CHAPTER-6

ISOZYME PROFILE OF ANTIOXIDATIVE ENZYMES AND CELLULAR LOCALIZATION OF PEROXIDASE AND ACCUMULATED COPPER IN TEA SEEDLINGS

CHAPTER-6

ISOZYME PROFILES OF ANTIOXIDATIVE ENZYMES AND CELLULAR LOCALIZATION OF PEROXIDASE AND ACCUMULATED COPPER IN TEA SEEDLINGS

In order to tolerate environmental fluctuations, plant metabolism must be flexible and dynamic. However, the metabolic balance of cells is often disrupted by biotic and abiotic stresses, resulting in accumulation of ROS and oxidative burst (Gill and Tuteja, 2010). ROS are produced as unavoidable by products of aerobic metabolism. When present in optimal concentrations, ROS play crucial role in the normal development and response of the plant to the environmental stresses. The lifespan of ROS within the cellular environment is determined by numerous antioxidative systems, which provide crucial protection against oxidative stress imposed by these molecules. A variety of gene and protein expression signatures are involved in ROS generation and inactivation which is a complex protective metabolic adaptation that alter physiological reactions in the whole plant.

Isozymes or isoenzymes, are enzymes that catalyze the same reaction, but exist in multiple molecular forms, possess different properties, and show different tissue distributions. Isozymes are the different gene products. They are usually recognized by the different electrophoretic mobilities they possess. Anti-oxidative enzyme isozymes have a number of roles in the growth and development of plants. Isozyme analysis of some ROS scavenging enzymes in plants exposed to different levels of metal stress might throw light on the physiological, biochemical and genetic changes that accompany adaptive metabolism against such stress. Thus, changes in activities of some antioxidant enzymes during copper stress were monitored.

Apart from the biosynthetic and scavenging functions carried out by the different isozymes, there is little information available concerning their precise localization on the organ tissue at cellular or sub-cellular level. To distinguish between several isoenzymes and to identify peroxidases involved in a specific stress response, proteomic approaches are state of the art (Meisrimler *et al.*, 2014). Since plant peroxidases are highly polymorphic, enzyme cytochemical probes are insufficient in most cases to establish the exact cellular localization of the particular isozyme. Therefore most

appealing approach for such study is to employ immunological assays (Repka *et al.*, 1997). The use of polyclonal and monoclonal antibodies and immunocytochemistry has allowed the detection and localization of enzymes such as peroxidase within cells in the tissue sections.

For cellular location of different proteins or antigens, immunogold labeling followed by electron microscopy is a powerful tool (Lee *et al.*, 2000; Trillas *et al.*, 2000; Nahalkova *et al.*, 2001). To visualize immunogold labels in light microscope, silver enhancement is essential. Colloidal gold labels are normally visible only at electron microscope level. Silver enhancer enhances the colloidal gold label by precipitation of metallic silver to give a high contrast signal visible under light microscope. Fluorescent antibody labelling with fluorescein isothiocyanate (FITC) is also known to be one of the powerful techniques to determine the cell or tissue location of antigens or proteins. However, autofluorescence present in the plant tissues may mislead the proper understanding of the actual cellular location of proteins. Moreover, a fluorescence microscope is more expensive than light microscope. Hence it was considered worthwhile to perform immunogold labeling followed by silver enhancement for tissue location of peroxidase under light microscope.

Generally, metals are absorbed by roots in cationic form from the soil water or nutrient media where they are cultured by a non-metabolic passive process driven by diffusion or mass flow (Marschner, 1995). The metal cations are first taken into the apoplast of the roots. Then some of the total amount of the metal is transported further into the cells some is transported further in the apoplast, and some become bound to cell wall substances. How the total amount of the metal taken up is distributed between these three depends on the metal and the plant genotype as well as on external factors (Greger, 2004). Since, during our early studies, we observed that copper is mainly accumulated in the roots and very little is transported to the leaves of the tea seedlings, it seemed worthwhile to study the cellular location of copper in the root by transmission electron microscopy. Also a study has been conducted on the isozyme profiling of the antioxidative enzymes to support the observations in previous studies. Further peroxidise expression was studied by immunoblotting and immunocytochemical detection of peroxidase in roots was done to confirm the findings.

6.1. MATERIALS AND METHODS

6.1.1. Stress induction in hydroponic cultures

Three month old tea seedlings of cultivar TS-462 was exposed to different concentrations of copper sulphate (200 µM, 500 µM and 700 µM) under hydroponic culture system as described in section 5.1.2. Control sets comprised of only nutrient solution without excess copper sulphate.

6.1.2. Polyacrylamide gel electrophoresis (PAGE) and in-gel enzyme assay

Native polyacrylamide gel electrophoresis (PAGE) was carried out for analysis of isoforms of the different enzymes under study. For studying the isozyme patterns, leaf samples were collected from treated and control tea-seedlings under experiment in hydroponic system. Total soluble proteins or specific enzyme extracts of the sampled leaves were separated on non-denaturing gels. These gels were subjected to biochemical treatments specific for a particular enzyme for the development of coloured isoform bands. Protein was estimated following Lowry's method (Lowry *et al.*, 1951) using BSA as standard. GelQuant.NET software provided by biochemlabsolutions.com was used for densitometric analysis of the bands produced in the gels following activity staining.

6.1.2.1 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was done following the method as described by Davis (1964) with some modifications. A mini slab gel (8 X 5 cm) was prepared for which, two glass plates ware thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with 1% agar solution. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. A 10% resolving gel was prepared by mixing the acrylamide stock solution (for resolving gel), Tris-HCl (pH 8.9, for resolving gel), APS (freshly prepared) and distilled water in the ratio 1:1:4:1 and carefully dispensing the mixture by a pasture pipette between the glass slabs leaving sufficient space for the stacking gel (1.5 cm). After pouring the resolving gel solution, it was immediately over layered with water and allowed to polymerize for 1.5 - 2 h. After polymerization was complete, the

water over layer was poured off and the gel was washed with water to remove any unpolymerized acrylamide.

