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Responses of *Oryza sativa* L. towards Azo Functionalised Schiff base Cu(II) Complexes and $CuSO_4$: A Comparative Biochemical Study

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ABSTRACT

Azo fuctionalised Schiff base ligands having N_2O_2 donor binding sites are capable of forming metal complexes and can be used as potential plant micronutrient supplier. Two different ligands and their copper complexes were synthesised by conventional protocols and then characterised by both spectroscopic and elemental analysis. Investigations were done by taking rice seeds as plant material. Various growth and biochemical parameters were monitored by taking different concentrations of $CuSO_4$, prepared ligands and their Cu(II) complexes. Analysis of various biochemical results reveal that Schiff base Cu(II) complexes have less toxic effects than copper sulphate on rice seedlings and thus facilitates better tolerance to copper toxicity than copper sulphate.

Keywords: Plant growth, Cu(II) chelates, copper tolerance, electrolyte leakage, rice seeds, azomethine group.

INTRODUCTION

Copper having the most stable oxidation state of +2, is one of the important redox active transition metal for plants[1]. The optimum content of Cu^{2+} in plant tissue is around 10µg g^{-1} dry weight as reported by Baker and Senef in 1995.[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, laccase, cytochrome C oxidase[3]. It is required in the pathway of photosynthesis, respiration and associated in carbohydrate and protein metabolism[4]. In plants, Cu is necessary for cytosol, endoplasmic reticulum, chloroplast stroma, thylakoid lumen etc. To make Cu available in plants generally Cu contained fertilisers of inorganic origin (mainly $CuSO_4$) are used. These types of compounds being ionic in nature are responsible for the alteration of the pH of the medium[5,6]. Owing to that more emphasis is given to metal chelates which are less reactive but can solve the deficiency problem for longer period of time[7,8]. Inspired by the facts we have synthesised two azo functionalised Schiff base ligands and their Cu^{2+} complexes. The important feature of these ligands and complexes is that they contain azomethine group (RHC=N-R/, The R and R/ are various alkyl or aryl groups) in which the nitrogen atom is sp^2 hybridised and the presence of a lone pair make it a good donor especially when one or more donor atoms are present adjacent to it. Complexes of these types draw the attention because of their catalytic power in oxygen atom transfer reaction, mediating organic redox reactions[9-12]. In our study, we have evaluated the responses of rice seedlings to metal toxicity while being exposed to Copper Sulphate ($CuSO_4$) and two Copper Schiff base complexes and the results are compared in terms of pigments, biochemical components, osmolyte accumulation, oxidative stress markers and overall tolerance level of plants.

MATERIALS AND METHODS

Synthesis of ligands and complexes

0.1 mol of aniline (A.R grade, procured from S. D. Fine Chemicals, India) was dissolved first in concentrated HCl without any further purification. 8g of analytical grade $NaNO_2$ (in water) was then added to aniline solution drop wise with constant stirring for 1 h keeping the reaction temperature throughout 0^0C. A solution of 0.1 mol salicyladehyde (A.R grade, procured from S. D. Fine Chemicals, India of purity level > 99%), sodium carbonate (36 g) and water was added drop wise in the above mixture with stirring. The reagents were allowed to react for 5 h at 0^0C. After the completion of reaction the light red precipitate of 4-(Benzeneazo) salicylaldehyde is fitered off and recrystalised in ethanol. The melting point was 118^0C with yield of 80%. Two different diamines (ethylenediamine and o-phenylene diamine) were treated with the azo compound synthesized earlier in 1:2 ratios in ethanol medium for 3 h at ambient temperature resulting two ligands. Ligands were washed and recrystalised in ethanol medium having yield 70%. The prepared two ligands were alternatively refluxed with $Cu(AcO)_2.H_2O$ (A.R grade, purity level > 99%) in ethanol medium in 1:1 ratio for 2 h at ambient temperature resulting two Cu complexes. They were filtered, washed and recrystalised and the yield was 70% almost. The two ligands L1 and L2 { [N,N/-bis[4-(benzeneazo) salicylaldehyde]ethylenediamine and [N,N/-bis[4-(benzeneazo) salicylaldehyde]-o-phenylenediamine respectively}and two complexes C1 and C2 {[N,N/-bis[4-(benzeneazo) salicylaldehyde]ethylenediamine Copper(II) and [N,N/-bis[4-(benzeneazo) salicylaldehyde]-o-phenylenediamine Copper(II) respectively} were characterised by elemental analysis (Perkin Elmer 2400 CHN analyzer), IR spectroscopy (Perkin Elmer FT/IR-RX 1 spectrometer), conductance measurement (Shanghai DDS-11A conductivity apparatus at 25^0C) and electronic spectra (Perkin-Elmer Lambda 7 spectrophotometer). All results are quite identical with the literature[13].

