Copper Phytotoxicity and its Role on the Growth, Metabolism and Productive Potential of *Vigna radiata* (L). Wilzek plants

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ABSTRACT

Copper (Cu) is an essential micronutrient for plant growth, but excessive accumulation can lead to phytotoxic effects, impacting plant development and yield. This study investigated the phytotoxic effects of copper on *Vigna radiata* (L.) Wilczek *(*mungbean) plants at three different concentrations (1 mM, 4 mM, and 7 mM). The experiments were conducted in a controlled environment. Various growth parameters, including seedling growth, chlorophyll content, and oxidative stress indicators, were assessed to determine the impact of copper toxicity. Results showed that increasing copper concentrations negatively affected mungbean seed germination and seedling growth. There was a significant reduction in seedling vigor was observed at 7 mM copper concentration compared to lower concentrations. Additionally, chlorophyll content decreased with increasing copper concentration, indicating impaired photosynthetic efficiency. Furthermore, oxidative stress markers, such as malondialdehyde (MDA) content and antioxidant enzyme activities (superoxide dismutase, catalase) were significantly altered in mungbean plants exposed to higher copper concentrations, indicating oxidative damage. In conclusion, copper phytotoxicity adversely affected mungbean growth and physiological processes, with higher concentrations exacerbating the negative effects. Understanding the mechanisms underlying copper toxicity in mungbean plants, it is essential for developing strategies to mitigate its impact on crop productivity and sustainability.

1. INTRODUCTION

Environmental pollution is a major issue that concerns humans today. Heavy metals are a particularly problematic type of pollutant because they are found in various parts of the environment, including soil, water, and air. This is largely due to unrestricted mining, manufacturing, municipal waste disposal, and other industrial activities. This has led to a significant accumulation of heavy metals in the biosphere which can persist indefinitely, posing a severe threat to agriculture and human health. The total toxicity of heavy-metal pollutants added to the environment each year now exceeds the toxicity of all organic and radioactive wastes combined (Nriagu and Pacyna, 1988).

Copper (Cu++) is a transition element and heavy metal, with a density greater than 5 g/ml. It is one of the four essential micronutrients, along with Cobalt, Nickel, and Zinc, required in trace amounts by both plants and animals. Unlike non-essential heavy metals such as cadmium, lead, and mercury, copper is not easily bioaccumulated and hence has a relatively low toxicity to humans and other animals. However, plants are highly sensitive to copper toxicity and display severe metabolic disturbances and growth inhibition even at concentrations only slightly higher than normal trace amounts (Woolhouse, 1966).

Plants need minerals to grow and develop properly. These minerals are mainly obtained from the soil, but they can also be applied directly to the leaves. Scientists are interested in studying how plants absorb and distribute these nutrients because they play important roles in plant

physiology, biochemistry, and molecular biology. Copper is one such mineral that is essential for plants and all living organisms. It is involved in many physiological processes due to its ability to exist in different oxidation states in living systems. In normal conditions, copper exists as Cu2+ and Cu+. The positively charged ion, Cu2+, is commonly attracted to nitrogen in histidine side chains, while Cu+ tends to interact with the sulfur in cysteine or methionine. Copper plays a crucial role as a structural component in certain metalloproteins, many of which are involved in electron transport in chloroplasts and mitochondria, as well as in the response to oxidative stress. Copper ions also act as a cofactor in various enzymes such as Cu/Zn-superoxide dismutase (Cu/ZnSOD), cytochrome c oxidase, ascorbate oxidase, amino oxidase, laccase, plastocyanin, and polyphenol oxidase. At the cellular level, Cu plays an essential role in cell wall metabolism, signaling the transcription and protein trafficking machinery, oxidative phosphorylation, iron mobilization, and the biogenesis of molybdenum cofactor (Raven et al. 1999; Yruela 2005; Gratão et al. 2005; Pilon et al. 2006; Krämer and Clemens 2006; Puig et al. 2007).

Plants need Cu (copper) for their normal growth and development. However, when this element is not available, plants develop specific deficiency symptoms, which mostly affect young leaves and reproductive organs. It is worth noting that the redox properties that make Cu an essential element also contribute to its inherent toxicity. Redox cycling between Cu 2+ and Cu+ can catalyze the production of highly toxic hydroxyl radicals. These radicals can cause damage to cells at the level of lipids, membranes, nucleic acids, proteins, and other biomolecules (Halliwell and Gutteridge, 1984). Although Cu usually binds to proteins it can initiate oxidative damage and interfere with important cellular processes such as photosynthesis, pigment synthesis, plasma membrane permeability, and other metabolic mechanisms, causing a strong inhibition of plant

development (van Assche and Clijsters 1990; Marschner 1995; Küpper et al. 2003; Bertrand and Poirier 2005; Yruela 2005). Cu in excess can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, and inhibition of root and shoot growth. At the cellular level, excess Cu can inactivate and disturb protein structure due to unavoidable binding to proteins. Toxicity may result from binding to sulfhydryl groups in proteins, thereby inhibiting enzyme activity or protein function, inducing a deficiency of other essential ions; impaired cell transport processes, and oxidative damage.

Both copper (Cu) deficiency and excess copper can negatively impact plant growth and development as they affect significant physiological processes in plants. To ensure healthy growth and development, plants need to acquire copper from the soil and distribute it throughout the different tissues and compartments while regulating its content within cells and organelles. Maintaining the right concentration of copper within tissues and cells is crucial for proper plant growth and function, and plants have homeostatic mechanisms to achieve this. These mechanisms enable plants to acquire appropriate amounts of copper in different environmental conditions and transport it precisely to specific compartments and target metalloproteins, while avoiding toxic effects. Thus, the acquisition and assimilation of Cu must be coordinated with mineral supply and plant demand in a complex and regulated interacting network. Cu homeostasis processes are dynamic and respond to metal availability, annual cycles, and growth phases.

The mineral nutrition of higher plants is crucial to agriculture and human health. However, there are still many unresolved questions about the accumulation of essential heavy metals in plants. These questions are fundamental to the field of plant biology and have become an emerging

area of research with the advent of complete genome sequencing and molecular tools. Researchers are using genomic approaches to understand nutrient acquisition, assimilation, and metabolism processes. In particular, studies on the yeast *Saccharomyces cerevisiae* have contributed to progress in understanding the basic cellular components of copper homeostasis in eukaryotic organisms. Various genetic and molecular techniques like sequence comparison, functional complementation of yeast mutants, and plant transformation have played a crucial role in the development of identifying transporters and regulating gene activities. Many gene families and proteins are being discovered in plants that are likely to be involved in maintaining Cu homeostasis. Furthermore, Cu homeostasis is gaining more interest in the field of plant research as it is implicated in the adaptive responses of plants to oxidative damage caused by environmental stress. It is imperative that mechanisms exist to fulfill the requirements of cellular metabolism while simultaneously protecting cells against toxic effects. At the cellular level, specific transporters are responsible for taking in and expelling metal ions, and there may be additional transporters that allow sequestration into organelles. The interaction of metal chaperones with transporters is particularly noteworthy, as it has important implications for the sequestration of metals within intracellular stores. Over the last decade, significant progress has been made in this area. Heavy metal homeostasis is a rapidly developing field in plant biology. Copper is an essential element for plants, as it is a key component of enzymes such as polyphenol oxidase, ascorbic acid oxidase, tyrosinase, cytochrome oxidase, and superoxide dismutase. Copper can be found in three different forms within proteins: blue proteins without oxidase activity (e.g. plastocyanin), non-blue proteins, which produce peroxidases and oxidize monophenols to diphenols, and multicopper proteins containing at least four copper atoms per molecule, which act as oxidases (e.g. ascorbate oxidase and laccase) and catalyze the reaction, 2AH2 + 02 —>■ 2A + 2H20. Cytochrome oxidase

is a mixed copper-iron protein while superoxide dismutase (CuZnSOD) is a mixed copper and zinc protein. Copper is present in the earth's crust at a concentration of 70 ppm and occurs as a constituent of several different ores. Chalcopyrite (CuFeS2) is thought to account for about 50% of the total world copper deposits. However, the extraction of copper from these sources often leads to pollution problems around sites of copper extraction.

Excessive amounts of copper can be found in the environment due to various sources, with the primary ones being atmospheric and hydrospheric sources (Alloway, 1994). Other sources include fossil fuel combustion (Morgan and Stumm, 1991), the use of agricultural materials like 'Bordeaux mixture' fungicides (Alloway et al. 1979; Yang et al. 1993), metallurgical industries (Rao and Dubey, 1992), waste disposal (Jenner et al. 1993), and vehicular traffic (Cook et al. 1994; Hsu and Chang, 1992). The bioavailability of copper, its uptake, and accumulation in plants can take place primarily in three ways through the atmosphere, through the aquatic and marine environments, and the soil. Copper particles can enter plant tissues through absorption by the cuticle when they fall onto foliage from the atmosphere. In the atmosphere, copper particles associated with particulate matter are mainly in the liquid phase as ionic species. Studies conducted on hydrophytes have reported that copper uptake through roots is higher than through leaves.

Copper pollution has the most long-lasting effects on soil due to its strong adsorption onto the humic and clay colloids in the soil. Toxicity problems are likely to be more severe in arid environments. It has been suggested that there is an interaction between the copper-uptake mechanisms, plasma-membrane H^+ ATPase, and root membrane permeability. Copper toxicity is generally manifested as a general chlorosis and stunting of growth. Epidermal traits such as

stomatal indices, trichome length, and trichome frequency in some plants are modified. The necrotic process has been seen to be initiated in *Nicotiana glutinosa* leaves. Reports of copper excess affecting the enzymatic activity of plants are common. Mukherji and Dasgupta (1972) invoked an enhancement of the activity of catalase, lAA-oxidase and peroxidase in lettuce seedlings and reduced a-amylase, ribonuclease and protease activities in rice seedlings. Copper is a potent inhibitor of photosynthesis and the inhibition sites range from photosynthetic electron transport, and photophosphorylation to dark reactions (Angelov et al. 1993). Other toxic effects of copper include reduced stomatal resistance and increased transpiration (Angelov, 1993), loss of permeability barriers to root cells (De Vos et al. 1990), inhibition of nitrogen fixation in lentil plants (Sarada and Polasa, 1992) and disturbance of plant water status. It is now generally believed that one of the most important effects of copper is the blocking of photosynthetic electron transport. This leads to the production of free radicals, which in turn, through lipid peroxidation bring about membrane damage and ultimately cell death (Luna et al. 1994). Thus, at the whole plant level, copper is an effective inhibitor of vegetative growth and induces general symptoms of senescence.

Plants have evolved several strategies of self-defense to counteract the high toxicity of copper. One of the most important is the production of copper-complexing compounds which can be divided into two main groups, viz., metallothionein-like compounds and phytochelatins. Studies on the effects of heavy metal toxicity in plants gain relevance when one considers the fact that plants are primary producers. Any damage caused by pollutants at this level leads to disruption in the entire food chain and energy flow.

Copper functions, acquisition and transport

2.1 Copper Bioavailability

The concentration of copper (Cu) in the tissues of plants varies depending on different factors such as the plant species, developmental stage, and environmental conditions like nitrogen supply and soil properties. Plants that are grown under a high nitrogen supply require more copper. Additionally, copper bioavailability is higher in acidic soils. Studies show that the copper concentration in plant tissues ranges between 1 and 5 µg g-1 dry weight, while the average composition of copper in leaves is 10 µg g-1 dry weight (5-20 µg g-1 dry weight). However, these concentrations may differ among plant species and varieties. Copper levels in cells must be kept low since it is highly toxic due to its high redox properties. The critical free Cu concentration in the nutrient media (below which Cu deficiency occurs) ranges from 10 -14 to 10 -16 M. Plants usually find a variable supply of Cu in the soil since typically soil solution concentrations range from 10 -6 to 10-9 M (Marschner 1995), but plants may still need to solubilize and reduce the metal.

The amount of free metal ions or metal chelates in the soil solution is usually low, but this can vary depending on the soil properties. Copper (Cu) is mainly associated with inorganic and organic matter in both the soil solution and the solid phase. Cu ions have a high affinity for binding sites in soil components and can be absorbed onto surfaces of clays, iron (Fe) or manganese (Mn) oxides, co-precipitated with carbonates and phosphates or present in the lattice of primary silicate minerals. Cu ions can be also bound to cell walls and the outer membrane surface of plant root cells. The distribution of Cu among these various solid and plant components will greatly influence the chemical mobility and hence the amount of Cu potentially taken up by plants. At acidic pH, dissolved Cu will increase because of its weaker adsorption and so will increase the free Cu ion

activity. As the pH increases, organic matter in the solid phase and dissolved organic carbon compete for adsorption, leading to an increase in the concentration of Cu in the soil solution. This happens due to the rise in dissolved organic carbon. The increase in pH causes a significant decrease in the activity of Cu ions while increasing the presence of organically bound complex species in the soil solution.

