DISCUSSION

4. Discussion

4.1. Suppression Subtractive Hybridization (SSH) Library

Generation of ESTs, as a genomic approach for identification of expressed genes, has been widely used in genome-wide gene expression studies in various organisms (Adams et al., 1995; Nathalie et al., 2005). It has been employed to identify the genes that are expressed in various tissues, cell types, or developmental stages (Ogihara et al., 2003; Ronning et al., 2003). The SSH method is an efficient approach to isolate differentially expressed genes in different tissues and different stages. This technique can suppress the rate of sequences presented in equal amounts in both populations and enrich the differentially expressed sequences (Diachenko et al., 1996; Paul and Michelle, 2002). The application of SSH and EST cloning can be used to maximize the identification of genes involved in host responses to pathogen infection and disease development (Birch et al., 1999; Degenhardt et al., 2005). Thus SSH technique has been adopted in this study as an attempt to identify genes involved in the resistance against the fungal pathogen *Exobasidium vexans* in tea.

From the forward SSH library, one of the up-regulated gene sequence is the early light inducible protein (ELIP) (GenBank Accn no: JG463735). These are a class of thylakoid proteins involved in a number of cellular processes and structurally related to light harvesting complex (LHC) proteins (Grimm et al., 1989 and Jansson, 1999). RNA hybridization, microarray, and cDNA analyses show that ELIP mRNA increases in abundance in response to a variety of stress-related signals. These include treatment with salt, heat, abscisic acid, cold, desiccation, aluminium, high CO$_2$, and senescence (Montane´ et al., 1997; Kro´l et al., 1999; Shimosaka et al., 1999; Adamska, 2001; Harari-Steinberg et al., 2001; Bhalerao et al.,
2003; Binyamin et al., 2001; Provart et al., 2003). A role for ELIP in resistance to drought stress is indicated by several studies. Expression of an ELIP-like gene in Craterostigma plantagineum, a plant that can survive and recover from extreme dryness, is influenced by abscisic acid and light levels and is induced by drought. Constitutive expression of the ELIP gene in Helianthus annuus confers drought tolerance (Alamillo and Bartels, 1996; Ouvrard et al., 1996).

Another sequence in the FSSH is Chloroplastic m-type thioredoxins (TRX m) (GenBank Accn no: JG463738) that are essential redox regulators in the light regulation of photosynthetic metabolism. The role of thioredoxin m is documented in the regulation of NADP-malate dehydrogenase (NADP-MDH) in vivo in corn and spinach (Crawford et al., 1986) and by the observations of Jacquot et al. (1984) in the activation of the enzyme in corn leaves. Malate dehydrogenase (MDH) is an enzyme of the tricarboxylic acid cycle and catalyses the conversion of malate into oxaloacetate, producing sufficient quantity of NAD(P)H, that can then be used to form H$_2$O$_2$ (Gross et al., 1977 and Ishida et al., 1987). In addition to central metabolic pathways, other energy generating pathways are also up-regulated during the defence response. For example, malate metabolism by NADP-MDH enzyme is up-regulated in response to pathogen infection and has been speculated to be involved in energy for plant defence through the generation of pyruvate and NADPH (Casati et al. 1999; Schaaf et al. 1995; Widjaja et al. 2009; Zulak et al. 2009). Several studies have demonstrated the increase in abundance of malate dehydrogenase in response to biotic and abiotic stresses (Cushman., 1993 and Subramanian et al., 2005). The levels of MDH were increased 2.5 fold only in the resistant Charlton in response to the pathogen challenge (Garg et al., 2013).
An EST with similarity to 2 oxoglutarate dehydrogenase (GenBank Accn no: JG463734) was obtained from the forward SSH library. It is the key enzyme in TCA cycle and the up-regulation of its abundance could speed up the energy metabolism and provide more energy for heat stress resistance (Li et al., 2013). Oxoglutarate dehydrogenase complex (OGDHC) is a crucial target of reactive oxygen species (ROS) and also able to generate ROS, which make it distinctly important for bioenergetics (Tretter and Adam., 2005). A sequence similar to cyclin dependent kinase (CDK) E (GenBank Accn no: JG463731) obtained from the forward SSH library belongs to a class of CDKs, a group of protein kinases that are key regulators of the cell cycle (De Veylder et al., 2003). An Arabidopsis A type CDK is known to be induced by wounding in the absence of cell division. This induced expression was correlated to an increased competence to cell proliferation.

