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RESEARCH ARTICLE

EFFECT OF COPPER ON LIPID PEROXIDATION AND ENZYMATIC ANTIOXIDANTS IN SORGHUM BICOLOR.

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Abstract

Copper (Cu) is one of the micronutrient needed by plants which activates some enzymes in plants, also required in the process of photosynthesis and is essential in plant respiration and assists in metabolism of carbohydrates and proteins. However excess of copper inhibits plant growth and impairs important cellular process (i.e. photosynthesis electron transport). In industrial effluent. Here we reviewed the adverse effects of Cu on *Sorghum bicolor* and was investigated. Copper was applied in the form of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in four levels (0,5,10.0 and 15.0ppm). We observed visible symptoms of Cu toxicity in this plants. The increased concentrations of Cu caused oxidative stress in plants and subsequently increased the antioxidant responses due to increased production of highly toxic oxygen free radicals which further increased the defensive mechanisms through Superoxide dismutase (SOD), Ascorbateperoxidase (APX), Guaiacolperoxidase (GPX), Catalase (CAT), activities in leaves compared with that of control group.

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Introduction:-

Copper is an essential metal for normal plant growth and development having Atomic number, Density and Weight respectively 29, 8.92 g/cm^3 and 63.55. Copper also occurs naturally in all plants and animals. It is an essential element for all known living organisms including humans and other animals at low levels of intake. In particular, free copper ions can catalyze the formation of highly toxic reactive oxygen species (ROS) such as hydroxyl radicals (OH^\bullet) from superoxide anions ($\text{O}_2^{\bullet-}$) or hydrogen peroxide (H_2O_2) via the Haber-Weiss reaction. However, in excess, copper can interfere with numerous physiological processes such as enzyme activity, DNA alterations, proteins oxidation, and membrane integrity, all of which could lead to growth inhibition of plant [1].

Plant concentrations of essential elements may exceed the critical the minimum concentrations required for growth and may vary somewhat from species to species. Nonetheless, the following value gives the general requirement of plants. Typical concentrations sufficient for plant growth. Copper 6mg/kg relative number of atoms 100. Toxicity to humans: 700-2100 mg/g dry liver tissue = lethal.

The permissible limit of copper for plants is 10mg/kg recommended by WHO[2]. Contamination of drinking water with high level of copper may lead to chronic anaemia. Copper accumulates in liver and brain. Copper toxicity is a fundamental cause of Wilson's disease. Copper particulates are released into the atmosphere by windblown dust; volcanic eruptions; and anthropogenic sources, primarily copper smelters and ore processing facilities. The fate of elemental copper in water is complex and influenced by pH, dissolved oxygen and the presence of oxidizing agents

and chelating compounds or ions. Concentration of copper in all the soil samples was above the maximum permissible limit set by WHO. Concentration of copper ranged between 0.536-1.504mg/kg. Copper is an essential trace element in plants and animals, but not some microorganisms. The human body contains copper at a level of about 1.4 to 2.1 mg per kg of body mass [3].

Toxic levels of Cu occur naturally in some soils whereas others may contain high levels of Cu as a result of the anthropogenic release of heavy metals into the environment through mining, smelting, manufacturing, and agriculture waste disposal technologies. At concentrations above those required for optimal growth Cu was shown to inhibit growth and to interfere with important cellular processes such as photosynthesis and respiration.[4] Hence, the presence of excess Cu can cause oxidative stress in plants and subsequently increase the antioxidant responses due to increased production of highly toxic oxygen free radicals. Accordingly, it was observed that excess Cu in plants led to oxidative stress inducing changes in the activity and content of some components of the antioxidative pathways (i.e., ascorbate peroxidase (APX), monodehydroascorbatereductase (MDHAR), dehydroascorbatereductase (DHAR), glutathione reductase (GR), superoxide dismutases (SODs), guaiacol peroxidase) [5]. Considering that Cu is an efficient catalyst in the formation of reactive oxygen species (ROS), it was suggested that the increased Cu toxicity by light during photo inhibition is due to production of hydroxyl radicals [6]. Reactive oxygen species which include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen (1O_2) are amongst the most reactive compounds known to be produced during heavy metal stress [7]. These protective enzymes include antioxidant enzymes such as superoxide dismutase (SOD), peroxidases like ascorbate (APX) and guaiacol (POD) peroxidase. SOD is located in various cell compartments and catalyzes the disproportionation of two O_2^- radicals to H_2O_2 and O_2 . Ascorbate peroxidase is primarily located in chloroplast and cytosol and it is the key enzymes of the ascorbate glutathione cycle that uses ascorbate as reducing substrate for H_2O_2 detoxification. However, hydrogen is also toxic to cells. In plant cells the most important reducing substrate for H_2O_2 detoxification is ascorbate [8].