In the rhizosphere, the activities of roots and microbes can have an impact on the movement of metal ions and their eventual uptake by plants. This is because the soil pH or dissolved organic carbon may change due to these activities, leading to changes in the chemical mobility of the ions. (Hinsinger and Courchesne, 2007). For instance, in the case of Graminaceous species, the increased root secretion of Fe-chelating compounds (phytosiderophores) under Fe deficiency has been reported to increase Cu uptake in calcareous soil (Chaignon et al. 2002). It is noticeable that soil chemical properties can differ between the bulk soil and the rhizosphere, so considering only properties in the bulk soil might be a poor predictor of Cu bioavailability and ultimately Cu uptake which rather depends on the particular properties induced by roots in the rhizosphere. Accordingly, contradictory results concerning the effect of pH on Cu uptake by plants are found in the literature. In very acidic soils, plant Cu concentration increased compared to calcareous soils in rape (*Brassica napus L.)* and tomato (*Lycopersicon esculentum L.)* (Chaignon et al. 2003; Cornu et al. 2007). On the contrary, Cu accumulation in maize (*Zea mays L.)* was as high in calcareous soils as in acidic soils (Brun et al. 2001). Michaud et al. (2007) did not find a clear relationship between Cu uptake and soil pH in durum wheat (*Triticum turgidum durum L*.) in Cu-contaminated soils, probably due to the implication of root-induced changes of pH and dissolved organic carbon in the rhizosphere. At low pH, alkalization in the rhizosphere was observed compared with the bulk soil,

which may result in reduced Cu bioavailability. In calcareous soils, a larger chemical mobility may be related to phytosiderophore secretion leading to greater Cu uptake in plants.

Copper function in plants

Copper (Cu) is an essential element for plant cells and is needed in at least six different locations, including the cytosol, the endoplasmic reticulum (ER), the mitochondrial inner membrane, the chloroplast stroma, the thylakoid lumen, and the apoplast (Marschner, 1995). Compared to other metal-dependent proteins (metalloproteins), the number of proteins in plants that require copper is generally smaller. In *Arabidopsis*, a type of plant, 105 proteins can be identified by searching for the term "copper protein," and 21 proteins that can be identified by searching for the term "copper-binding protein" (Krämer and Clemens 2006). The most common copper proteins found in the plant's green tissues are plastocyanin and Cu/ZnSOD. Cu/ZnSOD has three different forms in *Arabidopsis*, with the main forms being found in the cytosol (CSD1) and the chloroplast stroma (CSD2), and the third form is found in the peroxisome (CSD3) (Kanematsu and Asada 1989; Bueno et al. 1995). In maize, four cytosol Cu/ZnSOD isoenzymes have been found (Kernodle and Scandalios 2001). In addition to plastocyanin and Cu/ZnSOD there is a large number (>32) of related proteins (blue-copper binding proteins) with unknown functions encoded in the *Arabidopsis* genome (Nerissian et al. 1998). For instance, the existence of a Cu protein involved in photosynthetic reactions of photosystem II (PSII) non-dependent on plastocyanin was reported earlier (Lightbody and Krognann 1967; Barr and Crane 1976). More recently, Burda et al. (2002) found that Cu in an equimolar concentration to PSII reaction center stimulated in vitro the oxygen-evolution activity of PSII. Nevertheless. An important characteristic of Cu + is its ability to bind small molecules such as O_2 as ligands. Thus, Cu is a cofactor of a large number of oxidases. The

best-known oxidase is the mitochondrial cytochrome c oxidase. Other members of this enzyme group are: i) amine oxidase enzymes associated with the cell wall that catalyze the oxidation of putrescine that produces H_2O_2 involved in lignification, cross-linking of cell wall proteins, and programmed cell death (Moller and McPherson 1998); ii) multi-copper oxidases such as ascorbate oxidases that localize in the apoplast and regulate its redox state, and laccases also localized in the apoplast but not functionally well understood although a role in lignification has been proposed (Ramocha et al. 2002); iii) multi-copper oxidase-like proteins such as SKU5, which are involved in cell wall formation (Sedbrook et al. 2002); iv) polyphenol oxidase found in the thylakoids of some plants, such as spinach (Kieselbach et al., 1998) but not in other species such as *Arabidopsis* (Schubert et al. 2002) that is involved in ROS defense. Cu + can also bind ethylene. Accordingly, the ethylene receptor ETR1, which localizes in the endoplasmic reticulum (ER), is dependent on Cu^+ (Rodriguez et al. 1999). The role of copper (Cu) in the synthesis of a molybdenum cofactor has been proposed in a study by Kuper et al. (2004). This discovery links copper metabolism to nitrogen assimilation and phytochrome biosynthesis, as indicated by Mendel (2005).

Copper acquisition and transport:-

Cu acquisition and transport into and within cells is relatively little known in plants but in the last ten years, rapid progress has been made in understanding these processes within plant cells, particularly with the application of the knowledge in yeast to other eukaryote organisms. Consequently, several families of heavy metal transporters involved in intracellular homeostasis have been identified in plants (Fox and Guerinot 1998; Himelblau and Amasino 2000; Williams

et al.2000; Markossian and Kurganov 2003; Krämer and Clemens 2006; Colangelo and Guerinot 2006; Puig et al. 2007). However little results have been obtained concerning long-distance transport or transport processes taking place at the root level. For instance, at present, it is not clear how plant roots actively mobilize Cu ions. Phytosiderophore secretion by monocots is known to enhance Cu mobilization (Römheld 1991) but there is no evidence for the uptake of Cu-phytosiderophore complexes by plant roots.

Excess copper (Cu) in soils results not only from its increasing use industry like mining and smelting but also from its use as a presence in sewage sludge amendments (Nicholson et al., 2003, Xiong and Wang, 2005). Cu is an essential micronutrient for the growth and development of plants. At the cellular level, Cu also plays an essential role in the signaling of transcription and protein trafficking machinery, oxidative phosphorylation, and iron mobilization (Yruela, 2005). Moreover, Cu is required in biological systems as a structural component and catalytic enzyme activity as a cofactor, however, it can be a stress factor causing physiological responses that can inhibit plant growth at higher concentrations in soil (Monnet et al. 2001). Excessive amounts of heavy metals can cause the generation of free radicals and reactive oxygen species (ROS), leading to oxidative stress. Copper (Cu) specifically has been linked to damage to proteins and lipids due to its ability to generate ROS. Studies on various plant species have shown that Cu can cause oxidative stress by affecting the activities of antioxidative enzymes. The antioxidant protection in plant cells is complex and compartmentalized. Superoxide dismutases (SOD) are a group of metalloenzymes that catalyze the dismutation of $O2^{\cdot-}$ H_2O_2. The majority of H_2O_2 is removed by catalases (CAT), which are localized in peroxisomes and peroxidases that are found in vacuoles,

cell walls, and cytosol. Ascorbate peroxidase (APX) allows the scavenging of small amounts of H_2O_2 in specific parts of the cell, such as chloroplasts and mitochondria.

Plants that are grown in the presence of high levels of copper (Cu_2^+) usually exhibit chlorotic symptoms and reduced biomass production (Bernel et al. 2004; Yruela, 2005). Proline accumulation in plants is a common response to abiotic stress (Jain et al. 2001). Therefore, it is reasonable to link proline metabolism and heavy metal stress in plants. Free amino acids and proline have been found to accumulate in response to exposure to $Cu2^+$ (Chen et al. 2004, Fariduddin et al., 2009, Al-Hakimi and Hamada 2011). Excess copper concentrations can lead to oxidative stress by increasing the levels of reactive oxygen species (ROS) within subcellular compartments. ROS include the superoxide radical ($O2-\bullet$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\bullet OH$), which mainly affect lipids, proteins, carbohydrates, and nucleic acids (Mittler et al., 2004). However, cells have enzymatic mechanisms to eliminate or reduce the damaging effects of ROS.

The antioxidant enzymes, including catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD), play a crucial role in scavenging reactive oxygen species (ROS), which helps prevent oxidative damage. The balance between ROS generation and removal determines the system's survival (Khatun et al. 2008; Golshan et al. 2011). Cu+2 toxicity can cause problems with the uptake of other essential elements (Pätsikkä et al. 2002) and affect membrane properties by oxidizing membrane lipids. The harmful effects on the membrane can be estimated by measuring the increase in MDA, a product of lipid peroxidation

(Jouili and El Ferjan, 2003). Copper ion activity is not always an accurate predictor of copper toxicity because other cations, such as Ca^{2+} and Mg^{2+}, can compete with copper (Campbell, 1995).

Between 2010 and 2015, the production of refined copper in the world increased from 18,987 to 22,842 thousand metric tonnes. However, the repeated application of Bordeaux mixture ($CuSO_4.5H_2O+Ca(OH)_2$) as a fungicide, along with hog manure and sewage sludge amendments, can lead to an excess of copper in the soil, causing toxicity (Sharma et al. 2007). Copper is an essential mineral nutrient for plants and is typically found in plant tissue in concentrations ranging from 4-20 ppm. It plays a vital role in various metabolic activities. The presence of Cu is essential in the cytosol, endoplasmic reticulum, inner membrane, chloroplast stroma, thylakoid lumen, and apoplast for their normal function (Marschner, 1995). Additionally, Cu at the cellular level plays a role in signaling of transcription, protein trafficking machinery, oxidative phosphorylation and ion mobilization (Yruela, 2005). Cu also plays a significant role in PSII activity in ensuring the correct content and composition of pigments and polypeptides in PSII and in maintaining the lipid microenvironment in the thylakoid and serving as a structural component of active PSII complexes (Droppa et al. 1984). Cu is a redox-active transitional metal as it can exist in multiple oxidation states i.e. $Cu2^{+}$ and Cu+ which may catalyze the formation of hydroxyl radicals (OH·) from the non-enzymatic chemical reaction between superoxide (O_2^{-}) and H_2O_2 (Hydrogen peroxide) through Haber-Weiss reaction (Halliwell and Gutteridge, 1984) which is known to the damage cell membrane by binding to a SH group of membrane proteins and induces lipid peroxidation Supraoptimal concentration of Cu proves to be phytotoxic (Dewez et al. 2005) by causing electrolyte leakages, affecting the synthesis of phenolic compounds (Sanchez-Viveros et al. 2010), altering protein synthesis, chromatin structure and enzyme activity by binding to the thylakoid

membrane proteins (Vierke and Struckmeier, 1977 and Madejon et al. 2009) and alters the lipid-protein pigment complexes (Baszynski et al. 1988). Excess Cu also interferes with the uptake of other elements like N, P, S, Ca, and Mg (Cambrolle et al. 2011).

Responses to copper toxicity

Copper (Cu) can be found in some soils at naturally occurring toxic levels. However, some soils may contain high levels of Cu due to human activities that release heavy metals into the environment. These activities include the application of Cu-rich pig and poultry slurries, the accumulation of fertilizers and fungicides, and industrial and urban activities such as metalliferous mining, metal processing, and waste disposal technologies. According to Kabata-Pendias and Pendias (2001) and Pilon-Smits and Pilon (2002), the concentration of Cu in non-contaminated soils and natural waters is around 20-30 mg kg-1 and 2 µg kg-1, respectively. However, in contaminated soils and waters, the levels of Cu can be one hundred times higher (Fernandes and Henriques 1991). Additionally, atmospheric heavy metal emission has also been identified as an important source of heavy metal contamination in plants (Friedland 1990; Salim et al. 1992). At concentrations above those required for optimal growth, Cu can be toxic for most plants except a few plant species that can hyperaccumulate metals (i.e., *Arabidopsis halleri L*., *Silene vulgaris* (Moench) Garcke, *Thalspi caerulescens L*.). It is worth mentioning that this toxicity is dependent on plant species, the concentration of metal supplied, exposure time, and soil properties. In sensitive plant species or ecotypes, Cu was shown to inhibit growth and interfere with important cellular processes such as photosynthesis and respiration (Marschner 1995; Prasad and Strzalka 1999, Yruela 2005). In the presence of high levels of Cu (3-100 µM) plants normally show reduced biomass (reduction of the root and shoot volume, stem size, and leaf size), chlorotic symptoms,

necrosis, and inhibition of shoot and root growth. Lower content of chlorophyll and alterations of chloroplast structure and thylakoid membrane composition have been found in leaves of spinach, rice, wheat (*Triticum durum L. cvv. Adanello and Ofanto*), bean *(Phaseolus coccineus L*. cv. *Piekny*) and oregano (*Origanum vulgare L.*) in such growth conditions (Baszynski et al. 1988; Lidon and Henriques 1991; 1993; Ciscato et al. 1997; Pätsikkä et al. 1998; Quartacci et al. 2000; Panou-Filotheou et al. 2001).

Particularly, degradation of grana stacking and stroma lamellae, increase in the number and size of plastoglobuli, and appearance of intra thylakoidal inclusions were observed. It has been proposed that Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes (Maksymiec et al. 1994). Pätsikka et al. (2002) attributed the reduction of chlorophyll content to a Cu-induced Fe deficiency. The substitution of the central Mg ion of chlorophyll by Cu in vivo has also been proposed as a damage mechanism leading to the inhibition of photosynthesis (Küpper et al. 2003; Küpper and Kroneck 2005). Besides, lipid peroxidation, a decrease in lipid content, and changes in the fatty acid composition of thylakoid membranes were also shown (Sandmann and Böger 1980; Luna et al. 1994; Maksymiec et al. 1994). As a consequence of those modifications, an alteration of PSII membrane fluidity was found (Quartacci et al. 2000). On the other hand, the decrease of the photochemical activity caused by Cu is accompanied in vivo by an alteration of the structure and composition of the thylakoid membranes, which can influence the conformation and function of the photosystems (Baszynski et al. 1988, Ouzounidou et al. 1992, Lidon and Henriques 1993).

