CHAPTER 4

RESULT

Tea is the most economically important plant but prone to fungal attacks. Cu based fungicides are used to prevent the plants from the damage caused by pathogens. The five cloned (TV-28, TV-17, TV-21, TV-1 and S_3A_3) cultivars and one seed sampling (TS-491) were treated with different concentration of Cu (control, 0µM, 50µM, 200µM, 300µM, 400µM, 500µM and 600µM) at different exposure periods (2^{nd}, 4^{th} and 7^{th} day) to observe the effect of Cu on the physiological and biochemical characters of cultivars.

Identifying the tolerant cultivars which is associated with chelation mechanism by phytochelatin synthase (*PCS1*) and metallothionein gene were isolated and characterized. With this aim we conducted this study and the findings of the study have been presented in different chapters namely:

4.1. To study the effect of heavy metal Copper (through the application of pesticides) in tea plants (in different cultivars).

4.2. Analysis of the effect of physiological changes brought about by Cu stress in tea plants.

4.3. Analysis biochemical changes brought about by heavy metal (copper) stress in the tea plants.

4.4. Isolation, characterization and study the expression of the genes (phytochelatin synthase *PCS*1, metallothionein) in the heavy metal stressed tea plants.

4.5. Preparation of cDNA library of the overexpressed metallothionein genes from the TV-1 cultivar.

Chapter 4.1

Study the effect of Cu in tea cultivars

4.1.1. Effect of Cu on metal accumulation:

Accumulation of Cu by *C. sinensis* at different concentration and duration has been presented in Figure 4.1.1.A-4.1.1.F. Plants accumulated good amount of metal. Accumulation of metal was found to be dependent on both the dose and duration. There was a positive correlation between the concentration of Cu and accumulation of Cu. In all the cultivars there was a significant increase in the Cu accumulation with increase in the concentration of Cu and its exposure period, except S_3A_3.

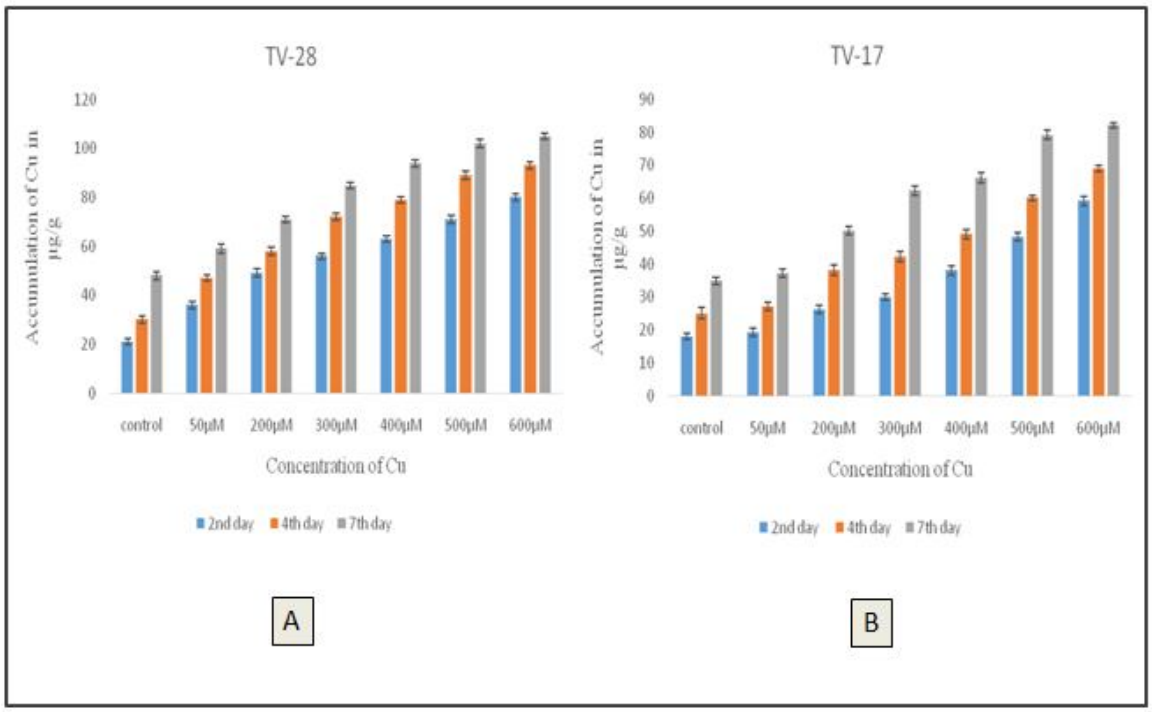

Figure4.1.1. A & B. Accumulation of Cu by *C. sinensis* (TV-28 & TV-17) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From Figure 4.1.1.A&B we can observe the same trend of increase in the content of Cu with the increasing Cu concentration.

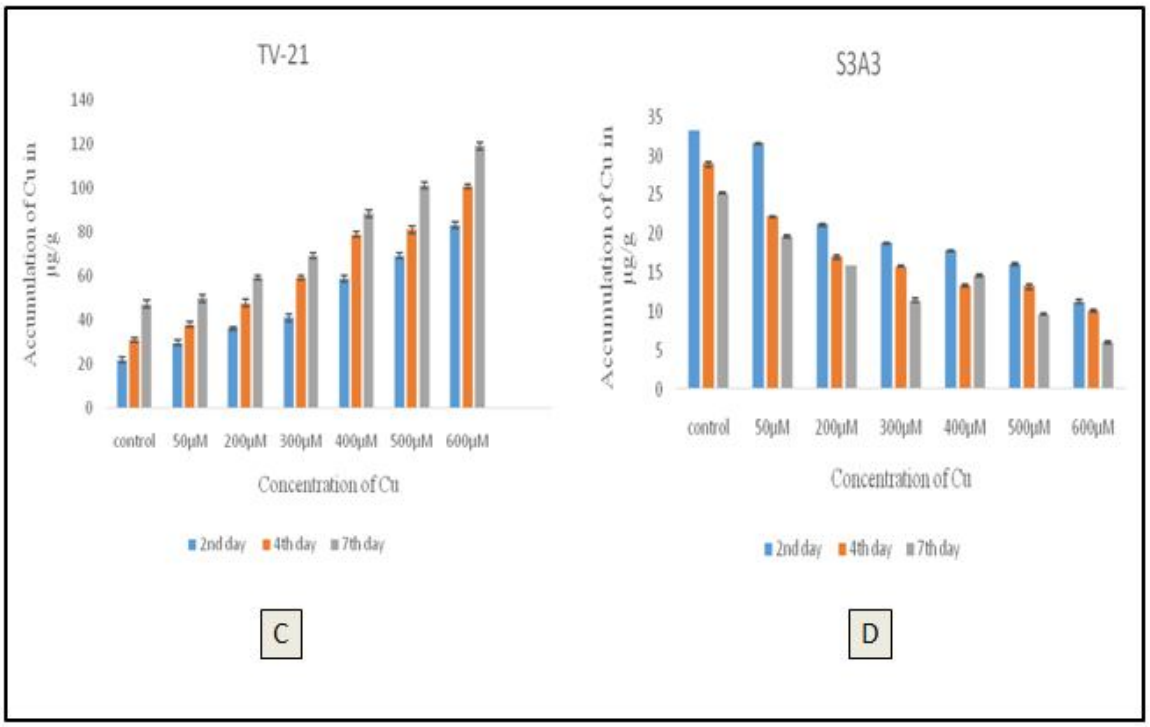

Figure4.1.1. C&D. Accumulation of Cu by *C. sinensis* (TV-21 & S_3A_3) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

In figure 4.1.1.C&D we can observe the different trend of respond by TV-21 & S_3A_3 in accumulation of Cu with the increasing concentration of Cu. While S_3A_3 showed a significant negative correlation between the content of Cu and increasing concentration of Cu. In case of S_3A_3, with respect to control the amount of Cu was decreased with the increase in the concentration of Cu along with the exposure periods.

Amongst the five cloned varieties TV-21 was recorded with the highest amount of accumulation of Cu. In TV-21 the repair accumulation was observed even at the highest concentration of Cu, 600 µM (4.1.1.C). With respect to control the accumulation was about 35.3 % on the 2nd day of 50µM, where the prolonged exposure leads to the increase in the

amount of accumulation. Increase in exposure concentration of Cu resulted in the 438.61% higher accumulation at 7th day of 600µM with respect to control.

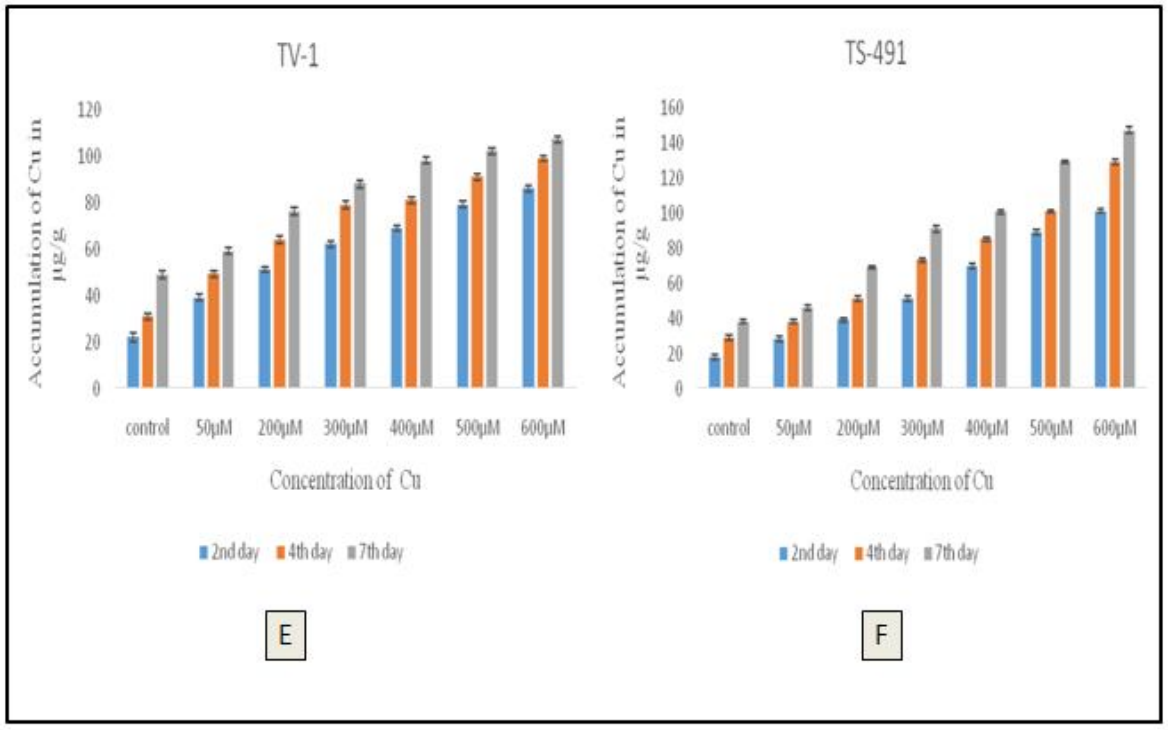

Figure4.1.1. E&F. Accumulation of Cu by *C. sinensis* (TV-1 &TS-491) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From figure 4.1.1.E&F we can observe the same trend of accumulation of Cu by TV-1 & TS-491 respectively cultivar exposed to different concentration and exposure duration, but the level of increase was different in both the cultivars.TS-491 was the seed germinated cultivar, when compared at highest exposure concentration 600 µM, the rate of accumulation was highest compared to all other cultivars.

4.1.2. Effect of Cu on Root length

Tea plant is a very slow growing plant. From the figure 4.1.2.A-4.1.2.F we can observe that with respect to control there was very slight increase in the length of root in all the cultivars. But at higher concentration there was low or no increase in the length of the roots of the cultivars.

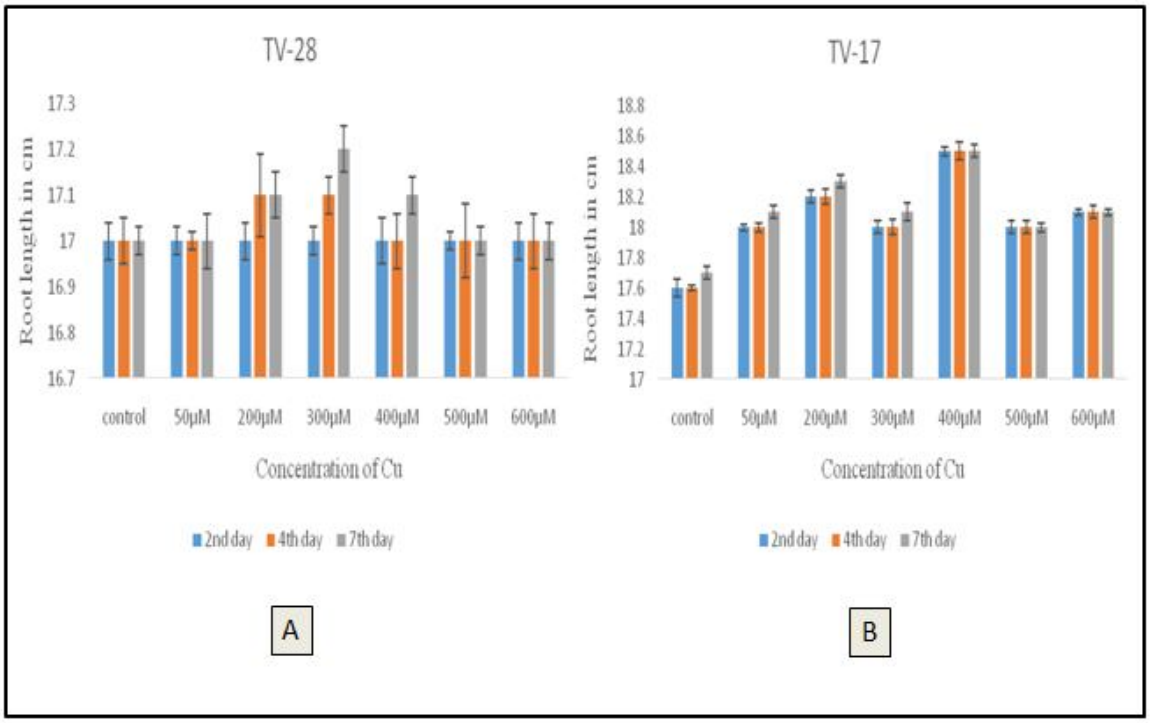

Figure4.1.2. A&B. Effect of Cu on root length on *C. sinensis* (TV-28 &TV-17) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From figure 4.1.2.A&B. we can observe the effect of Cu on root length on TV-28 & TV-17 cultivars exposed to different concentration and exposure duration. A very low or no growth was marked at the higher concentration of Cu with a very gradual increase in the length of root at the lower concentration.

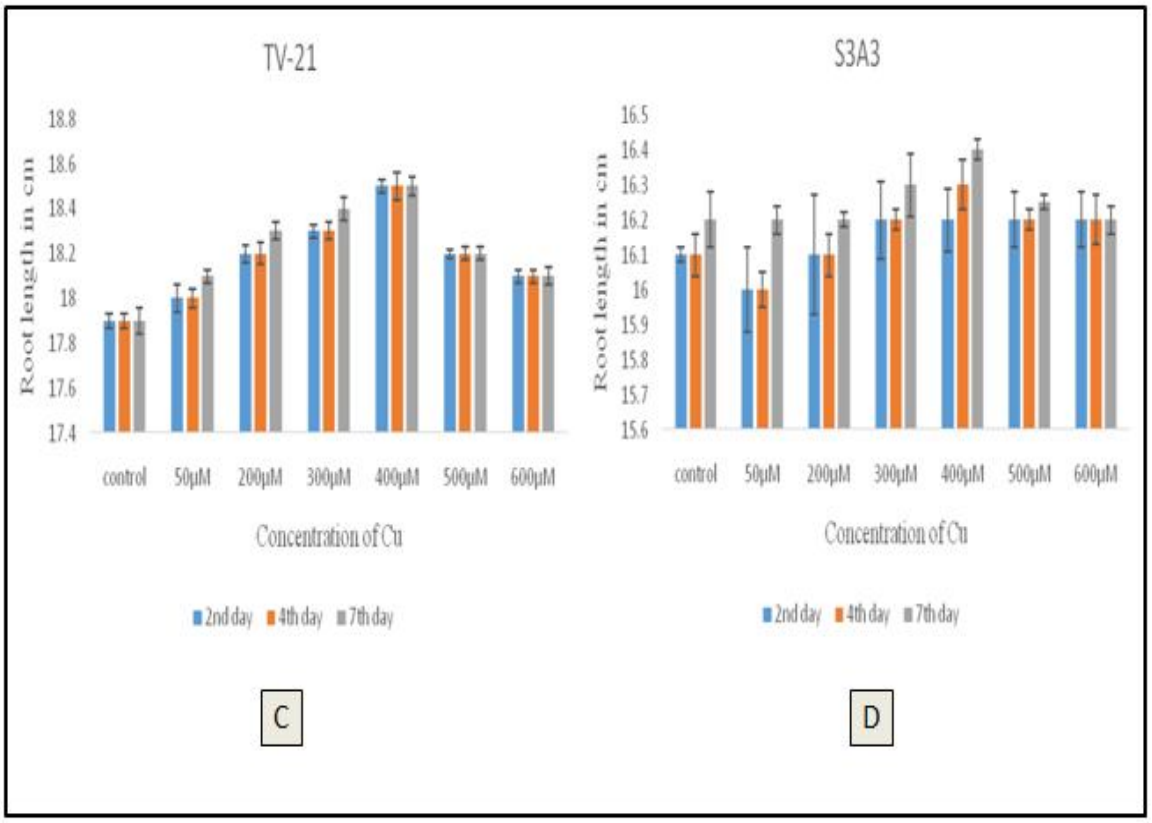

Figure4.1.2. C&D. Effect of Cu on root length on *C. sinensis* (TV-21 & S_3A_3) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From figure 4.1.2.C&D we can observe the effect of Cu on root length onTV-21 & S_3A_3 cultivars exposed to different concentration and exposure duration. In TV-21 the growth was marked up to 7^{th} day of 300 µM whereas in S_3A_3 there was significant growth up to 7^{th} day of 400 µM and the level of increase in root length was very low on 500 µM and which followed with no changes in the root length in 600 µM.

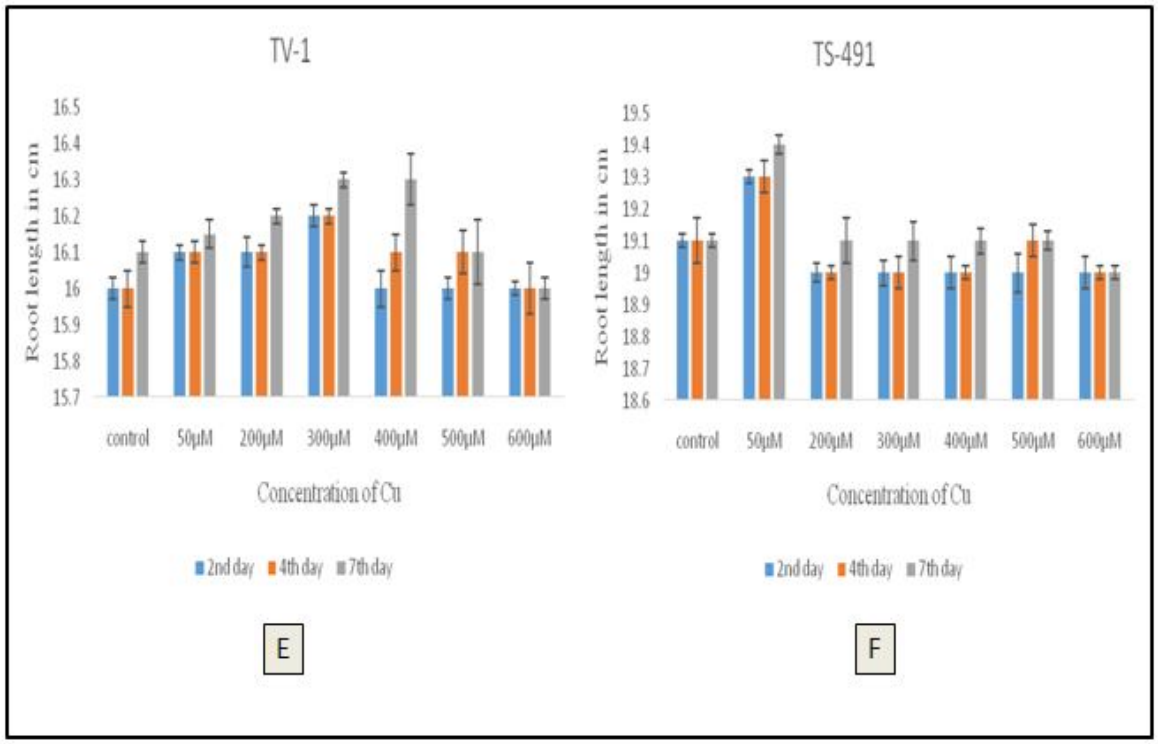

Figure4.1.2. E&F. Effect of Cu on root length on *C. sinensis* (TV-1 & TS-491) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From the figure 4.1.2.E&F we can view the effect of Cu on root length onTV-1 & TS-491 plant exposed to different concentration and exposure duration. In TV-1 there was gradual increase in the growth of roots up to 4th day of 500µM after that no increase was observed. The same trend of result was found in TS-491 too, where a very less increase in the root length was marked at lower concentration of Cu which followed with no increase in the length at higher concentration.

