Discussion

Plants, being immobile are constantly subjected to adverse environmental conditions such as extreme temperatures, water scarcity, flooding, heavy metals, excessive salts, high intensity irradiation and infection by pathogenic agents. Besides, various activities of human beings also impose different stresses on plants. Plants react to such stresses by various adjustments to their metabolic processes leading either to avoidance strategies, tolerance or in extreme cases they are victims of the stress. Not all metabolic responses are deleterious or injurious to the plants and most changes represent the adaptations of the plants, to withstand the particular stress. Hence, genetic programme in normal plant is altered by the stress stimuli to activate the biochemical pathway that ensure survival.

Tea (*Camellia sinensis* L.) is grown in rain fed eco-systems in specific regions of India in the hilly regions of Darjeeling and Nilgiri, Terai and Dooars of West Bengal, Brahamputra valley in Assam and certain other areas. Since the plant is grown in non-irrigated eco-systems they are subjected to the different environmental stresses which modify morphology and rate of development, limit yield and quality and reproduction (Leinhos and Bergman, 1995). Huge amounts of pesticides, fungicides and chemical fertilizers are used in tea plantations to ensure the production of disease free leaves for the planters. Soil pollution is caused as a run out of these chemicals used which also contribute to the anthropogenic stress on tea. Activities such as mining and smelting as well as agriculture have contaminated extensive areas of world mostly by heavy metals such as Cd , Cu, Zn, Pb, Cr, Ni (Smith *et al.* 1996; Zantopolus 1999; Herawati *et al* . 2000). Inorganic and organic fertilizers are most important sources of heavy metals to agricultural soil which include liming, sewadge/ sludge, irrigation water and pesticides (Sharma and Agarwal,2005).

In the present study investigations have been done on the biochemical changes induced in the tea leaves by an insecticide (Acephate) and a fungicide (Hexaconazole) which are commonly used in the plantations. Besides, effects of two heavy metals i.e. Cu (taken as $CuSO_4$) and a Cd (taken as $CdNO_3$) were also studied on the various biochemicals process in the leaves. Changes in the biochemical components of tea leaves could alter the flavour components in tea which in turn would affect the quality of made tea. It was observed that in general, plats sprayed with insecticide or fungicide were healthier due to less infestation by pests. However, the biochemical changes induced by the treatements which were considered as anthropogenic stress were determined. At the onset the effect of these chemicals on accumulation of phenol content in tea leaves of different varieties was investigated. Phenols are major components of tea leaves and some of the phenols are responsible for the flavour of tea. An increase in total phenols as well as o-phenol was observed in all treatments in cut shoots, potted plants as well as bushes. In higher concentration of heavy metals leaves from cut shoot showed a decline in phenol content. Phenols are considered to be involved in plant's defense to various stresses (Leinhos and Bergman, 1995). In case of tea, polyphenols are major components and their biosynthesis seems to be well regulated to help the plant to overcome various stresses. Kotasthane and Vyas, (1992) reported quantitative changes in phenol content in mustard following application of synthetic fungicide. Alteration of phenol metabolism following infection has been observed in many diseases and phenolics have been implicated in defense reaction in several instances (Zaichuk *et al.* 1988; Nicholson and Hammerschimidt, 1992; Chakraborty *et al.* 1996). The activation of defense reaction in plants is associated with increased expression of a number of genes that encode enzymes involved in the biosynthetic pathway of phenolic compound. It has been suggested that, certain fungicides used to mitigate or prevent pathogen attack may be involved in certain defense responses in plants (Gracia *et al.* 2003). Increased accumulation of polyphenols to heavy metal stresses has also been reported by several previous workers. Tripathy and Tripathy (1999) reported that phenol contents in *Albizzia lebek* increased with concentration of Ni, Hg and Cr . On the other hand Basak *et al* .(2001) also reported increased accumulation of phenols in tea leaves following Cu treatment but decreased accumulation due to Hg and Ni treatments. Phenolics have been also been implicated in diverse functional roles such as antioxidants and metal chelators as UV-light screens and signaling agents both above and below ground (Cooper *et al.* 1998) . Thus , results of the present study as well as those of previous workers suggest that, phenolics accumulate in plants as a response to various kinds of stresses and they play important roles in the resistance or tolerance of plants to various stresses.