Baszynski and Kruppa (1995) suggested that Cu-induced processes could either destroy the oxygen-evolving complex polypeptide composition or interact with ions required for the complex's proper functioning such as Mn, Ca, and Cl. Plant cell cultures are widely used as a model system to analyze cell stress response and adaptation and for various other studies on plant physiology. Studies on cell culture from mesophyll cells have provided information on the functional changes in cell organization induced by excess Cu that can be extrapolated to leaf cells in plants. Soybean cell suspensions exposed to excess Cu (10 μM) maintained the general cell organization pattern of the untreated soybean cultures, but Cu exposure induced changes in specific subcellular structures. Smaller chloroplasts with a rounded shape and more numerous, with denser structured internal membranes, no starch granules within chloroplasts, and larger cytoplasmic vacuoles were observed (Bernal et al. 2006b, 2006c). Similarly, chloroplasts of seven-week-old *Arabidopsis thaliana* plants exposed to 50 μM Cu for 2-14 days showed a rather circular than ellipsoidal shape (Wójcik and Tukiendorf 2003). Starch grains disappeared and plastoglobuli became larger in chloroplasts from leaves of oregano exposed to excess Cu (10-25 μM g-1) (Panou-Filotheou et al. 2001). Roots and shoots also sense the phytotoxicity of Cu. Roots of oregano plants exposed to 13-25.5 μM g-1 Cu. (Panou Fitlotheou and Bosabalidis 2004) revealed a destroyed epidermis and a cortex of large cells with folded walls. Cortical cells exhibited a metamorphosis of the amyloplasts into leucoplasts. In the root vascular cylinder, the diameter of the xylem vessels increased. It is well known that transition metals like Cu catalyze the formation of hydroxyl radicals (OH·) from the non-enzymatic chemical reaction between superoxide (O_2.) and H_2O_2 (Haber-Weiss reaction) (Halliwell and Gutteridge, 1984). 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 (ascorbate peroxidase (APX), catalase, dehydroascorbate reductase (DHAR), guiacol peroxidase, glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), superoxide dismutases (SODs)) (De Vos et al. 1992 Luna et al. 1994; Stohs and Bagchi 1995; Navari-Izzo et al. 1998; Gupta et al. 1999; Drazkiewicz et al. 2003; Wang et al. 2004; Lombardi and Sebastiani, 2005). The ascorbate-glutathione cycle has been reported to be involved in response to excess Cu (Gupta et al. 1999; Drazkiewicz et al. 2003).

The antioxidant responses have been observed in leaves and roots being either copper concentration or time-dependent as well as plant species or ecotype dependent. The mechanism of Cu toxicity on photosynthetic electron transport has extensively also been studied in vitro, and it was found that PSII is the most sensitive site to Cu toxicity. Both the acceptor and the donor sides of PSII were suggested as the main targets of Cu toxic action. On the PSII reducing side, the Q B binding site and the Pheo-Fe-Q A domain have been reported as the most sensitive sites for Cu toxicity (Barón et al. 1995; Yruela 2005). The interaction of Cu toxicity with photoinhibition and recovery processes on PSII has been also investigated (Yruela et al. 1996, Pätsikkä et al., 1998) demonstrating that Cu enhances the adverse effects of light.

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 photoinhibition is due to the production of hydroxyl radicals (Yruela et al. 1996). A different proposal was given by Pätsikkä et al. (2002) suggesting that the reduced chlorophyll content observed in plant leaves grown in the

presence of high Cu concentrations made leaves more susceptible to photoinhibition as a consequence of a Cu-induced Fe deficiency. Susceptibility to excess Cu varies with plant species and ecotypes. For instance, alfalfa and barley are highly tolerant to excess Cu, but rice (*Oryza sativa*) and potato are less tolerant (Jones 1998). Mungbean plants were chosen for this research as they are one of the most important crops of India, ranking third in pulses production after chickpea and pigeon pea (Singh and Yadav, 1978) and an inexpensive source of vegetable dietary protein.

Significance of work:

Research on the effect of copper on plants holds significant importance for several reasons. Copper is an essential micronutrient for plants, playing a crucial role in various metabolic processes, including photosynthesis, respiration, and enzyme activation. Understanding the optimal levels of copper uptake by plants is essential for agricultural practices to ensure adequate growth and productivity. Copper contamination in soil and water can occur due to various human activities such as mining, industrial discharge, and the use of copper-based pesticides and fungicides in agriculture. Research helps in understanding the impacts of excess copper on plant health and ecosystem dynamics, aiding in the development of strategies to mitigate environmental pollution. Copper can accumulate in edible plant parts, and excessive intake of copper through the food chain can pose health risks to humans, including liver and kidney damage. Studying the transfer of copper from soil to plants and assessing its potential health implications is crucial for food safety and human health. Some plants can absorb and accumulate heavy metals like copper from contaminated soils-a process known as phytoremediation. Research in this area helps identify plant species with high copper accumulation potential and optimizes phytoremediation techniques

for cleaning up polluted environments. Copper toxicity can adversely affect plant growth and soil microbial communities, leading to changes in ecosystem structure and function. Understanding how different plant species respond to copper stress can provide insights into ecosystem resilience and help conserve biodiversity in copper-contaminated environments. In essence, research on the effect of copper on plants is critical for sustainable agriculture, environmental management, human health protection, and biodiversity conservation.

We have observed several reports on copper phytotoxicity in plants. Most of them discussed how copper pollution in soil affects various crop plants through their roots. However, we couldn't find any research on the effect of atmospheric copper pollution on plants, particularly mungbean plants. So, we are interested to know if excess copper in the atmosphere, as found in industrial areas, can cause toxic symptoms in the whole plant. Specifically, we want to study whether 1, 4, and 7 mmol-1 concentrations of Copper Sulphate can transport excess copper to all parts of the plant and disturb their normal metabolism. Additionally, we are curious to know if copper phytotoxic effects can cause a decline in the yield of an agricultural crop like mungbean.

Research Gaps

Research on copper toxicity in plants has made significant strides, but there are still several gaps in our understanding. It's known that copper can be toxic to plants, but the specific molecular mechanisms underlying its toxicity are not fully understood. Further research is needed to elucidate the pathways through which copper interferes with essential physiological processes in plants. Plants are often exposed to multiple stressors simultaneously, such as heavy metals, drought,

salinity, etc. More research is needed to understand how copper toxicity interacts with other stress factors and how these interactions affect plant health and productivity. Many studies focus on short-term effects of copper toxicity, but there is a need for research into the long-term impacts. This includes understanding the persistence of copper in soils, its accumulation in plant tissues over time, and its effects on soil microbial communities and ecosystem functioning. While high levels of copper can cause obvious toxicity symptoms in plants, sublethal concentrations may also have significant impacts on plant growth, development, and metabolism. Further research is needed to understand these sublethal effects and their implications for plant fitness and ecosystem dynamics. Copper hyperaccumulating plants have been explored for their potential use in phytoremediation of copper-contaminated soils. However, there is still much to learn about the factors influencing their efficacy, including the role of soil properties, plant-microbe interactions, and the long-term stability of remediated sites. Addressing these gaps will not only deepen our understanding of copper toxicity in plants but also inform strategies for mitigating its adverse effects on crop productivity, environmental health, and ecosystem functioning.

OBJECTIVES

The objective of this study is to evaluate the phytotoxicity of copper and its impact on the growth, metabolism, and productive potential of mungbean plants *Vigna radiata* (L.) Wilczek. The study will analyze various below parameters to understand the damaging, protective and adaptational response of the plants to copper metal.

- Plant growth analysis
  - Photosynthetic efficiency
  - Relative water content
  - Biomass
- Quantitative analysis of different biochemical and physiological parameters and essay of enzymes
  - Pigment analysis
  - Total sugar and Reducing sugar content.
  - Protein content
  - Glycolipid and Phospholipid content
  - Proline content
  - Lipid peroxidation content

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions:-

Vigna radiata (L.) Wilczek, a high yielding variety, widely grown in Goa, India, procured from ICAR, Goa, were washed thoroughly under running tap water followed by surface sterilization with an aqueous solution of 4% sodium hypochlorite for 5 min and rinsed five times with tap water and soaked for 4 days. Seeds were grown in plastic pots of 15 cm diameter containing vermiculite in a growth chamber for 20 days under control conditions of temperature (25 ± 2°C) and light (200 µmolm^{-2} s^{-1} PAR) provided by cool white fluorescent tubes for 16 h photoperiod. Pots were carefully irrigated with Hoagland's solution (Hoagland and Arnon, 1950) containing copper treatment in the form of copper sulfate ($CuSO_4$) of different concentration 1 mmol l^{-1}, 4 mmol l^{-1} and 7 mmol l^{-1}.

Physiological and Biochemical analysis:-

2.2. Relative water content:-

Relative water content (RWC) of mung bean leaf was determined according to Barrs and Weatherley (1962). The first leaf of randomly selected plants was used for analysis. The fresh weight (FW) of the leaf was immediately recorded. The leaf samples were then soaked in distilled water containing a few drops of Tween 20 for 4 h at room temperature, under constant light conditions to obtain the Turgid Weight (TW). On placing the leaves in the oven at 80℃ for 24 h,

the Dry Weight (DW) of the leaves was recorded. On obtaining the above values of FW, TW and DW, RWC was calculated according to the following formula:

$$RWC = [(FW-DW) / (TW-DW)] \times 100$$

2.3. Total biomass

Biomass analysis was carried out according to Chen et al., (2014) using ten random plantlets that were harvested and weighed to obtain the shoot and root's fresh weight (FW). The samples were then dried at 80℃ for 48 h and weighed to record their dry weight (DW). The total biomass was determined using the following formula:

$$\text{Total biomass} = (FW-DW)$$

2.4. Pigment analysis by spectrophotometry:-

Chlorophyll a, Chlorophyll b and Carotenoids content were measured according to Arnon (1949). 0.2 g of tissue was homogenized with 2 ml of 80% acetone containing a few crystals of BHT, making the final volume 2 ml. The extract was kept overnight for incubation at 4℃. After 24 h the homogenate was centrifuged at 7000-8000 rpm for 10 min at 4℃. The supernatant was used to measure the absorbance at 663, 645 and 470 nm using a UV-visible spectrophotometer (UV-2450, Shimadzu).

$$\text{Chlorophyll a (Chl a) (mg/g FW)} = 12.27 \times A663 - 2.69 \times A645$$

$$\text{Chlorophyll b (Chl b) (mg/g FW)} = 22.9 \times A645 - 4.86 \times A663$$

$$\text{Carotenoids (mg/g FW)} = 4.7 \times A443 – 0.27 \times (20.2 \times A665 + 8.02 \times A663)$$

2.5. Measurements of photosynthetic efficiency:-

Photosynthetic efficiency was measured using a chlorophyll fluorescence monitoring system according to Sharma et al. (1997). Sorghum leaves were adapted to dark for 5 min to inhibit light-dependent reactions by oxidizing PSII electron acceptor molecules. Initial fluorescence (Fo) was measured by focusing on weak light beam modulation with an intensity of 3-4 μmol $m^{-2}s^{-1}$. Maximum fluorescence (Fm) was measured by exposing the sample to a saturation light pulse (≈ 4000 μmol $m^{-2}s^{-1}$ for 0.06 s). Variable fluorescence (Fv) was calculated as Fv = Fm – Fo and the maximum quantum yield (Fv/Fm) ratio. Actinic light of ≈ 600 μmol $m^{-2}s^{-1}$ was allowed to reach the steady fluorescence yield (Fs), followed by a far-red pulse for 5 s.

2.6. Total sugars content:-

2.6.1. Extraction of total sugars:-

Total sugars were estimated with slight modifications according to Dubois et al. (1956). 0.5g of leaf tissue was weighed, cut into small pieces and hydrolyzed in 5 ml of 2.5 N Hydrochloric acid by placing in a boiling water bath for 3 h and cooled at room temperature. The solution was neutralized with sodium carbonate until the effervescence ceased. The final volume was made to 15 ml and centrifuged at 5000 rpm for 10 min. The supernatant was used to estimate total carbohydrates.

2.6.2. Estimation of total sugars:-

0.5 ml of sample was taken, making the final volume to 1 ml using double distilled water. 1 ml of 5% phenol solution was added, followed by 5 ml of concentrated sulphuric acid by gentle mixing. The test tubes were allowed to cool down for 10 min at room temperature. Further, the tubes were placed in the hot water bath for 20 min at 30℃ and allowed to cool down at room temperature. A tube without the sample served as blank. The absorbance of the orange colour formed was recorded at 490 nm against a reagent blank. The amount of sugar in the unknown sample was read from a calibration curve using D- glucose as the standard solution (1mg/1m).

2.7. Protein Content

2.7.1. Extraction of Proteins:

Proteins were determined using Lowry et al. (1951). 0.5g of leaf tissue was homogenized in phosphate buffer saline (pH 7.4) using mortar and pestle making. The final volume was made to 10 ml, and the extract was centrifuged at 5000 rpm for 20 min at 4℃. The supernatant was used to estimate protein content.

2.7.2. Estimation of proteins:-

0.5 ml of the sample was used, making up the final volume to 1 ml using double distilled water. 5 ml of alkaline copper sulphate reagent was added, including the blank, with proper mixing. The solution was incubated at room temperature for 10 min. 0.5 ml of Folin-Ciocalteau reagent was added with appropriate mixing. The reagent mix was further incubated for 30 min at room

temperature. A tube without the sample served as blank. The absorbance of the blue-coloured complex was recorded at 750 nm. The protein content in the unknown sample was calculated from a calibration curve using Bovine serum albumin (BSA) (1mg/1ml) as standard.

