CHAPTER 5

DISCUSSION

5.1. Effect of Cu

Number of proteins and enzymes present in heavy metals, make them essential for the growth and development of plants and also maintain the optimum metabolism. The deficiency of these metals has direct impact on the process of photosynthesis, but with the increase in concentration of these metals become toxic to plant and affects photosynthesis. Heavy metal generally inhibits the plant metabolism, but some metals like Cu, Fe, and Mn are essential for the growth and other metabolic functions. Heavy metal plays a vital role in the physiological process of plants. In trace amounts, several ions required for metabolism, growth and development which is present in soil or as in growth media. The excess amount of metal creates problem in the plants by leading to cellular damage (Avery, 2001; Schützendübeland Polle, 2002; Gaetke and Chow, 2003). Though Cu is an essential component for both the photosynthetic and respiratory electron chains but excess of Cu can cause changes in permeability in membrane, chromatin structure, protein synthesis, enzyme activity, photosynthesis and respiratory processes through its phytotoxic effect and also can causes lipid peroxidation and lead to activation of senescence (Baryla *et al.*, 2000).

5.1.1. Metal accumulation

The bio accumulation of heavy metals by plants induces a series of changes in their metabolic functions. Some of which are directly connected to the mechanisms of metal tolerance by the plants (Liu *et al.*, 2010).The accumulation of Cu content in tea leaves were compared in the different cultivars (Fig 4.1.1A-4.1.1F).The different concentration of Cu with

respect to time exposure of treatment in the different cultivars of tea plants were studied. In our study the accumulation of Cu by the different cultivars of tea plants is accompanied by many physiological changes.

Our result showed a positive correlation in all cultivars except S_3A_3(4.1.1.D) between the accumulation of Cu and increase in the concentration of Cu. Accumulation potential of Cu in TS-491was found to be highest with compare to other cultivars (4.1.1.F). In cloned cultivars TV-21 was marked with the highest content of Cu (4.1.1.C). The level of increase in Cu content was different in different cultivars with respect to its exposure time. Tea have potentiality to bio accumulate Cu to several folds. Heavy metal accumulation by various plants have been reported, Cr by *C. sinensis* (Tang *et al.*, 2012), Cd by *Triticum aestivum* and *Zea mays* (Zhao, 2011), Cd by *Lonicera japonica* (Liu *et al*., 2010), Cu by *Alyssum montanum* (Ouzounidou, 1994), Cu by *C. sinensis* (Saha *et al*., 2012), Mn by *C. sinensis* (Wrobel *et al*., 2000). The significant increase in Cu concentration was observed in leaves blades, shoots and roots in rice plants with the application of $CuCl_2$ (Sudo *et al*., 2008). The trend of increase in the content of heavy metal was also observed with the increase in the concentration of Cr in the tea plants (Tang *et al*., 2008, 2012).

5.1.2. Effect of Root length

At higher concentration 400 µM, 500 µM, 600µM with the exposure time the Cu treated plants showed adverse effect by declined in the growth of shoot, decrease in length of root, growth of new leaves are very low, bleaching of leaves and fall off. After one day of metal treatment the growth inhibition starts in the plants (Weckx and Clijsters, 1996). In our study we observed the reduction in the growth of root with the increase in the concentration of Cu. the same trend of result was observed in Cr treated C.sinensis (Tang *et al*., 2008, 2012).

Our result is persistent with the findings of Cd treated corn and wheat (Zhao *et al.*, 2011). Zhao et al., 2011 observed that with the increase in concentration of Cu the root growth were restricted in both the plants and it was strongly reduced at the highest concentration.

5.1.3. Effect of Shoot length

In our study we observed the reduction in the growth of shoot along with the root with the increase in the concentration of Cu. Our result is persistent with the findings of Cd treated corn and wheat (Zhao *et al.*, 2011), Cr treated *C.sinensis* (Tang *et al*., 2008, 2012). Zhao et al., 2011 observed that with the increase in concentration of Cu the root growth were restricted in both the plants and it was strongly reduced at the highest concentration.

5.2. Study on physiological effects

5.2.1. Morphological analysis

The plants have different symptoms at different concentration of Cu with the time of exposure. In our study we observed declination in the growth rate of plants with the increase in the concentration. From we observed that at the lower concentration (50-300µM), there were less or no effects in the leaves pattern whereas the prolonged exposure of higher concentration was observed with brown and yellow patches (Figure 4.2.1.3.A-D). At the later phase, typical symptoms of Cu toxicity developed in the different cultivars after the beginning of treatment. Above 500µM, necrotic lesions were seen on the leaves. These symptoms led to a decline in the growth of tea and its production. The same result was observed on tea leaves with the increase in the concentration of Cr (Tang *et al*., 2008; 2012). The yellowing of leaves due to the interaction between the Cu and Fe present in the pigment (Tang *et al*., 2008). Cu toxicity was also found to affect the growth of *Alyssum montanum* (Ouzounidou, 1994), *Brassica*

juncea (Singh and Tewari, 2003), Cr on *C. sinensis* (Tang *et al*., 2012). Due to the Cu stress many oxidative stress also get generated and this as a whole cause the inhibition in the photosynthetic reactions which has been observed by many authors (Rocchetta and Kupper, 2009).At higher concentration of Cu (700 µM) were also tested the plants experienced severe fragmentation of leaves and it was not possible to carry out the experiments (Figure 4.2.1.3.B). That is why the concentration up to 600 µM of Cu was selected for the study.The high dosage of heavy metal leads to the speedier metabolism which increases the entrance of heavy metals in to cells. As a result if the metabolism is inhibited, toxicity to the plants is revealed (Zhao and Bi, 1999).

