamate is produced as an intermediate of phenylpropanoid pathway through deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) and regulates immediate as well as systemic stress responses in plants. During the induction of systemic acquired resistance (SAR), salicylic acid (SA) production is regulated by PAL gene expression (Mauch-Mani & Slusarenko, 1996; Nugroho, Verberne, & Verpoorte, 2002; Chaman, Copaja, & Argandona, 2003; Huang et al., 2010; Vogt, 2010). Benzothiadiazole (BTH), an analogue of SA was used as a potential chemical inducer for plant defence mechanism by inducing SAR pathway (Zimmerli, Metraux, & Mauch-Mani, 2001). Several scientists (Dann, Diers, Byrum, & Hammerschmidt, 1998; Perez-de-Luque, Jorrfn, & Rubiales, 2004) reported reduced disease severity in tomato, pea, *Arabidopsis*, bean and cucumber following the exogenous application of BTH in field or greenhouse condition. However, high concentration of BTH showed negative impact on plants (Azami-Sardooei, Seifi, De Vleesschauwer, & Hofte, 2013). The objectives were to improve resistance in tea plants against *L. theobromae* by the application of BTH.

2 | MATERIALS AND METHODS

2.1 | Maintenance of plant materials and fungal strain

One-year-old seedlings of moderately susceptible tea variety TV-26 were planted in earthen pots, maintained in the experimental garden of Department of Botany, University of North Bengal and were used for in vivo experiments. The fungus was isolated from infected tea plants of sub-Himalayan West Bengal showing necrotic spots on leaf and the pure culture was maintained in potato dextrose agar medium supplemented with tea root extract. The culture was identified as *L. theobromae* from Indian Type Culture Collection (ITCC), New Delhi [ITCC Accession No. 4151.2K]. Spore suspension (1×10^6 conidia/ml) was prepared from 10-day-old sporulated culture and used as the source of inoculum.

2.2 | Pathogen inoculation and BTH treatment

The plants were treated following the whole plant inoculation technique of Dickens and Cook (1989). Young test 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 hrs of inoculation up to 4 days, and 1 g leaf sample from each replica was collected and dipped in liquid nitrogen, and total RNA was isolated immediately for expression analysis.

2.3 | Disease assessment

Disease assessment was carried out following the method of Sinha and Das (1972). The number of lesions developed on the leaves after 4 and 8 days of inoculation was counted, and diameters of each lesion were measured. On the basis of lesion diameters leaves were differentiated into four different groups as suggested by Sinha and Das (1972). The groups were designated as very small (1–2 mm), small (2–4 mm), medium (4–6) and large (>6 mm) and values of 0.1, 0.25, 0.5 and 1.0 were assigned to each group, respectively. The number of lesions in each group was multiplied by the value assigned to it, and the sum total was calculated to obtain a disease index based on the mean of ten plants per treatment.

2.4 | Isolation of PAL gene and semi-quantitative RT-PCR

Total RNA was isolated from the leaf samples following the method of Ghawana et al. (2011) and treated with DNase I to remove genomic DNA contamination. The extracted RNA was quantified in a UV-VIS spectrophotometer (Systronics, India). The isolated RNA was resolved in 1% agarose gel for verification of the quality of isolated RNA. Reverse transcription-PCR (RT-PCR) was performed by one step M-MuLV RT-PCR kit (Genei, Bangalore) following manufacturer's protocol and using the PAL gene-specific primer, forward (5′-ACAACA ATGGGTTGCCATCGAATC-3′) and reverse (5′-ACTTGGCTAACAC TGTTCTTGACA-3′). The thermal condition for reverse transcription step was performed at 50°C for 30 min, and then PCR amplification was performed at 94°C for 3 min (for initial denaturation) and then allowed to run for 35 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for

1 min with a final extension at 72°C for 5 min. Amplified products obtained from the RT-PCR were subjected to electrophoresis at 5 V/cm through 1% agarose gel and quantified in UV-VIS spectrophotometer for semi-quantitative studies. The interpretation for gene transcripts level corresponds to the intensity of the amplicons.