Materials And Methods:-

Experimental setup and design:-

Sorghum bicolor were pot cultured in department of biochemistry and biochemical technology SHIATS Allahabad (deemed university) Allahabad. Treatment was given with different concentrations of Cu i.e. (5.0ppm, 1.0ppm, 15.0ppm) in the form of copper sulphate. Hoagland solution is supplemented as a nutrient solution for every 2 days interwell. Leaves were harvested for every 15 days interwell (3 harvests).

Malondialdehyde (MDA) content:-

The lipid peroxidation in the plant tissue was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) reaction following the method of Heath and Packer, 1968 [24]. The plant samples (leaves, 300 mg each) were homogenized in 3 ml of 0.2%, trichloroacetic acid.

Estimation of antioxidants:-

Plant tissues, leaves (200 mg each) were homogenized in 2 ml of 100 mM potassium phosphate buffer, pH 7.5 containing 1 mM of EDTA in presence of pinch of polyvinyl polypyrrolidone (PVP). The homogenate was centrifuged at 10000 g for 15 min at 4°C. All steps in the preparation of enzyme extract were carried out at 0–4°C. This supernatant was used to measure the activities of superoxide dismutase, ascorbate peroxidase and guaiacol peroxidase.

Superoxide dismutase (EC 1.15.1.1):-

The assay system for superoxide dismutase was adopted from the method of Nishikimi and Rao, 1972 [25] and activity was expressed as units per g fw. Assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml 186 μ M phenazinemethosulphate, 0.3 ml 300 μ M nitrobluetetrazolium, 0.2 ml NADH (780 μ M), 50 μ l plant extract and water (1.15 ml) in a total volume of 3 ml.

Ascorbate peroxidase (EC 1.11.1.11):-

The activity of ascorbate peroxidase was measured of the method of Nakano and Asada, 1981 [26] by estimating the rate of ascorbate oxidation at 290 nm. Enzyme activity was calculated in terms of μ mol of ascorbate oxidized min⁻¹ g⁻¹ fresh weight at 25 \pm 2°C.

Guaiacol peroxidase (EC 1.11.1.7):-

Guaiacol peroxidase was measured in plant parts, following the method of Curtis, 1971[27], modified by Kato and Shimizu, 1987 [28]. Activity was calculated using the extinction coefficient of 26.6 mM⁻¹ cm⁻¹ at 470 nm for oxidized tetraguaiacol polymer. One unit of peroxidase activity was defined as the calculated consumption of 1 μmol of H₂O₂ min⁻¹ g⁻¹ fresh weight.

Chlorophyll and carotenoid content:-

Plant tissues, leaves (200 mg) were crushed in 5 ml of (80%, v/v) chilled acetone by the method of Arnon, 1949; Duxbury and Yentsch, 1956 [29,30]. Extract was centrifuged at 10,000 g for 10 min and absorbance of the supernatant was read at 510 and 480 nm using GBC Cintra 10e UV-VIS Spectrophotometer. Carotenoid content was calculated in mg g⁻¹ fw by the formula as given below (Duxbury and Yentsch, 1956) [31].