2.8. Total lipid content

2.8.1. Extraction of total lipids:-

Total lipids were extracted according to Turnham and Northcote (1984). 2 g of leaf tissue was cut into small pieces and boiled in a sufficient amount of isopropanol for 10 min to inhibit lipase activity. The excess isopropanol was drained, and the tissue was dried using tissue paper. Further, the samples were homogenized in Chloroform: Methanol (1:2 v/v) containing 0.01% BHT and making the final volume 10 ml. The mixture was transferred into a separating funnel and was kept undisturbed for 1 h at 4℃. The supernatant was collected, and the residue was washed with Chloroform: Methanol (1:1 v/v). The same was repeated, and the supernatant was pooled. Extracted lipids were purified as described by Folch et al., (1957). The lipid extract was centrifuged for 5 min at 2000-3000 rpm to get rid of cell debris. Further, the supernatant was transferred into a separating funnel, followed by the addition of 2 ml double distilled water and 2.5 ml chloroform. The mixture was shaken for 2 min, and 2.5 ml of 0.88% potassium chloride was added. On vigorous shaking for 5 min, the extract was kept for separation for 30 min. The lower phase contains appreciable amounts of lipids. The extract was stored at -20℃ until further use. The entire extraction and purification process was carried out in diffused light to protect lipids from photo-oxidation.

2.8.2. Quantitative Estimation of glycolipids:-

Glycolipids were determined using phenol-sulphuric acid, according to Kushawa and Kates (1981). 0.1 ml of lipid sample was used, making the final volume 2 ml using double distilled water. 1 ml of 5% phenol solution was added to the solution, followed by gentle mixing, making sure that the film of lipid at the bottom of the tube was undisturbed. To this, 5 ml of concentrated sulphuric acid was added, followed by heating in a boiling water bath for 5 min and later allowed to cool for 15 min at room temperature. The orange colour absorbance was read at 490 nm against a reagent blank. The amount of sugar in the unknown sample was read from a calibration curve using D glucose as the standard solution (1mg/ml).

2.9. Lipid peroxidation

The level of lipid peroxidation is estimated in terms of MDA content determined by thiobarbituric acid (TBA) reaction following the methods of Sankhalkar and Sharma, 2002. Leaf tissue (0.5g) was homogenized with 5 ml of 1% TCA. The homogenate was centrifuged and supernatant incubated for 30 min at 95°C containing incubation buffer (50 mM Tris HCl + 150 mM NaCl, pH. 8.0) and freshly prepared 0.5% TBA in 20% TCA. The absorbance was recorded at 532 nm and nonspecific turbidity was corrected by subtracting absorbance at 600 nm. The concentration of MDA was calculated using the extinction coefficient of 155 mM-1 cm-1.

2.10. Determination of proline content

Proline concentration was determined following the method of Bates et al. (1973). Leaf tissue (0.2 g) was homogenized with 5 ml of 3% sulfosalicylic acid using mortar and pestle and homogenate centrifuged at 5000 rpm for 5 min. The supernatant was incubated with glacial acetic acid and acid ninhydrin reagent incubated at 100°C for 1 h in a water bath. After cooling the reaction mixture, 10 ml of toluene was added, vortexed and absorbance was read at 520 nm. The proline concentration was calculated using the proline standard graph.

2.11. Determination of SOD antioxidant activity

Total antioxidant activity was determined according to Boveris, (1984). Leaf tissue (0.2 g) extracted in 1.5 ml of 50 mM sodium phosphate buffer pH = 7.8 centrifuged at 4°C for 1 min. The supernatant is used to carry out the antioxidant activity. Reaction one consisted of 10 mM Na2CO3, 10 mM sodium phosphate buffer, 6 mM disodium EDTA, and 4.5 mM epinephrine. Reaction two consisted of sample extract instead of buffer. Reaction with water was considered blank. Enzyme kinetics was carried out at 480 nm. The protein concentration of enzyme extract was measured using the Bradford method at 595 nm. SOD activity was calculated as SOD activity (A) mg^{-1} protein min^{-1}.

3. RESULTS

3.1. Determination of Biomass

The effect of increasing concentrations of copper sulphate shows decrease in height of a mungbean plant **Fig.1.** The effect of increasing concentrations of copper sulphate on the average biomass of shoot and root (measured separately) of a mungbean plant has been presented in **Fig. 2 and Table No. 1** All the plant parts showed a progressive reduction in fresh weight. Plants sprayed with 7 mM concentration of CuS04 were most affected with a 60% and 49.8% reduction compared to control in the biomass of shoot and root respectively. Plants treated with CuS04 concentrations of 1mM, 4mM, and 7mM showed 47%, 23% and 63% decreases in the biomass of a mungbean plant respectively.

3.2. Determination of Photosynthetic efficiency (Fv/Fm ratio)

The effect of Cu treatment on Fv/Fm ratio, which is indicative of photosynthetic efficiency is presented in **Fig.3 and Table No. 1**. The ratio of the variable fluorescence to maximal fluorescence (Fv/Fm) was reduced by 3.91% and 8.61% at 1mM and 7 mM Cu treatment respectively as compared to its control.

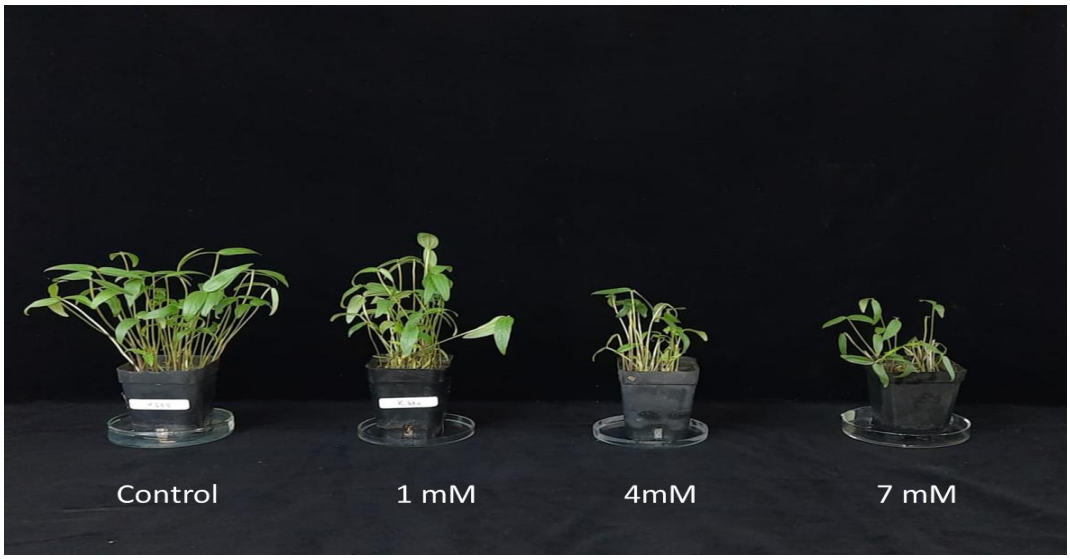

Fig.1. Effect of different concentrations of CuS04 on *Vigna radiata* (L.) Wilczek plants

Table 1. Effect of different concentrations of CuS04 treatment on photosynthetic efficiency and Biomass in *Vigna radiata* (L.) Wilczek plants. where ± indicates standard deviation, n=3.

Treatment	Fv/Fm ratio	Fv/Fm ratio % change	Biomass		Biomass % change	
			Shoot	Root	Shoot	Root
Control	0.702 ± 0	0	0.389 ±0.02	0.164±0.01	0	0
1 mM	0.69 ± 1.63	-1.635	0.307 ±0.00	0.086±0.00	-21.1407	-47.3957
4 mM	0.675 ± 2.31	-2.315	0.284 ±0.01	0.061±0.00	-26.8482	-63.001
7 mM	0.642 ± 4.88	-4.889	0.155 ±0.01	0.043±0.01	-60.0172	-49.8356

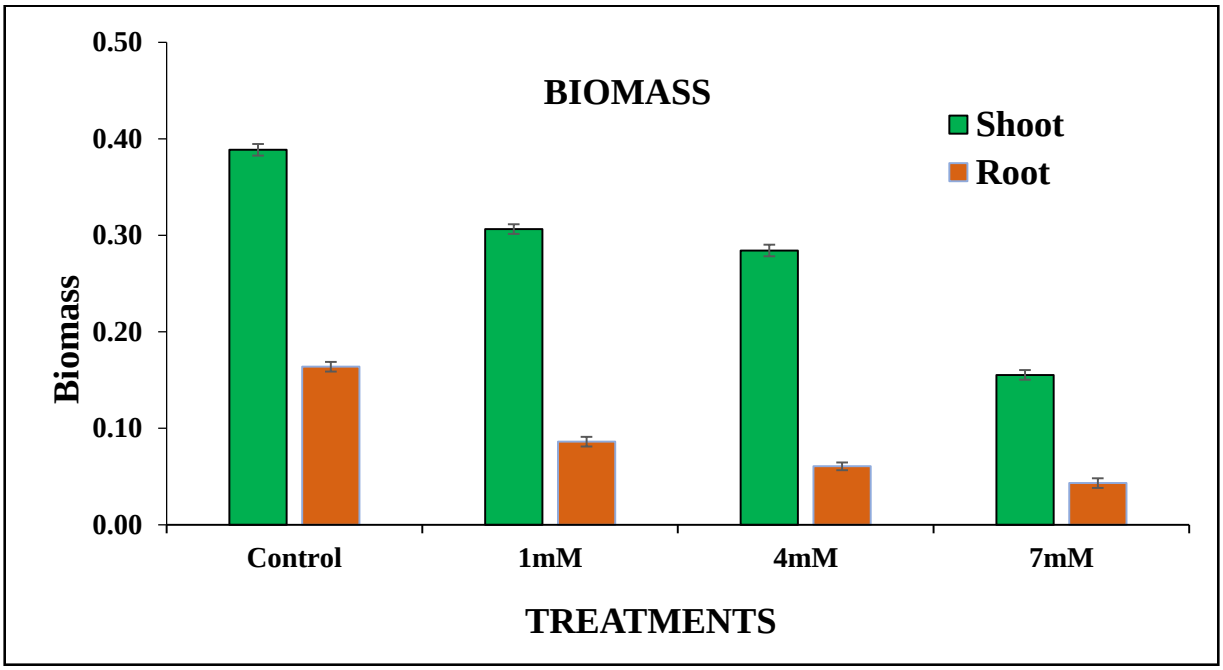

Fig. 2. Effect of different concentrations of CuS04 on Biomass in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

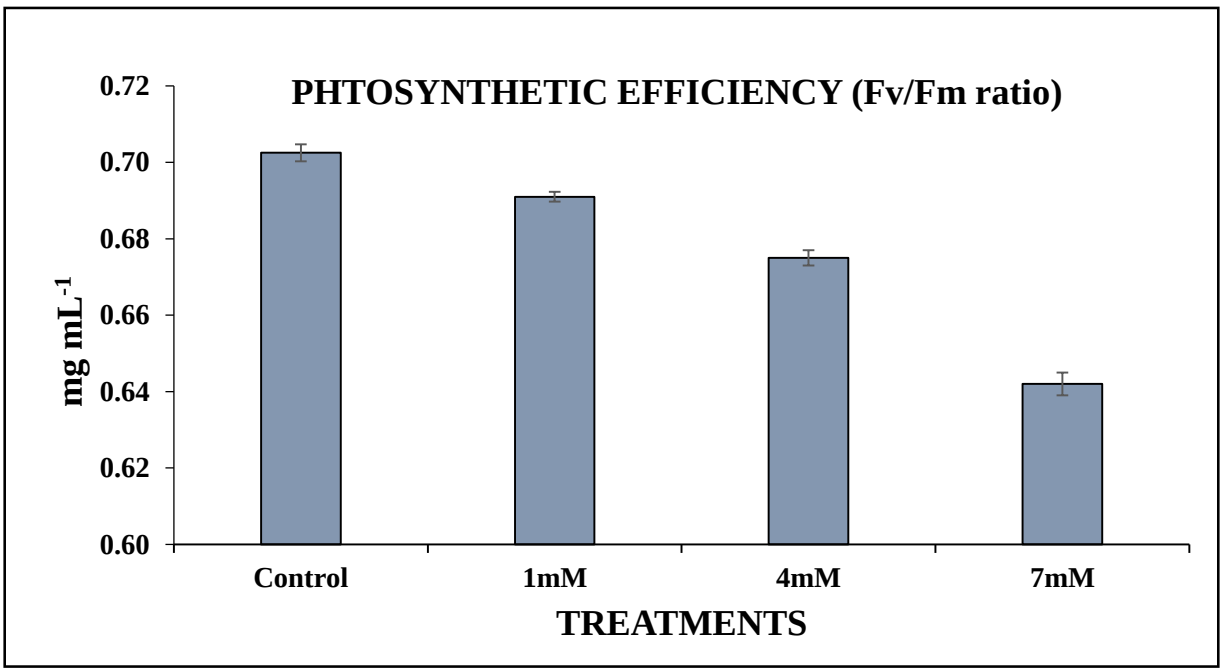

Fig. 3. Effect of different concentrations of CuS04 on Photosynthetic efficiency in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

3.2. Determination of Relative water Content (RWC)

Relative water content analysis data with increasing effect of CuS04 showed in **Fig.4 and Table No. 2**. The data showed a significant decrease in the relative water content in mungbean plant when grown over Cu treatment of 1 mM and above as compared to control. Plants sprayed with 1mM, 4 mM and 7mM concentration of CuS04 shows 15%, 28% and 53% decrease as compared to its control. Plants treated with 7 mM CuS04 concentration shows greater declined in relative water content.