4.1.3. Effect of Cu on Shoot length

From the figure 4.1.3.A-4.1.3.F we observed that with respect to control there was increase in the length of shoot in all the cultivars. But the same trend of low or no increase was observed at the higher concentration which was observed in case of root length.

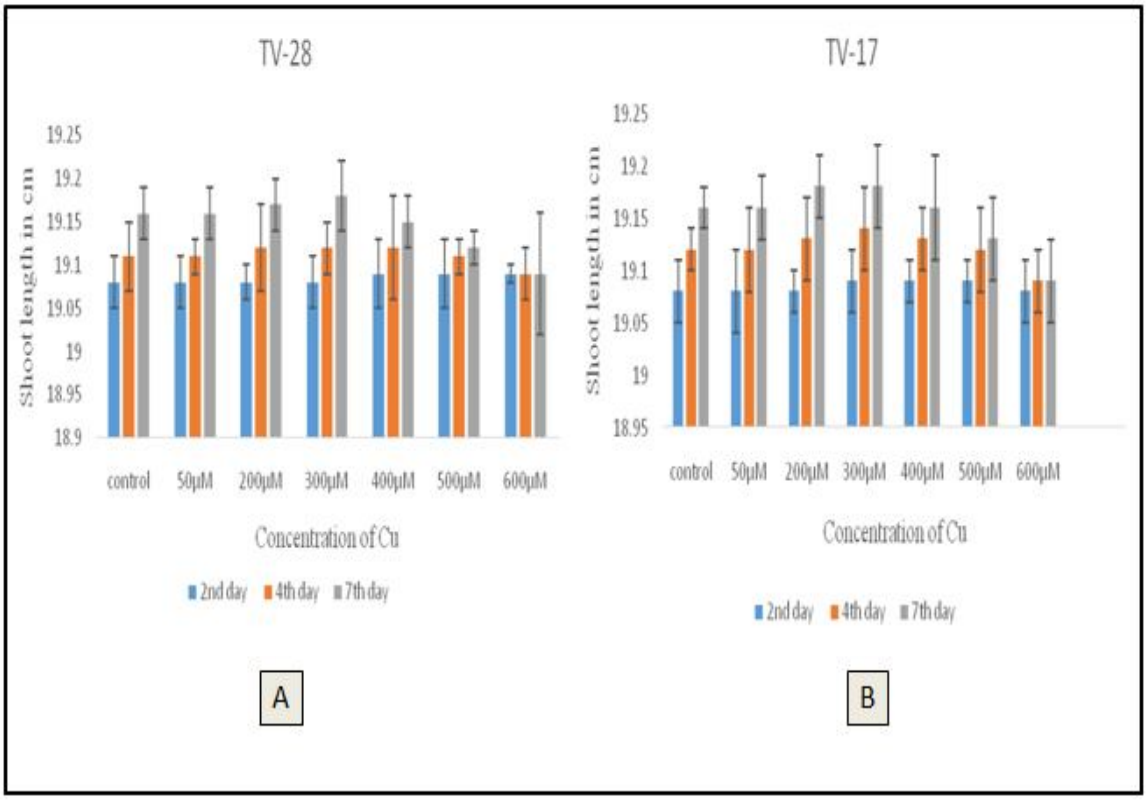

Figure4.1.3. A&B. Effect of Cu on shoot length on *C. sinensis* (TV-28 & TV-17) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From figure 4.1.3.A we can observe that in TV-28 there was significant increase in the length up to 7th day of 400 µM but very low increase in shoot length was observed in 500 µM which was followed with no increase in 600µM. In TV-17 the growth was marked up to 7th day of 500 µM (Figure 4.1.3.B).

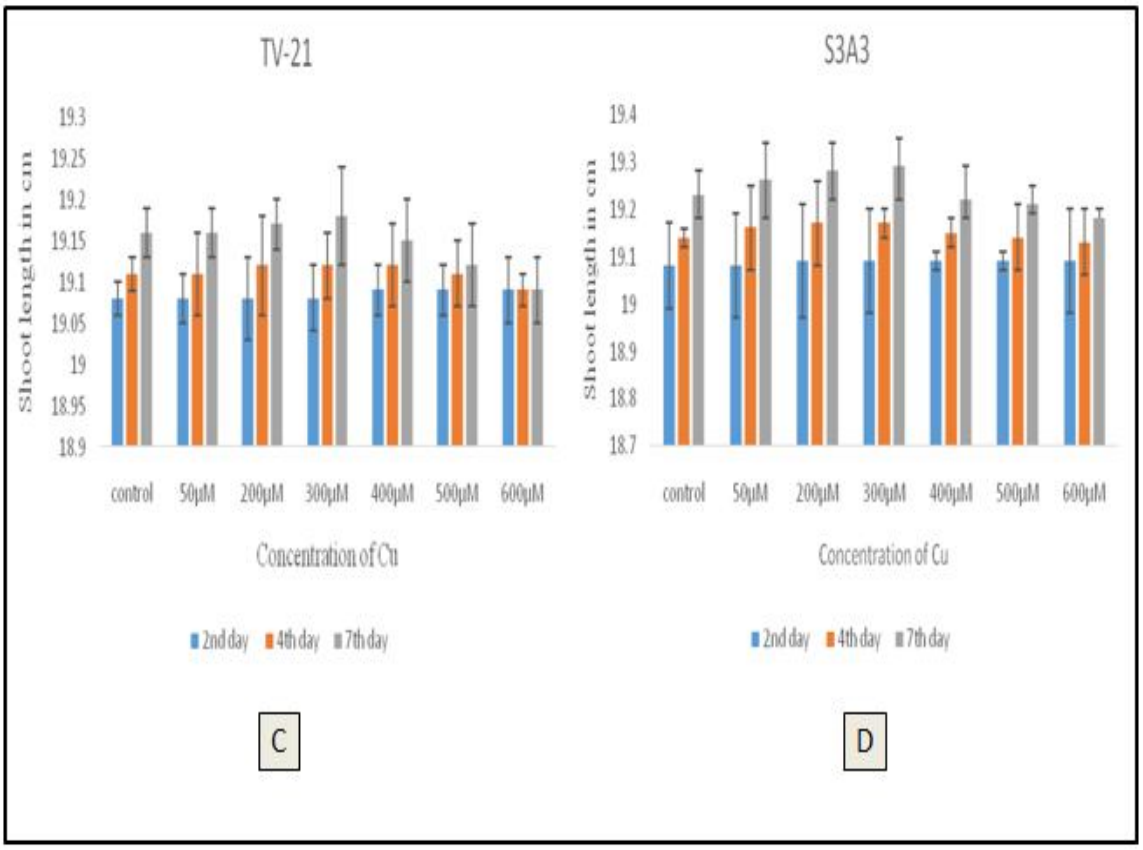

Figure4.1.3. C&D. Effect of Cu on shoot length on *C. sinensis* (TV-21 & S_3A_3) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

From figure 4.1.3.C we can observe the gradual increase in the growth at lower concentration of Cu in TV-21. In $S_3A_3$the increase was marked up to 300µM and very low increase was observed from 400-600µM (Figure 4.1.3.D).

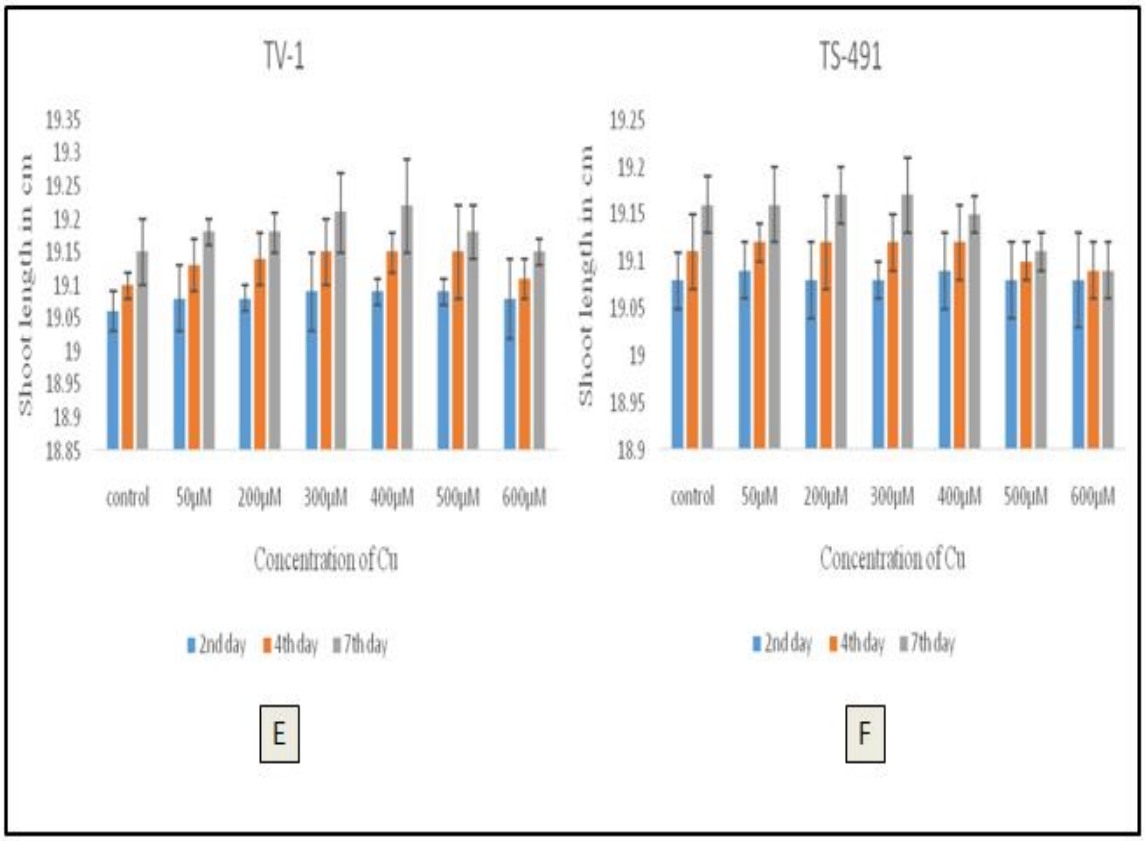

Figure 4.1.3.E&F. Effect of Cu on shoot length on *C. sinensis* (TV-1 & TS-491) plant exposed to different concentration and exposure duration. All the values are mean of triplicates ± SD.

In TV-1 there was significant growth of roots up to 400µM after that low increase was observed from 500-600µM (Figure 4.1.3.E).The same trend of result of increase in the length in TS-491 was observed at low concentration where no increase was marked from figure 4.1.3.F in TS-491.In TS-491 the significant increase in the shoot length was observed up to 7th day of 300 µM, very low increase was marked from 400-500 µM and no increase was observed with the prolonged exposure of 600µM concentration of Cu (Figure 4.1.3.F).

Chapter 4.2

Effect on physiology of plants due to Cu exposure

4.2.1. Morphological analysis

Morphological symptoms of toxicity were also noticed. Figure 4.2.1.1.A, B, C represent a demo of aclimitation of cultivar in Hoagland solution, growth of cultivar in Hoagland solution with Cu Concentration and growth of cultivar in Hoagland solution without Cu (Control).

Tea plants showed different degrees of symptoms in different concentrations of Cu stress. With the increase in the concentration of copper the roots suffered mostly which is followed by leaves and then new stems (Figure.4.2.1.2.A). Cu beyond 600µM resulted in fragmentation of leaves after 2nd day which become severe with the exposure time, for which it was impossible to carry out the experiment (Figure 4.2.1.3.A, B, C & D). The leaves first developed some brown spots, yellow patches, become dry and then fall off. At the highest concentration of 500 µM 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. In the highest concentration (600µM) the inhibition was the strongest with the smallest growth rate of roots, leaves and stem compared to tea plants treated with lower concentrations. This phenomenon indicated enhanced senescence by Cu.

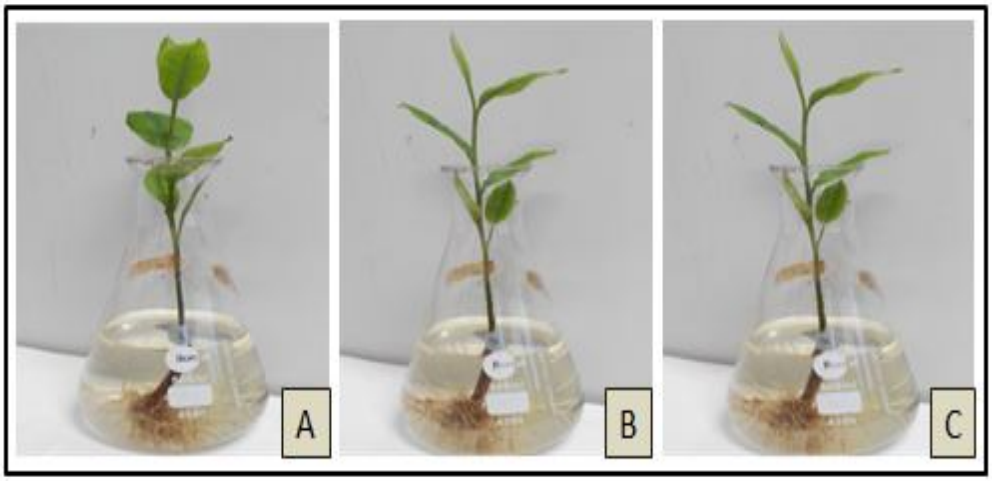

Figure4.2.1.1. A, B, C Represent a demo of aclimitation of cultivar in Hoagland solution, growth of cultivar in Hoagland solution with Cu Concentration and growth of cultivar in Hoagland solution without Cu.

Figure4.2.1.2. A, B & C. Effect of Roots at higher concentration of 400, 500, 600µM

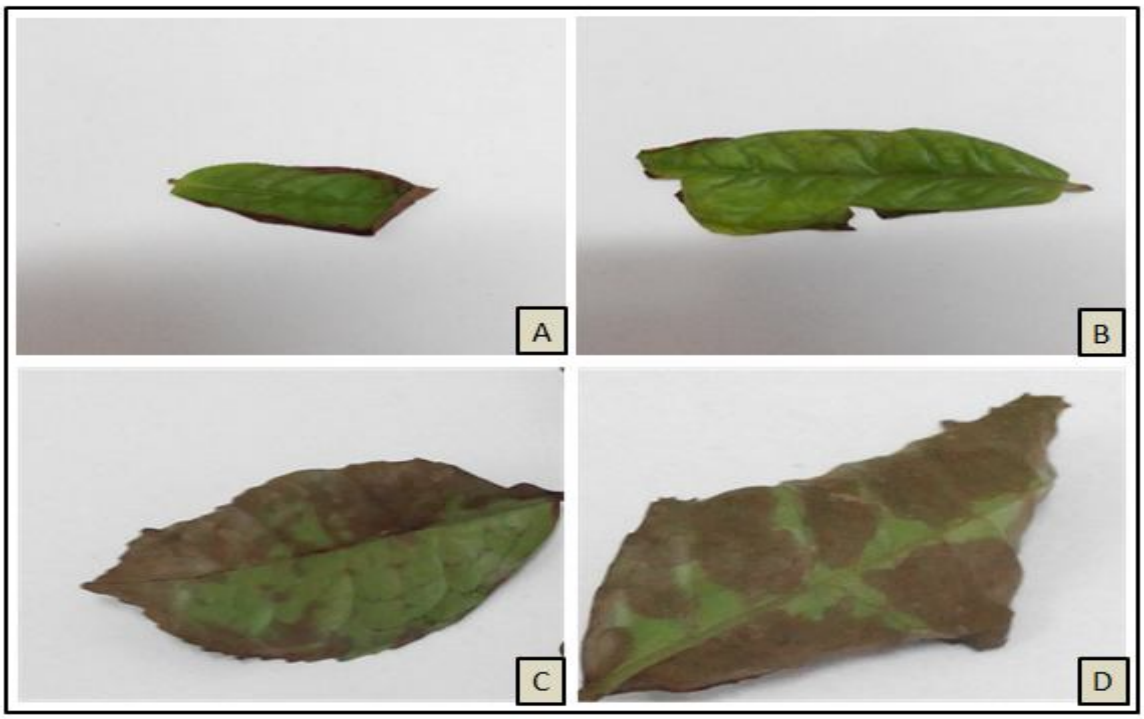

Figure4.2.1.3. A, B, C & D. Effect of Leaves at higher concentration of 400, 500, 600µM. Figure4.2.1.3. B represents the fragmentation in leaves beyond 600µM

4.2.2. Effect of Cu on Biomass

The effect on biomass was observed on the different cultivars of *C. sinensis* after Cu treatment (Figure 4.2.2.A-4.2.2.F). Due to Cu treatment biomass accumulation was affected in the cultivars. The fresh weight of the organs of the treated plants decreased with the increase in the concentration of the copper in both the cultivars with respect to exposure days.The plants treated with Cu accumulated lesser biomass with respect to control. With respect to control, there was slight increase in the biomass at the first phase ofincreasing concentration of Cu but the prolonged exposure of the same concentration lead to the decrease in the accumulation of biomass. Same trend of decrease in the biomass was observed in all the cultivars.

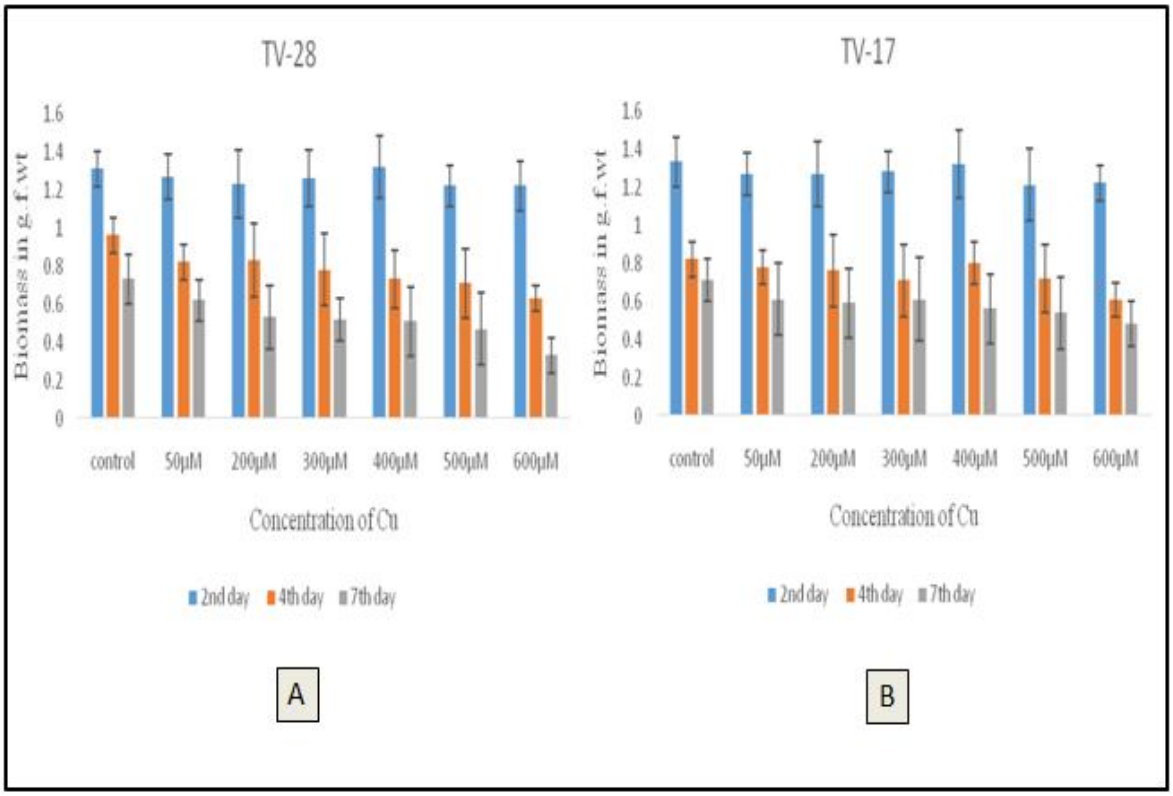

Figure4.2.2. A&B. Effect of Cu on biomass on TV-28& TV-17 cultivars exposed to different concentration and exposure duration. All the values are mean of triplicates ±SD.

Figure 4.2.2.A&B showed the same trend of decreasing in the biomass with the increasing concentration of Cu along with the exposure period in TV-28 and TV-17 respectively**.** From the figure we can marked the differences in the level of decreasing in the biomass in both the cultivar. The level of decrease in the content was higher in TV-28 cultivar with compare to TV-17.