A sequence similar to NAD+ transporter 1 (GenBank Accn no: JG463723) was obtained in the forward SSH. Reports for two mitochondrial NAD+ transporters, named Ndt1p and Ndt2p, found to import NAD+ into the mitochondria of Saccharomyces cerevisiae have been obtained earlier (Todisco et al., 2006). NAD and NADP could be the fundamental common mediators of nearly all major biological activities, including mitochondrial function, energy metabolism, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death. NAD may also affect antioxidation and generation of oxidative stress through several pathways. (Ying., 2008). These nucleotides also play vital roles in signalling via the generation and scavenging of reactive oxygen species (ROS; Mittler et al., 2004) and in systems controlling adaptation to environmental stresses such as UV irradiation, salinity, heat shock and drought (Amor et al., 1998; Chai et al., 2005, 2006).
In the forward SSH library a sequence similar to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Accn no: JG463719) was obtained which is a central glycolytic protein with pivotal role in energy production. It is a NAD(P)(+)-binding protein (Mittler et al., 2004) and has been reported to be involved in photosynthetic metabolism and responses to abiotic (Yang et al., 1993 and Jeong et al., 2001) and biotic stresses (Laxalt et al., 1996).

One of the forward SSH sequences is that similar to the gene encoding photosystem II 5kDa protein (GenBank Accn no: JG463703). In contrast, studies by Espinoza et al. (2007), Albrecht and Bowman (2008) and Nwugo et al. (2013) show that the gene encoding photosystem II 5kDa is down regulated during different plant-pathogen interactions supported by a whole lot of reports that evidence the down-regulation of photosynthetic genes (Berger et al., 2007 and Zou et al., 2005) though suggested and reported the other way by Trumble et al., (1993).

Fructose 1, 6-biphosphatase (GenBank Accn no: JG463680) is one of the up-regulated gene in our study. This enzyme catalyses a reaction that directs sucrose biosynthesis. It is also one of the four up-regulated genes isolated from Rhizoctonia solani - infected tomato plants treated with B.subtilis (Hafez et al., 2013). While sugar accumulation could potentially increase the osmotic potential within the cell or fuel respiration through glycolysis, the tricarboxylic acid (TCA) cycle and mitochondrial oxidative phosphorylation, a third fundamental consequence is the participation of sugars in specific signal transduction mechanisms in stressed plants (Jordan et al., 2013). Since tolerance must depend on the energy status of cells in which appropriate responses are induced, many tissues of stressed plants are likely to have an increased demand for rapidly metabolizable carbohydrate (Hare et al., 1998).
Several of the other forward SSH sequences are those involved in the general metabolism and photosynthesis while the reverse SSH did not yield any of the functional gene sequences except for a acyl-coA dehydrogenase involved in lipid catabolism. The construction of SSH library did not provide any fruitful insight into the resistant mechanism of the tea cultivar against blister blight pathogen except for the identification of the above discussed genes found to be up-regulated during infection.

4.2. Expression of pathogenesis related (PR) protein encoding genes during different stages of infection

Among the three major PR proteins encoding genes selected for the study, induction of CHIT expression was found to be several folds higher in the blister infected leaves of the resistant cultivar SA 6 than in the susceptible cultivar TES 34. The anti-fungal potential of this enzyme in plant-pathogen interactions are well documented (Punja and Zhang, 1993) and accordingly our findings supported the role of tea chitinase in defence against the fungal pathogen. In the resistant cultivar (SA 6), the expression level of the gene was high and reached to maximum level in the fourth stage of infection whereas the susceptible cultivar (TES 34) showed very low constitutive level of expression of the gene at each stage of disease development.

