

CHAPTER IV

Morphological and Biochemical Effects of two azo-functionalized Schiff base Cu(II) complexes on Rice plants*

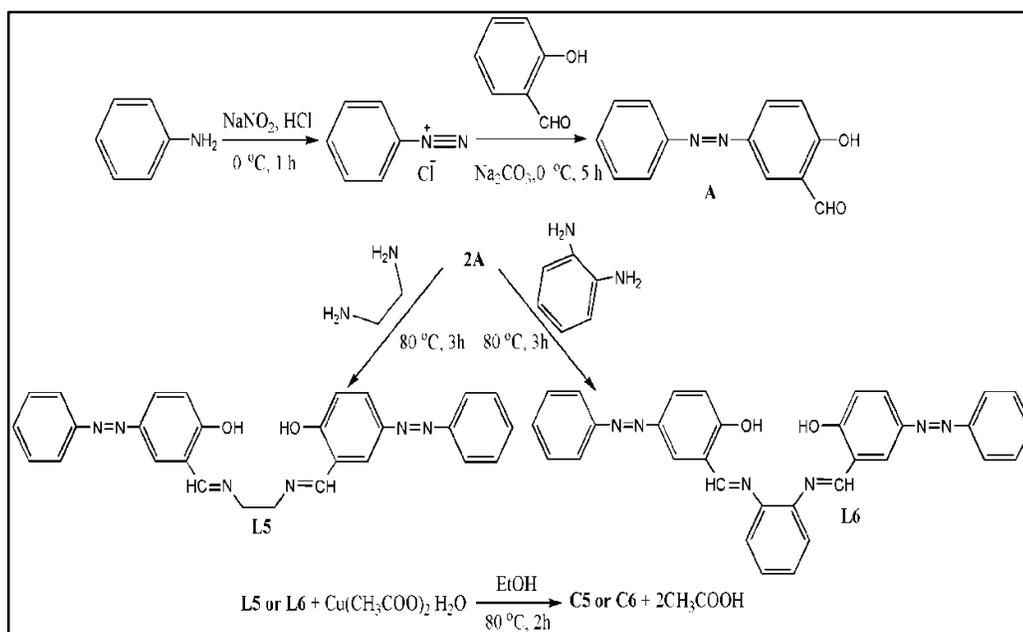
4.1. Introduction

Copper is one of the important redox active transition metal for plants.¹ The optimum content of Cu²⁺ ion in plant tissue is around 10 µg per gram dry weight of the plant.² It serves as a component of different enzymes (plastocyanin, cytochrome oxidase, *etc.*) which are mainly associated in electron transfer chain. It acts as cofactors in several enzymes such as Cu/Zn superoxide dismutase, lactase, cytochrome C-oxidase.³ It is required in the pathway of photosynthesis, respiration and is also associated with carbohydrate and protein metabolism.⁴ In plants, copper is necessary for cytosol, endoplasmic reticulum, chloroplast stroma, thylakoid lumen, *etc.* To make copper available in plants generally copper containing fertilizers of inorganic origin (chiefly CuSO₄) are used. These types of compounds being ionic in nature may alter the pH of the medium.^{5,6} Therefore more emphasis is being given recently to the metal chelates that are less reactive but such compounds can solve the deficiency problem for longer period of time.^{7,8} Inspired by these facts herein this chapter two azo-functionalized Schiff base ligands and their Cu(II) complexes were synthesized and applied as copper supplement to rice plants. The important feature of these types of ligands and their complexes is that they contain azomethine group (RHC=N-R', where R and R' stand for various alkyl or aryl groups) in which the N-atom is *sp*² hybridized and the presence of a lone pair of electrons makes it a good donor especially when one or more donor atoms are remain adjacent to it. Complexes of this type have draw much attention because of their diverse applications like their catalytic power in O-atom transfer reactions and organic redox reactions as well as their use as metallodrug.⁹⁻¹² Actually in this chapter the responses of rice seedlings to metal toxicity while being exposed to Copper Sulphate (CuSO₄) and two synthesized Schiff base complexes (containing Cu²⁺ion) are thoroughly explored. The experimental results are compared in terms of pigments, biochemical components, osmolyte accumulation, oxidative stress markers and overall tolerance level of the rice plants (*Oryza sativa* L.).

4.2. Materials and methods

4.2.1. Synthesis of the ligands and their Cu(II) complexes

The syntheses of azo-functionalized Schiff base ligands (**L5** and **L6**) and their Cu(II) complexes (**C5** and **C6**) have been detailed in the chapter II. These compounds were prepared by following methods as described in the literature¹³ with required alteration (illustrated in Scheme 4.1).



Scheme 4.1. Preparation of the ligands (**L5** and **L6**) and their Cu(II) complexes (**C5** and **C6**).