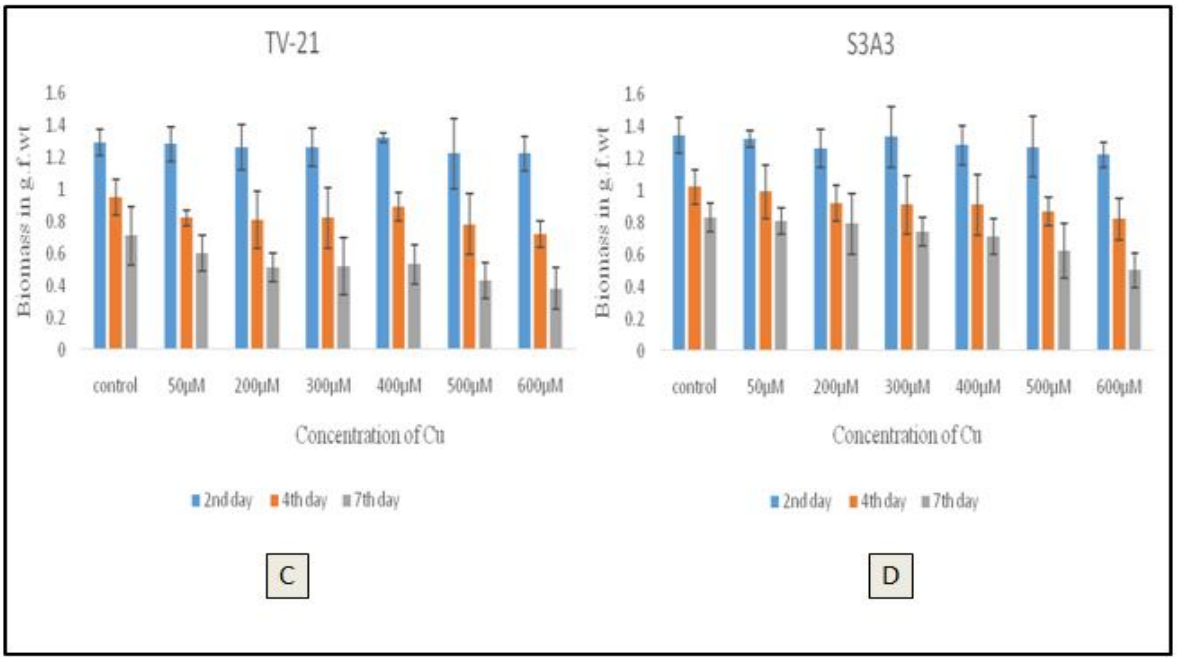

Figure4.2.2. C&D. Effect of Cu on biomass on TV-21 & S_3A_3 cultivars exposed to different concentration and exposure duration. All the values are mean of triplicates ±SD.

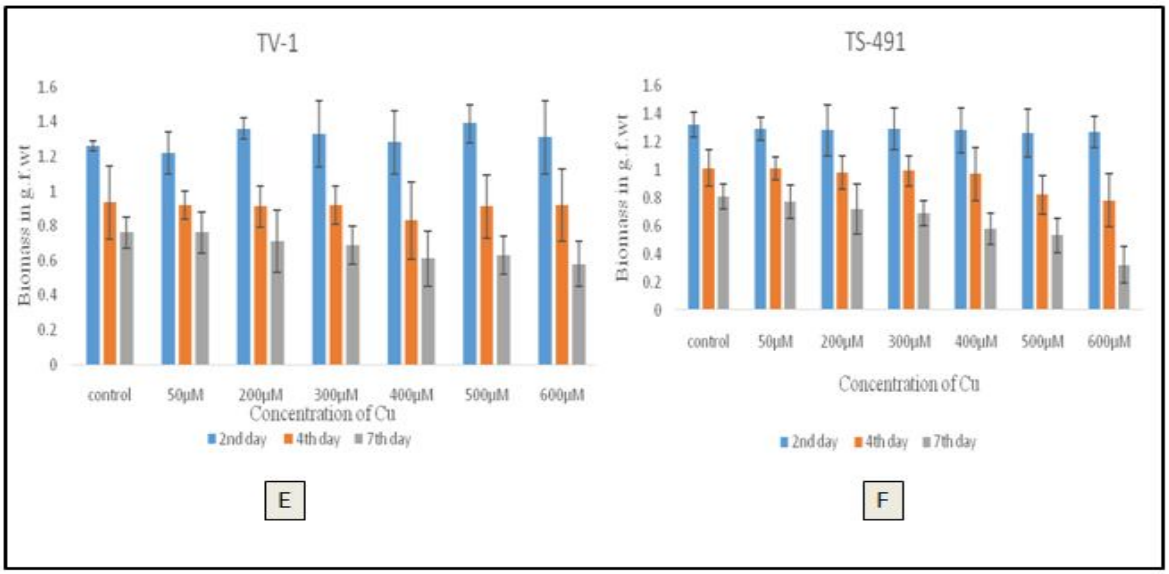

Figure4.2.2. E&F. Effect of Cu on biomass on TV-1 & TS-491 cultivars exposed to different concentration and exposure duration. All the values are mean of triplicates ±SD.

Maximum decrease in biomass was observed in TV-21 among the cloned cultivars (fig 4.2.2.C), where compared to biomass at the start of the experiment, the degradation in biomass was greater at their respective highest concentration with theduration of exposure. But TS-491 the seed sample was recorded with highest (75%) decline in the biomass with respect to control (4.2.2.F).

4.2.3. Effect of Cu on Total Chlorophyll Content:

Chlorophyll is an important pigment which plays an important role in photosynthesis. From figure 4.2.3.A-4.2.3.F we can observed that there was significantly negative correlation between the chlorophyll content with the increase in the concentration of Cu. In the present study total chlorophyll content was decreased in all the cultivars with the increase in the concentration of Cu and with the time of exposure.

The results indicate the degree of damage to the chlorophyll content varied with different concentration of Cu stress in different cultivars. The growth of tea has close relations with photosynthesis. The result suggests that the photosynthesis and growth of tea plant were inhibited by high concentration of Cu.

The chlorophyll content in the youngest leaves decreased more with compare to control. At the lower concentration of Cu the level of decrease in the content was low but at the higher concentration the level of decrease in the content was more. The content of chlorophyll lowered by higher concentration of Cu which indicated that low dosage of Cu may be beneficial to the tea plants. But high dosage of Cu causes toxicity. Though the cultivars showed the same trend of decrease in the chlorophyll content with the increase in the concentration but the level of decrease was different in different cultivars.

Table10. Effect of Cu on Chlorophyll content exposed to different concentration and exposure duration onTV-28. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	85.15±1.28	85.65±0.99	83.79±0.56
50 µM	84.81±1.82	83.65±1.02	80.09±1.33
200 µM	81.23±1.33	77.96±0.92	73.28±1.08
300 µM	73.67±0.92	65.01±1.29	60.81±1.21
400 µM	62.48±0.82	52.38±1.36	43.22±0.92
500 µM	54.23±0.78	45.19±1.48	39.13±0.81
600µM	51.68±0.65	42.97±1.09	32.19±1.47

Table11. Effect of Cu on Chlorophyll content exposed to different concentration and exposure duration onTV-17. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	88.73±1.27	88.01±1.33	87.27±1.42
50 µM	87.98±1.33	86.32±1.22	84.65±1.38
200 µM	85.92±1.42	82.95±1.72	79.29±1.22
300 µM	82.58±1.82	77.89±0.98	72.68±1.38
400 µM	79.85±1.08	73.96±1.21	68.47±1.09
500 µM	71.79±1.29	64.56±1.29	58.92±1.48
600µM	61.08±1.62	50.83±0.98	39.34±1.84

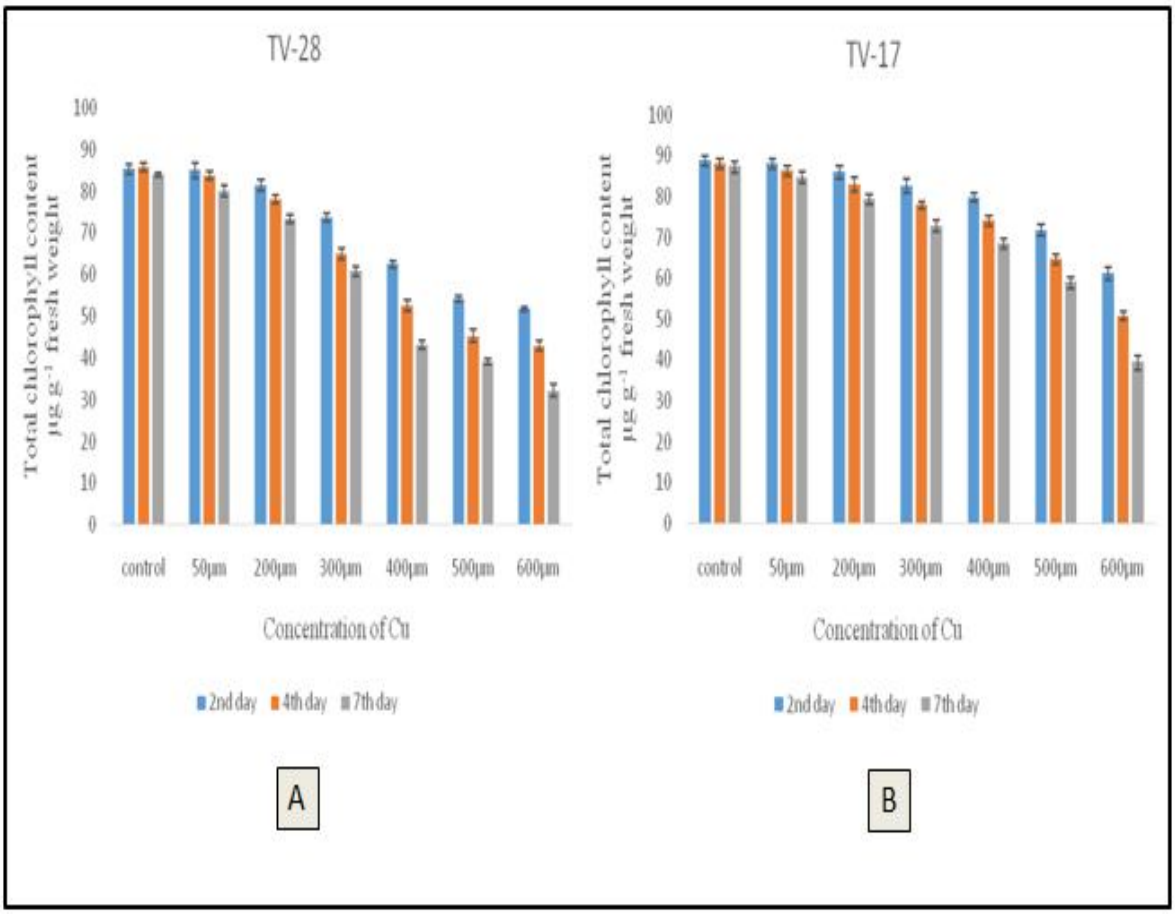

Figure4.2.3. A&B. Effect of Total chlorophyll contenton TV-28 & TV-17 cultivars exposed to different concentration and exposure duration. All the values are mean of triplicates ±SD.

From Figure 4.2.3.A we can observe the effect of total chlorophyll content of TV-28. With respect to control the content decreased by 61.58% at the later phase of 600µM. Where the decrease was marked by 54.92% at the highest concentration of 600µM with respect to control.

Table12. Effect of Cu on Chlorophyll content exposed to different concentration and exposure duration onTV-21. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	82.32±1.22	81.01±1.38	79.02±1.59
50 µM	80.21±0.92	78.19±1.56	74.02±0.82
200 µM	77.28±1.56	72.77±1.28	64.89±0.71
300 µM	72.04±1.52	65.93±1.33	56.29±0.68
400 µM	65.28±1.48	53.27±0.72	44.37±1.38
500 µM	52.13±0.82	43.11±1.29	32.19±1.43
600µM	40.18±1.52	31.22±0.86	19.63±1.38

Table13. Effect of Cu on Chlorophyll content exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	90.12±0.52	88.92±0.78	86.58±0.93
50 µM	86.25±1.38	85.65±0.67	82.29±1.4
200 µM	83.31±1.26	82.62±0.81	80.19±1.36
300 µM	81.02±1.29	79.93±0.51	75.14±0.86
400 µM	71.92±1.00	68.23±0.86	63.89±0.99
500 µM	65.23±1.36	62.18±0.91	56.12±0.69
600µM	54.23±1.39	50.19±1.57	42.13±0.86

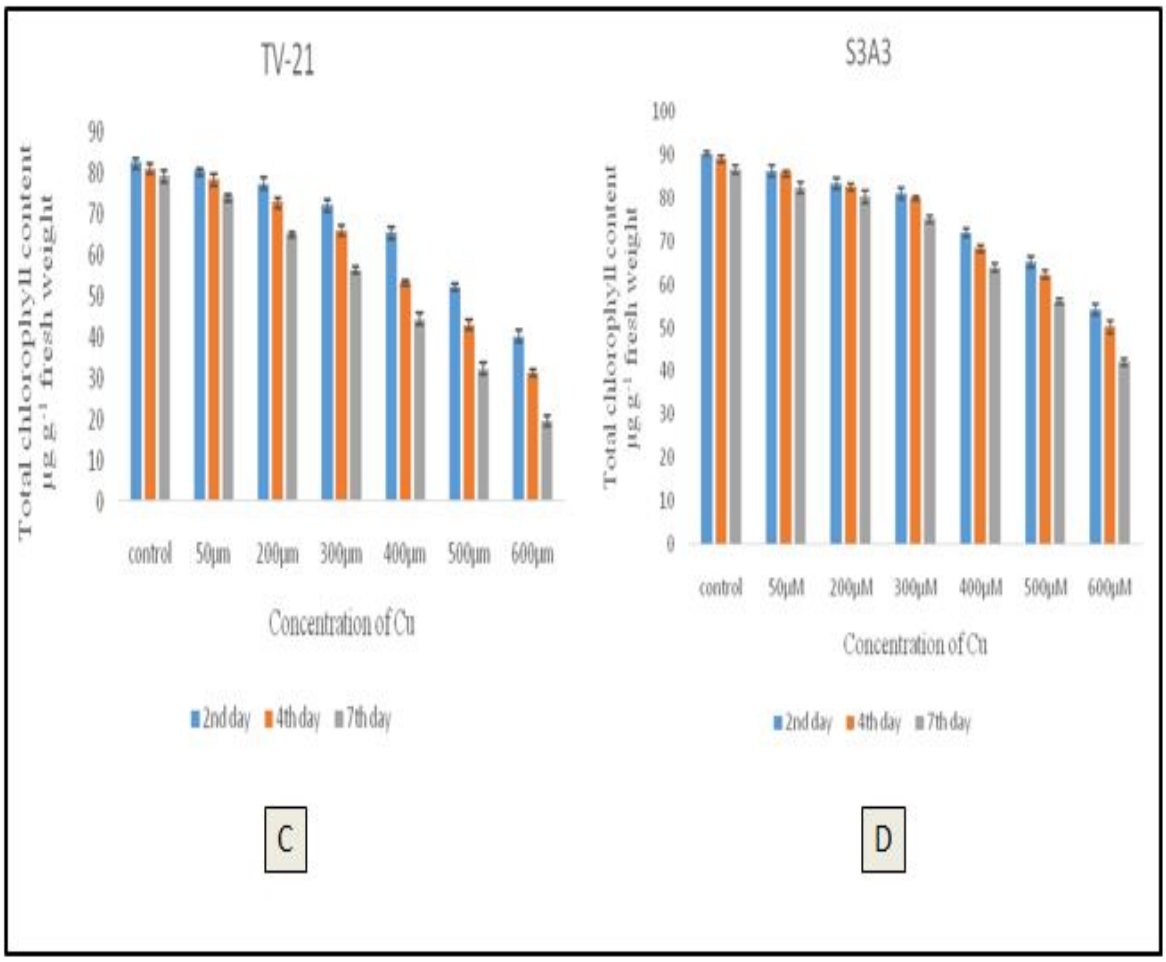

Figure4.2.3. C&D. Effect of Total chlorophyll contenton TV-21 & S_3A_3 cultivars exposed to different concentration and exposure duration. All the values are mean of triplicates ±SD.

From the figure 4.2.3.C&D we can observedthe effect of total chlorophyll content on TV-21 & S_3A_3 cultivars exposed to different concentration and exposure duration respectively. 75.15% of decrease was depicted in TV-21 where in S_3A_3 the decrease in chlorophyll was marked by 51.33% at 600µM with respect to the control.

Table14. Effect of Cu on Chlorophyll content exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	91.03±0.83	89.89±0.66	87.88±0.73
50 µM	88.65±0.864	86.92±0.933	85.38±0.92
200 µM	86.82±0.746	83.62±1.395	81.28±0.81
300 µM	84.21±0.698	81.02±0.737	78.72±1.29
400 µM	78.28±0.722	73.22±0.822	68.01±0.97
500 µM	71.09±0.739	67.21±0.938	60.03±0.88
600µM	63.07±0.933	56.92±0.893	45.92±1.28

Table15. Effect of Cu on Chlorophyll content exposed to different concentration and exposure duration on TS-491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	94.14±1.08	92.01±1.46	89.03±1.49
50 µM	90.13±1.09	87.32±1.26	84.28±0.99
200 µM	83.93±0.92	80.02±1.21	78.21±1.22
300 µM	73.32±1.03	70.89±1.22	67.12±1.36
400 µM	61.07±0.99	52.87±0.87	49.89±1.32
500 µM	49.27±1.29	36.87±1.27	32.13±1.36
600µM	36.23±1.28	23.98±0.99	18.09±1.21

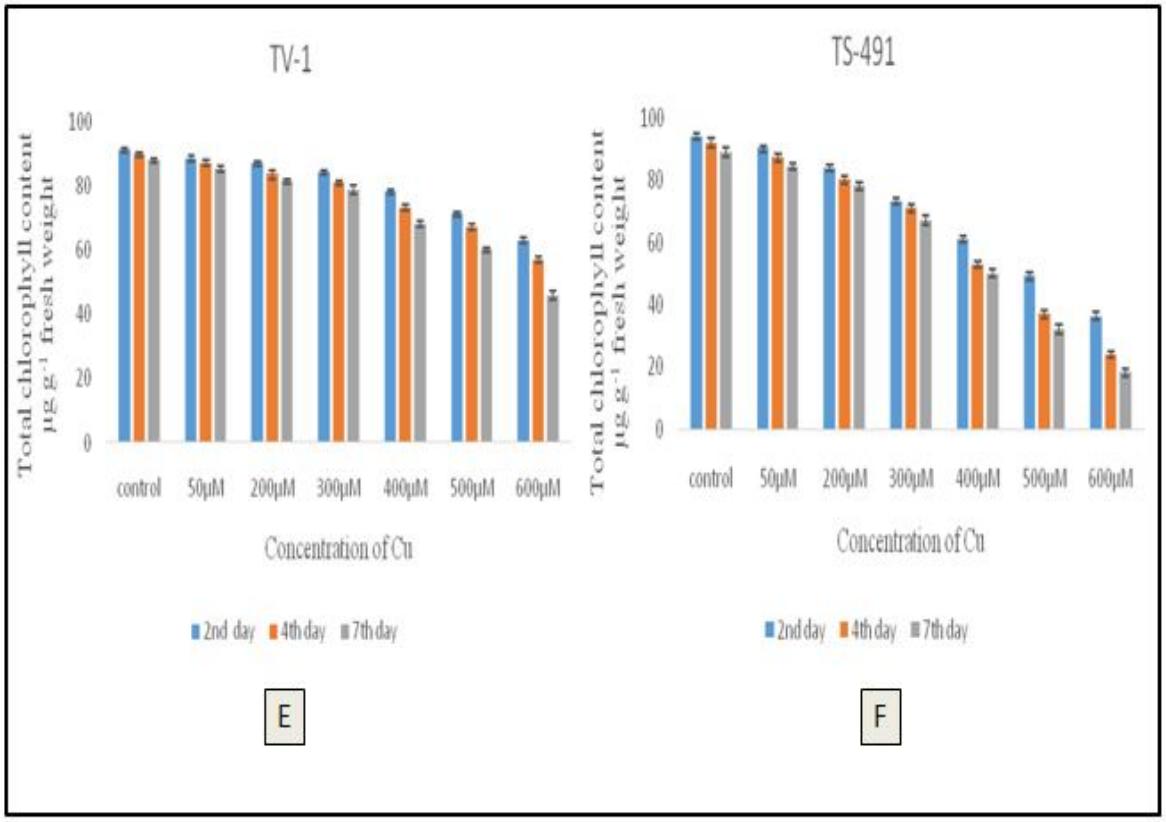

Figure4.2.3. E&F. Effect of Total chlorophyll contenton TV-1 & TS-491 cultivars exposed to different concentration and exposure duration. All the values are mean of triplicates ±SD.

Figure 4.2.3.E&F represent the effect of total chlorophyll content on TV-1 & TS-491 cultivars exposed to different concentration and exposure duration. 47.74% of decrease in chlorophyll content was marked in TV-1 where TS-491, the seed germinated cultivar was marked with highest level of decrease in chlorophyll content by 79.68%. Among the cultivars TS-491 was more sensitive cultivar.

Chapter4.3

Biochemical analysis

4.3.1. Effect of Cu on Malondialdehyde content

The oxidative damage done by Cu was measured as changes in thiobarbituric acid reactive substances like Malondialdehyde (MDA). MDA is a byproduct of lipid peroxidation, which results into membrane damage. The results of MDA have been presented in Figure 4.3.1.A-4.3.1.F. Cu produced a marked enhancement in MDA content. The MDA content increased progressively in concentration and duration dependent manner. A significant positive correlation was observed between increase in the metal and MDA content. With respect to control there was a significant increase in the MDA content in all the cultivars.

