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EFFECT OF COPPER ON GROWTH AND CHLOROPHYLL CONTENT IN TEA PLANTS (*CAMELLIA SINENSIS* (L.) O. KUNTZE)

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ABSTRACT

Tea is the oldest, most popular, non alcoholic caffeine containing beverage, in the world. The presence of heavy metal even in trace amount causes adverse effect both in quality and quantitative production. The accumulation of heavy metal causes health issues also. In the present work we have studied the effect of chlorophyll content which is an important photosynthetic parameter with the high concentration of copper stress with respect to the days of the treatment in two different cultivars *viz.* TV-23, TV-17 in hydroponically system. Both shoot and root weight decreased progressively with increasing Cu concentration for *Camellia sinensis*. Negative linear relationships were observed between total Chlorophyll contents and Cu concentration. With the increase in the concentration of Cu even at trace level causes deleterious effect in the metabolism of plant especially photosynthetic activity which caused remarkable breakdown in the photosynthetic parameters. A significant decrease of chlorophyll started for both the cultivars was observed at concentrations above 300µM. From this study, we found the photosynthetic activity of TV-17 is more sensitive to Cu stress than that of TV-23.

KEYWORDS: Tea Plants, Copper Stress, Physiological Characters, Chlorophyll Pigments

INTRODUCTION

In physiological process of plants heavy metal plays a vital role. In trace amounts, several ions required for metabolism, growth and development which is present in soil or as in growth media. But with the increase in the amount, creates problem by leading to cellular damage of plants (Avery, 2001, Schutzendubel and Polle, 2002, Gaetke and Chow, 2003). This leads to the inactivation of biomolecules by blocking essential functional groups or by displacing the essential metal ions (Goyer, 1997). Due to industrialization and globalization the heavy metal contamination became a serious problem (Boominathan and Doran, 2003; Plunkett, 1987; Seiler et al., 1988; Donmez and Aksu, 2002). And heavy metal became a serious threat for the environment due to their extreme toxicity (Kaewsarn & Yu, 2001). Leaves of tea plants are great source of mineral such as Zn, Cu, Mn, F, P etc. Cu is essential for plant growth but increase in the concentration of both the essential and non essential metals will result in inhibition of growth (J.L.Hall, 2002). In the presence of excessive amount of heavy metal shows the toxicity symptoms due to interactions of ions at cellular level. Much of the soluble copper in the apoplast is complexed with organic ligands. Much of Cu^{2+} is tightly bound in the cell wall of roots and can only be replaced by other Cu^{2+} ions (Iwasaki et al., 1990; Walker & Webb, 1981). Carotenoids are the pigments which are more or less produced by all photosynthetic and non photosynthetic organisms. The carotenoids are very important for plants, lack of carotenoids do functioning both in the acquisition of light energy and in protection of photosynthetic apparatus against excessive light damage (Demmimng-Adams et al., 1996). Chlorophylls are essential components for photosynthesis, and occur in chloroplasts as green pigments in all photosynthetic plant tissues. They are bound loosely to proteins but are readily extracted in organic solvents such as acetone or ether. In this study we have investigated the physiological mechanism of Cu induced inhibition of photosynthesis with accumulation of Cu at different concentration.

MATERIALS AND METHODS

Experimental Condition for the Growth of the Plants

Three months old plants of the two cultivars viz. TV-23, TV-17 were collected from the Rosekandy Tea Estate, Assam. Then transferred the plants to the Hoagland solution and allowed them to get stable for 7 days. Then we have treated the plants with $CuSO_4$ at different concentration of 50µM, 200µM, 300µM,400µM, 500µM, 600µM in the nutrient solution. The control plants were left as untreated, comprising only the nutrient solution. The top four leaves were collected for measuring the chlorophyll pigmentations after 2nd, 4th and 7th day of the treatment.

Determination of Chlorophyll

Both the treated leaves and the control leaves at the respective days were harvested and kept in liquid nitrogen for frozen and then lyophilized. The content of chlorophyll of the mature leaf of the plant was determined in 80% acetone extract of 0.1 g leaf and expressed as µg/g fresh weight (Hegedus et al., 2001).

Based on the data obtained from the experiment, the results presented are the mean ± standard deviation (SD) gained from at least three replicate samples. Statistical analysis has done by the least significant difference (LSD) for multiple comparisons, taking $P<0.05$ as significant.

RESULTS

Effect of Copper on Plant Growth

From the above experiment we have observed that at the different concentration of Cu plants are showing different symptoms. With compare to control there is a significant retardation in the growth of the Cu treated plants. At lower concentration leaves are least effected than that of roots and shoots (Figure 1). But with the increase in the concentration of Cu which severely inhibit the root growth with accumulation of Cu in leaves (Figure 2). Black brown spots were observed in the leaves at higher concentration. The roots were suffered a lot by retarding the growth of the length of roots. The number of new leaves slowed down with the less arrival of new stems. As a whole the growth has slowed down. The dry weight of shoots of both the cultivars was retarded with respect to control (Figure 4 & 5). With the increase in the concentration there was a significant decrease in the weight. Through the physiological study we have observed the slow growth rate of the treated plants with respect to control and at higher concentration of 500 µM & 600 µM were almost negligible.

Effect of Copper on Photosynthetic Pigments

There was a significant decrease in the total chlorophyll pigment in both the treated cultivars with respect to control. But the retardation level is high at TV-17. The expanding leaves of TV-23 had higher total chlorophyll than those of TV-17 (Figure 6 & 7).

The contents of pigment in plants showed an almost linear decrease in response to the increase in the concentration of Cu which ranges from 0 µM (control) to 600 µM. At higher concentration the level of decline in the content of chlorophyll has observed more than compare to lower concentration. With the exposure days the chlorophyll content was decreased.

Figure 1: Small Yellow Patches on Leaves at 200µM of Cu in TV-23

Figure 2: Effects of Leaves at 500µM of Cu in Tv-17

Figure 3: Effects of Leaves at 500µM of Cu in Tv-23

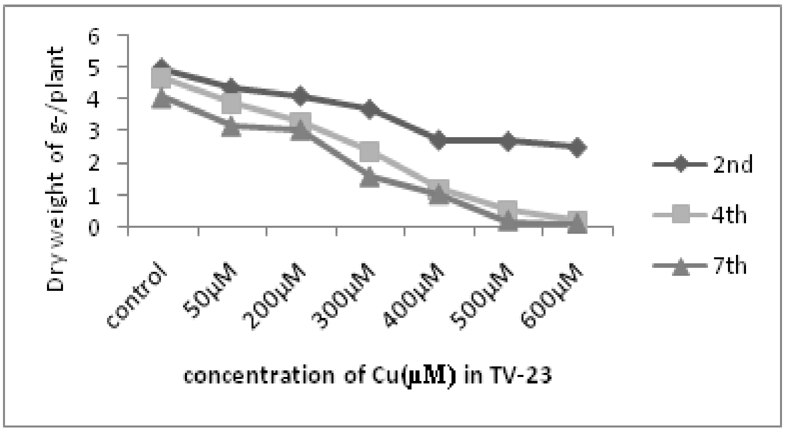

Figure 4: Impacts of Cu on the Dry Weight of Shoot of TV-23. Values are Average of Three Replicates

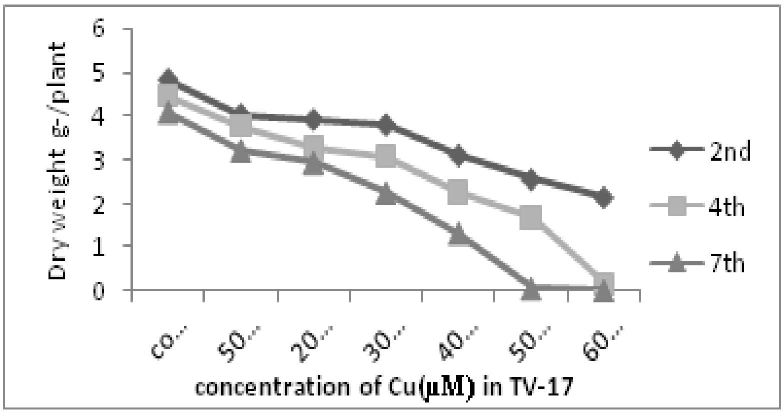

Figure 5: Impacts of Cu on the Dry Weight of Shoot of TV-17. Values are Average of Three Replicates

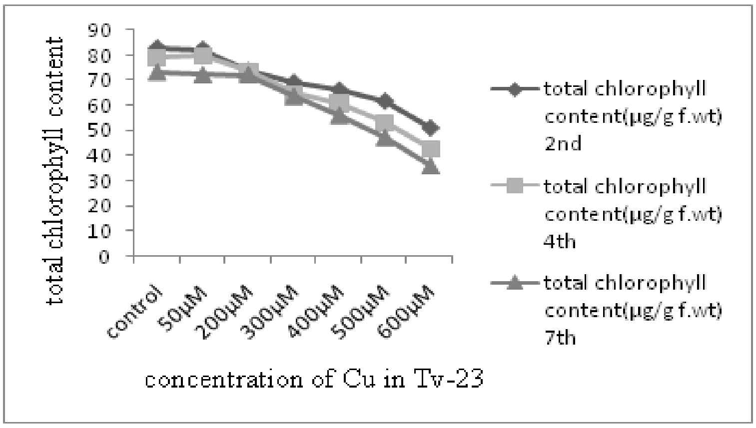

Figure 6: Impacts of Cu on the Total Chlorophyll Content of TV-23. Values are Average of Three Replicates