Table 2. Effect of different concentrations of CuS04 on Relative water content in *Vigna radiata* (L.) Wilczek plants. Where ± indicates standard deviation, n=3.

Treatment	Relative water content	Relative water content % change
Control	4.585 ± 0.040	0
1mM	3.869 ± 0.037	-15.616
4mM	3.288 ± 0.026	-28.288
7mM	2.137 ± 0.018	-53.391

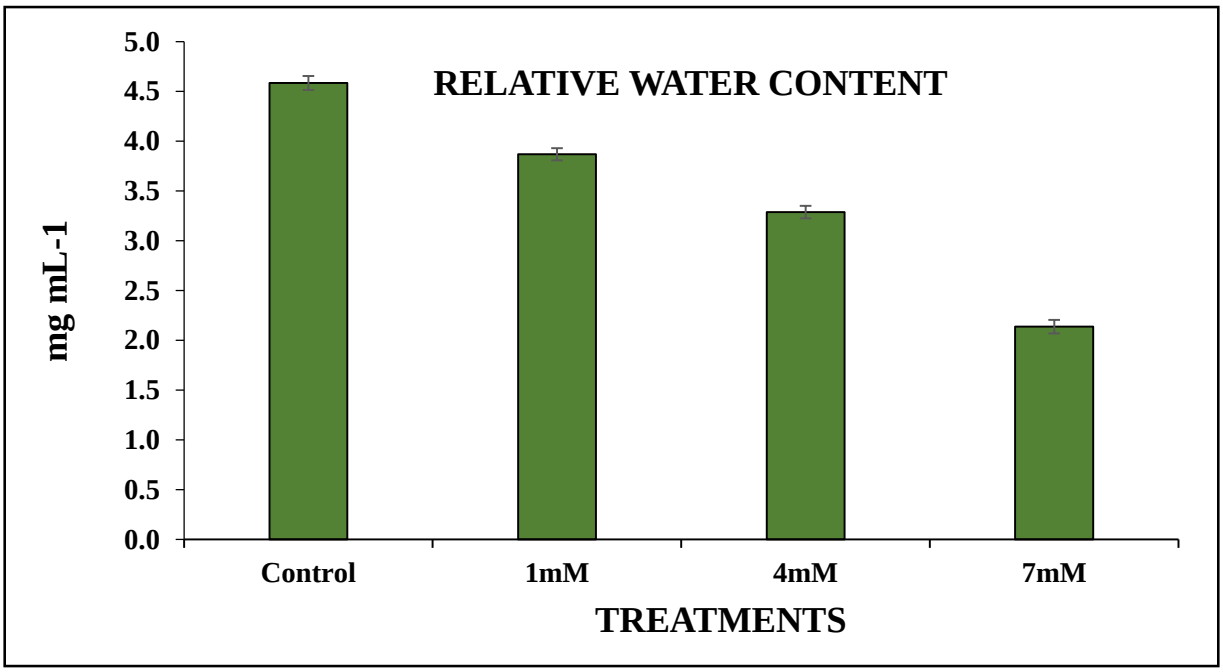

Fig.4. Effect of different concentrations of CuS04 on Relative water content in *Vigna radiate* (L.)Wilczek plants. Each bar represents the mean ±S.D. n=3.

3.4. Estimation of Photosynthetic Pigments

The effect of Cu treatment on photosynthetic pigments is shown in **Fig.5 and Table No.3.** Increasing concentration of CuS04 brought about a progressive decrease in the chlorophyll content of mature leaves of the mungbean plants. Treatment of 4 mM and 1mM CuS04 showed an 18 % decrease in chlorophyll a, a 9.21% and 18% decrease in chlorophyll b, and a 28%, 11% decrease in total chlorophyll. The highest dose of 7 mM CuS04 caused a 39% decrease in chlorophyll a and, 18% decrease in chlorophyll b as compared to its control.

The carotenoid content in mungbean leaf was also drastically reduced as concentration increases compared to control **(Fig.6 and Table No. 3)**. The lowest dose of 1 mM CuS04 brought about a 9% decrease in carotene, 4mM shows 28%, while the highest dose of 7 mM CuS04 resulted in a reduction of the carotene content by 45% as compared to its control.

Table 3. Effect of different concentrations of CuS04 on photosynthetic pigments in *Vigna radiata* (L.) Wilczek plants. Where ± indicates standard deviation, n=3.

Treatments	Chlorophyll a	Chlorophyll a % change	Cholorophyll b	Chlorophyll b % change	Carotenoids	Carotenoids % change
Control	19.508±0.875	0.000	6.269±0.578	0.000	10.306±0.728	0.000
1mM	18.266±0.845	-6.367	6.143±0.546	-2.010	9.355±0.663	-9.228
4mM	15.846±0.798	-18.772	5.392±0.514	-9.225	7.390±0.340	-28.294
7mM	11.896±0.715	-39.020	3.654±0.498	-18.668	5.655±0.478	-45.129

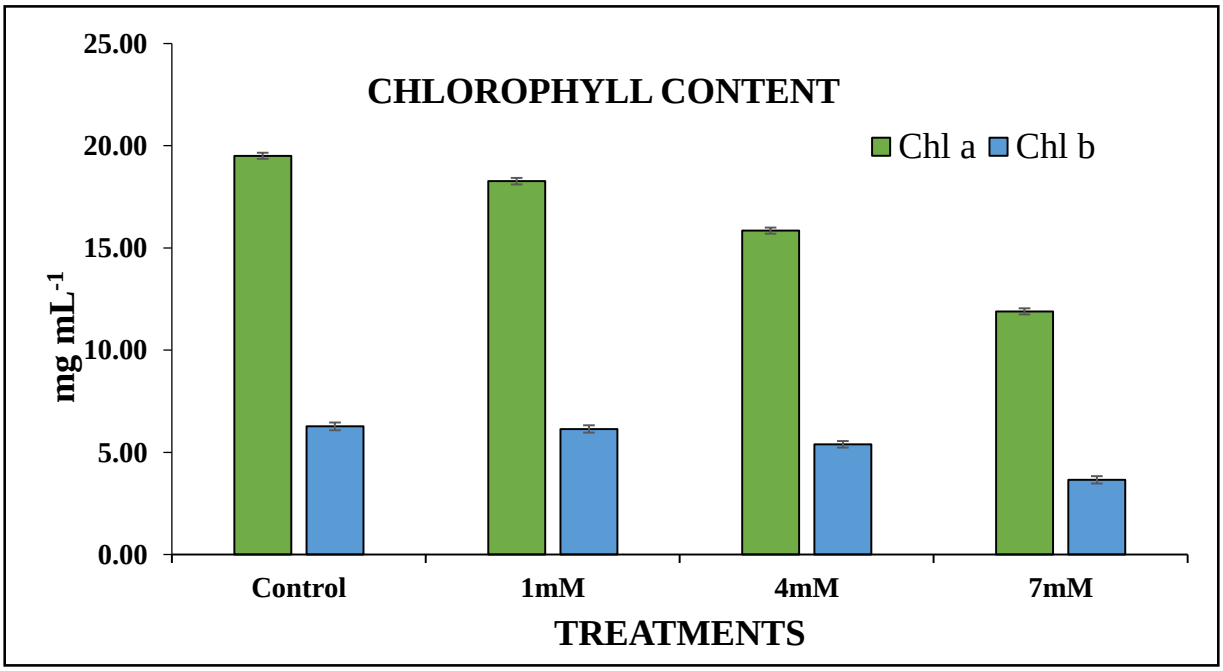

Fig. 5. Effect of different concentrations of CuS04 on pigment analysis in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

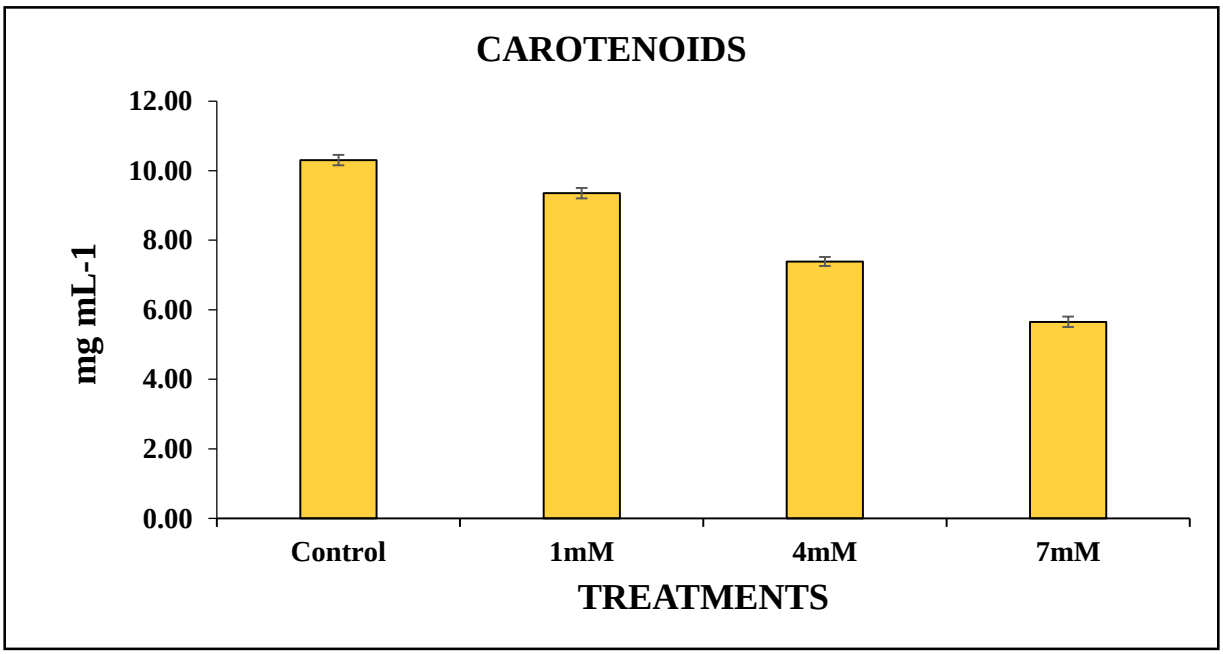

Fig. 6. Effect of different concentrations of CuS04 on carotenoids in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

3.5. Estimation of Total and Reducing sugar

The effect of toxic concentrations of CuS04 on the total sugars and reducing sugars in the leaves and roots of mungbean have been presented in **Fig. 7 and 8 and Table No. 4**. Total sugars decreased by 28% in 1 mM $CuS0_4$ concentration, 4mM CuSO4 showed 43% while 7 mM $CuS0_4$ caused 59% decreased in total sugar as compared to their control. Reducing sugars decreased by 18% in 1 mM CuS04 treatment, 37% by 4 mM and by 60% with 7 mM CuS04 treatment as compared to control.

Table 4. Effect of different concentrations of CuS04 on Total sugar and reducing sugar content in *Vigna radiata* (L.) Wilczek plants. Where ± indicates standard deviation, n=3.

Treatment	Total sugar content (mg/ml)	Total sugar content % change(mg/mL)	Reducing sugar content (mg/mL)	Reducing sugar content (mg/mL) % change
Control	5.029 ±0.019	0	4.458 ± 0.011	0.000
1mM	3.579 ±0.017	-28.838	3.633 ± 0.034	-18.502
4mM	2.829 ±0.015	-43.754	2.776 ± 0.012	-37.724
7mM	2.015 ± 0.032	-59.945	1.799 ± 0.008	-59.652

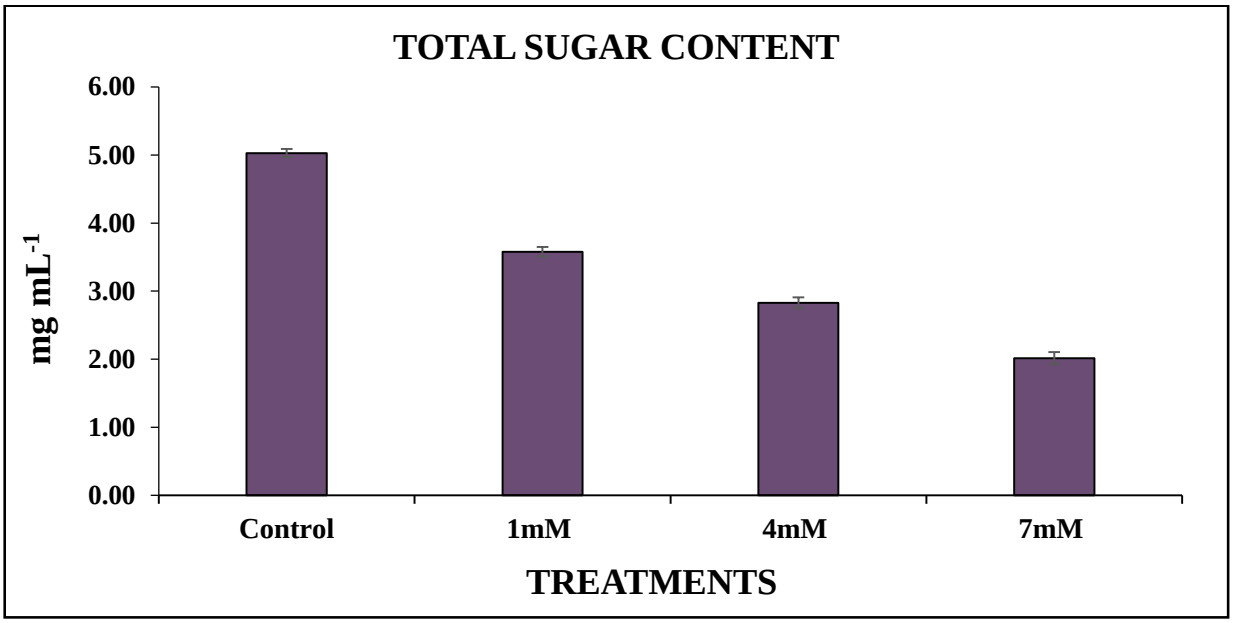

Fig. 7. Effect of different concentrations of CuS04 on Total sugar content in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