Treatment with ligands and complexes

To investigate the effects of prepared complexes, ligands 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 min. and then they were washed 3 times with distilled water14 and soaked for 12 h with various concentrations (10, 50, 100, 200 ppm) of $CuSO_4$, L1, L2, C1 and C2 along with a control (no treatment of micronutrients). After soaking was done the seeds were allowed to germinate on sterile petri plates. From each treatment we had chosen twenty germinated seeds which were transferred to plastic pots containing garden soil, well rotten cow dung and sand in 1:1:1 ratio under controlled conditions with a 8 h light period at 28-35^{o}C day/night temperature and 65-75 % relative humidity. 45 days old seedlings were subjected to biochemical analysis.

Growth parameters

To determine fresh weight, the harvested plants were rinsed with distilled water and blotted on paper towels before being weighed. Dry matter yields of the seedlings were determined after drying the seedlings in an oven at 80^{o}C to a constant weight. Relative water content (RWC) was measured according to the protocol described by Farooqui *et al.,*[15]

Copper tolerance index

Copper tolerance index (TI) was calculated as the quotient of the dry weight of plants grown under copper treated and control conditions[16] according to this formula:

$$\text{Tolerance index } (\%) = \frac{\textit{Dry weight of treated plants} \times 100}{\textit{Dry weight of control plants}}$$

Electrolyte leakage

Ion leakage was measured as electrical conductivity (EC %) according to Yan *et al.,*[17] The percentage of electrolyte leakage was calculated according to this formula:

$$EC(\%) = \frac{A1}{A2} \times 100$$

Where A1 and A2 are the electrolyte conductivities measured before and after boiling, respectively.

Determination of free amino acids and proline

Free amino acids were detected according to the method of Lee and Takahashi[18]. Proline content was determined by ninhydrin method[19].

Lipid peroxidation

It was measured as the content of malonyldialdehyde (MDA) using the thiobarbituric method of Heath and Packer[20].

H_2O_2 content

H_2O_2 levels in the leaves were estimated according to Jena and Choudhuri[21] with minor modifications. H_2O_2 levels were calculated using extinction coefficient 0.28 µmol^{-1} cm^{-1}.

Chlorophyll content

Chlorophyll was estimated according to the method of Harborne[22]. Estimation of chlorophyll was done by measuring the absorbance at 645 nm and 663 nm respectively in a UV-Vis spectrophotometer against a blank of 80% acetone and calculated using the formula as given by *Arnon*[23].

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg (g tissue)$^{-1}$ F.W

Carotenoid content

The extraction and quantification of carotenoids was completed by following the method of Litchtenthaler[24]. The absorbance was then assessed at 480 nm, 645 nm and 663 nm in UV-Vis spectrophotometer and the carotenoid content was estimated using standard formula:

$A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})$ µg (g tissue)$^{-1}$ F.W

Statistical analysis

Data were analysed by using Standard Error and LSD tests at $P \leq 0.05$ probability level using IBM SPSS statistics 21 software.