5.2.2. Biomass

In our study we observed the initial increase in the biomass with the increase in Cu concentration but the prolonged exposure of Cu led to the decline in the biomass of cultivars with respect to control. Accumulation of Cu at higher concentration (500µM, 600µM)) resulted in greater toxicity to the plants, which was evident by the reduced biomass (Figure 4.2.2.A-F).The same trend of result observed by many authors. The shoot and root biomass of corn and wheat decreased with the increase in the concentration of Cd in the nutrient media. The shoot biomass of corn and wheat were significantly decreased by treatment of Cd of 45.65% to 73.18% and 36.18 to 61.3% with respect to control. The biomass of root in corn decreased by 78.08% and in wheat the biomass decreased by 78.57% (Zhao, 2011). The biomass of leaves and root increased to 5mg/L of Cd with respect to control but further increase in the concentration of Cd in the nutrient media led to decrease in the biomass (Liu et al., 2011). The decrease in the biomass with the increase in Cr concentration in *C. sinensis* (Tang et al., 2012),

and Cu in *C. sinensis* (Saha et al., 2012) was observed.The growth parameters like biomass have been shown to very sensitive to heavy metals (Arun *et al.*, 2005).

5.2.3. Effect on chlorophyll content.

The physiological symptoms of toxicity in plants showed the reduction in chlorophyll content in plants (Lidon and Henriques, 1992). In our result we established a negative relationship between the content of chlorophyll and increasing concentration of Cu (Fig 4.2.3A-F). We observed that in all the cultivars there was a significant decrease in the chlorophyll content with the increase in the concentration of Cu. Among the cloned cultivars TV-21 was the most sensitive, showed highest level of degradation in the chlorophyll content (Figure 4.2.3.C), whereas TS-491 (seed germinated) showed maximum level of declination in the chlorophyll content amongst all the cultivars (figure 4.2.3.F). Several authors have reported the same pattern of decrease in the chlorophyll content with the increase in the Cu concentration (Rama Devi, 1998; Mohanpuria *et al*., 2007; Tang *et al*., 2008, Saha *et al*., 2012, Wang *et al*., 2008). Decrease in chlorophyll content was observed in Cu treated *Cladonia rangiformis* (Chettri *et al*., 1998), in *Crassula helmis* (Kupper *et al*., 2009). The photosynthesis of woody plants decreased under Cd contamination (Huang and Zang, 1986). Decrease in chlorophyll content in plant was also observed under other stress condition (Mobin and Khan, 2007; John *et al*., 2009; Wang *et al*., 2009).

Exposure of Cr in *Hydrocharis dubia* at 4-8 mg/kg the chlorophyll content increased but with higher concentration the content reduced (Yang *et al*., 1999a). The same result was observed in *B. schreberi* (Yang *et al*., 2001). With the increase in Cd the chlorophyll content decreased in macrophytes (Sun and Wang 1985). Decline in the chlorophyll content in tea

plants due to Cd was reported (Mohanpuria *et al*., 2007), in *Phragmites australis* (Pietrini *et al*., 2003), *Vigna mungo* (Singh *et al*., 2008) and *Camellia sinensis* (Saha *et al*., 2012).

Declination in the chlorophyll content is the primary bio indicator of toxicity of Cu (De Vos *et al*., 1992).The content of chlorophyll has a close relation with photosynthesis (Liu *et al*., 2001).Exposure of Cu, may be due to the blocking of enzymes involved in chlorophyll synthesis, can change the pigment and protein composition of photosynthetic membranes and interferes with the biosynthesis of photosynthetic activity, cause the destruction of chlorophyll content (Van Assche and Clijsters, 1990 and Luna *et al*., 1994). As a whole it causes damages in the structure and function of the chlorophyll (Kupper *et al*.; 2003). It was documented that at high concentration of heavy metals, the activities of photosynthetic enzymes degrade which results in the reduction of chlorophyll content (Thapar *et al*., 2008). It gave us an evident that excess Cu has a strong effect on chloroplast fine structure which results in degradation of grana stacks and stroma lamellae (Baszynski *et al*.; 1988). Various authors gave evidences that the toxic effect of metal on phototropic organisms strongly appears to be related to the increase in the levels of lipid peroxidation and protein carboxylation as well as the production of antioxidant defense systems (Gallego *et al*.; 1996, Tripathi *et al*.; 2006).

In most of the plants Cu is found associated with plastocyanin, an important component of the electron transport chain of PSI in the chloroplast. Cu ion insert directly to the reaction center specially PSII which due to the high irradiance causes direct damage to the reaction center (Kupper *et al*., 1996, 1998, 2002).Copper interferes with the biosynthesis of the photosynthetic activity by changing the pigment and protein composition of photosynthetic membranes.

5.3. Effect on Biochemical charcters

Though the metals are required by the plants, but higher concentration of metals can be toxic which have been reported to affect variety of processes in plants. One of the major consequences is enhanced production of reactive oxygen species (ROS). Due to the emergence of reactive oxygen species they generate of oxidative stress (Suzuki *et al*., 2011; Gill and Tuteja *et al*., 2010). These can be generated either directly by redox-active metals, such as Cu and Fe or indirectly by reduction of enzymatic and non-enzymatic antioxidants. Many oxidative stresses also get generated due to the accumulation of Cu in higher concentration and this as a whole cause the inhibition in the photosynthetic reactions which has been observed by many authors (Rocchetta and Kupper, 2009). The decreased concentration of the chloroplastic pigments may be the result of reduced synthesis and/or enhanced oxidative degradation of these pigments by the enhancement of oxidative stress (Romero-Puertas *et al*., 2002).

