

## Copper induced oxidative stress in tea (*Camellia sinensis*) leaves

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### Abstract

Tea [*Camellia sinensis* L. (O.) Kuntze] is an economically important plantation crop of India but is prone to attack by several fungal pathogens. Copper based fungicides are being used for decades to control fungal diseases in tea which may lead to accumulation of copper in the soil. The biochemical responses to increasing concentrations of copper (50 to 700  $\mu\text{M}$ ) were investigated in the leaves of two cultivars of tea commonly grown in the Darjeeling hills. Exposure to excess Cu resulted in increased lipid peroxidation (level of TBARS increased from 3.5  $\mu\text{mol g}^{-1}$  f.wt. in control to 12  $\mu\text{mol g}^{-1}$  f.wt. in TS-520 plants exposed to 700 mM of Cu), reduced chlorophyll content (from 83.7  $\mu\text{g g}^{-1}$  f.wt. in control to 22.5  $\mu\text{g g}^{-1}$  f.wt. in TS-520 plants exposed to 700 mM of Cu), higher levels of phenolic compounds (total phenol content increased from 4.54  $\text{mg g}^{-1}$  f.wt. in control to 5.79  $\text{mg g}^{-1}$  f.wt. in TS-520 plants exposed to 400 mM of Cu) and an increase in peroxidase enzyme levels. Two new peroxidase isozymes (POD1 and POD2) were detected in plants exposed to Cu. In addition, biochemical responses in two tested cultivars, TS-462 and TS-520 differed significantly. TS-520 was found to be more sensitive to increasing concentrations of Cu. Superoxide dismutase activity increased progressively from 2.55  $\text{U mg}^{-1}$  protein in control to 5.59  $\text{U mg}^{-1}$  protein in TS-462 but declined from 4.75  $\text{U mg}^{-1}$  protein in control to 3.33  $\text{U mg}^{-1}$  protein in TS-520 when exposed to Cu concentrations higher than 400  $\mu\text{M}$ . A sharp increase in the activity of ascorbate peroxidase (from 0.53 units in control to 2.37 units in plants exposed to 400 mM of Cu) was noticed at the 10<sup>th</sup> day of exposure in the more tolerant cultivar. On the other hand, catalase levels increased only marginally (from 8.4 to 10.1 units in TS 520 and 8.7 to 10.9 units in TS 462) in both the cultivars. From this study, it appears that Cu exposure led to the production of reactive oxygen species in the leaves resulting in significant lipid peroxidation. Tea plants try to mitigate this oxidative damage through accumulation of phenolic compounds and induction of antioxidant enzymes.

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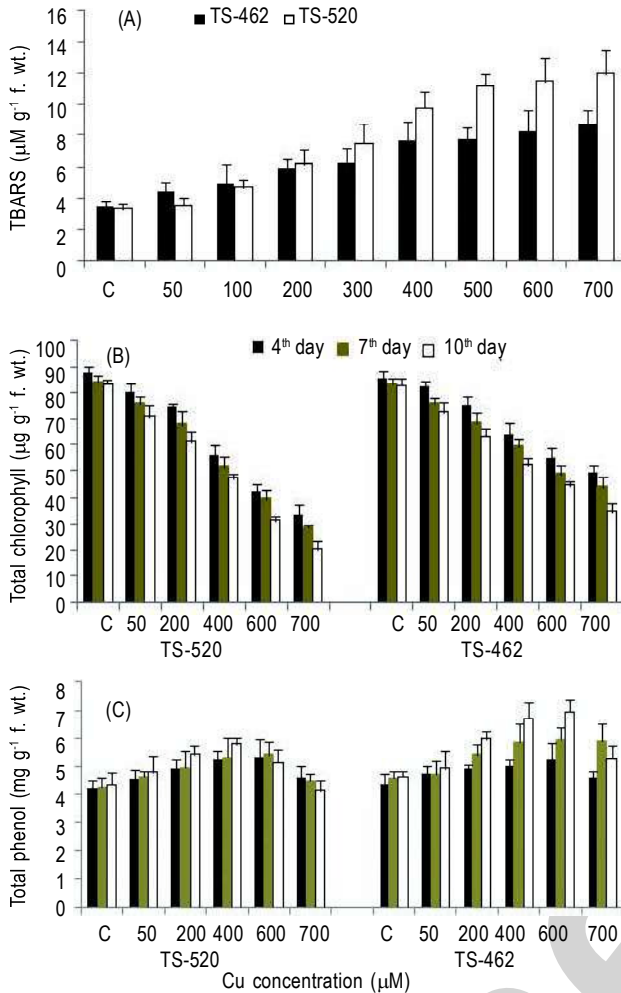
### Key words