RESULTS AND DISCUSSION

Characterisation of the prepared ligands and Cu (II) complexes

The analytical data and corresponding Infrared spectra of the prepared ligands and the corresponding Cu(II) complexes are almost identical with the literature[13] and are provided in the supplementary (Table 1, Table 2, Table 3 and Scheme of reaction). The prepared two ligands show characteristic bands in the region of 1280 cm^{-1} (for O-H bon), 1605-1635 cm^{-1}(for C=N bond vibration). A broad band in the region of 2800-2700 cm^{-1} signifies a strong hydrogen bonding between the hydroxyl hydrogen and nitrogen atom. For the prepared Cu complexes characteristic ν(C=N), ν(phenolic C-O), ν(Cu-N), ν(Cu-O) were observed. All Infrared spectra exhibit parity with literature.

The ligand L2 shows 3 main peaks 254.0, 318.0 and 368.5 nm in the electronic spectrum. The first and second peaks are due to benzene $\pi-\pi^*$ and imino $\pi-\pi^*$ transitions where as the third peak is due to $\pi-\pi^*$ transition. For C2 the third peak is shifted towards longer wave length due to donation of lone pair to Cu. The ligand L1 shows mainly two peaks and they are 270.5 and 362.5 nm (first one for $\pi-\pi^*$ and second one for $\pi-\pi^*$). In C1 the $\pi-\pi^*$ peak shifted to 396 nm.

Growth and tolerance to different copper complexes

Cu^{2+} can hamper growth and development of seedlings by causing damage to epidermal cells and cell membranes.[25] The outcomes reveal that plants treated with C1 and C2 complex 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 (Figure 1, Figure 2 and Figure 3).

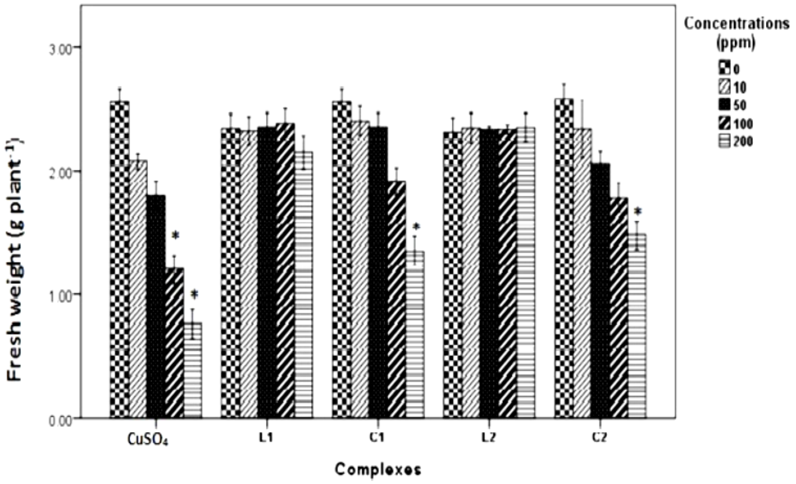

Fig. 1. Fresh weight (g plant-1) of 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

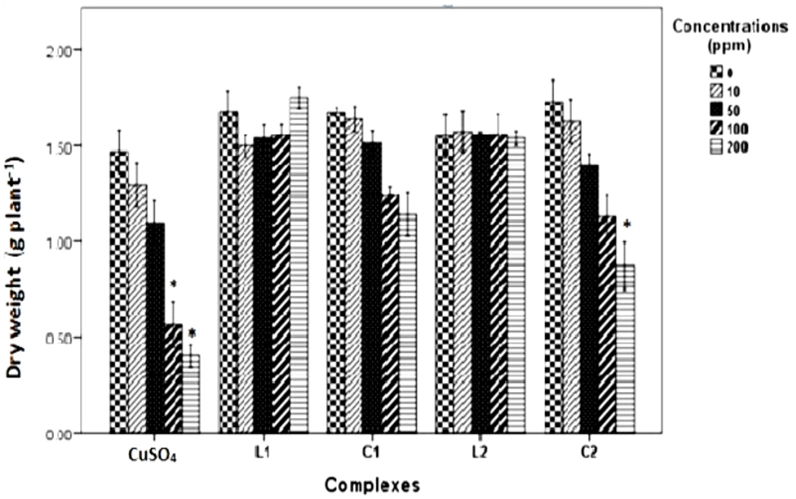

Fig. 2. Dry weight (g plant-1) of 45days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05