Carotenoid (mg g⁻¹fw) = $[[7.6 (A_{480}) - 2.63 (A_{510})] \times V] / 1000 \times W$

Where:

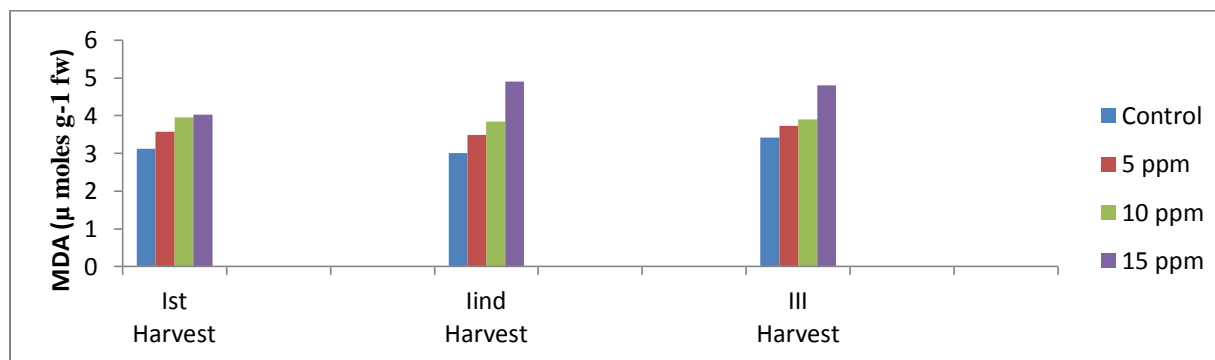
A₅₁₀ and A₄₈₀ = Absorption at the

Protein content:-

Protein was estimated by the method of Lowry et al. [32], using BSA as a standard protein, is a globular protein of molecular weight 68,000. Leaves (100 mg) of treated and control plants were crushed in 3 ml of 10% chilled trichloroacetic acid (TCA) and centrifuged at 10,000 g for 10 min and then last, the absorbance was recorded at 660 nm using bovine serum albumin (BSA) as a standard.

Activity of MDA:-

The results were compared between different exposure periods, which showed increased in MDA content of in the leaves as 29.038%, 63.12% and 40.63% after 1st, 2nd and 3rd harvest respectively when compared to control. MDA is the by-product of lipid per oxidation was considerably increased by heavy metals, which was also reported by (Gallego et al., 1996). According to (Sarmishtadey et al., 2015) the increase in the concentration of Cu, the MDA content increased and showed a significant positive correlation with Cu concentration. This shows that high concentration of Cu can lead to lipid per oxidation which causes damage to the balance of ROS scavenging activities in the experiment copper-induced changes in growth and antioxidative mechanisms of tea plant *Camellia Sinensis* (L.). Lipid per oxidation in roots of seedlings may be due to membrane degeneration. This result supports the possibility that the increase in peroxidation is due to the inhibition of ROS or by the enzyme lipoxygenase that are activated under metal stress (Dietz et al., 1999). Showed that Cd causes an enhanced lipid peroxidation and consequently degradation of chlorophyll by lipid peroxides.

**Activity of Superoxide dismutase:-**

Superoxide dismutase is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide (O₂⁻) to hydrogen peroxide (H₂O₂). Thus, SOD acts supportive antioxidative enzyme which provide protective defense against reactive oxygen species (Mansor, 2009). In the present study, specific activity of SOD expressed higher at 10ppm Cu treated plants (14.58% ,1st harvest) ,(14.60%,2nd harvest) and (16.25% ,3rd harvest) as compared to control shown in figure 5.2 table 5.2. According to S.Gao et al., (2008) excess of copper increases SOD activity in the experiment. Effects of copper on growth, antioxidant enzymes and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. seedling. The SOD activity of a plant is increased by the use of high concentration of heavy metal ions, by an increase in SO₂ concentration (Ashraf et al., 2009). A general plant

response to stress in activation of the system of antioxidative defense, which prevents each cell components from oxidative injury. One of the cell responses to the increased metal concentration in the environment is the increase in the capacity of antioxidative enzymes such as POD, SOD, and CAT (Vangronsveld and Clijsters, 1994). The changes of antioxidative enzymes level in a plant depends on duration of plant exposure to the metal stress and on metal concentration.

