CHAPTER-7

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Heavy metal stress is one of the important environmental factors which induce significant alterations in plant physiology and biochemistry. Plants respond to such stress by primarily through the generation of reactive oxygen species. Various metals either generate ROS directly through Haber-Weiss reactions or overproduction of ROS and occurrence of oxidative stress in plants could be the indirect consequence of heavy metal toxicity (Yadav, 2010). Changes occur as part of the tolerance mechanism which is an inherent capacity of the plants that enables to minimize the detrimental effects through detoxification mechanisms.

Tea has gained the world's taste in the past 2000 years as one of the most popular non-alcoholic beverage. The economic and social interest of tea is easily understood from the fact that about 18 to 20 billion cups of tea are consumed daily in the world. Tea is cultivated extensively in north east India and provides livelihood to a large population. To maintain productivity and minimize loss due to fungal diseases, a huge amount of copper based fungicides are used in the tea gardens of this region. Tea is woody perennial and plantation crop with a productive lifetime of 30 years. Therefore, it seemed very likely that long term use of copper based agrochemicals can cause copper accumulation in the tea garden soils and thereby affect the plants detrimentally. In order to confirm the exact status of fungicide usage and also disease occurrence in the tea estates of sub-Himalayan West Bengal and Assam, a survey work was conducted based on specific questionnaire. The result clearly revealed the excessive use of copper based fungicides in all the tea gardens that responded to our query. Thus, at the onset of this study, it was proved beyond doubt that chances of accumulation of copper in the soil might be a possibility and it was important to study the effect of excess copper in tea plants.

Inhibition of germination, growth and reduction of biomass production are general responses of higher plants to heavy metal toxicity. In the current study, a decrease in germination percentage, early root and shoot growth and biomass production was noticed in response to excess copper in three

cultivars, TS-462, TS-463 and TS-520. Long term exposure to copper caused serious morphological aberrations in the roots such as stunted growth and blackening of root tips. Of the three cultivars, TS-463 was found to be most sensitive to copper induced inhibition of germination and biomass production. The decline in biomass production could explain, in part, due to inhibition of both cell elongation and division by heavy metals (Arduini *et al.*, 1994). Ouzounidou *et al.* (1992) reported that metal affects ultrastructure of meristematic cells altering the ribosomal RNA precursor biosynthesis, thus affecting the plant growth.

Copper induced changes in several physiobiochemical parameters was studied under hydroponic culture system using three month old tea seedlings of TS-462 and TS-520 cultivars. These two cultivars were chosen because stress related modifications in the plant are best studied on cultivars which are more resistant. Results were noted on the 4^{th}, 7^{th} and 10^{th} day after start of treatment with increasing concentrations of copper (50 to 700 µM). Control plants were exposed to nutrient solution only without excess copper. Though the results from hydroponic experiments may sometimes be different from field experimental data, but solution culture is a model of the interaction between plant and soil solution and is widely used by scientists to study metal induced stress physiology.

Excess copper is toxic because as a redox active metal, it directly increases production of ROS through copper catalyzed Fenton reaction, where hydroxyl radicals are produced from superoxide and hydrogen peroxide. This ROS damages the biomolecules like proteins lipid and DNA, leading to multiple changes in plant physiology and biochemistry (Ducic and Polle, 2005). With the help of these changes, the plant is able to avoid excessive uptake of copper ions, detoxify copper ions by complexation and sequester ROS through multiple regulated pathways. In the present study, exposure to Cu resulted in an increase in superoxide anion (O_2^-) generation. Additionally, excess accumulation of products of lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS), in the leaves of tea seedlings was evident in comparison to control. The level of both O_2^- and TBARS increased steadily with Cu concentration and time of exposure in both cultivars until 500 to 600 µM and upto 7 days beyond which the rate of increase declined. This provided a clear indication of the oxidative damage

induced by high Cu concentrations. Other authors (Mazhoudi *et al.*, 1997; Rama Devi and Prasad, 1998; Chen *et al.*, 2000; Drazkiewicz *et al.*, 2004; Nie *et al.*, 2012) have reported similar increase in lipid peroxidation and O_2^- levels in plants exposed to Cu. The peroxidation of unsaturated lipids in biological membranes is the most prominent symptom of oxidative stress in animals and plants (Yamamoto *et al.*, 2001), and is considered a biomarker of metal-induced oxidative stress (Ferrat *et al.*, 2003).