The mRNA level of GLUC did not increase upon pathogen infection in SA 6 as rapidly as in TES 34 where the level increased in the initial infection stage and then decreased upto the third stage and again increased. Such low levels of the GLUC mRNA could lead to the prediction that the half-life of the tea GLUC mRNA could be relatively short for their increased accumulation to be detected by the RT-PCR in SA 6. This prediction out of this study is supported by the work on the GLUC from the genomic library of peach in
response to treatment with a bacterial pathogen *Xanthomonas campestris* pv. pruni and ethephon (Thimmapuram *et al.*, 2001). The expression of *GLUC* was transient with very low levels of the transcript accumulation at 7 h after the treatment with ethephon and decreased by 48 h. The *GLUC* mRNA of bean leaves has also been reported to have a relatively short half-life by the studies of Vogeli-Lange *et al.* (1988). This prediction is supported by the protein estimation data discussed later where the levels of glucanase enzyme increase with increasing incidence of disease in the resistant cultivar SA 6. The tea *GLUC* mRNA induced as a result of pathogen invasion is translated immediately into the respective protein in the resistant cultivar (SA 6) and accumulates in the leaf. In contrast, the cultivar TES 34 showed detection of *GLUC* mRNA during the infection stages that was not successfully translated as in the case of the resistant cultivar (SA 6). This is reflected in the protein levels where there is no much accumulation of the protein during infection in TES 34. This could be due to the interference of the pathogen in the translation machinery of the susceptible cultivar that prevents the accumulation of the protein. Further investigations in this area are needed to support this prediction derived out of the results obtained from this study.

Changes in chitinases and glucanases of crude leaf extracts were detected in incompatible and compatible *Coffea arabica - H. vastatrix* interactions (Maxemiuc-Naccache *et al.* 1992). These researchers observed an increase in the activities of the enzymes during the first day after inoculation, in the incompatible interactions but not in the compatible one. Guerra-Guimaraes et.al. (2009) have detected a high constitutive level of chitinase activity in the intracellular fluid of healthy *C. arabica* leaves and increased chitinase activity in the early stages of the I1 and I2, in association with restriction of fungal growth which is in coincidence to cell death (HR) (Rijo *et al.* 1991, Coutinho *et al.* 1993, Martins and Moraes 1996, Silva *et al.* 2002), suggesting that chitinases take part in this response.
Local and systemic de novo expression of class III chitinase was also induced by tobacco mosaic virus infection in tobacco (Lawton et al., 1992). Transgenic wheat lines carrying a barley-seed class II chitinase exhibited enhanced resistance to powdery mildew (Bliffeld et al. 1999; Oldach et al. 2001). Varying amount of resistance towards powdery mildew were observed in transgenic wheat lines carrying a barley chitinase or a barley $\beta$-1,3-glucanase (Bieri et al. 2003). Transcripts encoding chitinases and glucanases accumulated in wheat seeds and / or heads following infection with either Fusarium graminearum or Fusarium culmorum (Caruso et al., 1999; Pritsch et al., 2000; Li et al., 2001). The encoded enzymes could directly (via degradation of the fungal structural barrier) or indirectly (via elicitor activity of fungal cell wall degradation products) play a role in defence and resistance against Fusarium. Within poplar leaf transcripts with the highest rust-induced accumulation (.10-fold) in the incompatible interaction were the several encoded PR proteins including PR-2 (1,3-b-glucanase), PR-3 (acidic chitinase) and PR-8 (basic chitinase) (Cecile Rinaldi et al., 2007). Genetic analysis of resistance to Phoma tracheiphila in three Citrus poncirus progenies has been reported where the pathogenesis-related proteins and chitinase appear more frequently in resistant plants than in susceptible ones (Recupero et al. 1997). In some plant species, like the pea plant, the accumulation rate of chitinases and their final concentrations are greater in resistant than in susceptible tissues following inoculation with pathogenic fungi (Vad et al., 1991). In maize several PR proteins have been found to accumulate in leaves after treatment with mercuric choride or brome mosaic virus (BMV) infection, out of which several glucanases and chitinases have been identified and purified. (Nasser et al., 1988).

Phenylalanine ammonia-lyase (PAL) are the key enzymes that are involved in phenylpropanoid biosynthesis (Smith and Banks, 1986) and thus indirectly aids in the supply
of precursors for flavanoid pigments, lignin, UV protectants and phytoalexins (Hahlbrock and Grisebach, 1979; Vance et al., 1980; Grisebach, 1981; Hahlbrock and Scheel, 1989; Lewis and Yamamoto, 1990). In SA 6 the PAL mRNA transcripts were observed to decrease upon infection until the third stage and then a mild increase in the fourth infection stage, still lesser than that in the healthy leaves. The transcript levels in TES 34 saw a dramatic increase in the first stage of infection and then gradually decreased till the fourth stage but always higher than that in the healthy leaf. These observations are in hand with the protein estimation results of both the cultivars.