Tea, Copper stress, Reactive oxygen species, Antioxidant enzymes, Lipid peroxidation

### Introduction

Tea (*Camellia sinensis* L. (O.) Kuntze) is an economically important crop in India but some of its elite varieties are highly prone to fungal attack by several types of fungi (Singh, 2005). Copper (Cu) based fungicides are cheaper and effective in controlling fungal diseases in tea (Singh, 2005) and are often sprayed indiscriminately in the tea gardens. Agricultural practices with a long history of copper fungicide application have resulted in accumulation of high levels of copper in soil (Brun *et al.*, 1998). Copper in trace amounts is essential for various metabolic processes in the plant, but at higher

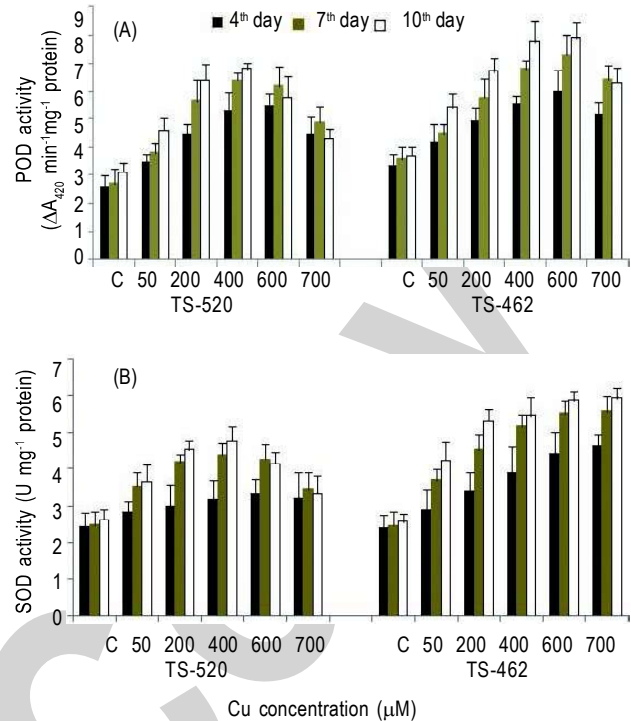
concentrations it causes physiological stress through generation of free radicals that induce the production of reactive oxygen species (ROS) via Haber-Weiss and Fenton reactions (Dat *et al.*, 2000). Copper-induced generation of hydrogen peroxide, hydroxyl radicals, or other reactive oxygen species are directly related with the damage to protein and lipids that may lead to reduced growth and even death (Dat *et al.*, 2000). Plants have a range of detoxification mechanism that includes enzymatic scavengers of ROS such as superoxide dismutase (SOD), peroxidase (POD) ascorbate peroxidase (APX), catalase (CAT) and enzymes of the ascorbate glutathione cycle (Dat *et al.*, 2000; Arora *et al.*, 2002).



**Fig. 1:** Effect of increasing concentrations of copper (Cu) on (A) the level of lipid peroxidation measured as thiobarbituric acid reactive substance (TBARS) on the 10<sup>th</sup> day of exposure, (B) total chlorophyll content and (C) total phenol content on the 4<sup>th</sup>, 7<sup>th</sup> and 10<sup>th</sup> day of exposure in leaves of two cultivars of tea (TS-462 and TS-520). Values are mean of three replicates  $\pm$ SE

Stress condition may enhance the protective processes including increased activities of these detoxifying enzymes (Chen *et al.*, 2000; Lombardi and Sebastiani, 2005). However, the response of the antioxidative system varies with the plant species (Ke *et al.*, 2007). Hence knowledge on key parameters associated with metal stress is essential to understand the level of tolerance which the plant has evolved.