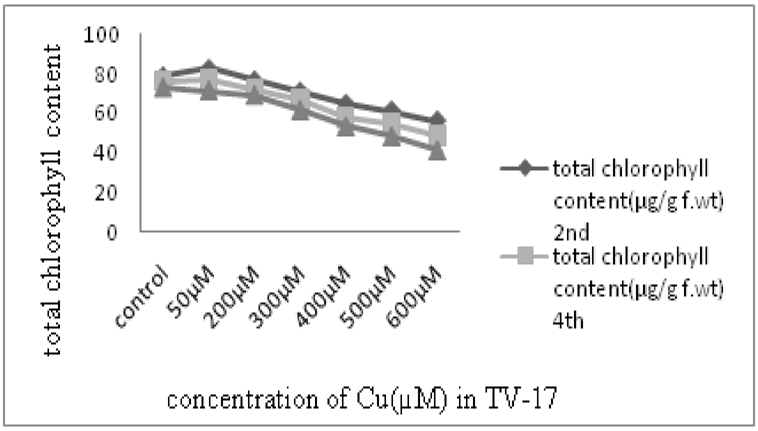

Figure 7: Impacts of Cu on the Total Chlorophyll Content of TV-17. Values are Average of Three Replicates

DISCUSSIONS

Heavy metal inhibits the plant metabolism, but some metals like Cu, Fe, Mn are essential for the photosynthesis. There are number of proteins and enzymes found in heavy metals and so they are essential for the growth and development of plants and also maintain the optimum metabolism. The deficiency of these metals has direct impact on the process of photosynthesis, but with the increase in concentration of these metals become toxic to plant and affects photosynthesis. The growth parameters like biomass have been shown to very sensitive to heavy metals (Arun *et al*., 2005). Our research has illustrated that Cu inhibited the plant growth by retarding the growth of root, shoot, and decline in the numbers of arrival of new leaves. The dry weight decreased progressively with the increase in the Cu concentration (Figure 4 & 5). At lower concentration the dry weight decrease with less or without showing toxic in leaves (Figure 1). But at higher concentration severely inhibit the root growth with accumulation of Cu in leaves (Figure 2). The chlorophyll contents in the cultivars TV-23, TV-17 has decreased with the increase in the concentration (Figure 6 & Figure 7).

With high concentration of heavy metals, the activities of photosynthetic enzymes degrade which results in the reduction of chlorophyll content (Thapar et al., 2008). In most of the plants Cu is found associated with plastocyanin, an important component of the electron transport chain of PSI in the chloroplast. Copper deficiency reduces PSI electron transport due to decreased formation of plastocyanin (Baszynski et al.; 1988). A decrease in PSII is also observed in Cu deficient plants (Henriques; 1989). The biochemical and photochemical reaction centres of photosynthesis are the important sites of inhibition by the action of heavy metals especially by Cu. Cu ion insert directly to the reaction centre specially PSII which due to the high irradiance causes direct damage to the reaction centre (Kupper et al., 1996, 1998, 2002). Copper interferes with the biosynthesis of the photosynthetic activity by changing the pigment and protein composition of photosynthetic membranes. A lower content of chlorophyll, inactivation of enzymes and proteins linked to photosynthesis process and modification of thylakoid membranes occurs under copper toxicity. Copper-induced chlorosis can result from the inhibition of pigment accumulation and decrease in the chlorophyll integration into photosystems. As a whole it causes damages in the structure and function of the chlorophyll (Kupper et al.; 2003).

Due to the Cu stress many oxidative stress also get generated and this as a whole cause the inhibition in the photosynthetic reactions which has been observed by many authors (Rocchetta and Kupper, 2009). With the increase in the concentration there is remarkable decrease in the chlorophyll pigment with respect to the control. At higher concentration 400µM, 500µM, 600µM with the exposure time the Cu treated plants are showing adverse effect by decline in the growth of shoot, decrease in length of root, growth of new leaves are very low, bleaching of leaves and fall off. All these result in the degradation of chlorophyll pigments. By binding to the various sensitive sites of the photosynthetic apparatus heavy metals can also directly affect the photosynthetic machinery. It gives us an evident that excess Cu has a strong effect on chloroplast fine structure which results in degradation of grana stacks and stroma lamellae (Baszynski et al.; 1988). Many authors have concluded the same in their observation.

Cu stress has decreased the photosynthetic level of *Crassula helmis* (Kupper *et al.*, 2009). Various authors gave evidences that the toxic effect of metal on phototropic organisms strongly appears to be related to the increase in the levels of lipid peroxidation and protein carbonylation as well as the production of antioxidant defense systems (Gallego et al.; 1996, Devi and Prasad; 2005, Tripathi et al.; 2006). In the different season the chlorophyll pigment shows different adverse action. At summer the degradation level is very high with respect to other season like autumn and winter. In high irradiance conditions Cu stressed plants showed different results in leaves of *Triticum aestivum* with the decline in the Fv/Fm (Lanaras *et al.*, 1993). The accumulation of Cd decreased net photosynthesis and transpiration in excised Acer sacchanium L. leaves (Lamoreaux R J et al.; 1978). From the above study it can be concluded that certain concentration of Cu inhibit the growth of plant, cause chlorophyll loss and can affect the photosynthetic activities.

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Copper-induced changes in growth and antioxidative mechanisms of tea plant (*Camellia sinensis* (L.) O. Kuntze)

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Tea is the oldest, most popular, non-alcoholic caffeine containing beverage in the world. Tea plants are prone to the attack of many diseases which can be controlled by the treatment of pesticides. These pesticides contain heavy metal which prolonged accumulation can lead to the damage of crop yield both in quantity and quality. In the present study, we observed the effects of high concentration of Cu stress on physiological and biochemical parameters. The tea cultivars (S_3A_3 and TS-491) were collected from the Rosekandi Tea Estate, Silchar, Assam. The accumulation of Cu in the different parts of the tea plants had a positive correlation with the Cu stress. The accumulation of Cu was higher in roots than in leaves and also the new stems. The results show gradual decrease in the photosynthetic activity with the increase in the concentration of the Cu stress in both the cultivars. The activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) increased with the increase in the Cu concentration but remarkably in different manner in both the cultivars. The activities of antioxidants ascorbate peroxidase (APX), CAT, SOD and POD in cultivar S_3A_3 increased up to 500 µM and at 600 µM showed a low rate of increase whereas TS-491was tolerant up to 400 µM. The responses in the oxidative stress were characterized by an accumulation of malondialdehyde (MDA). Phenol content has also positive correlation with increase in the concentration in both the cultivars up to 500 µM. Finally it was concluded that Cu is tolerant to the cultivars of tea plant to some extent (specifically at lower concentration), but at higher concentration (beyond 400 µM) of Cu with exposure time, tea plant had a strong inhibition of growth by damaging the normal metabolism.

Key words: Tea plants, copper stress, physiological characters, lipid peroxidation, reactive oxygen species.

INTRODUTION

Camellia sinensis (L.) O. Kuntze is an economically important crop for production of tea leaves. Tea is the oldest, most popular, non-alcoholic caffeine containing these are toxic to plants when they are present in soil or the growth media is above the permissible level (Xu and Shi, 2000). Sometimes, heavy metals like Cu, Cd, As, Zn, beverage in the world. Copper (Cu), cadmium (Cd), nickel (Ni), manganese (Mn) and zinc (Zn) are the essential micronutrients required for the plant life but concentra-Mn, Ni, etc. are hazardous pollutant even at very low con-tion (Ross 1994, Prasad and Strzalka 2002). Due to rapid industrialization and urbanization, there is an elevated emission of toxic heavy metals which enters the biosphere and affects the growth and development of plants (Nriagu and Pacyna, 1988; Kabata- Pendias, 2001). Pesticides and fertilizers are the other major sources of heavy metal which are directly taken up by plants. As Cu is an essential element, it participates in number of physiological processes, and is an essential cofactor for many metalloproteins, but due to excess amount of copper present in cells, causes problems by inhibiting the plant growth which impairs important cellular processes like photosynthetic electron transport (Demirevska-kepova et al., 2004). Since copper is both an essential cofactor and a toxic element, involving a complex network of metal trafficking pathways that must prevent accumulation of the metal in the freely reactive form (metal detoxification pathways) and ensure proper delivery of this element to target metalloproteins is necessary. Cu as fungicides and pesticides are very effective in the control of the diseases in tea plants and are widely used (Gallagher et al., 2001; Singh, 2005), but higher concentration can cause adverse effect in plants by lowering the chlorophyll content, delay in flowering, reduction in the number and quality of shoots which leads to fall down in the quality of tea (Setia, Kaur, and Setia, 1989). The accumulation of Cu at the higher concentration through direct contact or from the food chain is harmful to human beings who consume tea. Heavy metals such as iron, copper, zinc, nickel, manganese, lead and cadmium can cause oxidative stress, producing enzymatic and non-enzymatic antioxidative reaction responses and lipid peroxidation in plants. The exposure of Cu can cause toxicity to the plants which is due to oxidative damage to the biological macromolecule by redox cycling, depletion of glutathione and alteration of homeostatis (Stohs and Bagchi, 1995). Due to the accumulation of Cu in excessive manner leads to the production of reactive oxygen species (ROS) which can damage lipids, nucleic acids, proteins, amino acids, carbohydrates and other complex molecules produced from all these in cells (Pietrini et al., 2003). To overcome such stress, plants evolved much effective mechanism to detoxify the ROS (Dat et al., 2000). A group of effective antioxidants are present in plants to maintain the antioxidant potential in the cells, which detoxify the production of reactive oxygen species. Many researches showed that amounts of antioxidant enzymes like superoxide dismutase (SOD) and peroxidase (POD) increase in plants when highly exposed to heavy metals (Acar et al., 2001). Peroxidase plays a crucial role in physiological events which are related to diminishing growth of plants by lignification, cross-connection of cell wall polysaccharides, oxidation of indole-3-acetic acid (IAA), cell elongation and phenol oxidation (Mocquot et al., 1996). Rate of ROS formation and efficiency, and capacity of detoxification mechanisms in plants determine the level of damage of cells under stress conditions. Hence in this study, we observed the effects of Cu stress at different concentrations on some physiological indicators such as growth (shoot and root length), rate of photo-synthesis, enzymatic activity of scavengers of ROS such as SOD, peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), malondialdehyde (MDA) on two cultivars of tea plants *viz.* clonal tea plants, S_3A_3 and the tea plants produced from seeds, TS-491.