4.2.2. Treatment with the ligands and their Cu(II) complexes

To investigate the effects of prepared ligands (**L5** and **L6**), complexes (**C5** and **C6**), and CuSO_4 , rice seeds were selected as plant material. Grains of rice (*Oryza sativa* L.) were surface sterilized with sodium hypochlorite (0.1%) for 1 minute and then they were washed thrice with de-ionized distilled water and soaked for 12 hours with various concentrations (10, 50, 100, 200 ppm) of CuSO_4 , **L5**, **L6**, **C5** and **C6** along with a control (*i.e.*, no treatment with micronutrients). After soaking was done, the seeds were allowed to germinate on sterile petri plates. From each treatment twenty germinated seeds were selected and transferred to plastic pots containing garden soil, well rotten cow dung and sand in 1:1:1 ratio under controlled conditions with 8 hour light period at 28-35 °C day/night temperature and 65-75% relative humidity. 45 days old seedlings were then subjected to morphological and

biochemical analyses. Different growth and biochemical parameters as detailed in chapter II were subsequently monitored.

4.3. Results and Discussion

4.3.1. Characterization of the prepared ligands and their Cu(II) complexes

The analytical data and corresponding Infrared spectra of the prepared ligands and their Cu(II) complexes are almost identical with those available in the literature¹³ and some characteristic physico-chemical and spectral data are furnished in Tables 4.1-4.3. The two ligands have characteristic bands around 1280 cm⁻¹ due to H-bonded O-H in-plane bending vibrations, around 1610-1640 cm⁻¹ due to C=N bond stretching vibrations. Also a broad but weak band around 2800-2700 cm⁻¹ due to strong intra-molecular interactions. For the prepared Cu(II) complexes characteristic bands due to C=N, phenolic C-O, Cu-N and Cu-O stretching vibrations were observed (listed in Table 4.2). The ligand L5 shows mainly two peaks in its electronic spectrum at 270.5 and 362.5 nm, respectively for the π - π^* and n - π^* transitions. In C5 the peak for the n - π^* transition shifted from 362.5 to 396 nm due to coordination bond formation. Similarly, The ligand L6 shows three peaks at 254.0, 318.0 and 368.5 nm in its electronic spectrum, respectively for benzene π - π^* , imino π - π^* and n - π^* transitions. For C6 the peak due to the n - π^* transition shifted from 368.5 to 398 nm indicating donation of nitrogen lone pair of electrons to the Cu²⁺ ion.

Table 4.1. Analytical data and some physical properties of the Schiff bases and their complexes

	Molecular Formula	Colour	Yield (%)	Found (Calcd) %			
				C	H	N	M
L5	C ₂₈ H ₂₄ N ₆ O ₂	Orange	84	70.60 (70.57)	5.20 (5.08)	17.58 (17.64)	
C5	C ₂₈ H ₂₂ N ₆ O ₂ Cu	Green	73	62.55 (62.50)	4.02 (4.12)	15.53 (15.62)	11.76 (11.81)
L6	C ₃₂ H ₂₄ N ₆ O ₂	Orange	80	73.02 (73.26)	4.53 (4.61)	15.98 (16.03)	
C6	C ₃₂ H ₂₂ N ₆ O ₂ Cu	Red	75	65.64 (65.57)	3.69 (3.78)	14.42 (14.34)	10.78 (10.84)

Conductivity (ohm⁻¹·cm²·mol⁻¹): C5, 5.6 and C6, 5.1

Table 4.2. Some characteristic infrared (in cm^{-1}) and electronic spectral (in nm) data of the Schiff bases (L5 and L6) and their Cu(II) complexes (C5 and C6).

Compound	$\nu_{\text{C=N}}$	$\nu_{\text{phenolic C-O}}$	$\nu_{\text{M-N}}$	$\nu_{\text{M-O}}$	λ_{max}
L5	1640	1588	1290		362, 270.5
C5	1627	1578	1257	514 449	396, 266
L6	1610	1578	1285		368.5, 318, 254
C6	1630	1592	1258	530 430	398, 318, 251

4.3.2. Application of the ligands and their Cu(II) complexes to the plant material

Copper (Cu) being an indispensable trace element for the plant development, plays an important role in the photosynthetic reactions and is required for cell signaling, transcription, protein trafficking, oxidative phosphorylation. But at the same time over indulgence of Cu negatively impacts plants growth, inhibits photo system, electron transport and generates oxidative stress within plant cells and diminishes the content of photosynthetic pigments. In the present chapter, the response of rice seedlings in presence of different concentrations of Copper Sulphate (CuSO_4), the Schiff base ligands (**L5** and **L6**) and their Cu(II) complexes (**C5** and **C6**) are evaluated. The results were compared on the basis of various biochemical and growth parameters to see the efficacy of the copper complexes (**C5** and **C6**) over CuSO_4 .

4.3.2.1. Growth and tolerance to the copper complexes (C5 and C6)

Cu^{2+} can hamper growth and development of seedlings by causing damage to epidermal cells and cell membranes.¹⁴ The outcomes reveal that plants treated with C5 and C6 complexes were able to retain a higher percentage of fresh weight, dry weight and relative water content (RWC) than CuSO_4 treated plants with increasing concentration suggesting less toxicity of Schiff base complexes (illustrated in Figs 4.1-4.3).