Our observations with regard to PAL are in contrary to those in different plant-pathogen interactions where there was increase in both the transcript levels and the protein in the resistant hosts and decrease in the susceptible hosts like in Arabidopsis (Dong et al., 1991), barley (Kervinen et al., 1997), bean (Angela Diniz Campos et al., 2003), tobacco (Fritig et al., 1973), alfalfa (Nichole et al., 1994), potato (Fritzemeier et al., 1987), rice (Ikram Blilou et al., 2000) and so on. PAL activity has been observed to increase even in the studies on tea plant during diseases like grey blight disease (Senthilkumar, 2011), bird’s eye spot disease (Mythili Gnanamangai et al., 2011) and sclerotial blight disease (Indramani Bhagat and Chakraborty, 2010). Nevertheless our observation was supported by the work in triticale and wheat (Krystyna Rybka et al., 1998) where the activity of PAL was observed to be transient and decrease when there was a rise in chitinase, glucanase and peroxidase activities during Stagonospora nodorum infection and in tea leaves infested with tea mosquito bug (Chakraborty and Chakraborty, 2005) which showed decreased PAL activity while the activities of peroxidase, ascorbate peroxidase and polyphenol oxidase increased.

From these observations it could be derived that the resistance against blister blight disease shown by the cultivar SA 6 and other cultivars is not entirely PAL reliant but is
dependent on the activation of other PR proteins like chitinase and glucanase. The increased expression of \textit{PAL} in TES 34 with decreased chitinase and glucanase activity indicate that the induction of \textit{PAL} alone is not sufficient to resist the pathogen entry.

4.3. Isolation and characterization of the gene encoding chitinase

Rapid amplification of cDNA ends (RACE) reactions performed with gene specific primers for the isolation of full length gene encoding the major PR protein chitinase resulted in an 1181bp sequence with an ORF of 969bp and was found to encode a protein of 322 amino acids with 48bp 5’ UTR (untranslated region) and a 164bp 3’ UTR. This PR protein encoding gene is one of the highly explored candidate genes used in a large number of phytopathogen interactions for induced resistance. The constitutive overexpression of chitinases in transgenic plants increases resistance to pathogens in vivo (Broglie \textit{et al.}, 1991, Grison \textit{et al.}, 1996). The resistance levels of strawberry, grapevine, cucumber and rice against fungal diseases have been found to increase by the constitutive expression of a class I chitinase from rice (Azao \textit{et al.}, 1997, Yamamoto \textit{et al.}, 2000, Tabei \textit{et al.}, 1998, Nishizawa \textit{et al.}, 1999) and by the expression of barley class II chitinase in tobacco and wheat (Jach \textit{et al.}, 1995, Oldach \textit{et al.}, 2001).

Full length isolation of chitinase gene has been performed in a number of plant species including \textit{Arabidopsis thaliana} (Deborah \textit{et al.}, 1990), \textit{Benincasa hispida} (Chao-Yun \textit{et al.}, 2001), cucumber (Metraux \textit{et al.}, 1989), strawberry (Anwar and Ding; 2004), \textit{Nepenthes khasiana} (Haviva Eilenberg \textit{et al.}, 2006), rice (Qun Zhu and Christopher; 1991), tobacco (Jean-Marc Neuhaus \textit{et al.},1991), winged bean (Rogaya Sekeli \textit{et al.}, 2003), faba bean (Attia \textit{et al.}, 2007) to name a few, in addition to the genes isolated from microbes and entomopathogenic fungi.
Having documented the induced expression of chitinase gene during blister blight incidence, the full length isolation of the gene would be a valid tool for long-term applications in the improvement of tea cultivars for disease resistance.

4.4. Characterization of purified chitinase and documentation of its anti-fungal properties

Followed by the expression studies and full length isolation, the chitinase enzyme was purified from the healthy leaves of SA 6 through ammonium sulphate precipitation, dialysis and gel filtration chromatography. As revealed by SDS-PAGE electrophoresis, the purified fraction responded to a single polypeptide of 34kDa. This is well within the range of molecular weights of almost all known plant chitinases that generally lie between 25kDa to 35kDa (Collinge et al., 1993). However it is exactly of the same size of chitinase enzyme isolated from wheat (34kDa) (Broekaert et al., 1988), tobacco 34kDa (Hideaki Shinshi et al., 1987) and almost similar to that isolated from thorn-apple (35kDa) (Broekaert et al., 1988), pineapple (33kDa) (Toki Taira et al., 2005a), sorghum (33kDa) (Krishnaveni et al., 1999) and gazyumaru (33kDa) (Toki Taira et al., 2005b).