Table16. Effect of Cu on MDA Content exposed to different concentration and exposure duration on TV-28. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.267±0.28	1.436±0.16	1.736±0.17
50 µM	1.873±0.21	2.437±0.13	2.937±0.21
200 µM	2.638±0.18	3.026±0.24	3.937±0.17
300 µM	2.903±0.12	3.578±0.51	3.978±0.32
400 µM	3.567±0.28	4.1787±0.31	4.783±0.23
500 µM	4.027±0.38	4.692±0.27	5.063±0.28
600µM	4.892±0.19	5.367±0.19	5.928±0.13

Table17. Effect of Cu on MDA Content exposed to different concentration and exposure duration on TV-17. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.234±0.19	1.461±0.18	1.737±0.16
50 µM	2.236±0.37	2.282±0.28	2.432±0.21
200 µM	2.411±0.18	2.673±0.18	2.932±0.12
300 µM	2.857±0.19	3.375±0.11	3.945±0.12
400 µM	3.372±0.18	3.586±0.21	4.052±0.28
500 µM	3.8237±0.22	4.278±0.19	4.687±0.21
600µM	4.679±0.18	4.957±0.12	5.488±0.16

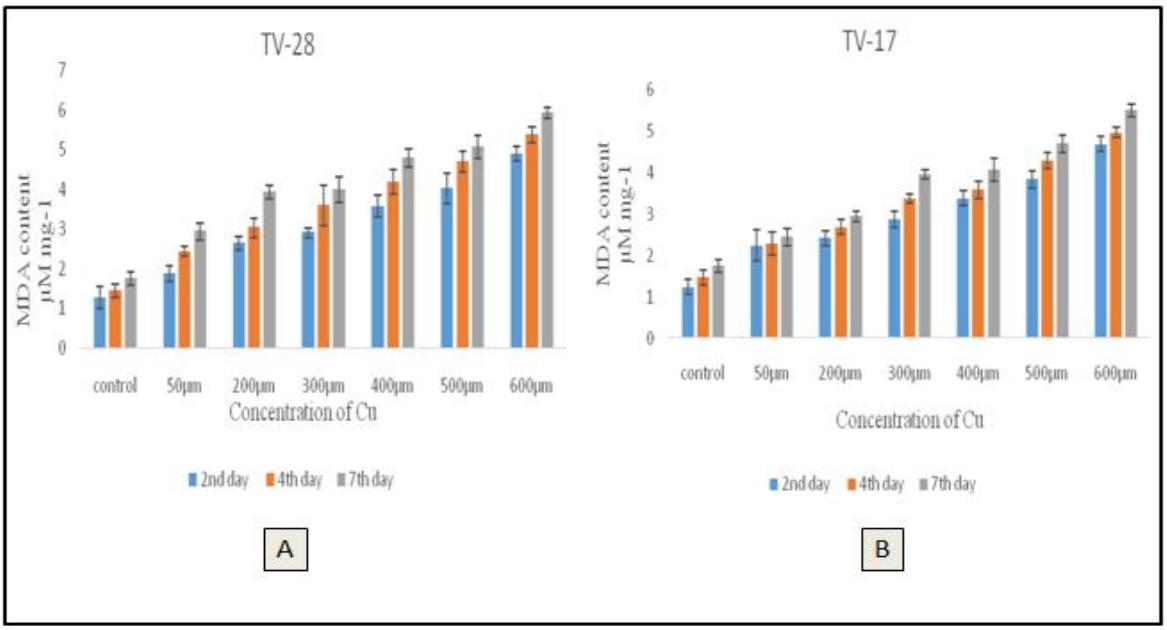

Figure4.3.1. A & B. Effect of Cu on MDA content exposed to different concentration and exposure duration on TV-28 & TV-17. All the values are mean of triplicate± SD.

Table18. Effect of Cu on MDA Content exposed to different concentration and exposure duration on TV-21. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.562±0.32	1.672±0.17	1.826±0.18
50 µM	1.892±0.22	2.013±0.13	2.382±0.22
200 µM	2.192±0.19	2.437±0.24	2.836±0.18
300 µM	2.326±0.13	2.721±0.59	3.017±0.27
400 µM	2.726±0.19	2.98±0.03	3.439±0.13
500 µM	3.426±0.23	3.982±0.02	4.426±0.12
600µM	3.928±0.22	4.627±0.19	5.231±0.13

Table19. Effect of Cu on MDA Content exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.225±0.14	1.465±0.22	1.817±0.26
50 µM	2.20±0.09	2.267±0.11	2.432±0.19
200 µM	2.45±0.16	2.673±0.17	2.913±0.08
300 µM	2.857±0.19	3.375±0.23	3.945±0.21
400 µM	3.372±0.02	3.586±0.18	3.749±0.15
500 µM	3.967±0.17	4.278±0.09	4.502±0.18
600µM	4.672±0.11	4.959±0.05	5.213±0.13

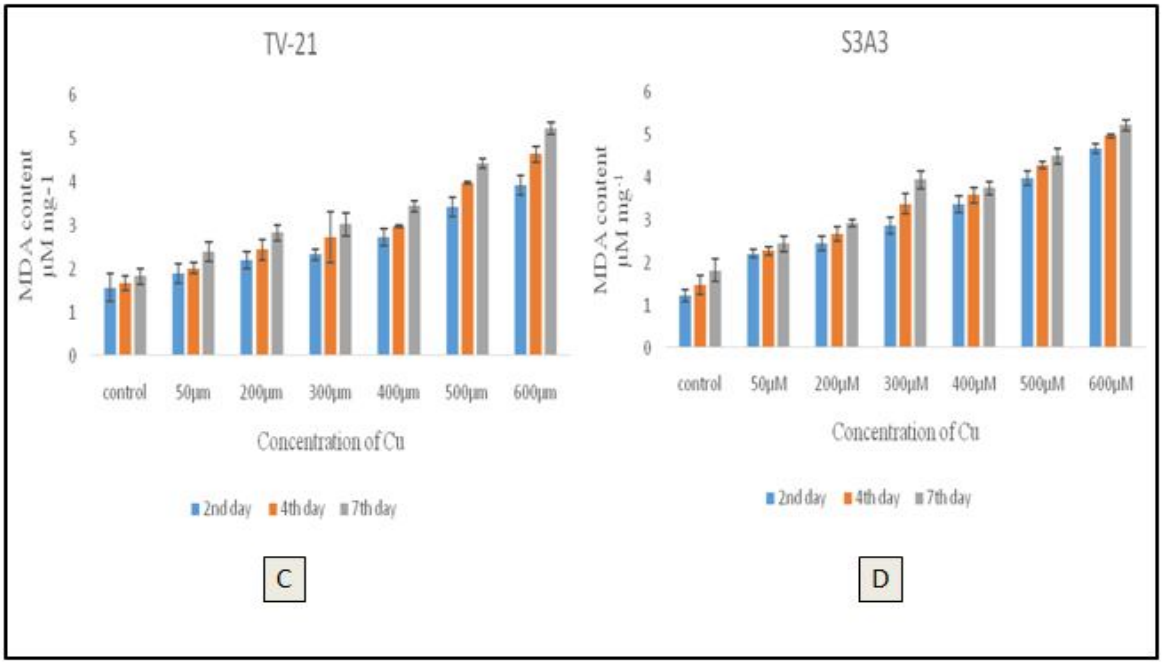

Figure4.3.1. C & D. Effect of Cu on MDA content exposed to different concentration and exposure duration on TV-21 & TV-17. All the values are mean of triplicate± SD.

Table20. Effect of Cu on MDA Content exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.989±0.19	1.221±0.22	1.518±0.28
50 µM	1.478±0.22	1.938±0.19	2.499±0.11
200 µM	1.928±0.11	2.592±0.19	2.983±0.17
300 µM	2.474±0.12	2.98±0.28	3.32±0.11
400 µM	2.962±0.22	3.327±0.19	3.728±0.18
500 µM	3.418±0.19	3.973±0.29	4.263±0.11
600µM	3.998±0.18	4.473±0.11	4.902±0.13

Table21. Effect of Cu on MDA Content exposed to different concentration and exposure duration on TS-491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
control	4.231±0.26	4.562±0.25	4.835±0.19
50µm	4.476±0.32	4.633±0.18	4.926±0.21
200µm	4.824±0.19	5.238±0.36	5.908±0.07
300µm	5.875±0.18	6.238±0.22	6.879±0.23
400µm	6.032±0.15	6.585±0.19	7.097±0.18
500µm	6.932±0.21	7.321±0.18	7.821±0.22
600µm	7.358±0.18	7.664±0.09	8.2845±0.24

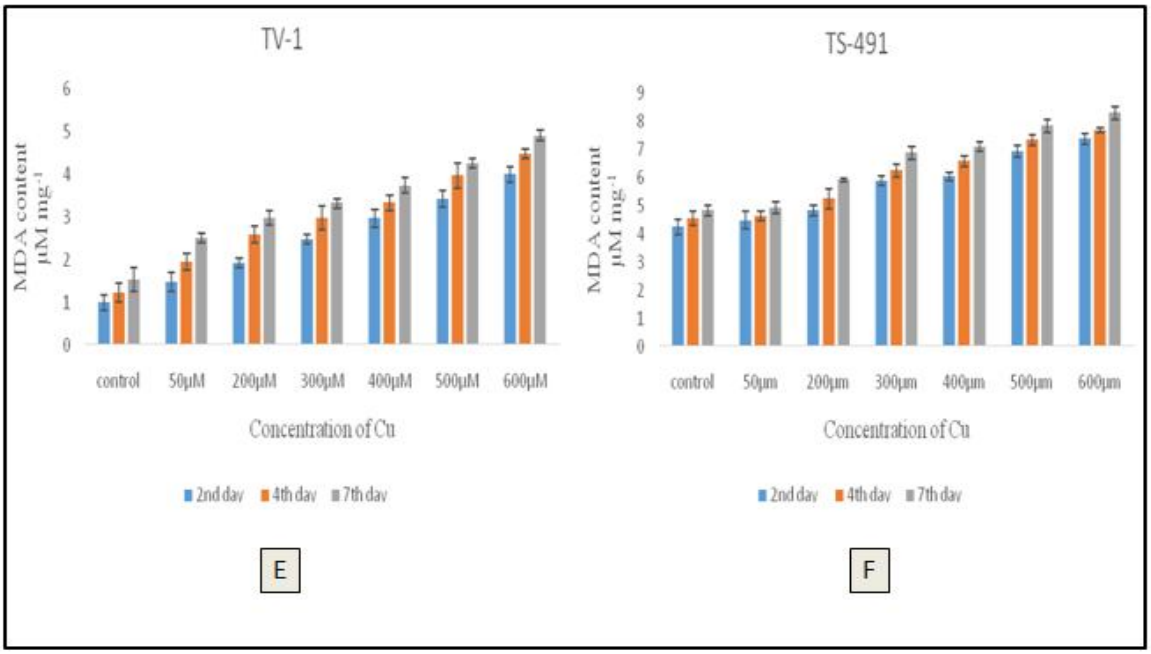

Figure4.3.1. E&F. Effect of Cu on MDA content exposed to different concentration and exposure duration on TV-21 & TS-491. All the values are mean of triplicate± SD.

From the entire figure we can observe the trend of increase in MDA content though the level of increase was different in different cultivars.This showed that high concentration of Cu can lead

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

4.3.2. Enzymatic Antioxidants:

Plants are exposed to abrupt daily and seasonal changes in the environment and they have a wide spectrum of developmental responses and biochemical adaptations to stress condition. The response of various antioxidants enzymes in cultivars against the different concentration of Cu has been shown in figure 4.3.2.1.A-4.3.2.5.F.Various antioxidant enzymes exhibited different responses upon Cu exposure. In general the activities showing increase at lower concentration and duration followed by decline at higher concentration and duration of exposure. The activities of APX, CAT, SOD, and POD gradually decreased with increase in the concentration of Cu.

4.3.2.1. Effect of Cu on SOD Activity

Duration wise in all cultivars Cu showed similar effects on SOD activity. Amongst all the cloned cultivars TV-28, TV-17, TV-21 showed similar effect of gradual increase in the activity at lower concentration up to 400 µM which followed by the decline in the activity with further increase in the concentration. In TV-28 and TV-21 SOD activity increased up to 7^{th} day of 300 µM (Figure 4.3.2.1.A and 4.3.2.1.C respectively). At 400 µM the level of increase in the activity slowed down compare to below 400 µM and from 7^{th} day of 400 µM the activity was declined, whereas in TV-17 declination in the activity started at the later phase of treatment of 500 µM concentration of Cu (4.3.2.1.B).

Table22. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-28. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.106±0.18	2.426±0.21	2.638±0.19
50 µM	2.392±0.14	2.673±0.18	2.937±0.17
200 µM	2.728±0.12	3.192±0.19	3.549±0.188
300 µM	3.283±0.18	3.735±0.19	4.027±0.12
400 µM	3.576±0.19	3.638±0.17	3.439±0.17
500 µM	3.247±0.13	3.038±0.18	2.839±0.16
600µM	2.938±0.19	2.736±0.17	2.462±0.13

Table23. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-17. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	3.362±0.12	3.6345±0.17	3.934±0.21
50 µM	3.6378±0.28	3.9382±0.21	4.362±0.28
200 µM	3.983±0.16	4.453±0.12	4.982±0.18
300 µM	4.3679±0.18	4.745±0.17	5.038±0.17
400 µM	4.773±0.17	5.032±0.18	5.735±0.19
500 µM	4.623±0.18	4.783±0.13	4.281±0.14
600µM	4.362±0.17	4.028±0.11	3.264±0.23

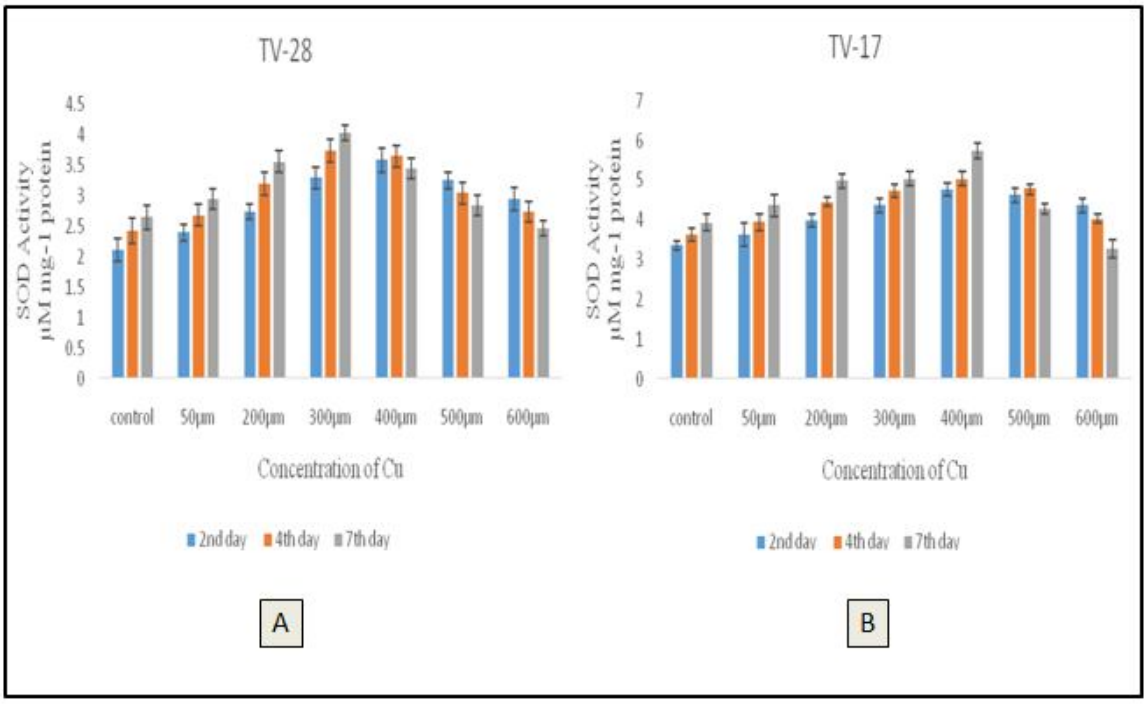

Figure4.3.2.1. A&B. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-28 &T17. All the values are mean of triplicate± SD.

Table24. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-21. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.223±0.08	2.526±0.23	2.712±0.11
50 µM	2.462±0.19	2.778±0.26	2.998±0.21
200 µM	2.716±0.21	3.203±0.10	3.549±0.28
300 µM	3.312±0.12	3.746±0.11	4.1198±0.18
400 µM	3.618±0.21	3.738±0.17	3.219±0.11
500 µM	3.108±0.18	3.012±0.08	2.739±0.12
600µM	2.519±0.19	2.136±0.22	1.462±0.28

Table25. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.832±0.22	2.065±0.18	2.234±0.16
50 µM	2.108±0.18	2.365±0.19	2.547±0.13
200 µM	2.289±0.26	2.356±0.1	2.657±0.21
300 µM	2.576±0.13	2.769±0.16	2.912±0.17
400 µM	2.839±0.31	3.109±0.28	3.387±0.17
500 µM	3.076±0.23	3.347±0.18	3.629±0.19
600µM	3.643±0.17	3.932±0.15	4.067±0.11

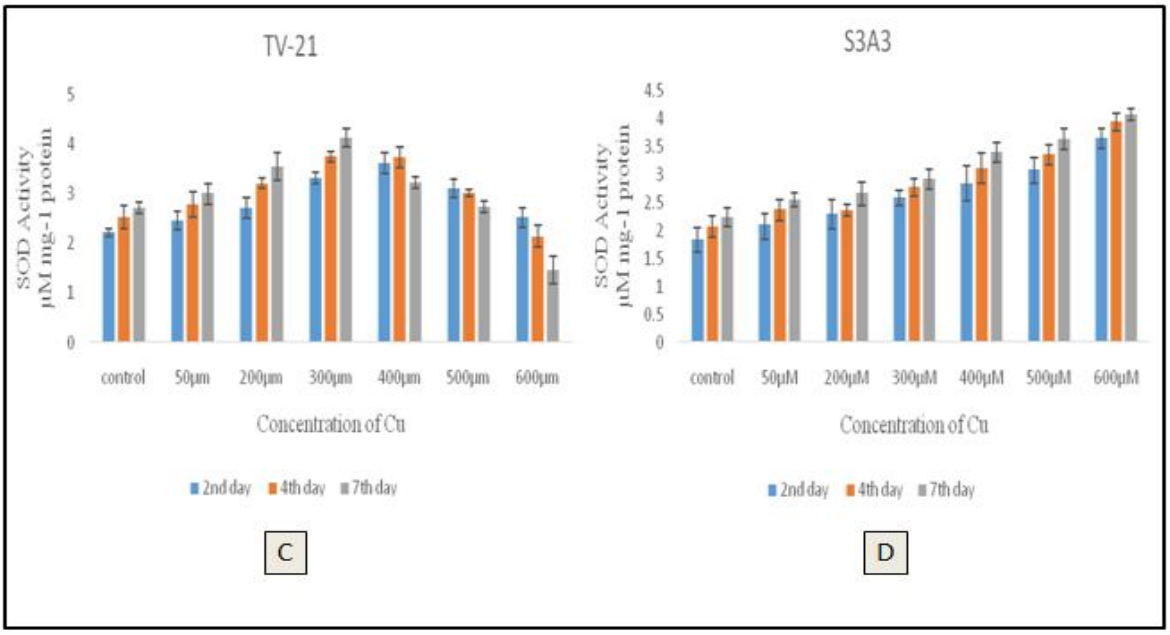

Figure4.3.2.1. C&D. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-21 & S_3A_3. All the values are mean of triplicate± SD.

