CHAPTER-5

PHYSIOBIOCHEMICAL RESPONSES IN TEA PLANTS TO EXCESS COPPER

CHAPTER-5

PHYSIOBIOCHEMICAL RESPONSES IN TEA PLANTS TO EXCESS COPPER

The physiobiochemical responses of higher plants to toxic doses of copper are very complex with multiple protection strategies being involved in the process. These strategies include complexation of metal ions, reduced influx of metals and enhanced production of antioxidants that detoxify reactive oxygen species (ROS). Copper being a redox active metal, forms an essential component of many electron carriers. However, within cells, it is found only in protein bound form, since as a free ion, it may generate oxidative stress and cause serious damage to organic molecules. Thus the reactivity of copper that makes it so useful in redox reactions also makes it toxic.

The principle mechanism behind copper toxicity involves Fenton and Haber Weiss reactions which generates hydroxyl radical, the most damaging ROS in cells, from superoxide and hydrogen peroxide. This radical causes maximum harm by initiating lipid peroxidation and also damage DNA, proteins and many essential smaller molecules (Arora *et al.*, 2002). Just like copper, ROS are also well recognized for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentration it acts as second messenger in intracellular signalling cascades that mediate several responses in plant cells (Sharma *et al.*, 2012). Though biochemical responses to copper is increasingly well understood, but the complete mechanism of tolerance in plants to excess copper still remains unknown (Ducic and Polle, 2005).

Copper in excess amounts cause various effects in the sensitive plants which depend strongly on the plant growth stage and on the duration of exposure (Maksymiec, 1997). Excess copper, when applied at initial stages of the plant life, strongly affects growth and metabolism such as inhibition of leaf expansion and reduced root biomass. Longer exposure to copper causes reduction in chlorophyll content in the leaves which is associated with simultaneous destruction of inner structure of chloroplast (Eleftheriou and

Karataglis, 1989). Further, due to generation of ROS; lipid peroxidation, decrease of lipid content and changes in fatty acid composition of thylakoid membrane was observed (Yruela, 2005).

Scavenging or detoxification of excess ROS, produced due to copper excess, is achieved by an efficient antioxidative system comprising of the nonenzymic as well as enzymic antioxidants. All these responses have been found to depend on both time and concentration of copper. Nonenzymic components of the antioxidative defence system as mentioned earlier include the major cellular redox buffers ascorbate and glutathione as well as tocopherol, carotenoids, and phenolic compounds. They interact with numerous cellular components and in addition to crucial roles in defence and as enzyme cofactors, these antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death (Sharma *et al.*, 2012). The enzymatic components of the antioxidative defence system comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), enzymes of ascorbate-glutathione cycle, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Arora *et al.*, 2002; Sharma *et al.*, 2012). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress.

In the current work, studies on the level of physiobiochemical changes that occur in tea plants in response to excess copper have been undertaken in a hydroponic set up. The extent of copper accumulation in leaves and roots and changes in protein, carbohydrate, chlorophyll, carotenoids, phenol, proline, non-protein thiols and lipid peroxidation levels have been measured in a time and concentration depended manner. Also the changes in the activities of four important antioxidant enzymes, SOD, POD, APX and CAT have been estimated.

5.1. MATERIALS AND METHODS

5.1.1. Raising of seedlings

Freshly harvested tea seeds of two biclonal seed stocks, TS-462 and TS-520, were obtained from Gayaganga Tea Estate (Darjeeling, West Bengal) as

described earlier (section 4.1.1). The seeds were sterilized with 0.01% $HgCl_2$, washed thoroughly and then submerged in excess distilled water. The floating seeds were removed with a strainer. The sunken seeds were sowed in moist clean sand taken in aluminium trays (22 cm in diameter x 25 cm in height) at roughly three inch distance from each other. Each seed was covered with a thick plug of moist cotton in order to retain the moisture. The cotton plugs were regularly wetted with sterile tap water. The seeds cracked after 5-6 days. The cotton plugs were then removed and regular water spraying was done to keep the seeds moist. The shoot was clearly visible after another 20 days. These germinated seedlings were transplanted to plastic cups (7 cm diameter x 12 cm height) containing wetted sand soil mixture. Each cup contained one seed. Seedlings were raised in the net house under natural conditions of day light and temperature. After approximately two months, the mature seedlings were used for experimental purposes in hydroponic cultures.

5.1.2. Stress induction in hydroponic cultures

Three month old tea seedlings of two cultivars (TS-462 and TS-520), which were raised under natural conditions of light and temperature in the net house, were transferred to hydroponic culture set up in Hoagland's and Knop's nutrient solution and allowed to be stable for 7 days. Next, the seedlings were placed in nine square- shaped glass containers each of 3 L capacity for treatment. The experimental jars contained eight different concentrations of copper sulphate taken in duplicate i.e. 50 µM, 100 µM, 200 µM, 300 µM, 400 µM, 500 µM, 600 µM and 700 µM which were added to the nutrient solution. Control sets comprised of only nutrient solution without excess copper sulphate. Leaves and/or roots from each set of plants were collected for measuring various biochemical parameters after the 4th, 7th and 10th day of treatment. The experiments were repeated thrice.

5.1.3. Determination of copper content in plant tissue

The leaves and roots of both tested cultivars were harvested on the 10^{th} day. Root cuttings (0.5 inches) and freshly detatched leaves were washed thoroughly in ice cold sterile distilled water thrice and blotted dry using blotting paper. The samples were freeze-dried using a lyophilizer (Eyla – freeze dryer FDU-506). Dried plant samples were weighed and subsequently

1 g dry powder was digested with 3:1 HNO_3:HCl for 24 h. The copper content was determined by atomic absorption spectroscopy (Thermo Scientific CE 3000). The copper concentrations in root and shoot samples were expressed as µg g^{-1} dry weight (DW). The data from three replications was averaged.

5.1.4. Measurement of lipid peroxidation

The level of products of lipid peroxidation in root tissue was determined as the thiobarbituric acid (TBA) reactive substance (TBARS) following the method of Heath and Packer (1968). Frozen root tissue (200 mg) was homogenized in 4.0 ml of TBA reagent and the resultant homogenate was heated for 30 min at 95^{0}C in a water bath, cooled for 10 min in ice and centrifuged at 10000g for 15 min. The amount of TBARS in the supernatant was measured by its specific absorbance at 532 nm and by subtracting the non-specific absorbance at 600 nm. The level of lipid peroxidation was expressed as µmol g^{-1} freshweight by using an extinction coefficient of 155 mM cm^{-1}. Each root extract was assayed twice and the results of three independent experiments were averaged.

5.1.5. Extraction and Estimation of superoxide anion radical (O_2^-) release

Superoxide anion radical (O_2^-) production rate in leaves were determined by the method of Doke (1983). One gram leaves were chopped and placed in a test tube and to this, a solution containing 50 mM potassium phosphate buffer (pH 7.8), 0.05% nitroblue tetrazolium (NBT) and 10 mM sodium azide (NaN_3) was added. The tubes were incubated for 5 min in dark, and subsequently, 2ml of the solution was removed from the tubes and heated for 10 min at 90^{0}C in a water bath. Then the samples were cooled and the final volume was assayed spectrophotometrically at 580 nm (Systronics, visiscan, model no.167, India). Superoxide anion released was expressed as A_{580} g^{-1} FW.

5.1.6. Extraction and estimation of chlorophyll a, chlorophyll b and carotenoids

Pigments such as chl a, chl b and carotenoids were estimated by the method of Dere *et al.* (1998) with minor modifications. Fresh tea leaves (1 g) were taken and washed with double distilled water and homogenized with 100%

acetone (5 ml). The homogenate was then filtered and the filtrate was centrifuged at 2500 rpm for 10 min. The supernatant was separated and the residual amount was grounded again with 2 ml of 100% acetone and centrifuged. The supernatants were pooled and the final volume was adjusted to 10 ml with 100% acetone. Absorbance was read at 470, 645 and 662 nm in a UV-VIS spectrophotometer (Systronics, Visiscan Model no.167, India). The amount of chl a, chl b and carotenoids were estimated by the formula given below (Lichtentaler and Wellburn, 1985) and expressed as µg in per g FW of sample.

Chl a (µg/ml) = (11.75 x A_{662}) - (2.35 x A_{645})

Chl b (µg/ml) = (18.61 x A_{645}) - (3.96 x A_{662})

Total carotenoids (µg/ml) = (1000 x A_{470}) - (2.270 x chl a) - (81.4 x chl b)/227

For converting µg/ml into µg per g of FW = µg/ml x final vol made/weight of tissue taken in grams

5.1.7. Extraction and estimation of protein

Soluble proteins were extracted from both leaves and roots. Fresh leaves or root samples (2 g) were ground in 5ml of 0.05 M phosphate buffer (pH 7.2), containing 1% polyvinyl pyrrolidone phosphate (PVPP) and sea sand. The extracts were centrifuged at 12000 g at 4^0C for 10 min and the supernatant were used as crude protein extract. Protein content was estimated following Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as standard. Briefly, reaction mixture was prepared by mixing 0.1 ml of protein sample, 0.9 ml water and 5 ml of alkaline mixture and incubated for 10 min. Subsequently 0.5 ml Folin-Ciocalteau's phenol (diluted 1:1 with distilled water) was added, incubated for 15 min and the absorbance was read in a UV-VIS Spectrophotometer (Systronics, Model no.118, India) at 710 nm and expressed as mg protein in g^{-1} FW of sample.

5.1.8. Extraction and estimation of total carbohydrate

5.1.8.1. Extraction

Fresh tea leaves (1 g) were taken and washed with double distilled water and homogenized with 6ml of 90% ethyl alcohol. Then the leaf extracts were filtered or strained through muslin cloth and the filtrates were boiled in

water bath for 10 minutes to remove the excess alcohol. Finally 4ml of distilled water was added to each sample.

5.1.8.2. Estimation

Carbohydrate was estimated by following standard procedures (Hansen and Moller, 1975). One ml of each sample was taken into test tubes and 0.1 ml of barium hydroxide [$Ba(OH)_2$] and 0.1 ml of zinc sulphate ($ZnSO_4$) were added. Then tubes were incubated for 10 minutes in water bath. After incubation, these were centrifuged and the supernatants (0.2 ml) were taken, in fresh test tubes to which 1.8 ml of distilled water and 6 ml of anthrone reagent was added. Then the tubes were again incubated for 10 minutes in water bath. Finally after cooling the tubes, the colour intensity were measured in a photoelectric colorimeter (Systronics 101) using red filter (630 nm). These OD readings were then plotted on a standard curve of D-Glucose and the concentration of each carbohydrate sample was calculated and expressed as mg g^{-1} FW.

5.1.9. Extraction and estimation of proline

Proline concentration in leaves of tea was determined spectrophotometrically by the method of Bates *et al.* (1978). Freshly sampled tea leaves (500 mg) were instantly crushed in 10 ml of 3% sulphosalicylic acid and the homogenate was then filtered through four-layered muslin cloth and the filtrate was centrifuged at 15,000 g for 30 min at 4^0C. After centrifugation, supernatant was collected and used for proline estimation. The reaction mixture contained 2.0 ml of supernatant, 2 ml of glacial acetic acid and 2 ml of ninhydrin. It was then heated upto 1 h in boiling water bath and change in colour was seen. The reaction was stopped by incubating in ice for few minutes. Following incubation, 4.0 ml of acetone was added to the cooled sample and shaken for 30 sec, then absorbance was measured at 520 nm in spectrophotometer (Systronics, visiscan, model no.167, India). Proline content was estimated from a standard curve of known proline and expressed as µmole in per g FW of sample.

5.1.10. Extraction and estimation of phenol and orthodihydroxyphenol content

5.1.10.1 Extraction

Total phenol and orthodihydroxyphenol were extracted from tea leaves by the method of Mahadevan and Sridhar (1982). Fresh leaves (1 g), collected from the experimental glass jars, were immersed into 20 ml of absolute ethanol and boiled for 10 min. After boiling, the leaves were cooled, crushed with cold 80% ethanol and centrifuged at 10000 rpm for 20 min at 4^0C. The supernatant was collected and the final volume was adjusted to 5ml with 80% ethanol.

5.1.10.2. Estimation of total phenol

The total phenol was estimated by Folin-Ciocalteau's method as described by Bray and Thorpe (1954). One milliliter sample (0.1 ml leaf extract + 0.9 ml distilled water) was added to 1 ml Folin-Ciocalteau's phenol reagent (diluted 1:1 with distilled water) and 2 ml of 20% of Na_2CO_3 solution taken in a test tube. The tube was placed in a boiling water bath for 1 min and then cooled under running tap water. The final volume was adjusted to 25 ml with distilled water. A blank was prepared with all reagents except Folin-Ciocalteau's to adjust the 'zero' reading. Quantity of total phenol was estimated with caffeic acid standard curve in a UV-VIS Spectrophotometer (Systronics, Model no.118, India) at 520 nm and expressed as mg phenol in g^{-1} FW of sample.

5.1.10.3. Estimation of orthodihydroxyphenol

The quantity of orthodihydroxyphenol was measured by the method of Arnow (1937). Initially 0.1ml of leaf extract was mixed with 0.9 ml distilled water taken in a take in a 10 ml test tube and the following reagents were added : 2ml of 0.05N HCl; 1ml of Arnow's reagent and 2ml of 1N NaOH. A blank containing all the reagents except Arnow's was used to adjust the absorbance to 'zero'. The volume was raised to 25 ml and absorbance was estimated by using a UV-VIS Spectrophotometer (Systronics, Model no.118, India), at 520 nm. Estimation was done by comparing the absorbance value with known amount of caffeic acid in a standard curve and expressed as mg g^{-1} FW of tissue.

5.1.11. Extraction and Estimation of non-protein thiols

5.1.11.1. Root

Non-protein thiols (NPT) was extracted and assayed as described earlier by Hartley-Whitaker *et al.* (2001). Five to 10 mg of lyophilized root material was ground using mortar and pestle in 2 ml of 5% (w/v) sulfosalicylic acid with 6.3 mM diethylene triamine pentaacetic acid (DTPA) (pH <1) at 0^0C (De Vos *et al.*, 1992). The ground material was centrifuged at 10,000g for 10 min at 4^0C. The clear supernatants were immediately assayed for thiols using Ellman's reagent (Ellman, 1959). For this, 300 µl supernatant was mixed with 630 µl of 0.5 M K_2HPO_4 and the absorbance was measured after 2 min at 412 nm (30 ^{0}C). After addition of 25 µl of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution known as Ellman's reagent, the A_{412} was measured again after 2min. The increase in absorbance was corrected for the absorbance of DTNB. Values were calculated using the molecular extinction coefficient, 13,600 M^{-1} cm^{-1} (Harmens *et al.,* 1993) and expressed as µmol/g DW.

5.1.11.2. Leaf

Leaf tissue (0.5 g) was ground using mortar and pestle in 5 ml ice-cold 5% (w/v) sulfosalisylic acid solution. The homogenate was then filtered through four-layered muslin cloth and the filtrate was centrifuged at 10,000 g for 30 min at 4^0C. Then, 2 ml of the supernatant was mixed with 1 ml of 0.1 M potassium phosphate buffer (pH 7.6). After a stable absorbance reading of 412 nm was obtained, 0.5 ml DTNB solution (2 mM DTNB dissolved in 5 mM EDTA, 0.1 M PBS, pH 7.6) was added and increase in absorbance at 412 nm was monitored (Zhou *et al.*, 2009). The values were expressed as µmol/g FW.

5.1.12. Studies on enzyme response due to copper induced oxidative stress

For determining enzyme activities, fresh leaf and root samples (500 mg) were collected and instantly dipped in liquid nitrogen and the frozen samples were homogenized in a pre-chilled mortar and pestle in 5 ml of 50 mM cold phosphate buffer (pH 7.8) containing 2% w/v PVP. The homogenate was filtered through four-layered muslin cloth and the filtrate was centrifuged at 13,000 g for 20 minutes at 4^0C and the supernatant after dialysis was used

for enzyme assay. Protein was measured following the method of Lowry *et al.* (1951) taking bovine serum albumin as standard. For all experiments, each extract was assayed twice and the results of three independent experiments were averaged.

5.1.12.1. Estimation of superoxide dismutase

Total SOD activity was determined according to the method of Giannopolities and Ries (1977). Reaction mixture (3 ml) contained 13µM methionine, 63 µM p-nitroblue tetrazolium chloride (NBT), 1.3 µM riboflavin, 50 mM phosphate buffer (pH 7.8) and enzyme extract. The reaction mixture was incubated for 10 min under white fluorescent light and subsequently assayed spectrophotometrically at 560 nm. One unit of SOD activity was defined as the amount of enzyme required for the inhibition of the photochemical reduction of NBT by 50%.

5.1.12.2. Estimation of peroxidase

Peroxidase activity was determined by the method of Hammerschmidt *et al.* (1982). Tissue samples were homogenized as described before in ice cold 50 mM sodium phosphate buffer (pH 7.0) containing PVP and the supernatant was used directly for enzyme assay. The reaction mixture of 3 ml contained 10 mM guaiacol, 2 mM H_2O_2 and 100 µl of enzyme. Change in absorbance was measured at 420 nm for 2 min at intervals of 30 sec. The enzyme activity was expressed as ΔA_{420} min^{-1} mg^{-1} protein.

5.1.12.3. Extraction and estimation of ascorbate peroxidase

Ascorbate peroxidase activity was measured following the method of Nakano and Asada (1981). Fresh tissue samples were homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 1mM ascorbate and 1mM EDTA and after centrifugation, the supernatant was used for enzyme activity assay. The assay solution contained 3ml 0.05 M sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 ml enzyme extract. The oxidation rate of ascorbic acid was estimated by following decrease in absorbance at 290 nm and enzyme activity was expressed as µM $min^{-1}mg^{-1}$ protein (extinction coefficient, 2.8 mM cm^{-1}).

5.1.12.4. Estimation of catalase

Catalase activity was determined using hydrogen peroxide as substrate following the modified method of Aebi (1984). The reaction was initiated by adding 200 µl of enzyme extract in an assay buffer of 3 ml volume containing 10 mM H_2O_2 in 50 mM K-phosphate buffer (pH 7.0) at 30°C. CAT activity was determined by measuring the rate of decrease in absorbance at 1 min interval for 5 min at 240 nm. The enzyme activity was expressed as ΔA_{240} $min^{-1}mg^{-1}$ protein.

5.1.13. Statistical analysis

Statistical analysis was done with the help of Smith's statistical package (version 2.5), developed by Dr. Gray Smith, Pomona College, Claremont-91711, USA. and Statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois.