MATERIALS AND METHODS

Experimental condition

Three months old plants of the two cultivars viz. S_3A_3 (clonal propagated) and TS-491(developed from seeds) were collected from the Rosekandy Tea Estate, Assam, India. The plants were then transferred to Hoagland solution as nutrient media and allowed to get stable for seven days. The plants were treated with $CuSO_4$ at different concentrations of 50, 200, 300, 400, 500 and 600 µM in the nutrient solution (Hoagland and Amon, 1938). The control plants were left as untreated, and allowed to grow only in Hoagland solution. The top four leaves were collected for measuring the various enzymatic activities after 2^{nd}, 4^{th} and 7^{th} days of the treatment.

Morphological and growth analysis

The roots, stem and leaves of the two cultivars S_3A_3 and TS-491 were collected and analyzed for the morphological characters like growth of the different parts of the plants, appearance of new leaves, new stems etc.

Determination of chlorophyll content

The chlorophyll content of the matured leaves of both treated and control plants of both cultivars was determined following the protocol of Hegedus et al. (2001). 0.1 g leaf extract was dissolved in 80% acetone and optical density was determined using UV -VIS spectrophotometer (Thermo Fisher) at 645 and 663 nm, respectively. The values of optical density was assayed and

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Abbreviations: ROS, Oxygen species; **POD,** peroxidase; **APX,** ascorbate peroxidase; **CAT**, catalase; **SOD,** superoxide dismutase; **PVP,** polyvinylpyrrolidone; **NBT,** nitrobluetetrazolium; **MDA,** malondialdehyde expressed as µg g^{-1} fresh weight.

Assay of enzyme activity

For determining the enzyme activities, 0.5 g leaves were dippedi

liquid nitrogen and then homogenized in a chilled mortar and pestle in 5 ml 50 mM cold phosphate buffer of pH 7.8 containing 2% polyvinylpyrrolidone (PVP). The filtrate homogenate was centrifuged at 13000 *g* for 20 min at 4°C and the supernatant were used for determining the enzyme activities. The antioxidant ability of leaves was determined by measuring the POD activity at absorbance of 470 nm due to the oxidation of guaiacol (Wu and von Tiedemann, 2002). SOD activity was measured by the inhibition of nitrobluetetrazolium (NBT) reduction (Krivosheeva et al., 1996). CAT activity was measured with the help of the absorbance at 240 nm (Pinhero et al., 1997).

Determination of lipid peroxidation and phenolic content:

The 0.5 g fresh weight of leaves was homogenized in a pre-chilled mortar pestle in 5 ml of 50 mM cold Na-phosphate buffer (pH 7.8), with 0.1 mM EDTA and 1% (w/v) PVP. After centrifugation at 13,000 *g* for 30 min at 4°C, supernatant was used for further analysis. The level of peroxidation was determined in terms of 2-thiobarbituric acid (TBA) (Liu et al., 1996). To determine the total phenolic components, the 0.5 g of leaves were extracted in ethanol and then the content was estimated with the changes in absorbance at 520 nm (Mahadevan and Sridhar, 1996).

Statistical analysis

All the experiments were done in triplicate and the mean were taken. Statistical analysis of mean values and standard deviations (SD) were performed for all the data. The significant difference was set between treatments at $p < 0.01$ or $p < 0.05$.

RESULTS

Effect of high concentration of copper on tea morphological characters

Tea plants showed different degrees of symptoms in different concentrations of copper stress. With the increase in the concentration of copper, the roots suffered mostly which is followed by leaves and then new stems (data was not given). The leaves first developed some brown spots, yellow patches, become dry and then fell off. At the highest concentration of 500 and 600 µM, less number of new leaves appeared, and most of the leaves became withered in both the cultivars. The number of new stems was very less and gradually the growth rate decreased. The fresh and dry weights of roots and leaves were analyzed and the results reflected that growth rate of the copper treated plants with respect to the control was less. The dry weight of the organs of the treated plants decreased with the increase in the concentration of the copper in both cultivars with respect to exposure days. In the highest concentration (600 µM), the inhibition was the strongest with the smallest growth rate of roots, leaves and stem compared to tea plants treated with lower concentrations.

Effect of Cu on chlorophyll content

Chlorophyll is an important pigment which plays a vital role in the process of photosynthesis. Table 1 shows that there was a significant negative correlation between the concentration of Cu and the chlorophyll contents in the tea plants. With the exposure of high concentration of copper, the total chlorophyll content of both the cultivars decreased. The level of decrease of chlorophyll content is high in TS-491 than the cultivar S_3A_3. From Table 1A, we can conclude that at higher concentration of 400, 500 and 600 µM, there was a gradual decrease in the chlorophyll content in cultivar S_3A_3 whereas from Table 1B, we observed the decrease was more than two fold in cultivar TS-491 compared to S_3A_3. In S_3A_3, at control, the total chlorophyll content was 90.12 µg g^{-1} fresh weight on 2nd day and chlorophyll content decreased gradually as the Cu concentration increased; whereas, maximum decrease in chlorophyll content was found to be started from 400 µm onwards where the content has degraded and ended up at 42.13 µg g^{-1} fresh weight on 7th day at 600 µm Cu concentration. Whereas in TS-491, the chlorophyll content was 94.12 µg g^{-1} fresh weight on 2nd day at control, and from 300 µM concentration onwards, the content degraded more than two folds and ended up at 18.09 µg g^{-1} fresh weight on 7th day at 600 µm Cu concentration. The observation indicates that compared to cultivar S_3A_3, cultivar, TS-491 was more sensitive to copper stress.

Effect of high concentration of Cu on antioxidative enzymes

Plants are exposed to abrupt stress daily and seasonal changes in the environment, and they have a wide spectrum of developmental responses and biochemical adaptations to stress condition. During normal metabolic activities and due to the consequences of environmental condition, O_2 is capable of giving rise to reactive oxygen species (Mittler et al., 2004). ROS include the superoxide radical, hydroxyl radical and hydrogen peroxide which are toxic to plants (Dismukes et al., 2001, Vellosillo et al., 2010). The activities of APX, CAT, SOD and POD gradually decreased with increase in the concentration of Cu.

It was observed that with respect to control, there was a significant increase in the APX activity in S_3A_3 cultivar up to the 4th day of 600 µM concentration, but there was a slight decrease in the APX activity on the 7th day of600 µM (Figure 1). On the other hand, there was only a

Table 1. Effects of different concentration of Cu on total chlorophyll contents of cultivar (A) S3A3 (B) TS-491.