The stacking gel (4%) solution was prepared by mixing the acrylamide stock solution (for stacking gel), TrisHCl (pH 6.7, for stacking gel), riboflavin (freshly prepared) and distilled water in the ratio 2:1:1:4. The mixture was poured over the resolving gel and the comb was inserted leaving a gap of approximately one cm below the well in the stacking gel. The gel was kept under bright sunlight or fluorescent light for polymerization. After solidification of the stacking gel, the comb was removed and the wells were washed thoroughly. Following casting, the gel with the glass slabs was fitted into the electrophoresis apparatus. Chilled tris-glycine running buffer (pH 8.4) was added sufficiently in both upper and lower reservoirs of the gel apparatus. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe. The gel was loaded with protein/enzyme samples and incubated at 4^0C supplying a constant current of 2.5 mA per well continuously for 3-4 h until the dye front reached the bottom of the gel. After electrophoresis, the gel was removed from the glass plates and then the stacking gel was cut off from the resolving gel and finally processed for activity staining of the antioxidative enzymes.

6.1.2.2. Sample preparation and activity staining

6.1.2.2.1. Superoxide dismutase

Superoxide dismutase extraction was done following the method of Burke and Oliver (1992). Fresh tea leaves (1 g) were taken from the plants of experimental jars (treated and control) and instantly dipped in liquid nitrogen. After 10 min, the frozen leaves were crushed in 5 ml buffer (containing 96 mM Tris-HCL, pH 6.8, and 13.6% v/v glycerol). The homogenate was then filtered through four-layered muslin cloth and the filtrate was centrifuged at 14,000 rpm for 15min. The supernatants were collected and protein concentration was measured. Samples (25 µg protein) were loaded for PAGE analysis at 4°C. Following electrophoresis, the gels were incubated in 50 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.2% v/v N,N,N',N'-tetramethylethylenediamine, 3 mM riboflavin, and 0.25 mM nitrobluetetrazolium in the dark at room temperature for 30 min with agitation. Following incubation, the gels were rinsed with and placed in

deionised water and exposed to light under a 400-W high-pressure sodium lamp 60 cm above the gel, generating high levels of 300 µE m^{-2} s^{-1} for 10 min at 25°C. White bands appeared in purple background gel.

6.1.2.2.2. Peroxidase

For studying the different forms of POD isozymes, total soluble proteins were extracted as described (Sadasivam and Manickam, 1996) and loaded (25 µg) onto the wells of non-denaturing gels and electrophoresed at 4°C. To determine POD activity, the gels were incubated for 30 min in 0.25% guaiacol solution and then in 0.3% H_2O_2 for 15 min for the development of reddish brown bands.

6.1.2.2.3. Ascorbate peroxidase

Fresh leaves (0.5 g) sampled from treated and control sets were homogenized in 5ml of 50 mM ice-cold phosphate buffer (pH 7.0, containing 1mM ascorbate, 1mM EDTA and 2% w/v PVP). The homogenates were centrifuged at 13000 g at 4°C for 20 minutes. The supernatants were ready to use for enzyme activity assay following protein quantification. The enzyme samples were loaded on native PAGE gels and processed after electrophoresis according to the method of Rao *et al.* (1996). The gels were prerun for 30 min with addition of ascorbate (2 mM) in the carrier buffer since APX is stable only in presence of ascorbate. After electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. The gels were again incubated in the above buffer containing 4 mM ascorbate and 2 mM H_2O_2 for another 20 min. After a brief wash in the buffer for 1 min, the gel was submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM tetramethylethylenediamine and 2.45 mM nitrobluetetrazolium with gentle agitation. The reaction was continued for 10 to 15 min and stopped by washing with distilled water. White bands appeared in blue background.

6.1.2.2.4. Catalase

Catalase isoform were detected by native polyacrylamide gel electrophoresis following the method described by Keissar *et al.*, (2002). The enzyme samples were prepared as described earlier (section 5.1.12.4) and loaded onto 10% polyacrylamide gels after protein estimation. After electrophoresis, gels were

incubated in 10mM hydrogen peroxide solution for 10 minute and stained with a mixture of 1% ferric chloride and 1% potassium ferricyanide in distilled water and shaken gently until achromatic bands were developed. The catalase activity was revealed as light zones on a deep green background.

6.1.3. Electrophoresis and Western blotting

To understand the differential expression of proteins in copper treated and control seedlings, analysis of crude protein extract was first carried out on 10% SDS-PAGE gels following Laemmli's (1970) method. Leaf samples were collected from the experimental and control jars, washed thoroughly with cold water and instantly dipped in liquid nitrogen. After 10 min, the frozen leaves were crushed in 5 ml buffer (containing 0.1 M Tris-HCL, pH 6.8, and 13.6% v/v glycerol) and centrifuged at 14,000 rpm for 15min. The homogenate was then filtered through four-layered muslin cloth and the filtrate was centrifuged at 14,000 rpm for 15min. Protein was extracted (Sadasivam and Manickam, 1996) from the supernatants and the concentration was measured by Lowry's method (Lowry *et al.*, 1951) using BSA as standard.

6.1.3.1. SDS-PAGE

Leaf and root sample containing 50µg protein were mixed with equal volume of sample buffer and heated for 5 min at 95^0C. Gel was made according to the method of Laemmli (1970). Separating gel (10%) was used for resolving the polypeptides whereas a 4% stacking gel was used to concentrate the polypeptides. Protein as well as standard markers were loaded on the gel and electrophoresis was accomplished in electrode buffer at 30mA for 4h using a Bio-Rad, Mini PROTEAN Tetra Cell Electrophoresis system. Following electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) for overnight and destained. The gel was photographed by Sony cyber-shot digital camera. The standard proteins marker (97.4 kDa-16 kDa) (Bangalore Genei, India) was used for determining the molecular masses of specific proteins.

6.1.3.2. Western Blotting

For determining the level of expression of peroxidase enzyme, the separated proteins were transferred from the gel to a sheet of PVDF membrane by a wet blotting system (BioRad, USA) at 90 V, 350 mA for 1 h following the method of Towbin *et al.* (1979) with modifications. Following transfer, the PVDF membrane was blocked for 5 h with blocking buffer containing 5% casein. Antiserum (antiperoxidase developed in goat fractioned antiserum, Sigma) diluted (1:1000) in 0.15 M PBST (pH 7.2) was applied to the PVDF membrane and incubated overnight at 4°C. The membrane was then washed 3 times (5 minutes for each) with PBST and incubated in a 1:10000 dilution (in PBST) of secondary antibody (goat antirabbit IgG-HRP conjugate) for 2 h at room temperature. Unbound conjugate was removed by washing twice (7 minutes for each) with PBST and finally the membrane was washed with TBS (pH 7.5) for 10 min. The blot was developed using the chromogenic substrate tetramethylbenzidine/hydrogen peroxide and finally photographed.