2.5 | Sequence analysis

The amplified fragments were purified using Genei Quick PCR purification kit (Genei, Bangalore), cloned into pGEM-T vector (Sambrook and Russel, 2001), and three clones were sent for sequencing to Chromous Biotech Pvt. Ltd., Bangalore, India. The nucleotide (nt) and deduced protein sequences were aligned using CLUSTALW 1.6 (Thompson, Higgins, & Gibson, 1994) and submitted in the GenBank after BLASTn analysis (Altschul et al., 1997). Nucleotide identity was calculated using BIOEDIT 7.2.5 (Hall, 1999), and phylogenetic tree was generated through neighbour-joining method with Kimura-2 parameter using MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Secondary structure of CsPAL protein was characterized using the SOPMA (Geourjon & Deleage, 1995). Motif analysis and physiochemical characterization of deduced protein were carried out by MEME (Bailey & Elkan, 1995) and ProtParam tool of ExPASy (Gasteiger et al., 2005), respectively. Homology modelling of the 3D structure of deduced protein was carried out using MODELLER 9.16 (Webb & Sali, 2014).

2.6 | Estimation of PAL activity

PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Sadasivam & Manickam, 1996). For PAL activity, 1 g leaf tissue was homogenized in 5 ml of 0.25 M borate buffer, pH 8.7 containing 0.1 g insoluble polyvinylpyrrolidone (PVP) in a mortar and pestle at 4°C and centrifuged at 12,000 g at 4°C for 15 min. The yellowish green supernatant was used as crude enzyme extract. Enzyme activity was expressed on fresh weight basis (μmol min^{-1} g^{-1} using trans-cinnamic acid as standard).

3 | RESULTS

3.1 | Disease assessment

Disease assessment was carried out in case of BTH-treated and untreated plants inoculated with *L. theobromae*. From the results, it was evident that BTH-treated plants showed reduced disease index (3.8 ± 0.02) in comparison with untreated control (14.6 ± 0.05) after 4 days of treatment. This clearly indicated the disease reducing ability of BTH in *C. sinensis* against *L. theobromae*.

3.2 | RNA quantification and semi-quantitative RT-PCR

Total gene expression (as represented by total transcript accumulation) was obtained from quantification of total RNA in the four sets of plants (Figure 1a). The concentration of total RNA increased from 1.50 μg/μl to 2.74 μg/μl in BTH-treated plant during these 4-day post-treatment. But in case of BTH-treated and *L. theobromae*-inoculated samples total RNA concentration increased from 1.43 μg/μl to 2.80 μg/μl and in pathogen-inoculated plants increase of RNA concentration varied from 1.04 μg/μl to 2.51 μg/μl. Whereas, in case of untreated-uninoculated control transcript accumulation remains constant. Semi-quantitative RT-PCR of expected PAL-specific fragment (~333 bp) for all the four sets of plants showed similar type of response (Figure 1b) where the highest induction of PAL gene was noticed in treated-inoculated plants (3.81 μg/μl DNA) followed by the treated-uninoculated (3.37 μg/μl DNA) and untreated-inoculated (3.26 μg/μl DNA) plants.

