CHAPTER 3

MATERIALS AND METHODOLOGY

3.1. Chemicals

All the chemicals used were of analytical grade (AR).

3.2. Plant material

Three months old plants of the cloned variants *viz.* (TV-1, TV-17, TV-21, TV-28 and S_3A_3) and the seed germinated sample (TS-491) were collected from the Rose Kandy Tea Estate, Assam, and India.

3.3. Plant growth condition

The plants were transferred to Hoagland solution as nutrient media and allowed them to get stable for 7 days.

3.4. Treatment Conditions for plants

Plants were acclimatized in 10% nutrient media were treated with different concentration of Cu *viz*. 50µM, 200µM, 300µM, 400µM, 500µM, 600µM maintained in 10% Hoagland solution. The control plants were left as untreated, and allowed to grow only in Hoagland solution.

3.5. Exposure days of treatment

The exposure days of treatment were 2^{nd}, 4^{th} and 7^{th} day after the treatment of Cu at different concentration.

3.6. Experimental set up

Each flask contained 3 plants of equal size. Flasks were aerated for an hour per day. The nutrient media without metal were kept as control with each set of treated one. Three replications were used for each concentration and duration. After harvesting, plants were washed with water, blotted and used for the study of various parameters.

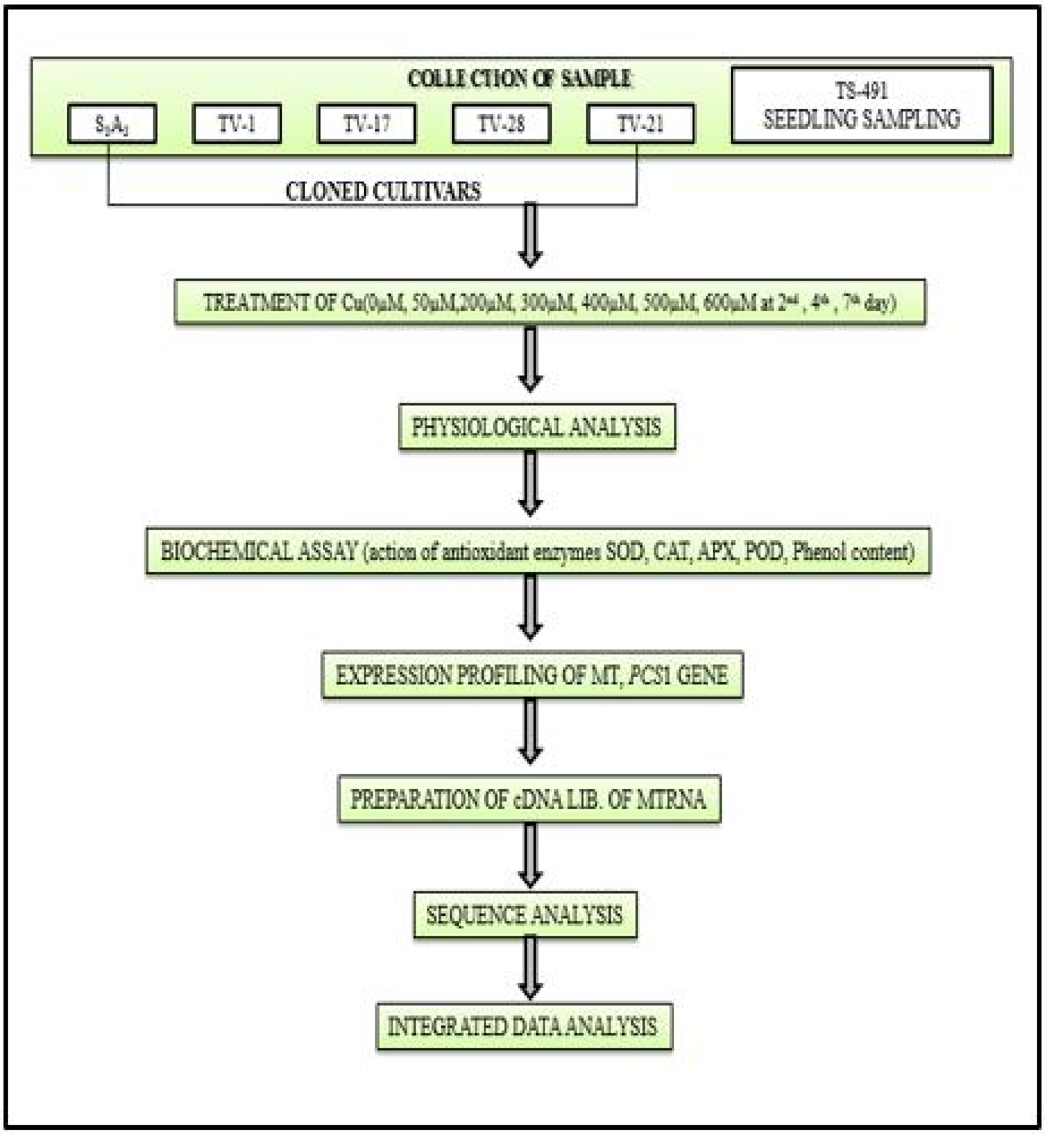

Figure3.1. Schematic representation of the methodology.