6.1.4. Immunogold labeling and silver enhancement

Immunogold labeling was performed following the method as described by Santen *et al.* (2005) with necessary modifications. The root samples of tea were collected, after 10 days of exposure to copper, washed thoroughly and kept at 4^0C before use. Fine cross sections of roots were cut manually and placed on clean grease free slides. Water drops (100 µl) were mounted on each section. The slides were incubated at 2-5^0C for 30 min and excess water surrounding the sections was blotted off. Thirty microlitre of blocking buffer (0.15 M PBS pH 7.2 containing 5% normal sera of goat) was placed on the cross sections and incubated for 10 min. Excess solution was wiped off and diluted in PBS (1:100) antiperoxidase primary antibody (Sigma, USA) was applied on the sections and incubated overnight at 4^0C. After incubation, the sections were carefully rinsed in 0.15 M PBS (pH 7.2) for 5 min and the excess buffer was poured off. The sections were then flooded with 100 µl of diluted (1:50) immunogold reagent containing 0.5 nm gold particle (Sigma, USA) and incubated for an hour. The sections were again rinsed for 4 minute with PBS and subsequently fixed in 200 µl of PBS-glutaraldehyde (2.5% glutaraldehyde solution in PBS) for 15 min. Next they were washed in distilled water and further processed for silver enhancement

using silver enhancement kit (Sigma). Initially solution A (silver salt) and solution B (an initiator) were mixed (1:1) according to the manufacturer's instruction. Mixed solution (100 µl) was used to flood each section. After 5 min of incubation, the cross sections were washed with distilled water. Distilled water was poured off and 100 µl of sodium thiosulphate solution (2.5% aqueous) was placed on the sections and allowed to incubate for 3 minutes. The sections were again washed in distilled water and counterstained with saffranin before mounting on to the slides. Immediately after the staining, the sections were viewed in a binocular light microscope (Leica, Germany) and photographed using digital camera (Canon A530 Powershot) with appropriate attachment system.

6.1.5. Cellular localization of copper by transmission electron microscopy (TEM)

For electron microscopic study, roots were collected from control and treated (700 µM) seedlings grown in hydroponic culture containing Hoagland and Knop's (nutrient) solution. Both control and treated roots of 10th day were used. Root sections of 1-2 mm length were cut and primary fixation was done using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours at 4°C followed by three washings with 0.1M phosphate buffer (1st change for overnight at 4°C and 2nd and 3rd change for 1 h each at 4°C). The fixed samples were sent to IARI, New Delhi, where the samples were further processed for electron microscopy. The samples were examined under JEOL JEM-2010 electron microscope at an accelerating voltage of 80.0 kV.

6.2. RESULTS

6.2.1. Isozyme patterns of antioxidative enzymes

Activity staining on non-denaturing polyacrylamide gels was done for each of the four enzymes, SOD, POD, APX and CAT. Tea leaves were sampled at 10 days after exposure to excess copper from treated and control sets and used for isozyme analysis.

6.2.1.1. SOD

Activity staining of SOD enzyme in non-denaturing polyacrylamide gels showed clear bands in a deep purple background. A single band was visible with the R_f value of 0.033 in each treatment along with control (Fig. 6.1).

However the intensity of the bands varied with the treatments. Highest intensity of the band was recorded in samples extracted from plants that were treated with 500 µM copper. A lower intensity was noted in other treated plants as well as in control (Fig. 6.1).

6.2.1.2. POD

The isoenzymes of peroxidase were visible as reddish brown bands in the polyacrylamide gels following activity staining. Two isoforms, POD 1 (R_f 0.15) and POD 2 (R_f 0.29) were found to be present after POD activity staining in the leaves of TS-462 cultivar of tea from both treated and control sets (Fig. 6.2). However, two new isozymes POD 3 (R_f 0.42) and POD 4 (R_f 0.51) were induced in the leaves of tea exposed to 500 µM of Cu (Fig. 6.2). These extra bands were not seen in the lower dose treatments and at the highest dose (700 µM) but the intensity of the isozymes were higher in treated sets than in control.

6.2.1.3. APX

On gel staining for APX activity revealed the occurrence of two isozymes, APX 1 (R_f 0.08) and APX 2 (R_f 0.32) in samples extracted from both treated and control plantlets which appeared as clear bands in deep blue background (Fig. 6.3). The intensity of APX 1 increased from control to 200 µM treated plants and the levels remained equally high with increasing copper concentrations upto 700 µM. APX 2 was also found to be regulated, but densitometric analysis showed that the levels did not rise at 200 µM but showed highest intensity at 500 µM and then sharply declined at 700 µM (Fig. 6.3).

6.2.1.4. CAT

Catalase activity staining in polyacrylamide gel is shown in Figure 6.4. Expression of catalase were observed as bands that were achromatic zones (or faint green zones) on a deep green background whose colour intensity varied with the treatments. A single band was visible at R_f value of 0.25 in each treatment. Strongest band was found in samples recovered from plants that were treated with 500 µM copper solutions (Fig. 6.4). The intensity was lower in both control and 200 µM treated plants. The plants treated with 700 µM copper also showed much reduced intensity and was almost invisible

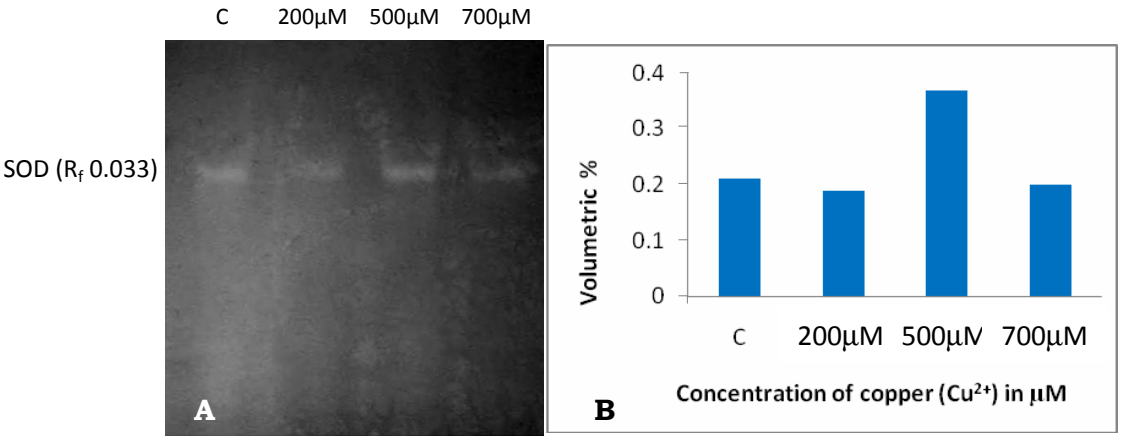

Fig. 6.1: (A) Isozyme pattern of superoxide dismutase in response to copper stress in leaves of tea (cultivar TS-462) compared to control (C); (B) Densitometric analysis of isozyme bands.