3.3 | Characterization and analysis of PAL gene

The BLASTn analysis showed high nt identity with *Camellia chekiangoleosa* (99%) and *C. sinensis* (91%) and comparatively low similarity with *Malus* sp. (85%) *Vitis vinifera* (84%), *Lonicera japonica* (82%) and *Medicago sativa* (81%) and the nucleotide (nt) sequences were submitted to GenBank (Acc. no. KF303590). Nt sequence identity matrix of the isolated PAL gene with that of the others have been presented in Table 1. Finally, phylogenetic tree was constructed (Figure 2) to determine the relationships between the present PAL sequence with other PAL gene families obtained from GenBank. Nevertheless, our PAL gene was closely related to *C. chekiangoleosa* and *C. sinensis* and clustered together, whereas, other PAL families (*i.e.*, PAL1, PAL2, PAL3) formed different clusters in phylogeny reconstruction. The secondary structure of PAL protein was dominated by α-helices with a contribution of 67.57% where random coil possessed approximately 22.52% in the secondary structure (Fig. S1a). The partial protein contains three conserved motifs (Fig. S1b). The 3D structure of PAL also revealed the

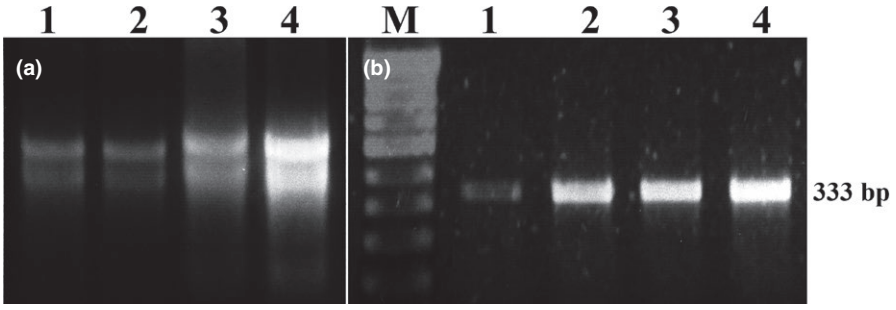

FIGURE 1 Agarose gel electrophoresis of (a) total RNA: Lane 1—control, lane 2—treated, lane 3—inoculated and lane 4—treated-inoculated; (b) RT-PCR products: lanes 1–4 expected product of phenylalanine ammonia-lyase (PAL) gene using PAL gene-specific primer, M: molecular marker

TABLE 1 Nucleotides sequence identity matrix of present isolated phenylalanine ammonia-lyase (PAL) gene sequence with other PAL gene sequences

	Camellia_ sinensis|KF303590	Camellia_ chekiangoleosa|JN944578	Camellia_ sinensis|AY694188	Camellia_ sinensis|D26596	Vitis_ vinifera|JN858957	Vitis_ vinifera|EF192469	Vitis_ vinifera|X75967
Camellia_sinensis|KF303590	100						
Camellia_ chekiangoleosa|JN944578	98	100					
Camellia_sinensis|AY694188	91	89	100				
Camellia_sinensis|D26596	91	89	100	100			
Vitis_vinifera|JN858957	84	83	83	83	100		
Vitis_vinifera|EF192469	84	83	83	83	100	100	
Vitis_vinifera|X75967	84	83	83	83	100	100	100
Vitis_vinifera|JN858963	83	82	82	82	99	99	99
Lonicera_hypoglauca|JX068619	81	80	82	82	78	78	78
Lonicera_ macrantho1es|JX068629	81	81	82	82	78	78	78
Lonicera_japonica|JX068602	81	81	82	82	78	78	78
Medicago_sativa|JN849763	80	80	80	80	80	80	80
Medicago_sativa|JN849771	80	80	80	80	80	80	80
Medicago_falcata|JN849768	80	80	80	80	80	80	80
Lonicera_japonica|JX068603	80	80	81	81	77	77	77