Being a redox metal, Cu can interfere with various physiological processes including lipid peroxidation, a toxicity indicator for plants exposed to Cu (Baryla *et al.*, 2000). The primary site of Cu toxicity lies at the cell membrane level including the photosynthetic membranes (De Vos *et al.*, 1992; Sandmann and Boger, 1980). A comparison of lipid peroxidation and superoxide generation revealed significant differences between the two cultivars of tea in response to Cu. TS-520 was found to be more sensitive to Cu as it produced significantly higher concentration of TBARS and O_2^- at high exposure concentrations ($P<0.05$). Differences among cultivars in response to Cu stress have been found in other plants such as *Triticum durum* (Ciscato *et al.*, 1997), *Holcus lanatus* (Hartley-Whitaker *et al.*, 2001) and *Kummerowia stipulacea* (Xiong *et al.*, 2008). Lipid peroxidation and O_2^- generation in tea plants have been reported in response to drought stress (Upadhyaya and Panda, 2004; Upadhyaya *et al.*, 2008) and to cadmium (Mohanpuria *et al.,* 2007) and zinc exposure (Mukhopadyay *et al.*, 2013). However, there is no previous study on the Cu induced lipid peroxidation in tea.

In the present study, total chlorophyll and carotenoid content was found to decrease significantly with increasing Cu concentrations and exposure times in both the cultivars with the more sensitive cultivar (TS-520) recording a significantly higher decrease ($P<0.05$). Several authors (Ouzounidou *et al.*, 1994; Ciscato *et al.,* 1997; Rama Devi and Prasad, 1998; Mohanpuria *et al.,* 2007) have also reported a decrease in chlorophyll content in plants when exposed to Cu. Leaf chlorophyll concentration is crucial for the susceptibility of the plants to photoinhibition and should be considered when an effect of environmental stress is under study (Patsikka *et al.*, 2002). The present study indicates that excess of Cu causes inhibition to the photosystem in tea leaves. A steady dose and time dependent decrease in carbohydrate content

noted in this study may be due to decrease in photosynthetic activity. However the decrease in carbohydrate content may also be attributed Cu interference in polymerization of glucose into carbohydrates (Azmat and Riaz, 2012). Carotenoids are known to function as collectors of light energy for photosynthesis and as quenchers of triplet chlorophyll and O_2^-. Moreover, they dissipate excess energy via the xanthophyll cycle and can act as powerful chloroplast membrane stabilizers that partition between the light-harvesting complexes and the lipid phase of thylakoid membranes, reducing membrane fluidity and susceptibility to lipid peroxidation (Havaux, 1998). In our results, increasing concentration of Cu significantly inhibited carotenoid concentration in tea seedlings. Protein content was also found to decrease significantly but only at higher exposure concentrations that may be due to breakage in polypeptides due to oxidation or due to damage to the genetic material by chromosome fragmentation caused by oxidative disruption of DNA structure (Olteanu *et al.*, 2013). A comparative study of proteins in the seedlings at different tested concentrations and control showed that a particular 77 kDa band in the leaves and 46 kDa band in the roots appeared only at high Cu concentrations but were not present in control. The level of proline, which is regarded as an important molecule in redox signaling and also a scavenger of ROS, increased steadily with increasing concentration of copper but marginally decreased at high exposure concentrations (> 500 μM) in the nutrient solution in both cultivars. The levels of phenolics and o-dihydroxyphenolics increased similarly in the leaves with increase in concentration of copper in comparison to control. The content of non-protein thiols recorded an increase with time upto 7th day beyond which it declined at all exposure concentrations in both root and leaf. Glutathione is a major cellular non-protein thiol and a well-known antioxidant playing a prominent role in the defense against free radicals in plants (Alscher, 1989). Glutathione is also an important precursor of phytochelatins that help in the binding and compartmentalization of metal and prevent further injury. The metal-induced depletion of glutathione in plants due to phytochelatin synthesis may therefore increase the susceptibility of cells to oxidative stress, especially in the case of the redox cycling metal copper. In addition, copper

may catalyze the oxidation of cellular thiols, resulting in the production of free radicals and subsequent lipid peroxidation (De Vos *et al.*, 1992).