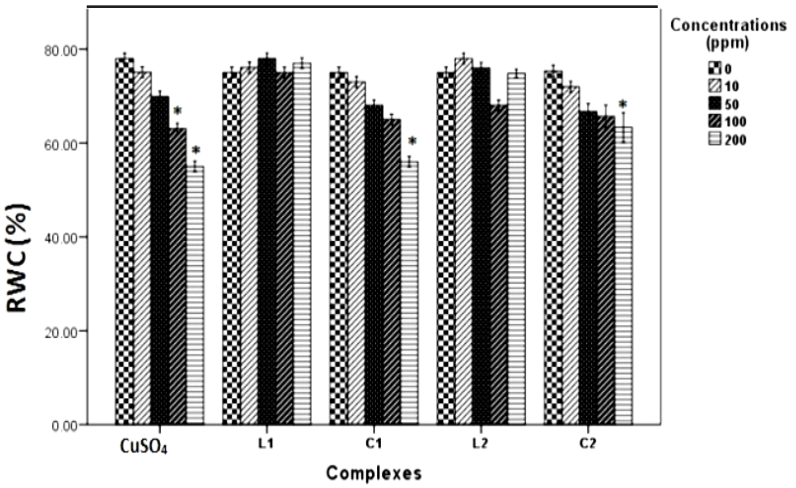

Fig. 3. Relative water content (RWC) of 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

For instance at 100 and 200 ppm $CuSO_4$ significantly reduces RWC by 22.99% where as C1 and C2 complex reduce only by 12.01% and by 12.34% respectively over control. Greater negative impact of $CuSO_4$ than the schiff base complexes on seedlings was further approved by Tolerance index. TI for C1 and C2 complex treated plants appeared to be significantly higher than that of $CuSO_4$ treated plants (Figure 4).

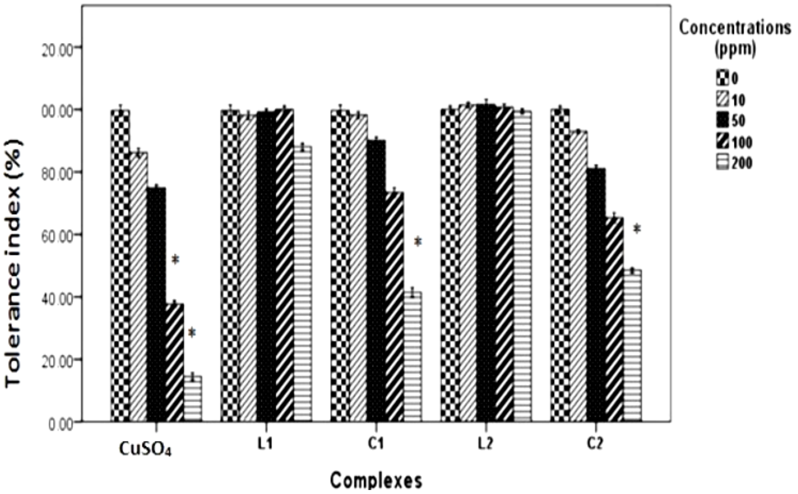

Fig. 4. Tolerance index (TI) of 45days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

Negative effect of $CuSO_4$ was also reported by Azooz *et al.,*[26] in wheat seedlings. Verma *et al.,*[27] reported application of copper at lesser concentrations enhanced the plant's dry biomass, while excess of copper reduced the biomass production of these plants.

Membrane damage and ion leakage

Copper can distress the membrane permeability by oxidation of membrane lipids which can be accessed from the increase of MDA, one of the lipid peroxidation products. Data obtained from this study revealed copper induced membrane damage which is expressed in terms of electrolyte leakage, lipid peroxidation enhanced significantly in $CuSO_4$ treated plant with rising concentration but in case of Schiff base complex treated plants malonyldialdehyde accumulation as well as electrolyte leakage were less as compared to $CuSO_4$ treated plants in relation to control indicating comparatively less membrane damage in Schiff base complex treated plants.