5.2. RESULTS

5.2.1. Accumulation of copper in leaves and roots

The copper concentration in the leaves and roots of the tea seedlings after exposure to different solution of copper was estimated by atomic absorption spectrometry. Results showed that there was a steady increase in mean copper concentration in both leaf and root tissue with increasing copper concentrations in the nutrient solution (Fig. 5.1 and 5.2). However, amount of copper accumulation in root tissue was far more than that of the leaf in both tested cultivars. TS-462 roots took up slightly more copper than TS-520 when copper concentrations in the nutrient solution was low (<400 µM); but, at higher concentrations, TS-520 accumulated more copper than TS-462).

5.2.2. Lipid peroxidation

The level of lipid peroxidation, expressed as thiobarbituric acid reactive substances (TBARS) showed that the treatment of plants with low amount of copper (0-100 µM) did not change significantly in the leaves of both cultivars when compared to control situation (Tables 5.1 and 5.2). However, a significant progressive increase of TBARS content was found at 200 µM and higher Cu concentrations. Highest increment of TBARS (31%) was noted at 300 to 400 µM increase in Cu^{2+} in TS-520. The extent of dose dependent increase in TBARS declined at the highest concentrations. Among the two

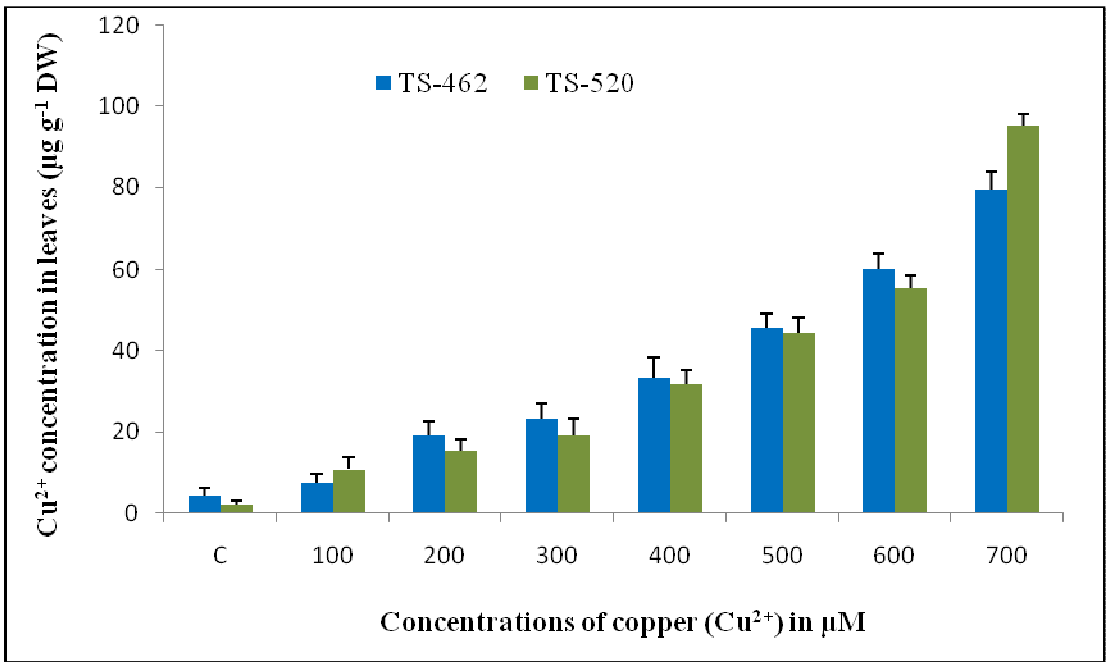

Fig. 5.1: Cu^{2+} concentration in leaves of tea seedling (cultivars TS-462 and TS-520) after 10 days of treatment with increasing concentration of $CuSO_4$ (100µM-700µM) compared with control. Data are mean of three replicates ± SE. C = Control.

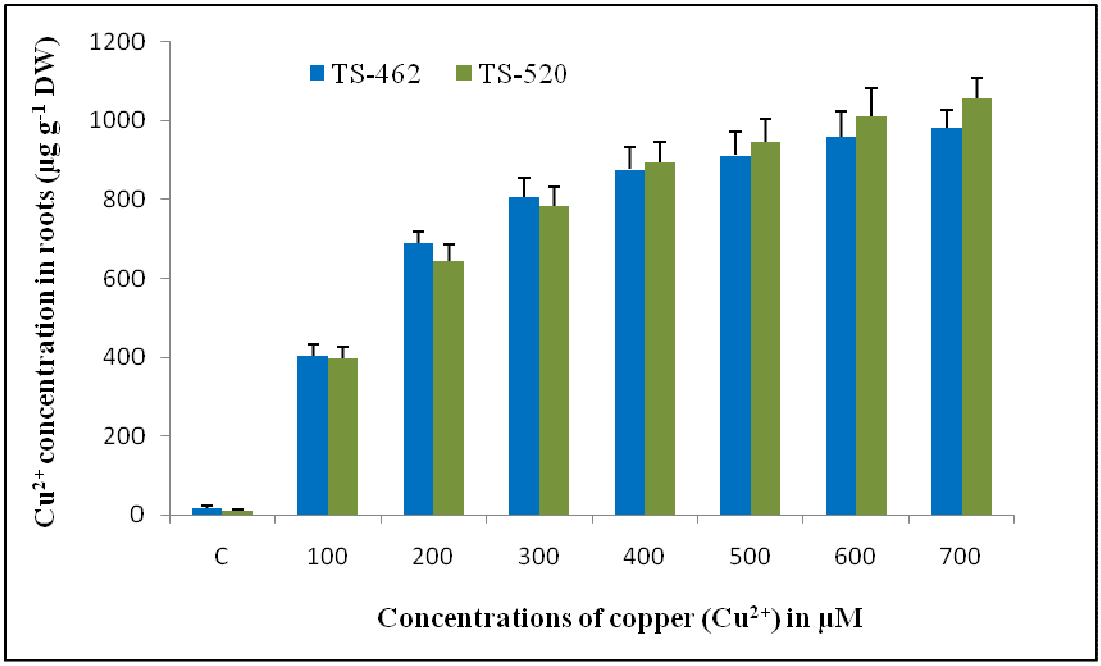

Fig. 5.2: Cu^{2+} concentration in roots of tea seedling (cultivars TS-462 and TS-520) after 10 days of treatment with increasing concentration of $CuSO_4$ (100µM-700µM) compared with control. Data are mean of three replicates ± SE. C = Control.

tested cultivars, TS-520 produced much higher TBARS values than TS-462 (Fig. 5.3). A 27% difference was recorded among the cultivars when 700 µM of copper was present in the nutrient solution.

Table 5.1: Effect of different concentration of copper on level of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	TBARS (µM g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	3.2±0.27	3.1±0.23	3.5±0.29
50	3.6±0.44	3.9±0.48	4.5±0.52
100	4.0±0.31	4.8±0.46	5.0±1.15
200	4.7±0.42	5.6±0.48	6.0±0.50
300	5.2±0.39	5.9±0.52	6.3±0.87
400	5.8±0.45	6.3±0.66	7.7±1.19
500	6.7±0.56	7.0±0.78	7.8±0.69
600	7.1±0.48	7.8±1.07	8.3±1.33
700	7.8±1.02	8.1±0.95	8.8±0.84
CD (5%)	0.20	0.24	0.23

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.2: Effect of different concentration of copper on level of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	TBARS (µM g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	3.3±0.25	3.2±0.27	3.3±0.35
50	3.4±0.37	3.3±0.42	3.5±0.56
100	4.0±0.35	4.4±0.38	4.7±0.49
200	4.9±0.54	5.8±0.40	6.2±0.87
300	6.0±0.52	6.6±0.49	7.5±1.27
400	8.2±0.79	9.0±0.56	9.8±1.05
500	9.3±0.65	10.6±0.71	11.2±0.81
600	9.7±0.72	10.9±1.21	11.5±1.45
700	10.5±0.80	11.7±1.04	12.0±1.44
CD (5%)	0.22	0.27	0.26

Data are mean of three replications; Data after ± indicate standard error values.

5.2.3. Superoxide ion (O_2^-) released

The leaf superoxide anion concentrations increased progressively in the two cultivars of tea following Cu treatment (Table 5.3 and 5.4) at lower doses. However, at higher exposure concentrations that is at 500 µM and above, there was a reduction in both cultivars when compared to control. The levels of O_2^- were always higher in TS-520 than in TS-462 (Fig. 5.4).

Table 5.3: Effect of different concentration of copper on release of superoxide anion in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Superoxide anion release (A_{580} g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.47±0.05	0.56±0.05	0.59±0.05
100	0.64±0.03	0.70±0.02	0.83±0.05
200	0.76±0.05	0.79±0.04	0.91±0.05
300	0.82±0.05	0.89±0.05	0.98±0.03
400	0.93±0.08	0.98±0.05	1.16±0.05
500	1.02±0.06	1.30±0.06	1.20±0.06
600	1.12±0.08	1.26±0.05	1.14±0.05
700	0.65±0.06	0.68±0.05	0.43±0.03
CD (5%)	0.023	0.025	0.035

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.4: Effect of different concentration of copper on release of superoxide anion in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Superoxide anion release (A_{580} g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.49±0.06	0.58±0.05	0.64±0.05
100	0.68±0.05	0.73±0.05	0.88±0.05
200	0.85±0.05	0.94±0.05	0.98±0.04
300	0.91±0.03	0.98±0.05	1.14±0.05
400	0.97±0.05	1.12±0.08	1.37±0.09
500	1.23±0.03	1.41±0.06	1.59±0.07
600	1.31±0.06	1.50±0.06	1.43±0.03
700	0.77±0.06	0.82±0.03	0.61±0.05
CD (5%)	0.029	0.028	0.036

*Data are mean of three replications; Data after ± indicate standard error values.

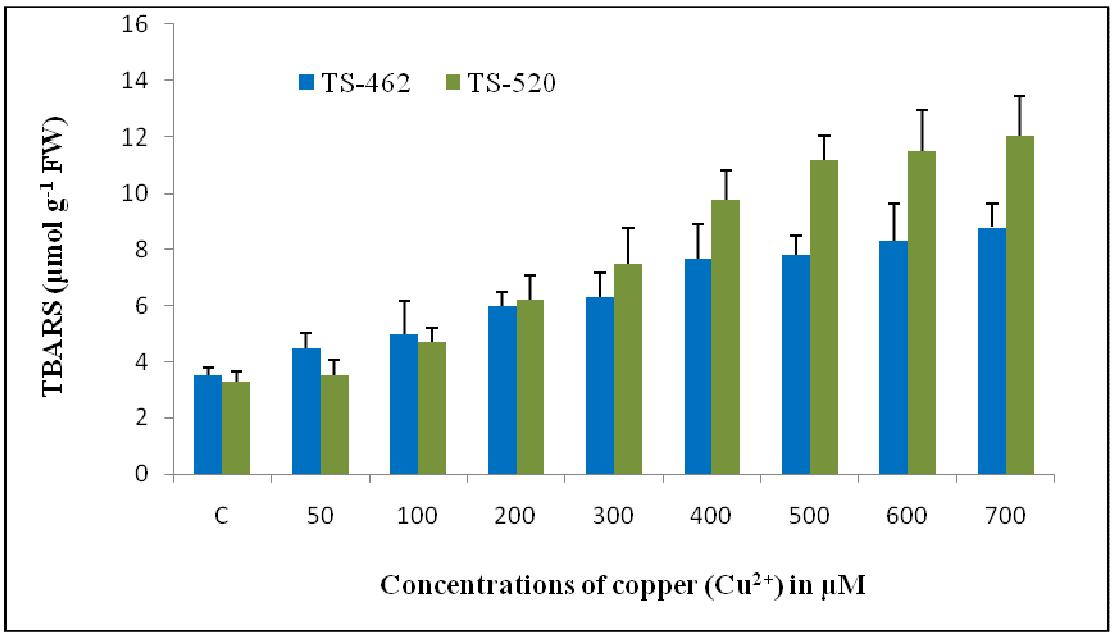

Fig. 5.3: Effect of increasing concentrations of copper (Cu^{2+}) on the level of lipid peroxidation measured as thiobarbituric acid reactive substance (TBARS) on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

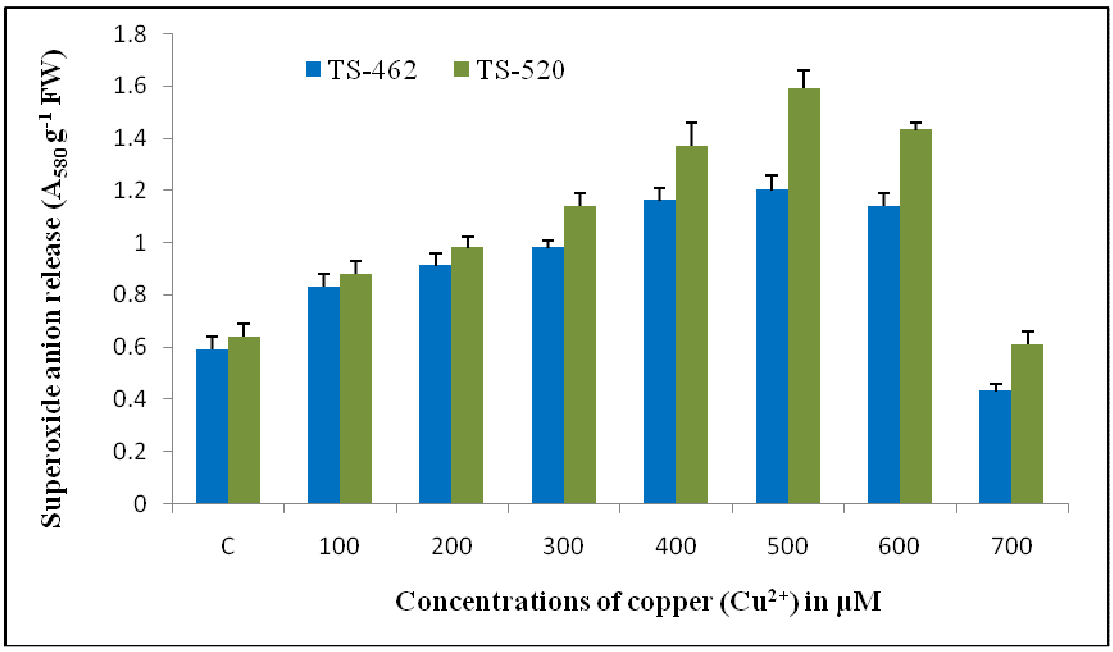

Fig. 5.4: Effect of increasing concentrations of copper (Cu^{2+}) on superoxide anion (O_2^-) release on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

5.2.4. Chlorophyll a, chlorophyll b and total carotenoid content

Chlorophyll a, chlorophyll b and total carotenoid contents were found to decrease significantly with increasing Cu concentrations in both the tested cultivars (Tables 5.5, 5.6, 5.7, 5.8, 5.9 and 5.10). There was also progressive decrease in the chlorophyll and carotenoid levels with increase in duration of exposure at 4th, 7th and 10th days. Although both cultivars showed a decreased activity in comparison to control plants, but the reduction was more in TS-520 when analyzed after 10 days of treatment (Figs. 5.5, 5.6 and 5.7). For example, the chlorophyll and carotenoids contents of TS-520 were significantly lower than TS-462 at 700 µM copper sulphate treatment. Control plant showed more or less similar chlorophyll and carotenoid levels within 10 days.

Table 5.5: Effect of different concentration of copper on chlorophyll-a content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Chlorophyll-a content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	3.21±0.11	2.92±0.13	2.81±0.07
50	3.05±0.09	2.29±0.12	2.27±0.09
100	2.74±0.08	2.13±0.10	2.23±0.10
200	2.46±0.08	1.84±0.10	1.80±0.08
300	2.27±0.06	1.75±0.08	1.78±0.12
400	2.15±0.09	1.65±0.09	1.55±0.13
500	2.05±0.10	1.61±0.10	1.52±0.12
600	2.04±0.07	1.52±0.06	1.41±0.09
700	1.5±0.07	1.3±0.08	1.23±0.10
CD (5%)	0.04	0.04	0.03

*Data are mean of three replications; Data after ± indicate standard error value

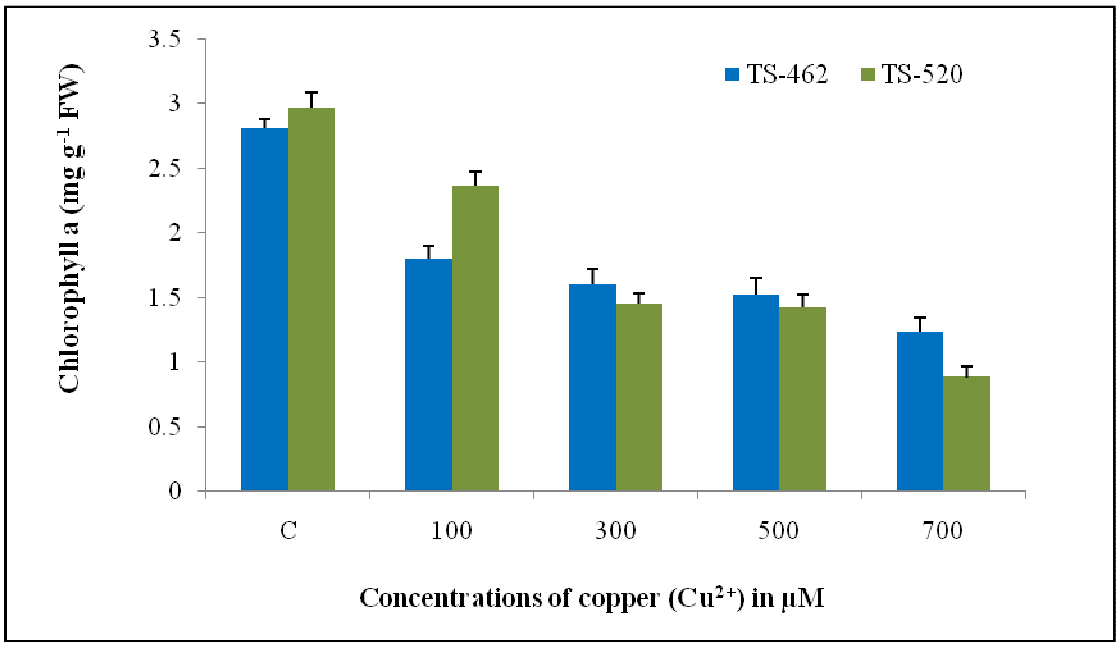

Fig. 5.5: Effect of increasing concentrations of copper (Cu^{2+}) on chlorophyll-a content on the 10th day of exposure in leaves of two cultivars of tea seedlings (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