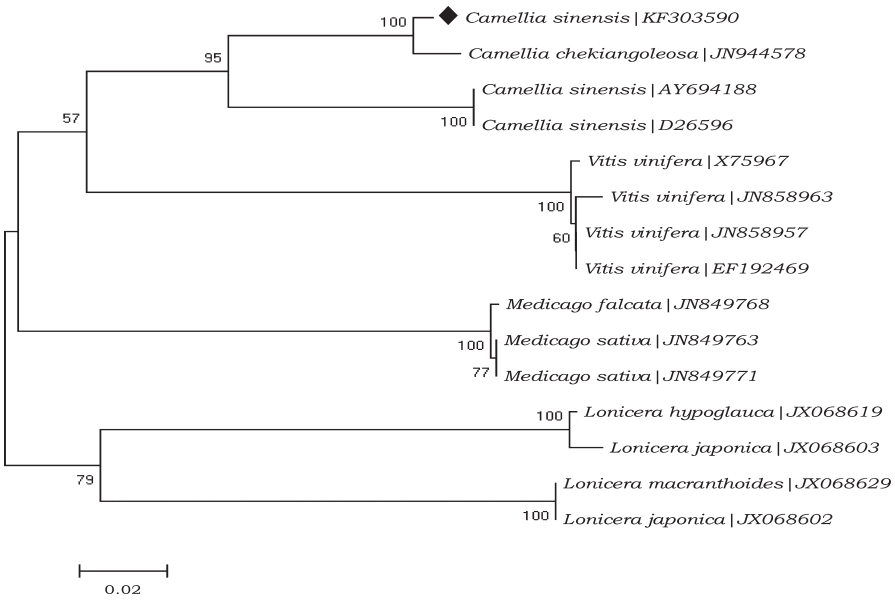

FIGURE 2 Phylogenetic tree was generated using the neighbour-joining method showing the relationship of present isolated phenylalanine ammonia-lyase (PAL) gene (KF303590) with the other PAL gene sequences publish in the GenBank. Numbers at the nodes indicate the bootstrap percentage scores out of 1,000 replicates

dominance of α-helices in the protein (Fig. S2). ProtParam analysis revealed that PAL protein showed pI in the acidic range, that is 5.85 and negative hydropathy (GRAVY) index of −0.225 (Table S1).

3.4 | Estimation of PAL activity

PAL is one of the key enzymes in the phenylpropanoid pathway and is highly responsive to various stimuli. The constant increase in PAL activity is being observed in treated (from 2.5 to 4.1 μmol min^{-1} g^{-1}) and treated-inoculated plants (from 2.5 to 4.4 μmol min^{-1} g^{-1}) in comparison with healthy control (from 2.5 to 2.7 μmol min^{-1} g^{-1}) and inoculated (from 2.5 to 3.1 μmol min^{-1} g^{-1}) one after 4 days of treatment. The highest PAL activity was observed on the 4th day of treatment (Figure 3).

4 | DISCUSSION

Plants are sensitive to various biotic and abiotic stresses (Oostendorp, Kunz, Dietrich, & Staub, 2001), and a network of induced signal pathways has been recognized by resistance spectra and marker proteins that are characteristics for activation of defence systems in different plants. In the present study, isolated PAL gene sequence was analysed, and phylogenetic tree was constructed. Our PAL gene showed

Vitis_ vinifera|JN858963	Lonicera_ hypoglauca|JX068619	Lonicera_ macrantho1es|JX068629	Lonicera_ japonica|JX068602	Medicago_ sativa|JN849763	Medicago_ sativa|JN849771	Medicago_ falcata|JN849768	Lonicera_ japonica|JX068603
100							
77	100						
78	81	100					
78	81	100	100				
79	79	79	79	100			
79	79	79	79	100	100		
80	79	79	79	99	100	100	
77	99	81	81	79	79	79	100