Superoxide dismutase activity increased with increasing Cu concentrations during the first seven days. The more tolerant cultivar, TS-462 recorded a higher increase in enzyme activity, however, with the longer exposure time and above 400 µM Cu concentrations, the activity levelled off. In TS-520, SOD activity decreased above 400 µM but after 10 days of exposure at the highest tested concentration (700 µM), the activity returned almost to the original level. The present results are in agreement with the previous studies on response of SOD to Cu stress (Ke *et al.*, 2007; Hartley-Whitaker *et al.*, 2001; Wang *et al.*, 2004). SOD enzymes provide the first line of defence against ROS by removing superoxide radicals and protect plant tissue from oxidative injury. SOD isozymes are metalloenzymes which utilize metal cofactors like Cu, Zn, Fe and Mn for activity (Alscher *et al.*, 2002). A deficiency or excess of these metals can therefore affect their activity (Xiong *et al.*, 2008). Upadhyaya and Panda (2004) observed that SOD activity increased significantly in tea leaves subjected to drought stress and decreased when rehydration was imposed. Aluminium exposure also caused an increase in SOD activity in cultured tea cells (Ghanati *et al.*, 2005). A single isozyme of SOD was detected during this study which showed maximum intensity at 500 µM concentration. Multiple SOD isozymes are recorded in plants such as Fe-SOD, Mn-SOD and Cu/Zn-SOD (Bowler *et al.*, 1994), however, only one Fe-SOD isozyme was visible under copper stress in *Solanum nigrum* plants and the rest were very weak (Fidalgo *et al.*, 2013). Other authors have suggested that excess copper increases Cu/Zn-SOD and suppresses the expression of other SOD enzymes (Tewari *et al.*, 2006). Thus, it may be probable that during the current study, except one, all other isozymes were suppressed to such levels that remained below the limit of detection.

Peroxidase activity increased with Cu concentration to more than two fold in both the tested cultivars. TS-520 recorded a significantly lower POD activity than TS-462. The activity increased in TS-520 plants at lower exposure concentrations but subsequently declined at concentrations higher than 400 µM. This lowering of activity may be due to complete inhibition of growth in the sensitive cultivar exposed to Cu concentrations higher than this

threshold level. On the other hand, in TS-462, POD activity showed an all through increase with increase in Cu concentrations and time of exposures except at the highest concentration where it recorded a sharp decline. It has been shown that excess Cu induces POD activity in several plant species (Mocquot *et al.,* 1996; Diaz *et al.*, 2001). Results of isozyme analysis of POD revealed that two new isozymes POD 3 and POD 4 were induced in the leaves of tea exposed to high concentration of Cu. Diaz *et al.* (2001) also detected two new POD isozymes in pepper hypocotyls while Fang and Kao (2000) observed one new POD isozyme in rice leaves induced by Cu treatment. POD induction by copper excess was further confirmed by immunogold labeling followed by silver enhancement in roots tissues of tea seedlings exposed to 500 μM and 200 μM Cu^{2+}. Stronger labeling was detected in the treated roots in comparison to control. Peroxidases take part in defense mechanism against heavy metal toxicity through lignification of cell walls (Diaz *et al.*, 2001) that confers rigidity and prevent growth. In the present study, growth parameters could not be investigated under hydroponic system because tea is a very slow growing plant (Mohanpuria *et al.,* 2007) and growing tea plants in a simple salt solution for long period is impractical (Ghanati *et al.*, 2005). Peroxidase also acts as efficient H_2O_2 scavenger in a process that involves phenolic compounds as electron donors in the apoplast and plant vacuoles (Morina *et al.*, 2008). In the present study, we measured total phenolics content and the results showed an increased content of phenolic compounds in both the cultivars at Cu concentrations below 400 μM. At higher concentrations, the activity either changed insignificantly or decreased. Thus the results show that there was simultaneous increase in POD activity and total phenolic content due to increasing concentrations of Cu. Phenolic compounds are important antioxidant chemicals of plants which generally act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al.*, 1997) that protect plants from oxidative damages. The hydroxyl and carboxyl groups of phenols may be involved in binding heavy metals like iron and copper (Jung *et al.*, 2003). It has been reported that defense related genes involved in phytoalexin and lignin biosynthesis are the most sensitive among all genes that are upregulated in response to Cu (Sudo *et al.*, 2008). Tea is a tannin rich plant and is tolerant to excess Aluminium (Morina *et al.*, 2008) and Manganese (Alscher *et al.*, 2002). Protection against