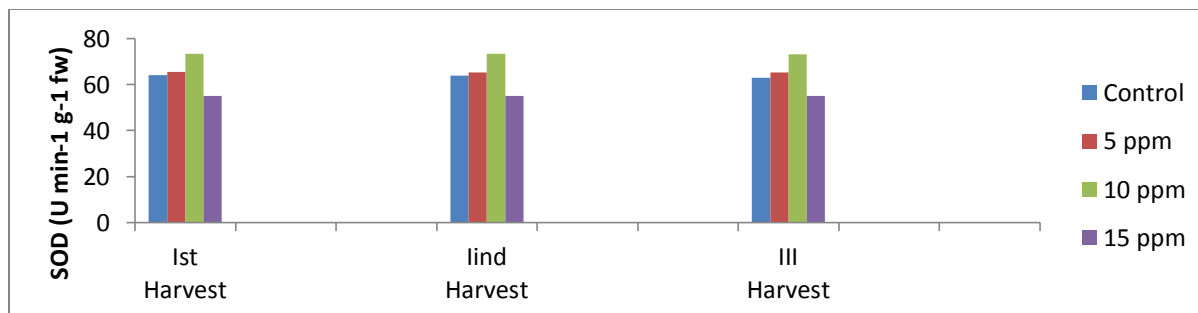


Figure 5.2:-Effect of Cu on the SOD content ($\mu \text{ min}^{-1} \text{ g}^{-1} \text{ fw}$) of the *Sorghum bicolor* Leaves (on 15th, 30th and 45th days from day of germination).

Activity of APX:-

There was an increasing trend in ascorbate peroxidase activity when subjected to different concentration Cu stress treatments. Ascorbate peroxidase activity increased to the maximum when the subjected to 5.0 ppm Cu (39.41%, at 1st harvest), (37.69% at 2nd harvest) and (37.78% at 3rd harvest) as compared to control. Sarmishta et al., (2014) also reported that there was a significant increase in the APX activity in the initial level of treatment with 200 μM , 300 μM of Cu respectively but there was a slight decrease in the APX activity with 600 μM Cu treatment. which supports this current research. APX: Recent studies have focused on the changes in activity of APX in higher plants subjected to several environmental stresses such as ozone, high lights, extremes of temperature, salts, heavy metals etc (Yoshimura et al., 2000). APX activities generally increased along with activities of other antioxidant enzymes like CAT, SOD, GPOD and GR in response to heavy metal stress factors, suggesting that the component of ROS scavengers are co-regulated. APX is a component of ascorbate-glutathione pathway, which plays a role in scavenging H_2O_2 because it is a systemic signal for the induction of APX (Morita et al., 1999). In well agreement with the results presented in this work, Hegedus et al., (2001) found that activities of APX were increased in roots and leaves of barley and bean plants.