Proline content in all tea varieties increased following insecticide /fungicide or heavy metal treatments. Proline is an important amino acid which has gained prominence over the years due to its phenomenal accumulation in plants subjected to different stressed conditions. Most of the early reports where proline has increased related to water stress.Proline being an osmoregulator may accumulate due to different stresses which ultimately create osmotic stress in the cell. Schat *et al.*(1997) reported that massive accumulation of proline occurs in leaves of *Silene vulgaris* in response to Cu, Cd and Zn . Increased accumulation proline due to heavy metal stress has also been reported by other workers (Tripathy and Tripathy 1999). Barman *et al.* (2002), and Panda and Khan,(2003), also reported that high concentration Zn and Cr induced accumulation of proline. Siddiqui and Ahmed, (2002) also reported increased accumulation of proline in varieties of *Triticum aestivum* following application of synthetic fungicides. In tea plants, Basak *et al* .(2001) reported increased accumulation of proline following Cu, Hg and Ni treatments while Chakraborty *et al.* (2002), also observed increased accumulation of proline following Cu stress . In a study involving heavy metal stress of okra, Chakraborty and Lahon (2007) reported increased accumulation of proline. However, in their study it was observed that the degree of enhancement varies with both varieties and the types of heavy metals. Different types of stresses which ultimately lead to osmotic stresses in the cells induce proline accumulation. At least 3 possible mechanisms has been proposed by various authors i.e. stimulation of enzymes of proline synthesis such as glutamic dehydrogenase, the inhibition of enzymes of proline catabolism and inhibition of protein synthesis. According to Yoshiba *et al.* (1997) synthesis of proline from L-glutamic acid via pyroline 5-carboxylate is mainly regulated by two enzymes pyrolline carboxylate synthese and pyrroline carboxylate reductase . Proline is metabolized to L-glutamate by two enzyme i.e. proline dehydrogenase and pyrroline carboxylate dehydrogenase and such metabolism of proline is inhibited when dehydration occurs. During dehydration the gene for pyrroline carboxylate synthase is strongly induced while the expression of the gene 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 re-hydration.

Proteins, being major biochemical components of plants, influence of the heavy metals and pesticides on protein contents were investigated. Spraying with insecticide and fungicide led to an initial increase in protein content in some of the varieties. However, further application of the chemicals resulted in decrease in the protein content. Similarly , in case of heavy metals though the first treatment induced the accumulation of protein to a certain extent further treatment led to decline.Dewan and Dhinghra (2004), reported that Cd treatment in general did not affect the seed protein appreciably in two parent cultivars of pea and their hybrid. However, high doses of Cd decreased protein content in one of the cultivar HFP4. Reports of induction of phytochelatin synthesis by Cd are numerous (Raineri *et al.*2005; Mishra *et al* . 2006). Giannaza *et al.* (2007) reported inhibition of storage protein catabolism and plant protein anabolism in ***Lipidum sativum*** plantlets exposed to Cd stress. Besides, they also reported that the appearance of two proteins may be related to cellular stress and another two which may be involved in embryogenesis. Siddiqui *et al.* (2002) reported that , application of systemic fungicide to susceptible and resistant variety of wheat caused a significant decrease in total protein content which was maximum in susceptible cultivar.