The optimal activity conditions for the purified enzyme were a pH of 5.8 and a temperature of 25°C. The optimal pH suggests that the purified tea chitinase is active in acidic environments in vivo such as a vacuole or apoplast. Several other plant species encoding for chitinase with optimal pH in the range of 5 to 6 like Phaseolus vulgaris (Wang et al., 2009), Citrus sinensis (Mayer et al., 1996), Nepenthes alata (Ishisaki et al., 2012), cabbage (Chang et al., 1996), sorghum (Krishnaveni et al., 1999), table grapes (Fernandez-Caballero et al., 2009) to name a few have been characterised. The optimal temperature of the purified enzyme is similar to those characterised from papaya (Chen et al., 2007) and wheat (Molano et al., 1979) but is in contrast to most of the chitinases that are active at high
temperatures of about 60°C (Mayer et al., 1996, Ishisaki et al., 2012, Fernandez-Caballero et al., 2009, Toki Taira et al., 2005a, Chang et al., 1996). The inhibitory effect of metal ions and inhibitors was similar to that shown by chitinases from other sources like cabbage that had inhibition by metal ions at 0.5mM (Chang et al., 1996), rice chitinase inhibited at 1mM (Hee-Young Park et al., 2002), legume chitinase strongly inhibited by metal ions (Wang et al., 2009) and chitinase from papaya inhibited at 10mM concentrations (Chen et al., 2007). The most potent inhibitors were p-nitrophenol (93.48%) and EDTA (91.8%) among all the chemicals tested. The degree of inhibition varies depending on the inhibitory agent employed. SDS and other surfactants used in this study are known to modify the tertiary and quaternary structures of proteins, resulting in almost 87% inactivation of tea chitinase.

The anti-fungal potential of the purified enzyme was documented against the germination of spores of two potent fungal pathogens of tea ecosystem, Exobasidium vexans and Pestalotiopsis thea. The inhibitory action was shown at enzyme concentrations as low as 3µg with explicit morphological distortions and abruptions of the mycelia followed by necrosis of the spores of both the pathogens. Combined with the fact that similar observations were seen in the chitinases of other sources against fungal pathogens (Mauch et al., 1988, Krishnaveni et al., 1999, Toki Taira et al., 2005a, Wang et al., 2008, Wang et al., 2009, Fernandez-Caballero et al., 2009) the purification and characterization of tea chitinase has proved it to be a valid gene that can be used for breeding programmes in tea.

4.5. Detection of chitinase activity in tea cultivars for screening their resistance levels against the disease

Having known that there is abundant chitinase mRNA transcript accumulation in the resistant cultivar during disease induction and that the protein has potent anti-fungal activity
in vitro, screening the tea cultivars for their constitutive chitinase expression could be a criteria for categorizing them under various resistant/susceptible groups.

There have been many attempts and methods devised to detect the chitinase activity using a range of chemicals and techniques. Vipul Gohel et al., (2005) devised a method for detection of chitinase activity on chitin agar plate after polyacrylamide gel electrophoresis using different staining dyes such as calcofluor white M2R, fluorescein isothiocyanate, rhodamine B, ruthenium red and congo red. All of these fluorescent dyes require preparation steps for the chitin agar plate. Zou et al., (2002) and Luis and Ray (2004) developed a quantitative assessment method for chitinase activity from crude plant extracts using glycochitin as enzyme substrate which is based on the affinity of calcofluor and fluorescent brightener 28 respectively with undigested glycochitin. Anil et al., (2007) reported the use of a less expensive fluorescent brightener Ranipal for detection of chitinases.

Many of the above said screening techniques for chitinase quantification in plants, though satisfactory, involve the use of hazardous chemicals, equipments and take longer time. Therefore, in the present study, Gram’s iodine based detection assay for chitinase production in tea cultivars has been attempted to screen and validate the data available on the resistance level of the cultivars against blister blight disease. The assay and screening method developed herewith can be employed for screening a large number of samples in lesser time and can be performed with small sample quantities, as crude extract without any specific preparation steps. The method has been proven to be very reliable, reproductive, cost-effective, involving lesser man power and more importantly environment-friendly and non-hazardous. A basal expression of pathogenesis related proteins like chitinase has been linked directly to the resistance of fungal infestation in tea plant. Therefore, plant breeders could potentially screen the tea germplasm for promising resistant varieties using this method. In
addition, this method can also be utilized in the studies of the enzyme induction in plant–pathogen interactions, acquired resistance and plant defence deployment.