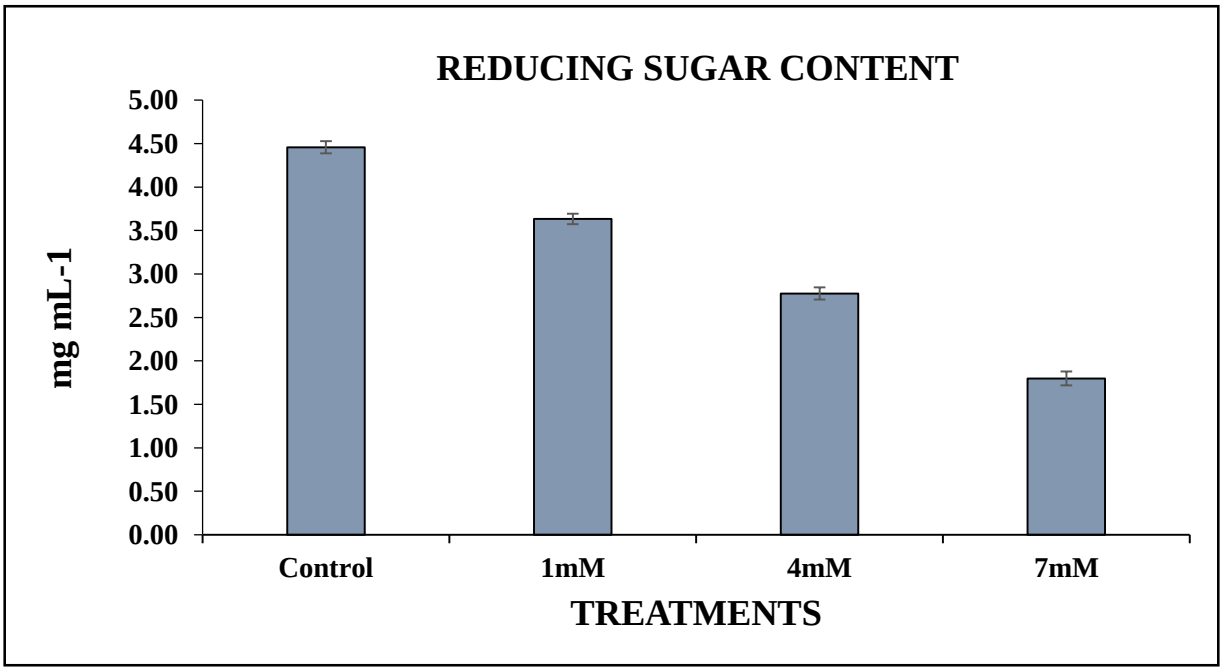

Fig. 8. Effect of different concentrations of CuS04 on Reducing sugar content in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

3.6. Estimation of Protein Content

Fig. 9 and Table No. 5 represents the effect of increasing concentrations of CuS04 on the buffer-soluble protein content in leaves and roots of mungbean plant. 1 mM and 4mM CuS04 treatment caused 40% and 71% increase in protein content respectively, as compared to control and 7 mM CuS04 treatment, on the other hand, brought about a much greater increase of 115% in the mungbean plant as compared to their control.

3.7. Estimation of Glycolipid Content

Glycolipid content was measured in control and treated plants. **Fig.10 and Table No. 5** showed the effect of increasing concentrations of $CuS0_4$ on the glycolipid content in mungbean plant. 1 mM CuS04 and 4 mM treatment caused 62% and 68% decreased respectively, and 7 mM CuS04 caused 76% decreased in glycolipid content as compared to their control.

3.8. Estimation of Phospholipid Content

The effect of toxic concentrations of phospholipid content in mungbean plant have been represented in **(Fig.11 and Table No.5)**. Phospholipid content decreased by 36% and 52% in 1 mM and 4mM CuS04 concentration respectively, while 7mM CuS04 caused 76% decreased as compared to their control.

Table 5. Effect of different concentrations of CuS04 on Protein, Glycolipid and Phospholipid content in *Vigna radiata* (L.) Wilczek plants. Where ± indicates standard deviation, n=3.

Treatment	Protein content (mg/mL)	Protein content (mg/mL) % change	Glycolipid content (mg/mL)	Glycolilpid content (mg/mL) % change	Phospholipid content (mg/mL)	Phospholipid content (mg/mL) % change
Control	1.972 ±0.020	0	346.25±0.02	0	367.68±0.016	0
1mM	2.777 ±0.020	40.829	129.48±0.03	-62.604	234.47±0.029	-36.230
4mM	3.390 ±0.020	71.918	109.40±0.04	-68.405	175.18±0.031	-52.355
7mM	4.245 ±0.190	115.263	79.96±0.015	-76.905	86.57±0.023	-76.453

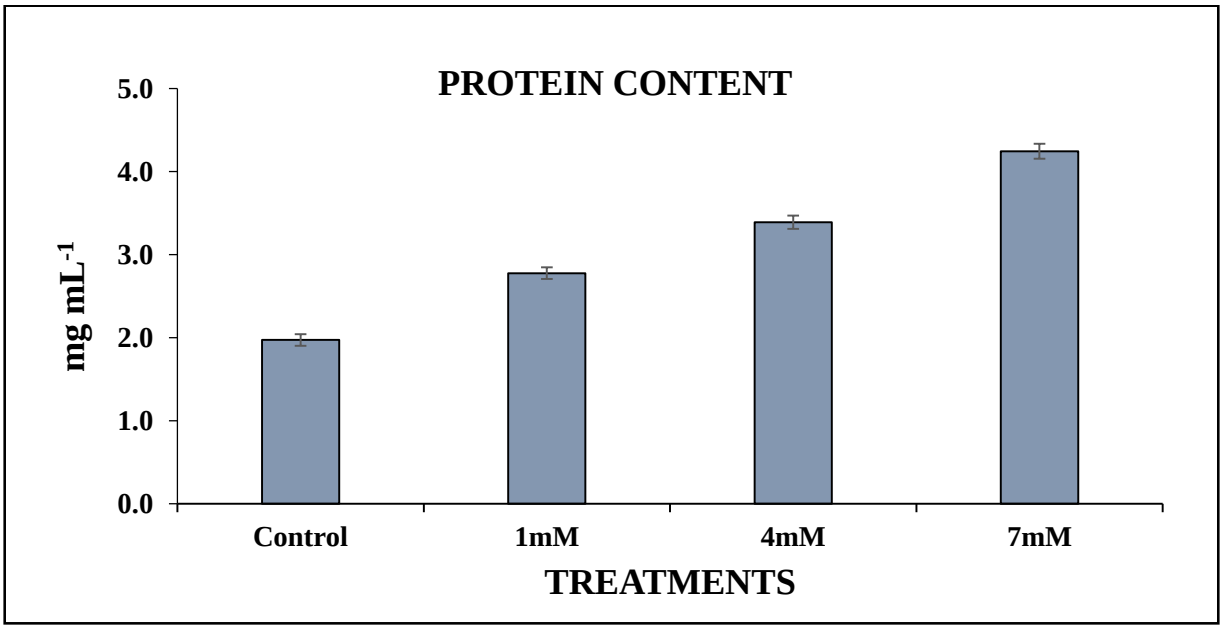

Fig. 9. Effect of different concentrations of CuS04 on protein content in *Vigna radiate* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

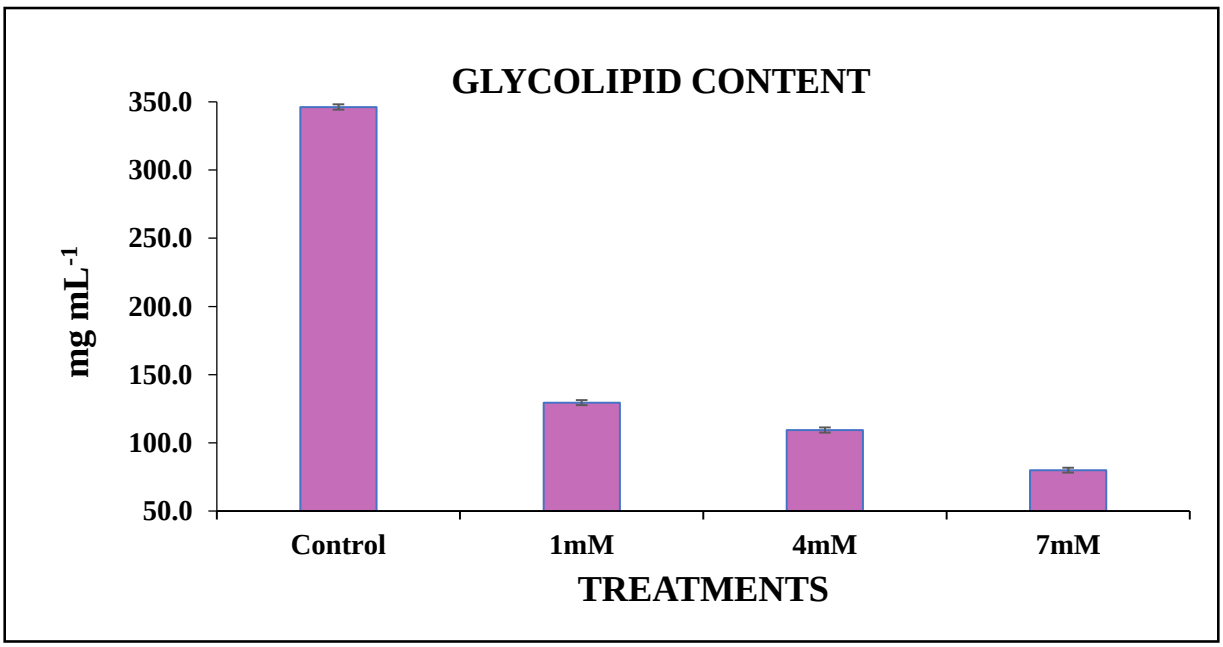

Fig. 10 Effect of different concentrations of CuS04 on Glycolipid content in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

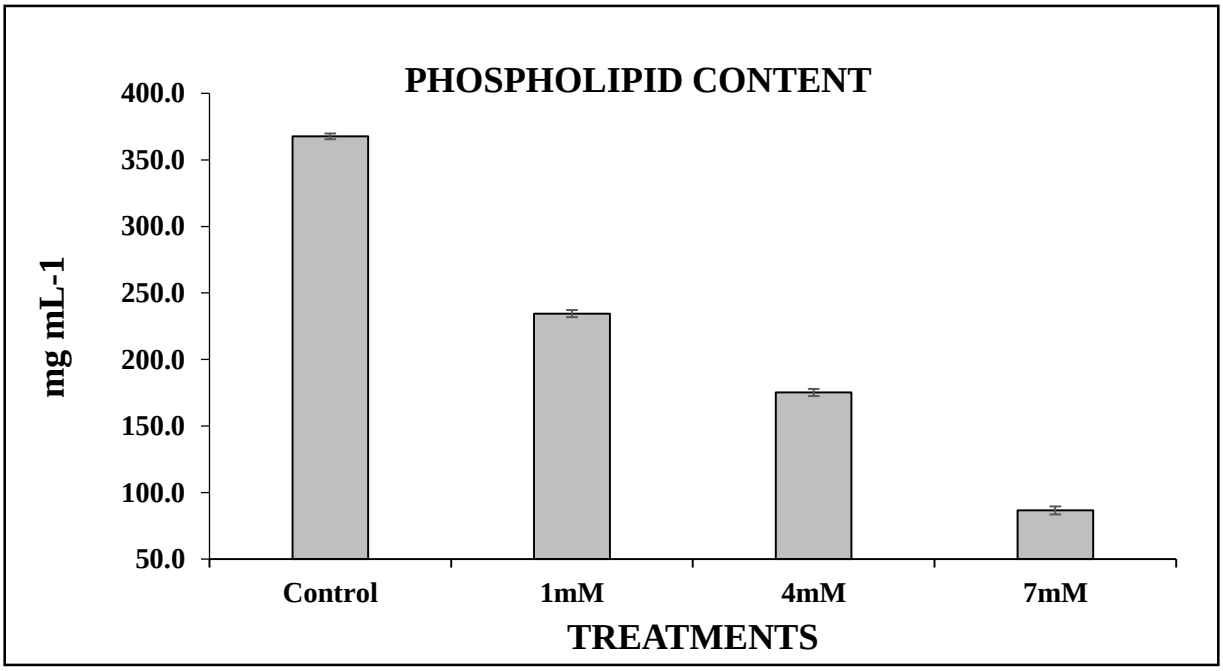

Fig. 11. Effect of different concentrations of CuS04 on Phospholipid conntent in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

3.9. Estimation of Proline Content.

The proline content in the leaves and roots of the mungbean plant also increased with increasing copper toxicity. As demonstrated in **Fig.12 and Table No.6** 1 mM and 4mm CuS04 caused a 140% and 71% increase in mungbean plants respectively, whereas the highest dose of 7 mM caused an increase of 115% in mungbean plant as compared to its control.