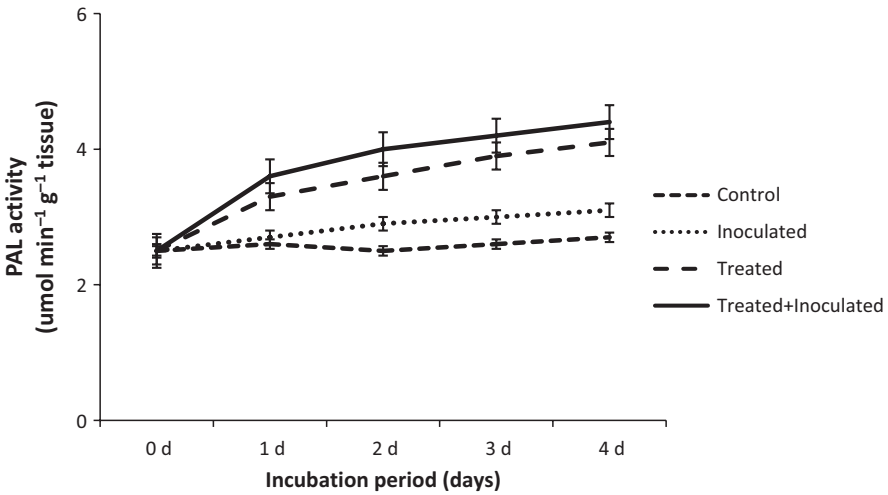

FIGURE 3 Estimation of phenylalanine ammonia-lyase activity following treatment with benzothiadiazole in inoculated and uninoculated tea plants against untreated controls

99%, 91% and 84% nt similarity with *C. chekiangoleosa*, *C. sinensis* and *V. vinifera*, respectively. Similar studies were carried out by Jeong et al. (2012), where the deduced amino acid sequence from kenaf showed 86% nt similarity to those of PAL from *V. vinifera*. Singh, Kumar, Rani, Gulati, and Ahuja (2009) analysed amino acid sequence of CsPAL from *C. sinensis* var. UPASI-10 which showed 85% similarity with *V. vinifera*. In the present PAL protein sequence, all the three motifs belong to the lyase superfamily (cl26059). Similar types of motifs were also identified by Dehghan et al. (2014) in the PAL family. Purwar, Gupta, Vajpayee, and Sundaram (2014) suggested that hydrophilic or hydrophobic nature of any enzyme or protein is reflected in the hydropathy (GRAVY) index. The negative hydropathy (GRAVY) index of existing tea-PAL indicated the hydrophilic nature of the enzyme.

PAL was estimated following BTH treatment with an objective to find a suitable inducer of PAL in tea. PAL is believed to be one of the key enzymes of secondary metabolic pathway and protection of plants against virulent pathogens. Hence, elevation of PAL level in tea plants after BTH treatment was taken into consideration. Here, it was observed that BTH may successfully increase PAL level after induction. Similar results were obtained by Conrath, Thulke, Katz, Schwindling, and Kohler (2001) where pretreatment of cultured parsley cells with BTH leads to direct activation and strong elicitation of sets of defence-related genes including PAL. In our study, PAL-specific PCR amplicons were quantified and increase in the transcript accumulation significantly coordinated with the elevated PAL activity after BTH induction in tea plants. Pretreatment of plants with chemical compounds can enhance resistance in tissues both at the site of infection as well as

at distant sites from the initial infection (Ryals et al., 1996). Different chemical elicitors including BTH may activate various defence-related enzymes that are effective in producing phenolic compounds (Thakur & Sohal, 2013).

Increased resistance of tea plants following challenge inoculation with *L. theobromae* was also seen in BTH-treated plants as compared to the untreated control in the present study. The augmented PAL gene expression and PAL-mRNA accumulation in *Arabidopsis* (Conrath et al., 2001; Kohler, Schwindling, & Conrath, 2002) and grape berry (Wen et al., 2005) with BTH (synthetic SAR inducer) and SA treatment respectively have also been reported previously. PAL transcript levels were also found to increase in *Salvia miltiorrhiza* against abscisic acid treatment in comparison with untreated one (Song & Wang, 2009). The accumulation of phenol synthesized by PAL via phenylpropanoid pathway (Hahlbrock & Scheel, 1989) was higher in the roots and shoots of chickpea treated with SA (Raju, Jayalakshmi, & Sreeramulu, 2008).

