DISCUSSION

Field grown plants are constantly subjected to adverse environmental conditions such as extreme temperatures, drought, flooding, excessive salts, heavy metals, high intensity irradiation and infection by pathogenic agents. Because of their immobility, plants have to make necessary metabolic and structural adjustments to cope with the stress conditions (Ho and Sachs, 1989). These responses can be regarded as departure from the metabolic norm where the norm is the metabolic state expressed under the condition of " no stress". However the latter condition itself may be highly elusive. Not all metabolic responses will be deleterious or injurious and most changes represent adaptations of the plants to withstand the particular stress. To this end, the genetic program in normal plants is altered by the stress stimuli to activate biochemical pathways that ensure survival.

Tea (*Camellia sinensis*) being a perennial, is subjected to varying environmental conditions through out its life. Being a cultivated crop, it has been brought from its source of origin and planted in different regions. It is also subjected to constant pruning, spraying of chemicals and other activities of man, all of which led to various stress conditions, in addition to the naturally occurring environmental stresses. A large number of varieties of tea are available and their responses to the environmental variables are known to vary. In the present study, investigations have been carried out on the biochemical response of tea plants to different environmental stress conditions such as elevated temperatures, drought, chemical spray and infection with a fungal pathogen (*Exobasidium vexans).*

At the onset changes in three enzymes – phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (PO) of different tea clones in response to all the different stresses were determined. Previous reports indicate that oxidative enzymes such as polyphenol oxidase and peroxidase as well as those involved in phenolic biosynthesis such as phenylalanine ammonia lyase and tyrosine ammonia lyase are involved in defense reaction in plants (Chen *et al.*, 2000). Considering the importance of phenol metabolism in tea plants, these three enzymes were selected for studies. Results showed that the constitutive enzyme activities under no stress conditions of the different clones varied. The different clones

considered here, could be differentiated into three groups depending upon their origin i.e. the Tocklai varieties from the North Eastern region, subjected to long spells of rain and humid conditions, the Darjeeling varieties from the hilly terrain of Darjeeling and the UPASI varieties from the peninsular southern part of India subjected to high temperatures in summer. In spite of these varieties being grown in the same experimental conditions their responses to stress varied. Elevated temperature treatments showed that in all Darjeeling varieties and in a few UPASI varieties PAL activity declined whereas in the others there was an increase at 40^{0}C after which there was a decline. In case of water stress all six varieties studied showed an initial increase after 4 days of water stress followed by a decline when drought was prolonged. A decrease in PAL activity was noticed following spray with both fungicide and insecticide as well as following blister blight infection. Matsumato *et al* (1994) reported that Japanese green tea cultivars belonging to variety 'sinensis' could be divided into three groups on the basis of their PAL cDNA cloning. Assam hybrids could not be placed into any specific groups because complex patterns were produced. They confirmed the existence of many kinds of PAL gene, expression of which varied depending on the varieties. An elevation in the level of activity of PAL has been frequently demonstrated to be one of the earliest responses of plants to biotic (Southerton and Deverall, 1990; Chakraborty *et al* 1993; Shiraishi *et al* ,1995) or to other environmental stresses (Kuhn *et al* , 1984; Eckey- Kaltenbach *et al* 1997). It was reported by Orczyk *et al.*, (1996) that in sorghum, naturally occurring high levels of PAL activity induced by light should be differentiated from the activity induced as a response of attempted fungal infection. Bhattacharya and Ward (1987) reported that PAL activity of soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma* f.sp. *glycinea.* Considering that PAL is a key enzyme in the biosynthesis, not only of phytoalexins, but also of phenolic compounds in general, and melanins, all of which have been associated with resistant responses in various host plants, the authors suggested that activity of PAL could be useful indicator of the activation of defense related enzymes. They also demonstrated that induction of the susceptibility to the pathogen, by changes in temperature conditions was associated with the suppression

of PAL activity. In the present study too in most varieties PAL activity decreased with temperature. In case of infection the observed decrease in PAL activity also supports the view of the role of PAL as a defense enzyme since in this case the plants were already infected and hence susceptible.

