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*.