3.91. Determination of Lipid Peroxidation

The effect of Copper toxicity on lipid peroxidation in plants was studied **Fig.13 and Table No.6**. Cupper treatment caused increased lipid peroxidation. Toxic concentrations of CuS04 increased the total malondialdehyde content (a measure of lipid peroxidation) in mungbean plant. Treatment with 1 mM CuS04 concentration resulted in 195% increase and 7 mM concentration elicited a massive increase of 269% and 113% in the mungbean plants.

Table 6. Effect of different concentrations of CuS04 on proline and lipid peroxidation content in *Vigna radiata* (L.) Wilczek plants. Where ± indicates standard deviation, n=3.

Treatment	Proline content (mg/mL)	Proline content (mg/mL) % change	Lipid peroxidation (mg/mL)	Lipid peroxidation(mg/mL) % change
Control	1.096 ± 0.016	0	1.217 ± 0.017	0.000
1mM	1.816 ± 0.015	65.664	3.601 ± 0.011	195.903
4mM	3.563 ± 0.042	140.280	3.919 ± 0.025	222.070
7mM	4.364 ± 0.027	225.024	4.497 ± 0.034	269.610

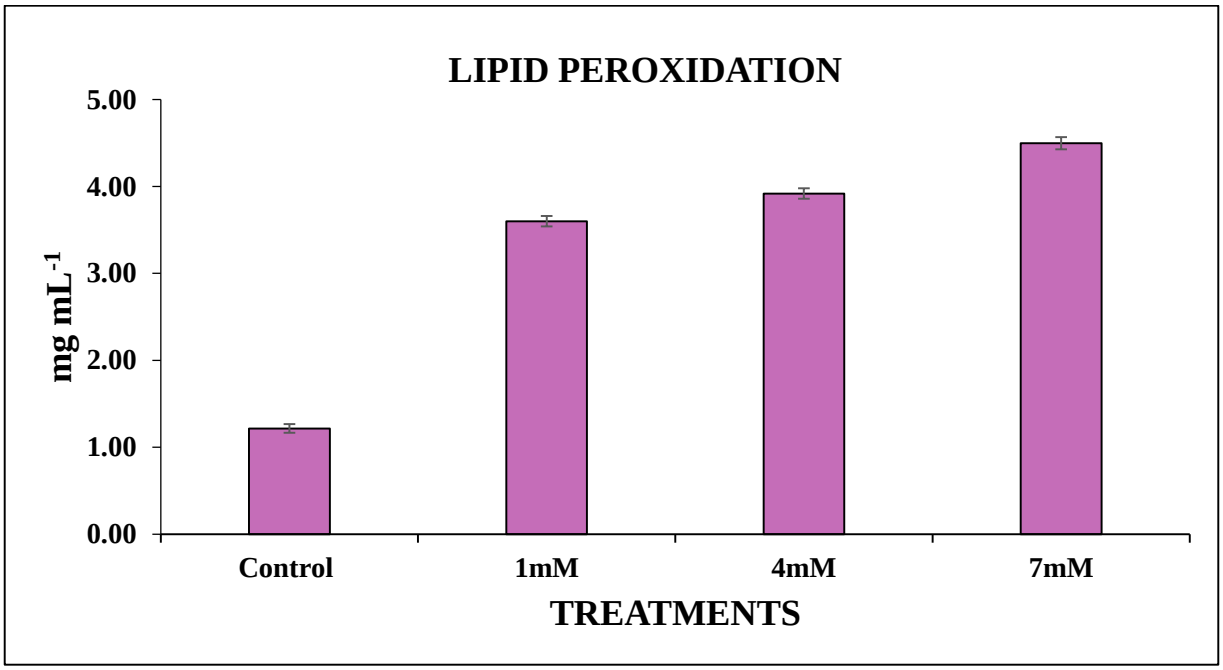

Fig. 12. Effect of different concentrations of CuS04 on proline content in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

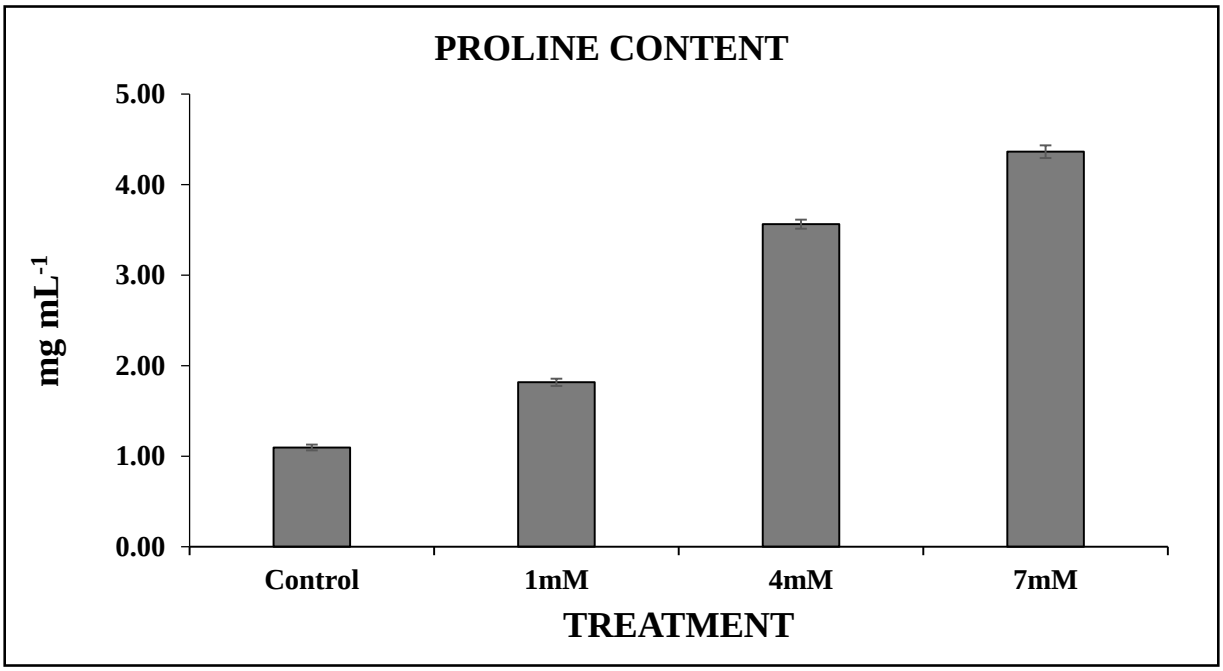

Fig. 13. Effect of different concentrations of CuS04 on lipid peroxidation content in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

3.92. Determination of SOD

Copper toxicity caused increase in SOD content as demonstrated by the following results **(Fig.14 and Table No. 7).** 1mM CuS04 caused 183 % increased, 4mM caused 227 % increased and 7 mM caused 366% increase in the mungbean plant.

Table 7. Effect of different concentrations of CuS04 on SOD content in *Vigna radiata* (L.) Wilczek plants plants. Where ± indicates standard deviation, n=3.

Treatment	SOD content	SOD content % change
Control	1.216 ± 0.09	0
1mM	1.216 ± 0.12	183.799
4mM	1.216 ± 0.14	227.878
7mM	1.216 ± 0.16	366.941

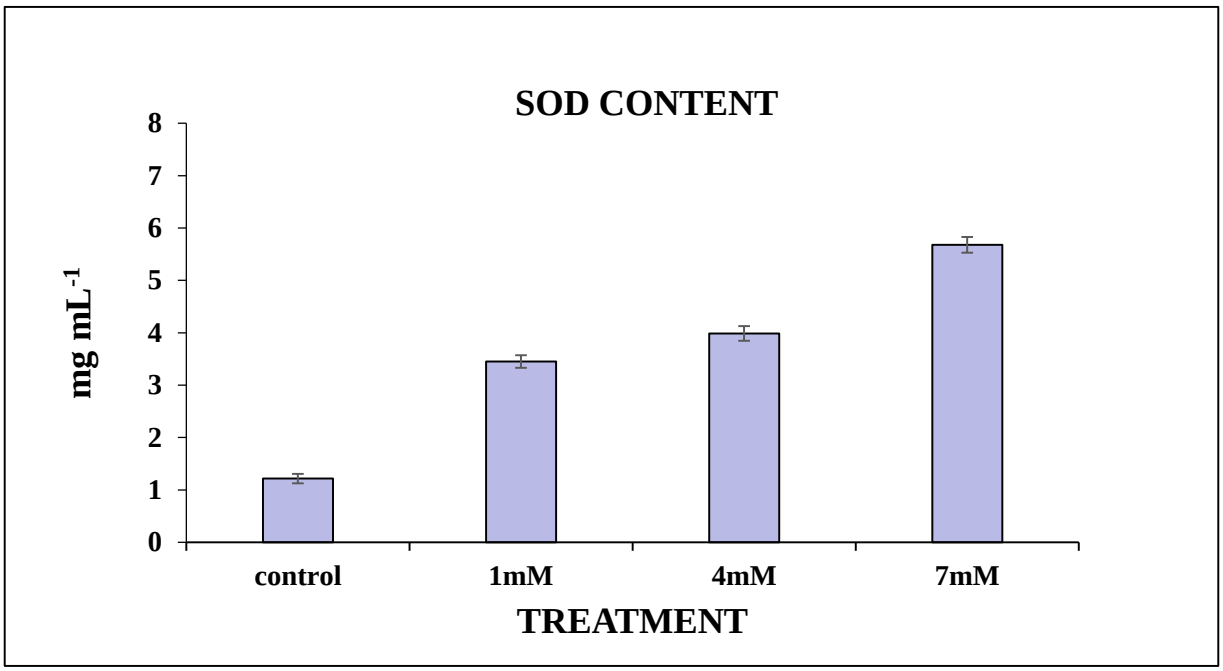

Fig.14. Effect of different concentrations of CuS04 on SOD content in *Vigna radiata* (L.) Wilczek plants. Each bar represents the mean ±S.D. n=3.

DISCUSSION

In this study, we found that mungbean plants are highly susceptible to copper toxicity. We observed that various aspects such as growth, metabolism, risking the survival of the plants **(Fig. 1,2 and Table 1).** Interestingly, our findings also revealed that the plants were making efforts to resist copper toxicity, as evidenced by several parameters studied. After analyzing all relevant parameters, it was found that increasing concentrations of CuSO4 led to retardation in the overall growth of mungbean plants. This growth inhibition is indicative of impairment in some physiological processes. Alia and Saradhi (1991) reported significant reduction of shoot length, root length, fresh weight and dry weight of roots and shoots in *Cajanus cajan, Vigna mungo* and *Triticum aestivum*, under heavy metal stress. They ascribed the reduction in growth to be a consequence of interference with normal development of plants, especially (i) synthesis of proteins (Stiborova et al. 1987), (ii) the activities of some important enzymes by binding free amino carboxylate or side groups replacing some important ions (Nath, 1986; Van Assche and Clijsters, 1990) and (iii) various photosynthetic processes like chlorophyll biosynthesis (Stobert et al. 1985), activities of photosystems and photophosphorylation or electron transport (Mohanty and Mohanty, 1988; Murthy et al. 1990). Sarada and Polasa (1992) also reported a reduction in growth of lentil plants with copper excess.

In this study, we observed that the growth of mungbean plants sprayed with copper was stunted. This was indicated by a gradual decrease in the height of the plants **Fig. 1.** We believe that this could be due to a modification of the plant's structure in response to the copper spray, resulting in a reduction of surface area of the plant parts. Additionally, we found that both the fresh

and dry weight of the treated plants were reduced. Interestingly, *Silene cucubalus*, a copper-tolerant plant population, has been shown to maintain its water content while non-tolerant populations experience a rapid decrease in water content. This tolerance is achieved through water conservation in response to low pH (Lolkema and Voojis, 1986). The dry weight of *Hyptis* sp. and *Helianthus annus* decreased when they absorbed 1200 ppm of Cu2+/g dry weight (Pillay, 1994). The area of leaves was reduced with increasing concentrations of copper sprayed, thus decreasing the total light receiving areas and subsequently hampering photosynthesis. Runner bean plants (*Phaseolus coccineus*) treated with excess Cu at different stages of growth showed a strong reduction in the area and fresh weight of leaves (Maksymiec et al. 1994).

In our study, there was a reduction in the chlorophyll content (chl a, chl b) in mungbean leaves with toxic concentrations of copper **(Fig. 5 and Table No. 3).** Heavy metals have been reported to induce changes in the chlorophyll content of various plants (Foy et al. 1978) and these changes were accounted for by decreased Fe2+ contents of leaves (Haghiri, 1973). Inhibition of chlorophyll synthesis by Cd2+ is achieved both by reaction with constituent biosynthetic enzymes as well as peroxide-mediated degradation (Somashekaraiah et al. 1992). A decrease in chlorophyll had been reported in sunflower leaves treated with Fe (II), Cu (II), and Cd (II) (Gallego et at. 1996). In oat leaves, it was found that exposure to high concentrations of Cu2+ ions caused the breakdown of chlorophyll and carotenoids (Luna et al. 1994). Treatment of pea plants with 1000 pM Cu2+ for four days showed lower chl a, and carotenoid contents and a complete disintegration of the chloroplast lamellar system (Angelov et al. 1993).