Chlorophyll plays important role in plant metabolism as it controls photosynthetic activity of a plant and thereby is one of the determinants of the productivity of the plant. In case of tea, chlorophylls also contribute 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). Differences in chlorophyll content among different tea clones have been reported previously. In a study by Desilva and Shivapalan (1982), it was reported that, chlorophyll content of different Srilankan clones varied and varieties were observed with climatic changes. Increase in chlorophyll content was correlated with rainfall by Wikramasingha and Perara (1996). The present study was undertaken to determine the effect of the various anthropogenic stresses on chlorophyll content of tea leaves. Results showed that, while low concentration of Cu increased chlorophyll content to certain extent, higher concentration of Cu inhibited chlorophyll. The effect of Cd was more pronounced as even lower concentration of Cd inhibited chlorophyll accumulation. Chlorophyll content was also observed to decrease in tea cultivars following insecticide / fungicide spray. In a similar study, Upadhyay and Pandey (2004), reported that treatment with a pesticide showed decrease in chlorophyll content of seedlings. There are several reports on the effect of Cd influencing chlorophyll content of plants. Keshan and Mukherjee (1992) reported that chlorophyll content of *Vigna radiata* leaves declined at all concentrations of Cd and the reduction in chlorophyll b was higher than that of chlorophyll a. However, in the present study, the reduction in the chlorophyll b was greater than chlorophyll a leading to higher a/b ratio. The results are in confirmity with those of Bhattercharjee and Mukkherjee (2003) , who also reported that , $CdCl_2$ treatment of *Amaranthus lividus* seedlings induce slightly higher chlorophyll a/b ratio and the loss of chlorophyll b was greater than that of chlorophyll a . Other heavy metals like Ni, Mn and Zn have also been reported to decrease chlorophyll content of plants (Prathiva and Rathore ,2002 ; Sinha *et al.* 2002; Singh *et al.* 2005 ; Hou *et al* .2007). The impairing of chlorophyll development by heavy metal may be due to interference with the synthesis of proteins which are structural component of chloroplasts. On the otherhand reduction in chlorophyll content might be due to stimulation of enzymes like chlorophyllase which degrade chlorophyll (Keshan and Mukherjee (1992); Kaur and Deshmukh , 1980) . Janave (1997) suggested that, chlorophyll degradation during senescence in cavandish bananas was a result of two types of catabolic pathways-chlorophyllase pathway and chlorophyll bleaching pathway. 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 seasons has been previously reported (Bera *et al.* 1997). In the present study it was also observed that carotenoids showed an initial increase due to heavy metal treatment but, with further treatment or increasing concentration of heavy metal the carotenoid content decreased. However, spraying with insecticide/ fungicide enhanced carotenoid content to certain degree. In many of the previous studies it has been reported that , heavy metals decrease carotenoid content of leaves. Mathur *et al.*(2006) reported that Cd induce decline in carotenoid pigment in moth bean cultivars. However, carotenoids being antioxidants it might be possible that plants respond to stresses initially by enhancement of antioxidants which led to certain tolerance. The increase in carotenoid content in the study may be explained by the above. Chlorophyll biosynthesis was inhibited by Cu stress in *Thalaspi ocrolucum* (Ouzounibou, 1992) and *Phaseolus vulgaris* (Gadallah, 1995). In general, it has been reported that chlorophyll accumulation is highly sensitive to heavy metal toxicity (Gupta and Chandra,1996).

Since carbohydrates form important biochemical constituents of any plants, in the present study, the influence of the various stresses on total soluble sugar and reducing sugar were determined. Total soluble sugars showed an increase in initial treatment but with increase in concentration the total sugar decreased. Reducing sugars also showed an increased accumulation at lower concentration which however declined with higher concentrations. Spraying with insecticides / fungicides led to an decrease in total and reducing sugar content. Shukla *et al.* (2003) reported that Cd altered the levels of several biochemical constituents including starch and soluble sugars in wheat seedlings. It was also reported that, higher concentrations of arsenate induce a decrease in total soluble sugar in *Vigna radiata* seedlings (Debnath and Srivastava ,2003).

Phenols being the most abundant and one of the important biochemical constituent of tea leaves enzymes involved in their metabolism i.e. phenylalanine amminia lyase (PAL) , polyphenol oxidase (PPO)and peroxidase (PO) also play important role in tea plant's metabolism. Hence, in the present study activities of these 3 enzymes under controlled and stressed conditions were analysed. Results revealed that the activity of the constitutive enzymes in different tea varieties also varied. PAL activity increased in 100and 500 µg /ml treatments of Cu and Cd but at 1000 µg /ml there was a decline. A decrease in PAL activity was noticed following spray with both fungicide/ insecticide. Matsumato *et al.* (1994) reported that Japaneese green tea cultivars belonging to variety 'sinensis' could be divided into 3 groups on the basis of their PAL c DNA cloning. They confirmed the exsistance of many kinds of PAL gene, the 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 plant to biotic stress (Southerton and Deverall ,1990; Chakraborty *et al* . 1993) or to other environmental stresses (Eckey-Kaltenbach *et al.* 1997). Yan *et al.* (2008) reported that Ni toxicity induced PAL activity which had a positive correlation to Ni concentrations.Gracia *et al.*(2001) determined the effect of application of different concentration of carbendazim on the metabolism of the phenolic compounds in tobacco plants not suffering biotic or abiotic damage. Their results indicated that, the application of carbendazim increased PAL activity as well as accumulation of phenolics. PAL plays a key role in linking primary metabolism to phenyl propanoid metabolism by converting L-phenylalanine to trans cinnamic acid. This reaction provides an entry point for the biosynthesis of large number of defense related functional products and PAL is considered a part of defense mechanism. The regulation of PAL activity in plants is made more complex in many species due to the exixtance of multiple PAL encoding genes some of which are expressed only in specific tissues or only under certain environmental conditions (Macdonald and D'cunda , 2007) . It has also been shown previously that PAL is generally stimulated in plant tissues exposed to heavy metal stresses (Santiago *et al* .2002). PPO activity was also found to be enhanced in the lower concentration of heavy metals and also due to spraying of in secticide/fungicide. Heighest concentration of heavy metals reduced PPO activity . Increased activity of PPO was demonstrated in cucumber leaf caused by some foliar pathogen or by phosphate application (Avdinshko *et al*, 1993). More over, polyphenol oxidase could be induced by abiotic inducer such as jasmonic acid (Constabel and Ryan, 1998). Increased accumulation of polyphenol oxidase induced by Cd was reported in *Arabidopsis thaliana* by Saffar *et al.* (2009). In the present study the isozyme patterns of PPO were also found to be affected by various heavy metal treatments as also treatment with insecticide / fungicide . However, changes in isozymes could not be correlated with overall change in activity.