Table26. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.816±0.39	2.122±0.22	2.329±0.28
50 µM	2.137±0.19	2.389±0.11	2.642±0.32
200 µM	2.307±0.33	2.539±0.19	2.722±0.22
300 µM	2.582±0.11	2.799±0.28	2.989±0.31
400 µM	2.882±0.22	3.266±0.28	3.433±0.22
500 µM	3.089±0.28	3.426±0.33	3.711±0.38
600µM	3.672±0.19	4.017±0.22	4.281±0.19

Table27. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TS-491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.319±0.11	2.521±0.13	2.689±0.18
50 µM	2.678±0.26	2.895±0.25	3.076±0.18
200 µM	2.986±0.18	3.328±0.15	3.468±0.17
300 µM	3.245±0.21	3.465±0.16	3.627±0.22
400 µM	3.867±0.32	4.329±0.26	4.527±0.28
500 µM	4.213±0.24	4.526±0.18	4.512±0.21
600µM	4.342±0.29	4.4933±0.25	4.436±0.23

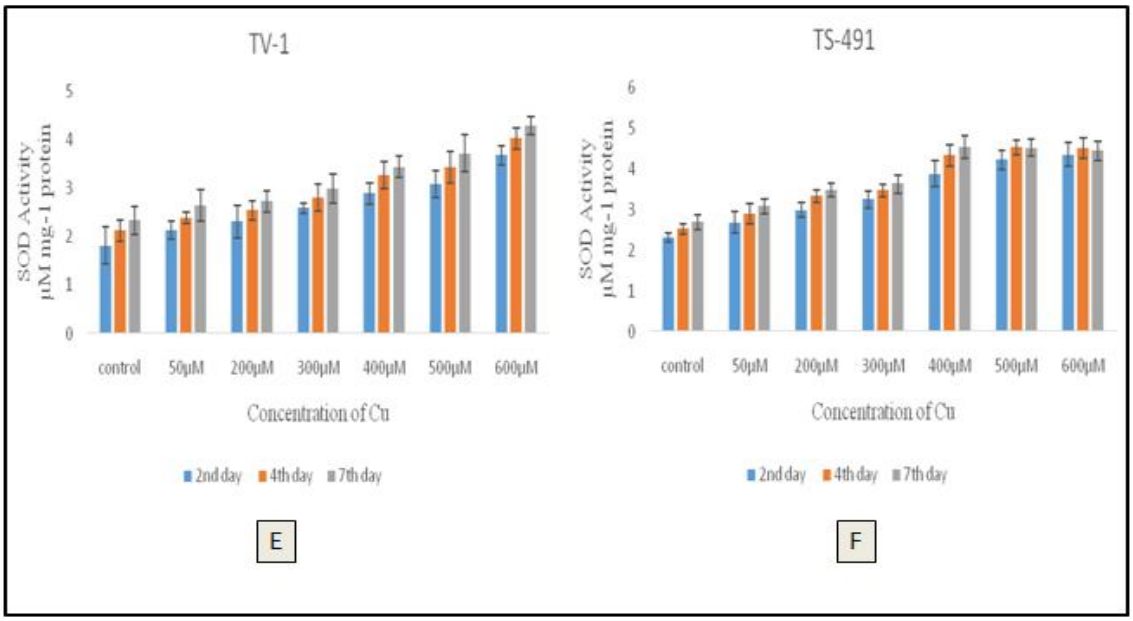

Figure4.3.2.1. E&F. Effect of Cu on SOD Activity exposed to different concentration and exposure duration on TV-1 &TS-491. All the values are mean of triplicate± SD.

In TV-1 and S_3A_3 the activity increased up to highest concentration of Cu (600 µM) though the level of increase in the activity slowed down beyond 400 µM (4.3.2.1.F and 4.3.2.1.D respectively). In TS-491 the activity increased up to the mid phase of exposure of Cu at 600 µM (Figure 4.3.2.1.F). After the 7^{th} day the activity was decreased. In TV-1 the increase in the activity was 135.7% and in S_3A_3 was 121.99%.

4.3.2.2. Effect of Cu on POD Activity

POD plays an important role in plant respiratory metabolism and physiological resistance. In POD activity same trend of increase at lower concentration and duration was observed which followed by decline at higher concentration and duration of exposure (4.3.2.2.A-4.3.2.2.F). In TV-28 and TV-21 the increase was up to 4^{th} day of 300 µM (4.3.2.2.A and 4.3.2.2.C), though the level of increase in the activity was different in both the cultivars.

Whereas in TV-17 the trend of increase in the activity was up to 7th day of 400 µM (4.3.2.2.B). The other two cloned varieties showed tolerance even up to highest concentration.

Table28. Effect of Cu on POD Activity exposed to different concentration and exposure duration on TV-28. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.02±0.11	2.318±0.27	2.589±0.11
50 µM	2.563±0.23	2.835±0.28	3.023±0.11
200 µM	2.632±0.21	2.762±0.32	3.226±0.21
300 µM	2.784±0.182	2.9845±0.22	3.34±0.18
400 µM	2.827±0.28	2.783±0.32	2.537±0.19
500 µM	2.625±0.21	2.439±0.18	2.176±0.11
600µM	2.376±0.18	2.067±0.22	1.536±0.29

Table29. Effect of Cu on POD Activity exposed to different concentration and exposure duration on TV-17. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.376±0.21	2.528±0.28	2.735±0.21
50 µM	2.634±0.32	2.837±0.38	3.276±0.22
200 µM	2.946±0.18	3.239±0.22	3.548±0.18
300 µM	3.284±0.22	3.635±0.32	3.937±0.28
400 µM	3.583±0.22	3.937±0.18	4.473±0.38
500 µM	3.865±0.22	3.673±0.32	3.352±0.29
600µM	3.537±0.27	3.236±0.43	2.96±0.22

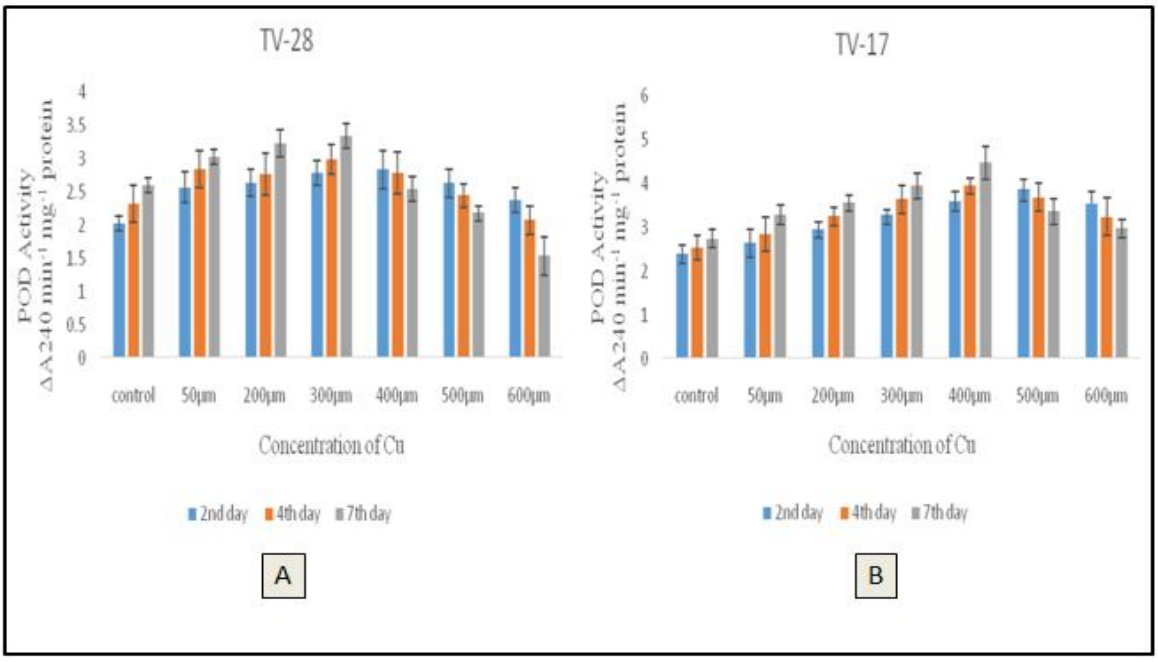

Figure4.3.2.2. A &B. Effect of Cu on POD Activity exposed to different concentration and exposure duration onTV-28& TV-17. All the values are mean of triplicate± SD.

Table30. Effect of Cu on POD Activity exposed to different concentration and exposure duration on TV-21. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.217±0.27	2.489±0.08	2.728±0.06
50 µM	2.589±0.18	2.817±0.03	3.192±0.11
200 µM	2.978±0.08	3.427±0.18	4.192±0.23
300 µM	3.562±0.27	3.983±0.08	4.423±0.33
400 µM	3.726±0.09	3.7728±0.25	3.526±0.05
500 µM	3.527±0.07	3.189±0.32	2.678±0.02
600µM	3.018±0.05	2.673±0.18	2.018±0.27

Table31. Effect of Cu on POD Activity exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.431±0.18	2.541±0.09	2.931±0.16
50 µM	2.953±0.22	3.298±0.18	3.574±0.28
200 µM	3.276±0.29	3.982±0.11	4.278±0.13
300 µM	3.657±0.25	3.926±0.17	4.238±0.13
400 µM	3.956±0.31	4.243±0.l8	4.673±0.27
500 µM	4.267±0.15	4.536±0.17	4.729±0.21
600µM	4.762±0.17	5.187±0.21	5.387±0.16

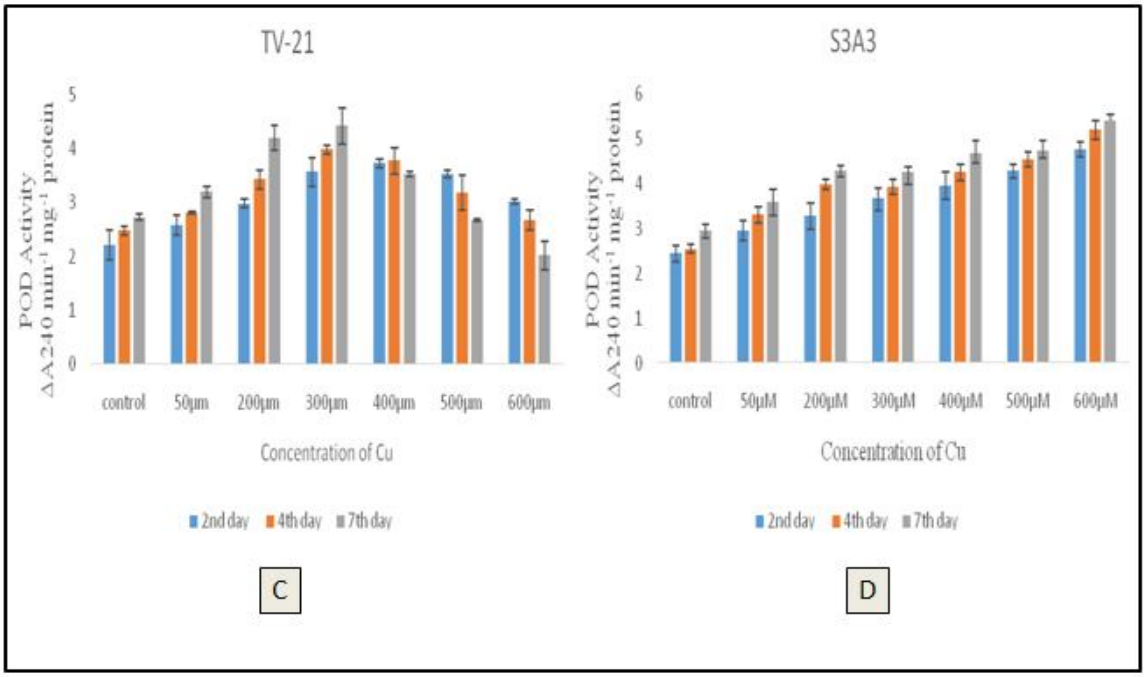

Figure4.3.2.2. C&D. Effect of Cu on POD Activity exposed to different concentration and exposure duration onTV-21 &S_3A_3. All the values are mean of triplicate± SD.

Table32. Effect of Cu on POD Activity exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	3.018±0.11	3.219±0.28	3.6831±0.19
50 µM	3.533±0.29	3.898±0.22	4.174±0.21
200 µM	3.892±0.11	4.2982±0.27	4.635±0.31
300 µM	4.218±0.18	4.789±0.13	5.228±0.19
400 µM	4.561±0.21	4.939±0.11	5.438±0.22
500 µM	4.977±0.22	5.439±0.25	5.929±0.18
600µM	5.628±0.19	6.018±0.18	6.431±0.11

Table33. Effect of Cu on POD Activity exposed to different concentration and exposure duration on TS-491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	1.893±0.16	2.01±0.18	2.325±0.13
50 µM	2.265±0.22	2.657±0.19	2.769±0.25
200 µM	2.573±0.17	2.773±0.11	3.224±0.13
300 µM	2.846±0.21	3.281±0.16	3.915±0.19
400 µM	3.286±0.29	3.781±0.27	4.535±0.26
500 µM	3.792±0.31	4.025±0.29	4.392±0.17
600µM	3.732±0.12	3.834±0.15	3.632±0.18

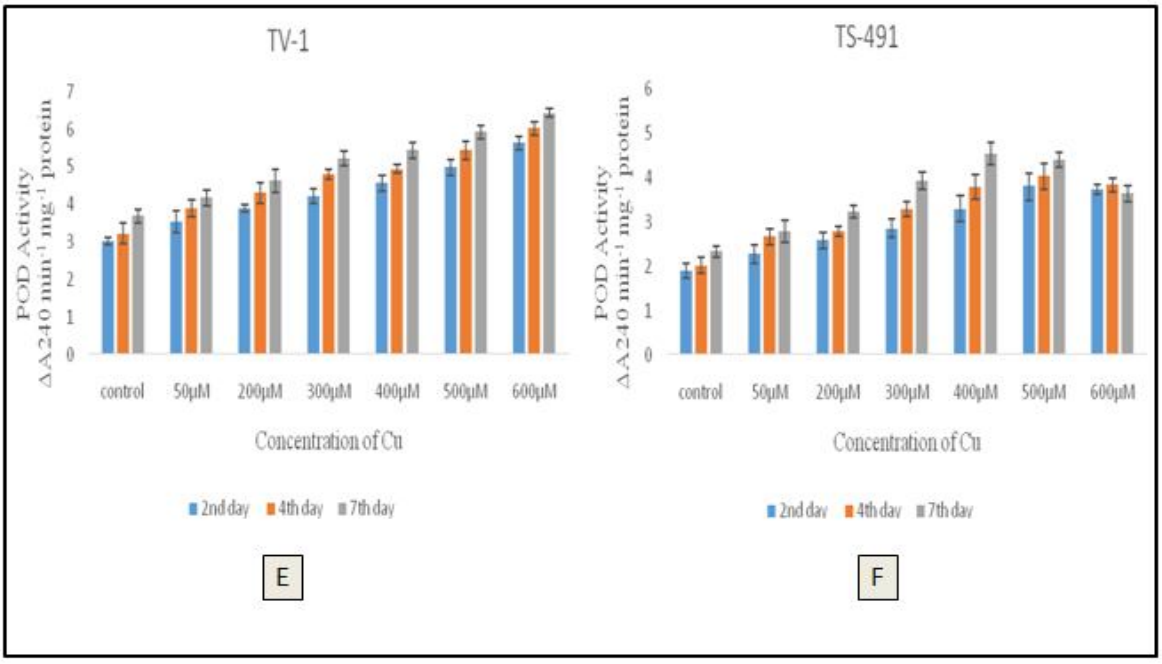

Figure4.3.2.2. E &F. Effect of Cu on POD Activity exposed to different concentration and exposure duration onTV-1 & TS-491. All the values are mean of triplicate± SD.

In S_3A_3 there was significant increase up to 400 µM where from 500 µM the level of increase in the activity slowed down. On the other hand TV-1 showed a sharp increase in the activity up to 600 µM (4.3.2.2.E). In TS-491 the increase in the activity was observed up to 500 µM with gradual fall in the level of increase in 500 µM which followed by the decrease in activity at 600 µM (4.3.2.2.F).

4.3.2.3. Effect of Cu on APX Activity

With respect to control there was a significant increase in the APX activity in all the cultivars (4.3.2.3.A-4.3.2.3.F). In TV-28 and TV-17 there was increase in the activity up to 400 µM and from 500 µM there was decrease in the activity (4.3.2.3.A and 4.3.2.3.B). In cultivar TV-21 the increase in activity was observed up to 300 µM and further increase in the concentration lead to the decrease in the activity (4.3.2.3.C). In S_3A_3 and TV-1 the increase in the activity was observed up to 4^{th} day of 600 µM (4.3.2.3.D and 4.3.2.3.E). We can observe

from figure 4.3.2.3.F, there was only a marginal increase in the APX activity with the exposure of Cu up to 300 µM in TS491 cultivar but further increase in the concentration of Cu there was 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 compare to the lower concentration, finally the APX activity started to decline from 7th day of 500µM in TS-491.

Table34. Effect of Cu on APX Activity exposed to different concentration and exposure duration on TV-28. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.22±0.021	0.368±0.045	0.437±0.032
50 µM	0.33±0.018	0.489±0.013	0.572±0.011
200 µM	0.42±0.011	0.592±0.017	0.687±0.031
300 µM	0.53±0.023	0.682±0.024	0.751±0.031
400 µM	0.51±0.021	0.627±0.033	0.652±0.022
500 µM	0.48±0.018	0.387±0.04	0.2983±0.037
600µM	0.43±0.029	0.361±0.036	0.238±0.028

Table35. Effect of Cu on APX Activity exposed to different concentration and exposure duration on TV-17. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.35±0.003	0.44±0.018	0.50±0.011
50 µM	0.42±0.007	0.56±0.019	0.62±0.032
200 µM	0.51±0.002	0.63±0.005	0.70±0.028
300 µM	0.63±0.011	0.71±0.029	0.83±0.029
400 µM	0.74±0.021	0.80±0.02	0.89±0.021
500 µM	0.65±0.008	0.61±0.019	0.56±0.032
600µM	0.57±0.0092	0.46±0.032	0.32±0.029

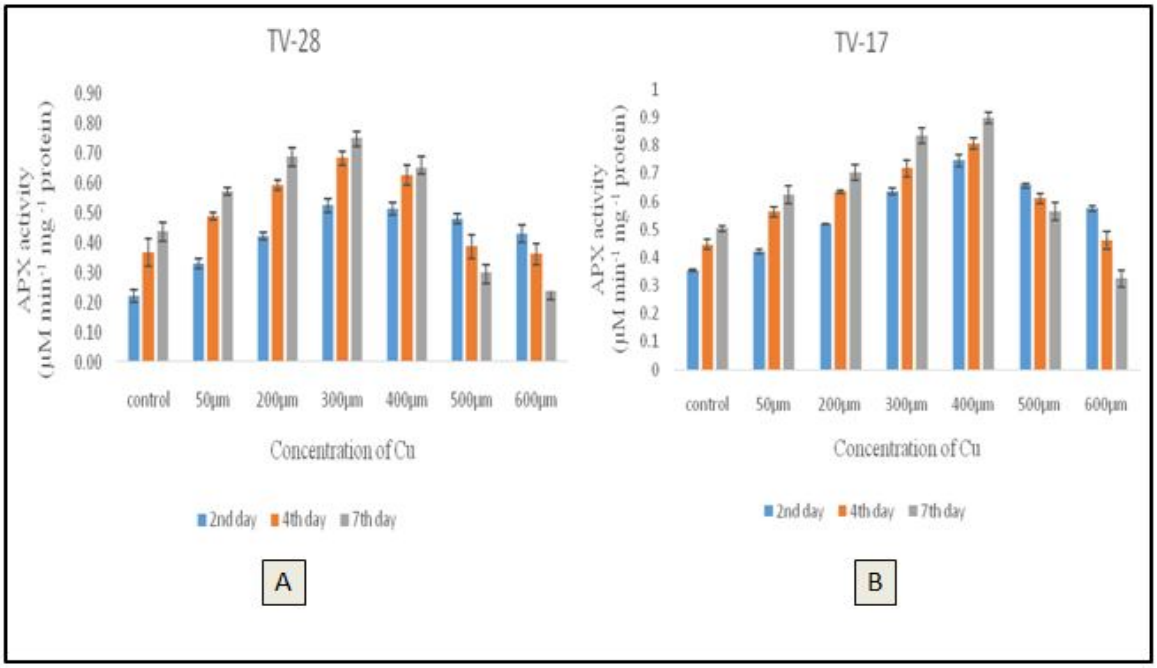

Figure4.3.2.3. A&.B. Effect of Cu on APX Activity exposed to different concentration and exposure duration onTV-28 & TV-17. All the values are mean of triplicate± SD.

Table36. Effect of Cu on APX Activity exposed to different concentration and exposure duration on TV-21. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.24±0.023	0.33±0.016	0.41±0.11
50 µM	0.28±0.012	0.35±0.018	0.46±0.021
200 µM	0.35±0.013	0.42±0.011	0.51±0.013
300 µM	0.41±0.007	0.56±0.013	0.637±0.011
400 µM	0.49±0.021	0.44±0.019	0.37±0.009
500 µM	0.33±0.012	0.29±0.011	0.20±0.019
600µM	0.24±0.011	0.21±0.017	0.14±0.014

Table37. Effect of Cu on APX Activity exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.27±0.02	0.33±0.0421	0.49±0.08
50 µM	0.40±0.09	0.52±0.03	0.64±0.07
200 µM	0.56±0.11	0.73±0.09	0.855±0.02
300 µM	0.68±0.12	0.810.0523	0.94±0.024
400 µM	0.79±0.085	0.85±0.0175	1.01±0.014
500 µM	0.74±0.02	0.88±0.109	1.010.012
600µM	0.76±0.102	1.01±0.05	0.91±0.09

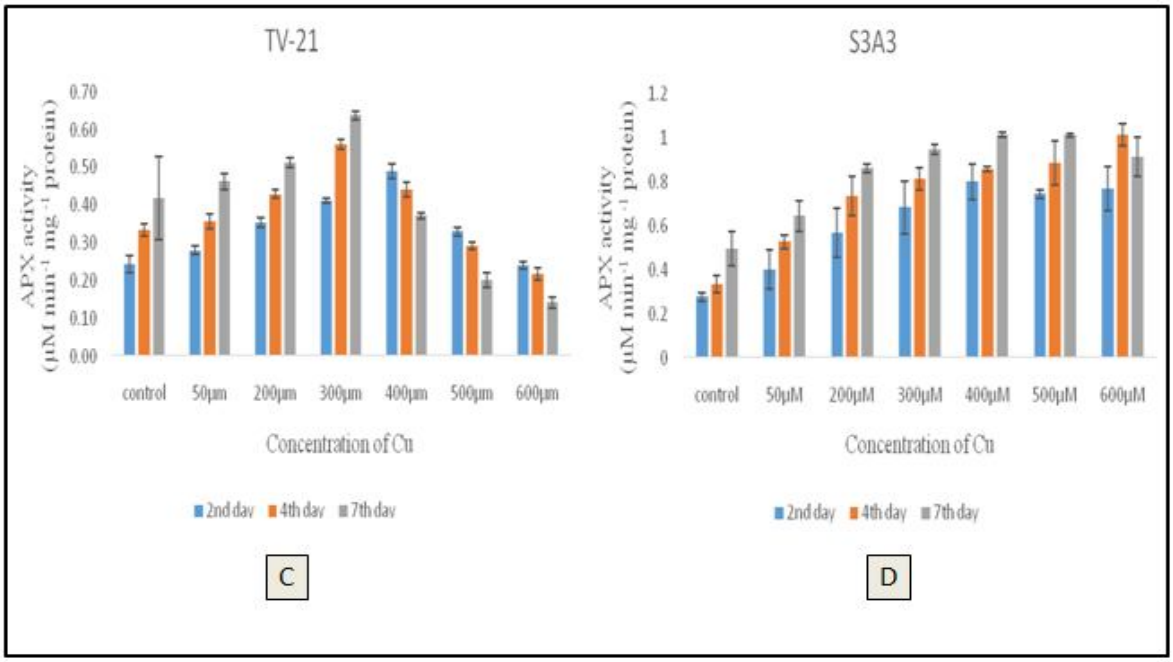

Figure4.3.2.3. C&D. Effect of Cu on APX Activity exposed to different concentration and exposure duration onTV-21 &S_3A_3. All the values are mean of triplicate± SD.