3.7. Appearance assessment and growth assay

Shoot length, root length, plant height were measured by simple standard scale instruments.

3.8. Metal Quantification

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 converted to ashes at 50°C for several hours and then cooled down. 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 colorless and then allowed for cooling and transferred it into conical flask. The container was washed with nitric acid solution. To dilute the same solvent we added the washing solution was added in the flask by adjusting it to 20ml and shaked 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 (AAS) as per protocol of Lee *et al*., (2003a, b). Data were rounded off suitably according to the value of standard deviation from measurements in triplicates.

The concentration of the metal was calculated according to the formula:

$$X.V/W$$

X----- Reading in ppm on AAS

V-----Final volume

W----Dry weight of the plant material (g).

3.9. Estimation of Biomass

Plant biomass was measured on fresh weight basis. Plants were blotted gently to remove excess water and weighed prior to treatment at different concentration of Cu at different incubation period of treatment. The leaves were kept for 48 hrs. in dry hot air oven at 50ºc, then the dry weight was measured by electronic balance.

3.10. Estimation of Photosynthetic pigment

Photosynthetic pigment of treated and untreated leaves of the samples were extracted in 5ml of 80% chilled acetone and centrifuged at 10,000rpm for 10 min at 4 ºC (Hegedus *et al*., 2001). The absorbance was taken at 645 nm and 663 nm. Chlorophyll contents were calculated according the following formula:

Total chlorophyll content = $20.2(A_{645}) + 8.02\ (A_{663})$ X V/ D X 1000 X V

A----- Absorbance in 645 and 663 nm

V-----Final volume

3.11. Determination of lipid peroxidation

Lipid peroxidation was determined by estimation of the MDA content. The 0.5g fresh weight of leaves was homogenized in a pre-chilled mortar pestle in 5ml of 50mM cold Na-phosphate buffer (pH 7.8), with 0.1mM EDTA and 1% (w/v) polyvinylpyrrolidone (pvp). After centrifugation at 13,000 x g for 30 min at 4^0C, supernatant was used for further analysis. The level of peroxidation was determined in terms of 2-thiobarbituric acid (TBA). MDA was measured as described by Liu *et al*., (1996).

3.12. Determination of phenol content

Total phenolic components were extracted in ethanol and the content was estimated by Folin Ciocalteau's method (Mahadevan and Sridhar, 1996) with absorbance changes at 520nm.

3.13. Assay of Antioxidant enzymes

Preparation of extract for SOD, APX, POD

For determining the enzyme activities, 0.5 g leaves were dipped in liquid nitrogen and then homogenized in a chilled mortar and pestle in 5ml of 50mM cold phosphate buffer of pH 7.8 containing 2% PVP. The filtrate homogenate was centrifuged at 13000 x g for 20 min at 4^0C and the supernatant were used for determining the enzyme activities. The protein content in the supernatant was measured according to Lowry *et al*., (1951).

3.13.1. Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined by the method ofKrivosheeva *et al.*, 1996. The required cocktailfor SOD activity was prepared by mixing of 27ml sodium phosphate buffer (pH 7.8), 1.5ml of methionine, 1ml of NBT, 0.75ml of triton-X-100 and 1.5 ml of 2mM EDTA. 10 unit of riboflavin and 50 unit of protein were added to 1 ml of the above cocktail. The content was taken in cuvette. The temperature was maintained at 25°C, a protein contained tube was kept in dark which was taken as a blank. The control tube was without enzyme and kept in light. The absorbance was measured at 560 nm. NBT reduction was measured both in absence and in presence of enzyme.

3.13.2. Ascorbate Peroxidase (APX)

Ascorbate peroxidase was assayed by the method of Nakano and Asada (1981). The reaction mixture for measuring APX activity contained 50mM sodium phosphate buffer (pH

7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250mM H_2O_2 and 50 (ig of protein). The activity was recorded as decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized. Non denaturing electrophoresis was performed in a buffer containing 2mM ascorbate. Subsequent to electrophoresis the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2mM ascorbate for a total of 30 min with the equilibration buffer changed every 10 min. the gel was then incubated with 50 mM sodium phosphate buffer containing 4mM ascorbate and 20mM H_2O_2 for 20 min. H_2O_2 was added to the solution just prior to the incubation of the gel. The gel was subsequently washed with sodium phosphate buffer (pH 7.8), 28 mM TEMED and 24mM NBT with gentle agitation for approximately 10 min and stopped by brief wash with distilled water.