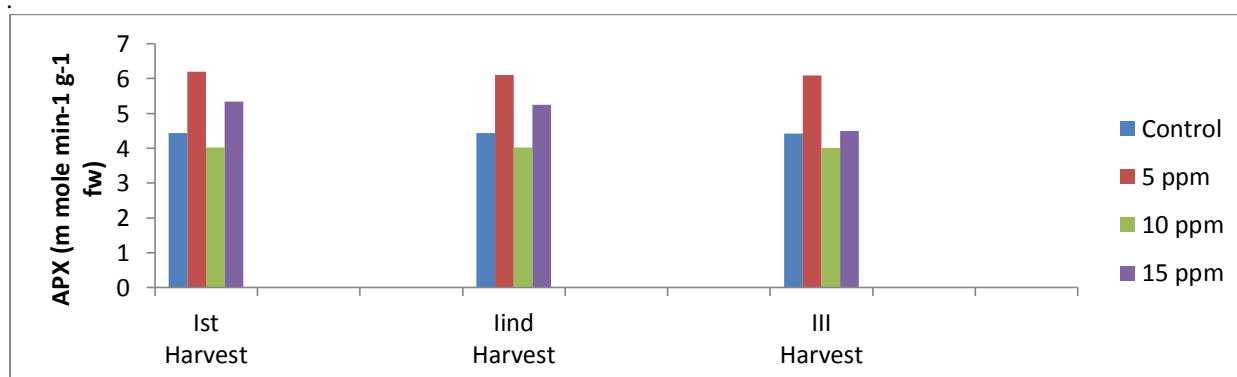


Figure 5.3:-The effect of Cu on APX content in leaf ($\mu \text{ moles g}^{-1} \text{ fw}$) of *Sorghum bicolor*. (on 15th, 30th and 45th days from day of germination).

Activity of GPX :-

The concentration wise analysis of the data of guaiacol peroxidase activity in the leaves showed reduction with increasing Cu concentration as compared to control. The maximum increase of (45.23% at 1st harvest), (29.31%, at 2nd harvest) and (36.31% at 3rd harvest) at 10.0ppm concentration of Cu as compared to control shown in figure 5.3 and table 5.3. The induction of a particular group of enzyme activities is considered to play an important role in the cellular defence strategy against oxidative stress, caused by toxic metals concentrations Clijsters et al., (1994). According to Thorny et al., (2012) GPX activity has been increased with increasing copper concentration in the

experiment Excess copper induced oxidative stress and response of antioxidants in rice. An increase in the GPX activity was found in the leaves. **Sinha et al.,(1997)**. : Induction in GPOD activity has been documented under a variety of stressful condition under toxic levels of Al, Cu, Cd, Zn(**Chaouiet al .,1997**). As GPOD are located in cytosol, cell wall, vacuole and in extracellular spaces, increased peroxidase activity in Pb stressed seedlings, might be possibly due to increased release of peroxidases localized in the cell walls. Under sublethal salinity and metal toxicity conditions, level of peroxidase activity has been used as potential biomarker to evaluate the intensity of stress (**Shah et al., 2001**).

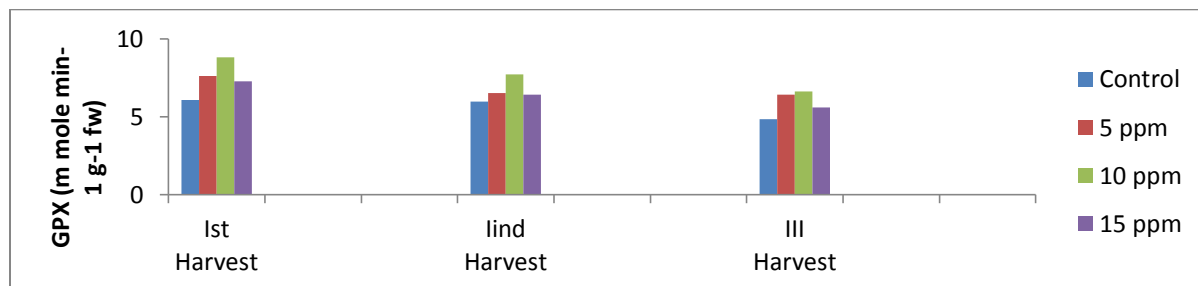


Figure 5. 4:-The effects of Cu on the GPX content ($\mu\text{mol min}^{-1}\text{g}^{-1}\text{fw}$) in *Sorghum bicolor*(15th, 30th and 45th days from day of germination).

