CHAPTER 6

SUMMARY AND CONCLUSION

The present study deals with the analysis of phytoremediation capacity and its biochemical and molecular basis of the different cultivars of *C. sinensis* under controlled laboratical conditions. The objective of study considers accumulation of Cu at the different dose concentration and durations, which were found to be dependent on both the concentration of dose and its exposure duration. They have effect on physiological and biochemical parameters. The study includes the estimation of detoxification metabolism of metal and metalloids by analyzing the expression profiling of both *PCS*1 and MT genes in all the cultivars. In vitro analysis of MT which include the cloning and characterization of MT gene was also studied.

Tea plant is the world second most popular beverage. Many regions climate are suitable for the growth of tea cultivars. They are very much prone to fungal attack, to prevent from the very same many pesticides, fungicides are used which are generally contains high concentration of heavy metal especially Cu. For the estimation of Cu accumulation the different cultivars were treated with different concentration of Cu at the different exposure time. The plant leaves were harvested and the amount of Cu content was estimated by AAS. Except S_3A_3 all the cultivars showed a positive relation of Cu content with the increase in the concentration. TV-1 and TV-21 showed maximum amount of content among the cloned cultivars. TS-491 was recorded with the highest content of Cu.

The physiological changes exerted by the metal in cultivars, were analyzed in terms of biomass, photosynthetic pigment and protein. The accumulation of Cu in the different cultivars of *C. sinensis* lead to different symptoms in the different parts of the plants, demonstrate that Cu has different poisonous effect on tea plants with the increase of Cu concentration. With respect to control the Cu treated cultivars showed significantly less biomass. Maximum decrease in biomass was observed in seed germinated cultivar TS-491 with compared to the cloned cultivars.

Cu causes many morphological, physiological and biochemical changes in plants. Cu beyond 600μM resulted in fragmentation of leaves after 2^{nd} day, which became severe after 4^{th}day with chlorosis. Decrease in the chlorophyll content was the first indication of toxicity of Cu. when compared at their respective highest treatment concentration and duration the total chlorophyll content was affected more. Among the cloned cultivars TV-21 was observed with maximum declination of total chlorophyll content which was 75.5% with respect to control and TS-491 was recorded with highest level of declination in the chlorophyll content amongst all the cultivars. The % of decrease in chlorophyll content was 79.68 with respect to control. The effects of heavy metal Cu depends on environmental conditions such as pH, plant species, organic substances present in media, fertilization, etc. Protein content showed induction to various levels in plants treated with Cu at various concentrations of exposure and duration. The high accumulation of Cu in *C. sinensis* indicated that it had a potential as a phytoremediation of Cu contaminated condition and the plant used for medicine can be toxic for human also.

The tea plant encounters stress conditions that induce loss in yield. Heavy metal disturbs the metabolic pathways especially in thylakoid membrane which results in formation of free radical and ROS, confirmed by the elevated content of MDA. All the cultivars showed a marked enhancement in MDA with increase in concentration and duration. Maximum increase in MDA content was observed in TV-28. Cu cause damage to membrane. The level of MDA in tissue is considered a measure of lipid peroxidation status. The increase in MDA content was significantly correlated with increase in accumulation of Cu at different concentration in different cultivars. Increase in MDA contents with increase in metal concentration is indicative of correlation with the generation of free radicals.

The plant capacity to combat oxidative stress was analyzed by response of various enzymatic antioxidants. Different responses were analyzed by enzymatic antioxidants. Antioxidant enzyme exhibited different responses upon metal exposure. ROS are toxic byproducts which are the important component which plays a defense response during stress, which directly attributed to metal tolerance ability by plants. ROS interact selectively with the target molecule that perceives an increase in ROS concentration which translates information into signals that leads the plant to response to stress the cultivars showing increase at lower concentration and durations followed by decline at higher concentration and durations. Heavy metals mainly inactivate the antioxidant enzymes which are responsible for free radical detoxification, the accumulation of metal results in the depletion of low molecular weight antioxidants under phytochelate formation.