3.13.3. Activity of peroxidase (POD)

Peroxidase (POD) was measured by the method of Wu and von Tiedemann, 2002. 50mM phosphate buffer, 0.2mM guaiacol, 10mM H_2O_2 and distilled water was the mixture in a total volume of 3 ml. the reaction was started by adding 50µg of protein. The change in absorbance was measured at 470nm.

3.14. Preparation of extract for catalase

For analyzing the CAT activity, extraction was done in the buffer containing 50 mM Tris-HCl (pH 7), 0.1 mM EDTA, 1mM PMSF and 0.3 g^{-1} f.wt PVP. Homogenate was squeezed through four layers and extract thus obtained was centrifuged at 15000 x g for 15 min at 4^0C. The protein content was measured according to Lowry *et al*., (1951).

3.14.1. Assay of CAT

The assay system contains 50mM of sodium phosphate buffer (pH6.8) 20mMH_2O_2 and a suitable aliquot of enzyme in the final volume of 3 ml. decrease in the absorbance were taken at 240nm.For analyzing the CAT activity we followed the protocol by Pinhero e*t al.,* 1997.

3.15. Statistical Analysis

Experimental data were analyzed using the statistical package. A one-way ANOVA procedure followed by Least Significant difference ($P<0.05$) between the treatment means. Each mean was calculated from triplicate values.

3.16. Relative Gene Expression profile

3.16.1. Total RNA isolation

The total RNA was isolated from Cu treated and untreated *Camellia sinensis* leaf tissues.

3.16.2. RNA Extraction buffer

1.1 M Tris pH 8.0

0.4 M Nacl

1.2 M EDTA pH 8.0

CTAB

3.16.3. Method

The isolation of RNA was done by Cold extraction buffer.

1. 1g of leaves was grinded in mortar pestle.
2. The powdered form of 1g of tissue was transferred to a tube containing 5ml CEB+5ml Phenol at 4 ºC.
3. 15ml of ice cold phenol was added and kept for 45 min at 4 ºc. Then centrifuged at 12000rpm for 20min at 4 ºC.
4. The supernatant was collected and equal volume of phenol: chloroform: indoleacetic acid was added.
5. The supernatant was transferred to a fresh tube then 0.3 volume of LiCl was added and kept at 4 ºC overnight.
6. Again spinned at 12000rpm for 20min. the supernatant was discarded.
7. The pellets were washed in 80% ethanol and again spinned for 10 min.
8. Dry the pellets at 37 ºc for 5-15 min. Suspended in 50µl of HPLC nuclease free water.
9. Then 3µl of sample was taken and RNA dye was added, spinned, denatured at 65ºC for 10min. the sample was run in the gel at 100v for 1hr.
10. The quality and quantity of RNA was checked on UV visible spectrophotometer and Nano-spectrophotometer at 260 and 280 nm as well as gel electrophoresis.

3.16.4. RT-PCR Amplification

Degenerate primers were designed. Nucleotide sequences having maximum homology was selected to design the oligonucleotides. The forward and reverse primers were designed after the analysis. These primers were used for RT-PCR reaction.

Primer for metallothioniens genes:

Forward 5'' AGAAGACCACCACTGAGACC 3'

Reverse 3'-TCCATACCCTCAAACGCAGA-5'.

The primer sequences for phytochellatin synthase genes:

Forward 5'' TTGAGTTGGTTCGATGATTCAATG 3'

Reverse 3'-CCGCTTCATAGCACCGATAGG-5'.

3.16.5. Gene Expression study by qRT-PCR SYBR green based method:

Content	**Amount**
Template(firstcDNA)	4.0 µl
Forward Primer	2µl
Reverse Primer	2µl
2X PCR SYBR green ready mix	25µl
Water	17µl
Total volume	50µl

All reactions were kept in triplicates and run was taken on ABI Step One™ Real-Time PCR System (Applied Biosystem). The PCR conditions were a two-step program: 95ºC for 10 min and 40 cycles of 95ºC for 15 sec, 58ºC for 30 sec and 72ºC for 30 sec.