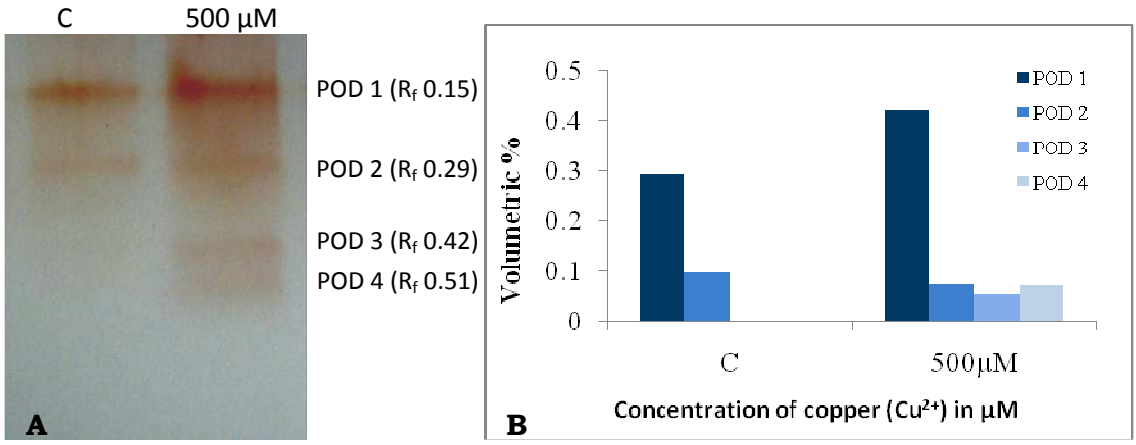

Fig. 6.2: (A) Isozyme pattern of peroxidase in response to copper stress in leaves of tea (cultivar TS-462) compared to control (C); (B) Densitometric analysis of isozyme bands.

and therefore not presented in the figure. In case of untreated-inoculated plants, similar single band was present but with much less intensity (Fig. 6.4).

6.2.2. Protein profile and peroxidase expression

SDS-PAGE analysis of leaf and root proteins were done using total protein extract from treated and control tea seedlings. Results showed that a particular 77 kDa band was visible in leaves of tea seedlings treated with 500 μM of Cu^{2+} (Fig. 6.5A). Similarly a 46 kDa band appeared in the roots at high exposure of copper concentration (Fig. 6.5B). However, Western blotting revealed a 65 kDa peroxidase whose expression levels increased with increasing levels of copper (Fig. 6.6).

6.2.3. Tissue and cellular localization of peroxidase activity

Immunogoldlabeling followed by silver enhancement was performed to locate the regions of roots that showed increased levels of peroxidase. The results revealed that the labeling was strongest in the cortical regions of the roots that indicated high peroxidase activity in those regions (Fig. 6.7). Epidermal regions also showed some extra gold particles in the treated sets. The labelling was also found to be intense in the roots treated with 500 μM and 200 μM copper solutions when compared to the untreated roots (control).

6.2.4. Cellular localization of copper in tea roots by transmission electron microscopy (TEM)

The cellular localization of copper in the roots of tea seedlings exposed to 700 μM of copper was studied by TEM. Several dark precipitates were detected along the cell wall in the roots of treated sets (Fig. 6.8). The particles appeared essentially as isolated circular dark spots scattered in the region adjacent to cell walls. Some electron dense precipitates were found inside the vacuole. Such precipitation was not found to be present in control plants.

6.3. DISCUSSION

A great deal of research has established that the induction of the cellular antioxidant machinery in the plant is important for their protection against ROS. Overexpression of ROS scavenging enzymes like isoforms of SOD (Mn-SOD, Cu/Zn-SOD, Fe-SOD), CAT, APX, and POD resulted in abiotic stress

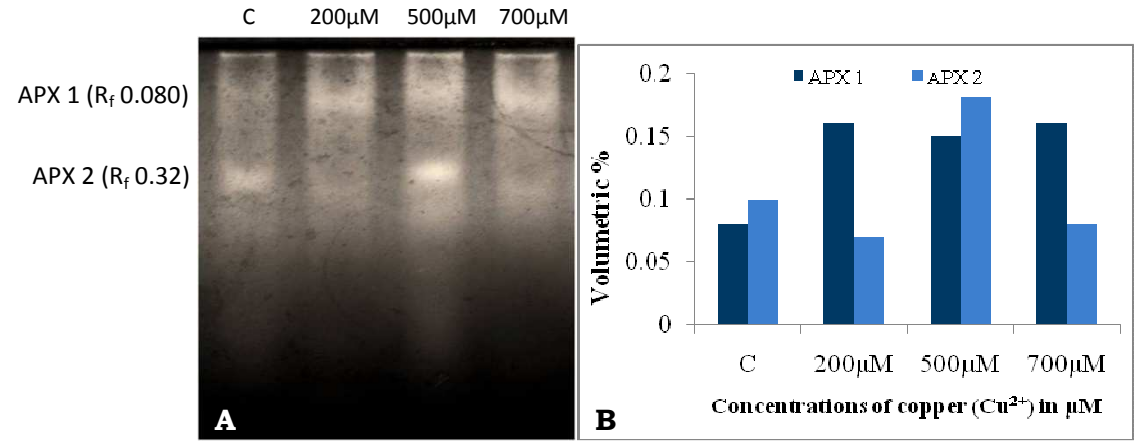

Fig. 6.3: (A) Isozyme pattern of ascorbate peroxidase in response to copper stress in leaves of tea (cultivar TS-462) compared to control (C); (B) Densitometric analysis of isozyme bands.