Table38. Effect of Cu on APX Activity exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.36±0.032	0.38±0.038	0.50±0.029
50 µM	0.46±0.021	0.56±0.029	0.68±0.010
200 µM	0.57±0.036	0.69±0.044	0.92±0.032
300 µM	0.70±0.028	0.89±0.021	0.98±0.019
400 µM	0.87±0.083	1.08±0.043	1.32±0.037
500 µM	0.90±0.053	1.10±0.054	1.23±0.049
600µM	0.93±0.029	1.11±0.0119	1.00±0.037

Table39. Effect of Cu on APX Activity exposed to different concentration and exposure duration on T491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	0.18±0.02	0.28±0.02	0.33±0.04
50 µM	0.32±0.04	0.59±0.06	0.61±0.08
200 µM	0.49±0.03	0.68±0.04	0.71±0.02
300 µM	0.58±0.06	0.81±0.03	1.00±0.07
400 µM	0.67±0.04	0.89±0.06	0.93±0.05
500 µM	0.77±0.02	0.88±0.04	0.72±0.03
600µM	0.82±0.06	0.84±0.08	0.61±0.02

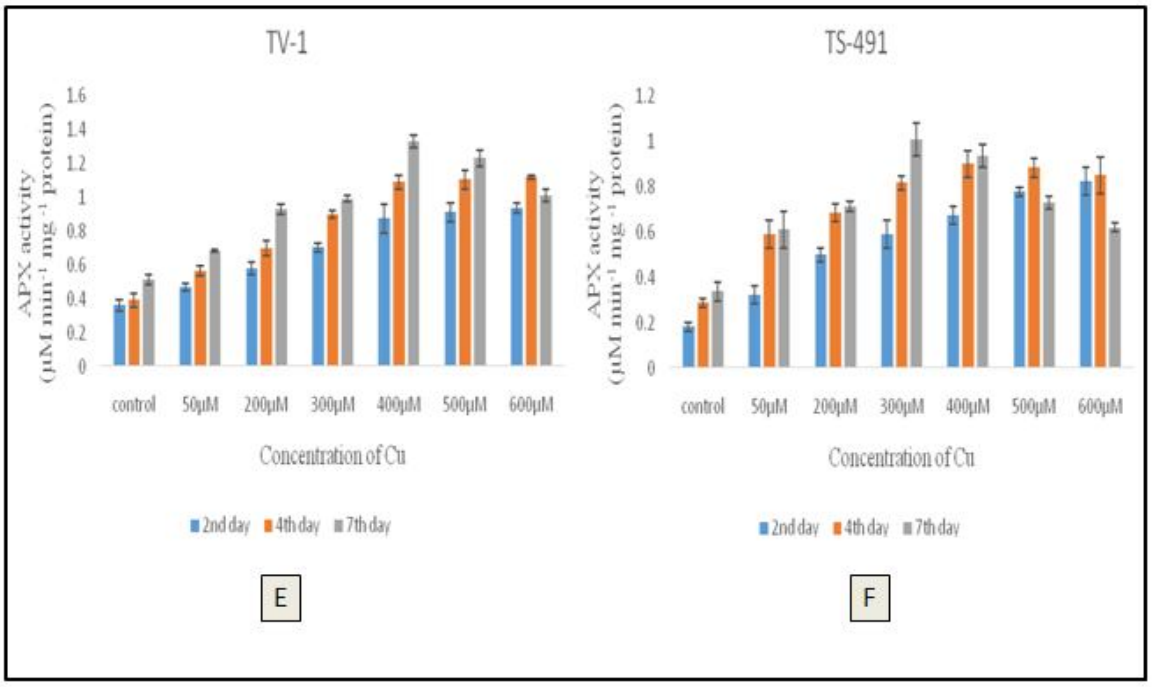

Figure4.3.2.3. E&F. Effect of Cu on APX Activity exposed to different concentration and exposure duration onTV-1 & TS-491. All the values are mean of triplicate± SD.

4.3.2.4. Effect of Cu on CAT Activity

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 catalyzes the oxidation of H donors with the consumption of one mole of peroxide. In TV-28 and TV-21 the activity of Cat increased up to 300 µM, where a sharp declination in the activity was observed from 400 µM (4.3.2.4.A and 4.3.2.4.C). In TV-17 the increase was upto 400 µM (4.3.2.4.B) and from 500 µM there was decrease in the CAT activity. The increase in the CAT activity of S_3A_3 cultivar continued even at higher concentration of Cu was observed up to 4^{th} day of 600 µM. But there was slight decrease in the activity on the 7^{th} day of 600 µM (4.3.2.4.D). In TV-1 the increase in the activity was continued till the later phase of the treatment i.e. 7^{th} day of 600 µM (4.3.2.4.E). Whereas in TS-491 the decrease in the CAT activity was observed from 400µM itself (4.3.2.4.F).

From the result we can observe that with respect to control the CAT activity increased by 413.9% in S_3A_3 and in TV-1 the increase in the activity was by 613%. S_3A_3 and TV-1 cultivar is more tolerant to higher concentration of Cu compare to other cloned cultivars and TS-491.

Table40. Effect of Cu on CAT Activity exposed to different concentration and exposure duration on TV-28. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	3.36±0.08	3.48±0.15	3.61±0.18
50 µM	3.52.08	3.68±0.23	3.83±0.32
200 µM	3.73±0.08	3.94±0.119	4.10±0.202
300 µM	4.01±0.22	4.43±0.156	4.63±0.211
400 µM	3.92±0.182	3.72±0.22	3.56±0.218
500 µM	3.76±0.119	3.53±0.171	3.34±0.131
600µM	3.50±0.172	3.28±0.138	2.18±0.264

Table41. Effect of Cu on CAT Activity exposed to different concentration and exposure duration on TV-17. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	5.52±0.029	5.86±0.19	6.20±0.23
50 µM	5.72±0.091	6.21±0.21	6.83±0.32
200 µM	6.49±0.28	7.20±0.31	7.92±0.22
300 µM	6.86±0.218	7.48±0.228	7.87±0.262
400 µM	7.28±0.39	7.84±0.28	8.12±0.318
500 µM	7.11±0.211	6.89±0.319	6.35±0.419
600µM	6.42±0.321	6.13±0.291	5.35±0.192

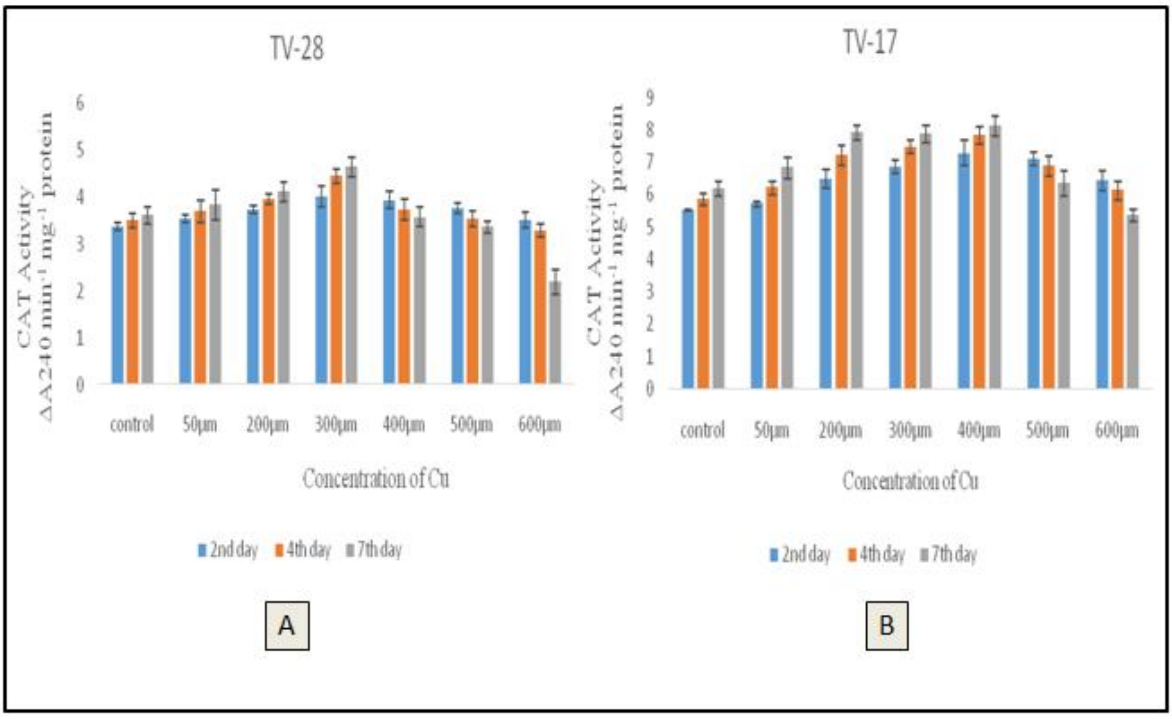

Figure4.3.2.4.A&.B. Effect of Cu on CAT Activity exposed to different concentration and exposure duration onTV-28& TV-17. All the values are mean of triplicate± SD.

Table42. Effect of Cu on CAT Activity exposed to different concentration and exposure duration on TV-21. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	2.98±0.03	3.19±0.12	3.32±0.18
50 µM	3.12±0.09	3.32±0.18	3.56±0.12
200 µM	3.43±0.07	3.73±0.23	4.12±0.21
300 µM	4.01±0.18	4.43±0.13	4.93±0.23
400 µM	4.12±0.17	3.98±0.21	3.72±0.21
500 µM	3.92±0.28	3.64±0.18	3.23±0.12
600µM	3.67±0.18	3.12±0.12	2.52±0.28

Table43. Effect of Cu on CAT Activity exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	6.52±0.32	6.81±0.28	7.11±0.17
50 µM	6.72±0.16	7.21±0.18	7.51±0.1
200 µM	6.89±0.11	7.31±0.13	7.62±0.11
300 µM	7.32±±0.2	7.48±0.17	7.87±0.19
400 µM	8.28±0.16	8.33±0.13	8.85±0.14
500 µM	8.71±0.15	8.91±0.07	9.19±0.21
600µM	9.42±0.11	9.53±0.14	9.22±0.09

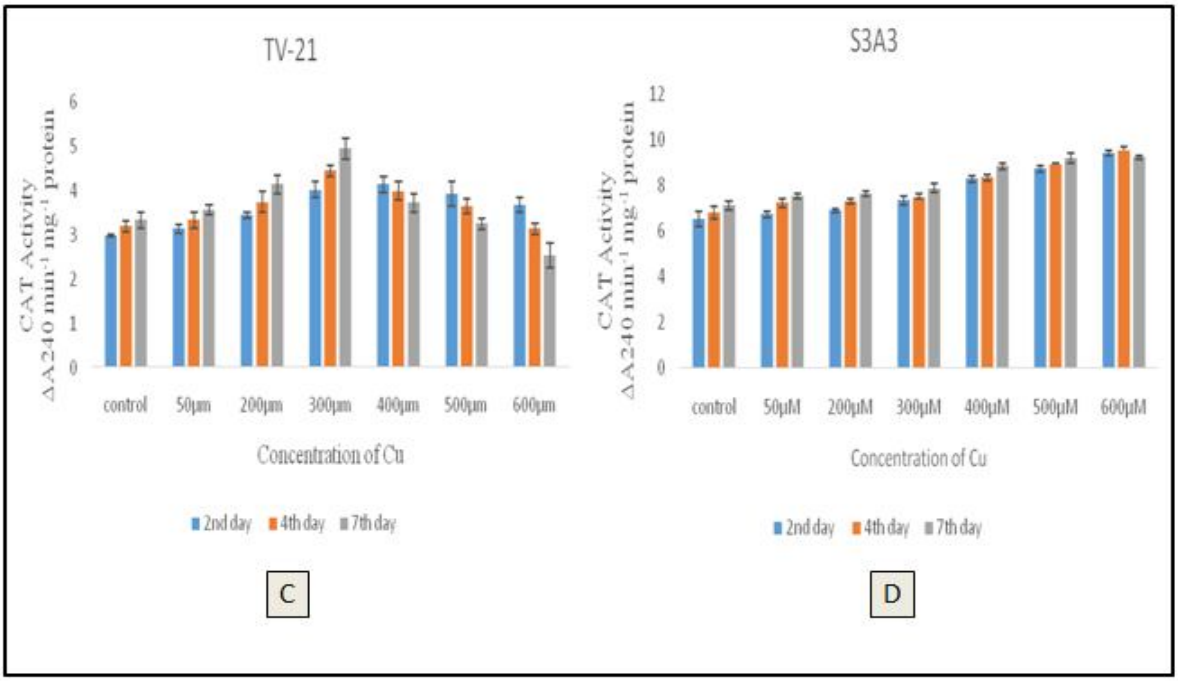

Figure4.3.2.4. C&D. Effect of Cu on CAT Activity exposed to different concentration and exposure duration onTV-21 & S_3A_3. All the values are mean of triplicate± SD.

Table44. Effect of Cu on CAT Activity exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	5.83±0.33	6.22±0.27	6.98±0.56
50 µM	6.37±0.18	6.91±0.27	7.32±0.33
200 µM	6.56±0.28	7.27±0.73	7.55±0.438
300 µM	6.98±0.22	7.49±0.63	7.83±0.528
400 µM	7.56±0.38	7.90±0.22	8.33±0.728
500 µM	8.19±0.33	8.58±0.38	8.89±0.39
600µM	9.01±0.18	9.34±0.32	9.41±0.438

Table45. Effect of Cu on CAT Activity exposed to different concentration and exposure duration on TS-491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	6.22±0.29	6.41±0.27	6.51±0.21
50 µM	6.82±0.19	6.990.l	7.25±0.22
200 µM	6.89±0.15	7.01±0.21	7.43±0.17
300 µM	7.12±0.11	7.43±0.15	7.87±0.08
400 µM	6.81±0.08	7.32±0.16	7.55±0.18
500 µM	6.71±0.14	7.11±0.17	6.98±0.11
600µM	6.42±0.1	6.03±0.21	5.32±0.18

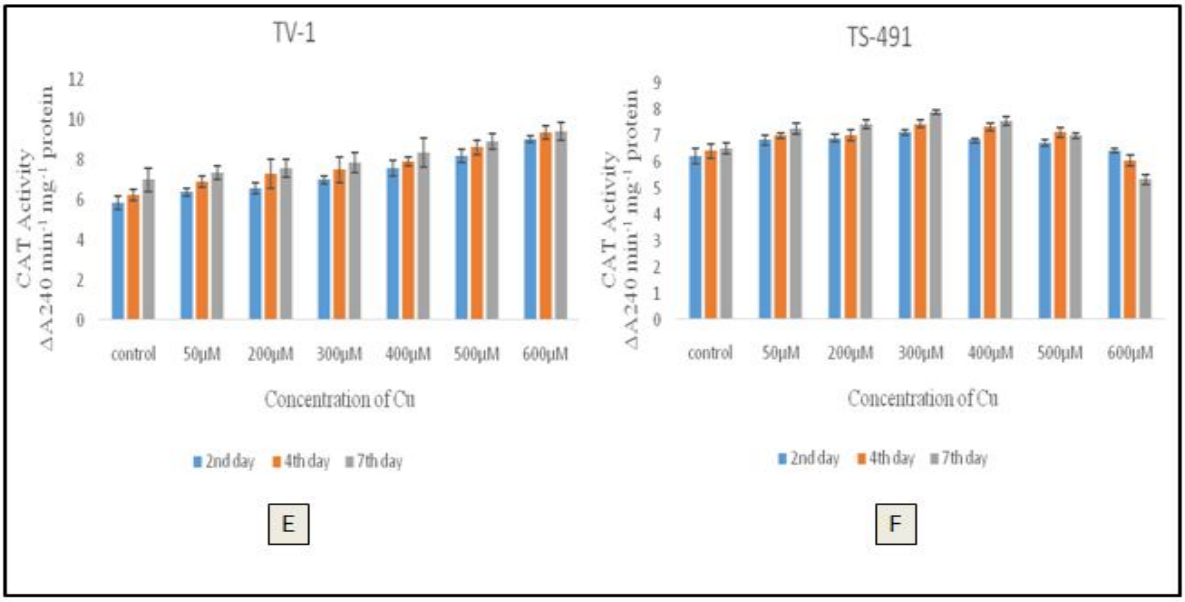

Figure4.3.2.4. E&F. Effect of Cu on CAT Activity exposed to different concentration and exposure duration onTV-1& TS-491. All the values are mean of triplicate± SD.

4.3.3. Effect of Cu on Phenol contents

Phenols are aromatic compounds with hydroxyl groups, offers resistance to diseases and pests in plants. From figure 4.3.3.A-4.3.3.F, it can be observed that the cultivars show a positive correlation between the phenol content and the increase in the concentration of Cu. From the figure 4.3.3.A and 4.3.3.C we can observe that in TV-28 and TV-21 the increase in the content was up to 300 µM which followed by the decrease in the content with the prolong exposure of higher concentration of Cu. In TV-17 the content was significantly increased up to 400 µM and from 500 µM there was a gradual decrease in the content (Figure 4.3.3.B). S_3A_3 has a significant increase in the total phenolic compound up to 500µM and at 600 µM the level of increasing in the content has slowed down slightly (Figure 4.3.3.D). In TV-1 there was a significant increase in the content even up to 600 µM which we can observe from figure

4.3.3.E. On the other hand in TS-491 there was increase in phenolic content up to 4th day of 500µM and then there was a decline in the activity from 7th day of 500 µM (Figure 4.3.3.F).

Table46. Effect of Cu on Phenol Content exposed to different concentration and exposure duration on TV-28. All the values are mean of triplicate± SD

Concentration	2nd day	4th day	7th day
0 µM	3.456±0.27	3.573±0.18	3.748±0.25
50 µM	3.516±0.12	3.673±0.15	3.827±0.17
200 µM	3.712±0.17	3.923±0.21	4.231±0.18
300 µM	3.936±0.18	4.276±0.21	4.453±0.12
400 µM	3.982±0.11	3.637±0.09	3.329±0.08
500 µM	3.635±0.18	3.463±0.21	3.209±0.39
600µM	3.243±0.27	3.018±0.22	2.748±0.21

Table47. Effect of Cu on Phenol Content exposed to different concentration and exposure duration on TV-17. All the values are mean of triplicate± SD

Concentration	2nd day	4th day	7th day
0 µM	3.897±0.19	4.01±0.28	4.365±0.11
50 µM	4.025±0.21	4.376±0.32	4.627±0.31
200 µM	4.326±0.28	4.674±0.32	4.913±0.31
300 µM	4.637±0.21	4.936±0.28	5.263±0.28
400 µM	4.926±0.09	5.273±0.12	5.56±0.21
500 µM	4.826±0.21	4.628±0.28	4.372±0.11
600µM	4.261±0.38	3.891±0.29	3.284±0.31

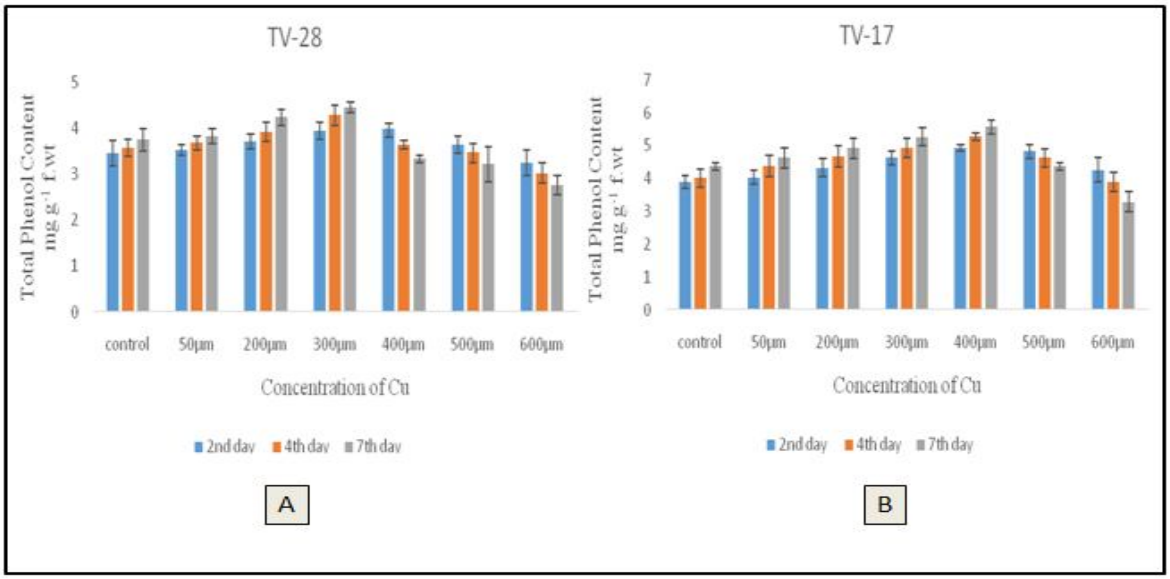

Figure4.3.3. A&B. Effect of Cu on Phenol content exposed to different concentration and exposure duration onTV-28 & TV-17. All the values are mean of triplicate± SD.