Treatment (μM)	2^{nd} day	4^{th} day	7^{th} day
	μg g^{-1} fresh weight		
(A) S_3A_3			
control	90.12±0.52	88.92±0.78	86.58±0.93
50	86.25±0.38	85.65±0.67	82.29±1.4
200	83.31±0.26	82.62±0.81	80.19±1.36
300	81.02±0.29	79.93±0.51	75.14±0.86
400	71.92±0.42	68.23±0.86	63.89±0.99
500	65.23±0.36	62.18±0.91	56.12±0.69
600	54.23±0.39	50.19±0.57	42.13±0.86
(B) TS491			
control	94.14±1.08	92.01±1.46	89.03±1.49
50	90.13±1.09	87.32±1.26	84.28±0.99
200	83.93±0.92	80.02±1.21	78.21±1.22
300	73.32±1.03	70.89±1.22	67.12±1.36
400	61.07±0.99	52.87±0.87	49.89±1.32
500	49.27±1.29	36.87±1.27	32.13±1.36
600	36.23±1.28	23.9±0.99	18.09±1.21

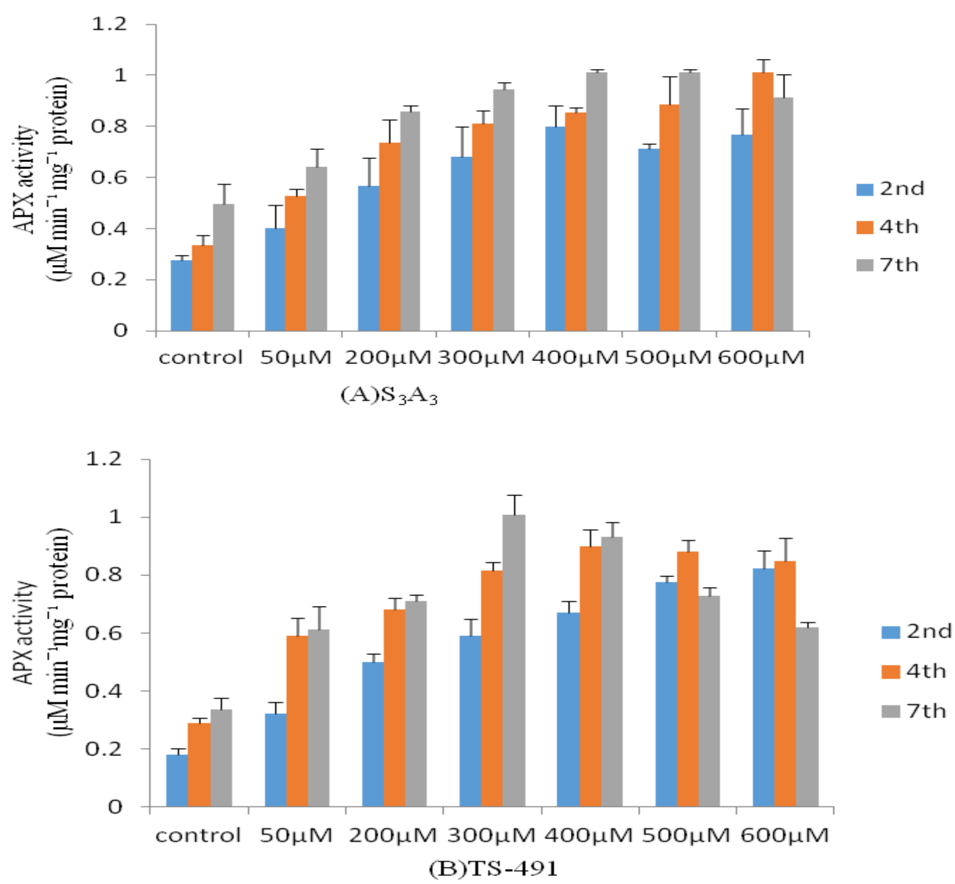

Figure 1. Effect of increasing concentrations of Cu on APX activity in leaves of two cultivars of tea S_3A_3 and TS-491.

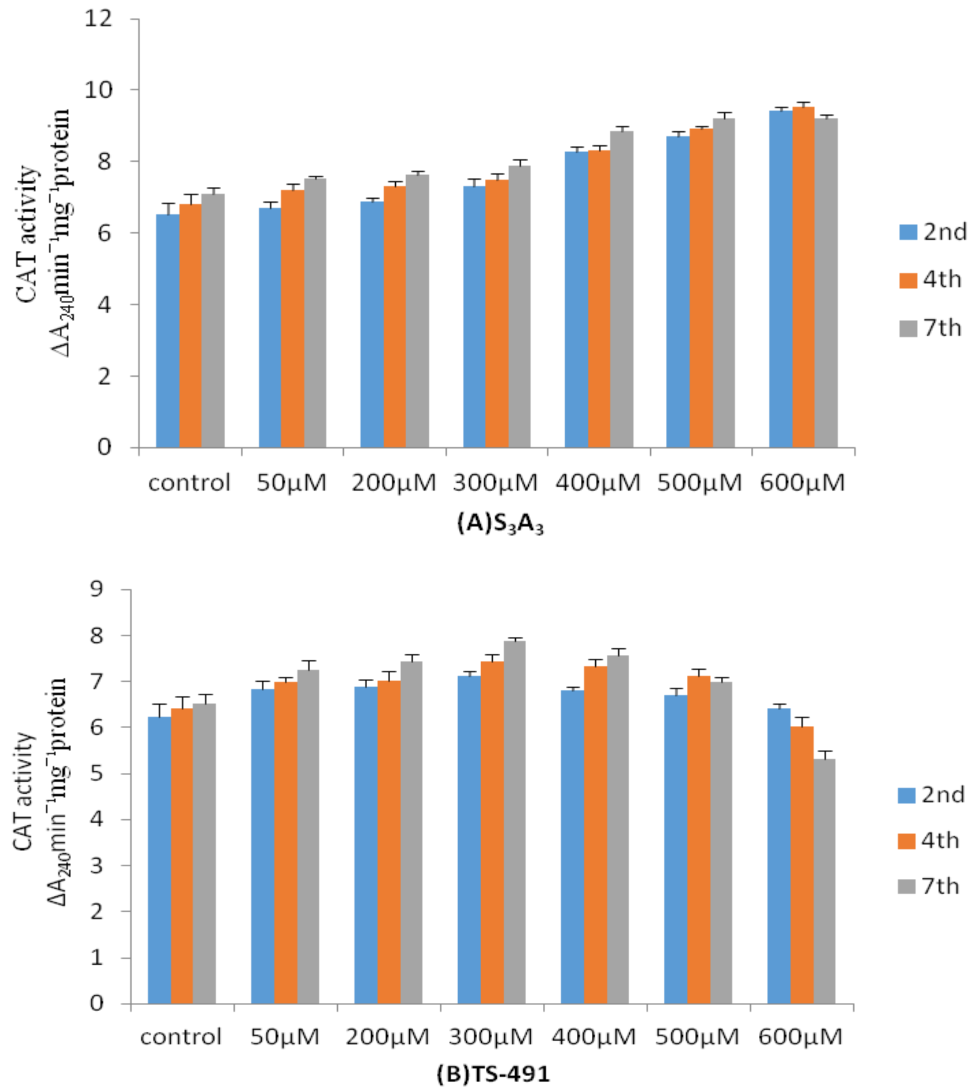

Figure 2. Effect of increasing concentrations of Cu on CAT activity in leaves of two cultivars of tea S_3A_3 and TS-491.

marginal increase in the APX activity with the exposure of Cu up to 200 µM in TS491 cultivar; but further increase in the concentration of Cu resulted in a significant increase (more than two fold) in the activity specially from the 2nd day of 300 µM and continued up to 4th day of 400 µM compared to the lower concentration, finally the APX activity started to decline from 7th day of 500 µM in TS-491. Catalase has double function, as a primary enzymatic mechanism it generally decomposes the toxic H_2O_2 generated during oxygen metabolism by the aerobic organisms and it also catalyses the oxidation of H donors with the consumption of one mole of peroxide (Havir and Mchale, 1987). From Figure 2A and B, it was observed that there was a significant increase in the CAT activity occurs in both the cultivars. S_3A_3 is found to be more tolerant compare to the cultivar TS-491. The increase in the CAT activity of S_3A_3 cultivar continued even at higher concentration of Cu up to 4th day of 600 µM; but, there was slight decrease in the activity on the 7th day of 600 µM. whereas in TS-491 the decrease in the CAT activity was observed from 400 µM itself. The same trend of result has been found in the activity of SOD in both the cultivars. From Figure 3A, it can be observed that in the cultivar S_3A_3 the activity showed a positive correlation with the increasing concentration. There was increase in the SOD activity by one fold with the subsequent increase in the concentration with respect to the exposure days. Whereas in cultivar TS-491, the increase in the activity was up to 400 µM concentration and with further increase in the concentration the activity was