Although Cu based fungicides are being used in tea gardens for several decades (Sarmah, 1960), we do not know how tea plants are affected by excess Cu and at what concentrations it may be considered as a pervasive threat. This prompted us to investigate whether oxidative stress is generated in young (three months old) tea plants exposed to varying concentrations of Cu. In this study we have studied levels of lipid peroxidation, phenolic compounds, total chlorophyll content and SOD, POD, APX and CAT activities in the plantlets of two cultivars of tea cultured in hydroponic system.

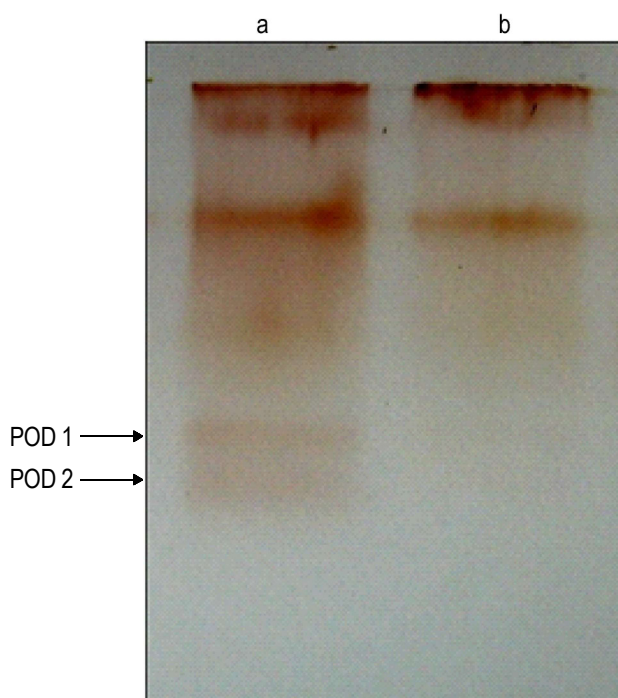


**Fig. 2:** Effect of increasing concentrations of copper (Cu) on (A) POD activity and (B) SOD activity in leaves of two cultivars of tea (TS-462 and TS-520) on the 4<sup>th</sup>, 7<sup>th</sup> and 10<sup>th</sup> day of exposure. Values are mean of three replicates  $\pm$ SE

## Materials and Methods

**Growth conditions:** Tea seeds of two different cultivars *viz.* TS-520, TS-462 were collected from Gayaganga Tea Estate, Siliguri, India and seedlings were raised under natural conditions of light and temperature in net house. Three-months-old healthy plants were transferred to hydroponic culture set up in Hoagland's nutrient solution and allowed to be stable for 7 days before placing them in separate 3l capacity square shaped glass containers for treatment. The plants were treated with eight different concentrations of  $\text{CuSO}_4$ , *i.e.* 50, 100, 200, 300, 400, 500, 600 and 700  $\mu\text{M}$  in the nutrient solution. Control sets comprised of nutrient solution without  $\text{CuSO}_4$ . Leaves from each set of plants were collected for measuring various biochemical parameters after 4<sup>th</sup>, 7<sup>th</sup> and 10<sup>th</sup> day of treatment.