ROS can lead to many effects such as membrane peroxidation, ions loss, and cleavage of proteins, inactivation and damage, breakage of DNA strand (Gille and Singler, 1995). The primary site of metal induced injury is cell membrane, ROS initiates lipid peroxidation due to removal of hydrogen from unsaturated fatty acids which lead to the formation of lipid radicals. The ROS are Superoxide radicals, hydroxyl radicals, singlet oxygen and H_2O_2, these damage the cells by degrading the nucleic acids (Pietrini *et al*., 2003). The phenomenon of stabilization of membrane components cause oxidative burst leads to inhibition of growth elongation in plants (Brisson *et al*., 1994).

5.3.1. Effect on Malondialdehyde content

There was increase in the fresh mass of the plant at the initial period of growth which leads to the intensified lipid peroxidation (Weckx and Clijsters, 1996). In the present study the

oxidative stress exhibited by Cu in *C. sinensis* was observed by increase in lipid peroxidation. From fig 4.3.1.A-4.3.1.F, we can observe that there was a significant increase in lipid peroxidation in all the cultivars. The highest level of increase in MDA was found in TV-28 (4.3.1.A) and TV-1 cultivar was the most tolerant one (4.3.1.E).Between the cultivars there was a significant differences in the Lipid peroxidation in response to Cu. Difference result among the cultivar in response to Cu stress has found in *Triticumdurum* (Ciscato *et al.*, 1997); *Holcus lanatus* (Hartley-Whitakeer, 2001) and *Kummerowia stipulaceae* (Xiong, 2008) *Camellia sinensis* (Saha *et al*., 2012).

The increase in MDA content was also observed in the TS-520 and TS-462 of tea plants up to 7 days of 600 µM of Cu (Saha *et al*., 2012). Increased in MDA content is an indicator of physiological stress and aging (Quariti *et al*., 1997). The same trend of increase in the lipid peroxidation in plants due to Cu was reported by Chen *et al*., 2000; Rama Devi and Prasad, 1998. Many authors also reported that exposure of heavy metals causes increase in MDA levels (Asada, 1994; Gille & Singler, 1995; Ozounidou, 1994).Increased lipid peroxidation has been reported in many plants under environmental stress condition (Prasad, 1996; Gulen, 2008; Xu *et al*., 2010), in response to drought stress (Upadhyaya and Panda, 2004).

When the plants are grown under some stress involved in the environment then excess of free radicals accumulate in the cells which results in the lipid peroxidation consequently there will be increase in the malondialdehyde (MDA) production as a product of this activity (Chaoui *et al*., 1997). The deleterious effect induced by the exposure of heavy metal in plant is lipid peroxidation which causes deterioration in biomembrane. MDA is a decomposition product of membrane which is an indicator of oxidative stress (Karuppanapandian and Manoharan, 2008; Karuppanapandian *et al*., 2011). The increase in the MDA content may be

due the reduced activity of SOD (Karuppanapandian *et al*., 2006, Karuppanapandian and Manoharan, 2008).

Exposure of stress results in the increased level ROS indicate the unsaturation in lipid membrane, modification on membrane composition, fluidity and permeability or differentiation in the activities of cell wall modifying enzymes led to the generation of lipid peroxidation (Horvath *et al*., 1998; Örvar *et al*., 2000; Brummell *et al*., 2004). Cu as redox metal can interfere with various physiological processes as an indicator of toxicity of plants due to Cu exposure (Baryla *et al*., 2000). At high concentration Cu promote lipid peroxidation and damage the balance of ROS in tea plants (Saha *et al*., 2012). Thus generation of ROS and damage of membranes is well documented and it suggests that increase in MDA observed in this investigation on *C. sinensis* is due to enhanced generation of toxicity. Other symptoms of toxicity as observed in terms of decrease in pigments, proteins and biomass might also be attributed to increased ROS generation.

5.3.2. Effect on enzymatic antioxidants

Cu is an efficient catalyst leads to the formation of reactive oxygen species (Maksymiec, 1997).Cu induced ROS generation has been supposed to be responsible for the toxicity caused in plants (Gallego *et al*., 1996, Aravind and Prasad, 2003).To overcome the problem of ROS and oxidative stress plant cells have developed a wide array of defense mechanisms involving enzymatic and non-enzymatic antioxidant systems which can regulated according to the environmental conditions (Foyer and Halliwell, 1976). Heavy metal induces oxidative stress in cells and tissues by transfer of electrons in single electron reactions which generate free radicals (Sahw *et al*., 2004). Enzymatic defenses are provided by Superoxide dismutase (SOD),

catalase (CAT), Peroxidase (POD), Ascorbate peroxidase (APX). Induction of these enzymes may be considered as mark of stress and defense against pollution (Chaoui and Ferjani, 2005).

As an adaptive response to metal toxicity the modulation of antioxidative system has been studied extensively. Cu is reported to produce oxidative stress and simultaneous increase in antioxidants in various plants (Saha *et al*., 2012). In the present study, Cu treated plants showed higher induction of the antioxidant enzymes viz., APX, POD, SOD and CAT.