3.17. Agarose Gel Electrophoresis

The following buffer and solutions was used:

3.17.1. TBE buffer

A 5X stock solution were prepared by dissolving 54 g of Tris base, 27.5 g of boric acid in 20 ml 0.5 M EDTA (pH 8.0) 0.09 M Tris borate and 0.001M EDTA was used to prepare 1 X solutions.

Agarose (0.8% w/v) was prepared in 1 X TBE buffer and 1 µl of ethidium bromide was added and heated in microvan to dissolved it completely. After cooling it about 60 ºC, it was poured in a casting trey, electrophoresis apparatus and allowed to set at room temperature. Comb was placed in a casting trey prior to pour the molten agarose. The comb was removed after 15-20 min when agarose was solidified. 1X TBE buffer was poured in the electrophoresis tank so that the gel gets completely immersed in it. Samples were loaded in the wells and allowed to run at 50v. After 1 hr, gel was visualized in gel DOC (Biomed)

3.18. Reverse Transcriptase Reaction

The tube was heated with RT buffer, Oligo dt Primer and RNase free waterto 75 ºc for 10 mins, and immediately chilled on ice and then MuMLV RT and dNTPS were added. Reaction mixture was incubated at 42 ºc for 1h. MuMLV RT was heated inactivated by incubating the reaction mixture at 95 ºc for 2 min.

Sterile micro centrifuged tubes were used for the followings:

Content	**Concentration**	**Amount**
Total RNA	(2µg)	2µl
RT buffer	10X	3µl
Oligo dt Primer	500mg	1µl
RNase free water	-	7µl
MuMLV RT	-	1µl
dNTPs	10mM	1µl
Total volume		15 µl

3.19. PCR reaction

Following components were added in sterile thin walled PCR tube for the PCR reaction:

Content	**Concentration**	**Amount**
DNA(cDNAfrom RT reaction)	-	2µl
Forward Primer	10 p mol/ µl	1µl
Reverse Primer	10 p mol/ µl	1µl
TaqDNApol Buffer	10X	2 µl
dNTPs	10mM	0.4µl
Sterile water	-	12.6 µl
TaqDNApolymerase	-	1 µl
Total volume		20µl

PCR was initiated by the step of denaturation at 94 ºc for 2min, followed by 35 cycles of amplification each consisting of denaturation at 94 ºc for 10 sec., annealing at 54 ºc for 15 se., and extension at 72ºc for 5min was carried and reaction mixture stored at 4 ºc.The amplified fragment was resolved on 0.8% Agarose gel and analyzed.

3.20. DNase treatment of the Isolated RNA sample:

To remove traces of genome DNA contamination in the isolated RNA sample before further downstream applications, RNA sample was treated with DNase as mentioned below:

Content	**Volume required**
Isolated RNA	2 µg
DNase I	1 µl
10X buffer	2 µl
DEPC-H2O	20 µl
Total Volume	25 µl

The mixture was mixed well and incubated at 37 ºC for 30 minutes. The enzyme was inactivated by incubating at 65 ºC for 10 minutes in presence of 2mM EDTA. The RNA was further purified using phenol extraction and ethanol precipitation as per standard protocol. The concentration of RNA aliquot was determined using Nanodrop instrument.

3.21. Determination of quality of RNA

The quality of the purified RNA sample was examined by using denaturing agarose gel electrophoresis. The RNA sample was mixed with the RNA loading dye, heated at 95 ºC for 2 minutes snap- chilled in ice for 2 minutes and loaded in 1.5% agarose gel containing formaldehyde in MOPS buffer. The bands for RNA were visualized using Ribostain. The pattern and the intensity of the bands ensured the proper quality of the sample.

3.22. Preparation of cDNA:

The cDNA pool was prepared from the purified RNA using oligo dT primer and M-MUL V Reverse Transcriptase (200u/µl) as per the following protocol:

1ug of DNase treated RNA was mixed properly with oligo dT primer (1 µl; 50 µg/ µl) and DEPC- H_2O to make final volume to 11 µl. The RT master mix was prepared by mixing the reagent as follows: 10X RT buffer: 2ul; M-MuLV Reverse transcriptase: 1 µl; dNTPs : 1 µl; DEPC H_2O : 5 µl. The RNA primer mixture was heated at 65 C for 5 minutes and snaps chilled at ice for 2 minutes. The master mix was added to it and incubated at 42 ºC for 2 hrs. Negative (- RT reaction without Reverse Transcriptase) was also included. The mixture was further incubated at 70 ºC for 15 minutes and used for PCR reaction after completion of incubation.