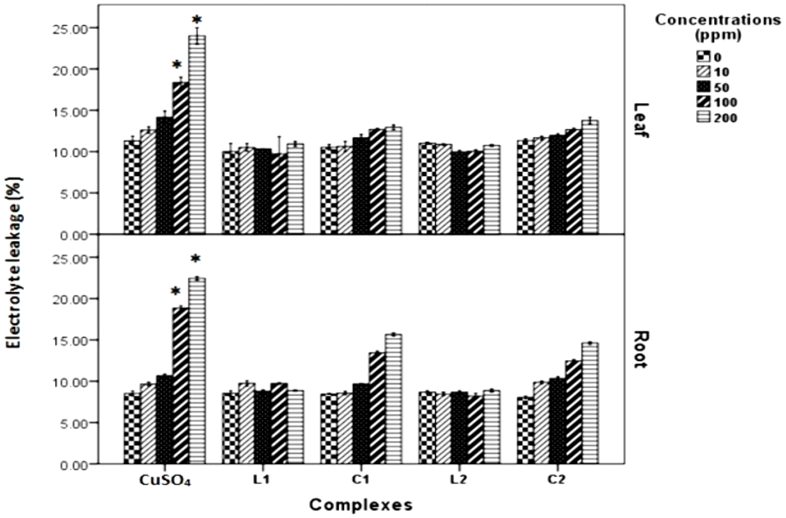

Fig. 5. Electrolyte leakage (%) 0f 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

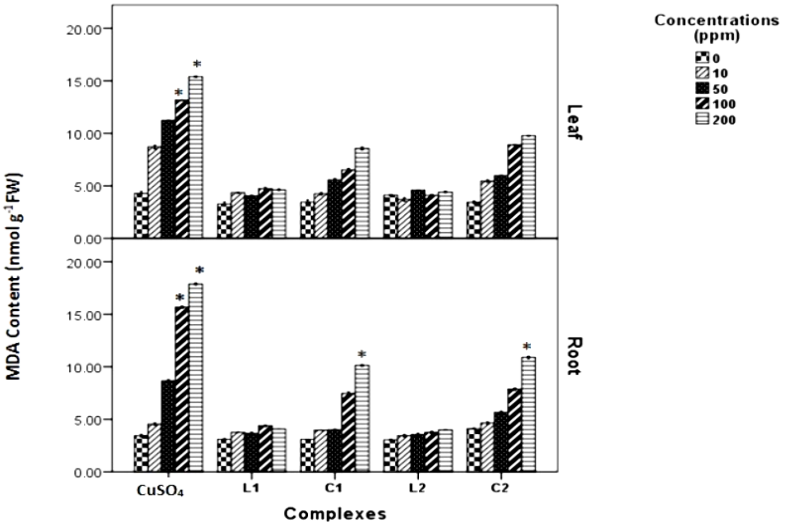

Fig. 6. MDA content of 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

The outcomes showed that the MDA content in leaf (Fig. 6) and electrolyte leakage in leaf and root (Fig. 5) of Schiff base Cu(II) complex treated plants increase with increasing dose but did not echo any significant changes. But in case of $CuSO_4$ treated plants ion leakage and oxidation of membrane lipid enhanced drastically at 100 and 200 ppm level in relation to control plants.

Reactive oxygen species induced plasma membrane damage increase MDA and ion leakage at the higher levels of Cu^{2+}.28 Hydrogen peroxide [one form of reactive oxygen species (ROS)] accumulation is minimum in case of schiff base Cu(II) complex treated plants than in $CuSO_4$ treated plants (Figure 7).