5.3.2.1. Effect of Cu on SOD activity

Due to abiotic stress the SOD activity suggests different mechanisms involved in oxidative damage (Jebara *et al*., 2005, Karuppanapandian *et al*., 2006, Karuppanapandian and Manoharan 2008).SOD provide a first line of defense against ROS. As an essential antioxidant component SOD catalyzes a disproportionate amount of superoxide anion (O^{2-}) to hydrogen peroxide (H_2O_2) (Bowler *et al*., 1992), can be used as biomarker of environmental stress (Dazy*et al*., 2009). As a stress resistant enzymes it act as precursor of other active species of oxygen, which get dismuted into H_2O_2 and O_2, which substantiates the importance of SOD in detoxification of ROS. In our study we observed increased SOD activity with the increase in the concentration of Cu in all the cultivars (Fig4.3.2.1.A-F). TheTV-1 and S_3A_3 cultivar was most tolerant amongst all the cultivars which was recorded higher increase in the activity of SOD (4.3.2.1.E and 4.3.2.1.D respectively). But the longer exposure of Cu above 600µM, the activity was levelled off. Many authors found the same result of increase in SOD up to a certain level of Cu stress (Ke *et al*., 2007, Wang *et al*., 2004). At higher concentration due to prolong exposure, slight depletion in SOD activity was observed in TV-21, TV-28, and TV-17. Such response of SOD activity has also been reported in pea leaves (Dixit *et al*., 2001) and *B. monnieri* (Mishra *et al*., 2006). Our result of increase in SOD activity at lower concentration is

persistent with the findings of other authors (Hartley-Whitaker *et al*., 2001; Wang *et al*., 2004;Saha *et al*., 2012).The high level of SOD activity was observed in the present study which demonstrate the efficient breakdown of O_2^-in the cultivars at the moderate concentration of Cu**.**It was observed that the SOD activity also increased in tea plant due to drought stress and increased due to rehydration (Upadhyaya and Panda, 2004). The exposure of Al also gave the same result of increased in SOD activity in cultured tea (Ghanati *et al*., 2005).

Increase in SOD activity appears to be probably attributed to superoxide radical accumulation, de-novo synthesis of the enzymaticproteins (Verma and Dubey, 2003). Possible explanations for the decrease in SOD activity above 300 µM of Cu in TV-28 and 400 µM in TV-17 and TV-28 may be linked to inactivation of enzyme by the production of excess RO(Filek *et al*., 2008) or the binding of nonessential heavy metals to the active center of the enzyme (Stroinski and Kozlowska, 1997; Fatima and Ahmad, 2004). When oxygen was higher than threshold level it can destroy the metabolic functions and can restrain SOD activity (Ding *et al*., 2004). The decrease or increase in the level of these metals causes adverse effect in the activity. Declination in SOD activity would have disrupted the balance between the generation of superoxide and its dismutation. Increased level of H_2O_2 is produced due to action on SOD on $O2^{\cdot -}$ or may be due to formation in biochemical pathway.

5.3.2.2. Effect of Cu on POD activity

POD plays a major role in plant respiratory metabolism and physiological resistance.Large number iso-enzymatic forms are present in Peroxidases (POD) which participates in a variety of cellular functions (Cui and Wang, 2006). The increased POD activity can catalyze H_2O_2 into H_2 and prevent the accumulation of H_2O_2 and O_2. This mechanism can also reduce the harm in the plants due to free radicals (Sun *et al*., 2006).POD

includes a group of specific enzymes such as NAD-peroxidase, NADP-peroxidase, fatty acid peroxidase etc. POD catalyzes the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinone etc., which are taken part in improving the mechanical protection in plants (Dong *et al*., 2005).

The present study observed the increase in the POD activity in the cultivars (Fig 4.3.2.2.A-F). The elevation in the activity of POD shows that it catalyzes the H_2O_2 into H_2O and prevents the accumulation of H_2O_2 and O_2. This may be due to ionic micro environment or tissue specific gene expression in plants. The increase in POD helps in reduction of harmful affect caused by the free redicals to structure and function of the membrane (Pauls and Thompson, 1984; Vetanovetz and Peterson, 1990; Sun *et al*., 2006). Saha *et al*., 2012 also reported the increase in the activity of POD with the increase in the Cu concentration up to 400 µM. Exposure of heavy metal lead to the formation of ROS where it can replace the essential metals, can block the essential functional groups, may result in changes in structure or the integrity of proteins and the interruption of signal transduction pathways of antioxidant which as a whole can lead to decrease in enzymatic activity (Alvarez and Lamb, 1997; Stroinski and Kozlowska, 1997; Schützendübel and Polle, 2002). With respect to control, peroxidase activity increased in all the cultivars but up to a certain level of Cu treatments. The increase in activity might be a due to increase in de novo protein synthesis or the activation of enzymes which is already present in plant cells to diminish the deleterious effects of ROS (Van Assche and Clijsters, 1990).

5.3.2.3. Effect of Cu on APX Activity

In the present study there was increase in APX activity in the cultivars. TV-1 and S_3A_3 was the more tolerant cultivar among the cultivars (4.3.2.3.E and 4.3.2.3.D). Many other

authors also observed the increase in APX activities due to accumulation of stress. Accumulation of Cu showed increase in APX activity in *Pheseolus vulgaris* (Gupta *et al*., 1999) and *Camellia sinensis* (Saha *et al.,* 2012).The increase in the APX activity was significantly found in citrus plants (Lu *et al*., 2007). There was late increase in APX activity in *Pheseolus vulgaris* due to Cu (Gupta *et al*., 1999).However the higher concentration showed decline in the activity. The overexpression of SOD and APX in chloroplast of transgenic tall fescue plants showed tolerance to abiotic stress (Lee *et al*., 2010). Increase in the APX activities in tea suggests that the antioxidative mechanism induced by Cu was invaded in detoxification of H_2O_2(Saha *et al*., 2012).