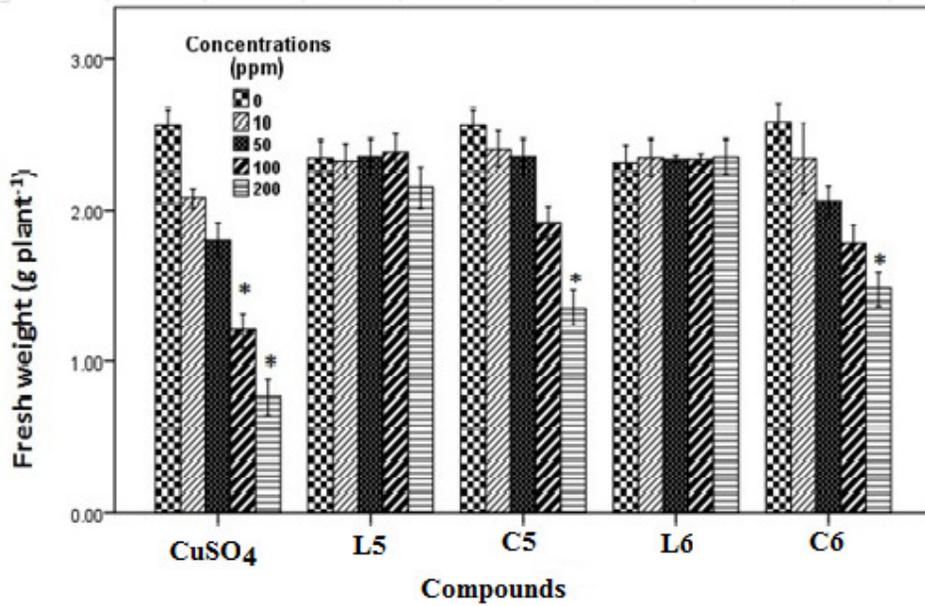


Fig 4.1. Fresh weight (g plant⁻¹) of 45 days old rice seedlings. Mean \pm SE, n = 3.
*The mean difference is significant at the 0.05 level.

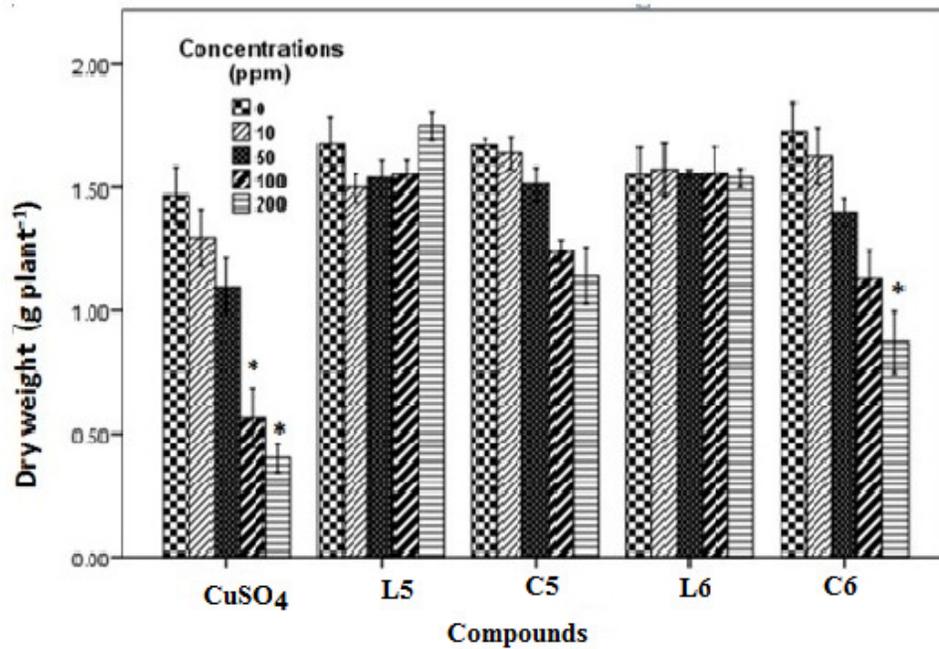


Fig 4.2. Dry weight (g plant⁻¹) of 45 days old rice seedlings. Mean \pm SE, n = 3.
*The mean difference is significant at the 0.05 level.