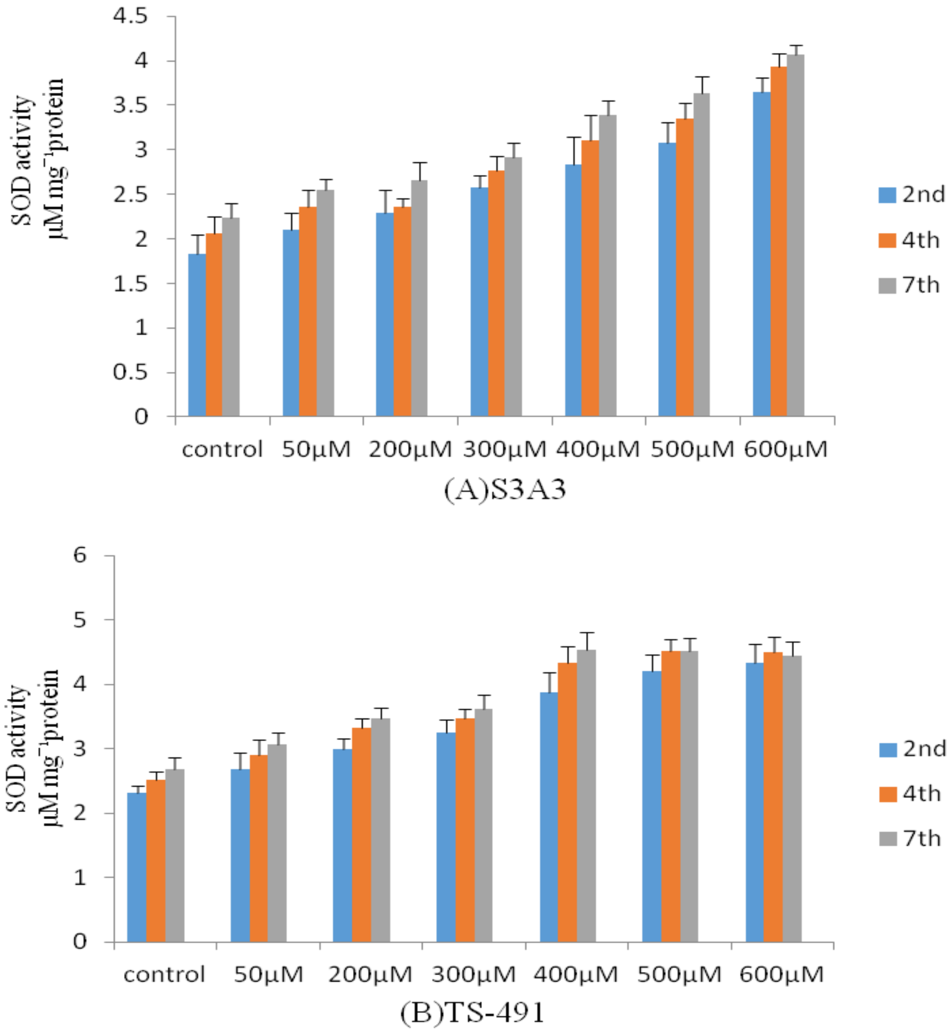

Figure 3. Effect of increasing concentrations of Cu on SOD activity in leaves of two cultivars of tea S_3A_3 and TS-491.

declined (Figure 3B). In cultivar S_3A_3, the POD activity has increased with the exposure of higher concentration of Cu (600 µM) (Figure 4A). In cultivar TS-491, the increase in the activity was more than two fold up to 400 µM concentration of Cu, but the level of increase in the activity lowered at 500µM whereas at 600µM there was total decline in the activity of POD from 7th day onwards. POD plays an important role in plant respiratory metabolism and physiological resistance. From the result, it can be observed that S_3A_3 cultivar is more tolerant to higher concentration of Cu compared to TS-491. The adverse action of biotic and abiotic stress affects the active oxygen metabolism of plants.

Effect of phenolic contents and lipid peroxidation

Phenols are aromatic compounds with hydroxyl groups, which offer resistance to diseases and pests in plants. From Figure 5A and B, it can be observed that the cultivar S_3A_3 has a significant increase in the total phenolic compound up to 500 µM and at 600µM; the level of increasing in the activity has slowed down slightly. On the other hand in TS491, there was increase in phenolic content up to 4th day of 500 µM and then there was a decline in the activity from 7th day of 500 µM. MDA is the final product of lipid peroxidation and its content reflect stress tolerance of plants (Liu et al., 2001; Sugiyama, 1994). It can be observed that with the increase in the concentration of Cu, the MDA content of tea cultivars increased and showed a significant positive correlation with Cu concentration. The level of increase in the peroxidation activity was less in S_3A_3 compared to TS-491 (Figure 6A and B). This shows that high concentration of Cu can lead to lipid peroxidation which

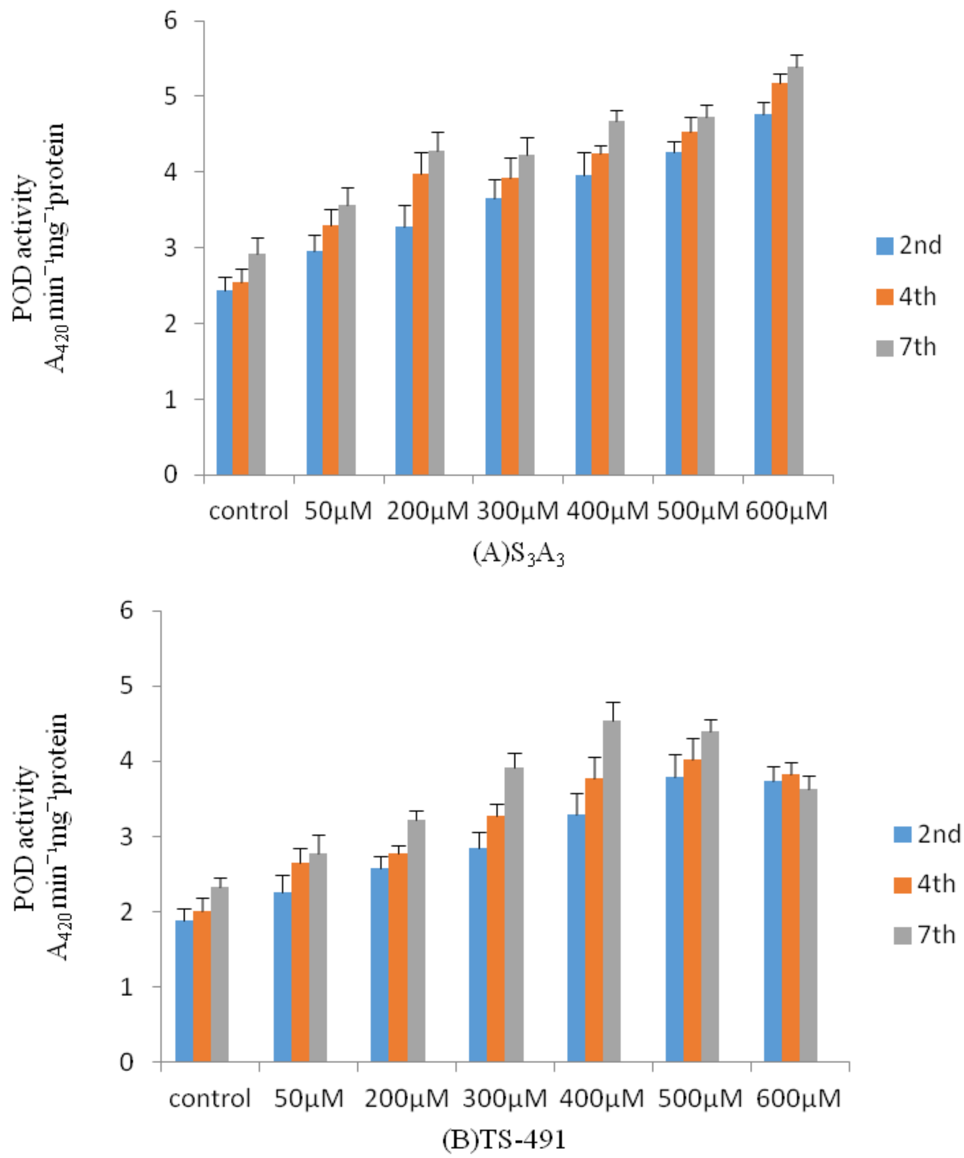

Figure 4. Effect of increasing concentrations of Cu on POD activity in leaves of two cultivars of tea S_3A_3 and TS-491.

causes damage to the balance of ROS scavenging activities of tea plants.

DISCUSSION

Heavy metals play a vital role in the physiological process of plants. In trace amounts, several ions are required for metabolism, growth and development which are present in soil or as in growth media. Number of proteins and enzymes are found to contain heavy metals which make them essential for the growth and development of plants and also helps in maintaining the optimum metabolism; but when the amount exceeds, it creates problem in the plants by leading to cellular damage (Avery, 2001; Schutzendubel and Polle, 2002; Gaetke and Chow, 2003). Though Cu is an essential component for both the photosynthetic and respiratory electron chains, but excess of Cu can cause changes in permeability in membrane, chromatin structure, synthesis of protein, enzyme activity, photosynthesis and respiratory processes through its phytotoxic effect and also can causes lipid peroxidation and lead to activation of senescence (Baryla et al., 2000). Cu in Free State binds irreversibly to SH group which are involved in the catalytic action of enzymes (Van Assche and Clisjters,