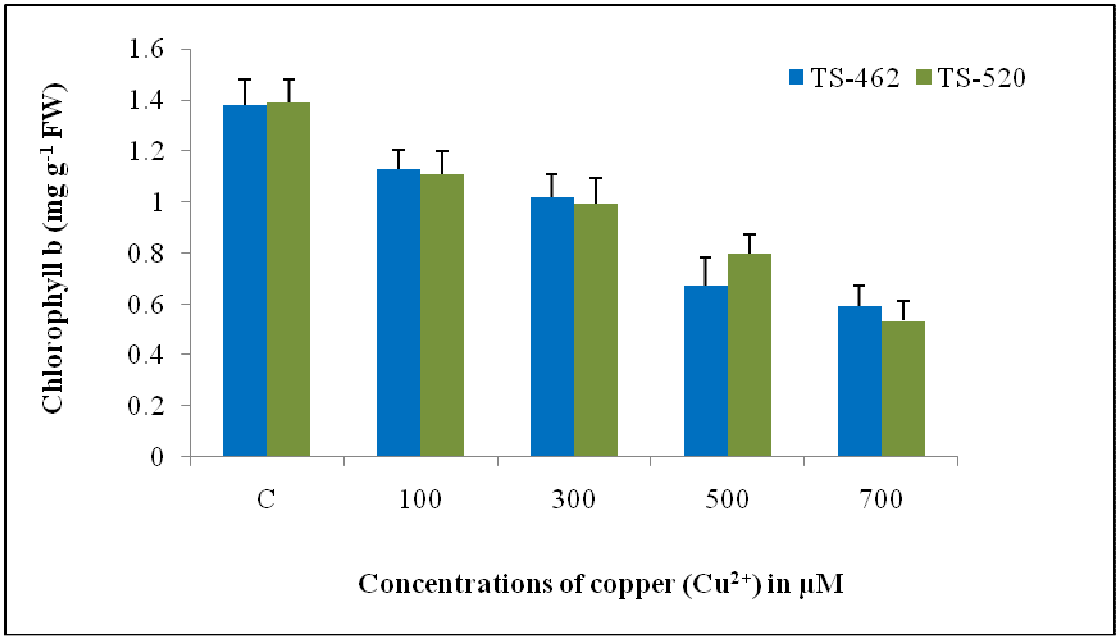

Fig. 5.6: Effect of increasing concentrations of copper (Cu^{2+}) on chlorophyll-b content on the 10th day of exposure in leaves of two cultivars of tea seedlings (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

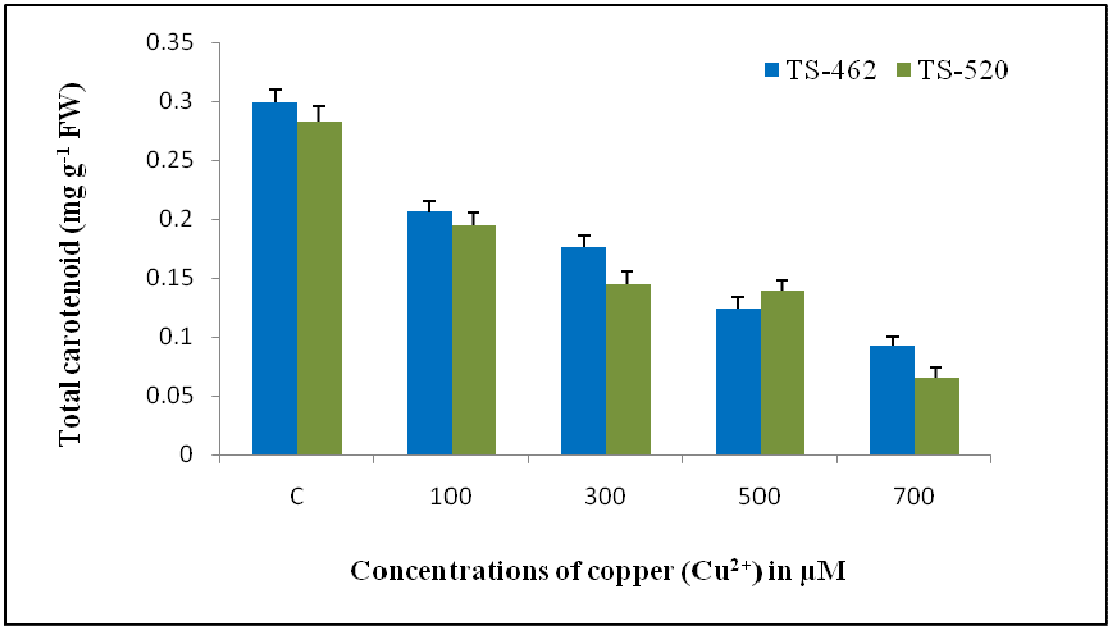

Fig. 5.7: Effect of increasing concentrations of copper (Cu^{2+}) on total carotenoid content on the 10th day of exposure in leaves of two cultivars of tea seedlings (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

Table 5.6: Effect of different concentration of copper on chlorophyll-a content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Chlorophyll-a content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	3.15±0.08	3.03±0.11	2.97±0.11
50	2.89±0.09	2.61±0.10	2.51±0.10
100	2.74±0.08	2.55±0.10	1.94±0.11
200	2.53±0.09	2.34±0.09	1.77±0.10
300	2.30±0.09	1.98±0.09	1.45±0.08
400	2.12±0.09	1.94±0.09	1.32±0.09
500	1.92±0.08	1.85±0.10	1.25±0.10
600	1.67±0.10	1.48±0.10	1.37±0.11
700	1.09±0.09	0.97±0.09	0.73±0.08
CD (5%)	0.01	0.02	0.02

*Data are mean of three replications; Data after ± indicate standard error values

Table 5.7: Effect of different concentration of copper on chlorophyll-b content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Chlorophyll-b content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	1.64±0.10	1.43±0.10	1.38±0.10
50	1.51±0.10	1.29±0.10	1.18±0.09
100	1.38±0.09	1.18±0.09	1.13±0.07
200	1.31±0.10	1.13±0.08	1.01±0.06
300	1.25±0.10	1.13±0.07	1.02±0.09
400	1.11±0.09	1.04±0.07	0.97±0.09
500	0.92±0.09	0.79±0.06	0.67±0.11
600	0.87±0.10	0.75±0.07	0.62±0.07
700	0.72±0.09	0.61±0.07	0.59±0.08
CD (5%)	0.15	0.12	0.16

*Data are mean of three replications; Data after ± indicate standard error values

Table 5.8: Effect of different concentration of copper on chlorophyll-b content in leaf tissue of TS-520

Concentration of $CuSO_4$ (μM)	Chlorophyll-b content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	1.49±0.07	1.41±0.05	1.39±0.09
50	1.32±0.06	1.29±0.08	1.22±0.07
100	1.27±0.08	1.14±0.09	0.98±0.09
200	1.26±0.08	1.18±0.07	1.15±0.09
300	1.09±0.07	1.07±0.09	0.81±0.10
400	0.97±0.09	0.87±0.08	0.87±0.10
500	0.96±0.09	0.83±0.08	0.53±0.07
600	0.91±0.08	0.86±0.09	0.63±0.09
700	0.76±0.08	0.65±0.09	0.46±0.08
CD (5%)	0.13	0.22	0.17

*Data are mean of three replications; Data after ± indicate standard error values

Table 5.9: Effect of different concentration of copper on total carotenoid content in leaf tissue of TS-462

Concentration of $CuSO_4$ (μM)	Carotenoid content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.32±0.008	0.30±0.009	0.29±0.011
50	0.28±0.010	0.25±0.010	0.23±0.010
100	0.26±0.010	0.26±0.010	0.21±0.009
200	0.24±0.011	0.23±0.008	0.20±0.009
300	0.23±0.008	0.18±0.009	0.17±0.010
400	0.18±0.011	0.15±0.012	0.13±0.011
500	0.16±0.010	0.13±0.011	0.13±0.010
600	0.14±0.008	0.12±0.008	0.10±0.009
700	0.10±0.009	0.09±0.009	0.09±0.008
CD (5%)	0.11	0.13	0.15

*Data are mean of three replications; Data after ± indicate standard error value

Table 5.10: Effect of different concentration of copper on total carotenoid content in leaf tissue of TS-520

Concentration of $CuSO_4$ (μM)	Carotenoid content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.31±0.009	0.29±0.009	0.28±0.013
50	0.29±0.010	0.27±0.009	0.25±0.009
100	0.27±0.011	0.23±0.010	0.17±0.010
200	0.26±0.010	0.21±0.008	0.17±0.014
300	0.23±0.008	0.19±0.008	0.14±0.010
400	0.18±0.009	0.14±0.010	0.16±0.013
500	0.15±0.010	0.17±0.009	0.11±0.009
600	0.14±0.009	0.15±0.009	0.14±0.008
700	0.10±0.010	0.07±0.008	0.05±0.009
CD (5%)	0.017	0.010	.019

*Data are mean of three replications; Data after ± indicate standard error values

5.2.5. Total protein content

Total soluble protein concentration was found to decrease considerably at high Cu exposure concentrations in both leaves and roots of the two tested cultivars in comparison to control. Protein content of leaves decreased in a time dependent manner to 5.8 mg/g FW on 10th day from 8.8 mg/g FW on the 4th day at 700 μM exposure concentrations in TS-462 cultivar (Table 5.11). TS-520 recorded lower protein content (3.12 mg/g FW) in the leaves than TS-462 at the highest exposure concentration (Fig. 5.8). However there were no noticeable changes in untreated controls which varied from 14.8 mg/g FW to 14.2 mg/g FW in TS-462 and 15.17 to 15.35 in TS-520 (Table 5.12). A similar response was noted in the root tissues, however, the decrease in protein content was lower in roots than leaves; and variation among the two tested cultivars was not detectable in roots (Fig. 5.9). Although there was an ultimate decrease in the protein content, but at lower exposure concentrations (50 to 300 μM), the protein contents in both leaves and roots either remained almost similar or a very marginal increase was detected with increasing copper concentrations in both tested cultivars.

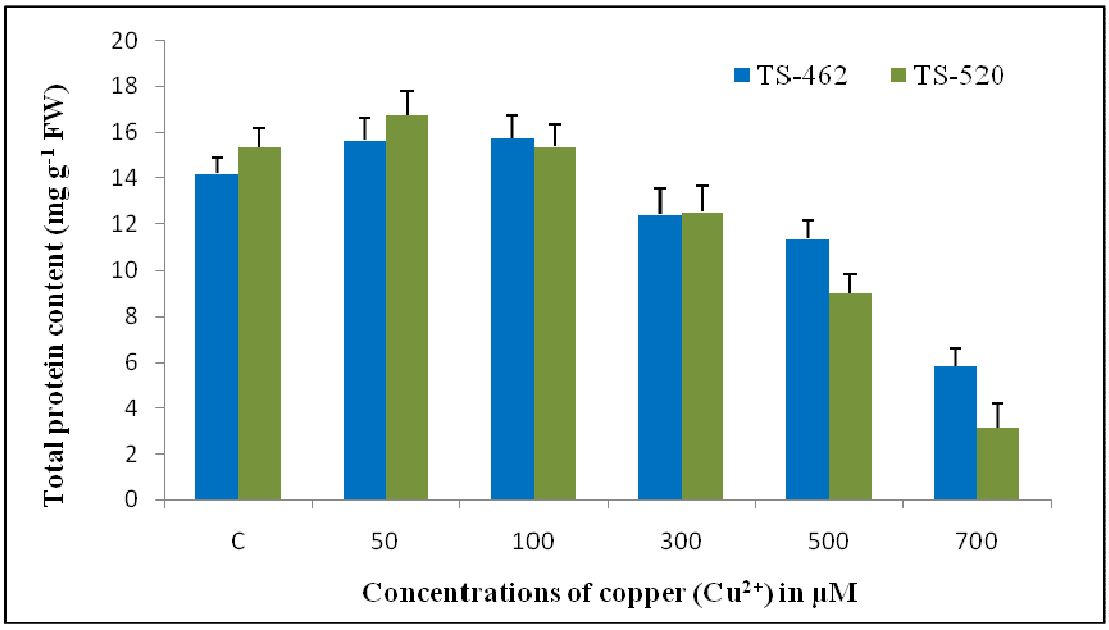

Fig. 5.8: Effect of increasing concentrations of copper (Cu^{2+}) on total protein content on the 10th day of exposure in leaves of two cultivars of tea seedlings (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

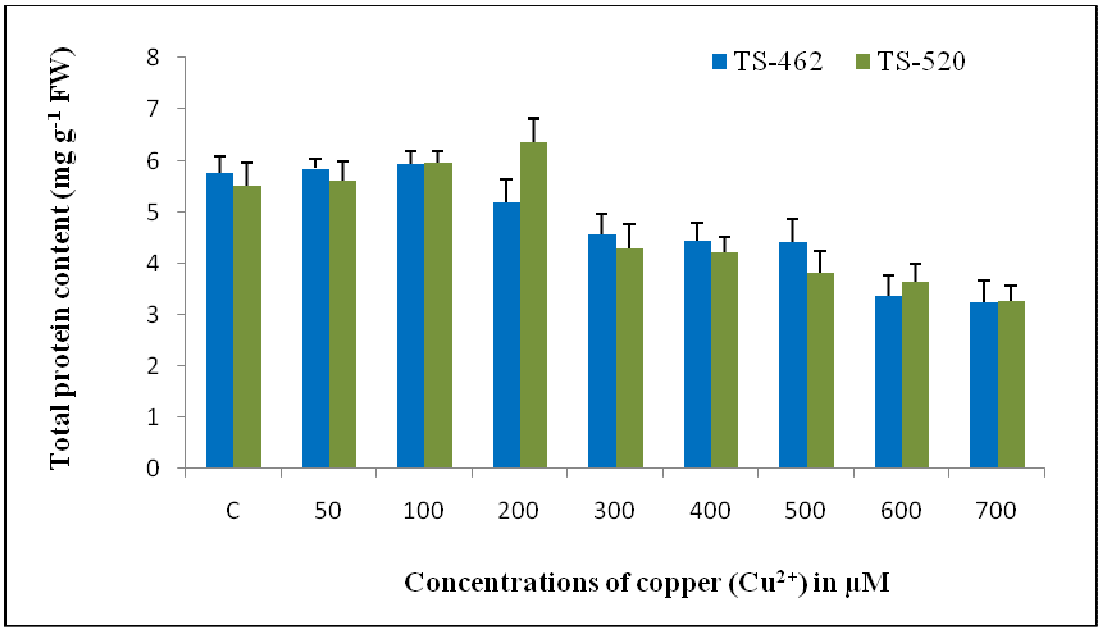

Fig. 5.9: Effect of increasing concentrations of copper (Cu^{2+}) on total protein content on the 10th day of exposure in roots of two cultivars of tea seedlings (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

Table 5.11: Effect of different concentration of copper on total protein content of leaf tissue in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Total protein content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	14.80±0.64	13.50±0.75	14.20±0.71
50	15.20±0.58	15.46±0.69	15.65±0.96
100	16.30±0.87	15.20±0.87	15.72±1.03
200	17.40±0.92	15.59±1.10	15.07±0.65
300	17.50±0.98	16.90±0.71	12.40±1.14
400	16.30±1.04	15.07±0.91	11.56±0.96
500	14.09±0.75	12.40±0.89	11.40±0.72
600	11.50±0.69	10.77±0.74	08.30±0.70
700	08.80±1.10	07.40±0.76	05.80±0.80
CD (5%)	0.32	0.22	0.30

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.12: Effect of different concentration of copper on total protein content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Total protein content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	15.17±0.83	15.58±1.14	15.35±0.84
50	15.85±0.88	16.24±0.91	16.74±1.03
100	16.88±0.98	15.65±1.15	15.37±0.96
200	17.20±1.01	16.17±1.14	15.02±0.90
300	17.61±0.78	15.20±0.79	12.52±1.11
400	15.74±1.09	14.07±0.84	10.45±1.02
500	13.42±1.15	11.35±0.95	09.03±0.80
600	10.70±0.97	07.67±0.70	05.31±0.79
700	07.10±1.10	04.20±0.73	03.12±1.08
CD (5%)	0.22	0.31	0.21

*Data are mean of three replications; Data after ± indicate standard error values.

5.2.6. Effect of copper on total carbohydrate content

The total carbohydrate content was extracted from the leaves of copper treated plants and estimated by using anthrone reagent. A steady decrease in carbohydrate concentration with increasing concentration of copper with respect to control was observed in both the tested cultivars. Additionally a time dependent decline in carbohydrate content was also noted. The lowest

carbohydrate content of 9.4 mg/g FW was observed in 700 µM copper treated tea seedlings of TS-520 cultivar after 10 days which was much lower than control which recorded 40.92 mg/g FW (Table 5.13). TS-462 recorded significantly higher values than TS-520 but only after 7 and 10 days of exposure (Table 5.14). Varietal difference was not prominent on the 4th day at any tested concentration (Fig. 5.10).

Table 5.13: Effect of different concentration of copper on total carbohydrate content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Total carbohydrate content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	39.61±2.30	40.30±1.84	41.53±2.30
50	37.53±1.73	36.61±2.88	35.15±2.77
100	35.69±2.88	32.38±2.77	30.92±2.94
200	33.07±1.73	32.15±2.36	28.49±1.44
300	31.61±2.25	29.53±1.84	27.10±2.88
400	29.15±2.13	28.53±2.77	26.07±2.30
500	27.20±2.36	26.53±2.48	23.15±1.73
600	25.46±2.42	20.30±2.88	18.53±2.19
700	22.50±2.40	19.60±2.36	12.50±2.71
CD (5%)	0.86	0.69	0.90

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.14: Effect of different concentration of copper on total carbohydrate content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Total carbohydrate content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	40.76±2.88	41.30±2.71	40.92±2.59
50	38.69±2.94	36.38±2.59	35.50±2.65
100	36.61±2.65	34.15±2.19	32.07±1.78
200	34.38±2.30	32.90±2.42	27.80±1.96
300	31.53±2.59	29.30±2.13	27.50±2.19
400	29.92±2.40	26.07±2.36	21.00±2.42
500	27.76±2.42	23.53±2.59	17.30±2.54
600	25.61±2.36	17.69±2.82	13.30±2.25
700	23.70±2.30	12.30±2.54	09.40±2.30
CD (5%)	0.33	0.35	0.50

*Data are mean of three replications; Data after ± indicate standard error values.