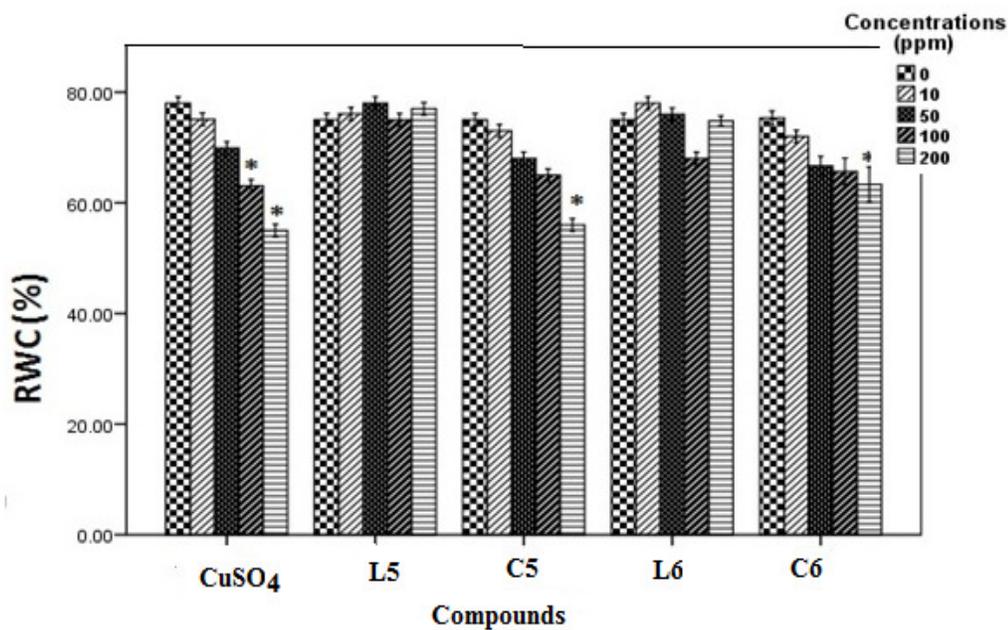


Fig 4.3. Relative water content (RWC) of 45 days old rice seedlings. Mean \pm SE, n = 3. *The mean difference is significant at the 0.05 level.

For instance at 100 and 200 ppm CuSO₄ significantly reduces RWC by 22.99% where as C5 and C6 complex reduce only by 12.01% and by 12.34%, respectively over the control. Greater negative impact of CuSO₄ than the Schiff base complexes (C5 and C6) on seedlings was further approved by the Tolerance index (TI). TI for C5 and C6 complex treated plants appeared to be significantly higher than that of CuSO₄ treated plants (illustrated in Fig 4.4). Similar type of negative effect of CuSO₄ was also reported by *Azooz et al.*¹⁵ in wheat seedlings. *Verma et al.*¹⁶ also reported that the application of copper at lesser concentrations enhanced the plant's dry biomass, while excess use of copper reduced the biomass production of these plants.

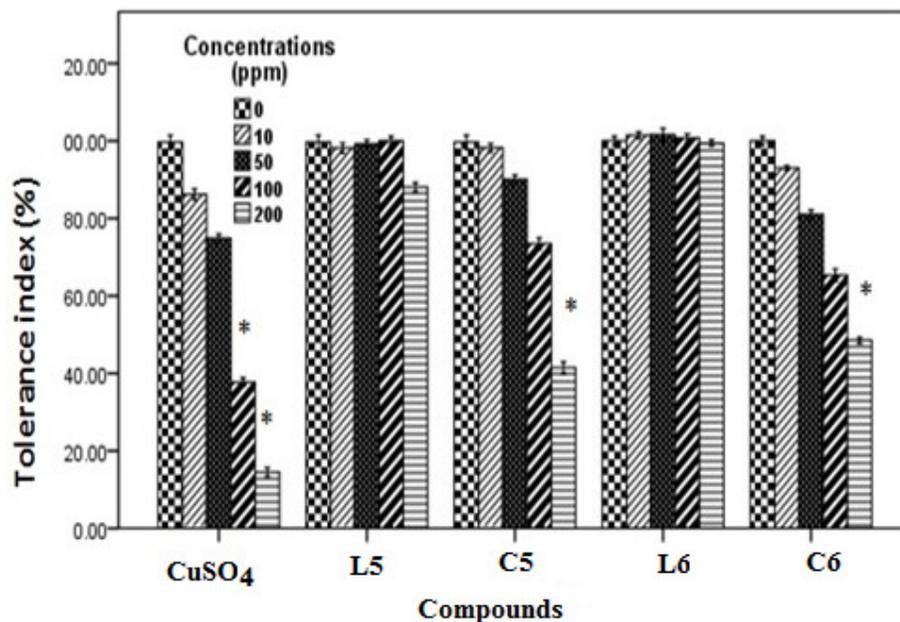


Fig 4.4. Tolerance index (TI) of 45days old rice seedlings. Mean \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

4.3.2.2. Membrane damage and ion leakage

Copper can distress the membrane permeability by oxidation of membrane lipids and this effect can be accessed from the increase of malonyldialdehyde accumulation (MDA), one of the lipid peroxidation products. Data obtained from this study revealed copper induced membrane damage which can be expressed in terms of electrolyte leakage. Lipid peroxidation enhanced significantly in CuSO₄ treated plants with increasing concentration but in case of the Schiff base complex (C5 and C6) treated plants malonyldialdehyde accumulation as well as electrolyte leakage were less as compared to CuSO₄ treated plants with respect to the control indicating comparatively less membrane damage in the Schiff base complex (C5 and C6) treated plants.