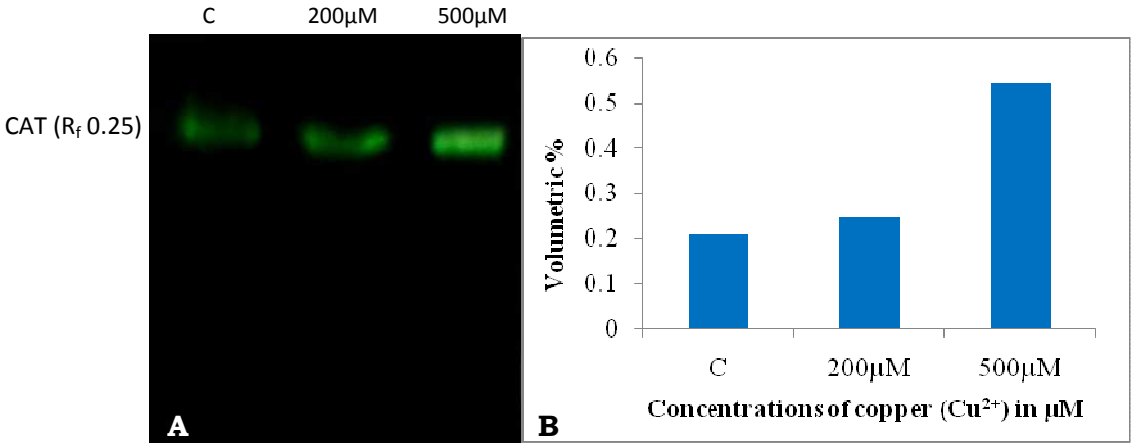

Fig. 6.4: (A) Isozyme pattern of catalase in response to copper stress in leaves of tea (cultivar TS-462) compared to control (C); (B) Densitometric analysis of isozyme bands.

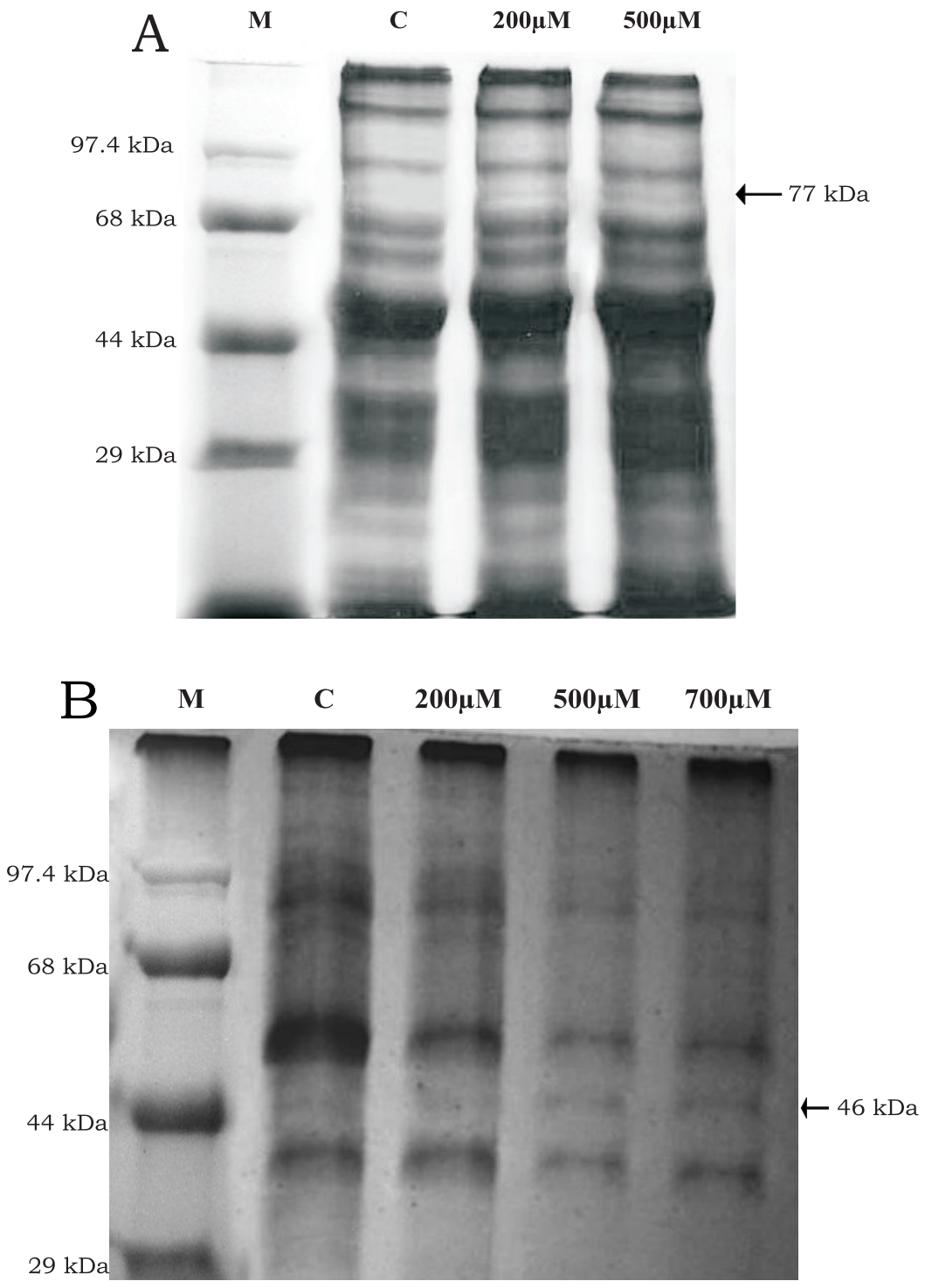

Fig. 6.5: SDS-PAGE analysis of total protein in leaf (A) and root (B) of tea seedlings (cultivar TS-462) exposed to excess Cu^{2+} for 10 days. Standard molecular weight markers (M) shown at the left. Specific 77 kDa band found in tea leaves at 500µM Cu exposure and 46kDa band found in the roots at higher exposure concentrations are indicated.