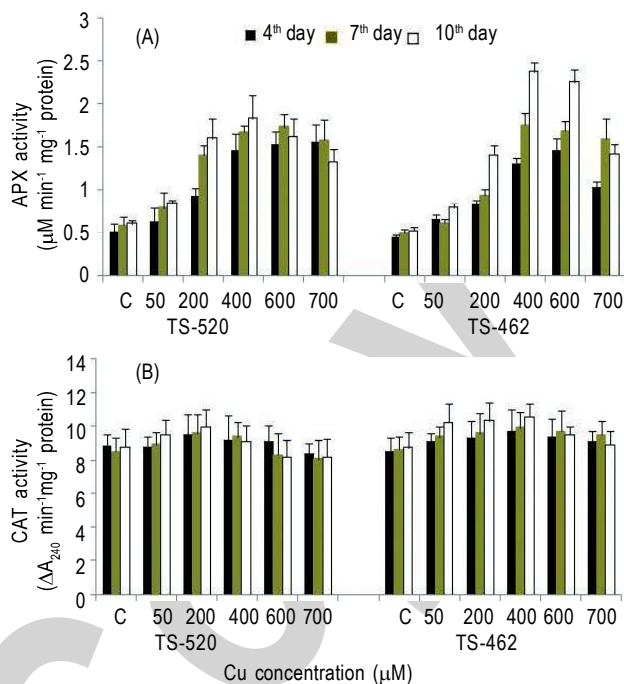
**Determination of lipid peroxidation, phenolics and chlorophyll content:** The level of products of lipid peroxidation in leaf tissues was determined as the thiobarbituric acid (TBA) reactive substance (TBARS) following the method of Heath and Packer (1968). Frozen leaf tissue (200 mg) was homogenized in 4.0 ml of TBA reagent containing 0.25% w/v TBA in 10% w/v trichloroacetic acid (TCA) and the resultant homogenate was heated for 30 min at 95°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  $\mu\text{M g}^{-1}$  fresh weight by using an extinction



**Fig. 3:** Isozyme pattern of peroxidase in response to copper stress in leaves of tea, (a) Cultivar TS-462 exposed to 500  $\mu\text{M}$  Copper; (b) unexposed control plants

coefficient of  $155 \text{ mM cm}^{-1}$ . Total phenolic compounds were extracted in ethanol and the content was estimated by Folin ciocalteau's method using a caffeic acid standard curve as described by Mahadevan and Sridhar (1996) with absorbance changes at 520 nm. For chlorophyll measurement, leaf tissue was homogenized in 100% acetone and estimated following the method of Dere *et al.* (1998).

**Measurement of enzyme activities and protein assay:** For determining enzyme activities, fresh leaf samples (500 mg) were instantly dipped in liquid nitrogen and the frozen leaves 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 13000g for 20 min at 4°C and the supernatant after dialysis was used for enzyme assay. Catalase activity was determined following the method of Aebi (1984) by measuring the rate of decrease in absorbance at 30 sec interval for 2 min at 240 nm of a solution of 10 mM  $\text{H}_2\text{O}_2$  in 50 mM K-phosphate buffer (pH 7.0) at 30°C. The enzyme activity was expressed as  $\Delta A_{240} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ . Total SOD activity was determined according to the method of Giannopolities and Ries (1977). Reaction mixture (3ml) contained 13  $\mu\text{M}$  methionine, 63  $\mu\text{M}$  p-nitroblue tetrazolium chloride (NBT), 1.3  $\mu\text{M}$  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 560nm. One unit of SOD activity was defined as the amount of enzyme required for the inhibition of the photochemical reduction of NBT by 50%. For estimation of POD activity, leaf samples were similarly homogenized in ice cold 50 mM



**Fig. 4:** Effect of increasing concentrations of copper (Cu) on (A) APX activity and (B) CAT activity in leaves of two cultivars of tea (TS-462 and TS-520) on the 4<sup>th</sup>, 7<sup>th</sup> and 10<sup>th</sup> day of exposure. Values are mean of three replicates  $\pm$ SE

sodium phosphate buffer (pH 7) containing PVP and the supernatant was used for enzyme assay. The reaction mixture of 3 ml contained 10 mM guaiacol, 2 mM  $\text{H}_2\text{O}_2$  and 100 ml of enzyme (Hammerschmidt *et al.*, 1982) Change in absorbance was measured at 420 nm for 2 min at intervals of 30 sec. The enzyme activity was expressed as  $\Delta A_{420} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ . The APX activity was measured following the method of Nakano and Asada (1981). Fresh 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 3 ml 0.05 M sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1mM  $\text{H}_2\text{O}_2$  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  $\mu\text{M min}^{-1} \text{ mg}^{-1} \text{ protein}$  (extinction coefficient,  $2.8 \text{ mM cm}^{-1}$ ). For all experiments, each leaf extract was assayed twice and the results of three independent experiments were averaged. Protein was measured following the method of Lowry *et al.* (1951) taking bovine serum albumin as standard. All data were analysed by analysis of variance using Statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois. Differences were compared by least significant difference (LSD) procedure at the level of  $P < 0.05$ .