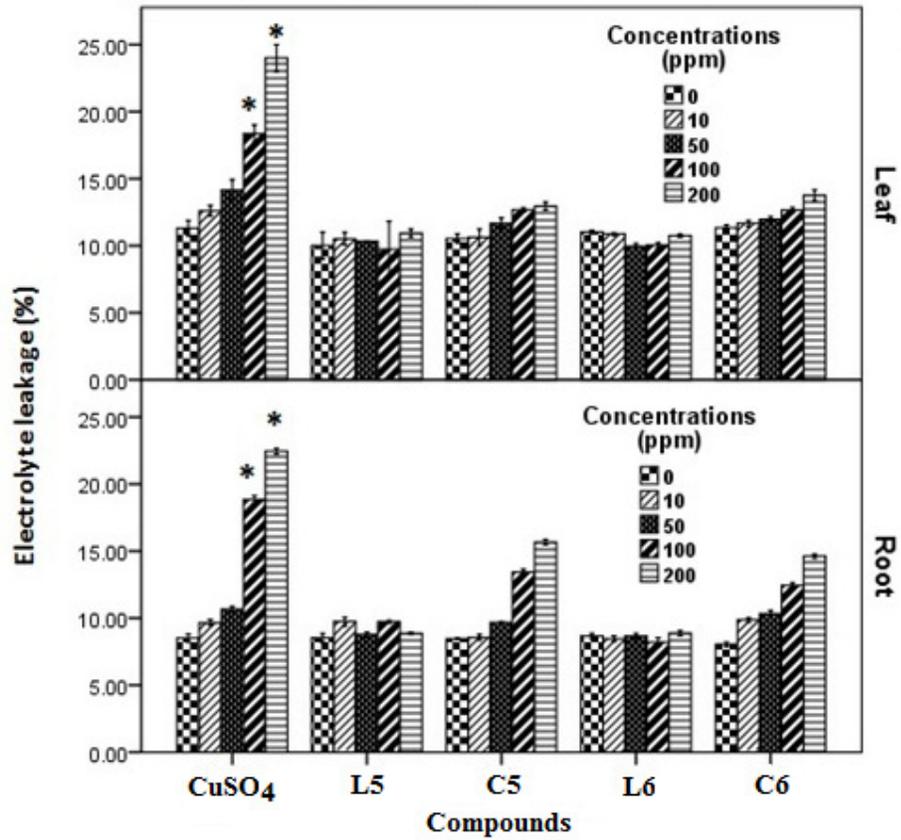


Fig 4.5. Electrolyte leakage (%) of 45 days old rice seedlings. Mean \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

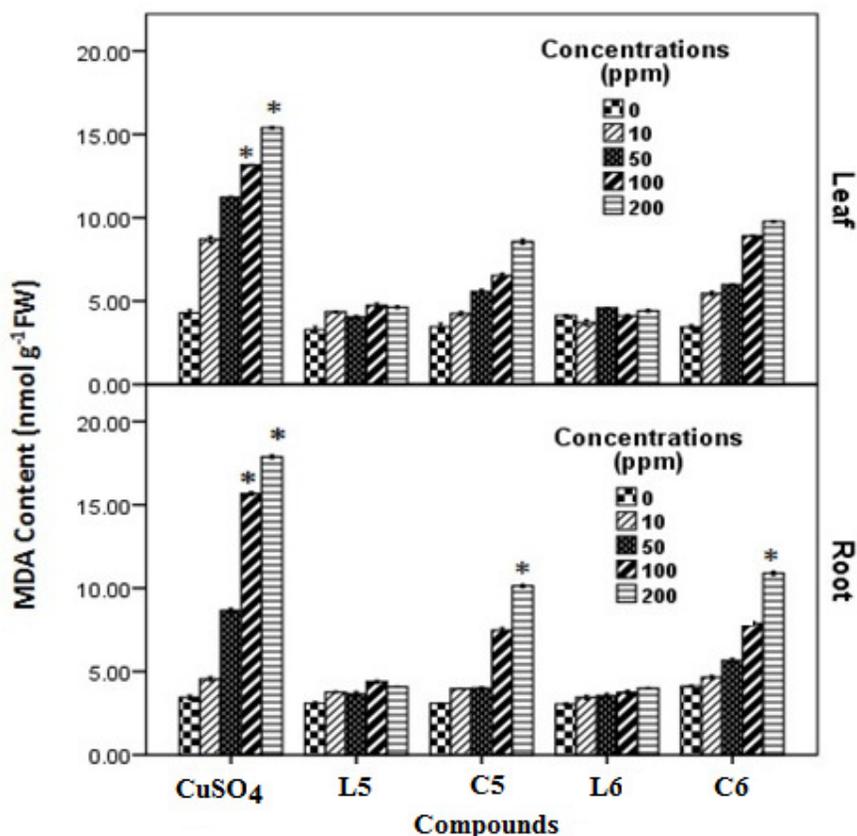


Fig 4.6. MDA content of 45 days old rice seedlings. Mean \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

The outcomes showed that the MDA content in leaf (illustrated in Fig 4.6) and electrolyte leakage in leaf and root (illustrated in Fig 4.5) of the Schiff base complex (C5 and C6) treated plants increase with increasing dose but did not echo any significant changes. But in case of CuSO₄ treated plants ion leakage and oxidation of membrane lipid enhanced drastically at 100 and 200 ppm level with respect to the control plants. Reactive oxygen species (ROS) induced plasma membrane damage increase MDA and ion leakage at the higher levels of Cu(II) ion.¹⁷ Hydrogen peroxide [one form of reactive oxygen species (ROS)] accumulation is minimum in case of the Schiff base Cu(II) complex (*i.e.*, C5 and C6) treated plants than in CuSO₄ treated plants (illustrated in Fig 4.7).