According to Stadnik and Buchenauer (2000), the PAL activity was increased in response to powdery mildew infection in wheat when the plants were treated with BTH. Paul and Sarma (2005) showed higher activity of defence enzymes like PAL, PO, PPO and CAT along with phenolic compounds and lignin in black pepper when induced with *Pseudomonas fluorescens*. The activities of PAL, PO, PPO, chitinase and β-1,3-glucanase enzymes were increased in cucumber, pepper and tomato plants when the plants sprayed with resistance inducers (El-Mougy, Abdel-Kader, Lashin, & Megahed, 2013). Raju et al. (2008) reported that the PAL activity was increased at 5th day after pathogen inoculation in comparison with control. Campos et al. (2003) observed that the PAL activity was increased in bean plantlets after 5 days of treatment with SA and challenged with virulent pathotype (33/95) of *Colletotrichum lindemuthianum*. In the present study, PAL was found to increase after 4th day post-treatment and challenge inoculation by *L. theobromae*.

The present study deals with the characterization and induction of PAL gene with chemical inducer BTH through elevated transcript accumulation locally or systemically. It might be one of the significant inducers of PAL which can be used subsequently for the induction of disease resistance in tea plants in sub-Himalayan West Bengal, India.

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REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402.

Azami-Sardooei, Z., Seifi, H. S., De Vleesschauwer, D., & Hofte, M. (2013). Benzothiadiazole (BTH)-induced resistance against *Botrytis cinerea* is inversely correlated with vegetative and generative growth in bean and cucumber, but not in tomato. *Australasian Plant Pathology*, *42*, 485–490.

Azzahra, L. F., Fouzia, H., Mohammed, L., & Noureddine, B. (2012). Antioxidant response of *Camellia sinensis* and *Rosmarinus officinalis* aqueous extracts toward H_2O_2 stressed mice. *Journal of Applied Pharmaceutical Science* , *02*, 70–76.

Bailey, T. L., & Elkan, C. (1995). The value of prior knowledge in discovering motifs with MEME. *Proceedings of the Third International Conference on Intelligent Systems for Molecular Biology* (pp. 21–29). Menlo Park, CA: AAAI Press.

Campos, A. D., Ferreira, A. G., Hampe, M. M. V., Antunes, I. F., Brancao, N., Silveira, E. P., … Osorio, V. A. (2003). Induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid and *Colletotrichum lindemuthianum* in common bean. *Brazilian Journal of Plant Physiology*, *15*, 129–134.

Chaman, M. E., Copaja, S. V., & Argandona, V. H. (2003). Relationships between salicylic acid content, phenylalanine ammonia-lyase (PAL) activity, and resistance of barley to aphid infestation. *Journal of Agricultural and Food Chemistry*, *51*, 2227–2231.

Chen, Z. (1999). Pharmalogical functions of tea. In N. K. Jain (Ed.), *Global advances in tea science* (pp. 333–358). New Delhi, India: Aravali Books International (P) Ltd.

Conrath, U., Thulke, O., Katz, V., Schwindling, S., & Kohler, A. (2001). Priming as a mechanism in induced systemic resistance of plants. *European Journal of Plant Pathology*, *107*, 113–119.

Dann, E., Diers, B., Byrum, J., & Hammerschmidt, R. (1998). Effect of treating soybean with 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by *Sclerotinia sclerotiorum* in field and greenhouse studies. *European Journal of Plant Pathology*, *104*, 271–278.

Dehghan, S., Sadeghi, M., Poppel, A., Fischer, R., Lakes-Harlan, R., Kavousi, H. R., & Vilcinskas Rahnamaeian, A. M. (2014). Differential inductions of phenylalanine ammonia-lyase and chalcone synthase during wounding, salicylic acid treatment, and salinity stress in safflower, *Carthamus tinctorius*. *Bioscience Reports*, *34*, art:e00114.