Polyphenol oxidase usually accumulates upon wounding in plants. PPO transcript levels systemically increased in tomato when mature leaflets were injured (Thipyapong and Steffens, 1997). In the present investigation, elevated temperatures led to an initial increase in PPO activity till 40^0C, 45^0C or 50^0C (depending on the varieties). Similarly all other stresses i.e. drought, spraying with fungicide and insecticide as well as blister blight stimulated an initial increase. Prolonging of the different stresses however led to a decline in activity. Increased activation of PPO and PO was demonstrated in the cucumber leaf in the vicinity of the lesions caused by some foliar pathogens or by phosphate application (Avdiushko *et al.*, 1993). Moreover PPO could be induced by jasmonic acid (Constabel and Ryan, 1998).

Among all the stress related enzymes, role of peroxidase has been most thoroughly worked out. PO is a metallo-enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols, aromatic amines, amino acids, and inorganic compounds (Balasimaha, 1982). These are ubiquitous to plants and are characterised by a large numbers of isozymes. The activity of these peroxidases is markedly influenced by various naturally occurring and synthetic substances, growth regulators and environmental factors. It was observed in the present investigation that elevated temperature decreased PO activity whereas water stress stimulated PO activity. In the Darjeeling and the Tocklai varieites after the initial increase at 4 days of water stress there was a decline in activity, whereas in the two UPASI varieties the activity was higher even after 12 days of water stress. These two UPASI varieties were the drought tolerant ones as exhibited by their morphological changes. Ability to withstand prolonged water stress could in part be due to the induction of high PO activity. Ashraf *et al.* (1995) also reported that water stress induced the activity of PO whereas the other enzymes like nitrate reductase and carboxylase were reduced in wheat genotypes. Brown *et al.* (1995) in their studies on the effect of moderate drought on ascorbate peroxidase, and glutathione

reductase activity reported no increase in ascorbate peroxidase in drought. Increased activity of PO was also obtained following chemical sprays as well as blister blight infection. In a previous study Akhtar and Garraway (1990) observed an increased PO activity susceptible cultivars compared with the resistant one when both were treated with sodium bisulphite prior to inoculation with *Botrytis maydis*. On the other hand there are also reports of increased PO activity due to induction of resistance (Ye *et al* 1990; Chen *et al.* 2000). Curtis *et al.*,(1997) also reported the induction of PO activity by pathogens and methyl jasmonate. Results of the present investigation taken with those of previous ones clearly demonstrate the induction of PO activity following different stresses.

The existence of multiple molecular forms of peroxidase in tea have been reported by previous authors (Takeo and Kato 1971; Gunashekhar *et al.*, 1996). In the present investigation, therefore, the isozymes of PO were analysed by PAGE to determine if the activity of any new isozyme was induced by different stresses. Result revealed no significant changes in isozyme patterns following temperature and water stress but biotic stress induced the activity of at least two new isozyme bands. Chen *et al.*(2000) also reported that inoculation with *Pythium aphanidermatum* induced the activity of new acidic isozymes of peroxidase.

Chlorophyll controls the photosynthetic activity process of a plant and thereby determines the productivity of the plant. It contributes to the 'blackness' of made tea that is considered to be one of the important criteria in the commercial evaluation of tea (Liyanage and Penyasiri, 1993). It has been suggested that the blackness of tea depend on the chlorophyll content and its transformation products (Wickramasinghe and Perera, 1972). Differences in chlorophyll content among different tea clones have been reported previously. De Silva and Sivapalan (1982) reported that the chlorophyll content varied among the different Sri Lankan clones. However, they could not correlate yield to chlorophyll content. They observed considerable variation in chlorophyll content associated with climatic variation. It has also been reported that chlorophyll content increased with rainfall (Wickramasinghe and Perera, 1996). In the present study, chlorophyll content decreased with elevated temperature that was more significant from 45^0C onwards.

In case of water stress also chlorophyll content decreased. The decrease after 4 days of drought was not significant but, after prolonged drought, significant reduction was observed. Du *et al* (1996) also reported that chlorophyll content as well as photosynthetic enzymes decreased with imposition of drought in sugarcane. Both anthropogenic and biotic stresses also reduced chlorophyll content. There are also previous reports on the reduction in chlorophyll accumulation following infection (Bhawani *et al.*, 1998; Singh *et al.*, 1998 a). Th reduction in chlorophyll contents might be due to stimulating of enzymes like chlorophyllase which degrade chlorophyll (Kaur and Deshmukh, 1980) or inhibition of chlorophyll synthesis caused by the pathogen. Janave (1997) suggested that chlorophyll degradation during senescence in Cavandish bananas was the result of two types of catabolic pathways – the chlorophyllase pathway and the chlorophyll bleaching pathway. In the present study, chlorophyll content has been shown to be reduced by all types of stress conditions. Variation in chlorophyll content due to a number of factors including variety, nature of leaf, increase in rainfall, shade, rainy weather, low elevation and different season has been previously reported (Bera *et al.*, 1997).