5.3.2.4. Effect of Cu on CAT activity

CAT is an important component of plant antioxidant enzymes which remove excess H_2O_2 and make H_2O_2 at normal level to prevent the damage by protecting the membrane structure (Li *et al*., 2008). In present study enhanced activity of CAT by Cu treated cultivars has been observed (4.3.2.4. A-F). In present work CAT activity increased in all treatments which can be considered as a circumstantial evidence for role of CAT in detoxification of H_2O_2that induced under heavy metals stress. In TS-491, TV-21 and TV-28 the increase in CAT activity was observed up to 300µM (4.3.2.4.F, 4.3.2.4.B and 4.3.2.4A respectively) in TV-17 the increased was up to 400µM where TV-1 and S_3A_3 was recorded with highest activity of CAT even up to 600µM (4.3.2.4.E and 4.3.2.4.D). In TS-491, TV-21 and TV-28 above 300µM the activity decreased, this indicate that increase in Cu concentration cause the inhibition of enzyme activity. At high concentration of metal stress CAT unable to protect cell against ROS. These results are in agreement with results of Verma and Dubey (2003). It was also reported that decrease in SOD and CAT activities cause increase in lipid peroxidation by convert $Fe^{+3}$to

$Fe^{2+}$for generation OH°. Alterations in the assembling of CAT subunits and inactivation of enzyme or proteolytic degradation are the reasons for decrease in CAT activity under stress conditions (Cakmak, 2000).

The significant increase in CAT activity at lower concentration with the Cu exposure has also been reported in tea plant (Saha *et al*., 2012). Tang *et al*., 2012, also observed reduced CAT activity in tea plants due to increase in the Cr concentration.The CAT activity was remained unaltered or marginal increased in response to Cu stress in Oat leaf segments (Luna *et al*., 1994), tomato seedlings (Mazhoudi *et al*., 1997), rice seedlings (Chen *et al*., 2000). In *Prunus cerasifera* plantlets the CAT activity was recorded significantly with the increase in the Cu concentration (Lombardi and Sebastiani, 2005; Saha *et al*., 2012). The increase in SOD and decrease in CAT activity in response to excess supply of heavy metals has been widely reported (Chaoui *et al*., 1997; Pandey and Sharma, 2002; Cho and Seo, 2005, Lombardi and Sebastiani, 2005). The decrease in CAT activity, observed at higher concentration which resulted in the inactivation of its reaction with superoxide ions which leads to weaken the effective detoxification of H_2O_2 (Kono and Fridovich, 1982). Degradation of enzyme by induced peroxisomal proteases may be other possible reason of decline in CAT activity (Sandalio *et al*., 2001).

SOD activity results in H_2O_2production from $O2^{-}$ that should be detoxified by some other oxidative enzymes such as APX, POD and CAT to H_2O and O_2 (Anuradha and Rao, 2007). Although any change in peroxidase activity can consider as a typical response to oxidative stress, but diversity in peroxidase activity under heavy metals stress depends on plant species, time of treatment and concentration of metal (Schützendübe and Polle, 2002).

Our result showed the increase in the antioxidant activities at lower concentration of Cu but higher concentration showed decline in the activity of SOD, POD, CAT. It is may be due to the fact that the resistance mechanism exceeds the threshold level with the increase in the concentration of Cu and the capacity of scavenging oxygen system weaken gradually. It seems that the heavy metal caused oxidative stress which affect the growth and productivity of plants. Higher induction of APX, SOD, POD in Cu treated cultivars (either at lower concentration in some cultivars or at higher concentration in some of the cultivars) was observed in the present study. This suggests that Cu is phytotoxic, produces higher amount of ROS at moderate exposures. However at higher concentration of Cu in TV-17, TV-21 and TV-28, enzymatic activity declined with significant built up of metal, which create imbalance between the generation of ROS and its detoxification, causes serious stress. Plants have several antioxidant enzymes and metabolites located in various cellular compartment helps in scavenging ROS production (Dat *et al*, 2000). The role of antioxidant enzymes under stress condition have demonstrated in several studies (Lee *et al*., 2000; Saha *et al*., 2012, Tang *et al*., 2012).

5.3.3. Effect on Phenolic Content

Phenolic compounds act as reducing agents, hydrogen donor and singlet oxygen quenchers which as a whole make it as an important antioxidant (Rice-Evans *et al*., 1997), participates in ROS scavenging in plant cells (Jung *et al*., 2003). They stabilize membranes by decreasing fluidity and it hindrance the diffusion of ROS (Arora *et al*., 2000). Phenolic have various functions in plants. In our study we observed the increase in phenol content in Cu treated cultivars with respect to control (Fig 4.3.2.5.A-F). The trend of increase in phenol content was different in different cultivars. TV-21, Tv-17, Tv-28 and TS-491 showed tolerance

up to a certain level by increasing in the phenol content at the lower concentration of Cu. On the other hand the increase in phenol content was up to 600 µM was observed in TV-1 and S_3A_3.

Under stress conditions an enhancement in the amount of phenolic compound was observed (Grace *et al*., 2000;Diaz *et al*., 2001; Sakihama *et al*.,2002). The biosynthesis of phenolic compound was observed in wheat in response to Ni (Diaz *et al*., 2001) and in maize in due to Al stress (Winkel-Shirley 2002). With respect to control the Cu treated *Phyllantus tenellus* leaves contain more phenolic (Diaz *et al*., 2001). An increase in the content of phenol was correlated to the increase in the enzymes involved in phenolic compounds under heavy metal stress. Increase in phenolic can reflect the anatomical change induced against harmful action of heavy metals (Diaz *et al*., 2001).