Activity of Catalase:-

Catalase is a peroxisomalheme protein that catalyses the removal of hydrogen peroxide formed during the reaction catalysed by SOD. Thus, CAT acts supportive antioxidative enzyme which provide protective defense against reactive oxygen species (**Mansor, 2009**). The concentration wise analysis of the data of catalase activity in the leaves showed increase in activity with increasing cu concentration as compared to control. The maximum increase of 70.21% , 71.11% and 72.92% for 1st , 2nd and 3rd harvest respectively at 10ppm concentration of cu was observed at days as compared to control as shown in **figure 5.5 and table 5.5**.CAT works more effectively at scavenging the H_2O_2 converting it into oxygen and water **Li et al.,(2004)** According to **Gaoet al.,(2008)** excess of copper increases **CAT** activity in the experiment Effects of copper on growth, antioxidant enzymes and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. seedling. Activity of CAT was examined as one of the major antioxidant enzyme that eliminates H_2O_2 by converting it into oxygen and water (**Foyer and Noctor, 2000**). Activity of CAT in response to Cr has been studied in many crop plants like rice, wheat, green gram and even in lower plants like mosses (**Choudhury and panda, 2004**). In rice, Cr can either induce CAT activity or suppress it. Treatment of developing wheat seedlings of different concentrations of Cr showed varied response.

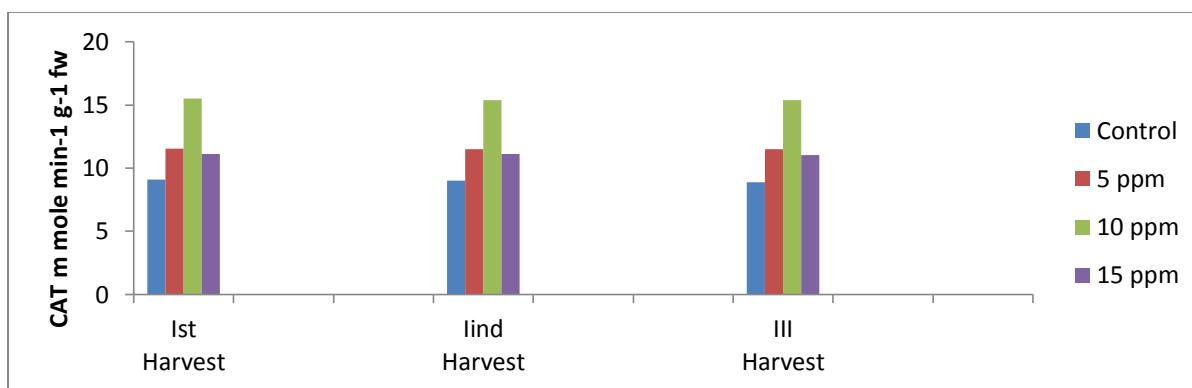
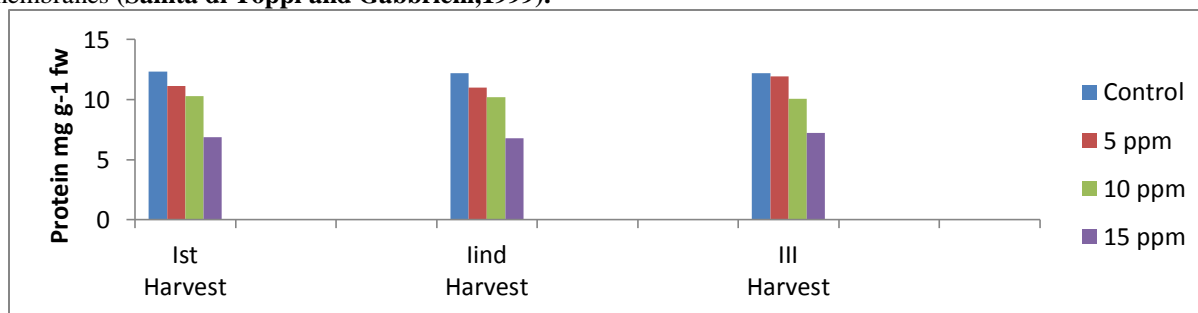


Figure 5. 5:-The effect of Cu on the catalase activity ($\mu\text{mol min}^{-1}\text{g}^{-1}\text{fw}$) in *Sorghum bicolor*. (on 15th,30th and 45th days from day of germination).