One of the most important responses of plants to environmental stresses is in their protein metabolism. They respond to environmental stress in two common ways i.e. either by disassembly of preformed polysome resulting in a decrease in translation of mRNAs present at the time of induction and a preferential synthesis of 'stress proteins' from newly transcribed stress induced mRNAs (Bonham-Smith *et al.*, 1987). The response of tea plants to the various stresses with respect to the quantitative changes in protein, as well as nature of proteins were analysed in this study. In case of elevated temperatures different varieties from the different geographical zones seemed to respond differently. Though there was a quantitative decrease in protein content in all varieties, new heat shock protein (HSP), of similar molecular weight were not detected. In the Darjeeling variety, a HSP of approximately 46KD molecular weight was detected while in the UPASI varieties the HSP had a molecular weight of 80Kda, while in the Tocklai varieties no HSP could be detected. Besides varietal differences, induction of HSP was also dependent on the seasonal changes. Several previous authors have reported the induction of

HSPs in plants and in each case the temperature or HSP induction is different. Vierling *et al.*, (1990) reported the induction of HSPs in wheat at a temperature of 37^0C. Both qualitative and quantitative diversion of HSPs was observed in different accessions of *Triticum monococcum.* In barley, Kruse *et al.*, (1993) reported the expression of several HSPs during development of which HSP 26 was a plastidic protein. The accumulation of 104KD protein in rice in response to several abiotic stresses including high temperature was reported by Singla *et al.* (1998). They showed that differential uninduced and induced levels of these proteins accumulated in various organs of the mature rice plant grown under field conditions. In a further study these authors (Pareek *et al.*, 1999) analysed alteration at cellular levels of the various stress associated proteins at four developmental stages in a high yielding rice. They found that the sum total of polypeptide alteration was different at various growth stages analysed. While the alterations in the levels of some proteins were found to be common at different stages, alterations in levels of some other proteins showed growth stage dependent differences. Thus it is clear that the induction of HSPs is governed by several factors.

Water stress also led to the accumulation of one new protein of molecular weight 74KD in tea along with increased accumulation of several other proteins of intermediate molecular weight. Induction of drought induced proteins has also been reported by previous workers. Rao *et al.* (1993) identified several stress responsive proteins in young rice seedlings of which a 23 KD protein was found to be heat stable. A 23 KD drought induced protein was also detected in lettuce by Leinhos and Bergmann (1995a). They also reported the induction of polypeptides of molecular masses of 24, 16 and 14 KD along with enhanced staining of polypeptides weights 50, 36, 16 and 14 KD in barley subjected to drought stress (Leinhos and Bergmann 1995b). Riccardi *et al.*, (1998) reported that in maize subjected to water stress for 10 days 78 proteins out of a total of 413 showed a significant quantitative variation with 38 of them exhibiting a different expression in the two genotypes. Sinha *et al.* (1999) reported the accumulation of polypeptides of 46, 40, 35, and 28 KD in *Lathyrus* seedlings subjected to water stress. Present study also confirms the results of previous workers but here the commonly reported 23KD drought induced protein

accumulated in enhanced quantity, but was already present in low quantities constitutively. Spray of chemicals did not induce any significant change in protein pattern as observed on SDS-PAGE gel even though slight quantitative differences was obtained. Biotic stress, on the other hand, decreased protein content as well as intensity of the bands on the gel. An interesting observation was that, while in the young and mature blisters a reduction in band intensity and band number was observed in relation to the healthy extract, in the very old blisters more intense staining of the band was observed and the appearance of new high molecular weight protein was also detected. Hence it is apparent that during the early stages of infection there is inhibition of protein synthesis which was also reversed when the blister dried up. The induction of pathogenesis related proteins following infection have been reported in a number of cases (Tahiri-Alaoui *et al.*,1993). Eckey- Kaltenbach *et al.*, (1997) in a very interesting study reported differential induction of pathogenesis related proteins in parsley by ozone and heat shock. The induction of same proteins following stresses has also been reported previously. Thus a plant's response to stress may be mediated by common signal pathways leading to similar expression in certain cases.