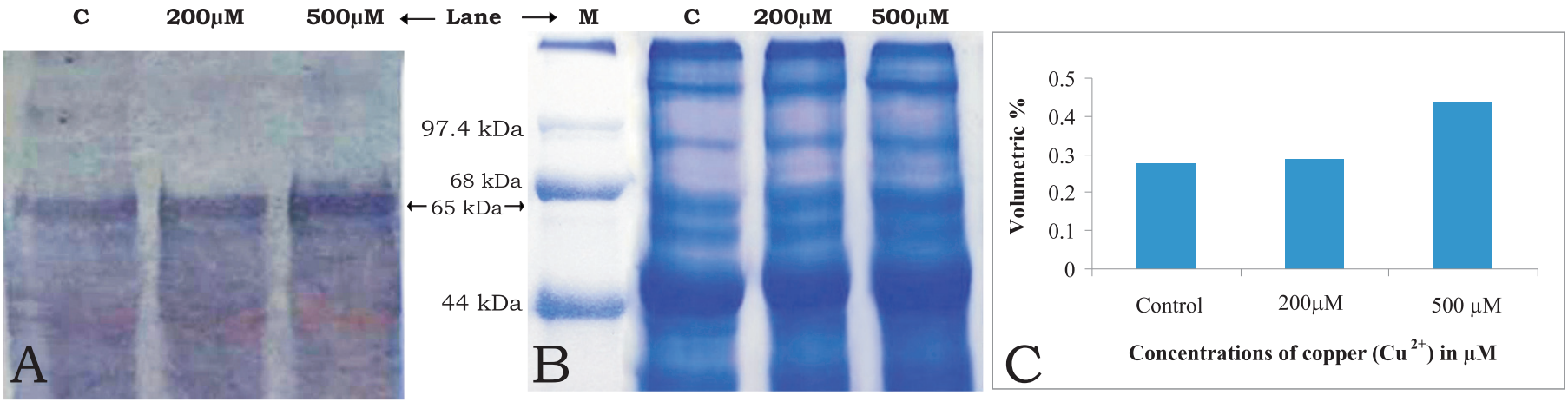

Fig. 6.6: (A) Western blot analysis using specific antibody against peroxidase induced in tea leaves (TS-462) by excess Cu^{2+}; (B) The corresponding SDS-PAGE gel is shown for comparison (M= standard molecular weight marker, C= Control) ; (C) Densitometric analysis of immunoblots.

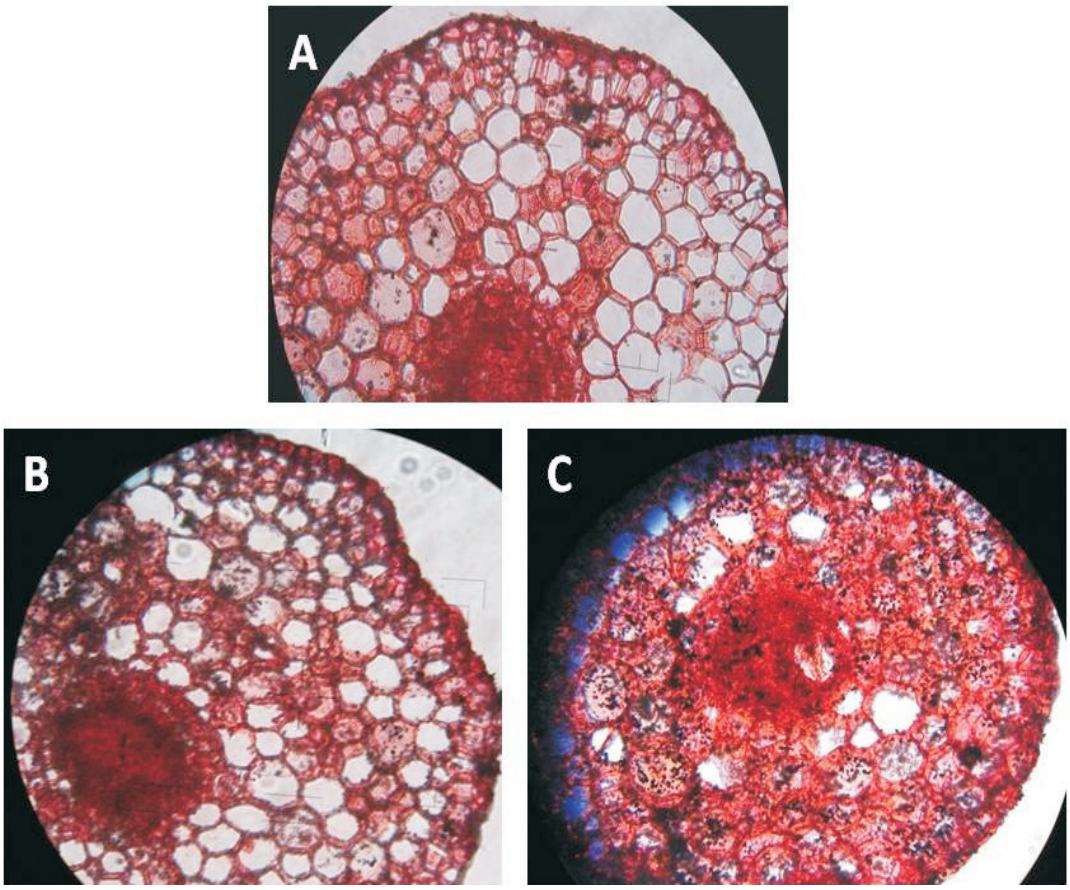

Fig. 6.7: Cellular localization of peroxidase in root tissues in TS-462 cultivar of tea treated with different concentrations of copper solution by immunogold labeling and silver enhancement. [A: Untreated control, B: treated with 200 μM Cu^{2+}, C: treated with 500 μM Cu^{2+}].