Protein Content:-

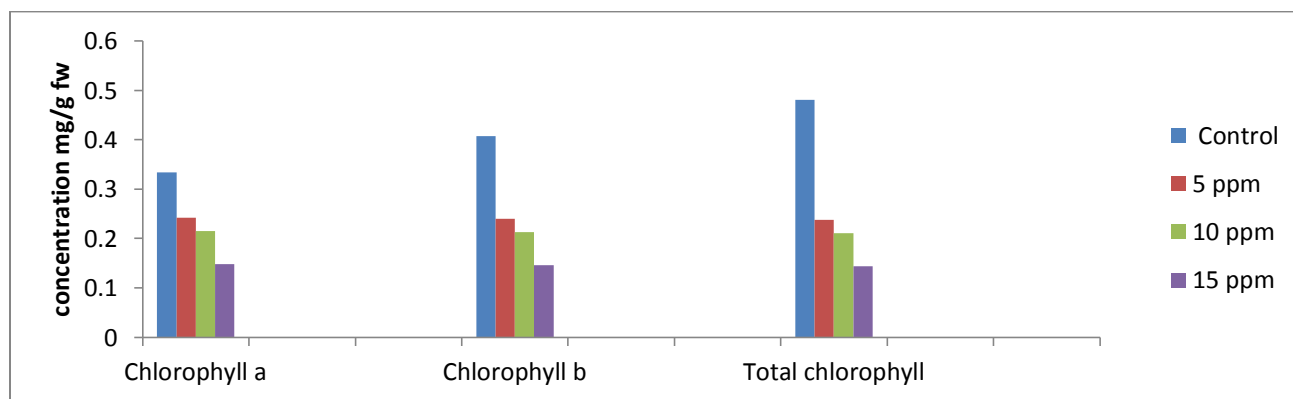
Data presented in **table no 5.6 and figure no 5.6** indicates that in leaves, protein content showed decrease at all concentration and maximum decrease was observed as 44.13%,44.4% and 40.74% after 1st , 2nd , and 3rd harvest respectively at 15.0ppm. The results were compared between different exposure periods, which showed decrease in protein content of in the leaves at all the concentration as compared to control. According to (**GUO et al., 2007**) the high levels of cu induced the reduction in leaf total soluble protein in barley plants .The mechanism by which copper

affects protein content is complex and needs a further study. Heavy metals induce the synthesis of stress proteins, which might limit and repair the damages caused by metals to cell proteins and exert protective effects on membranes (Sanita di Toppi and Gabbrielli,1999).



Effect of the Copper on Chlorophyll a, b, and total Chlorophyll content in *Sorghum bicolor* (L.) leaves:-

The concentration scrutiny of the data demonstrated decrease in chlorophyll a, b, and total chlorophyll content in the leaves, with increasing concentration of Cu upto 15.0 ppm after 15days from sowing as compared to control. The maximum decrease of 55.68%, 64.12% and 70.06% was observed at 15ppm concentration of Cu after the exposure period respectively in first harvest. However, during days of exposure period, the chlorophyll a, b and total chlorophyll content was found to decrease with increase in concentration of Cu. Maximum decrease of 51.36%, 52.29% and 55.71% was observed at 15.0 ppm after 30days(2nd harvest) of exposure as compared to respective control. A decrease in chlorophyll content was found to be significant on increasing copper concentration. This observation was also supported by Chandra and Alam, (2013) in the study copper scavenging potential and its effect on chlorophyll in seedlings of *Brassica juncea* (L.)The decrease in chlorophyll content may be due to reduced chlorophyll biosynthesis by inhibiting δ -amino levulinic dehydrogenase and protochlorophyllidreductase activities and breakdown of pigments or their precursor as reported by Teramura and Sullivan in 1994.



Effect of Cu on Chlorophyll content in *Sorghum bicolor* Leaves (1st harvest; 15th days from day of germination)

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