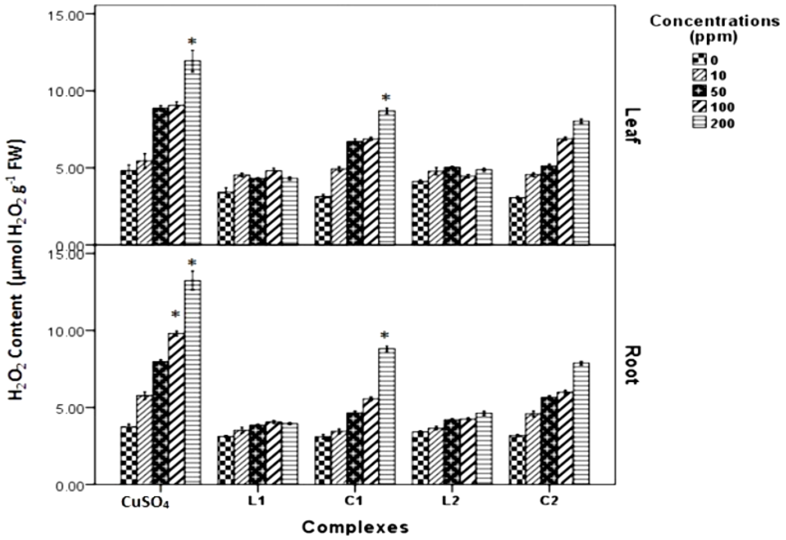

Fig. 7. H_2O_2 accumulation in 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05

In copper sulphate treated plants H_2O_2 increased 1.35 fold at 200 ppm where in Schiff base complex treated plants especially in case of C2 complex H_2O_2 accumulation was less (0.62 fold) in relation to control. This observation appears to be narrated the redox-active character of Cu^{2+} resulting in creation of extremely reactive hydroxyl radicals.[29] These results suggest that Schiff base Cu(II) treated plants had better ability to tolerate Cu^{2+} stress. The significant increase of MDA in plants, exposed to $CuSO_4$ indicated that increase of lipid peroxidation in Cu-treated plants led to disorder of plasma membranes on contrary plasma membrane damage was less in case of Schiff base Cu (II) complexes treated plants.

Total free amino acids and proline

Accumulations of total free amino acids (Fig. 8) were significantly increased in root and leaf tissue at higher $CuSO_4$ concentrations. However, low $CuSO_4$ up to 50 ppm had non-significant enhancement on 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 tissue. On contrary both the Schiff base Cu(II) complex treated plants showed insignificant enhancement of amino acids.

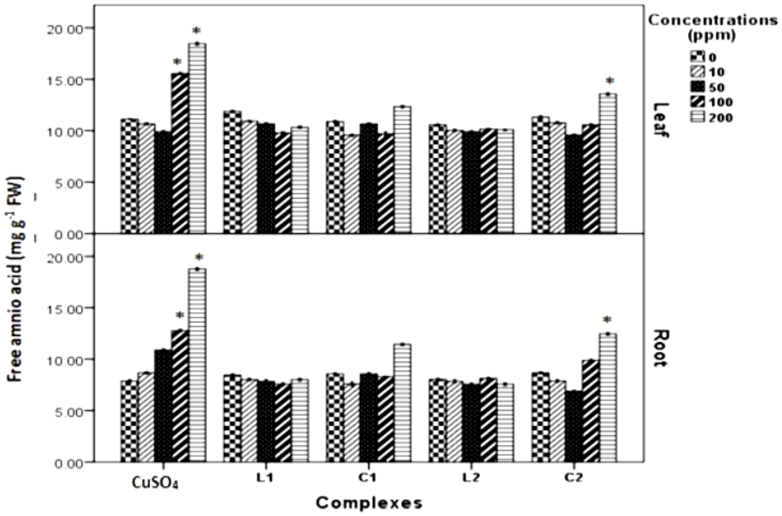

Fig. 8. Free amino acid accumulation in 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

Al-Hakimi and Hamada[30], in their study, suggested that free amino acids content enhances in plant tissues upon Cu^{2+} exposure. In agreement amino acids are looked upon as key player in metal chelation through which plant detoxify alleviate heavy metal stress.[31] Therefore, it might be suggested that plants experiencing higher amount of copper induced oxidative stress can accumulate greater amount of free amino acid to alleviate oxidative stress. In that scenario copper Schiff base complexes are proved to be less toxic than $CuSO_4$.