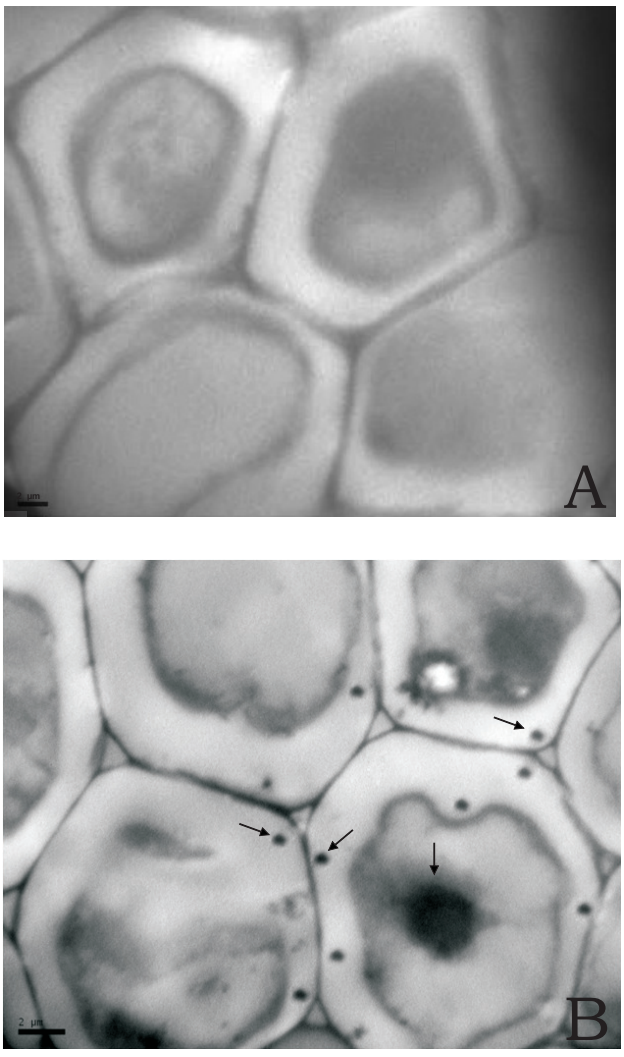

Fig. 6.8: TEM photos of tea root cell of cultivar TS-462 (A) Control (X3000); (B) treated with 700 µM copper (X3000) showing dark precipitates along cell wall and inside vacuole.

tolerance in various crop plants due to efficient ROS scavenging capacity (Gill and Tuteja, 2010). The upregulation of these enzymes is implicated in combating oxidative stress caused due to several different types of abiotic stresses and have a critical role in the survival of plants under adverse environmental conditions. Isoforms of SOD, APX and POD are localized in several subcellular compartments, whereas CAT is mostly localized in the peroxisome. They strongly differ in their substrate affinities and ensure a tight control of H_2O_2 concentrations at very low levels (Yadav *et al.*, 2012). Other enzymes, such as glutathione peroxidase (GPX) and glutathione-transferases (GSTs), also contribute to the redox homeostasis of the cell.

In the current study, the enzyme isoform patterns were studied in order to understand the involvement of the specific isozymes for control of ROS. A single SOD isozyme was evident to be present in native PAGE gels. Thounaojam *et al.* (2012) studied the effects of copper in rice plant and observed that three isoenzyme bands (i, ii, iii) were detected in the root and SOD iii was detected only at the higher concentration of Cu. Brahim and Mohamed (2011) studied the effect of excess Cu on *Atriplex halimus* grown in hydroponics conditions and observed the presence of three isoforms of SOD whose intensity increased with increasing copper dose. In a study on copper stress on *Withania somnifera* seedlings, Rout *et al.* (2013) observed a single SOD band in control plants while the treated plants showed two bands. *Solanum nigrum* plants were exposed for 28 days to 100 and 200 μmol/L copper (Cu) in a hydroponic system by Fidalgo *et al.* (2013). Five major bands corresponding to SOD isozymes were visible but differences in response could not be detected. One particulary prominent band of Fe-SOD was visible in roots while rest others were very weak. On the contrary it has been reported that excess copper increased or induced Cu/Zn-SOD activity and disrupted expression of other SODs in soybean and mulberry leaves (Chongpraditnum *et al.*, 1992; Tewari *et al.*, 2006). Unlike most other organisms, plants have multiple enzymic forms (isozymes) of SOD. The existence of SOD isozymes in plants and their genetic basis was first demonstrated in maize (Baum and Scandalios, 1979, 1982), and the first plant *Sod* gene to be cloned was from maize (Cannon *et al.*, 1987). The basis for the multiplicity of SOD in other plant species continues to be investigated, and indirect evidence suggests that multiple genes for SOD

exist in most plants (Lee *et al.*, 2007). Here, only one SOD was visible probably because of lower expression of other SOD isozymes or due to suppression of their expression to such levels that remained undetected in the present study. However, exact identification of the over-expressed isozyme requires further study.

Results of isozyme analysis of POD revealed that two new isozymes POD 3 and POD 4 were induced in the leaves of tea exposed to high concentration of Cu. Diaz *et al.* (2001) also detected two new POD isozymes (PRX-A2 and PRX-A4) in pepper hypocotyls while the other two isoperoxidases, PRX-B and PRX-A3, were enhanced. Fang and Kao (2000) observed that one new POD isozyme with a pI of 4.81 was induced by Cu treatment in detached rice leaves. Brahim and Mohamed (2011) observed five peroxidase isoforms in *A. halimus* leaves after 48 h treatment with copper of which the intensity of at least three were correlated with copper concentration. Two isozymes were observed in the roots of copper stressed *Withania somnifera* seedlings by Rout *et al.* (2013). The authors reported that, with the increase in Cu concentration, the number of isoenzymes increased, but after a specific concentration, the bands disappeared in both roots and leaves.

APX activity staining in native PAGE gels revealed that two isozymes were visible and both were regulated by fluctuating copper levels. APX 2 increased at 500µM and decreased at higher levels but APX 1 increased at lower concentrations but the levels did not fall at higher concentrations. APX belongs to the class I heme-peroxidases which are widely distributed in the plant kingdom. The enzyme utilizes ascorbic acid as specific electron donor to reduce H_2O_2 to water. APX exists in different isoforms classified according to their subcellular localization. Soluble isoforms are found in cytosol (cAPX), mitochondria (mitAPX) and chloroplast stroma (sAPX), while membrane-bound isoforms are found in microbody (including peroxisome and glyoxisome) (mAPX) and chloroplast thylakoids (tAPX) (Caverzan *et al.*, 2012). Lee *et al.* (2007) detected a new isoform of APX in transgenic plants when they were treated with copper or cadmium, suggesting that APX might have some level of sensitivity to abiotic or metal stresses. The authors generated tall fescue plants expressing the Cu/Zn-SOD and APX genes in chloroplasts under the control of the oxidative stress-inducible promoter, sweet potato peroxidase anionic 2 (SWPA2).