5.2.7. Proline content

The concentration of proline was found to increase with increasing copper concentration upto 500 µM exposure concentrations, then gradually declined in both cultivars (Table 5.15 and 5.16). TS-462 showed maximum increase of 1.85 µM g^{-1} FW in 500 µM concentration. Proline content decreased above 500 µM at all exposure times tested. TS-462 recorded higher proline accumulation than TS-520 and less decrease at high concentrations (Fig. 5.11).

Table 5.15: Effect of different concentration of copper in proline content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Proline (µM g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	5.67±0.16	6.64±0.11	7.89±0.12
100	6.30±0.10	6.99±0.12	8.24±0.10
200	7.61±0.06	8.45±0.13	9.07±0.15
300	8.17±0.11	8.86±0.08	9.14±0.10
400	8.83±0.09	9.28±0.09	9.90±0.16
500	8.93±0.08	11.5±0.08	11.9±0.05
600	5.26±0.12	5.40±0.12	6.99±0.14
700	4.43±0.10	5.12±0.12	4.36±0.12
CD (5%)	0.63	0.29	0.23

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.16: Effect of different concentration of copper in proline content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Proline (µM g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	4.84±0.16	5.36±0.13	5.95±0.14
100	5.88±0.15	6.64±0.09	7.82±0.06
200	6.99±0.09	7.96±0.06	8.79±0.03
300	8.45±0.14	9.35±0.09	9.76±0.07
400	8.72±0.09	11.15±0.07	9.83±0.05
500	9.14±0.11	9.83±0.05	10.94±0.03
600	9.55±0.09	9.76±0.06	8.93±0.03
700	6.17±0.14	7.13±0.05	6.09±0.13
CD (5%)	0.29	0.24	0.21

*Data are mean of three replications; Data after ± indicate standard error values.

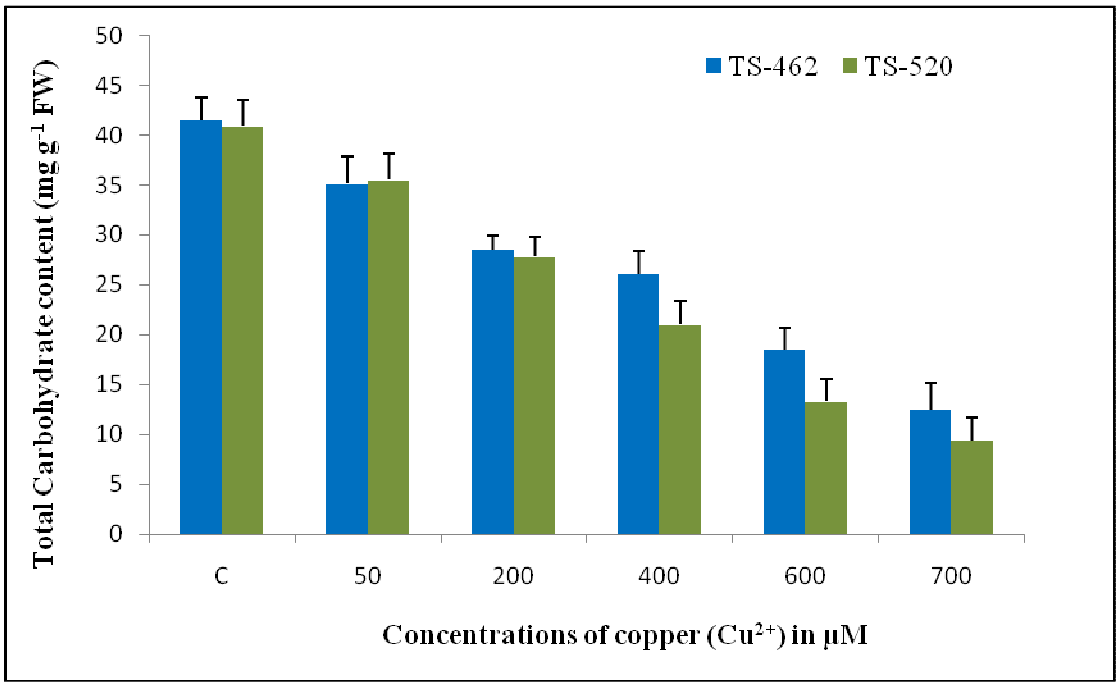

Fig. 5.10: Effect of increasing concentrations of copper (Cu^{2+}) on total carbohydrate content on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

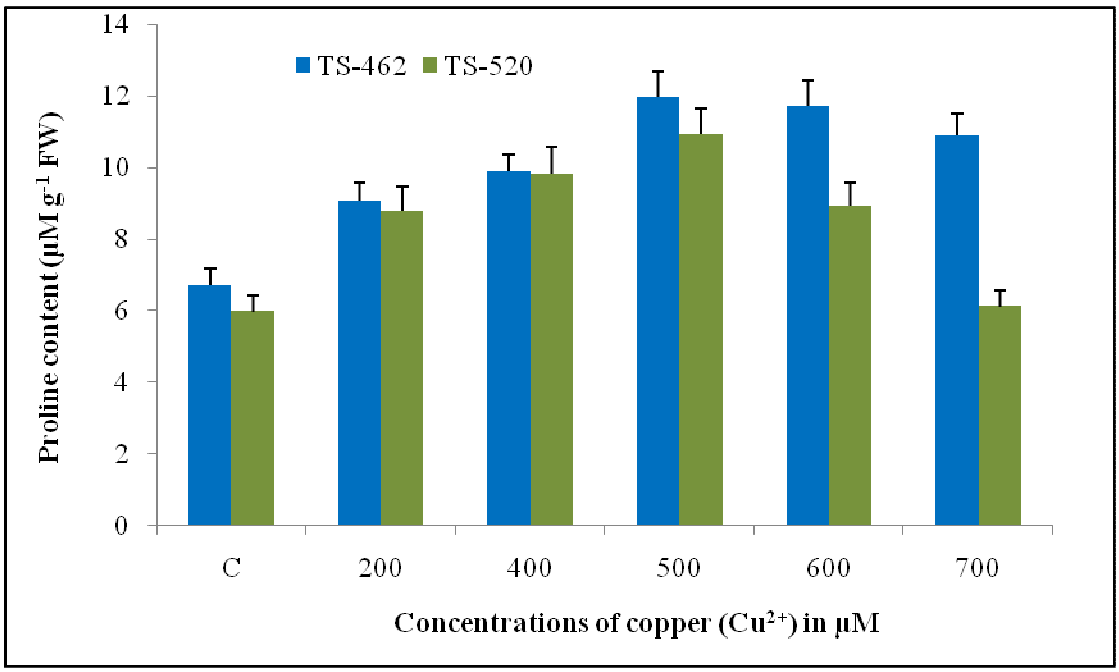

Fig. 5.11: Effect of increasing concentrations of copper (Cu^{2+}) on proline content on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

5.2.8. Phenolics and *o*-dihydroxy phenolics content

A measurement of the phenolics content in the tea seedlings exposed to excess copper revealed an increase in total phenolic compounds in the leaves of both cultivars of tea when compared to control. The significant increments of phenolics content were recorded at all concentration at 4th, 7th, and 10th day. In TS-520, amount of increase was found to be less at higher concentrations as shown in Table 5.18. Phenolics content was highest at 400 µM after which it showed a declining trend at higher concentrations. But in TS-462 (Table 5.17), the total phenolics content gradually increased with time and concentration upto 600 µM and then declined at the highest tested concentration of 700 µM although the values did not vary greatly among the tested cultivars (Fig. 5.12).

Total *o*-dihydroxyphenolics content showed similar trends of change (Table 5.19 and 5.20) which also exhibited time and concentration dependence when compared to control. However, there was marked variation among cultivars. TS-462 recorded much higher *o*-dihydroxyphenolics content at the highest exposure concentration than TS-520 (Fig. 5.13).

Table 5.17: Effect of different concentration of copper on total phenol content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Total phenol content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	4.52±0.40	4.59±0.20	4.61±0.20
50	4.69±0.20	4.73±0.50	4.92±0.60
100	4.73±0.20	4.85±0.30	5.02±0.30
200	4.87±0.20	5.42±0.70	5.73±0.50
300	4.92±0.50	5.63±0.40	5.97±0.40
400	4.98±0.25	5.84±0.60	6.23±0.50
500	5.23±0.40	5.21±0.40	5.87±0.30
600	4.72±0.40	5.56±0.30	5.78±0.20
700	4.57±0.40	5.01±0.40	4.85±0.30
CD (5%)	0.41	0.60	0.39

*Data are mean of three replications; Data after ± indicate standard error values

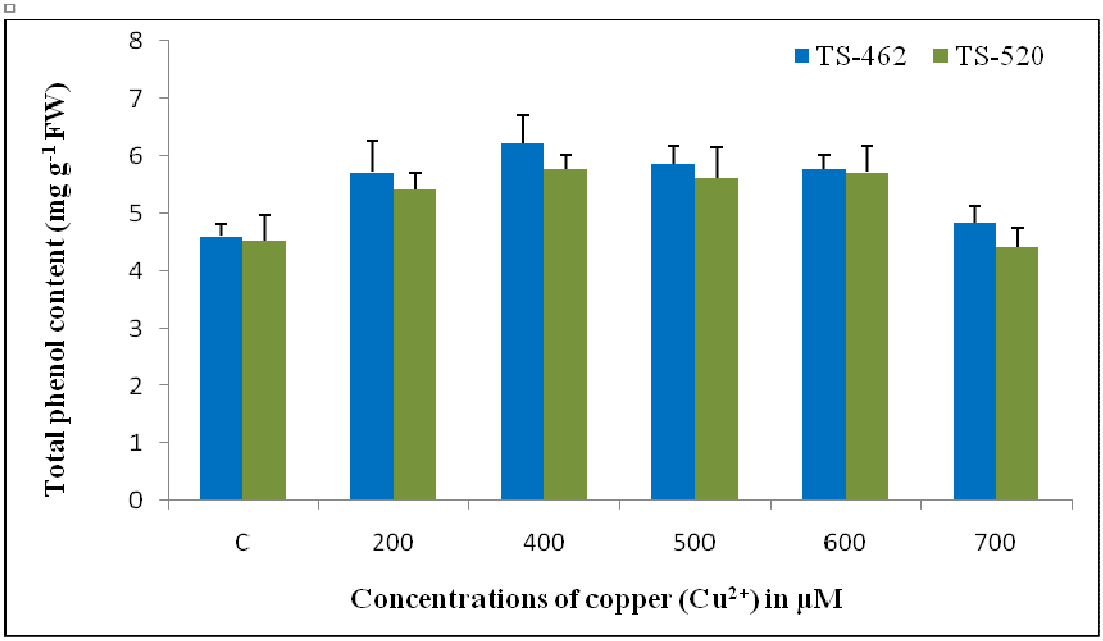

Fig. 5.12: Effect of increasing concentrations of copper (Cu^{2+}) on total phenol content on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

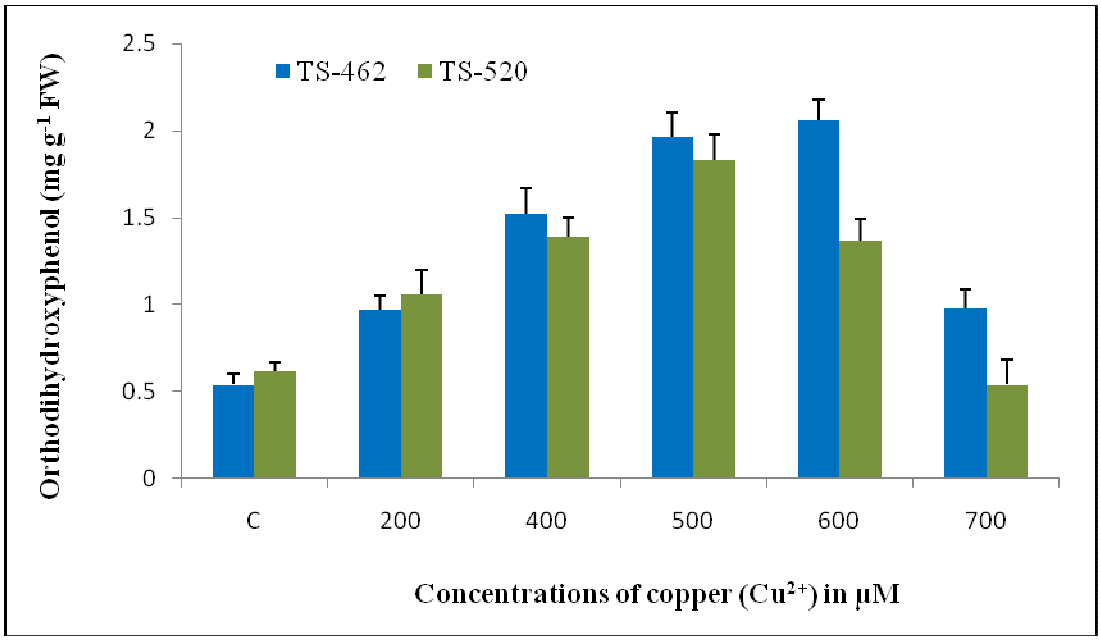

Fig. 5.13: Effect of increasing concentrations of copper (Cu^{2+}) on total ortho-dihydroxyphenol content on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

Table 5.18: Effect of different concentration of copper on total phenol content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Total phenol content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	4.43±0.30	4.52±0.30	4.54±0.40
50	4.52±0.35	4.62±0.20	4.78±0.60
100	4.67±0.40	4.78±0.35	4.83±0.40
200	4.87±0.40	4.93±0.60	5.42±0.30
300	4.95±0.30	5.12±0.50	5.64±0.30
400	5.2±0.35	5.32±0.70	5.79±0.20
500	5.25±0.40	5.39±0.40	5.63±0.50
600	5.31±0.60	5.42±0.45	5.73±0.45
700	4.62±0.40	4.78±0.20	4.42±0.30
CD (5%)	0.34	0.43	0.35

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.19: Effect of different concentration of copper on orthodihydroxyphenol content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	orthodihydroxyphenol content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.35±0.05	0.43±0.07	0.54±0.06
50	0.40±0.03	0.58±0.07	0.65±0.07
100	0.47±0.07	0.80±0.08	0.89±0.06
200	0.55±0.05	0.88±0.09	0.97±0.08
300	0.67±0.06	1.02±0.11	1.30±0.10
400	0.71±0.05	0.92±0.08	1.52±0.15
500	0.80±0.06	1.12±0.14	1.96±0.14
600	0.94±0.11	1.48±0.10	2.06±0.12
700	0.88±0.07	1.22±0.04	0.98±0.11
CD (5%)	0.11	0.17	0.06

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.20: Effect of different concentration of copper on orthodihydroxyphenol content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	orthodihydroxyphenol content (mg g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.48±0.03	0.50±0.07	0.62±0.05
50	0.51±0.05	0.54±0.05	0.68±0.05
100	0.54±0.03	0.60±0.12	0.72±0.06
200	0.57±0.05	0.84±0.08	1.06±0.14
300	0.63±0.04	0.90±0.12	1.14±0.13
400	0.89±0.12	1.17±0.08	1.39±0.11
500	1.32±0.18	1.58±0.16	1.83±0.15
600	0.76±0.11	1.04±0.13	1.37±0.12
700	0.46±0.05	0.50±0.12	0.54±0.14
CD (5%)	0.07	0.05	0.06

*Data are mean of three replications; Data after ± indicate standard error values.

5.2.9. Non protein thiols content

Non-protein thiols (NPT) content was measured in the leaves and roots of tea plants exposed to copper excess using Ellman's reagent. The leaves showed an increase in NPT content with time upto 7th day and declined thereafter in all exposed concentrations in both cultivars when compared to control (Tables 5.21 and 5.22). Steady increments were also recorded with increasing copper levels but only at lower concentrations and exposure durations. Beyond 500 µM, the NPT contents decreased drastically on the 10th day. The two cultivars showed some variation, TS-520 recorded higher values than TS-462 (Fig. 5.14). In the roots, NPT content increased with increasing Cu concentrations at lower exposure levels. At high concentrations (>400 µM) NPT content decreased considerably especially in TS-462 (Fig. 5.15).