Due to the tendency of metal chelating phenolic compounds exhibit antioxidant action. Hydroxyl and carboxyl group present in phenolic compounds able to bind iron and Cu (Jung *et al*., 2003). The nucleophilic character of the aromatic rings are also responsible for its chelating ability (Morgan *et al*., 1997). Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical, which depends on the structure of molecules, number an position of hydroxyl group (Millic *et al*., 1998).

Due to accumulation of Cu the phenolic content was high in *Phyllantus tenellus* with respect to control (Diaz *et al*., 2001). *Phaselus vulgaris* exposed to Cd^{2+} accumulate soluble and insoluble phenolics (Diaz *et al*., 2001). Phenolic antioxidants inhibits lipid peroxidation, it depends on the structure of molecules and the position of hydroxyl group in molecules (Millic *et al*., 1998). The roots of many plants exude high levels of phenolics due to the accumulation of heavy metal (Winkel, 2002). The chelating character of phenolic compounds may be related

to the high nucleophilic character of aromatic rings (Morgan *et al*., 1997). The flavonoids can directly scavenge active oxygen, the antioxidant activity are the ability to donate electrons (Khan *et al*., 2000). The significant increase in the activity shows that the compound helps in the binding of the heavy metal Cu. It was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen *et al*., 1993; Gülçin, 2005). The present study showed the resemblance with the result of Cu stress on tea plant where they showed significant increase in the phenol content below 400µM concentration (Saha *et al*., 2012).

From the biochemical studies we can conclude that the Cu induce oxidative stress in leaves of tea plants which leads to the formation of ROS. This in result produce enzymes which are involved in scavenging ROS. But when the ROS production is high it exceeds the threshold capacity of plant to damage the ROS leading to the inactivation of defense mechanism of plants (Shigeoka *et al*., 2002). Our result showed significant differences in the cultivars of tea where the more sensitive cultivar loose its antioxidative mechanism at higher concentration of Cu (300µM), whereas the most tolerant cultivar TV1 and S_3A_3 able to tolerate the Cu concentration even at 600 µM.

5.4. Expression Profile

ROS influence the expression of several genes involved in various metabolic (Mittler *et al*., 2004). Research has been further passed to study the expression profile of *PCS*1 and MT genes of Cu treated samples, and then proceed with the characterization of MT gene. Different gene and coding factors involved in signal transduction cascade are induced by stress treatments. Several methods are used to analyze gene expression profiles, in our current study the differential expression patterns on *PCS*1 and MT gene with respect Cu stress was analyzed

by RT-PCR. A partial cDNA sequence having high homology with known MT has been cloned in pGEM-T easy vector for increasing the number of desired MT gene which showed the maximum fold change in the expression profile for future prospect.

5.4.1. Expression Profile of Phytochelatin synthase gene

Phytochelatins (PCs) are oligomers of glutathione, produced by the enzyme phytochelatin synthase. Phytochelatins play major roles in metal detoxification in plants and fungi (Gallagher *et al.*, 2001; Singh, 2005). Phytochelatins are the principal metal binding ligands produced naturally by plants for detoxification of heavy metal. PCs are considered as biomarkers of metal toxicity and their synthesis is induced by many metals, such as Cu, Cd, Pb, Zn (Sneller *et al*., 1999). Plants respond to heavy metal toxicity in a variety of different ways. Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms such as ethylene and stress proteins. Though there has been considerable debate concerning the function of PCs, they are involved in homeostasis or trafficking of essential for tolerance of several non-essential metals (Cobbett *et al*., 1998).

Present investigation exhibited that the expression of phytochelatin synthase (*PCS*1) under the Cu stress increased significantly at the lower concentration of Cu at the respective days of treatment. From fig 4.4.3.A-Cwe can observed that there was up regulation of the gene at lower concentration whereas with the further increase in the concentration there was decrease in the activity of *PCS*1 and at the higher concentration of 500 µM, 600 µM there was down regulation of the gene at both the cultivars with negative value of fold change. But compare to cultivars the expression of fold change in gene was more at TV-1 than that of$S_3A_3$and TS-491.In TV-1, there was a sharp increase in the level of mRNA upto 7^{th}

day300µM (4.3.3.A). In **S_3A_3** there was a significant increase in the expression profile of *PCS*1 gene (Figure 4.3.3.B). There was a significant increase in the up-regulation of gene up to 4th day of 300 µM. Prolonged exposure of 300 µM lead to the declination in the expression of *PCS*1 gene. The same trend was observed at 400 µM too. After 2nd day at 500 µM and 600 µM the expression was positive but after 4th day there was down regulation in the expression of gene.Whereas in TS-491, the expression of *PCS*1 gene was highest at 7th day of 200 µM (4.3.3.C). Compare to the cultivars the level of fold change of *PCS*1 gene was higher at TV-1. In TS-491down regulation of expression of *PCS*1 gene started from 7th day of 400 µM and maximum down regulation was observed in 7th day of 600 µM.