Table 48. Effect of Cu on Phenol Content exposed to different concentration and exposure duration on TV-21. All the values are mean of triplicate± SD

Concentration	2nd day	4th day	7th day
0 µM	2.98±0.28	3.278±0.33	3.627±0.09
50 µM	3.27±0.11	3.567±0.28	3.982±0.29
200 µM	3.762±0.52	4.092±0.03	4.896±0.32
300 µM	4.298±0.23	4.918±0.18	5.536±0.05
400 µM	4.028±0.11	3.828±0.22	3.521±0.19
500 µM	3.738±0.06	3.281±0.31	2.673±0.23
600µM	3.217±0.21	2.721±0.07	1.324±0.19

Table49. Effect of Cu on Phenol Content exposed to different concentration and exposure duration on S3A3. All the values are mean of triplicate± SD

Concentration	2nd day	4th day	7th day
0 µM	3.84±0.26	3.996±0.32	4.201±0.28
50 µM	3.894±0.23	3.989±0.22	4.401±0.26
200 µM	4.225v0.19	4.379±0.26	4.489±0.17
300 µM	4.233±0.07	4.467±0.13	4.689±0.11
400 µM	4.32±0.21	4.587±0.21	4.867±0.22
500 µM	4.634±0.31	4.896±0.28	5.145±0.27
600µM	4.853±0.17	5.034±0.25	5.112±0.23

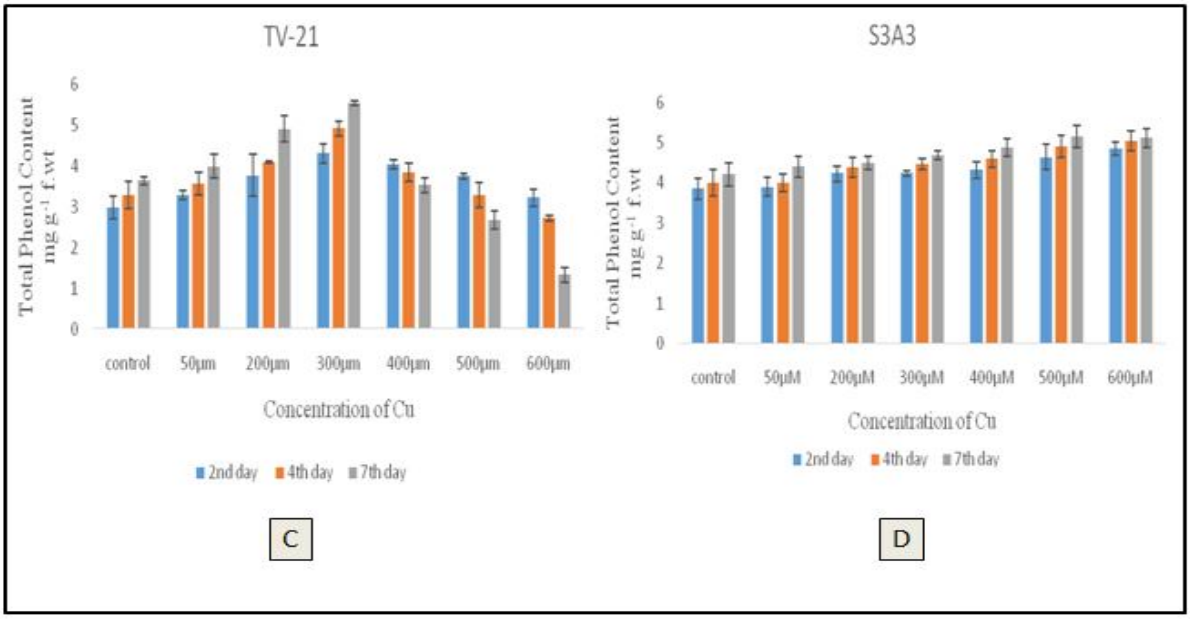

Figure4.3.3. C&D. Effect of Cu on Phenol content exposed to different concentration and exposure duration onTV-21&S_3A_3. All the values are mean of triplicate± SD.

Table50. Effect of Cu on Phenol Content exposed to different concentration and exposure duration on TV-1. All the values are mean of triplicate± SD

Concentration	2nd day	4th day	7th day
0 µM	3.88±0.22	4.026±0.26	4.223±0.33
50 µM	3.972±0.18	4.118±0.11	4.426±0.22
200 µM	4.366±0.28	4.462±0.12	4.622±0.19
300 µM	4.511±0.22	4.727±0.19	4.902±0.11
400 µM	4.721±0.19	4.928±0.21	5.109±0.29
500 µM	4.933±0.22	5.283±0.19	5.449±0.11
600µM	5.028±0.19	5.217±0.11	5.433±0.18

Table51. Effect of Cu on Phenol Content exposed to different concentration and exposure duration on TS-491. All the values are mean of triplicate± SD.

Concentration	2nd day	4th day	7th day
0 µM	3.217±0.15	3.384±0.04	3.521±0.13
50 µM	3.294±0.11	3.402±0.23	3.531±0.18
200 µM	3.421±0.18	3.469±0.22	3.679±0.17
300 µM	3.62±0.21	3.867±0.25	3.998±0.18
400 µM	3.815±0.27	4.256±0.38	4.465±0.19
500 µM	4.002±0.23	4.435±0.29	4.221±0.18
600µM	3.92±0.17	3.276±0.11	3.129±0.21

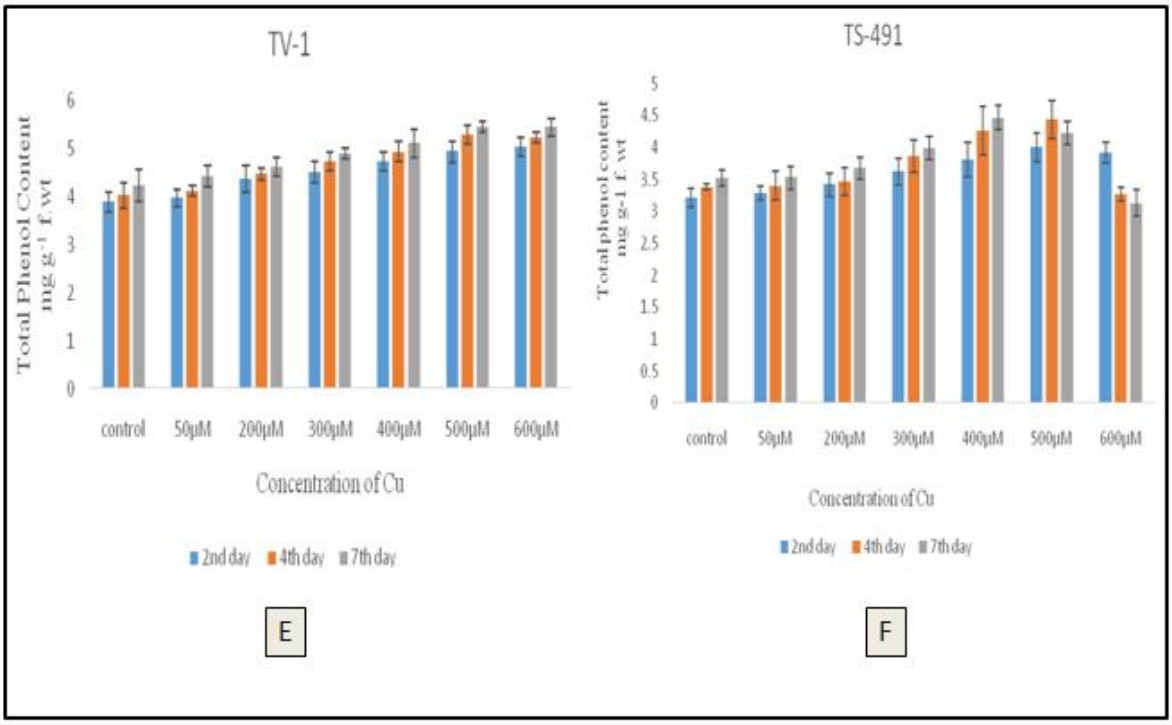

Figure4.3.3. E&F. Effect of Cu on Phenol content exposed to different concentration and exposure duration onTV-1& TS-491. All the values are mean of triplicate± SD.

Chapter 4.4

Relative Expression Analysis

4.4.1. Isolation of RNA:

The RNA was isolated from the leaves of the cultivarsTV-1, TS-491and S_3A_3.

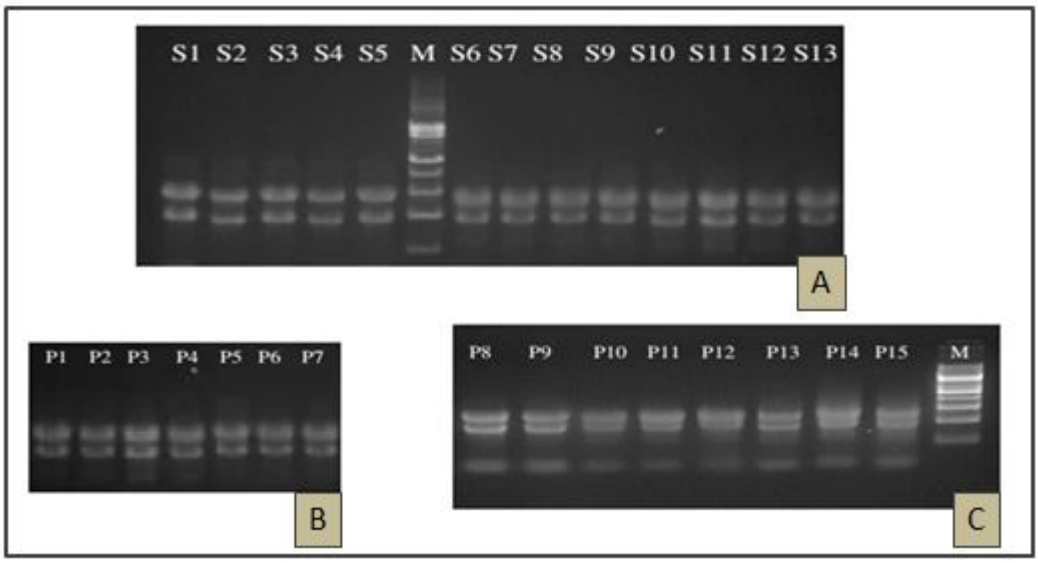

Figure4.4.1.1. A, B&C.Total RNA extraction from cultivars. Lane description: Marker 500bp Ladder.P1= 50µM, 2nd day; P2= 50µM, 4th day;P3=50µM, 7th day; P4=200µM, 2nd day; P5= 200µM, 4th day; P6=200µM, 7th day; P7=300µM, 2nd day; P8=300µM, 4th day; P9= 300µM, 7th day; P10=400µM, 2nd day; P11=400µM, 4th day; P12=400µM, 7th day; P13=500µM, 2nd day; P14=500µM, 4th day; P15=500µM, 7th day; S1=50µM, 2nd day; S2=50µM, 4th day; S3=50µM, 7th day; S4=200µM, 2nd day; S5=200µM, 4th day; S6=200µM, 7th day; S7=300µM, 2nd day; S8=300µM, 4th day; S9=300µM, 7th day; S10=400µM, 2nd day; S11=400µM, 4th day; S12=400µM, 7th day ; S13=500µM, 2nd day; R1=50µM, 2nd day; R2=50µM, 4th day; R3=50µM, 7th day; R4=200µM, 2nd day.

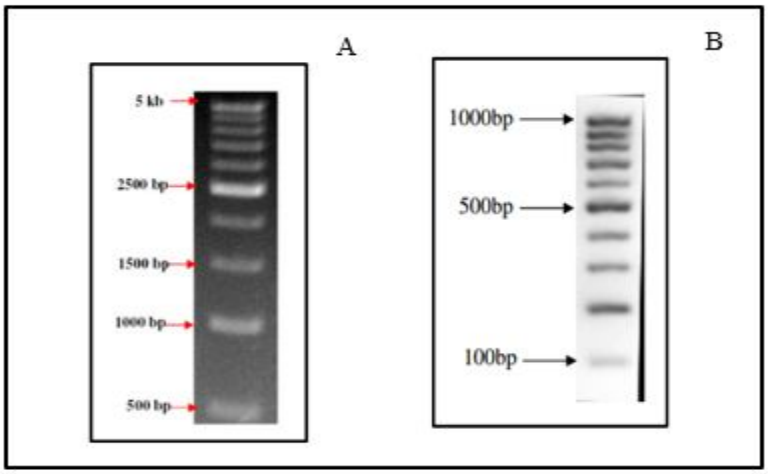

Figure4.4.1.2. A&B. Lane description: Marker 500bp Ladder, contains 10 DNA fragments of size 500bp, 1000bp, 1500bp, 2000bp, 2500bp, 300bp, 4000bp, 4500bp and 5000bp. And 100bp ladder contains 10 DNA fragments of Size 100bp, 200bp, 300bp, 400bp, 600bp, 800bp, 900bp and 1kb.

4.4.2. Amplification of RNA with Phytochelatin synthase (*PCS*1) and Metallothionein gene.

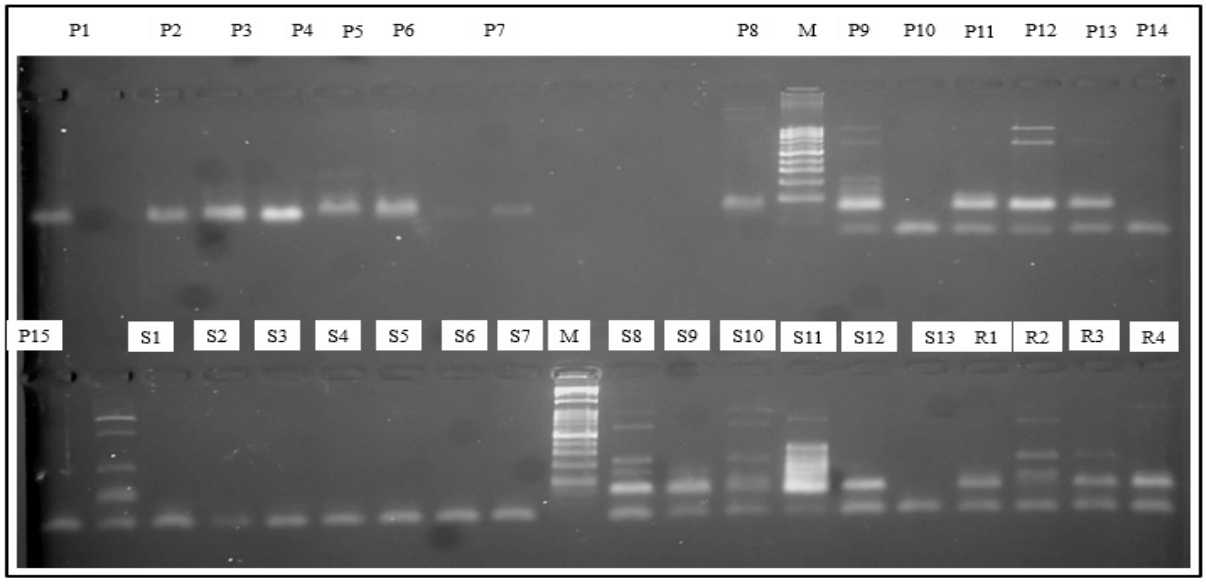

Figure4.4.2.1. Amplification of some of the samples with PCS1.

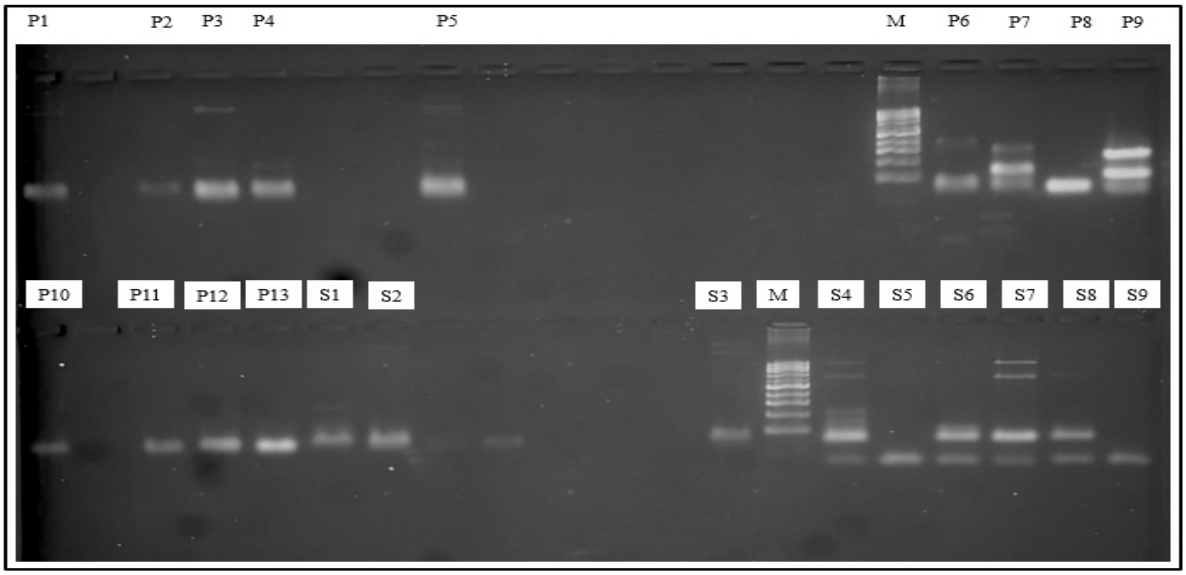

Figure4.4.2.2. Amplification of samples with MT

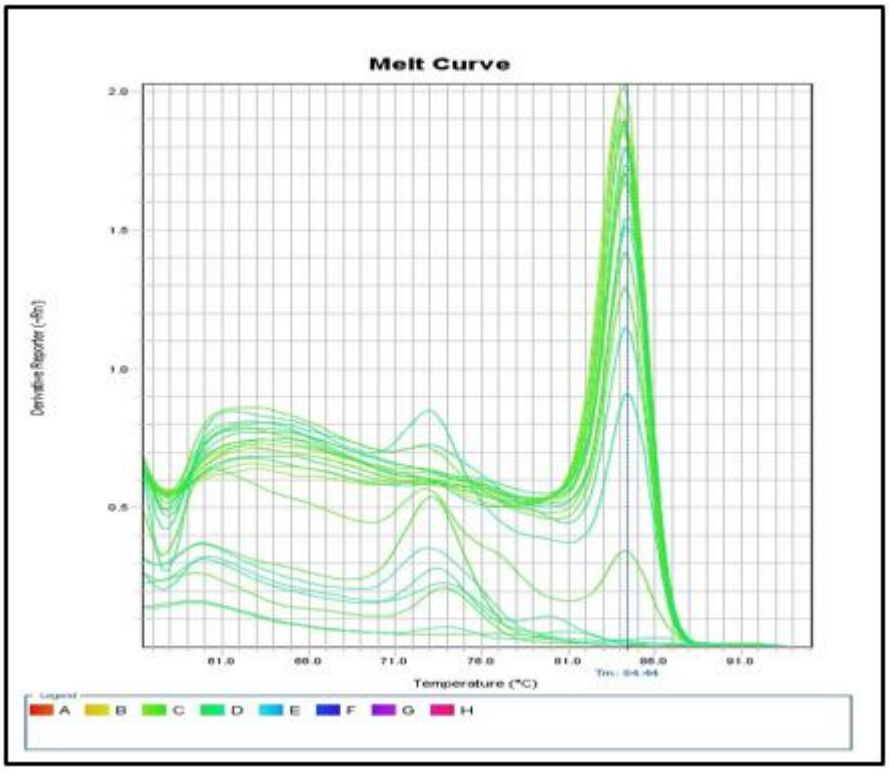

Figure4.4.2.3. Melting Curve of the expressed gene.