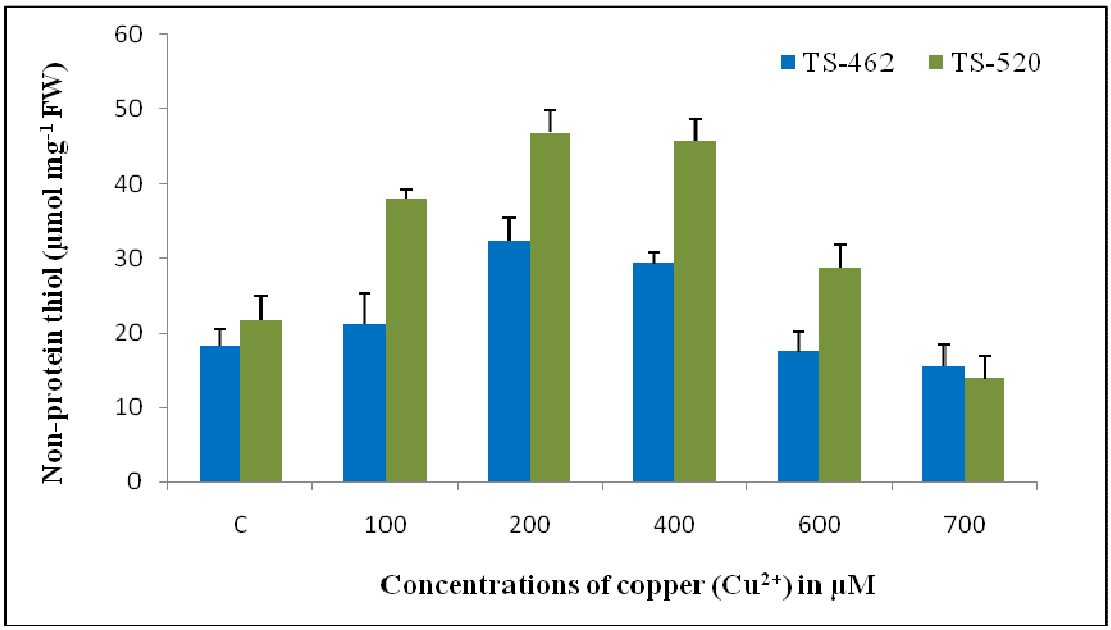

Fig. 5.14: Effect of increasing concentrations of copper (Cu^{2+}) on level of non-protein thiol on the 10th day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

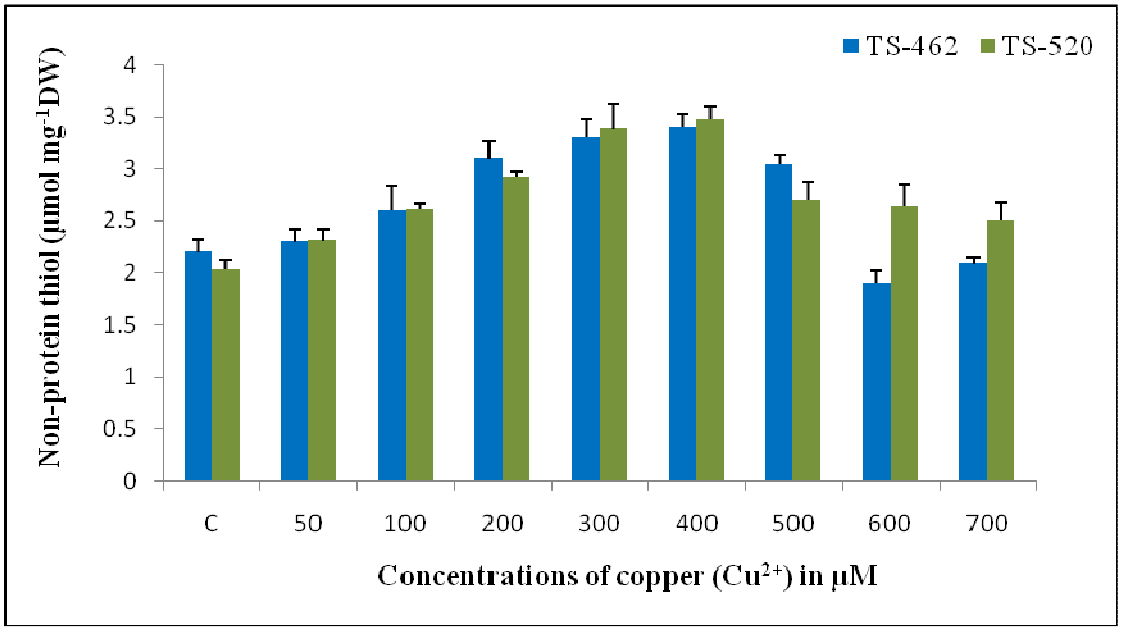

Fig. 5.15: Effect of increasing concentrations of copper (Cu^{2+}) on level of non-protein thiol on the 10th day of exposure in roots of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates ±SE. C = Control.

Table 5.21: Effect of different concentration of copper on non-protein thiol content in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Non-protein thiol (µM g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	16.5±3.06	16.2±2.82	18.3±2.17
100	18.9±1.20	31.0±3.00	21.2±4.10
200	21.4±3.01	38.1±1.36	32.5±3.08
300	23.3±1.40	42.4±2.94	35.3±1.67
400	25.8±2.42	49.7±3.18	29.4±1.46
500	19.0±1.67	42.1±2.08	27.4±2.31
600	12.7±3.41	35.8±2.44	17.5±2.62
700	11.02±1.84	26.8±1.21	15.5±3.06
CD (5%)	1.55	1.41	1.58

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.22: Effect of different concentration of copper on non-protein thiol content in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Non-protein thiol (µM g^{-1} FW)*		
	Incubation period (days)		
	4th	7th	10th
Control	19.3±2.02	21.0±1.21	21.7±3.29
100	21.2±0.98	34.3±3.41	38.1±1.13
200	25.7±2.66	43.5±3.75	46.8±3.00
300	27.1±1.36	51.6±2.59	47.9±1.14
400	29.9±2.54	56.5±1.33	45.7±2.89
500	27.9±1.25	48.3±2.74	41.9±3.23
600	20.1±1.56	45.3±3.23	28.8±3.07
700	11.7±2.31	29.6±1.27	13.9±3.06
CD (5%)	1.77	1.92	1.70

*Data are mean of three replications; Data after ± indicate standard error values.

5.2.10. Activity of antioxidative enzymes

Time dependent and concentration dependent changes in the activities of the enzymes, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) known for their antioxidative activity were measured in seedlings of two tea cultivars TS-462 and TS-520. Enzyme activity was assayed for the treated and control sets on the 4th, 7th and 10th day after start of treatment in case of leaves and on the 10th day in case of roots.

5.2.10.1. Superoxide dismutase

SOD activity increased progressively with increasing copper concentrations in both tested cultivars in the leaves of treated tea seedlings. Maximum rise in enzyme activity was visible in TS-462 on the 10th day when an increase from 2.55 units mg^{-1} protein in control to 5.95 units mg^{-1} protein at the highest tested concentration (700 µM) (Table 5.23) was recorded. Similarly a significant time dependent increase was also noted from 4th to the 10th day. However, in TS-520, the SOD activity increased steadily in a dose and time dependent manner at the lower exposure concentrations and declined at concentrations greater than 400 µM (Table 5.24) but this decline was not below control levels. In the roots, SOD levels increased in a similar way with increasing Cu concentrations and highest activity was recorded at 500 µM by TS-462 and at 400 µM by TS-520 (Fig. 5.16). A comparison among the cultivars revealed that TS-462 showed higher SOD levels at all tested concentrations (Fig. 5.17).

Table 5.23: Effect of different concentration of copper on superoxide dismutase activity in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Superoxide dismutase activity (µM $min^{-1}mg^{-1}$ protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	2.39±0.4	2.45±0.4	2.55±0.2
50	2.90±0.55	3.71±0.3	4.23±0.5
100	3.21±0.4	4.23±0.3	4.87±0.4
200	3.42±0.5	4.53±0.45	5.32±0.3
300	3.83±0.4	5.02±0.4	5.40±0.4
400	3.91±0.7	5.18±0.3	5.46±0.5
500	4.23±0.55	5.35±0.3	5.56±0.45
600	4.43±0.6	5.52±0.4	5.88±0.25
700	4.63±0.35	5.58±0.4	5.95±0.25
CD (5%)	0.32	0.40	0.16

*Data are mean of three replications; Data after ± indicate standard error values.

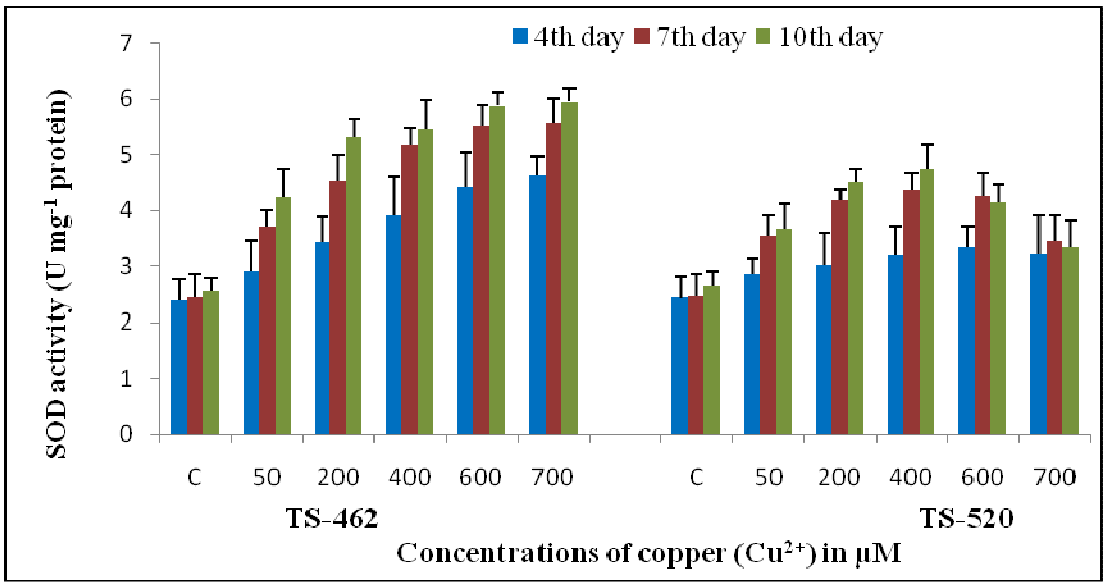

Fig. 5.16: Effect of increasing concentrations of copper (Cu^{2+}) on superoxide dismutase (SOD) activity in leaves of two cultivars of tea (TS-462 and TS-520) on the 4th, 7th and 10th day of exposure. Values are mean of three replicates ±SE. C = Control.

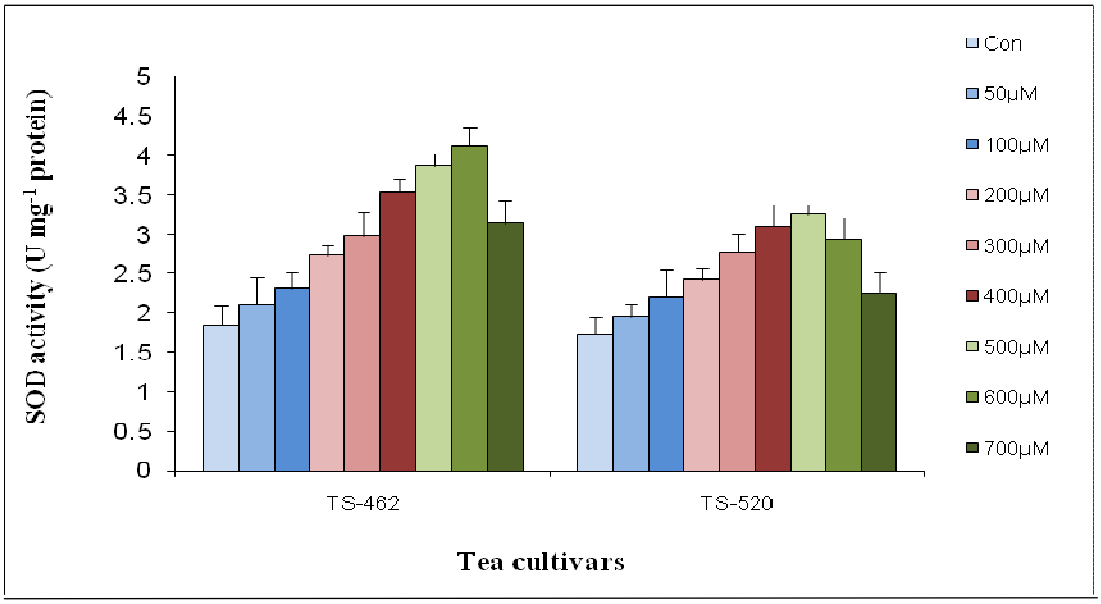

Fig. 5.17: Effect of increasing concentrations of copper (Cu^{2+}) on superoxide dismutase (SOD) activity in roots of two cultivars of tea (TS-462 and TS-520) on the 10th day of exposure. Values are mean of three replicates ±SE. Con = Control.

Table 5.24: Effect of different concentration of copper on superoxide dismutase activity in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Superoxide dismutase activity (µM $min^{-1}mg^{-1}$ protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	2.43±0.4	2.46±0.4	2.64±0.3
50	2.85±0.3	3.54±0.4	3.66±0.5
100	2.95±0.2	4.02±0.3	4.23±0.2
200	3.01±0.6	4.18±0.2	4.52±0.2
300	3.11±0.3	4.24±0.3	4.65±0.3
400	3.19±0.5	4.38±0.3	4.75±0.4
500	3.23±0.4	4.3±0.3	4.54±0.3
600	3.34±0.4	4.26±0.4	4.14±0.3
700	3.22±0.7	3.46±0.5	3.33±0.5
CD (5%)	0.24	0.14	0.20

*Data are mean of three replications; Data after ± indicate standard error values.

5.2.10.2. Peroxidase

Peroxidase activity also increased in the leaves of tea seedlings with time and with augmentation of copper concentrations in the nutrient solution in comparison to control (Tables 5.25 and 5.26). However, such increase was recorded upto 400 µM Cu^{2+} in TS-520 cultivar (Fig. 5.18). When the exposure concentration was higher, POD activity showed a declining trend. On the other hand, in TS-462, the POD level was higher than TS-520 and more than twice of control. Maximum value was recorded at 600 µM on the tenth day and declined at 700 µM exposure concentration (Fig. 5.18). In the roots, a similar concentration dependent rise in POD levels were noticed at lower doses which peaked at 500 µM in both cultivars and then declined (Fig. 5.19). However POD activity always remained above the control levels in both roots and leaves at all tested concentrations.

5.2.10.3. Ascorbate peroxidase

Ascorbate peroxidase recorded a very high increase in activity in a time and dose dependent manner in the leaves in both tested cultivars of tea (Tables 5.27 and 5.28). TS-520 showed a three-fold increase in APX activity on the 10th day at 400 µM of Cu^{2+}. However, TS-462 recorded almost 4.5 fold

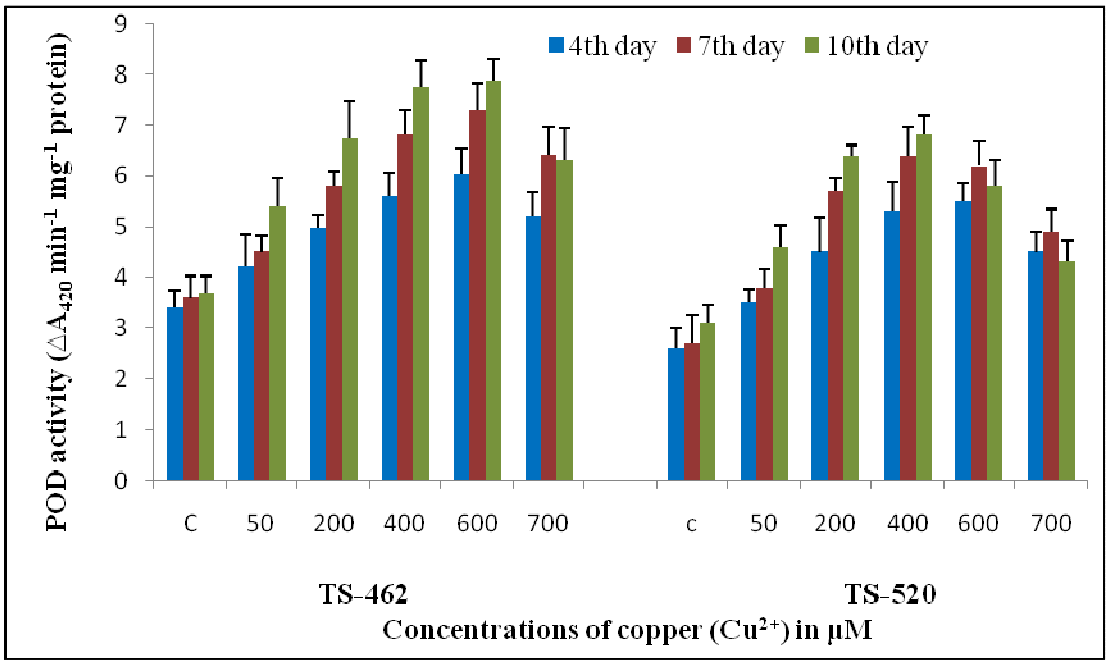

Fig. 5.18: Effect of increasing concentrations of copper (Cu^{2+}) on peroxidase (POD) activity in leaves of two cultivars of tea (TS-462 and TS-520) on the 4th, 7th and 10th day of exposure. Values are mean of three replicates ±SE. C = Control.

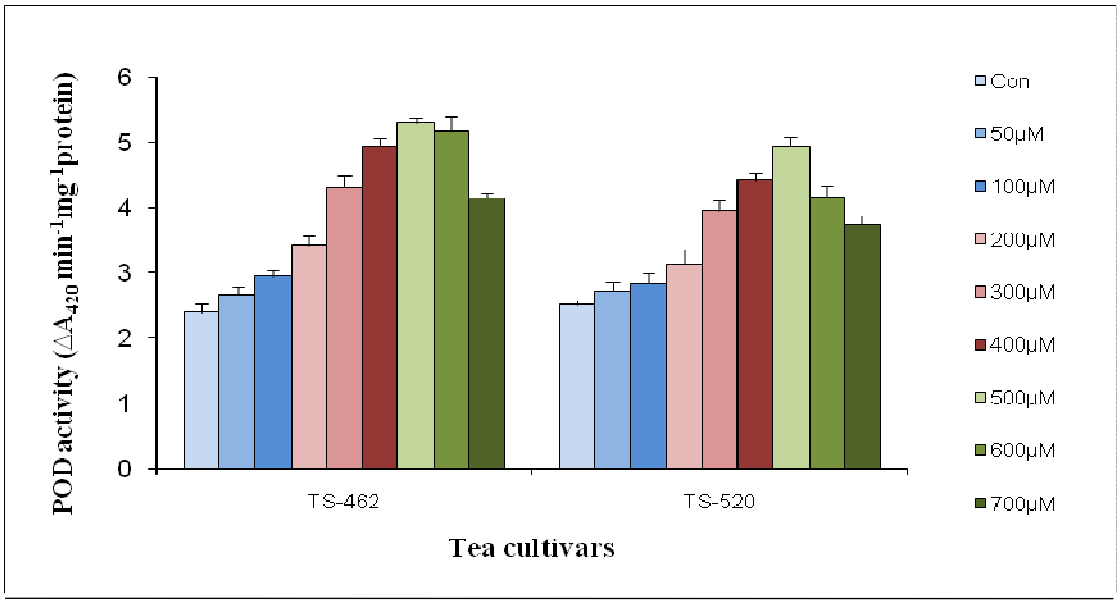

Fig. 5.19: Effect of increasing concentrations of copper (Cu^{2+}) on peroxidase (POD) activity in roots of two cultivars of tea (TS-462 and TS-520) on the 10th day of exposure. Values are mean of three replicates ±SE. Con = Control.

increase at similar time and dose. The activities declined at exposure concentrations higher than 500 µM in TS-462 and 400 µM in TS-520 (Fig. 5.20) but remained above control levels. Similar concentration dependent changes were noticed in the roots on the 10th day after start of treatment (Fig. 5.21).

5.2.10.4. Catalase

Catalase activity did not show significant change with change in Cu concentrations or with time ($P<0.05$) in the leaves or roots of tea seedlings of both tested cultivars (Tables 5.29 and 5.30). Further, the cultivars also did not show significant variation in CAT levels at all tested concentrations. Nevertheless, a slight increase was recorded in TS-462 cultivar at 50 µM concentrations when compared to control which did not show further change (Fig. 5.22). In roots, there was significant rise in CAT levels at 50 µM concentration in both cultivars but such change was not noted at other concentrations (Fig. 5.23).