Duration wise all the cultivars more or less showed similar effect on SOD activity. TV-28 and TV- 21 showed increase in activity up to 7^{th} day of 300µM, but the level of increase in the activity was different in the both. With respect to control in TV-28 there was increase in SOD activity by 91.2% whereas in TV-21 the increase was marked by 85% up to 7^{th} day of 300µM concentration of Cu. TV-17 showed increased in the activity up to 7^{th} day of 400 µM by 70.58%, after that the activity was decreased with the increase in the concentration of Cu whereas TV-1 and S_3A_3 which showed increase in the activity even up to 7^{th} day of highest concentration of Cu i.e. 600 µM. TV-1 showed increase in the activity by 135.71% where $S_3A_3$was marked with 121.99% of increase in the activity with respect to control.In seed germinated cultivar TS-491, there was significant increase up to 7^{th} day of 500 µM after that the stimulated level of SOD activity was declined. However with respect to control the activity was increased up to 600 µM in all the cultivars.

APX was found to be the most responsive enzyme in the plants treated with heavy metals. Among all the cultivars TV-1 and S_3A_3 was the best stimulator of APX activity even up to 7^{th} day of 600 µM concentration of Cu though the trend of increase in the activity was different in different cultivars. The maximum activity was observed in S_3A_3 with respect to control. In other cloned cultivars the activity increased up to a certain concentration (lower concentration viz. 300, 400µM) where the stimulated level of APX declined with the further increase in the concentration and exposure duration.

POD activity showed similar trend of increase in the activity at the lower concentration of Cu with respect to control where with the further increase in the concentration the activity declined. In TV-17 the activity increased up to 7^{th} day of 400 µM by 88.2%. In TV-28 and TV-21 the activity increased up to 7^{th} day of 300 µM. In TV-28 the increase in the activity was noted by 65.3%and in TV-21 the increase in the activity was by 99.5%. In TV-1 and S_3A_3 the activity increased up to 7^{th} day of 600 µM. In S_3A_3 with respect to control the level of increase in the activity was by 121.5% whereas in TV-1 the inclination in the activity was by 113.08.9%. In TS-491 the increase in the activity was observed up to 7^{th} day of 500 µM.

With respect to control the CAT activity was increased in all the cultivars with the increase in the concentration of Cu. In TV-17 the activity increased up to 7^{th} day of 400 µM. In TV-28 and TV-21 the activity increased up to 7^{th} day of 300 µM by 37.6% and 65.4% respectively. In TV-1 the activity was marked with highest increase in the activity (61.4%)up to 7^{th} day of 600 µM. In S_3A_3 the activity increased up to 4^{th} day of 600 µM by 46.15%. In TS-491 there was significant increase in the activity was up to 7^{th} day of 300 µM and on 400 µM there was a slight increase in the activity. And further increase in the concentration the activity was decreased.

All plants produce secondary metabolites and phenolic is one of them, bearing one or more hydroxyl groups at least one aromatic ring. Cu may be more damaging due to its redox nature, but certain level of tolerance are mediated by antioxidant chemicals are observed in the present study. In some cultivars we observed a significant increase in the content at lower concentration which followed by decrease in the content with prolonged exposure of Cu. In TV-28 and TV-21 the increase in the content was marked up to 7^{th} day of 300 µM. But the level of increase in the content in TV- 21 was 56.9% higher than that of TV-28. The increase in the phenol content was observed up to 7^{th} day of 400µM by 42.92% in TV-17. Where TV-1 and S_3A_3 was recorded with highest level of increase at lower concentration. Though the level of increase in the content reduced but increase in the content was observed up to 600µM.

TV-1 and S_3A_3 was the most tolerant cultivars among the cloned and TS-491, the seed germinated cultivar was taken further for the study of expression profile of *PCS*1 and MT gene under the different concentration of Cu. TV-1 was recorded with the highest level of expression of MT gene at 7^{th} day of 300 µM. There was significant increase in the up regulation of gene up to 7^{th} day of 400µM though the cultivar was recorded with positive value of fold change throughout the exposure period of Cu at different concentration. In TS-491, the expression of MT gene was recorded with the highest positive fold change at 7^{th} day of 200 µM which was followed with the declination in the rate of up regulation of gene expression. The further prolonged exposure of higher concentration of Cu led by the negative fold change of expression. The same trend of up regulation of MT gene at lower concentration of Cu was observed in S_3A_3.A significant increase in the level of up regulation of gene was observed up to 7th day of 300 µM. After 7th day at 500 µM and after 4th day at 600 µM there was negative fold change in the expression of MT gene. So we can conclude that the sensitivity of plant increased at the higher concentration (400-600µM).