Dickens, J. S. W., & Cook, R. T. A. (1989). *Glomerella cingulata* on *Camellia*. *Plant Pathology*, *38*, 75–85.

Dixon, R. A., & Pavia, N. L. (1995). Stress-induced phenylpropanoid metabolism. *Plant Cell*, *7*, 1085–1097.

Edwards, K., Cramer, C. L., Bolwell, G. P., Dixon, R. A., Schuch, W., & Lamb, C. J. (1985). Rapid transient induction of phenylalanine ammonialyase mRNA in elicitor-treated bean cells. *Proceedings of the National Academy of Sciences USA*, *82*, 6731–6735.

El-Mougy, N. S., Abdel-Kader, M. M., Lashin, S. M., & Megahed, A. A. (2013). Fungicides alternatives as plant resistance inducers against foliar diseases incidence of some vegetables grown under plastic houses conditions. *International Journal of Engineering and Innovative Technology*, *3*, 71–81.

Ferrera, J. L., Austinb, M. B., Stewart, C., Jr., & Noelb, J. P. (2008). Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiology and Biochemistry*, *46*, 356–370.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy Server. In: J. M. Walker (Ed.), *The proteomics protocols handbook* (pp. 571–607). Totowa, NJ: Humana Press.

Geourjon, C., & Deleage, G. (1995). SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Computer Applications in the Biosciences*, *11*, 681–684.

Ghawana, S., Paul, A., Kumar, A., Singh, H., Bhardwaj, P. K., Rani, A., … Kumar, S. (2011). An RNA isolation system for plant tissues rich in secondary metabolites. *BMC Research Notes*, *4*, 85–89.

Hahlbrock, K., & Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology*, *40*, 347–369.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y. H., … Chen, Z. (2010). Functional analysis of the *Arabidopsis* PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiology*, *153*, 1526–1538.

Jeong, M., Choi, B. S., Bae, D. W., Shin, S. C., Park, S. U., Lim, H., … Bae, H. (2012). Differential expression of kenaf phenylalanine ammonia-lyase (PAL) ortholog during developmental stages and in response to abiotic stresses. *Plant Omics*, *5*, 392–399.

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*, 323–329.

Kohler, A., Schwindling, S., & Conrath, U. (2002). Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPR1/NIM1* gene in *Arabidopsis*. *Plant Physiology*, *128*, 1046–1056.

MacDonald, M. J., & D'Cunha, G. B. (2007). A modern view of phenylalanine ammonia lyase. *Biochemistry and Cell Biology*, *85*, 273–282.

Macedo, R., & Oliveira, M. (2012). Quantification of catechins and caffeine from green tea (*Camellia sinensis*) infusions, extract, and ready-to-drink beverages. *Ciência e Tecnologia de Alimentos*, *32*, 163–166.

Mauch-Mani, B., & Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell*, *8*, 203–212.

Mondal, T. K., Bhattacharya, A., Laxmikumaran, M., & Ahuja, P. S. (2004). Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell, Tissue and Organ Culture*, *76*, 195–254.

Nugroho, L. H., Verberne, M. C., & Verpoorte, R. (2002). Activities of enzymes involved in the phenylpropanoid pathway in constitutively salicylic acid-producing tobacco plants. *Plant Physiology and Biochemistry*, *40*, 755–760.

Oostendorp, M., Kunz, W., Dietrich, B., & Staub, T. (2001). Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology*, *107*, 19–28.

Paul, D., & Sarma, Y. R. (2005). *Pseudomonas fluorescens* mediated systemic resistance in black pepper (*Piper nigrum* L.) is driven through an elevated synthesis of defence enzymes. *Archives of Phytopathology and Plant Protection* , *38*, 139–149.

Payyavula, R. S., Navarre, D. A., Kuhl, J. C., Pantoja, A., & Pillai, S. S. (2012). Differential effects of environment on potato phenylpropanoid and carotenoid expression. *BMC Plant Biology*, *12*, 39.