Since epigemetic function of gene encoding a functional *PCS* (*AtPCS*2) was observed in *A.thaliana* genome (Ca Zale and Clemens, 2001; Clemens, 2006b). The exposure of metal ions like Cu, Cd, Ag, Hg can induce PCS activity (Oven *et al*., 2002). A significant increase in *AtPCS*1 mRNA level was reported after first week of Cd treated adult plants (Semane *et al*., 2007). The expression of *AtPCS*1 allowed the higher concentration of Cd tolerance in Indian mustard where in tobacco; it resulted into Cd hypersensitivity (Wojas *et al*., 2008). A transcriptional induction of *TaPCS*1 was observed in roots of Cd treated 4 day old seedlings of wheat (Clemens *et al*., 1999). The disruption of *PCS* gene decreased the Cd tolerance in *S. pombe* (Clemens *et al*., 1999). The expression of *OsPCS*1 in rice was suppressed by RNA interfence, resulted in the reduction of Cd accumulation in rice without any toxicity symptoms (Li *et al*., 2007). It was observed that inactivation of *PCS* gene in *C. elegans* increased Cd sensitivity (Vatamaniuk *et al*., 2001). Though PCs play a vital role in chelating but have a very little influence on Cu treated plants (Cobbett and Goldsbrough, 2002).Many authors has suggested the catalytic role of *PCS* gene in Cd^{2+} sequestration which helps in phytochelatin

synthesis (Stephan *et al*., 1999). *PCS* showed significantly higher growth rate at 200µM of Cd^{2+} compare to 60 µM. In the study between the wild *S. pombe* and SpPCS, PC2 and PC3 were found in wild types which were control whereas absent in SpPCS (Clemens *et al*., 1999).Phytochelatin play a major role in Cd and Arsenate (Clemens *et al*., 1999; Bleeker *et al*., 2006). Many researchers tried to overexpress *PCS* genes in various plants to enhance heavy metal tolerance in plants (Vatamaniuk *et al*., 1999; 2001). The tolerance of heavy metal toxicity related to the number of phytochelatin (Staffens, 1990).

Gene expression profile has been used as various stress indicators (Lettieri, 2006). The upregulation of *PCS*1 gene in response to Cu stress are in agreement with the findings of upregulation of gene required for PC synthesis under Cd stress (Xiang and Oliver 1998, Rother *et al.*, 2006). The expression analysis of *PCS*1 gene in *C. sinensis* at different concentration of Cu has a significant fold change. These results suggested that the activation of PCS during the exposure to Cu may be responsible for accumulation of metal (Chen *et al*., 1997, Grill *et al*., 1989, Vatamaniuk *et al*., 1999, 2000, 2004). *PCS* genes directly mediated phytochelatin synthesis (Clemens *et al*., 1999). Overexpression of *PCS* genes in various plant species reported to increased Cd tolerance and accumulation (Lee *et al*., 2003, Pompani *et al*., 2006). The effect of *TaPCS*1 expression in *Saccharomyces cerevisiae* on Cd tolerance showed the expression of *PCS* genes which can enhance metal tolerance (Clemens *et al*., 1999).

5.4.4. Expression analysis of metallothionein gene

MTs can be induced by the accumulation of Cu which played a vital role in detoxifying the toxicity of metal and enhance the tolerance in fungi and animals (Hamer 1986, Giritch *et al*., 1998, Schat *et al*., 2000). MT2 mRNA was strongly induced in Arabidopsis by Cu compare to Cd & Zn (Zhou & Goldsbrough, 1994). There was a relation has been established between

Cu sensitivity of seedlings and the expression of MT2 in 10 ecotypes of Arabidopsis (Murphy and Taiz 1995).MTs gene get expressed not only present of essential metal like Cu but also due to induced nonessential metal like Cd (Goldsbrough, 1995). The role of MTs as metal metabolism and detoxification has been confirmed by expression and characterization of MT-metal complexes (Guo *et al*., 2003; Yuan *et al*., 2008).

In our study we observed a significant increase in the relative expression of MT gene which showed the up-regualtion of the genes till up to 7^{th} day of 600 µM (Figure 4.4.4.A). In S_3A_3, where it showed a significant increase in the level of up regulation of gene up to 7^{th} day of 300 µM (4.4.4.B) there was rapid fall in the value of fold change in the up regulation of MT gene at the later phase of the exposure of 400 µM i.e. 7^{th} day, after initial a slight increase in the fold change. Prolonged exposure of higher concentration of Cu lead to the negative expression of gene. The exposure of higher concentration led to the negative fold change in the value. In TS-491, the expression of MT gene was highest at 2^{nd}day of 200 µM (4.4.4.C). From the lowest concentration of Cu, 50µM itself the up-regulation of gene was marked with decline in the relative expression fold change value. The level of decline in the level of fold change value followed by the negative value of fold change. At the highest concentration a very low up regulation of gene was observed at 2^{nd} day of 600µM.

In *Arabidopsis thaliana* the expression of metal was due to Cu (Roosens *et al*., 2004). With respect to control, the expression profile of *Arabidopsis* metallothionein showed no effect of BSO on metallothionein dependent Cd^{2+} tolerace (Celemens *et al*., 1999). It was been documented that the MTs can protect plants against oxidative damage by removing the free radicals (Jia *et al*., 2012). MT with antioxidant properties can scavenges the ROS under drought and light stress (Akashi *et al*., 2004). Transgenic tobacco plants overexpressing MT3

showed increased tolerance to ROS stress compare to wild type (Jia *et al*., 2012). During the process of ROS scavenging metals may have released when the ROS bound to Cys residue of MTs (Jia *et al*., 2012). The accumulation of Cu, oxidative stress and the abiotic stress are responsible for the expression of MTs in plants (Jin *et al*., 2006). The hydroponically grown poplars showed different expression of *PtdMT1a, PtdMT1b, PtdMT2a* and *PtdMT2b* in response to Zn (Kohler *et al*., 2004). It was found that the overexpression of *PtdMT1a, PtdMT1b, PtdMT2a* and *PtdMT2b*increased the tolerance of Cd in transgenic yeast (Kohler *et al*., 2004). MT3 type not only scavenged ROS but also enhanced the tolerance of heavy metals (Yang *et al*., 2011).