In case of *PCS*1 gene expression, a different trend was observed in TV-1.With respect to control there was a significant increase in the expression of *PCS*1 gene up to 7th day of 300 µM, but prolonged exposure cause declination in the rate of positive fold change value. Higher concentration of 500 &600µM was also marked with negative fold change during the later phases of treatment. In S_3A_3 there was a significant increase in the expression profiling of PCS1 gene up to 4th day of 300 µM. Which further marked with declination in the expression of *PCS*1 gene. The further prolonged exposure of higher concentration of Cu lead to the negative expression of gene. In TS-491, the expression of *PCS*1 gene was highest at 7th day of 200 µM. From 300 µM there was decline in the level of expression of gene where the prolonged exposure of Cu at higher concentration (From 400 µM to 600 µM) leads to the negative expression of gene.

The first strand cDNA for cloning and characterization of MT gene was synthesized from total RNA of 7th day of 300 µM Cu treatedTV-1cultivar using oligo dT primers and cDNA synthesis system. Degenerate oligonucleotides synthesized on the basis of homology in different MT were used for PCR analysis. Two pairs of primers (1 forward and 1 reverse primer) were used for amplification. This pair of primers produced RNA fragment about 225 bp which was used for cloning and characterization. The isolated RNA was cloned in pGEMT easy vector and the positive clones were screened by digestion with *EcoRI*. The result depicted the presence of about 1 kb DNA of vector and about 100 bp fragment, which was confirmed by

PCR reaction using degenerating primers. The cDNA fragment was sequenced using clones and automated DNA sequencing system.

High homology of cDNA sequence in comparison to other plants MT analyzed through NCBI database using BLAST, suggested that cloned RNA sequence is partial cDNA from *C. sinensis.*The BLAST search indicated that the partial nucleotide sequence has homology with *Camellia sinensis*metallothionin 1 mRNA, | *Camellia oleifera* metallothionein mRNA.The gene bank accession number of the sequence of cloned MT gene of our sample is KT588832. The desired MT gene of our sample showed two matches one range is 118 to 182 and other is 1-67 respectively.

From the present investigation we can conclude that *C. sinensis* is a good plant accumulator which accumulated high amount of Cu. The work was based on the study of cellular and molecular mechanisms that are involved in the resistance and tolerance in response to different concentration of Cu in tea plants. The strategy which are adopted by plants prevent the onset of toxicity due to accumulation of heavy metals, which can be achieved by the various mechanism of plants. The cultivars tolerated metal stress up to a certain level by induction of various antioxidants and synthesis of Phytochelatins and MT gene. Thus plant can be efficiently used for remediation of moderately contaminated soil bodies. The characterization of gene can be used for genetic engineering for suitable production of plants to enhance the phytoremediation capacity.

The cultivars of *C. sinensis*accumulated high amount of Cu concentration along with exposure duration of treatment. Some of the cultivars tolerate up to a moderate concentration of Cu whereas TV-1 and S_3A_3 tolerated the metal up to 600μM without severe toxicity and showed stimulation of various enzymatic antioxidants. Though the photosynthetic pigment decreased with the increase in the concentration of Cu, but at the moderate concentration the decrease was not so high with respect to control. High concentration and prolonged exposure of the same resulted in toxicity, which was showed significant reduction in biomass, chlorophyll and protein. Cu treated cultivars showed higher induction of enzymatic antioxidants. Elevated MDA content in leaves indicated that the plants were subjected to Cu induced oxidative stress. As a defensive mechanism the cooperation of SOD, CAT, POD, and APX might have played a vital role in plant tolerance to Cu toxicity in the cultivars.These antioxidants showed significant increase with the exposure of Cu concentration, indicated that some acclimation effect, but decrease by exposure to high concentration of Cu implicating a potential injury to the plant. The relationship between lipid peroxidation, antioxidant enzyme activity and gene expression profile was found to be a very significant indicator of the effect of Cu stress in plants.

Metal toxicity in the cultivars are may be the result of complex interactions of toxic ions along with both the essential and non-essential ions with other environmental factors. Excess metal accumulation produces some common effects on the cultivars. In many cases the phytotoxic mechanisms of a given metal ion involve different biochemical pathways in different plant species and varieties. Once the genetic control mechanism of metal tolerance are identified, it can lead us to the way of combining the metal tolerance character with the other desired traits to produce plants that are better adopted to high metal soils. The study of synthesis of Phytochelatins and the cloning and characterization of MT gene revealed that the accumulation potential of Cu by *C. sinensis* can be used for phytoremediation purpose in moderately Cu contaminated soil bodies.