4.6. Analysis of anti-oxidative enzymes and PR proteins in leaves during disease incidence, different stages of infection and in different tissues of healthy and infected bushes of the resistant and susceptible cultivars with respect to the healthy leaf

4.6.1. Anti-oxidative enzymes – peroxidase, ascorbate peroxidase and superoxide dismutase

The activity of peroxidase (POX) and ascorbate peroxidase (APX) enzymes increased during disease incidence in all the cultivars, however the increase being more pronounced in the susceptible cultivars than the resistant ones. The role of the enzymes could more easily be predicted by looking at the status of the enzyme activity during different stages of infection in SA 6 and TES 34. There is increased activity in TES 34 during all stages but the resistant cultivar behaves in a way to minimise the accumulation and thus activity of POX and APX. The activity of the enzymes in the tissues of the infected bush is also higher in the susceptible cultivar than the resistant. All these findings suggest that increased POX and/or APX do not increase the resistance/tolerance of the cultivar against the fungal pathogen. The gray blight disease of tea is also known to show a similar trend in POX and APX activity. (Senthilkumar, 2011).

Bashan et al. (1987) reported that the increase in POX activity of tomato leaves in response to P. syringae infection is not directly involved in the resistance mechanism of the plant. Similar observations were noted by Nadolny and Sequeira (1980) in tobacco where there was no direct correlation between increased POX and resistance. The levels of POX during subsequent stages of infection in the resistant and susceptible cultivar suggest the possible involvement of reactive oxygen species (ROS) in the induction of resistance. ROS
are known to act as potent microbial agents where micromolar concentrations of H$_2$O$_2$ inhibited spore germination of a number of fungal pathogens in vitro (Peng and Kuc 1992). An accumulated level of H$_2$O$_2$ is known to activate the MAPK cascade that is known to have an important role in the transduction of signals for the activation of various defence pathways (Zwerger and Hirt, 2001) and is reported in Arabidopsis suspension cultures (Desikan et al. 1999). However, Shetty et al. (2007) found that, even though sporulation of S. tritici in wheat coincided with a massive accumulation of H$_2$O$_2$, removal of this H$_2$O$_2$ by infiltration of catalase resulted in increased pathogen growth, indicating that the pathogen can survive and tolerate the presence of H$_2$O$_2$ but still the growth was minimal in the presence of H$_2$O$_2$.

Upon pathogen attack, AOS including O$_2^-$, H$_2$O$_2$ and nitric oxide can help to induce cell death in infected cells or serve as a signal to activate defence responses in distant uninfected cells. APX expression in TMV-infected tobacco is suppressed by inhibition of protein synthesis in the polysomes (Mittler et al., 1998). Transgenic antisense tobacco with reduced APX suggests that the suppression of APX plays a key role in elevating cellular H$_2$O$_2$ levels and results in enhanced cell death in response to pathogen attack (Mittler et al., 1999). A response of the chickpea during Fusarium wilt in the compatible interactions (susceptible host) was an induction of APX (Carmen et al., 2002). The down-regulation of APX has been found associated with the expression of resistance, rather than susceptibility in some instances (El-Zahaby et al., 1995; Vanacker et al., 1998). The level of SOD in the infected leaves of both resistant and susceptible cultivars is seen to decrease in contrary to most of the reports. This was however in accordance with the observations during the gray blight disease of tea where disease induction decreased SOD levels in both resistant and susceptible cultivars (Senthilkumar, 2011).
4.6.2. Pathogenesis related (PR) proteins – chitinase, β 1, 3-glucanase and phenylalanine ammonia lyase

The accumulation of chitinase and β 1, 3-glucanase in tea leaves during diseased conditions increased rapidly in all the cultivars irrespective of that being resistant or susceptible, though higher increase was seen in the resistant cultivars. This is in accordance to the observations as discussed in section 4.2 where several of the reports in various plant-pathogen interactions documented higher production of these enzymes. The trend shown by these enzymes during different stages of infection in SA 6 and TES 34 is similar to the reports of Vad et al. (1991), Maxemiuc- Naccache et al. (1992), Recupero et al. (1997) and Cecile Rinaldi et al. (2007). Increased enzyme levels in the tissues of the infected bush indicate effective signalling from other parts of the plant upon pathogen infection. These reports are in hand with that given by the tea cultivars in response to Pestalotiopsis theae infection (Senthilkumar, 2011).