**In-gel assay of peroxidase:** For studying the different forms of POD isozymes, native polyacrylamide gel electrophoresis (PAGE) was carried out on 10% polyacrylamide gels. Total soluble proteins were extracted as described by Sadasivam and Manickam (1996) and loaded (25 g) 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<sub>2</sub>O<sub>2</sub> for 15 min for the development of reddish brown bands.

### Results and Discussion

Exposure to Cu resulted in accumulation of products of lipid peroxidation in the leaves (Fig. 1A). 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 (Chen *et al.*, 2000; Rama Devi and Prasad, 1998; Mazhoudi *et al.*, 1997) have reported similar increase in lipid peroxidation in plants exposed to Cu. Being a redox metal, Cu can interfere with various physiological processes including lipid peroxidation, a toxicity indicator for plants exposed to Cu (Baryla *et al.*, 2000). The primary site of Cu toxicity lies at the cell membrane level including the photosynthetic membranes (De Vos *et al.*, 1992; Sandmann and Boger, 1980). A comparison of lipid peroxidation 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, total chlorophyll content was found to decrease significantly with increasing Cu concentrations and exposure times in both the cultivars with the more sensitive cultivar (TS-520) recording a significantly higher decrease (Fig. 1B) ( $P < 0.05$ ). Several authors (Rama Devi and Prasad, 1998; Ciscato *et al.*, 1997; Mohanpuria *et al.*, 2007; Ouzounidou *et al.*, 1994) have also reported a decrease in chlorophyll content in plants when exposed to Cu. Leaf chlorophyll concentration is crucial for the susceptibility of the plants to photoinhibition and should be considered when an effect of environmental stress is under study (Patsikka *et al.*, 2002). The present study indicates that excess of Cu caused inhibition to the photosystem in tea leaves.

Peroxidase activity increased with Cu concentration to more than two fold in both the tested cultivars (Fig. 2A). 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  $\mu$ M. This lowering of activity may be due to complete inhibition of growth in the sensitive cultivar exposed to Cu concentrations higher than this threshold level. On the other hand, in TS-462, POD activity showed an all through increase with increase in Cu concentrations and time of exposures except at the highest concentration where it recorded a sharp decline. It has been shown that excess Cu induces POD activity in several plant species (Mocquot *et al.*, 1996; Diaz *et al.*,

2001). Results of isozyme analysis of POD revealed that two new isozymes POD1 and POD2 were induced in the leaves of tea exposed to high concentration of Cu (Fig. 3). Diaz *et al.* (2001) also detected two new POD isozymes in pepper hypocotyls while Fang and Kao (2000) observed one new POD isozyme in rice leaves induced by Cu treatment. Peroxidases take part in defense mechanism against heavy metal toxicity through lignification of cell walls (Diaz *et al.*, 2001) that confers rigidity and prevent growth. In the present study, growth parameters could not be investigated because tea is a very slow growing plant (Mohanpuria *et al.*, 2007) and growing tea plants in a simple salt solution for long period is impractical (Ghanati *et al.*, 2005). Peroxidase also acts as efficient H<sub>2</sub>O<sub>2</sub> scavenger in a process that involves phenolic compounds as electron donors in the apoplast and plant vacuoles (Morina *et al.*, 2008). In the present study, we measured total phenolics content and the results showed an increased content of phenolic compounds in both the cultivars at Cu concentrations below 400  $\mu$ M (Fig. 1C). At higher concentrations, the activity either changed insignificantly or decreased. Thus, the results show that there was simultaneous increase in POD activity and total phenolic content due to increasing concentrations of Cu. Phenolic compounds are important antioxidant chemicals of plants which generally act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al.*, 1997) that protect plants from oxidative damages. The hydroxyl and carboxyl groups of phenols may be involved in binding heavy metals like iron and copper (Jung *et al.*, 2003). It has been reported that defense related genes involved in phytoalexin and lignin biosynthesis are the most sensitive among all genes that are upregulated in response to Cu (Sudo *et al.*, 2008). Tea is a tannin rich plant and is tolerant to excess aluminium and manganese (Alscher *et al.*, 2007; Morina *et al.*, 2008). Protection against manganese has been attributed to direct chelation of manganese by the phenolic compounds (Michalak *et al.*, 2006). Copper may be more damaging due to its strong redox nature; nevertheless, from the present results, certain degree of tolerance mediated by antioxidant chemicals and enzymes is evident in the two tested cultivars of tea.