Proline is an important amino acid which has gained prominence over the years due to its phenomenal accumulation in plants subjected to different stress conditions (Kathiresen 1987; Machakova *et al* 1989). Most reports of increased accumulation of proline are related to water stress. In the present investigation, accumulation of proline in tea leaves subjected to the various stresses have been determined. In case of temperature stress it was observed that accumulation of proline increased up to a temperature of 50^0C and then declined. Most previous studies on the influence of temperature stress on proline metabolism has been concerned with low temperature responses. An accumulation of proline has been reported in many plants grown at low temperatures (Stewart and Larher, 1980). Das and Mukherjee (1994) also observed increased proline accumulation during early imbibition in seeds of *Vigna radiata* subjected to elevated temperatures ranging from 40^0C-60^0C. De *et al.* (1996) also reported that proline accumulated in the seedlings as well as cultured cells of tomato as a consequence of short-term heat shock and

cold shock treatment. Significant water stress induced accumulation was observed in tea plants. Accumulation of proline was extremely high when plants were subjected to prolonged water stress up to 12 days. This result is in conformation with those of all previous workers. One of the first reports that water stressed plants could accumulate proline was that by Burnett and Naylor (1966). Subsequently, several authors have obtained increased accumulation of proline in water stressed plants but the mechanism of accumulation still remains to be agreed upon. Andrade *et al.* (1995) reported increased accumulation of proline in both resistant and drought susceptible cultivars of *Phaseolus vulgaris*. However, the greatest increase in proline was observed to occur mainly in drought susceptible cultivar. Girousse *et al.* (1996) also reported significant increase in certain amino acids including proline in alfalfa subjected to water stress. An increase in free proline level ranging from 4-40 times was observed in 49 pea cultivars with different drought tolerance (Sanchez *et al.* 1998). They reported that the cultivars which accumulated more proline had low water contents upon turgor loss.

At least three possible mechanisms of proline accumulation has been proposed by various authors, i.e., the stimulation of enzymes of proline synthesis such as glutamate dehydrogenase, the inhibition of enzymes of proline catabolism, and the inhibition protein synthesis. Yoshiba *et al.*, (1997) observed that proline was one of the common compatible osmolytes in water stressed plants and accumulated in increased amounts, both by its activation of its biosynthesis and by inactivation of the degradation. They suggested that the synthesis of proline from L-glutamic acid via δ pyrroline 5- carboxylate is mainly regulated by two enzymes- pyrroline carboxylate synthase and pyrroline carboxylate reductase. Proline is metabolised to L-glutamate by two enzymes-proline dehydrogenase and pyrroline carboxylate dehydrogenase. Such metabolism of proline is inhibited when dehydration occurs. During dehydration the author suggested that the gene for pyrroline carboxylate synthase is strongly induced, while the expression of the gene for proline dehydrogenase is inhibited. Therefore, they suggested that the levels of proline are regulated in the transcriptional level of these two genes during dehydration and rehydration.

Chemical sprays also induced increased accumulation of proline in the present study with fungicide and insecticide showing similar responses. Aluminium induced increased accumulation of proline was reported by Zaifnejad *et al.* (1997). They reported that aluminium combined with water stress resulted in high proline in both shoots and roots than aluminium stress alone. Accumulation of free proline in response to Cu, Cd and Zn in *Silene vulgaris* was also reported by Schat *et al.* (1997). They demonstrated that metal induced accumulation of free proline depends on the development of metal induced water deficit in leaves. Proline was also found to increase significantly following infection with *Exobasidium vexans*. Accumulation of proline following infection has been reported previously (Stewart and Larhar, 1980).

It is apparent from the results of present study as well as those of all others that proline increases in plants subjected to various stresses of which drought is most important. Proline is thus , considered to be involved in adaptation mechanisms of plants subjected to various stresses (Aspinall and Paleg, 1981). It may have multiple functions – in osmotic adjustment, in the maintenance of protein stability and as storage of N and C to overcome the unfavourable conditions resulting from stress (Andrade *et al.* 1995).