Proline accumulation in plant tissue which is an indicator of oxidative stress[32] increased in all treatments. In $CuSO_4$ treated plants proline accumulation was maximum at 100 ppm and at 50 ppm in leaf and root tissue respectively. Beyond this concentration proline accumulation decreased. Decline in proline accumulation may be attributed to reduction competence of plants to withstand oxidative stress.[33] Conversely, in Schiff base Cu(II) complex treated plants, proline content continued to be increased up to 200 ppm signifying lesser oxidative stress in those plants (Figure 9).

Proline accumulation indeed stabilizes plasma membrane free radical scavenger and some macro molecules and facilitates rapid recovery from heavy metal stress.[34]

Photosynthesic 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[35]. Our study revealed that total chlorophyll content increased in case of all treatments up to 50 ppm. Beyond this concentration total chlorophyll content reduced, but the reduction was maximum in case of $CuSO_4$ treated plants and minimum for C2 treated plants (Fig. 10). Less reduction of chlorophyll may be attributed to less copper toxicity of Schiff base complexes to plants.

Carotenoid, a non enzymatic antioxidant involved in quenching of oxidizing species, participate in disrupting regular cellular functioning. In $CuSO_4$ treated plants carotenoid content increased gradually upto 100 ppm. At 200 ppm carotenoid content reduced drastically signifying the huge toxicity of CuSO4 but on contrary in C1 and C2 treated plants carotenoid content continued to increase up to 200 ppm (Figure 11).

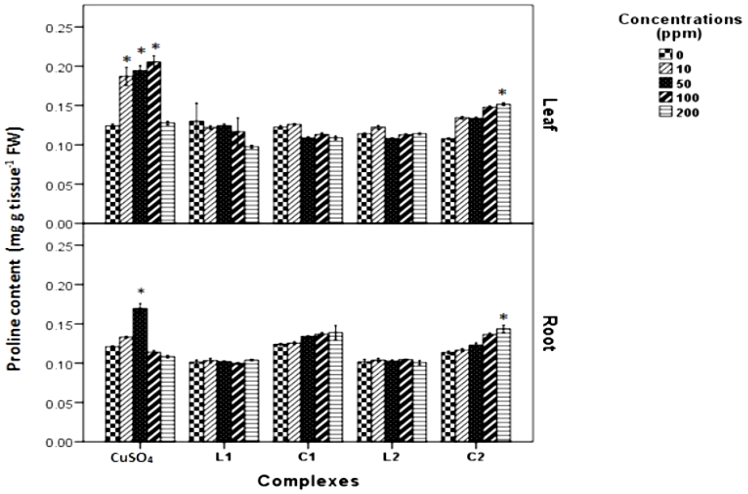

Fig. 9. Proline level in 45 days old rice seedlings. Means ± SE, n = 3. * The mean difference is significant at the 0.05 level

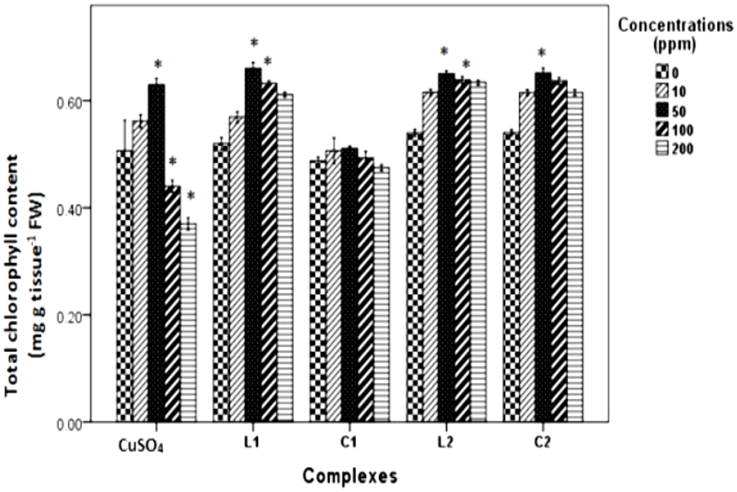

Fig. 10. Total chlorophyll content in 45 days old rice seedlings. Mean ± SE, n = 3. * The mean difference is significant at the 0.05 level