The SOD activity increased with increasing Cu concentrations during the first 7 days (Fig. 2B). The more tolerant cultivar, TS-462 recorded a higher increase in enzyme activity, however, with the longer exposure time and above 400  $\mu$ M Cu concentrations, the activity levelled off. In TS-520, SOD activity decreased above 400  $\mu$ M but after 10 days of exposure at the highest tested concentration (700  $\mu$ M), the activity returned almost to the original level. The present results are in agreement with the previous studies on response of SOD to Cu stress (Ke *et al.*, 2007; Hartley-Whitaker *et al.*, 2001; Wang *et al.*, 2004). The SOD enzymes provide first line of defence against ROS by removing superoxide radicals and protect plant tissue from oxidative injury. The SOD isozymes are metalloenzymes which utilize metal cofactors like Cu, Zn, Fe and Mn for activity (Alscher *et al.*, 2002). A deficiency or excess of these metals can therefore affect their activity (Xiong *et al.*, 2008). Upadhyaya and Panda

(2004) observed that SOD activity increased significantly in tea leaves subjected to drought stress and decreased when rehydration was imposed. Aluminium exposure also caused an increase in SOD activity in cultured tea cells (Ghanati *et al.*, 2005). 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  $\mu\text{M}$ . A sharp increase in APX activity was noticed at the 10<sup>th</sup> day of exposure at concentrations 400  $\mu\text{M}$  to 600  $\mu\text{M}$  in the more tolerant cultivar, TS-462 (Fig. 4A). However, at 700  $\mu\text{M}$ , the activity declined. Gupta *et al.* (1999) also observed a late increase in APX activity in *Phaseolus vulgaris* subjected to Cu stress. Increase in both POD and APX activities in tea leaves suggests that the antioxidative machinery induced by Cu was involved in detoxification of  $\text{H}_2\text{O}_2$ . In this study, CAT activity showed an insignificant increase in both the cultivars of tea (Fig. 4B). Moreover, no significant difference was observed between the cultivars exposed to excess Cu ( $P < 0.05$ ). Thus, CAT activity remained unaltered or was marginally increased in response to oxidative damage induced by Cu. The CAT activities were found to decrease or remain unaffected in Cu-stressed oat leaf segments (Luna *et al.*, 1994), rice seedlings (Chen *et al.*, 2000) and tomato seedlings (Mazhoudi *et al.*, 1997). On the other hand, CAT activity was reported at significantly higher levels in Cu-stressed *Prunus cerasifera* plantlets (Lombardi and Sebastiani, 2005). Catalase represents a primary enzymatic mechanism which is used by aerobic organisms for the decomposition of toxic  $\text{H}_2\text{O}_2$  generated during oxygen metabolism (Havir and Mchale, 1987). In the leaf cells, CAT is exclusively located in the peroxisomes, while  $\text{H}_2\text{O}_2$  mainly accumulates in the chloroplast through dismutation of superoxide anion radical generated due to photoreduction of dioxygen in a reaction mostly catalyzed by Cu-Zn SOD (Asada, 1992). Ascorbate peroxidase participates in scavenging of  $\text{H}_2\text{O}_2$  in the chloroplast using ascorbate as the electron donor (Asada, 1992; Shigeoka *et al.*, 2002). The present findings showed that excess  $\text{Cu}^{2+}$  ions caused considerable increase in APX activity along with SOD and POD activities which is evidently a consequence of an excess accumulation of  $\text{H}_2\text{O}_2$  in tea leaves. But it appears that CAT was not markedly mobilized for protection against this oxidative injury possibly because the excess accumulation of  $\text{H}_2\text{O}_2$  inactivated CAT (Wang *et al.*, 2004). 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  $\text{H}_2\text{O}_2$ . 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 defense enzymes (Shigeoka *et al.*, 2002). 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  $\mu\text{M}$  while the more tolerant cultivar being able to withstand a maximum of 600  $\mu\text{M}$  of Cu treatment.

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