Perez-de-Luque, A., Jorrfn, J. V., & Rubiales, D. (2004). Crenate broomrape control in pea by foliar application of benzothiadiazole (BTH). *Phytoparasitica*, *32*, 21–29.

Punyasiria, P. A. N., Abeysinghea, I. S. B., Kumarb, V., Treutterc, D., Duyd, D., Goschd, C., … Fischerd, T. C. (2004). Flavonoid biosynthesis in the tea plant *Camellia sinensis*: properties of enzymes of the prominent epicatechin and catechin pathways. *Archives of Biochemistry and Biophysics*, *431*, 22–30.

Purwar, S., Gupta, A., Vajpayee, G., & Sundaram, S. (2014). Isolation and *in-silico* characterization of peroxidase isoenzymes from wheat (*Triticum aestivum*) against Karnal Bunt (*Tilletia indica*). *Bioinformation*, *10*, 87–93.

Raju, S., Jayalakshmi, S. K., & Sreeramulu, K. (2008). Comparative study on the induction of defense related enzymes in two different cultivars of chickpea (*Cicer arietinum* L) genotypes by salicylic acid, spermine and *Fusarium oxysporum* f. sp. *ciceri*. *Australian Journal of Crop Science*, *2*, 121–140.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., & Hunt, M. D. (1996). Systemic acquired resistance. *Plant Cell*, *8*, 1809–1819.

Sadasivam, S., & Manickam, A. (1996). *Biochemical methods*. New Delhi: New Age International Ltd.

Sambrook, J., & Russel, D. W. (2001). *A laboratory manual* (2nd edn). Cold Spring Harbour, NY: Laboratory Press.

Sarmah, K. C. (1960). *Disease of tea and associated crops in North East*. Assam: Indian Tea Association, Tocklai Experimental Station.

Singh, K., Kumar, S., Rani, A., Gulati, A., & Ahuja, P. S. (2009). Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. *Functional & Integrative Genomics*, *9*, 125–134.

Sinha, A. K., & Das, N. C. (1972). Induced resistance in rice plant to *Helminthosporium oryzae*. *Physiological Plant Pathology*, *2*, 401–410.

Song, J., & Wang, Z. (2009). Molecular cloning, expression and characterization of a phenylalanine ammonia-lyase gene (SmPAL1) from *Salvia miltiorrhiza*. *Molecular Biology Reports*, *36*, 939–952.

Stadnik, M. J., & Buchenauer, H. (2000). Inhibition of phenylalanine ammonia-lyase suppresses the resistance induced by benzothiadiazole in wheat *Blumeria graminis* f. sp. Tritichi. *Physiological and Molecular Plant Pathology*, *57*, 25–34.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729.

Tea Board India (2016). 62nd Annual report 2015-16. Retrieved from http://www.teaboard.gov.in/pdf/Tea_Board_Annual_Report_2015_16_pdf3913.pdf.

Thakur, M., & Sohal, B. S. (2013). Role of elicitors in inducing resistance in plants against pathogen infection: A review. *ISRN Biochemistry*, *2013*, 1–10.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680.

Vogt, T. (2010). Phenylpropanoid biosynthesis. *Molecular Plant*, *3*, 2–20.

Webb, B., & Sali, A. (2014). Comparative protein structure modeling using MODELLER. *Current Protocols in Bioinformatics*, *47*, 5.6.1–5.6.32.

Wen, P. F., Chen, J. Y., Kong, W. F., Pan, Q. H., Wan, S. B., & Huang, W. D. (2005). Salicylic acid induced the expression of phenylalanine ammonia-lyase gene in grape berry. *Plant Science*, *169*, 928–934.

Zimmerli, L., Metraux, J., & Mauch-Mani, B. (2001). β-Aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. *Plant Physiology*, *126*, 517–523.

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