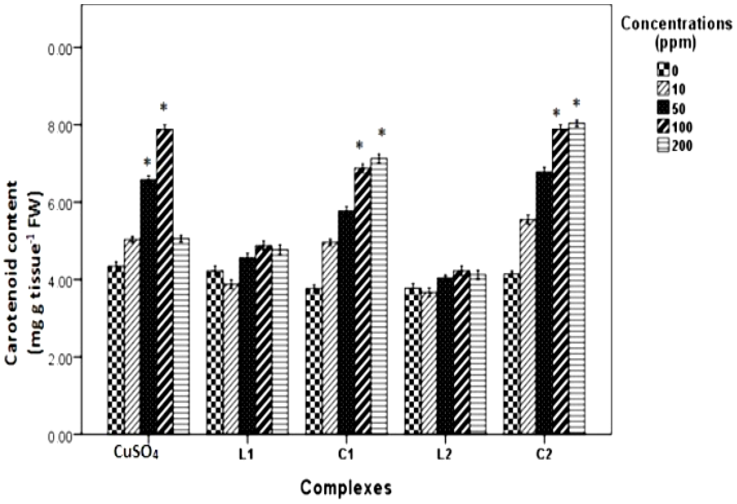

Fig. 11: Carotenoid content in 45 days old rice seedlings. Means ± SE, n = 3. * The mean difference is significant at the 0.05 level

Table 1: Elemental analysis data and some physical properties of the Schiff bases and their complexes

Formula		Colour	Yield (%)	Found (Calculated)%				Conductivity
				C	H	N	M	C ohm^{-1}.cm^{2}. mol^{-1}
L2	$C_{32}H_{24}N_6O_2$	Orange	80	73.02 (73.26)	4.53 (4.61)	15.98 (16.03)		
C2	$C_{32}H_{22}N_6O_2Cu$	Red	75	65.64 (65.57)	3.69 (3.78)	14.42 (14.34)	10.78 (10.84)	5.1
L1	$C_{28}H_{24}N_6O_2$	Orange	84	70.6 (70.57)	5.2 (5.0)	17.58 (17.64)		
C1	$C_{28}H_{22}N_6O_2Cu$	Green	73	62.55 (62.5)	4.02 (4.12)	15.53 (15.62)	11.76 (11.81)	5.6

Note : Decomposition occurs with conc. nitric acids, and the resultant solution was used after suitable dilution for metal analysis

Table 2: IR spectroscopic data of the Schiff bases and their complexes

Compound	ν(C=N)		ν(phenolic C-O)	ν(M-N)	ν(M-O)
L2	1610	1578	1285		
C2	1630	1592	1258	530	430
L1	1640	1588	1290		
C1	1627	1578	1257	514	449

Table 3: Electronic spectral data of the Schiff bases and their Cu(II) complexes

	λmax(ε, L/mol cm) in DMF		
L2	368.5	318	254
C2	398	318	251
L1	362.5	270.5	
C1	396	266	

CONCLUSION

Offshoots of the present study reveals that Schiff base Cu(II) complexes have less toxic effects than copper sulphate on rice seedlings and provide better tolerance to copper toxicity than copper sulphate. Maximum positive impact of 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 Schiff base Cu(II) complex treated seedlings but the optimum positive impact of these Schiff base Cu(II) complexes largely depends on dose. Beyond certain concentration these complexes may have inhibitory effects on rice. Cu(II) schiff base complexes can be used as a supplement to turn down micronutrient deficiency at the same time minimize the toxicity generated by application of different ionic form of Cu(II) may open a new direction research.

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