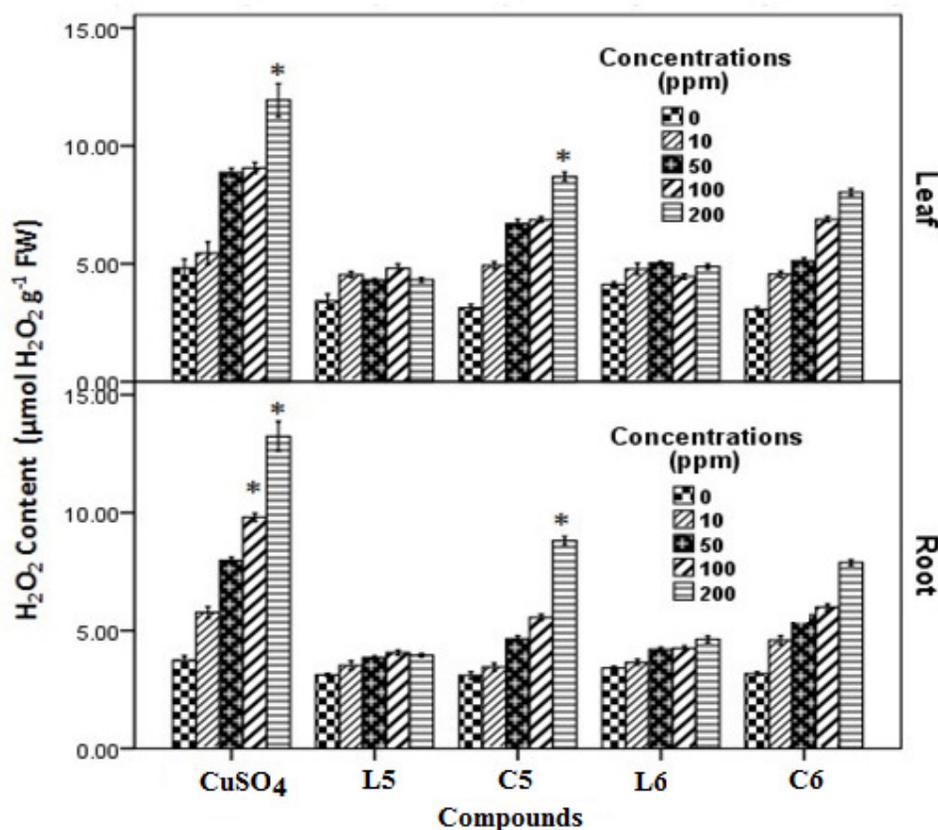


Fig 4.7. H₂O₂ accumulation in 45 days old rice seedlings. Mean \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

In copper sulfate treated plants H₂O₂ increased 1.35 fold at 200 ppm whereas in Schiff base complex treated plants especially in case of C6 complex H₂O₂ accumulation was less (0.62 fold) in relation to control. This observations appear to be due to the redox-active character of Cu²⁺ ion resulting in creation of extremely reactive hydroxyl radicals.¹⁸ These results suggest that the Schiff base Cu(II) treated plants had better ability to tolerate Cu²⁺ ion stress. The significant increase of MDA in plants, exposed to CuSO₄ indicated that the increase of lipid peroxidation in Cu-treated plants led to disorder of plasma membranes, whereas plasma membrane damage was less in case of the Schiff base Cu (II) complex treated plants.

4.3.2.3. Total free amino acids and proline

Accumulations of total free amino acids (illustrated in Fig 4.8) were significantly increased in root and leaf tissues at higher CuSO₄ concentrations. However, low CuSO₄ concentrations up to 50 ppm had non-significant enhancement