Activity staining of CAT enzyme revealed a single band whose intensity was correlated with copper levels in the nutrient medium. Rout *et al.* (2013) also reported that a single isoenzyme of CAT was observed in both the samples of control and all the concentrations (25–200 µM) of Cu treated *Withania somnifera* L. seedlings, but the intensities of band varied from one sample to another. Brahim and Mohamed (2011) observed three isoenzymes of CAT in *Atriplex halimus* leaves of which CAT-1 was very slow and present in all treatments. CAT-2 and CAT-3 bands appeared at 50 µM Cu and its intensity increased with copper amount. Fidalgo *et al.* (2013) observed that Cu excess did not change the isozyme patterns in *Solanum nigrum* plants, since two CAT isozymes were detected in both control and Cu-exposed plants. In this study although single band was visible at all treatments, the intensities were slightly increased in plants exposed to higher copper doses although our earlier studies indicated that catalase level was less modified in response to copper induced oxidative stress. Further studies especially at the genetic level may help to explain this incongruity.

Peroxidases have been involved in a broad range of physiological processes such as lignification, senescence, auxin metabolism, the cross-linking of cell wall proteins, defence against pathogenic attack and a variety of abiotic stress tolerances. Many workers have noted that POD has a large family of isoenzymes in a variety of higher plants (Bakalovic *et al.*, 2006). However, the inherent complexity of the physiological processes in which POD isoenzymes are involved makes understanding the specific function difficult (Guo *et al.*, 2012). In this study, we have attempted to study the expression level modifications in peroxidase by Western blotting as this was the only enzyme that showed the appearance of new isozymes under copper excess during activity staining. Prior to that, SDS-PAGE analysis of leaf and root proteins showed that a particular 77 kDa band was visible in leaves of tea seedlings treated with 500 µM of Cu^{2+}. Similarly a 46 kDa band appeared in the roots of tea seedlings treated with higher copper concentrations. Western blotting revealed a 65 kDa peroxidase whose expression levels increased with increasing levels of copper. In a study, Guo *et al.* (2012) isolated a peroxidase gene, named ThPOD1 from root tissue of a woody plant, *Tamarix hispida* that was exposed to 0.4 M NaCl. The gene was expressed in bacteria and the purified peroxidase was obtained as 42 kDa protein. Antiserum

raised against the protein was used in western blotting studies to study the expression levels of this peroxidase in *T. Hispida* under stress. Abiotic stress caused an increased expression of this peroxidase with a higher expression in roots than in the leaves. Meisrimler *et al.* (2014) observed that the strongest regulated band of peroxidase abundance was a particular 133 kDa band in stressed maize plants which was visible in the second dimension by Clear Native Electrophoresis.

As stated earlier, microscopic studies following immunogoldlabeling is a powerful tool for cellular location of different proteins or antigens. In the current study, gold particles were denser in the cortex and epidermis of treated plants in comparison to control plants. The immunolocalization and distribution of peroxidases from hyperhydrated leaves of carnation were studied by Olmos *et al.* (1997) where the sections were subjected to treatment with colloidal-gold-labelled antibodies. They showed different distribution pattern of the peroxidase activity in the xylem cell wall of the normal leaves and hyperhydric leaves, with a significant lower particle density in stressed leaves compared to normal leaves. The authors suggested that absence of peroxidase in places of peroxide formation has resulted in oxidative stress. Ramos *et al.* (2008) revealed the presence of Glutathione peroxidases (GPXs) proteins in root and nodule amyloplasts and in leaf chloroplasts of *Lotus japonicas* and other legumes by immunogoldlabeling under salt stress. The authors reported that labeling was associated with starch granules. Peroxidase localization during the current study revealed that the activity of this enzyme was increased due to copper excess which is essentially a common consequence of oxidative stress. Thus it is most likely that copper is generating oxidative stress resulting in higher H_2O_2 production that is controlled by the higher amount of peroxidase synthesised or mobilised due to excess stress.

Agricultural soil contamination with heavy metals is nowadays a problem because of its implication in the environment protection and human health. Copper accumulation in the soil due to spraying of copper based agrochemicals has been found to be hazardous to plants. However, some plants can assimilate copper to certain levels by multiple detoxification mechanisms and are capable of surviving in metal contaminated soils. The uptake and translocation may vary considerably among plant species and is

additionally dependent on the metal type (Prasad, 2004). Different metals are differentially mobile and copper is considered less mobile within plants among the heavy metals. After absorption, metals are apoplastically translocated in the plant tissue and have to pass the epidermal and other anatomical barriers in order to reach the xylem vessel for transport to the shoots and leaves. Therefore membrane bound uptake proteins which are designed for acquisition of nutrient metals plays a role in the transport from roots to the leaves.

Localization of copper in the plant cells can be studied best in species which are normally tolerant to high levels of copper. Thus, in a study on *Elsholtzia splendens*, a copper tolerant plant, Cai-ying *et al.* (2005) observed that vacuoles and cell-wall were compartments for copper tolerance. In the current study, copper was found to be associated with the cell wall as well as precipitated in vacuole of tea roots. During their transporation through the plants, metals get bound largely on the cell walls which explain why most of the metal taken up is commonly found in the roots (Prasad, 2004). Arru *et al.* (2004) in their study on *Cannabis sativa* L. grown in a copper-rich solution, observed that copper accumulated preferentially in the upper leaf epidermal cells. The authors found that a part of the absorbed Cu was detected in vacuolar inclusions. In other plant species Cu was found to be accumulated in the vacuole by chelation with poly-hydroxy phenolic compounds or phytochelatins (Neumann *et al.*, 1995).

In the current study, detection of copper precipitates in vacuoles and cell wall may suggest that tea is a fairly copper tolerant plant. However, this opinion is nullified by our earlier results where we found that several stress related metabolic and physicochemical changes are accompanied with exposure to the highest copper concentration tested (700 μM). Moreover, in case of copper, plants considered as hyperaccumulators (eg. *Acollanthus biformifolius*), can take up more than 1000 μg g^{-1} DW of copper inside the leaves. In this study, copper concentration in leaves was much less at the highest tested exposure concentration and this was related with severe disruption of redox poise of the leaves. Thus tea is not a copper tolerant plant and excess copper would definitely have negative consequences in overall metabolism and yield.