The trend of decreased PAL activity during disease incidence observed on our study is contradictory to the role of the enzyme in conferring resistance in various plant-pathogen interactions. The increase in PAL activity is seen in the susceptible cultivar but not in the resistant one during different infection stages. Krystyna Rybka et al. (1998) and Chakraborty and Chakraborty (2005) have evidences for such contradictory observations with regard to PAL in wheat and tea leaves respectively. We could assume from this trend that the enzyme PAL is not the major factor conferring resistance against the blister blight pathogen in tea as seen in other plant species. Further work would be required to ascertain our assumption out of this observation.
4.7. Effect of pathogen infection on flavonoid pathway specific genes

Flavonoid compounds are involved in a wide range of functions in plants, including plant protection. They are known to play a role in plant defence against both abiotic and biotic stresses (Dixon et al. 2002) and are one among the several factors contributing to plant resistance (Treutter 2005). The synthesis of flavonoids starts with the condensation of one molecule of cinnamic acid derivatives yielding flavanones after which the pathway diverges into several branches, each resulting in a different class of flavonoids (Bohm 1998).

Many of the flavonoid compounds can function as passive or inducible barriers against herbivores or microbial pathogens, and the flavanoid content can increase or the flavonoid composition can change in response to pathogen attack (Dixon and Paiva 1995; Miranda et al. 2007; Carlsen et al. 2008). Genes encoding almost the entire pathway from Phe and malonyl- CoA to proanthocyanidin synthesis were induced in poplar plants as a defense response to *M. medusae* rust infection (Miranda et al., 2007). In a similar way, up-regulation of genes involved in the phenylpropanoid pathway, particularly those leading to isoflavone and isoflavonoid compounds was reported in the response of *M. truncatula* to abiotrophic pathogen, *Erysiphe pisi*, the causal agent of powdery mildew (Foster-Hartnett et al., 2007). However, the involvement of flavonoids in plant defense depends on the species (Dixon and Paiva, 1995; Carlsen et al., 2008).

The expression of the genes involved in the pathway is highly influenced by the *E. vexans* infection in both SA 6 and TES 34 cultivars. In the present study it is observed that one of the genes involved in the early steps of flavanoid synthesis 4-Coumarate:CoA ligase (*4CL*) is highly induced during disease induction in the cultivar SA in contrast to TES 34 where there is very less induction during the second stage alone. This could be seen as an induction of resistance as seen in bean (Cramer et al., 1985) and in the studies by Bell et al.,
(1984) where there is increased induction of the 4CL and CHS mRNA levels in response to pathogen infection. 4CL plays a particularly important role in plant defense reactions because of its position joining the phenylpropanoid pathway with lignin and flavonoid branch pathways (Bourlaye Fofana et al., 2005)

The expression of CHI, F3H and ANS is induced in the cultivar TES 34 during various stages of infection whereas it is induced to a lesser extent and even suppressed in SA 6. These genes were observed to vary in their expressions in the studies conducted by Dixon and Lamb (1979), Chengjian Xie et al., (2012). The mere activation of the core gene of the flavanoid pathway F3H as seen in the cultivar TES 34, cannot be considered to confer resistance as the induction of the downstream steps of the pathway involving the genes DFR, ANS and ANR are required for the increased accumulation of proanthocyanidins (Winkel-Shirley., 2001) which are known to have anti-microbial properties and involved in plant resistance (Dixon et al., 2005). Thus the co-ordinated induction of the three afore-said genes along with F3H may confer the cultivar resistant to the fungal pathogen.

Polymerisation of catechins to oligomeric proanthocyanidins and 2, 3-cis isomerisation occurs in tea plants during infection by the fungal pathogen Exobasidium vexans (Punyasiri et al., 2004). Thus oligomeric proanthocyanidins might function as an important growth-limiting factor towards endophytic and pathogenic fungi, and play an important role in plant defence against fungal pathogens.

Further studies relating the changes in gene expression patterns, transcripts and protein accumulations of the flavanoid pathway genes in the resistant cultivar SA 6 would be required to address the down-regulation of these genes during infection. The protein levels would need to be analysed in the susceptible cultivar TES 34 to support the up-regulated expression of these genes during disease induction.