manganese has been attributed to direct chelation of Manganese by the phenolic compounds (Michalak, 2006). Copper may be more damaging due to its strong redox nature; nevertheless, from the present results, certain degree of tolerance mediated by antioxidant chemicals and enzymes is evident in the two tested cultivars of tea.

In the present study, APX activity recorded a four-fold increase in the more sensitive cultivar but the activity declined at concentrations higher than 400 µM. A sharp increase in APX activity was noticed at the 10th day of exposure at concentrations 400 µM to 600 µM in the more tolerant cultivar, TS-462. However, at 700 µM, the activity declined. Activity staining revealed the occurrence of two isozymes both of which were found to be regulated under copper excess. Gupta *et al.* (1999) also observed a late increase in APX acivity in *Pheseolus vulgaris* subjected to Cu stress. Increase in both POD and APX activities in tea leaves suggest that the antioxidative machinery induced by Cu was involved in detoxification of H_2O_2. In this study, CAT activity showed an insignificant increase in both the cultivars of tea. Moreover, no significant difference was observed between the cultivars exposed to excess Cu ($P<0.05$). Thus CAT activity remained unaltered or was marginally increased in response to oxidative damage induced by Cu. Catalase activities were found to decrease or remain unaffected in Cu-stressed oat leaf segments (Luna *et al.*, 1994), rice seedlings (Chen *et al.*, 2000), and tomato seedlings (Mazhoudi *et al.*,1997). On the other hand, CAT activity was reported at significantly higher levels in Cu-stressed *Prunus cerasifera* plantlets (Lombardi and Sebastiani, 2005). In activity staining studies, a single CAT isozyme was detected which was most intense at the highest exposure concentration. Catalase represents a primary enzymatic mechanism which is used by aerobic organisms for the decomposition of toxic H_2O_2 generated during oxygen metabolism (Havir and McHale, 1987). In the leaf cells, CAT is exclusively located in the peroxisomes, while H_2O_2 mainly accumulates in the chloroplast through dismutation of superoxide anion radical generated due to photoreduction of dioxygen in a reaction mostly catalyzed by Cu/Zn-SOD (Asada, 1992). Ascorbate peroxidase participates in scavenging of H_2O_2 in the chloroplast using ascorbate as the electron donor (Asada, 1992; Shigeoka *et al.*, 2002). The present findings showed that excess Cu^{2+} ions caused considerable increase in APX activity

along with SOD and POD activities which is evidently a consequence of an excess accumulation of H_2O_2 in tea leaves. But it appears that catalase was not markedly mobilized for protection against this oxidative injury possibly because the excess accumulation of H_2O_2 inactivated CAT (Wang *et al.*, 2004).

In this study, the tea plants exposed to the highest tested concentration (700 µM) was observed to take up almost 1000 µg g^{-1} DW of copper from the nutrient solution but this copper was mainly retained in the roots and the leaves recorded only 95 µg g^{-1} DW of copper in TS-520, the more susceptible cultivar. TEM analysis shows that copper was precipitated in the cell wall and vacuoles of the tea roots. Vacuoles and cell walls are regarded as the compartments for copper tolerance (Cai-ying *et al.*, 2005). However, the extent of oxidative damage in the leaves and roots shows that tea plants are quite susceptible to copper excess although some accumulation occurs in the roots upto a threshold level of approximately 500 µM Cu^{2+}. Beyond this limit, copper gets transported to the leaves in higher amounts and generates an overload of ROS that causes terminal injury to photosystems, membranes, accessory pigments and others. For exact understanding of the oxidative damage, further studies on the underlying mechanisms at the genetic level should be of interest.