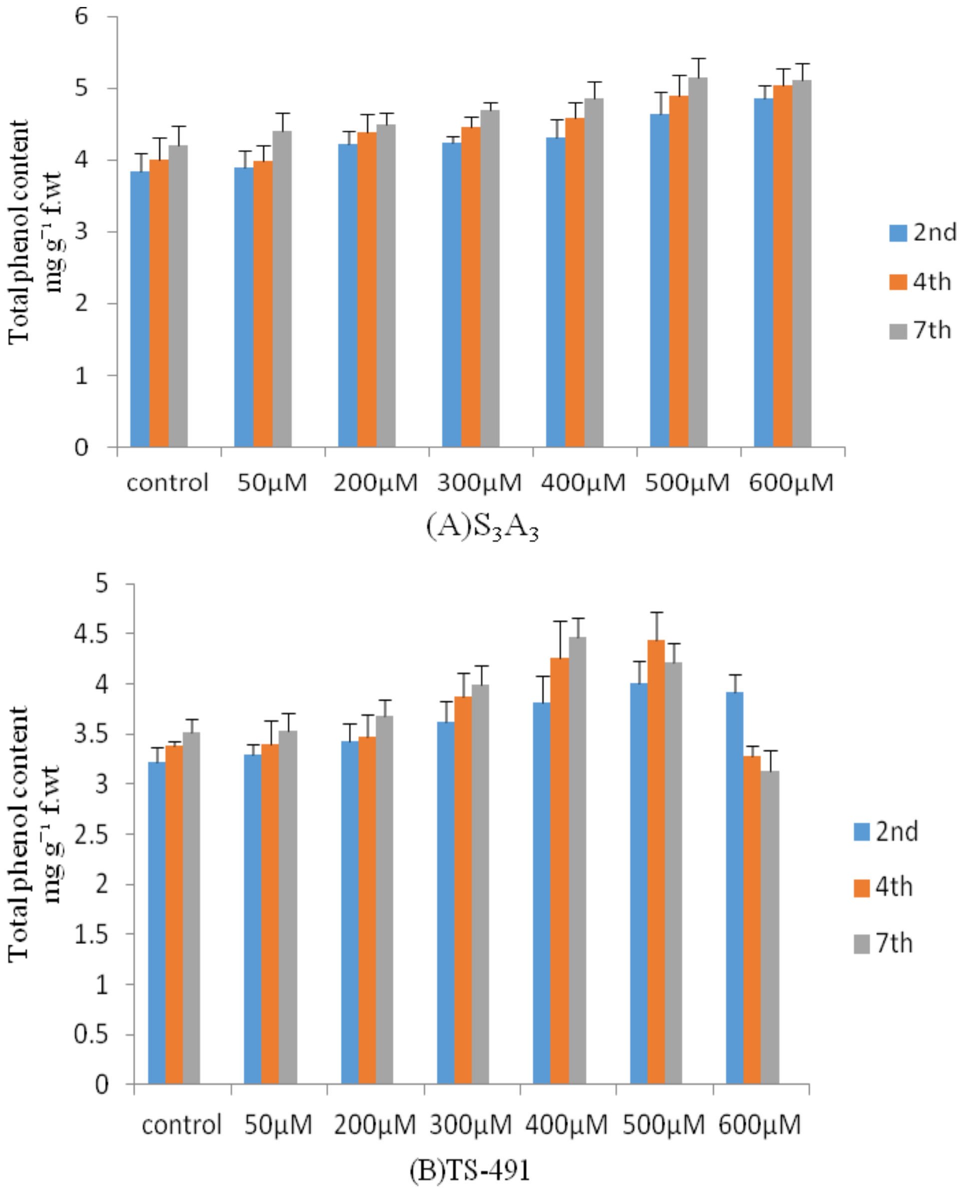

Figure 5. Effect of increasing concentrations of Cu on total phenol content in leaves of two cultivars of tea S_3A_3 and TS-491.

1990). In the present investigation, we observed that the chlorophyll content decrease with increase in the concentration of Cu. Other investigators also reported that with increase in the concentration of heavy metals, the activities of photosynthetic enzymes degrade which results in the reduction of chlorophyll content (Thapar et al., 2008). The content of chlorophyll has a close relation with photosynthesis (Liu et al., 2001), Cu ion is inserted directly to the reaction center especially PSII which due to the high irradiance causes direct damage to the reaction center (Kupper et al., 1996, 1998, 2002). Cu can change the pigment and protein composition of photo-synthetic membranes and interferes with the biosynthesis of photosynthetic activity. Declination in the chlorophyll content is the primary bio indicator of toxicity of Cu (De Vos et al., 1992). The same result of decrease in chlorophyll content was observed in tea plants when exposed to Cr stress (Tang et al., 2011). Many other authors also observed the same trend of decreasing of chlorophyll content with increase in Cu concentration (Rama Devi and Prasad, 1998; Mohanpuria et al., 2007; Saha et al., 2011). The present study indicates that the excess of Cu concentration decrease the chlorophyll content which in turn can cause inhibition to photosystem

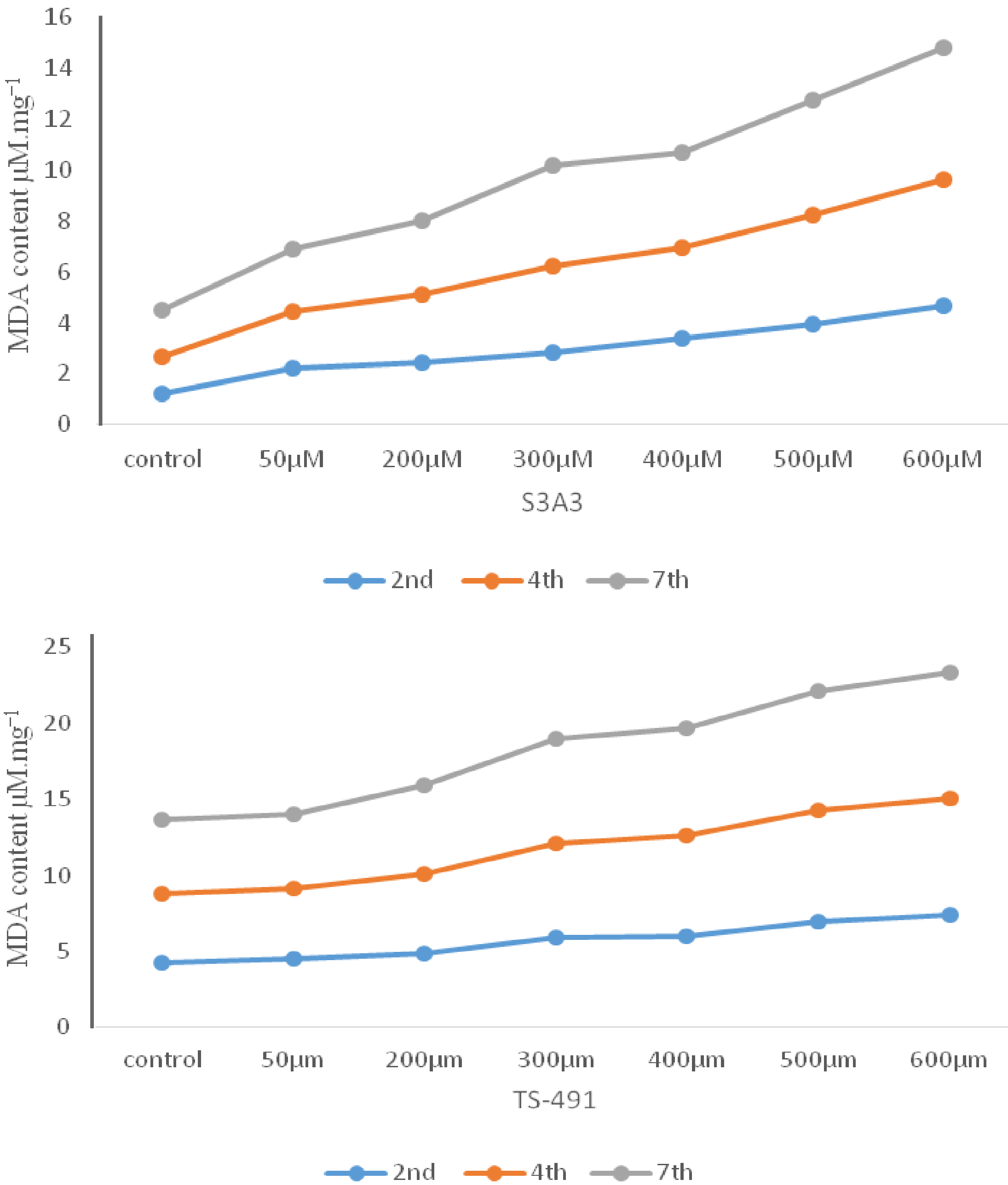

Figure 6. Effect of increasing concentrations of Cu on MDA content in leaves of two cultivars of tea S_3A_3 and TS-491

(Saha et al., 2012).

The different parts of the plants come in contact through the absorption of roots in the solution. In this study, we found that with the increase in the concentration of Cu, the level of it also increased in the different parts of the plants. The roots found to accumulate highest level of Cu as they are in direct contact with the Cu stress present in the nutrient media. The deposition of Cu in organ are in the following order roots> leaves> stems. The accumulating ability of root was stronger than other parts of the tea plants (Tang et al. 2011). In our study both the cultivars showed different symptoms due to different concentration of Cu and in the later phases of the experiment, the growth of the tea plants were inhibited. Tang et al., 2008 reported the same results when they grow tea plant in nutrient solution incorporated with Cu stress. Cu toxicity was found to affect the growth of *Alyssum montanum* (Ouzounidou, 1994), Singh and Tewari, 2003 also observed similar results in *Brassica juncea* when treated with Cu.

Many oxidative stresses also get generated due to the accumulation of Cu in higher concentration and this as a whole cause the inhibition in the photosynthetic reactions which has been observed by many authors (Rocchetta and Kupper, 2009). The decreased concentration of the chloroplastic pigments may be the result of reduced synthesis and/or enhanced oxidative degradation of these pigments by the enhancement of oxidative stress (Romero-Puertas et al., 2002). Due to the emergence of reactive oxygen species, there is occurrence of oxidative stress. The ROS are superoxide radicals, hydroxyl radicals, singlet oxygen and H_2O_2, these damage the cells by degrading the nucleic acids (Pietrini et al., 2003). Increase in the APX activities in tea suggests that the anti-oxidative mechanism induced by Cu was invaded in detoxification of H_2O_2. Many other authors also observed the increase in APX activities due to accumulation of stress. Accumulation of Cu showed increase in APX activity in *Pheseolus vulgaris* (Gupta et al., 1999) and *Camellia sinensis* (Saha et al., 2011).