Muthuchelian et al. (1988) observed the synthesis of chlorophyll 'a' in *Vigna sinensis* (L.) Savi seedlings was found to be inhibited more than that of chlorophyll 'b' at all concentrations of Cu^{2+} and Cd^{2+}), a report correlating to our findings where chl a decreased to the extent of 78% at the highest concentration while chl b was 64% reduced from the control. Metal-induced production of active oxygen species can cause oxidation of the chlorophyll molecule causing visible damage to leaves, if they are not properly scavenged (Asada, 1996). The inhibition of chlorophyll synthesis may also result from the Cu-induced inhibition of ALA-dehydratase as reported by Scarponi and Perucci (1984). Sandmann and Boger (1980) observed that the decrease in chlorophyll content was well correlated with ethylene production, suggesting that Cu-induced peroxidation could also affect chlorophyll.

In this study, the carotenoid content in the mungbean leaves was reduced with increasing concentrations of $CuSO_4$ sprayed **(Fig.6 and Table No.3).** Carotenoids play an important role as accessory light-harvester and energy-transforming pigment in photosynthesis. They also play an essential role in photoprotection. The photosynthetic membrane can easily be damaged by the large amounts of energy absorbed by the pigments. When the energy stored in chlorophylls in the excited state is rapidly dissipated by excitation transfer or photochemistry, the excited state is said to be quenched. If the excited state of chlorophyll is not rapidly quenched it can react with molecular oxygen to form an excited state of oxygen known as singlet oxygen (102*). This extremely reactive singlet species goes on to react with and damage many cellular components, especially lipids. Carotenoids exert their photoprotective action by rapidly quenching the excited state of chlorophyll. The excited state of the carotenoid does not have sufficient energy from singlet oxygen, so it decays back to its ground state while losing its energy as heat. Thus, the decline in

carotenoid content can lead to photooxidative damage (Goodwin and Mercer, 1983; Sharma and Hall, 1993), and their role as antioxidants is now well-recognized. Recently it has been found that xanthophylls are involved in non-photochemical quenching. Xanthophylls can be interconverted by epoxidase and de-epoxidase enzymes present in the chloroplast. Zeaxanthin is associated with the quenched state while violaxanthin is associated with the unquenched state. High light activates de-epoxidase that converts xanthophyll into the zeaxanthin form while low light conditions activate the epoxidase resulting in violaxanthin accumulation (Horton et al. 1996). Copper probably decreases the xanthophyll content by reducing the photon-utilizing capacity of the leaves and creating a condition of photo-inhibition (Asada, 1996).

In the breakdown pathway of chlorophyll, in senescent leaves, the first step is the removal of the phytol tail by the enzyme chlorophyllase, followed by the removal of magnesium by magnesium dechelatase (Holden, 1961; Matile et al. 1996). In our experiments with mungbean plants, exposure to toxic copper concentrations brought about a substantial increase in chlorophyllase activity. The activity of this enzyme is increased during leaf senescence bringing about chlorophyll degradation (Sabater and Rodriguez, 1978). Copper toxicity probably encouraged early senescence of mungbean plants by increasing the activity of this enzyme. From the above observations, it is now quite apparent that the decrease in chlorophyll content due to copper toxicity is due to both inhibition of its synthesis as well as promotion of its degradation.

The effect of copper stress on carbohydrate content in both the leaves and roots of the mungbean plants was reduced **(Fig. 7, 8 and Table 4).** Inhibition of photosynthesis at any point will lead to a reduction in carbohydrate content. The decrease and increase in carbohydrate content

is directly proportional to growth. Metal ions not only specifically affect carriers of electron transport but also inhibit the electron flow from H_20 to the PS II reaction center, thereby decreasing the energy level of the cells (Prasad et al. 1991). This inhibition would naturally lead to reduced ATP and NADPH generation and this might be the major cause of inhibition of $C0_2$ fixation and consequently low carbohydrate content (Rai et al. 1996; Plucinska and Ziegler, 1996). The decrease in accumulation of sugars in roots with elevated copper concentration can either be related to inhibition of sugar transport or diminished sugar formation leading to decreased phloem-loading. The sucrose transport in plants has been connected to $H7K^+$ pump (Malek and Baker, 1977). Heavy metals like Cd_2* and Cu_2^+ inhibit the metabolism-dependent K^+ uptake (Jensen and Adalsteinsson, 1989). In wild rice, total soluble carbohydrates were negatively correlated with Cu concentration (Pip, 1993).

This study resulted in increased soluble protein content in both leaves and roots of the mungbean plants with copper stress **(Fig. 9 and Table 5).** This increase may be due to the synthesis of new proteins (akin to heat-shock proteins) as a reaction to Cu stress. It has been shown that different environmental stresses affect the normal metabolism of growing plants and alter the level of extractable proteins in stressed plants (Chen and Kao, 1996; Taylor, 1996; Shah and Dubey, 1997). An increase in protein turnover in plants may be a non-specific response induced by stresses like metals (Brune et al. 1995), temperature (Howarth and Oucham, 1993), and air pollutants (Bahl et al. 1995). Since the protein content increased, enzyme activities in these experiments were calculated on a fresh weight basis rather than on a protein basis (Brune et al. 1995; Brunschon-Harti et al. 1995; Patra et al. 1997). The specific enzyme activities would appear to decrease if expressed on a protein basis (Patra et al. 1998). The biochemical responses to temperature,

oxidative or metal stresses, have in common the induction of some heat-shock proteins which may have a role in the adaptive response (Wollgiehm and Newman, 1995 and Patra et al. 1998). The total amino acid content of the mungbean plants also increased when exposed to increasing concentrations of copper. Higher amounts of free amino- acids have been observed in different parts of plants under stressful conditions (Dubey and Pessarakli, 1995; Shah and Dubey, 1995). It is suggested that different plant species differ in their capacity to accumulate specific amino acids under different stresses (Dubey and Pessarakli, 1995) along with other low molecular weight organic solutes like proline, glycinebetaine, sorbitol etc. (Ahmad et al. 1979 and Nan et al. 1991). Accumulated amino acids, along with other compatible solutes, in stressed plants may help in stabilisation of enzymes (Pollard and Wyn Jones, 1979) and membranes (Jolivet et al. 1982) and also in protection of the cellular constituents against free-radical induced damage (Smirnoff and Cumbes, 1989). Accumulation of amino acids, as observed in these experiments, with copper excess may be due to their increased synthesis, as observed by other workers under stressful conditions (Dubey and Pessarakli, 1995; Chen and Kao, 1995 and Taylor, 1996).

In this study, there was a substantial increase in proline content in the mungbean plants that were exposed to toxic concentrations of copper **(Fig. 12 and Table 6).** Proline plays an important role in osmoregulation, protecting enzyme denaturation, stabilization of the protein synthesis machinery, regulation of cytosolic acidity and acting as a storage compound for nitrogen (Nikolopoulos and Manetas, 1991 and Venekamp et al. 1989). The presence of proline can reduce the level of free radicals being generated by chloroplasts (Alia and Saradhi, 1991). Thus, proline plays an important role in non-enzymatic free-radical detoxification mechanisms possibly in a similar manner to other biological molecules such as ascorbate, glutathione and tocopherol (Alia

and Saradhi, 1991). Proline accumulates in plants exposed to various environmental stresses including heavy metal stress (Boggess et al. 1996; Alia et al. 1995). This type of increase in proline content could occur either due to fresh synthesis or inhibition in proline oxidation and reconversion of proline oxidation products to proline in stressed condition (Stewart and Boggess, 1978), or breakdown in cellular compartmentation of proline synthesis and proline oxidation (Singh et al. 1973). The accumulation of proline may be related to a decrease in the activity of the electron transport system, as reported in plants exposed to stress (Sawhney et al. 1990). Due to such decrease there would be an accumulation of NADH and H^+. Increase in NADH to NAD^+ ratio might affect substrate level phosphorylation besides inhibiting important metabolic reactions that need NAD+. Accumulation of organic acids such as citrate, malate and lactate might occur resulting in a decrease in cytosolic pH. The proline synthesis from glutamic acid might be an adaptive mechanism to reduce the accumulation of NADH and/or reduce acidity (Venekamp et al. 1989). The concentration of free proline significantly increased in the leaves of sunflower plants exposed to excess Cu (Kastori et al. 1992).

The excess copper caused enhanced lipid peroxidation in mungbean plants as indicated by the extremely high contents of malondialdehyde and total peroxide, in both leaves and roots **(Fig. 13 and Table 6).** Lipid peroxidation is the reaction of oxidative deterioration of PUFA and is identified as a basic cell-membrane damage in cellular mechanism (Andrews et al. 1965). Lipid peroxidation destroys membrane integrity, membrane fluidity and elasticity (Pauls and Thompson, 1984). MDA (malondialdehyde) is produced on oxidation of PUFA (polyunsaturated fatty acids) during the synthesis of thromboxanes. This MDA is bound to endogenous proteins and converted to CO2 by a mitochondrial aldehyde dehydrogenase. Further lipid peroxidation leads to damage

of DNA if MDA is not further degraded (Shigenaga and Ames, 1991). The increase in lipid peroxidation may arise either by (a) altering the antioxidant potentials of the tissues or (b) over production of free radicals due to exposure to pollutants or (c) both. Lipid peroxidation by Cu++ probably occurred by its blocking the electron flow in PS II which led to the formation of excited chlorophyll and in turn caused the production of free radicals (Kato and Simizu, 1985). Metals can influence lipid peroxidation through two ways (Smirnoff, 1993). Firstly, it enhances hydroxyl radical production in the Haber-Weiss reaction. Secondly, it reacts with lipid hydroperoxides to form alkoxy and peroxy radicals (Bowler et al. 1992). Both redox (Cu and Fe) and non-redox active metal ions (Zu and Cd) are reported to increase lipid peroxidation via ROS (reactive oxygen species) in plants (Shaw, 1995; Gallgo et al. 1996 and Chaoui et al. 1997). Cu is known to damage cell membranes by binding to the sulphydryl groups of membrane proteins and by inducing lipid peroxidation in both leaves and roots (DeVos et al. 1992; Chen et al. 2000). Malondialdehyde (MDA) is the most widely measured indicator of free radical production and oxidative damage from stress (Smirnoff, 1993). Free radicals and other active derivatives of oxygen are inevitable by-products of biological redox reactions. Reduced oxygen species such as hydrogen peroxide (H202), the superoxide radical anion (02*), and hydroxyl radicals (OH») inactivate enzymes and damage important cellular components. In addition, singlet oxygen produced via the formation of triplet-state chlorophyll is highly destructive. This oxygen species initiates lipid peroxidation and produces lipid peroxy radicals and lipid hydroperoxides that are also very reactive. The increased production of toxic oxygen derivatives is considered to be a universal feature of stress conditions (Foyer et al. 1994).

The activity of the enzyme superoxide dismutase (SOD) increased substantially with copper stress shown in **(Fig. 14 and Table No. 7)** SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H202. Scavenging of superoxide is essential, since the producing site of superoxide production is the same as that of the generation site of the reducing equivalents required for the operation of the C02 - fixation cycle (Asada, 1996). CuZn - SOD is the major SOD in gymnosperms and angiosperms. Increase in the activity of SOD as a response to excess Cu, is well documented (Luna, 1994; Gallego, 1996; RamaDevi and Prasad, 1998 and Chen et al., 2000). According to Chongpraditnum et al. (1992), copper induced an increase in SOD activity in soyabean roots via the synthesis of cytosolic Cu, Zn-SOD. The induction of SOD by such treatment may be the result of a direct effect of copper on the gene for SOD or of an indirect effect via an increase in levels of O2.

CONCLUSION

The effect of copper stress on various morphological, physiological, and biochemical parameters in *Vigna radiata* L. Wilczek was studied. In conclusion, the impact of copper stress on plants is multifaceted, affecting morphological, physiological, and biochemical parameters in various ways. Morphologically, plants under copper stress often exhibit stunted growth, reduced leaf size, and altered root architecture. Physiologically, copper stress disrupts essential processes such as photosynthesis, water and nutrient uptake, and overall metabolism, leading to decreased plant vigor and productivity. At the biochemical level, plants respond to copper stress by activating antioxidant defense mechanisms to mitigate oxidative damage, but prolonged exposure can overwhelm these defenses, leading to cellular damage and impaired function.

The excess copper has a detrimental effect on photosynthesis, Chlorophyll content (‘a’, ‘b‘ and total), carotenoid content (both carotene and xanthophyll), carbohydrate content (reducing and total sugars), while there was an increase in soluble protein content which is a heavy-metal stress reaction, increased total amino acids content, increased proline content and increased lipid peroxidation. Hence, as indicated by these results, copper phytotoxicity plays an inhibitory role in the growth, metabolism, and productive potential of mungbean plants, although an effort towards tolerance was displayed by the plants in certain cases. Overall, understanding the intricate interplay of morphological, physiological, and biochemical responses to copper stress is crucial for developing strategies to mitigate its negative effects on plant growth and productivity, thereby ensuring sustainable agriculture and environmental stewardship.

Hence, as indicated by these results, copper phytotoxicity plays an inhibitory role in the growth, metabolism and productive potential of mungbean plants, although an effort towards tolerance was displayed by the plants in certain cases.

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