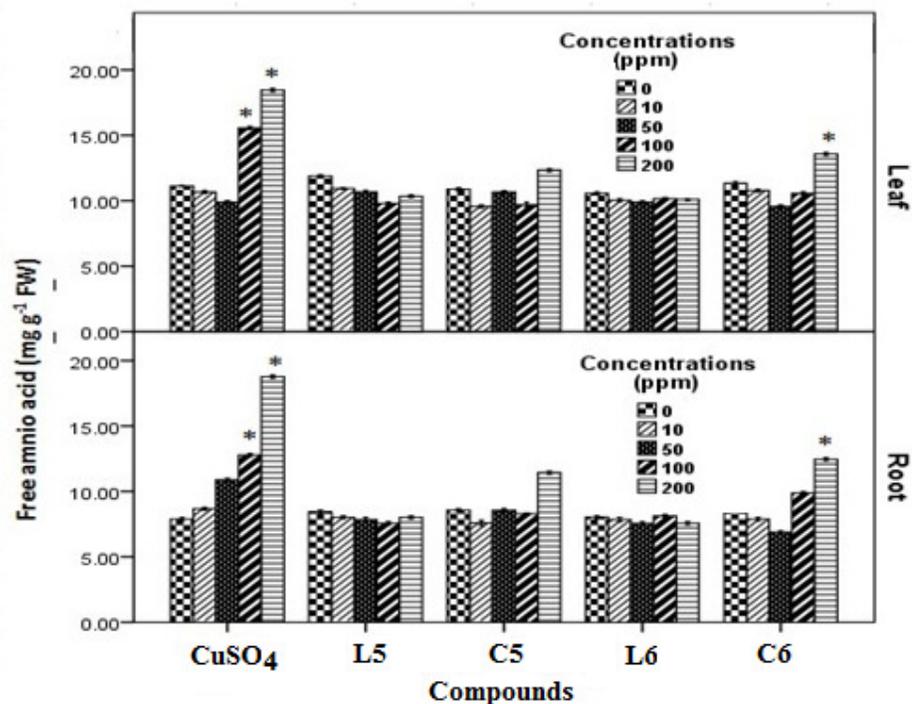


Fig 4.8. Free amino acid accumulation in 45 days old rice seedlings. Mean \pm SE, n = 3. *The mean difference is significant at the 0.05 level.

on the total free amino acids. The highest increase in free amino acids in case of CuSO_4 treatment was noticed at 100 and 200 ppm in both leaf and root tissues. On the contrary both the Schiff base Cu(II) complex treated plants showed insignificant enhancement of the amino acids. *Al-Hakimi and Hamada*¹⁹, in their study, suggested that free amino acids content enhances in plant tissues upon Cu^{2+} ion exposure. In agreement the amino acids are looked upon as key player in metal chelation through which plant detoxify/alleviate heavy metal stresses.²⁰ Therefore, it might be suggested that the plants experiencing higher amount of copper induced oxidative stress can accumulate greater amount of free amino acids to alleviate oxidative stress. In that scenario the copper Schiff base complexes (C5 and C6) are proved to be less toxic than CuSO_4 .

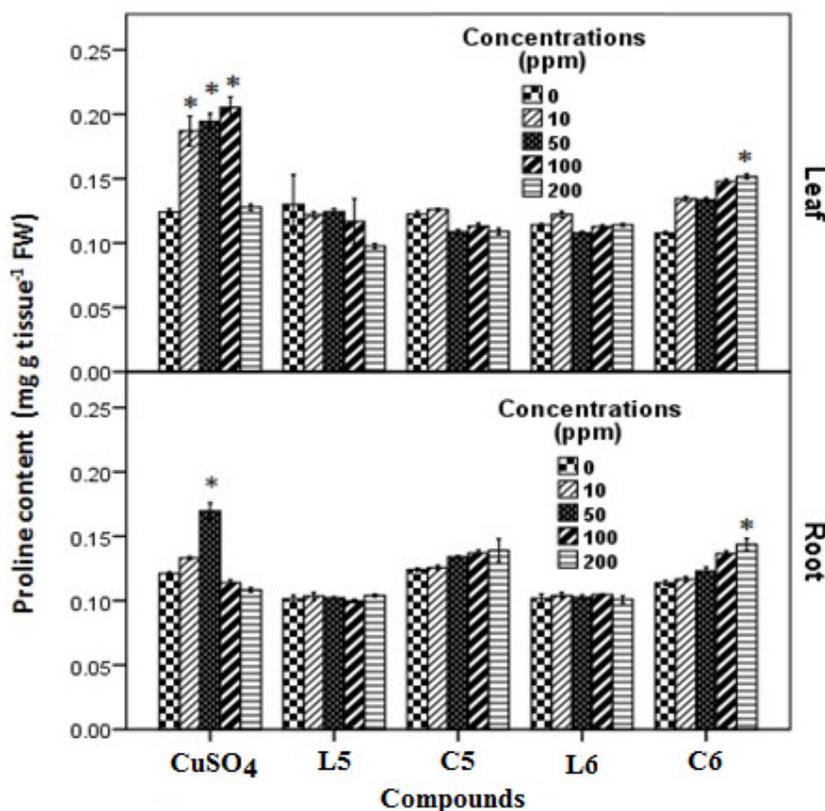


Fig 4.9. Proline level in 45 days old rice seedlings. Means \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

Proline accumulation in plant tissues, an indicator of oxidative stress,²¹ increased in all the treatments. In CuSO₄ treated plants proline accumulation was maximum at 100 ppm and at 50 ppm in leaf and root tissues, respectively. Beyond this concentration proline accumulation decreased. Decline in proline accumulation may be attributed to reduction competence of the plants to withstand oxidative stress.²² Conversely, in the Schiff base Cu(II) complex treated plants, proline content continued to be increased up to 200 ppm signifying lesser oxidative stress in those plants (illustrated in Fig 4.9). Proline accumulation indeed stabilizes plasma membrane free radical scavenger and some macro molecules and thus facilitates rapid recovery from heavy metal stress.²³

4.3.2.4. Photosynthetic pigments

Copper facilitates in the utilization of iron during chlorophyll synthesis and enhances photosynthesis at low concentration. But at higher concentration copper reduces photosynthetic competence, low quantum efficiency of Photosystem-II and reduced cell elongation.²⁴ The present study revealed that total chlorophyll content