Copper was inhibitory to CAT but relatively less effective in producing oxidative damage probably due to the fairly enhanced levels of carotenoids, Cu/Zn SOD, POD and glutathione reductase (GR). CAT is also an important enzyme which catalyses H_2O_2 by breaking down it directly to form H_2O and O_2. Initially there was increase in CAT activity in cultivar TS-491 but from 4th day, there was decline in the activity. The increase in SOD and decrease in CAT activity in response to excess supply of heavy metals has been widely reported (Chaoui et al., 1997; Pandey and Sharma, 2002; Cho and Seo, 2005; Lombardi and Sebastiani, 2005). The decrease in CAT activity results in the inactivation of its reaction with superoxide ions which leads to weaken the effective detoxification of H_2O_2 (Kono and Fridovich, 1982). Cho and Seo (2005) reported that oxidative stress in response to Cd toxicity is due to H_2O_2 accumulation. Cu toxicity causes oxidative stress by generating oxygen species ROS which as a result cause lipid peroxidation. The oxidative damage in the heavy metal-stressed tea plants could be due to accumulation of H_2O_2 as a consequence of enhanced activity of total SOD and inhibition of CAT. The significant increase in CAT activity at lower concentration with the Cu exposure has also been reported in tea plant (Saha et al., 2011). However, the decrease or unaffected CAT activity due to Cu was reported in oat leaf (Luna et al., 1994), tomato seedlings (Mazhoudi et al., 1997); whereas, significant increase in CAT activity was also reported in *Prunus cerasifera* plantlets due to Cu stress (Lombardi and Sebastiani, 2005). Tang et al., 2011, also observed reduced CAT activity in tea plants due to increase in the Cr concentration.

In the present investigation, we observed increase in SOD activity in both the cultivars with the increase in the concentration of Cu. SOD is one of the stress resistant enzymes which can catalyze O_2^- radicals to H_2O_2 and O_2. The decrease or increase in the level of these metals causes adverse effect in the activity. The same result has been observed by other authors (Saha et al., 2011; Wang et al., 2004; Hartley-Whitaker et al., 2001). SOD is an important antioxidant enzyme that catalyzes a disproportionate amount of superoxide anion (O^{2-}) to hydrogen peroxide (H_2O_2) (Bowler et al., 1992). Excess supply of heavy metals led to increased accumulation of ascorbate, which is associated with increased activities of APX and GR. The heavy metal-induced oxidative stress also triggers the ascorbate-glutathione cycle for detoxification of hydrogen peroxide which could be a common strategy for counteracting the over-production of the ROS (Foyer and NoCtor, 2005).

Peroxidase (POD) includes a group of specific enzymes such as NAD-peroxidase, NADP-peroxidase, fatty acid peroxidase etc. POD catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydro-quinone etc. POD catalyses H_2O_2 dependent oxidation of substrates which takes part in improving the mechanical protection in plants (Dong et al., 2006). The present study observes the increase in the POD activity in both the cultivars. In TS-491, increase in the activity was only up to 500 µM concentration whereas in S_3A_3 the increase was up to 600 µM. The elevation in the activity of POD shows that it catalyses the H_2O_2 into H_2O and prevent the accumulation of H_2O_2 and O_2; which may be due to ionic micro environment or tissue specific gene expression in plants. The increase in POD helps in reduction of harmful affect caused by the free redicals to structure and function of the membrane (Sun et al., 2006; Pauls and Thompson 1984; Vetanovetz and Peterson, 1990). Saha et al., 2011 also reported the increase in the activity of POD with the increase in the Cu concentration up to 400 µM.

The present study shows the resemblance with the result of Cu stress on tea plant where they showed significant increase in the phenol content below 400 µM concentration (Saha et al., 2011). Phenolic compounds act as reducing agents, hydrogen donor and singlet oxygen quenchers which as a whole make it as an important antioxidant (Rice-Evans et al., 1997). The significant increase in the activity shows that the compound helps in the binding of the heavy metal Cu. The hydroxyls groups of phenol help in chelation by binding heavy metal (Jung et al., 2003). It was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993; Gülçin, 2005).

When the plants are grown under some stress involved in the environment, then excess of free radicals accumulate in the cells which results in the lipid peroxidation consequently resulting to increase in the malon-dialdehyde (MDA) production as last product of this activity (Chaoui et al., 1997). Increased in MDA content is an indicator of physiological stress and aging (Quariti et al., 1997). Many authors also reported that exposure of heavy metals causes increase in MDA levels (Asada 1992; Gille and Singler 1995; Ozounidou 1994). In our study, we observed the increase in MDA content in both the cultivars with the increase in Cu concentration which shows similarity with the findings of Cu stress with other authors (Saha et al., 2011, Rama Devi and Prasad, 1998; Mohanpuria et al., 2007). The difference among the cultivars in response to Cu has also been reported by many authors (Ciscato et al., 1997)

Conclusion

In conclusion, our data demonstrates that among the two cultivars, TS-491 was more sensitive to the higher concentration of Cu compared to S_3A_3. The higher concentration beyond 400 µM of Cu became toxic to the tea plant. It leads to the decline in the growth by retarding many biological functions. The accumulation of Cu was higher in roots leading to the production of ROS, which are followed by lipid peroxidation and many more antioxidant enzymes. The cooperation and the interaction between the antioxidant enzymes detoxify the ROS.

Conflict of interests

The authors did not declare any conflict of interest.

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Accumulation of Cu at different concentration in tea plant (*Camellia sinensis*(L.)O.Kuntze).

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Abstract: *Tea is the most popular beverage in the world. Tea is considered a beneficial drink as it contains many essential nutrients. But the intake of heavy metals even in a small amount causes adverse action in human health along with the plants. The heavy metals are present in the nutrient media itself which are required for plant growth, other important sources are pesticides and fertilizers used in the field for better production. In the present study the tea cultivars (S_3A_3) were collected from the Rosekandy tea estate, Silchar, Assam and were analyzed for the accumulation of Cu at different level on the different organs of the plants. The accumulation of Cu had a strong positive correlation with the Cu concentration. The order of deposition of Cu are in following manner roots>leaves>stems with the exposure of treatment days. From the present study we have concluded that even low amount of deposition of Cu at higher concentration causes adverse affect by retarding the growth of a plant with many other physiological characters. And this may lead to the adverse action of biological characters.*
Key words: *Accumulation, adverse effects, Cu stress, oxidative stress, toxicity.*

I. Introduction

Plants require many heavy metals as their essential source of nutrition which is provided as growth media (e.g. Cd, Cu, Fe, Mn, Ni, and Zn)which play a vital role in metabolic processes. But excess uptake of these metals can cause adverse effect by inhibiting the plant metabolism [1]. With the development of modern industries and agriculture, the accumulation of heavy metal has become one of the most harmful pollutants. Tea is one of the most popular beverages in the world.A few publications are available on the presence of heavy metals in tea and analytical difficulties associated with the separation from tea leaves [2].Copper is an essential elements for growth of plants, lack of Cu can cause defects in growth [3][4].Trace level of copper is essential for human health also. But copper can occur in very high concentration which is used as pesticides in agriculture and field to protect the crops from the attack of pests[5]. Cu can translocate to the tissues through the roots and can damage the tissues by crossing the toxic level. Where it can inhibit the stomatal closure, chlorophyll pigmentation as a whole can affect the physiological character [6]. There is also much evidence showing soil microbial community changes due to metal contamination of soils [7]. Metal hyperaccumulation acts as a remedy for metabolic defects in plant. Plants exposed to metal toxicity suffer from oxidative stress. Not only Fenton-type metals, such as Cu, induce oxidative damage , but also metals like Cd,Al, or Zn that do not change valence within plant cells [8][9][10].

II. Materials And Methods:

2.1: Sample collection:
Three months tea cultivars (S_3A_3) were collected from the Rosekandy Tea Estate, Silchar, Assam.The collected cultivars were transferred to the Hoagland solution for allowingthem to get stable for 7 days [11].Then the plants were placed in a plastic platform in 500 ml jar containing 400 ml of Hoagland solution; so that the roots were immersed in the solution and the shoot were above the platform.Then the plants were treated with $CuSO_4$ at different concentration of 50µm, 100 µm 200µm, 300µm, 400µm, 500µm, 600µm in the nutrient solution. The control plants were left as untreated, comprising only the nutrient solution. The top four leaves were collected for measuring the accumulation of Cu concentration after 2^{nd}, 4^{th},and 7^{th}day of the treatment.