The study of expression of MTs genes helped us in the study of metal detoxification. The upregulated expression of MTs genes in plants with the increase in concentration of metal suggests the ability of plants to absorb the heavy metal ions due to the MT genes. The expression of transformed MTs genes in *Arabidopsis thaliana*, tobacco and yeast enhanced the ability of metal uptake, transport and sequestration (Kohler, 2004; Yang *et al*., 2011). Poplar species, transgenic forest tree plays a model role for forest tree biotechnology in the study of phytoremediation (Bradshaw *et al*., 2000).

The fields of genetic engineering enhanced the phytoremediation has environmental benefits as well as human (Linacre *et al*., 2003). Expression of MTs enhances the oxygen defence mechanism (Jia *et al*., 2012). The down-regulated if oxidative signal has been discussed in Poplar and rice due to MT genes (Smith *et al*., 2004; Wong *et al*., 2004). Different genes were analyzed in *Populus* exposed to 30 and 60 of Cu for 12 and 24 hrs found that the Cu increased the metal accumulation in roots suggested that *Populus* could be bred for phytoremediation (Guerra *et al*., 2009). Expression and characterization analysis of MT2 from

grey mangrove species due to accumulation of metal result in metal homeostasis (Huang and Wang, 2010). In *Arabidopsis thaliana* and *Gossypium hirsutum* have similar function of homeostasis due to metal (Xue *et al*., 2009). The expression of Lacti-dehydrogenase of *Vicia faba* L. under Pd, Cd, Hg stress gave different expression of isoenzymes at different concentration (Duan and Wang 1998).

5.5. Preparation of cDNA library of MT gene

Result of our present study provide a perspective about the relationship among the accumulation of ROS, action of enzymatic activity, generation of lipid peroxidation and gene expression profile in different cultivars of tea plants under different concentrations of Cu stress. These results suggest the regulation of *PCS* 1 and MT gene expression at translational or post translational levels. The sharp increase in mRNA level concludes that the transcriptional level of *PCS*1 and MT might have sustained at that period and after that the plant start to lose their viability. Both the PCS and MTs are capable in detoxifying the metal toxicity but the precise function was not clear; they may have different functions for different metals (Hamer, 1986). They can play a vital role as antioxidants (Dietz *et al*., 1999).

The gene expression profile showed maximum fold change of MT gene at 7^{th} day of 300 µM in TV-1, was preceded for preparation of cDNA library by cloning the desired gene in pGEM-T Easy vector. In the present study we cloned a partial cDNA of MT from TV-1. The partial sequence of 225 bp has high nucleotide homology to other known MT gene. The desired gene was cloned which can be preserved for further use in transgenic field to enhance the capacity of metal accumulation and to degrade the toxicity level. The sequence of the desired gene of our study has the resemblances with *Camellia sinensis*metallothionin 1 mRNA,

compl 422 2e-114 and gb|FJ965290.1| *Camellia oleifera* metallothionein mRNA, comple. 244 4e-61.

MT genes from many plants have been isolated and characterized from different species like Sweet potato (Chen *et al*., 2003), Barley (Heise *et al*., 2007), *Noccaea caerulescens* (Fernandez *et al*., 2012). Based on the analysis of MTRNA expression, it was observed that type 1 MT genes are predominantly expressed in roots, type 2 expressed abundantly in leaves (Zhou and Goldsbrough, 1994, 1995). Type 3 MT genes are found to be expressed in leaves or in ripening fleshy fruits (Ledger and Gardner, 1994) whereas expression of type 4 MTs are restricted to developing seeds (Kawashima *et al*., 1992; White and Rivin, 1995). It was observed that in *Arabidopsis* and *Vicia faba,* MTs are highly expressed in trichomes (Garcia-Hernandez *et al*., 1998; Foley and Singh, 1994). In rice MT genes are found to be expressed in stand nodes (Yu *et al*., 1998).

The different patterns of MT gene expression are due to the fact that the isoforms of MT may differ not only in sequence but also in functions (Cobett and Goldsbrough, 2002). In *Saccharomyces cerevisiae* MTs played a major role in detoxification of Cu (Ecker *et al*., 1986). MT genes are transcriptionally regulated by metals which play an important role in increase the tolerance level of metals in plants and regulate homeostasis (Cobett and Goldsbrough, 2002). It was found that MTRNA was strongly induced in Cu treated seedlings of rice and *Arabidopsis* compared to Cd and Zn (Zhou and Goldsbrough, 1994; Hsieh *et al*., 1995). A MT deficient yeast mutant was complemented by isolated MT genes from Arabidopsis which helped in restoring Cu tolerance (Zhou and Goldsbrough, 1994). It was documented that type 2 MT gene expression has a close relation to Cu tolerance across the ecotypes of *Arabidopsis* (Murphy and Taiz, 1995) and in *Silene vulgaris* (van Hoff *et al*., 2001). Increase in MTRNA level was

observed in the period of senescence in bean (Foley *et al*., 1997), Brassica (Buchanan-Wollaston, 1994), rice (Hsich *et al*., 1995) and *Arabidopsis* (Garcia- Hernandez *et al*., 1998). Several rice MT genes were reported to be exposed at very high levels in different tissues (Yu *et al*., 1998; Wong *et al*., 2004, Zhou *et al*., 2005). There was increase in mRNA levels was found in*OsMT*-1-1a, *OsMT*-1-1b, *OsMT*-1-2a, *OsMT*-1-4c in senescing leaves (Navabpour *et al*., 2003; Gisela *et al*., 2004).