Table 5.25: Effect of different concentration of copper on peroxidase activity in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Peroxidase activity (ΔA_{420} $min^{-1}mg^{-1}protein$)*		
	Incubation period (days)		
	4th	7th	10th
Control	3.4±0.35	3.6±0.42	3.7±0.32
50	4.2±0.65	4.5±0.32	5.4±0.55
100	4.4±0.44	5.1±0.64	5.6±0.44
200	4.9±0.26	5.8±0.29	6.7±0.75
300	5.1±0.70	6.1±0.74	6.8±0.60
400	5.6±0.46	6.8±0.49	7.7±0.53
500	5.6±0.41	7.1±0.47	7.8±0.54
600	6.0±0.50	7.3±0.53	7.9±0.43
700	5.2±0.48	6.4±0.52	6.3±0.62
CD (5%)	0.52	0.60	0.50

*Data are mean of three replications; Data after ± indicate standard error values.

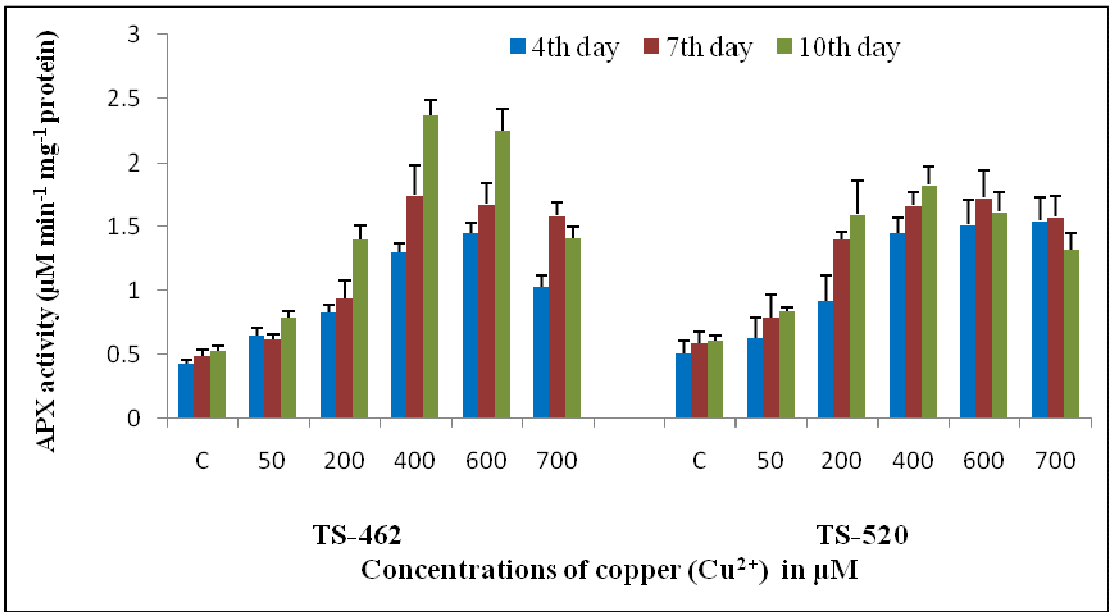

Fig. 5.20: Effect of increasing concentrations of copper (Cu^{2+}) on ascorbate peroxidase (APX) activity in leaves of tea (cultivars: TS-462 and TS-520) on the 4th, 7th and 10th day of exposure. Values are mean of three replicates ±SE. C = Control.

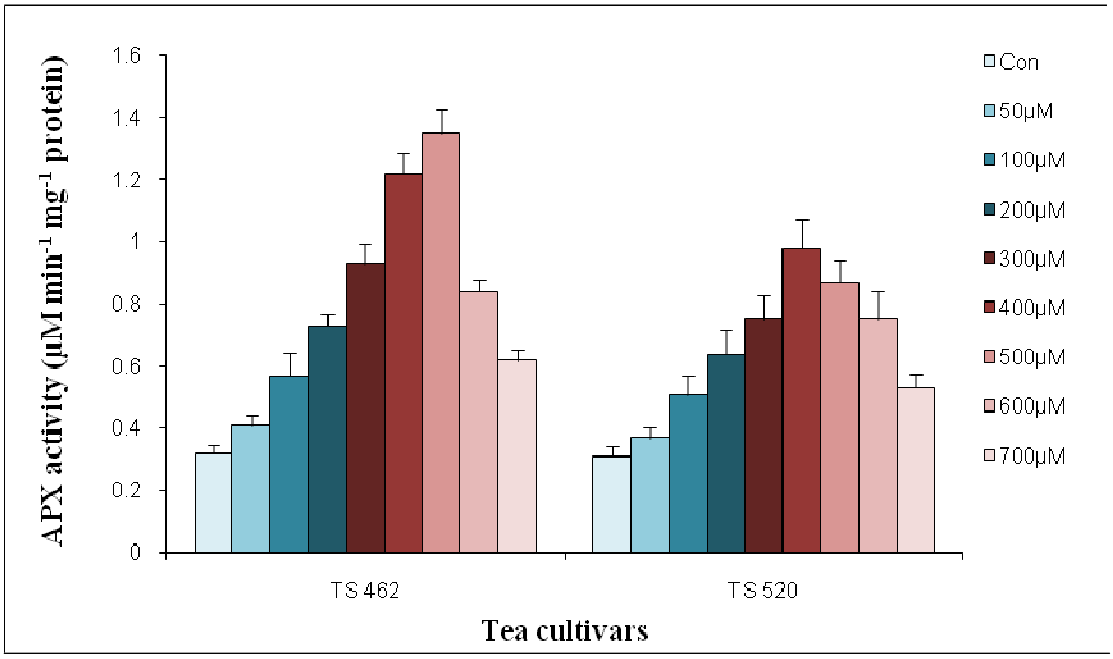

Fig. 5.21: Effect of increasing concentrations of copper (Cu^{2+}) on ascorbate peroxidase (APX) activity in roots of two cultivars tea (TS-462 and TS-520) on the 10th day of exposure. Values are mean of three replicates ±SE. Con = Control.

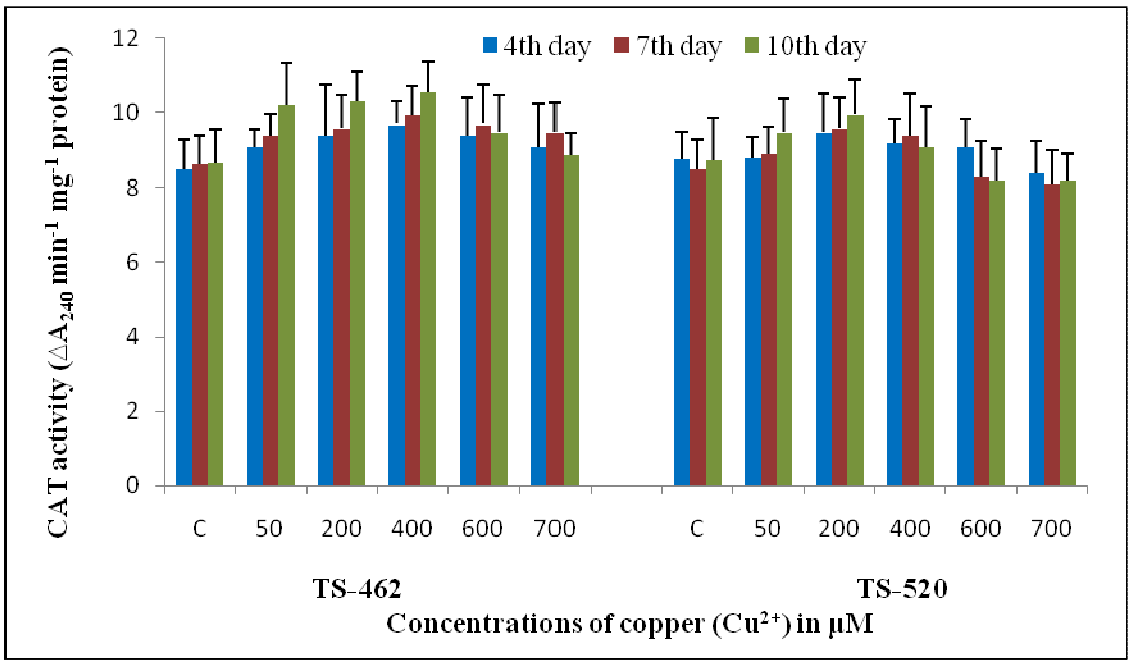

Fig. 5.22: Effect of increasing concentrations of copper (Cu^{2+}) on catalase (CAT) activity in leaves of two cultivars of tea (TS-462 and TS-520) on the 4th, 7th and 10th day of exposure. Values are mean of three replicates ±SE. C = Control.

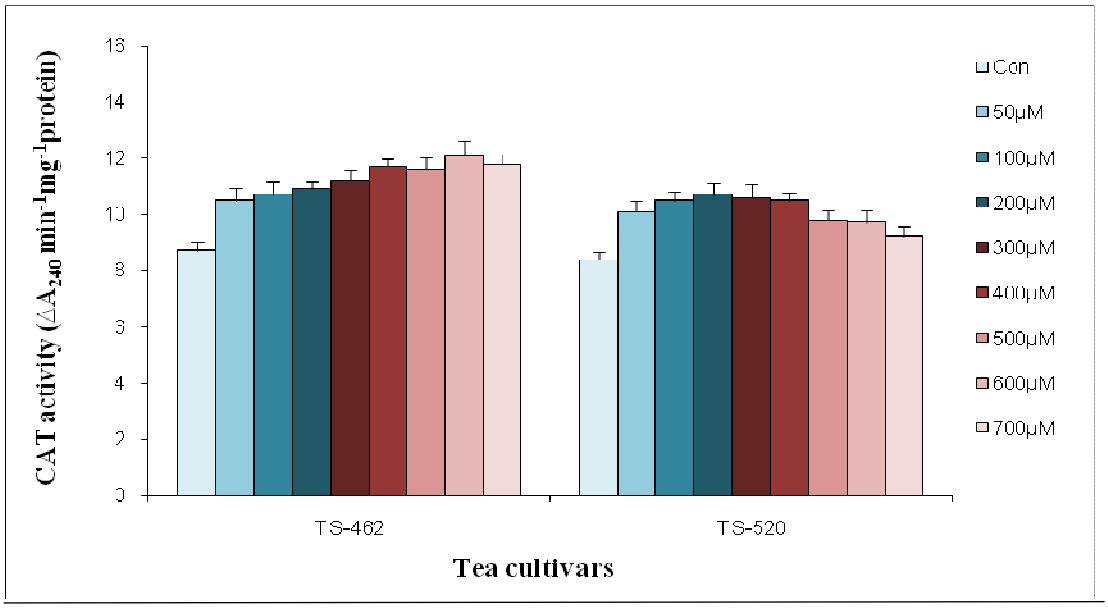

Fig. 5.23: Effect of increasing concentrations of copper (Cu^{2+}) on catalase (CAT) activity in roots of two cultivars of tea (TS-462 and TS-520) on the 10th day of exposure. Values are mean of three replicates ±SE. Con = Control.

Table 5.26: Effect of different concentration of copper on peroxidase activity in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Peroxidase activity (ΔA_{420} $min^{-1}mg^{-1}$protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	2.6 ±0.40	2.7±0.55	3.1±0.36
50	3.5 ±0.26	3.8± 0.36	4.6±0.44
100	3.8 ±0.35	4.2±0.70	5.3±0.59
200	4.5 ±0.69	5.7±0.25	6.4±0.20
300	4.9 ±.044	6.1±0.67	6.3±0.72
400	5.3 ±0.57	6.4±0.55	6.8±0.38
500	5.4 ±0.42	6.1±0.45	6.6±0.35
600	5.5 ±0.34	6.2±0.47	5.8±0.52
700	4.5 ±0.39	4.9±0.44	4.3±0.43
CD (5%)	0.51	0.48	0.26

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.27: Effect of different concentration of copper on ascorbate peroxidase activity in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Ascorbate peroxidase activity (µM min^{-1} mg^{-1} protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.43±0.03	0.49±0.05	0.53±0.04
50	0.65±0.06	0.62±0.04	0.79±0.05
100	0.75±0.05	0.86±0.07	0.95±0.12
200	0.83±0.06	0.94±0.14	1.40±0.12
300	0.97±0.14	1.02±0.11	1.57±0.14
400	1.30±0.07	1.75±0.23	2.37±0.12
500	1.47±0.12	1.69±0.14	2.39±0.13
600	1.45±0.08	1.68±0.16	2.25±0.17
700	1.03±0.09	1.59±0.10	1.41±0.09
CD (5%)	0.17	0.21	0.23

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.28: Effect of different concentration of copper on ascorbate peroxidase activity in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Ascorbate peroxidase activity (µM min^{-1} mg^{-1} protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	0.51±0.10	0.59±0.09	0.61±0.04
50	0.63±0.16	0.79±0.18	0.84±0.03
100	0.72±0.10	0.94±0.12	0.15±0.20
200	0.92±0.20	1.40±0.07	1.60±0.26
300	1.23±0.15	1.54±0.15	1.74±0.20
400	1.45±0.12	1.67±0.10	1.83±0.15
500	1.48±0.17	1.70±0.12	1.75±0.18
600	1.52±0.19	1.73±0.16	1.62±0.15
700	1.55±0.18	1.58±0.04	1.32±0.13
CD (5%)	0.23	0.26	0.32

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.29: Effect of different concentration of copper on catalase activity in leaf tissue of TS-462

Concentration of $CuSO_4$ (µM)	Catalase activity (ΔA_{240} $min^{-1}mg^{-1}$protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	8.5±0.8	8.6±0.8	8.7±0.9
50	9.1±0.5	9.4±0.6	10.2±1.0
100	9.2±1.0	9.5±1.0	10.24±1.0
200	9.3±1.0	9.6±0.9	10.3±0.8
300	9.6±1.0	9.8±1.0	10.4±0.5
400	9.7±0.6	9.9±0.8	10.5±0.8
500	9.5±0.8	9.7±1.0	9.8±0.85
600	9.4±1.0	9.7±1.0	9.5±1.0
700	9.1±1.0	9.5±0.8	8.9±0.6
CD (5%)	0.55	0.31	0.38

*Data are mean of three replications; Data after ± indicate standard error values.

Table 5.30: Effect of different concentration of copper on catalase activity in leaf tissue of TS-520

Concentration of $CuSO_4$ (µM)	Catalase activity (ΔA_{240} $min^{-1}mg^{-1}$protein)*		
	Incubation period (days)		
	4th	7th	10th
Control	8.8±0.7	8.5±0.8	8.76±1.0
50	8.83±0.5	8.94±0.7	9.5±0.9
100	9.3±0.9	9.5±1.0	9.8±1.0
200	9.5±1.0	9.6±0.8	9.94±0.95
300	9.7±0.9	10.2±1.0	10.3±0.95
400	9.2±0.6	9.4±1.0	9.1±1.0
500	9.2±0.6	9.0±1.0	8.8±1.0
600	9.1±0.7	8.3±1.0	8.2±0.9
700	8.4±0.9	8.1±0.7	8.2±0.7
CD (5%)	0.51	0.48	0.36

*Data are mean of three replications; Data after ± indicate standard error values.

5.3. DISCUSSION

Oxidative stress occurs in plants when they are exposed to adverse environmental conditions such as heavy metal stress. Copper is an essential micronutrient that is involved in the activity of several enzymes and is also a component of electron transport chain in chloroplast and mitochondria (Yruela *et al.*, 2000). Although Cu is usually present at low concentrations in surface waters, elevated concentrations of Cu may develop with time in agricultural soils due to several anthropogenic activities including prolonged use of copper containing pesticides to control diseases. At higher concentrations, Cu is highly toxic to plants as it can cause a range of morphological and physiological disorders (Vassilev *et al.,* 2003). Copper manifests its toxicity mainly through increased production of ROS which involves copper catalyzed Fenton reaction, characterized by production of hydroxyl radicals from superoxide and hydrogen peroxide. The ROS severely affects the biomolecules like proteins lipid and DNA, which induces significant alterations in plant physiology and biochemistry (Ducic and Polle, 2005). Changes occur as part of the tolerance mechanism which is an inherent capacity of the plants that enables to avoid excessive uptake of copper ions, detoxify copper ions by complexation and sequester ROS

through multiple regulated pathways. A clear understanding of cellular response to excess copper provides us the scope for deciphering the extent of cellular damage. Owing to the excessive use of copper fungicides in tea gardens, understanding of copper induced mechanism for selecting and/or developing tolerant cultivar with quality crop is an urgent need in modern tea industry.

The mechanisms of Cu tolerance in higher plants include two main strategies: (1) exclusion, whereby plants avoid excessive uptake and transport of metal ions, and (2) accumulation and sequestration, whereby plants detoxify free metals by compartmentation of metals in vacuoles, complexation of metal ions by organic ligands, such as organic acids, amino acids and metal-binding peptides (Clemens, 2001; Hall, 2002). There are, however, marked differences in Cu tolerance between plant species and between cultivars of the same species; these differences are often related to the copper content of roots and shoots (De Vos *et al.,* 1991; Rouphael *et al.,* 2008). In the present study, the tea cultivars showed some variation in Cu accumulation, TS-520 accumulating more in both leaves and roots at the highest tested concentration. The enhancement of Cu content in both the organs was proved to be dependent on the metal concentration in the nutrient solution. The roots retained most of the Cu taken up by the plants although a steady increment of copper content in leaves was noted with increase in the copper content in nutrient solution. The roots are in a direct contact with the metal-containing solutions and are known to act as storage for heavy metals and can restrict their translocation to the shoot system (Mazhoudi *et al.*, 1997). However, the increment of Cu content within the leaves of the tea plants indicated an efficient translocation. Presumably, Cu makes complexes with nicotinamine and is transported via xylem vessels into the shoot (Burkhead *et al.,* 2009). Peng *et al.* (2006) studied the accumulation of copper ions in *Elsholtzia splendens*, a native Chinese Cu-tolerant and accumulating plant species. Results showed that copper concentrations in roots, stems and leaves of *E. splendens* increased with increasing Cu levels in solution. After exposure to 500 µmol/L Cu for 8 days, about 1000 mg/kg Cu were accumulated in the stem and 250 mg/kg Cu in the leaf of *E. splendens*. Copper distribution in plant organs were high in root followed by stem and leaf. Ali *et al.* (2006) observed that roots of *Panax*

ginseng exposed to various concentrations of Cu (0, 5, 10, 25, and 50 µM) accumulated high amounts of Cu in a concentration-dependent and duration-dependent manner. In a study on five week old *Arabidopsis thaliana* plants, Lequeux *et al.* (2010) observed that copper was mainly retained in the roots in plants exposed to copper excess (5 µM). Copper levels in the shoots did not increase significantly with increasing copper concentrations. Eren *et al.* (2009) found that copper accumulation in cultured barley embryosis correlated with copper concentrations and higher accumulation was observed in roots compared to shoots. Yurekli and Porgali (2006) found that copper accumulated mainly in the roots of bean plants exposed to 100 µM Cu^{2+}. Feigl *et al.* (2013) studied the uptake and accumulation of Cu by Indian mustard (*Brassica juncea*) and rapeseed (*Brassica napus*) roots under Cu treatment. Both species showed similar accumulation rate. Cu was higher in the roots than in the shoots of both *Brassica* species.