3.23. Optimization of PCR reaction, Target specific amplification and gel electrophoresis:

The gene specific oligonucleotide primers were prepared. Theprimers sequence used

Forward: 5'- AGAAGACCACCACTGAGACC-3'

Reverse: 3'-TCCATACCCTCAAACGCAGA-5'

cDNA equivalent to 100ug of total RNA was used for PCR amplification of metallothionein gene. A PCR reaction was also set with RT sample to ensure amplification exclusively from RNA- derived cDNA. A NO TEMPLATE CONTROL (NCT) was also set to ensure quality of PCR reagents.

The final condition for the PCR reaction was optimized to be as follows:

Content	Amount
10X Buffer	5 µl
Metallothioneinspecific primer (F/R)	0.5/ 0.5 µl
10mM dNTP mix	0.5 µl
cDNA	2 µl
G9 Taq DNA polymerase	0.5µl
Nuclease- free H_2O	41 µl
Total Volume	50 µl

The thermal condition was optimized to be as mentioned:

Temperature	Time
95ºC	2 mins
95ºC	20 secs
57ºC	20 secs
72ºC	15 secs
Cycle	30
Final extension	
72°C	10 mins.

10 µl of each PCR product were electrophoresed through 1 % agarose gel and visualized by super stain nucleic acid gel stain. A band of size 225 bp was observed for RT-PCR sample only confirming exclusive RNA cDNA derived amplification. No band was observed for RT and NTC samples ensuing absence of contamination/ non-specific/gDNA derived.

3.24. Molecular Cloning of PCR amplified gene of interest in pGEM-T Easy:

The reaction was set as follows:

Content	Concentration	Amount
pGEM-T Easy vector	50 ng/ µl	1 µl
Purified PCR product	10 ng/ µl	2 µl
10 X ligation buffer	-	1 µl
T4 DNA ligase	4u/µl	0.5µl
Nuclease- free H_2O	-	5.5 µl
Total volume	-	10µl

The mixture was incubated at 22ºC for 15 minutes. The mixture was transformed in DH5 α super competent cell. The transformants were tested by blue white screening as per standard protocol. Tentatively positive clones (whites) were further screened by colony PCR using insert – specific primers. The NO- INSERT CONTROL with pGEM-T Easy vector DNA nullify possibility of false positives and No Template Control was also included in the reaction set up. The PCR condition was maintained the same as above PCR products were electrophoresed through 1 % agarose gel as previously.

3.25. Sequencing of the clones

3.25.1. Cycle Sequencing PCR:

The cycle sequencing is a method in which successive rounds of denaturation, annealing and extension in a thermal cycler results into a linear amplification of extension product. The ddNTPs were labeled with different colored florescent dyes like ddCTP were labeled with blue color dye, ddATP with green color dye, ddGTP with yellow and ddTTP were colored with red color dye. The four ddNTPs therefore produces blue, green, black and red color peaks respectively.

Sequencing reactions was carried out with both forward as well as reverse primers saperately for confirmation. A 15 µl sequencing reaction mix was prepared for both forward and reverse strand that consisted of 4ul sequencing ready mix 3.1, 4µl of 5X sequencing buffer, 1 ul of the amplicon of 2^{nd} PCR of the partial fragment of CuMT and 0.5 µl of respective forward and reverse primer. The sequencing ready mix contains unlabeled dNTPs, labeled ddNTPs, Taq polymerase and 10X buffer. Conditions for cycle sequencing remains same for both the mix: 96ºC for 2 min as initial denaturation followed by 30 cycles of denaturation 96ºC for 10 sec, annealing 54ºC for 15 sec and extension 60ºC for 4 min.

3.25.2. Purification of the sequencing product:

The PCR product of cycle sequencing was first purified to remove the unused dNTPs, DDNTPs, primers and various inorganic ions before subjecting them to capillary electrophoresis. The purification was carried out in two steps at first the amplicon of cycle sequencing PCR was taken in 0.5 ml sequencing tube. To the amplicon, 25ul of 100% alcohol was added. The tubes were kept at room temperature for 15 mins and then centrifuged at 4000 rpm for 20 mins. Supernatant was discarded and 100ul of 75% alcohol was added to the pellet. Tubes were again centrifuged at 4000 rpm for 15 mins. Supernatant was discarded and tubes were incubated at 56ºC for 10-20 mins to evaporate the alcohol. Finally, 15ul of Hi-di-formamide was added in each tube.

3.25.3. Analysis of sequencing data

The sequence analysis v 3.41 software (ABI) was used for data collection and sequence analysis. The software cells the nucleotide based on the color of the peak. The order of the nucleotides was then aligned with the known MT gene of the other plants by comparison with sequence protein and EST database from NCBI (National Center for Biotechnology Information) using the search programm blastn and blastx (Altschul et al.,1990; 1997).