Polyphenols are major components of tea plants and as such it is expected that they would be affected by the different stresses. In case of temperature stress it was observed that there was a correlation between the inherent phenol content in the tea variety and its increase following exposure to elevated temperatures. In general, in those varieties with high inherent phenol content exposure to higher temperatures led to a decline in phenol content, whereas in those varieties those with lower inherent phenol content, accumulation of phenols kept increasing till 50^0C. A wide variation in the phenol contents in the different varieties was also evident. The observed interesting trend could be explained by the fact that phenols are considered to be involved in plant's defense to various stresses (Leinhos and Bergmann, 1995a). When subjected to temperature stress, varieties with low inherent phenol content increased its accumulation while those that already had a higher content did not have to increase synthesis. In case of tea, polyophenols are also known to vary seasonally

(Zakoskiva *et al.*, 1991). Thus phenol biosynthesis seems to be well regulated to help the tea plant to overcome various stresses. Similarly, in case of drought too, phenol content increased initially up to 8 days of stress after which there was a decline. Even after prolonged stress the drought resistant varieties registered quite high level of phenol indicating their role in tolerance. In general O-dihydroxy phenol also showed similar trends to the total phenols. Initial increase in phenol accumulation was also observed in case of chemical sprays as well as following infection. Kotasthane and Vyas (1982) reported quantitative changes in phenol contents in mustard following application of systemic fungicides. They observed differences in rate of accumulation induced by the different fungicides. Alteration of phenol metabolism following infection has been observed in many diseases and phenolics have been implicated in the defense reaction in several instances (Zaichuk *et al.* 1988; Nicholson and Hammerschmidt, 1992; Chakraborty *et al.*, 1996). Phenolics have been implicated in diverse functional roles such as antioxidant and metal chelators, as UV-light screens and as signaling agent both above and below ground (Cooper *et al.*, 1998). Jones and Hartley (1999) proposed a protein competition model for predicting total phenolic allocations and concentration in leaves of terrestrial higher plants. They suggested that protein and phenol synthesis compete for the common limiting resource - phenylalanine and hence protein and phenolic allocations are inversely correlated. The observed increase in phenolic concentration, with a decrease in protein concentration in the present study could be explained by the above model.

The accumulation of oxidised phenol compounds in plants which are toxic to certain pathogens are believed to take part in the plants defense reaction. The success of a pathogen in establishing a disease could depend on its ability to metabolize the toxic compound. In the present study, results revealed the presence of antifungal phenolics in healthy tea leaves which was shown to be quantitatively lesser in the blister infected leaves. Though the antifungal compound from healthy and infected leaves show similar Rf value on TLC, their UV-spectrophotometric analysis showed the absorption maxima to be different. Chakraborty and Saha (1994) reported the presence of antifungal catechins in healthy leaf extracts which they suggested have

been broken down to catechol in the infected leaves. Nagahulla *et al.*, (1996) also reported the production of antifungal compounds in tea leaves following infection with the blister pathogen. Therefore it seems probable in the present study, the antifungal phenols present constitutively in the healthy leaf tissues are metabolized by the pathogen during the course of its infection into compounds which were less antifungal. In the present study it was also considered interesting to determine whether the constitutively present antifungal phenols were affected by other stresses. For this such compounds are extracted and analysed in case of one abiotic stress i.e. elevated temperatures. A decrease in the antifungal activity as observed in the TLC plate bioassay was noticed in all elevated temperature but no difference could be detected among the different temperature treatments. Since catechins are known to be involved in the flavor of tea, it was also considered worthwhile to analyze the changes in the catechins of tea leaves following biotic stress. Biotic stress was selected for detailed analysis, since, there was previous report on the breakdown of catechins by fungal pathogens (Chakraborty and Saha 1994). HPLC analysis revealed that no major changes were evident in the types of catechins of healthy and infected tea leaf extracts but some of the peaks showed quantitative differences.

In conclusion, it might be stated that the tea plant responds to various stresses by alterations of its metabolic processes in such a way that it becomes capable of withstanding stress. Several responses were found to be of a similar nature in all types of stress i.e. showing an initial increased accumulation followed by decline. The quantum of change in several cases was dependent on the inherent amount of that particular constituent required for defense. Varieties also showed differences among themselves in most of the biochemical constituents. Thus, tea plant which is a perennial during the course of its life, develops mechanisms to overcome the environmental stresses which would finally be regulated by the genetic make up of the particular variety. This is because although plant responses to environmental stresses involve adaptation at several levels of organisation they all must ultimately have a genetic basis. Several genes are known to respond to different stresses commonly encountered in agriculture. The genetic manipulation of these genes holds considerable promises as a first step towards increasing stress tolerance (Hare *et al.*,

1996). Since many of the overlapping responses to different environmental stresses maybe mediated by common cellular signal transduction pathways, in the long term, targeting the genes encoding components of stress related transduction pathways might be more profitable than manipulation of genes involved in intermediate metabolism.