The production of ROS is an unavoidable consequence of aerobic respiration. When the terminal oxidases - cytochrome C oxidase and the alternative oxidase react with O_2, four electrons are transferred and H_2O is released. However, occasionally O_2 can react with other ETC components. Here, only one electron is transferred, and the result is the O_2^- radical, a moderately reactive ROS with approximately 2-4 µs of half-life (Gill and Tuteja, 2010). It has been noted that O_2^- is usually the first ROS to be generated. About 1-2% of O_2 consumption within the plant tissues leads to the generation of O_2^- (Halliwell, 2006). Reduction of O_2 produces O_2^- during electron transport along the noncyclic pathway in the ETC of chloroplasts and other compartments of the plant cell. Reduction of O_2 to the O_2^- can occur in the ETC at the level of PSI. The generation of O_2^- may trigger the formation of more reactive ROS like OH^-, and more possibly $^1O_2$, each of which may cause peroxidation to membrane lipids and cellular weakening (Gill and Tuteja, 2010).

The peroxidation of lipids is considered as the most damaging process known to occur in every living organism. The overall process of lipid peroxidation involved three distinct stages: initiation, progression and termination steps. The initial phase of lipid peroxidation includes activation of O_2 which is rate limiting. O_2^- and OH^- can react with methylene groups of

polyunsaturated fatty acids forming conjugated dienes, lipid peroxy radicals and hydroperoxides. The peroxy radical formed is highly reactive and able to propagate the chain reaction. Peroxidation of polyunsaturated fatty acid by ROS attack can lead to chain breakage and, thereby, increase in membrane fluidity and permeability (Sharma *et al.*, 2012). Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction under various stresses. Now, it has been recognized that during lipid peroxidation, products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, malonaldehyde, etc. and compounds related to them (Gill and Tuteja, 2010; Sharma *et al.*, 2012). Some of these compounds react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS). Lipid peroxidation, in both cellular and organelle membranes, takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals.

In the current study, exposure to Cu resulted in accumulation of products of lipid peroxidation in the leaves. The level of TBARS increased steadily with Cu concentration and time of exposure in both cultivars 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) have reported similar increase in lipid peroxidation in plants exposed to Cu. A comparison of lipid peroxidation 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 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 in tea plants have been reported in response to drought stress (Upadhyaya and Panda, 2004) and to cadmium exposure (Mohanpuria *et al.,* 2007). However, there is no previous study on Cu toxicity in tea.

In the present study, assay of O_2^- release was done as a measurement of ROS production in response to excess copper. A dose dependent increase in

O_2^- production was observed in treated tea seedling cultivars in comparison to control. TS-520, which appeared to be more susceptible to copper excess than TS-460 as evident by higher TBARS accumulation, also showed a higher O_2^- level under excess copper. This showed that while ROS generation is taking place in both cultivars, TS-462 is better adapted to bear such stress. Upadhyaya *et al.* (2008) showed that O_2^- generation in four clonal cultivars of tea plants (TV-1, TV-20, TV-29 and TV-30) increased with increased imposition of drought stress. The authors further reported that an increase in O_2^- content and lipid peroxidation, as a consequent of stress imposition, was least in TV-1, which was therefore more tolerant in comparison with other tested clones. Mukhopadyay *et al.* (2013) reported the stimulation of O_2^- production and lipid peroxidation in tea plants (T-78) under Zn stress.

Lipid peroxidation and ROS generation have been reported to occur in response to excess copper in several plant species. In a study, *Solanum nigrum* plants were exposed for 28 days to 100 and 200 µM/L copper in a hydroponic system (Fidalgo *et al.*, 2013). The level of lipid peroxidation showed that the treatment of plants with 100 µM/L Cu did not change significantly in shoots when compared to control situation. However, about 33% increase in lipid peroxidation was found in shoots at 200 µM/L Cu. Hartley-Whitaker *et al.* (2001) observed that the level of TBARS increased with copper concentration in all four *Holcus lanatus* clones. However, the more copper-sensitive clones produced significantly higher concentrations of TBARS at all exposure concentrations. Drazkiewicz *et al.* (2004) found an elevated level of O_2^- content in leaves of *Arabidopsis thaliana* exposed to Cu excess (0-300 µM) for 7 days. Wang *et al.* (2004) found that exposure of *Brassica juncea* seedlings to excess Cu led to lipid peroxidation in roots which was enhanced with the increasing Cu applied. A peak was observed with Cu at 8 µM, with the lipid peroxides value being twofold higher than control. A further increase in Cu concentration to 16 µM failed to elevate the production of lipid peroxides. Additionally, a Cu-induced progressive increase in the lipid peroxidation level over the time was also observed but it declined after 6 days. In a study by Khatun *et al.* (2008), Indian ginseng (*Withania somnifera*), a medicinally important plant, was exposed to different concentrations of $CuSO_4$ (0, 10, 25, 50, 100 and 200 µM) for 30 d *in vitro*.

The authors observed that superoxide formation and lipid peroxidation increased significantly when concentrations were higher than 25 µM Cu. Ibrahim and Bafeel (2008) showed that lead toxicity increased lipid peroxidation and superoxide generation especially at higher exposure concentrations. Vestena *et al.* (2011) observed that superoxide concentrations increased, in addition to lipid peroxidation in the leaves of *Salvinia* and water hyacinth under Cadmium stress. Nie *et al.* (2012) also reported increase in lipid peroxidation and O_2^- production in leaves of maize variety Zhendan958 after the seedling was treated with 2 to 8 mM Cu for 96 h. Talukdar (2013) found that lipid peroxidation levels did not increase even at highest copper levels which allowed the *Canna indica* plants to overcome copper induced damage by oxidative stress.

At higher concentrations, copper is an effective inhibitor of photosynthesis for both algae and higher plants. The inhibitory effect of copper at different sites of the electron transport chain and on chloroplast lipid biosynthesis was studied by several authors (Droppa and Horvath, 1990; Baron *et al.*, 1995). Leaf chlorophyll concentration is crucial for the susceptibility of the plant's photosynthetic machinery to photo inhibition and should be considered when an effect of environmental stress is under study (Patsikka *et al.*, 2002). In the present study, chlorophyll a chlorophyll b and carotenoid content was found to decrease significantly with increasing Cu concentrations and exposure times in both the cultivars of tea with the more sensitive cultivar (TS-520) recording a significantly higher decrease ($P<0.05$). Studies have shown that carotenoid may play an important role in photoprotection of chlorophyll and chloroplasts against photooxidative damage (Gill and Tuteja, 2010). Therefore, the decrease in carotenoid content in the tea leaves may be responsible for the chlorophyll damage by the Cu treatments.

Tea plants have been known to be sensitive to heavy metal stress. In a study on heavy metal stress on tea plants, Mohanpuria *et al.* (2007) reported significant decrease in chlorophyll content even after 12 h of Cd (50 µM) treatment and it declined continuously with increase in time and concentration. In a similar study, Shi *et al.* (2009) found that chlorophyll contents were reduced in tea plants that were exposed to cadmium (0.50 mg/kg) or arsenic (50 mg/kg) at high soil concentrations. Yadav and

Mohanpuria (2009) observed that Cu and Al treatment (100 µM) decreased Chl a+b content by 8 % in Chinary and 18 % in Assamica cultivars of tea while Al treatment decreased chlorophyll content by 20 % in Chinary and 16 % in Assamica. In a study conducted on the seedlings of tea that were grown hydroponically for 30 d Li *et al.* (2011) observed that the chlorophyll content decreased with increasing, Fluorine concentrations. In another study, Mukhopadhyay *et al.* (2013) observed that Zn-stress (30 µM) decreased content of chlorophylls a and b and carotenoids in tea plantlets of cultivar T-78.

In general, decrease in chlorophyll concentrations under stress conditions in plants is regarded as direct indicator of injury to the plants. 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. The decline in chlorophyll content in plants exposed to heavy metals stress such us Cu is believed to be due to: (a) inhibition of enzymes associated with chlorophyll biosynthesis (John *et al.*, 2009); (b) inhibition of uptake and transportation of other metal elements such as Mn, Zn and Fe by antagonistic effects (Jayakumar *et al.*, 2009; John *et al.*, 2009). Similar decrease in chlorophyll content under heavy metal stress was reported earlier in cyanobacteria, unicellular chlorophytes (*Chlorella*), gymnosperms such as *Picea abies* and angiosperms such as *Zea mays*, *Quercus palustrus* and *Acer rubrum* (Siedlecka and Krupa, 1996). The decrease in chlorophyll content was also reported in sunflower (Zengin and Munzuroglu, 2006) and in almond (Elloumi *et al.*, 2007). In an investigation on *Ceratophyllum demersum* L. (Coontail), a free floating macrophyte, Rama Devi and Prasad (1998) observed that Cu uptake decreased the chlorophyll content in the plants. Ciscato *et al.* (1997) studied the effect of excess copper (3.6 µM/ Cu) on the photosynthetic apparatus of two cultivars of durum wheat *(Triticum durum*) and found that both chlorophyll *a* and *b* contents, expressed on a dry weight basis, were reduced. Patsikka *et al.*, (2002) found that Cu treatment lowered the leaf chlorophyll concentration in bean (*Phaseolus vulgaris*) plants grown in the presence of 0.3 (control), 4, or 15 µM Cu^{2+}. In contrast, Fidalgo *et al.* (2013) found that the total chlorophyll, as well as carotenoid content in shoots of *Solanum nigrum* plants exposed for 28 days to 100 and 200 µmol/L of Cu, did not present significant changes in

response to the Cu increase in the nutrient solution compared to shoots of the control plants.

Proteins are the most abundant cellular component oxidized by ROS constituting up to 68% of the oxidized molecules in the cell (Rinalducci *et al.*, 2008). Protein oxidative injury is a covalent modification induced by ROS generated under heavy metal stress. This refers to amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products and changes in electrical charge (Olteanu *et al.*, 2013). Protein oxidation mostly is irreversible, however, a few involving sulfur-containing amino acid are reversible (Ghezi and Bonetto, 2003). The most susceptible residues to oxidation are the sulphur containing cysteine and methionine residues (Boguszewska and Zagdanska, 2012). In some cases, oxidation of susceptible residues such as cysteine and histidine leads to the production of oxogroups which "mark" the protein macromolecules for an increased susceptibility to proteolysis (Davies, 2003).

In the present study, protein content decreased in a time and concentration dependent manner in comparison to control primarily at high exposure concentrations, that is, when the copper ions are in far excess. At lower copper levels, there was slight increase in the protein levels which was insignificant in the roots but significant in the leaves. A probable explanation for the increase in protein content could be the formation of carbonyl derivatives in presence of copper, or *de novo* synthesis of some stress proteins as result of exposure to exogenous factor (Verma and Dubey, 2003). The decline in soluble protein amount at high copper excess may be due to action of proteolytic enzymes or it may also be attributed to the loss of genetic material by chromosome fragmentation, micronuclei and lagging chromosomes, with repercussions on synthesis of proteins encoded by the genes lost in this way (Olteanu *et al.*, 2013). Among the two cultivars tested in this study, TS-520 recorded lower protein content than TS-462 especially in the leaves indicating that TS-520 is more vulnerable to copper excess.

Literature reports on the changes in protein content in response to stress are numerous and mostly report a decline in protein content with heavy metal excess but variations are also there. Additionally, varietal differences are also reported by several authors. Influence of Cd treatment on protein levels of

tea bud tissue was determined by Mohanpuria *et al.* (2007). Data obtained in their experiments indicated that decrease in protein levels were time dependent as there was not much decrease in protein levels after 12 and 24 h of 50–400 μM $CdCl_2$ treatments, while significant decrease in protein content was observed after 48 h even at 50 μM $CdCl_2$ exposures. The protein levels in bud tissues decreased from 12 mg/g FW to 9.2, 8.4, 7.5, and 6 mg/g FW after 48 h of 50, 100, 200, and 400 μM Cd treatments, respectively. In another study Yadav and Mohanpuria (2009) found that protein content decreased in apical buds of tea after exposure to excess Cu by 65 and 72 % in Chinary and Assamica types, respectively, as compared to control.

Zengin and Kirbag (2007) studied the effect of copper chloride (0.4, 0.5 and 0.6 mM) on the level of protein in sunflower (*Helianthus annuus*) seedlings under hydroponic conditions. The plants treated with high copper concentrations had significantly lower total protein contents than control plants. Total protein content in seedlings decreased, by 15%, 27.5% and 37.5% with increasing of Cu concentrations compared to the control seedlings. Similarly, Cu stress induced changes in the protein concentration of bean (Cuypers *et al.*, 2005). Guo *et al.* (2007) found that in comparison with the control, a significant reduction in protein content in roots and leaves was observed in 'Shang 70-119' genotype after the barley plants were exposed to excess metal solution in combination that included Cu, Al and Cd. The decrease was greater in roots than in leaves. Nevertheless, there was no significant difference in protein content both in roots and leaves among the treatments for 'Gebeina' genotype. Eren *et al.* (2009) found that total soluble protein content in cultured barley embryos decreased at high copper concentration in growth medium. Xing *et al.* (2010) observed that the synthesis of protein was inhibited significantly by excess copper in *Spirodela polyrrhiza*. Brahim and Mohamed (2011) found a reduction in leaf total soluble protein after 6 h of copper treatment in *Atriplex halimus* grown in hydroponics conditions (38% at 2000 μM). They further reported a recovery in protein biosynthesis after 24 h, which became more significant after 48 h (155% at 500 μM). Vinod *et al.* (2012) observed that protein content decreased with increasing Cu concentration in wheat plants exposed to high concentrations of copper. Olteanu *et al.* (2013) observed that there was no

direct relationship between copper concentration and protein level in *Triticum aestivum* seedlings. The authors reported that inhibition rates of protein synthesis ranged between 11 and 19%, except for 50 µM copper acetate and 100 µM copper citrate where small increases were noticed. The most important decline of soluble protein level was also registered in 25 µM copper citrate.

Carbohydrates are considered as an important macromolecule in plant metabolism not only because it is the first organic compound formed in the plants as a results of photosynthesis but also provide the major source of respiratory energy (Azmat and Riaz, 2012). Thus carbohydrate content in plant tissue directly reflects the energy level in the plants. Apart from the sugars and starches that meet this vital nutritional role, carbohydrates also serve as a structural material (cellulose), a component of the energy transport compound ATP, recognition sites on cell surfaces, and one of three essential components of DNA and RNA (Duffus and Duffus, 1984).

In the current study carbohydrate content was found to decrease in a time dependent and concentration dependent manner in both leaves and root of tea plants of both tested cultivars in response to copper excess. In our experiment a significant correlation was obtained between copper concentration and carbohydrate content suggesting that excess copper modified carbohydrate accumulation such that it was consumed in excess to maintain the basic physiological functions. Deef (2007) found that *Rosmarinus officinalis* subjected to low concentration of copper treatment exhibited an increase in the total carbohydrate values. However, the reverse was true at high concentration (3200 ppm) when the total carbohydrates decreased greatly as compared to the control. Xing *et al.* (2010) studied the effect of excess copper on the physiology of the aquatic plant *Spirodela polyrrhiza* which was exposed to three different concentrations (1, 10 and 100 mg L^{-1}). Results showed that the total carbohydrate content decreased significantly in the plant tissue after 24 h of treatment. Al-Hakimi and Hamada (2011) studied the physiological parameters in wheat seedlings under copper stress. It was clear from their results that the investigated levels of Cu that is, 20 and 40 mg/l generally induced an inhibitory effect on the accumulation of soluble and total carbohydrates in the roots but in shoots the accumulation of total carbohydrates was stimulated by all levels

of Cu. In a study to understand the effect of Cu on polymerization of glucose into carbohydrate under Cu stress in seedlings of 15 day old *Vigna radiata*, Azmat and Riaz (2012) observed a drastic decrease in carbohydrate contents which the authors related with Cu interference in polymerization of glucose in to carbohydrates. Deo and Nayak (2011) observed that copper exposure decreased carbohydrates content in *Musa acuminate* cv. Bantala grown *in vitro* at 100 µM of copper.

The proteinogenic amino acid, proline, is known to accumulate in many plants in response to environmental stress. Proline has long been considered an important compatible osmolyte, however, recent studies have proved its multiple functions in stress adaptation, recovery, and signaling (Gill and Tuteja, 2010). It is therefore being considered now as a potential nonenzymatic antioxidant. Proline is regarded as an important molecule in redox signaling and also a scavenger of ROS, which is capable of reducing the damage of oxidative stress induced by metal excess (Gill and Tuteja, 2010). Free proline accumulation has been observed in response to a wide range of biotic and abiotic stresses in plants (Ducic and Polle, 2005; Sharma and Dietz, 2006; Verbruggen and Hermans, 2008). It has also been found that overexpression of proline biosynthetic pathway genes enhance the abiotic stress tolerance in transgenic plants (Gill and Tuteja, 2010).