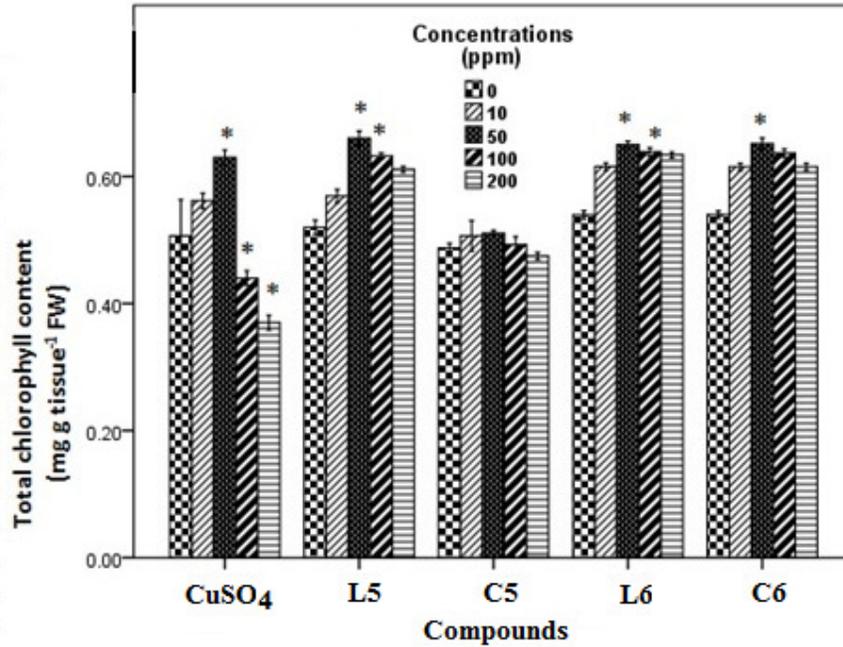


Fig 4.10: Total chlorophyll content in 45 days old rice seedlings. Mean \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

increased in case of all the treatments up to 50 ppm. Beyond this concentration total chlorophyll content reduced, whereas the reduction was maximum in case of CuSO₄ treated plants and minimum in case of C6 treated plants (illustrated in Fig 4.10). Less reduction of chlorophyll may be attributed to less copper toxicity of the Schiff base complexes (C5 and C6) to plants. Carotenoid, a non enzymatic antioxidant, involved in quenching of oxidizing species participates in disrupting regular cellular functioning. In CuSO₄ treated plants carotenoid content increased gradually up to 100 ppm. At 200 ppm carotenoid content reduced drastically signifying the huge toxicity of CuSO₄ but on the contrary for C5 and C6 treated plants the carotenoid content continued to increase up to 200 ppm (illustrated in Fig 4.11).

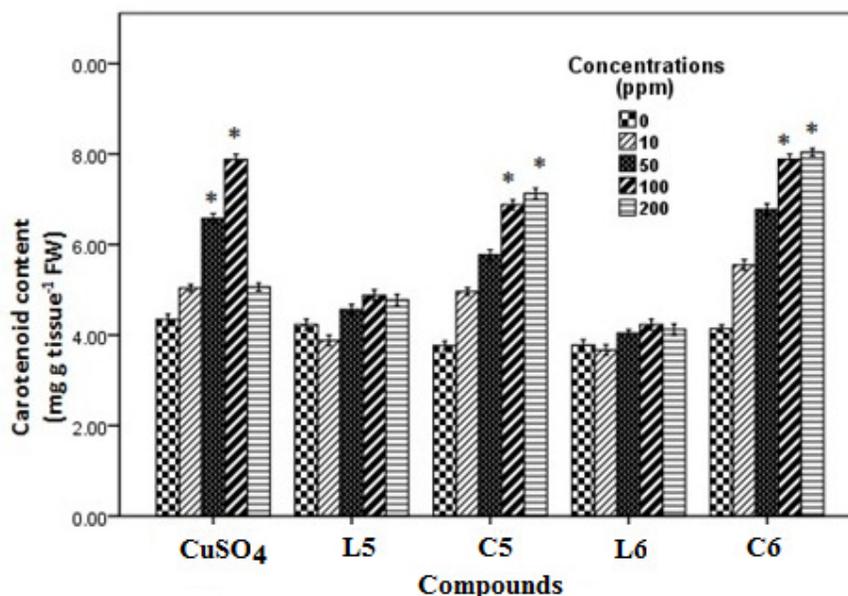


Fig 4.11. Carotenoid content in 45 days old rice seedlings. Means \pm SE, n = 3.

*The mean difference is significant at the 0.05 level.

4.4. Conclusions

The present study reveals that Schiff base Cu(II) complexes (C5 and C6) have less toxic effects than copper sulphate on rice seedlings and provide better tolerance to copper toxicity than copper sulphate. Maximum positive impact of the Schiff base complexes was found mostly at 50 ppm concentration. Though different stress marker and reactive oxygen species accumulation were less and minimum pigment damage was noticed in the Schiff base Cu(II) complex treated seedlings but the optimum positive impact of these Schiff base Cu(II) complexes largely depends on the dose and beyond certain concentration these complexes may have inhibitory effects on rice plants. The Schiff base Cu(II) complexes (C5 and C6) can thus be used as a potential supplement to meet up micronutrient deficiency and at the same time such complexes can minimize the toxicity generated by application of different ionic form of Copper (Cu^{2+}) ion.

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