4.4.3. Relative Expression Analysis of *PCS*1 gene

Biochemical assays confirmed that the clonal variants TV-1 and S_3A_3 was the best cultivars having the better tolerant level even at higher concentration of Cu. The expression profile of *PCS*1 gene of the TV-1,S_3A_3andTS-491cultivars found to be different (Figure 4.4.3.A-4.4.3C). In these cultivars, up regulation of gene was observed even at the higher concentration, though the level of expression at higher concentration was less than that of lower concentration.

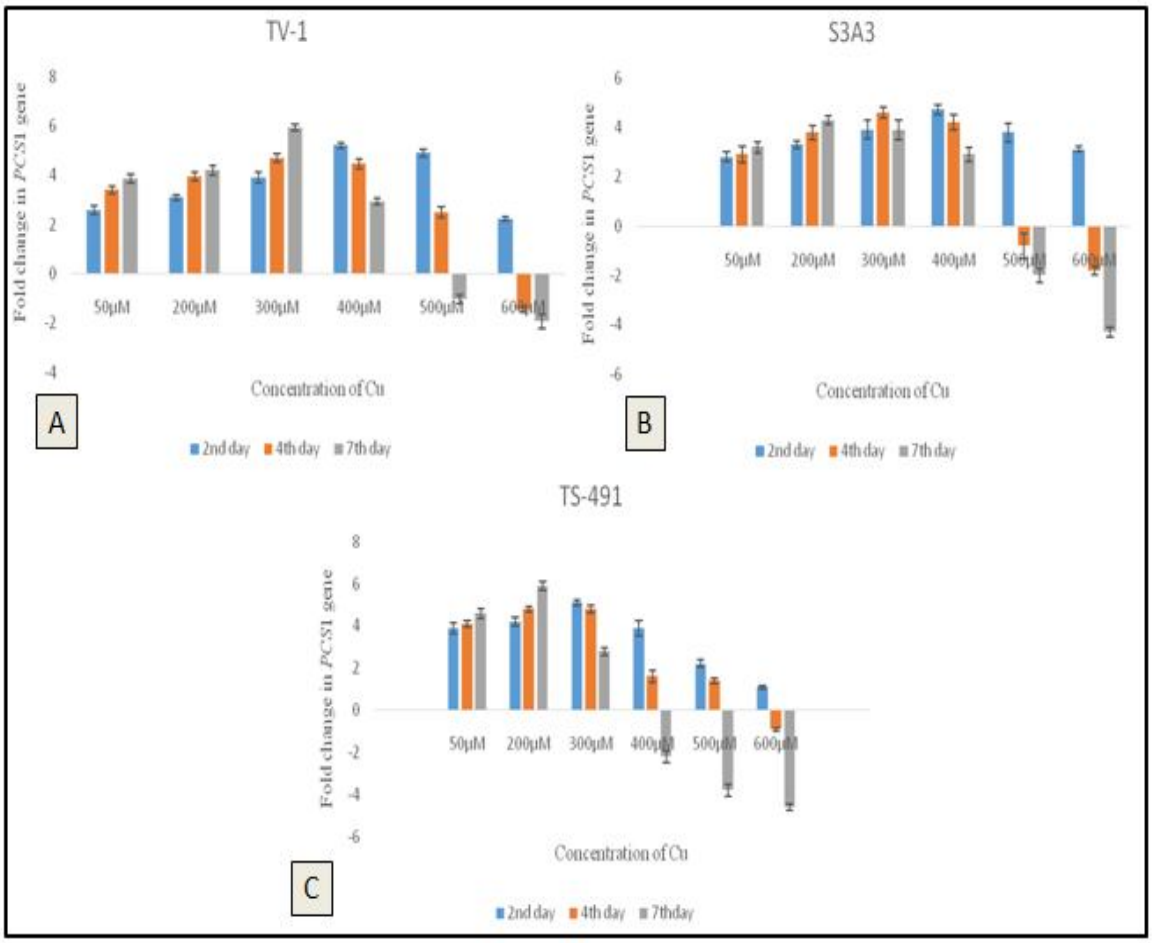

Figure4.4.3. A, B &C. Relative Expression fold change of *PCS*1 in TV-1, S_3A_3 and TS-491 respectively.

In TV-1 the expression profile of *PCS*1 gene showed a significant increase in up regulation of gene upto 7th day of 300 µM. At 400 µM there was decline in the value of positive fold change with the prolonged exposure of 400 µM. At the later phase of treatment 500 µM concentration of Cu was marked with down-regulation of gene. At 600 µM only the 2nd day was reported with positive fold change value where the mid and later phase of treatment of higher concentration led to the down regulation of gene with increased negative value in the fold change.

In **S_3A_3** there was a significant increase in the expression profile of *PCS*1 gene (Figure 4.3.3.B). There was a significant increase in the up-regulation of gene up to 4th day of 300 µM. Prolonged exposure of 300 µM lead to the declination in the expression of *PCS*1 gene. The same trend was observed at 400 µM too, where the early phase of exposure of Cu lead to the increase in the expression and from the 4th day there was declination in the expression profile. The further prolong exposure of higher concentration of Cu lead to the negative expression of gene. After 2nd day at 500 µM and 600 µM the expression was positive but after 4th day there was down regulation in the expression of gene.

Whereas in TS-491, the expression of *PCS*1 gene was highest at 7th day of 200 µM (4.3.3.C). 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. Compare to both the cultivars the level of fold change of *PCS*1 gene was higher at TV-1. In TS-491down regulation of expression of *PCS*1 gene started from 7th day of 400 µM and maximum down regulation was observed in 7th day of 600 µM.

4.4.4. Relative Expression analysis of MT gene

In TV-1, the exposure of Cu induces the activation of MT with respect to control there was a significant increase in the relative expression of MT gene which showed the up-regualtion of the genes till up to 7th day of 600 µM (Figure 4.4.4.A). The highest expression was observed at 4th day of 300 µM, after that there was decrease in the level of expression of gene by two fold, which was followed by the level of declination in the relative expression of gene from 500µM onwards.

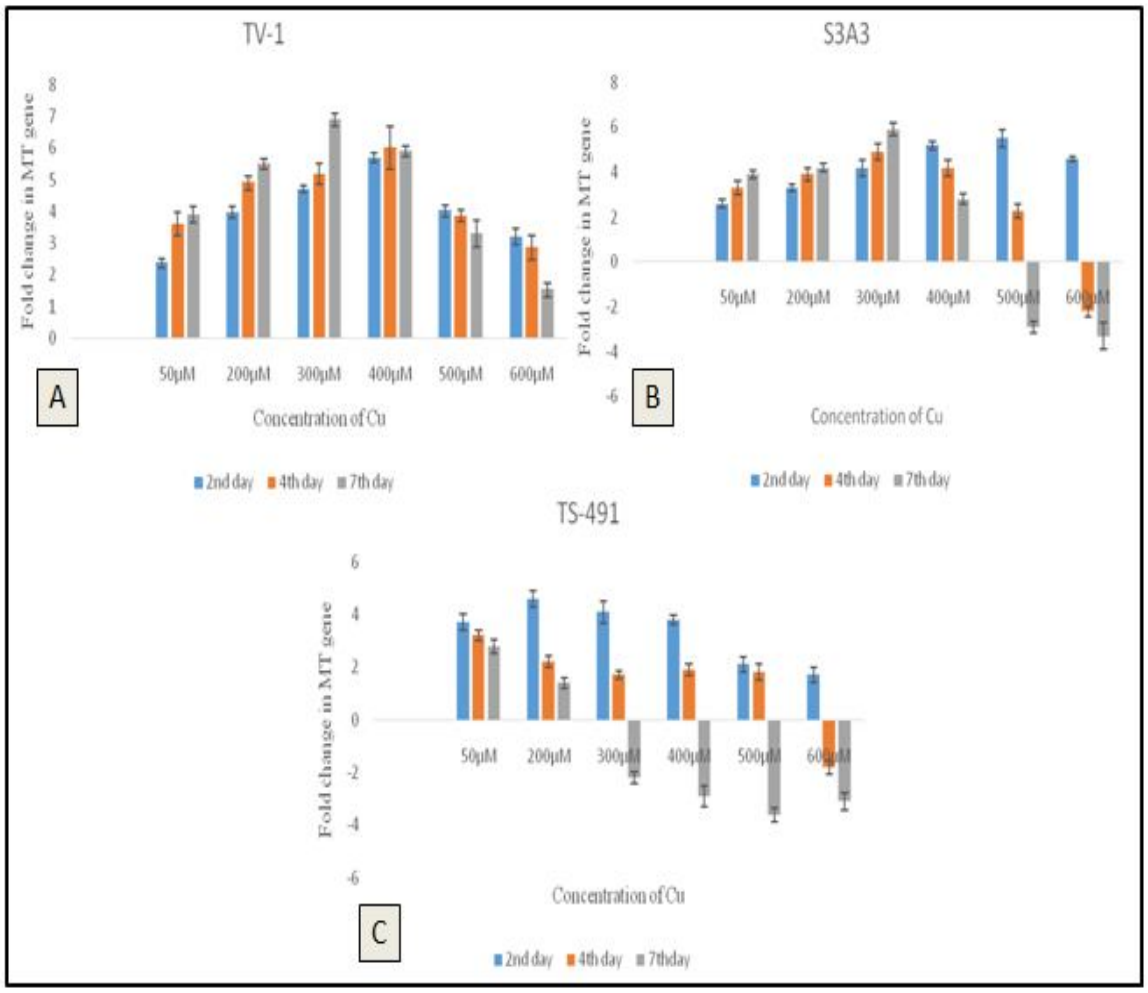

Figure4.4.4. A, B&C. Relative expression analysis of MT gene.

The same trend of up regulation of MT gene at lower concentration of Cu was observed in S_3A_3, where it showed a significant increase in the level of up regulation of gene up to 7^{th} day of 300 µM (4.4.4.B) there was rapid fall in the value of fold change in the up regulation of MT gene at the later phase of the exposure of 400 µM i.e. 7^{th} day, after initial a slight increase in the fold change. Prolonged exposure of higher concentration of Cu lead to the negative expression of gene. From the middle phase of exposure of 400µM there was decline in the up-regulation of MT gene. Which followed by down-regulation of gene by prolonged exposure of higher concentration of Cu. 7^{th} day of 500 µM was marked with down-regulation of MT gene. At the 2^{nd} day of 600µM S_3A_3 was viewed with up-regulation of MT gene. But from 4^{th} day at same concentration the MT gene was recorded with negative fold change value.

In TS-491, the expression of MT gene was highest at 2^{nd}day of 200 µM (4.4.4.C). From the lowest concentration of Cu, 50µM itself the up-regulation of gene was marked with decline in the relative expression fold change value. The level of decline in the level of fold change value followed by the negative value of fold change. 7^{th} day of 300 µM twas marked with down-regulation of MT gene. From 400 µM the level of decrease in the expression profile was increased and followed by the negative fold change of expression. At the highest concentration a very low up regulation of gene was observed at 2^{nd} day of 600µM. With the prolonged exposure the level of down regulation expression was increased with greater negative value.

Chapter 4.5

Preparation of cDNA library of MT gene

4.5.1. Determination of quality of RNA

Total RNA was isolated from Cu treated leaves of TV-1 cultivar of 300µM concentration of 7th day. 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 agarose gel electrophoresis of total RNA has been shown in figure 4.5.1. Two major bands corresponding to 28S and 18S rRNA was observed with a faint smear in the background. The pattern and the intensity of the bands ensured the proper quality of the sample.

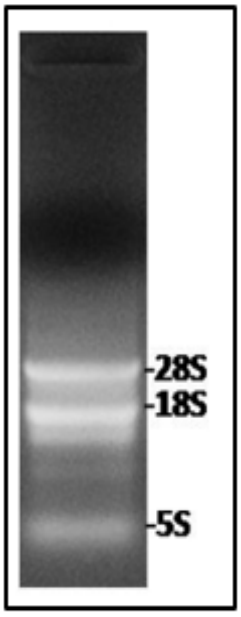

Figure4.5.1. 5% denaturing agarose gel electrophoresis for isolated RNA sample.

4.5.1.1. Preparation of cDNA:

The cDNA pool was prepared from the purified RNA using oligo dT primer and M-MUL V Reverse Transcriptase (200u/µl). The first strand cDNA was synthesized from the total RNA isolated from Cu treated TV-1 cultivar. Using oligo dt primers and cDNA synthesized on the basis of homology in different MTs were used for PCR analysis. One forward and one reverse primer used for amplification. The PCR amplification of products were resolved on agarose gel electrophoresis are shown in figure 4.5.1.1.

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. 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.

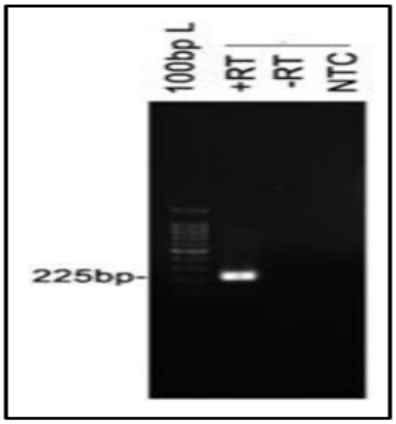

Figure4.5.1.1. 1% Agarose gel electrophoresis of PCR products.

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

The single fragment obtained from use of primers i.e. 225 bp was found in high quantity. Therefore the fragment of 225 bp was used for cloning. The isolated DNA was clonedinpGEMT- Easy vector.

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 pGEMT- 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. Amplification of 225 bp product was observed in each case. Two colonies selected as PCR-positive (Colony 3 and 6 as per lane 3 and 6 of figure 4.5.1.2.) were isolated and mini prep scale from the bacterial culture grown overnight.

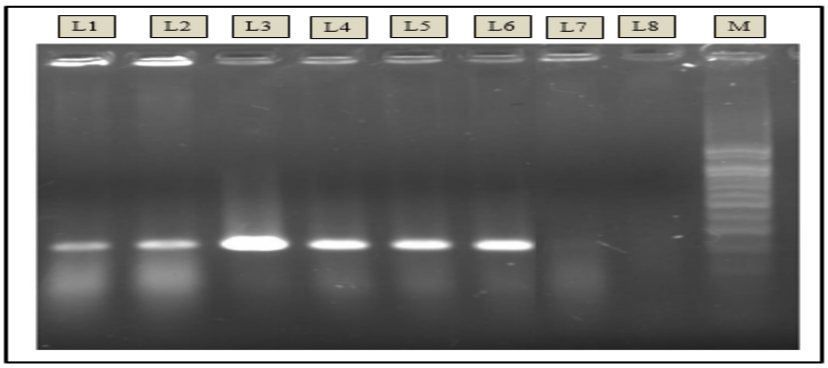

Figure4.5.1.2. 1% agarose gel electrophoresis of colony-PCR products. Lane1-6: Colony PCR products; Lane 7: No-Insert Control; Lane 8: NTC; Lane 9: 100 bp ladder.

4.5.1.3. Confirmation of Cloning:

The clone was further confirmed by digestion with restriction enzyme EcoRI. The vector pGEMT easy contains a couple of EcoRI, digestion sites flanking the 'T-A cloning site'. The purified plasmid DNAs were digested with EcoRI enzyme for 2 hrs and the digestion products were electrophoresed through 1 % agarose gel. A fragment of size about 225 bp was found to be dropped off the pGEMT easy vector backbone for both the plasmids (Figure 4.5.1.3.). The clone was also confirmed by capillary sequencing.

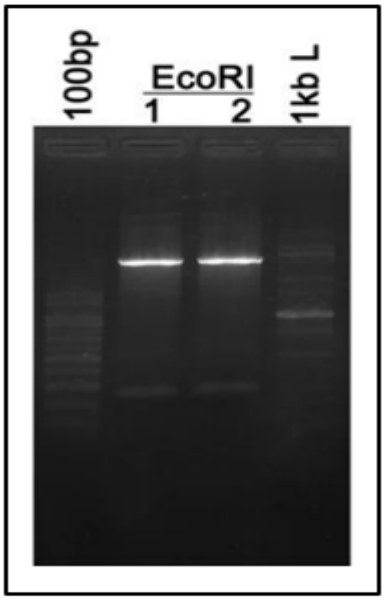

Figure 4.5.1.3. 1% agarose gel electrophoresis of restriction enzyme digestion products. The lanes ere as shown on the top of gel.

4.5.1.4. Sequencing of Desired cloned MT gene:

The nucleotide sequence of cDNA of 225bp was analysed through NCBI data base using BLAST for its identification and determination. The BLAST search indicated that the partial nucleotide sequence has homology with gb|DQ442270.2| *Camellia sinensis*metallothionin 1 mRNA, compl 422 2e-114 and gb|FJ965290.1| *Camellia oleifera* metallothionein mRNA,

comple. 244 4e-61.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 (figure 4.5.1.4. B).

Forward

CTATAGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGCGTGGCCGCG

GGAATTCGATTAGAAGACCACCACTGAGACCCTCATTGTTGGTCTTGCCCCAAAGA

AGACATTCTTTGAGGGAACTGAGATGGGTGAGGCAGCTGAGAATGGTTGCAAGTG

TGGAGCTAACTGCACCTGTGACCCTTGCACCTGCAAATGAGACCCAATCAAAATC

ATCAACCATAATATAGAAATATAATATATAATTAAGTAGTATTAGCAGTAGTGTCT

GCGTTTGAGGGTATGGAAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCA

TATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACC

TAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG

CTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG

CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG

TCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG

GGCGGTTTGCGTTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTC

GGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTA

TCCACAGAATCAGGGGATAACGCAGAAAGAACATGTGAGCAAA

Reverse

TATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGA

ATTCACTAGTGATTTCCATACCCTCAAACGCAGACACTACTGCTAATACTACTTAA

TTATATATTATATTTCTATATTATGGTTGATGATTTTGATTGGGTCTCATTTGCAGG

TGCAAGGGTCACAGGTGCAGTTAGCTCCACACTTGCAACCATTCTCAGCTGCCTCA

CCCATCTCAGTTCCCTCAAAGAATGTCTTCTTTGGGGCAAGACCAACAATGAGGGT

CTCAGTGGTGGTCTTCTAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATG

CGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCG

TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCA

GCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCC

CTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCA

TTAAGCGCGGCGGGGTGGTGGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCA

GCGCCCTTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCC

GGCTTTCCCCGTCAAGCTCTAAATCGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTT

TACGGCACCTCGACCCCAAAAAACTTG

CONSENSUS SEQUENCE ASSEMBLY

CAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAG

CCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG

AAGAAAGCGAAAGGAGCGGGCGCTAAGGGCGCTGGCAAGTGTAGCGGTCACGCT

GCGCGTAACCACCCACCACCCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCC

ATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCG

CTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAA

CGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATAC

GACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGCGC

GGCCGCGGGAATTCGATTAGAAGACCACCACTGAGACCCTCATTGTTGGTCTTGCC

CCAAAGAAGACATTCTTTGAGGGAACTGAGATGGGTGAGGCAGCTGAGAATGGTT

GCAAGTGTGGAGCTAACTGCACCTGTGACCCTTGCACCTGCAAATGAGACCCAAT

CAAAATCATCAACCATAATATAGAAATATAATATATAATTAAGTAGTATTAGCAGT
AGTGTCTGCGTTTGAGGGTATGGAAATCACTAGTGAATTCGCGGCCGCCTGCAGGT
CGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGT
GTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGT
TATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGG
GGAGAGGGCGGTTTGCGTTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGC
TGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT
ACGGTTATCCACAGAATCAGGGGATAACGCAGAAAGAACATGTGAGCAAA

Figure 4.5.1.4.A: Partial cDNA sequence of C. sinensis (TV-1) MT gene cloned in pGEM-T cloning vector.

AGAAGACCACCACTGAGACCCTCATTGTTGGTCTTGCCCCAAAGAAGACATTCTTT
GAGGGAACTGAGATGGGTGAGGCAGCTGAGAATGGTTGCAAGTGTGGAGCTAACT
GCACCTGTGACCCTTGCACCTGCAAATGAGACCCAATCAAAATCATCAACCATAAT
ATAGAAATATAATATATAATTAAGTAGTATTAGCAGTAGTGTCTGCGTTTGAGGGT
ATGGA

Figure 4.5.1.4.B. Sequence of desired MT gene of interest.

Vector screening

gnl|uv|NGB00072.2:12-193 PROMEGA pGEM-T easy vector T7 promoter-multiple cloning site-SP6 promoter

Sequence ID: Length: 182Number of Matches: 2

Range 1: 118 to 182Graphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
130 bits(65)	8e-28	65/65(100%)	0/65(0%)	Plus/Plus

```
Query  687  AATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGT  746
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118  AATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGT  177

Query  747  TGGAT  751
            |||||
Sbjct  178  TGGAT  182
```

Range 2: 1 to 67Graphics Next Match Previous Match First Match

Alignment statistics for match #2

Score	Expect	Identities	Gaps	Strand
122 bits(61)	2e-25	67/68(99%)	1/68(1%)	Plus/Plus

```
Query  391  ACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGCGCGGCCGCGGGA  450
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1    ACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATG-GCGGCCGCGGGA  59

Query  451  ATTCGATT  458
            ||||||||
Sbjct  60   ATTCGATT  67
```