In the present study, proline levels increased steadily with increasing concentration of copper in the nutrient solution. However, at high exposure concentrations (> 500 µM), there was a marginal decrease in both cultivars. Proline accumulation is recognized as the first metabolic responses to stress which functions as an osmoregulator, stabilizer of protein synthesis, a metal chelator, and a hydroxyl radical scavenger (Gill and Tuteja, 2010). The proline content at high exposure concentrations was lower but only when compared to low exposure concentrations and not in comparison to control. This lowering may be due to the overall breakdown of biosynthesis and metabolic processes in the plants exposed to very high copper concentrations. However, at lower concentrations proline does accumulate in the tea leaves and possibly contributes to the alleviation of copper toxicity. Yadav and Mohanpuria (2009) observed that, excess Cu exposure in tea plants increased proline content by 80% in apical buds of Chinary and Assamica types. Thounaojam *et al.* (2012) investigated the effects of copper

in rice plants that were treated with different Cu concentrations (0, 10, 50 and 100 mM) for 5 days in hydroponic condition. Results showed that the proline content increased by 26.89%, 45.58% and 67.60% in shoot while 19.12%, 54.81% and 110.43% in root at 10, 50 and 100 mM Cu respectively with respect to control after 1st day of treatment. Almost similar pattern of enhancement in the proline content was recorded after 5th day where maximum content was recorded at 100 mM Cu by 98.95% in shoot and 106.49% in root over control. The effects of copper sulfate on *Plantago psyllium* was studied by Mohammadi *et al.* (2013) five levels (0, 15, 30, 45 and 60 mg/lit). The highest proline content was observed in 60 mg/lit copper sulfate concentration.

Karimi *et al.* (2012) studied the effect of different copper concentrations at toxic levels on *Astragalus neo-mobayenii*, an endemic plant around the Cu-rich areas. Copper was applied in four levels (0, 50, 100, and 150 μM). The proline content was found to increase substantially with increasing Cu concentrations. Fidalgo *et al.* (2013) exposed *Solanum nigrum* plants to 100 and 200 µmol/L copper in a hydroponic system for 28 days to analyze the antioxidant defence response. The authors observed that the shoot proline levels did not show significant change but in the roots proline content increased at high Cu concentrations. Rastgoo *et al.* (2014) studied the effects of heavy metals including copper on *Aeluropus littoralis*, a halophytic plant, using two different concentrations (50 µM and 100 µM) for each metal. The results revealed that proline content increased significantly with increasing concentration in all treatments.

Phenolics are an important group of secondary metabolites that are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. They are mainly synthesized from cinnamic acid, which is formed from phenylalanine by the action of L-phenyl-alanine ammonia-lyase, the branch point enzyme between primary (shikimate pathway) and secondary (phenylopropanoid) metabolism (Dixon and Paiva, 1995; Michalak, 2006). Phenolics have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions. An increase of phenolics correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was

reported, suggesting *de novo* synthesis of phenolics under heavy metal stress. In contrast, some evidence indicates that the increase in flavonoid concentration is mainly the result of conjugate hydrolysis and not due to *de novo* biosynthesis (Parry *et al.,* 1994; Michalak, 2006). Increase in soluble phenolics such as intermediates in lignin biosynthesis can cause an increase in cell wall endurance and the creation of physical barriers preventing calls against harmful action of heavy metals which is the normal characteristic structural change induced by all kinds of stress (Diaz *et al.,* 2001).

Since polyphenols are major constituents of tea leaves, their involvement in the defence response mechanism against ROS induced damage caused by metal excess seemed highly probable. To explore this possibility, the level of phenolics in leaves of two cultivars of tea was estimated. Both phenolics and o-dihydroxyphenolics content increased with increase in concentration of copper in the nutrient solution. Orthodihydroxyphenols are mainly considered to play a significant role during biotic stresses (Bhagat and Chakraborty, 2010; Michalak, 2006). Some reports (Chakraborty *et al.,* 2002) also suggest its role in abiotic stresses. In a study on drought induced stress in tea plants, Chakraborty *et al.* (2002) observed that the contents of both total and *o*-dihydroxyphenols increased under low stress in the leaves of tea clonal cultivars but declined when the stress conditions were acute. Mukhopadhyay *et al.* (2013) treated young tea plants with zinc excess (30 µM) and found that the plants showed considerable increase in phenol content in both leaves and roots. Upadhyaya (2012) studied the effect of low temperature stress in five selected clones of tea. Total phenolic content in low temperature treated clones, TV-17, TV-19, TV-25 and TV-29 was found to be highest at 20ºC, but TV-30 showed low content of the same at 20ºC, which is only 0.75 fold and highest content at 30 µM. The author suggested that TV-30 is low temperature sensitive with respect to phenol metabolism.

Khatun *et al.* (2008) found that *Withania somnifera* plants exposed to different concentrations of $CuSO_4$ (0, 10, 25, 50, 100 and 200 µM) for 30 d *in vitro* showed an increase in the level of total phenols in metal-treated plants compared to the control. In a study on physiological responses of *Matricaria chamomilla* plants exposed to copper excess (3, 60, and 120 µM for 7 days), Kovacik *et al.* (2008) observed that Cu doses of 60 and 120µM led to increase in soluble phenolics in the leaf by 12 and 16%. Posmyk *et al.* (2009) found

that Cu^{2+} excess at low doses (0.5 mM), increased the levels of phenolic compounds in red cabbage. Vinod *et al.* (2012) observed that high concentration of Cu markedly increased the phenolic content of wheat leaves. The result of the present work is therefore in agreement with the finding of other workers and suggests that phenolics may play an important role in tea plants under copper stress.

Plants produce non-protein thiol compounds, comprised of several acid-soluble sulfohydryl components, such as cysteine, reduced form of glutathione (GSH) and phytochelatins, for detoxification or homeostasis of heavy metals (Cobbett, 2000). The process of detoxification includes formation of thiolate complex with GSH and cysteine, forming metal chelate complexes with phytochelatins and reducing their harmful activities. GSH can also alleviate oxidative stress with its strong antioxidative properties. Glutathione is the basic substrate for phytochelatin formation which consists of the three amino acids; cysteine, glycine and glutamic acid.

In the present study, non-protein thiol content showed an increase with time upto 7^{th} day after which, it declined at all exposed concentrations in both root and leaf when compared to control. Although there was a rise in NPT levels with increasing copper concentration at lower Cu levels; at higher levels, that is beyond 400 µM, the NPT contents decreased drastically especially on the 10^{th} day. Copper is bound as Cu^{+} in phytochelatins, and the stability of the complex depends on the chain length of the phytochelatins (Mehra and Winge, 1988; Reese *et al.*, 1988; De Vos *et al.*, 1992). Thus low amount of copper may induce synthesis of phytochelatins in tea plants. However, since total NPT levels was recorded during this study, the increase may also be due to increased production of GSH or cysteine, both of which are known to play major role in alleviating metal toxicity in plants (Yadav, 2010). Further studies on the individual levels of the different thiols produced under copper excess in tea plants should be of interest. The pattern of change in NPT levels in this study was found to be similar in root and leaf. However, a direct comparison between the root and leaf NPT levels could not be computed because they were expressed in separate units. This was due to the fact that the root tissue could not be homogenized appropriately in fresh condition, thus a dry weight was taken for the roots after lyophilization. Among the two tested cultivars, TS-462, that is the more

resistant one showed lower levels of NPT than TS-520. De-Vos *et al.* (1992) observed that both the rate of phytochelatin production upon an excessive supply of copper and the maximum level of phytochelatin-SH were higher in the roots of copper-sensitive *S. cucubalus* than in the roots of tolerant plants. According to the authors, this result indicated that copper tolerance in this species is not based on an elevated production of phytochelatins. They further suggested that phytochelatin synthesis cannot prevent copper toxicity and phytochelatins, regardless of their chain length, are not primarily involved in copper tolerance. In our work, tea plants of the more susceptible variety showed higher NPT levels. Thus there may be a possibility that apart from glutathione and cysteine which might have a role in mitigating oxidative stress, phytochelatins do not have any significant role in Cu excess physiology but this can only be ascertained after testing the different NPT compounds individually.

Murphy and Taiz (1995) observed that the correlation between copper tolerance and non-protein thiol levels in the 10 *Arabidopsis* ecotypes was not statistically significant. Nagalakshmi and Prasad (2001) observed that the non-protein thiol content in copper-treated *Scenedesmus bijugatus* cells decreased with increase in copper concentrations (0-150 µM). However, the total thiol content remained unaffected. The authors suggested that this shows the increase in protein thiol content and served as a marker for the synthesis of metal-binding peptides, phytochelatins, which are reported to have a role in sequestration and metal homoeostasis. Wang *et al.* (2004) studied the antioxidative reactions induced by copper excess (8 µM) in the roots of *Brassica juncea*. The authors reported that the total non-protein thiol content increased during the first eight days, but it declined at later stages. The response of the non-protein thiols to copper treatment was investigated in the marine diatom *Phaeodactylum tricornutum* by Morelli and Scarano (2004) by monitoring the cellular levels of phytochelatins (PCs), glutathione, γ-glutamyl cysteine and cysteine. The authors reported a rapid induction of the synthesis of PCs initially formed at the expense of the cellular pool of glutathione. Afterwards, cellular total non-protein thiols (the sum of γ-glutamyl cysteine, glutathione and PCs) increased with respect to the control. The authors suggested that as the PCs synthesis requires only glutathione as substrate, the accumulation of these peptides in the cell

indicated that Cu actively stimulated new synthesis of glutathione. In another study, Demirevska-Kepova *et al.* (2004) observed that the NPT levels decreased in the barley leaves at lower doses of copper but increased several folds at higher doses when compared to control. Srivastava *et al.* (2006) studied the responses induced by lower as well as higher doses of copper (0.1–25 μM) in an aquatic macrophyte, *Hydrilla verticillata* for a period of 1–7 days. Total non-protein thiols and cysteine levels increased significantly up to 5 μM copper exposures while at 25 μM, their level declined drastically. Kalinowska and Pawlik-Skowronska (2010) observed that low intracellular Cu-accumulation and maintenance of high GSH level concomitant with PCs production seem to be responsible for a higher Cu-resistance of the terrestrial green microalga *Stichococcus minor* and *Geminella terricola*.

The delicate equilibrium between generation and detoxification of ROS in the plant cell is disrupted by various abiotic stresses. As plants cannot escape exposure to adverse conditions, the well regulated array of antioxidant defence mechanisms keep the routinely formed ROS levels under control and prevent them from exceeding toxic thresholds. The antioxidant network consists of several enzymatic components that includes superoxide dismutase (SOD) which provides the first line of defence against ROS by scavenging O_2^- and ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT) that are predominantly associated with H_2O_2 decomposition and helps to maintain the cellular redox steady state. A literature study shows that the involvement of antioxidant enzymes in limitation of ROS production has been examined in tea cultivars against several different stress factors including drought stress (Chakraborty *et al.*, 2002; Damayanthi *et al.*, 2010), cold stress (Upadhyaya, 2012), cadmium stress (Mohanpuria *et al.*, 2007; Shi *et al.*, 2009), aluminium stress (Ghanati *et al.*, 2005), fluorine excess (Li *et al.*, 2011), boron deficiency (Hajiboland and Bastani, 2012), zinc deficiency and excess (Mukhopadhyay *et al.*, 2013), and copper excess (Basak *et al.*, 2001; Yadav and Mohanpuria, 2009). Additionally, investigations have been conducted on whether the ROS scavenging antioxidant enzymes maybe critical for protecting plants against metal deficiency in the soil and/or whether they may be responsible for the timely activation of the defence response.

In the present study, 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 leveled 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. A rise in SOD activity has been observed in several plants in response to copper stress. Hartley-Whitaker *et al.* (2001) observed that SOD activity increased with copper concentration in the four *Holcus lanatus* clones tested; the more tolerant clones showing a lower rise than the non-tolerant clones. Wang *et al.* (2004) observed a twofold increase in SOD activities with 8 µM Cu in the roots of *Brassica juncea* L. during the first 2 days. The stimulation lasted for 4 days and then gradually declined. Srivastava *et al.* (2006) showed that the activity of SOD increased up to 5 µM copper exposure till day 7 in a concentration–time dependent manner in an aquatic macrophyte, *Hydrilla verticillata.* Ke *et al.* (2007) showed that at 100 mg kg^{-1} Cu treatment, the SOD activity increased in the two *Daucus carota* populations and then, decreased gradually with the increase of Cu concentration in the soil. The authors found that the decrease of SOD activity differed among the populations. Gao *et al.* (2008) observed that under copper stress, SOD activity in leaves of *Jatropha curcas* seedlings increased concomitantly with increasing copper up to 400 µmol in pot experiments. However, SOD activity in stems and roots showed a slight increase. Karimi *et al.* (2012) investigated the effect of copper on *Astragalus neo-mobayenii*, an endemic plant around the Cu-rich areas and observed that with increasing copper concentrations, SOD activities increased significantly in leaves and roots compared with that of the control group. On the other hand, activity of SOD did not significantly increase in *Triticum aestivum* cv. Hasaawi at most Cu^{2+} concentrations, as compared with control when grown in various copper levels (0-100 mM) for 30 days (Azooz *et al.*, 2012). Rastgoo *et al.* (2014) observed that SOD activity increased under excess copper conditions (50 µM and 100 µM) in *Aeluropus littoralis* in all treatments.

Peroxidase activity increased with Cu concentration to more than two fold in both the tested cultivars of tea during the present study. TS-520 recorded a

significantly lower POD activity than TS-462 ($P<0.05$). 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. Cuypers *et al.* (2002) studied the contribution of peroxidases in roots and primary leaves of bean seedlings as a part of the defence mechanism against copper imposed oxidative stress. After application of 15 µmol/L Cu to the nutrient solution, the enzyme activities of the roots increased from 24 h onwards. However, a decrease in the GPOD capacity was noticed 96 h after the start of the treatment. In the leaves, the activities were significantly increased 24 h after the application of 50 µmol/L Cu to the nutrient solution and a stronger increase was observed after 96 h. Zhao *et al.* (2010) observed that POD activity increased at low Cu level and decreased at high Cu level in *Festuca arundinacea* L. roots under Cu stress in hydroponic experiments. Karimi *et al.* (2012) observed that with increasing copper concentrations, POD activities increased significantly in leaves and roots of *Astragalus neo-mobayenii*. Olteanu *et al.* (2013) found 70.51% increase in POD activity when 25 µM copper citrate was applied in *Triticum aestivum* seedlings. Liu *et al.* (2014) worked on copper stress on maize seedlings, and observed that POD activity was increased when the concentration of copper was higher than 10 µmol/L. Martins *et al.* (2014) evaluated the response of tobacco plants genetically engineered with the AtTPS1 (trehalose-6-phosphate synthase) gene, known to improve plant tolerance, from *Arabidopsis* to stress induced by excess Cu. Two transgenic lines, transformed with the AtTPS1, with different levels of trehalose-6-phosphate synthase expression (B5H, higher and B1F, lower), and a wild type (WT) were investigated. When plants were grown under high Cu concentrations (400 µM) in hydroponic solution, POD activity in WT and B5H plants increased significantly after a 48 h exposure.

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. Gupta *et al.* (1999) also observed a late increase in APX activity in *Pheseolus vulgaris* subjected to Cu stress. Chen *et al.* (2000) reported that APX activity showed an increase in copper stressed roots of rice seedlings. Ratkevicius *et al.* (2003) observed strong activation of APX in *Enteromorpha compressa*, an alga collected from copper enriched environment but no activation was seen in the same algae from non-impacted sites. In a study in *Zea mays* plants, Kumar *et al.* (2008) reported that the activity of APX was increased enormously at all the stages under study, after excess-Cu (100 µM) treatment. Wang *et al.* (2011) found that in comparison to control, activity of APX increased about 46.30% and 51.85%, respectively, in roots of maize seedlings under 2 and 4 mM and then declined to control levels. In a study on copper stress in *Triticum aestivum* cv. Hasaawi, Azooz *et al.* (2012) showed that APX levels increased at copper concentrations above 10 mM. Fidalgo *et al.* (2013) observed that APX activity was decreased in shoots of *Solanum nigrum* plants that were exposed for 28 days to 100 and 200 µmol/L copper in a hydroponic system. Total APX activity was lower in Cu-exposed plants than in control ones reaching a significant reduction of about 36% at 200µmol/L Cu. A similar effect was detected regarding related mRNA accumulation in roots and shoots of the Cu-treated plants.

CAT activity showed an insignificant increase in both the cultivars of tea during the present study. Moreover, no significant difference was observed between the cultivars exposed to excess Cu. Thus CAT activity remained unaltered or was marginally increased in response to oxidative damage induced by Cu. Reports by other authors showed variation in catalase activity in different plants in response to copper excess. Chen *et al.* (2000) reported that excess copper sulphate had no effect on CAT activity in the roots of rice seedlings. In another study, Thounaojam *et al.* (2012) showed that CAT activity did not show significant variation both in shoot and root of the rice plants exposed to excess copper. However, Lombardi and Sebastiani (2005) reported that total catalase in *Prunus cerasifera* plantlets grown on 100 µM Cu for 10 and 20 days was approximately five times greater than the control plantlets. Pourakbar *et al.* (2007) observed that CAT activity increased only slightly in maize seedlings exposed to excess copper for 15

days. In a similar study, Kumar *et al.* (2008) reported that the activity of CAT was not altered significantly by excess supply of Cu to maize plants. Iseri *et al.* (2011) observed that catalase activity did not change in the tomato seedlings exposed to high copper concentrations but recorded enormous increase in cucumber seedlings under similar conditions. In a study on seeds and germinated seedlings of *Vigna mungo*, Solanki *et al.* (2011) observed a decline in CAT activity under copper stress. Rout *et al.* (2013) observed that with increasing Cu concentration (up to 50 µM), a sharp increment of CAT activity was observed in root and shoot of 7 days treated *Withania somnifera* L. seedlings which was 2-folds more in comparison to control. But in 14 days old plants, the maximum activity was observed at 25 µM of Cu in leaf and root tissues, which was 2.66 and 2.81-folds over the control but then declined. Talukdar (2013) observed that CAT levels did not vary in the leaves of *Canna indica* plants grown in nutrient solution containing excess copper in comparison to control but increased significantly in the roots.

From the results of the present study it seems most likely that Cu induces oxidative damage in tea leaves leading to the formation of ROS. This in turn caused an induction of enzymes involved in the scavenging of superoxide radical and H_2O_2. But when the ROS production was very high, it exceeded the endogenous capacity of the plant to scavenge the ROS which upset the regulated balance between the scavenging system and the generating system leading to the inactivation of defence enzymes (Shigeoka *et al.*, 2002). In addition, our results showed a significant difference in the two Cu stressed cultivars of tea where the more sensitive cultivar seems to lose its antioxidative capacity at Cu concentrations higher than 400 µM while the more tolerant cultivar being able to withstand a maximum of 600 µM of Cu^{2+} ions. In order to evaluate the oxidative damage, further studies on the zymogram pattern of the antioxidative enzymes assayed in this study along with localization of peroxidase enzyme in both leaves and roots was conducted.