2.2: Plant harvest and Cu analysis:
During harvest the plants were rinsed with distilled water. The roots, shoots and the leaves were separated. Then they were oven dried at 70°C for 48 hrs to a constant weight, after which dry weight of roots, shoots and leaves were determined by electronic balance. The plant tissues were ashed at 500°C for several hours and then cooled down. The 0.5g of the coarse powder transferred into a beaker. Add 5-10ml of the mixture of nitric acid (HNO_3) and perchloric acid ($HCLO_4$) (4:1). The samples were then heated till the solution become colourless and then allowed for cooling and then transferred it into conical flask. Then we washed the

container with nitric acid solution. To dilute the same solvent we added the washing solution in the flask by adjusting it to 20ml and then shake vigorously. The blanks were treated in the same process to minimize the analytical errors. The samples were analyzed for the accumulation of Cu by flame atomic absorption spectroscopy. Data were rounded off suitably according to the value of standard deviation from measurements in triplicates.

Figures and Tables:

The results which were obtained for analysis of tea cultivars by AAS showed the accumulation of copper at different concentration in the different parts of the plant with respect to the treated days.

	2nd day of treatment		
Concentration	Roots (µg/g)	Leaves (µg/g)	Stems (µg/g)
control	36.48±0.06	33.26±0.01	30.18+0.16
50 µm	32.44±0.09	31.55±0.12	28.03±0.02
100 µm	29.70±0.02	25.71±0.08	22.98±0.23
200 µm	27.16±0.17	21.15±0.16	19.29±0.03
300 µm	24.78±0.08	18.75±0.04	15.98±0.13
400 µm	21.40±0.15	17.78±0.12	11.03±0.02
500 µm	19.80±0.01	16.11±0.15	10.98±0.13
600 µm	15.65±0.51	11.24±0.25	9.87±0.23

Table 1: Accumulation of Cu on roots, leaves and stems on 2nd day at different concentration.

	4th day of treatment		
Concentration	Roots (µg/g)	Leaves (µg/g)	Stems(µg/g)
control	32.32±0.17	28.93±0.31	25.03±0.17
50 µm	28.81±0.01	22.15±0.12	21.09±0.23
100 µm	27.15±0.07	16.31±0.33	15.98±0.21
200 µm	26.41±0.04	16.97±0.28	14.32±0.11
300 µm	21.23±0.11	15.78±0.04	13.32±0.14
400 µm	18.87±0.33	13.34±0.14	11.32±0.02
500 µm	15.93±0.42	13.21±0.32	10.03±0.04
600 µm	13.79±0.02	10.04±0.11	9.03±0.02

Table 2: Accumulation of Cu on roots, leaves and stems on 4th day at different concentration.

	7th day of treatment		
Concentration	Roots (µg/g)	Leaves (µg/g)	Stems (µg/g)
control	26.93±0.03	25.18±0.06	22.09±0.04
50 µm	21.39±0.01	19.60±0.12	16.21±0.02
100 µm	20.25±0.42	13.98±0.04	11.03±0.01
200 µm	18.90±+0.02	15.91±0.03	11.63±0.12
300 µm	14.13±.21	11.42±0.26	9.21±0.11
400 µm	13.38±0.54	14.55±0.16	9.03±0.25
500 µm	11.76±0.15	9.63±0.01	8.32±0.13
600 µm	9.17±0.02	6.02±0.20	3.02±0.04

Table 1: Accumulation of Cu on roots leaves and stems on 7th day at different concentration.

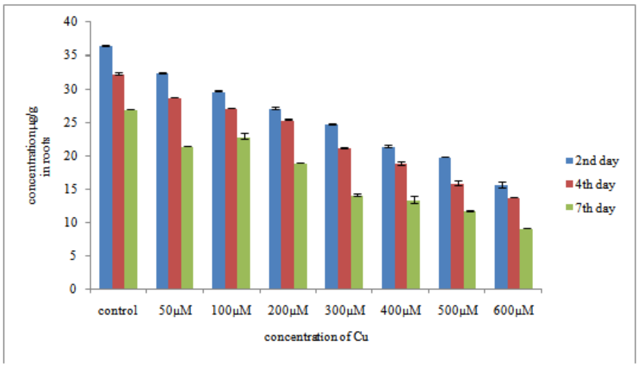


Fig1: concentration of Cu in roots of $S_3A_3$with respect of exposure days.

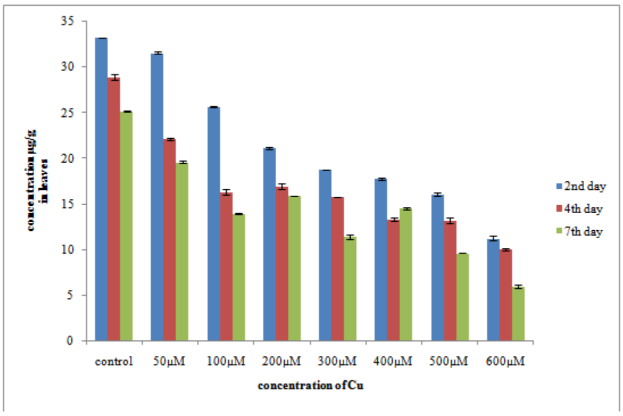


Fig2: concentration of Cu in leaves of S_3A_3 with respect of exposure days.

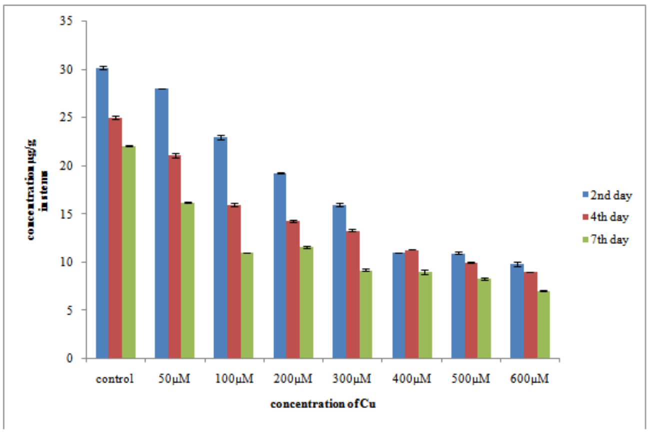
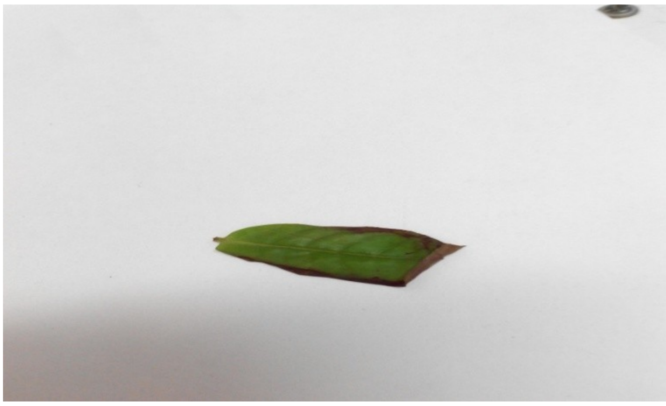


Fig3: concentration of Cu in stemsof $S_3A_3$with respect of exposure days.



(a)

(b)

(c)
Fig4: effect of leaves at higher doses. (a) At500µM on 7th day. (b) At 600 µM on 4th day (c)) At 600 µM on 7th day

III. Conclusion:

The pathogen is an obligate parasite and the crop has to be protected with frequent application of fungicides, pesticides, and fertilizers throughout the season. And these fungicides, pesticides contain a lot of heavy metals in it. Which lead to the adverse effect both in the quality and quantity of production. Many other authors have also determined the accumulation of Cu in tea leaves by Flame Atomic Absorption Spectrophotometry [12]. And many other authors have determined the accumulation of Cd, Cu, Ni, Pb in tea samples [13]. From the above experiment we have observed that with the exposure of days, at different concentration of Cu are showing different level of accumulation of Cu by the different parts of the tea cultivars. From the fig:1 we can observed that the roots are at highest levelof accumulating Cu as they are in direct contact with the stress present in the nutrient media.Form the fig:2 and fig:3 we can observed thatthe deposition of Cu in organ are in the following order roots> leaves> stems. At the higher concentration along with the span of days the deposition is at very low amount but is affecting a lot in the physiological characters. They showed different degrees of symptoms at different concentration with the exposure days. From fig: 4 the leaves first show some brown spots, yellow patches, become dry and then fallen off. From fig: 4(b)at the highest concentration less number of leaves come out, and most of the leaves became withered. The stress plants on the 2nd day is showing less amount of adverse effect whereas 4th day and 7th day plants are affected mostly. The roots suffered a lot by retarding the growth of root length. The number of new leaves slowed down with the less arrival of new stems. As a whole the growth has slowed down. The same results have been observed by many authors that the growth rate of plants slowed down with the increase in the Cr stress [14]. This adverse action of physiological characters may show adverse effect in biological characters with reduction in chlorophyll pigments.Many authors have observed the decreasing of chlorophyll content with increase in Cu concentration [15]. This also may lead to the action of ROS enzymes. With the adverse effect of CAT activity, SOD activity, POD activity so on. The decrease or unaffected in CAT activity by Cu on oat leaf was reported by [16]. The SOD activity has declined with Cr exposure [14]. There was a significant decrease in the activity of POD on exposure of Cr in tea plant [14]. But the judicious use of Cu fungicides may not cause or can cause less problems of public health and also can reduce environmental contamination. This analysis of Cu content in S_3A_3 cultivar indicated that the mean value of Cu ranged between 36.48±0.06 µg/g to 3.02±0.04 µg/g.

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