Peroxidase is one of the most worked out enzymes either in connection with its role in defense or as an antioxidative enzymes. Peroxidase is a metallo enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrate including phenols, aromatic amines, amino acids and inorganic compounds. These are ubiquitous to plants and are characterized by a large number of isozymes. The activity of peroxidase is markedly influenced by various naturally occurring synthetic substances, growth regulators and environmental factors. It was observed in the present investigation that peroxidase activity was greatly increased by anthropogenic stresses. Decline in activity was observed at high concentration of Cu or Cd. Chen *et al.*(2002) reported that, treatment of radish seedling with $CuSO_4$ solution increased peroxidase activity which was concentration dependent. Activities of both anionic and cationic peroxidases were found to be enhanced but differed in the time of enhancement. Mourato *et a.l* (2009) also reported that peroxidase and polyphenol oxidase activity increased in *Lupinus luteus* plants grown in hydroponic solution for 15 days under different Cu concentrations. Cd was also reported to enhance activity of peroxidase in *Arabidopsis thaliana* (Saffar *et al* .2009).

Isozyme analysis of the peroxidase in the present study revealed changes in isozyme pattern induced by chemical treatments. The existence of multiple molecular form of peroxidase in tea have been reported by previous workers (Takeo and Kato 1971; Gunashekhar *et al.* 1996) . Yan *et al* . (2008) detected atleast 5 peroxidase isoforms in *Jatropa curcas* cotyledons with different patterns. The staining intensity of 3 isoform bands were stimulated with increasing Ni concentrations. In the present study also staining intensity of certain bands increased in some treatments while in others there was an inhibition. Peroxidases are commonly found as several isozymes in plants because of its multiple functions. The pattern of expression of isoforms varies in the different tissues of healthy plants and is regulated at different time and places by various kinds of biotic and abiotic stress inducers (Passardi *et al.* 2005) .

Catechins are phenol flavonoid flavour components of tea and as such it was considered worthwhile to analyse the changes in catechins in tea leaves following the different stresses. HPLC analysis revealed that, some of the isoforms of catechins was suppressesd by the stresses but at the lower concentration , the pattern was similar to that of control. Some of the peaks showed quantitative differences in the treatments. Accumulations of various heavy metals in different tissues in organs of plants which are taken up by the plant are the important consideration for human beings. Dietery exposure to heavy metals mainly Cd, Pb, Zn and Cu has been identified as a risk to human through consumption of vegetable crops (Kachenko and Singh,2006). Accumulation of heavy metals in tea leaves is particular by important because these heavy metals are taken by the plant and translocated to the leaves and which may remain in the leaves used as beverage. Hence, in the present study the actual content of heavy metal in leaves of two varieties of tea plants subjected to heavy metal stress were determined. It was observed that significant accumulation of both Cu and Cd occurred in the leaves though, Cu accumulation was greater than Cd leaves of untreated plants were also found to contain certain amount of Cd and Cu specially more of Cu.

In conclusion, it can be stated that, either heavy metal treatment or spraying with insecticide/ fungicide induced definite metabolic changes in the tea plants. While the lower concentration of heavy metals or initial application of insecticide/ fungicide led to an initial increase in the metabolic products, higher concentration or prolonged treatments led to decline in metabolism. The plant's ability to withstand the stress was evident by enhanced antioxidative and other such responses of the different tea varieties.It is clear that tea plant which is a perennial develops mechanisms to overcome various environmental stresses including anthropogenic stresses. This would finally be regulated by the genetic make-up of the individual variety. Several genes are known to respond to different stresses commonly encountered in agriculture. The genetic manipulation of these genes holds considerable promise as a first step towards increasing stress tolerance (Hare *et al*.1996).