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Research Article

PHOTOSYNTHESIS IN COPPER MEDIATED PLANT IS AFFECTED DUE TO OXIDATIVE DAMAGE CAUSED BY REACTIVE OXYGEN SPECIES (ROS) GENERATION

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ABSTRACT

Rice *Oryza sativa* cv. Jaya grown in vermiculite and irrigated with the range of Cu treatments (0 to 3.0 mmol l^{-1}) in Hoagland solution were studied for its effect on photosynthesis and oxidative damage in twenty days old plants. Gradual decrease in plant growth and biomass was observed with the increase in Cu treatment. Treatments with 3 mmol l^{-1} Cu led to 69 times more accumulation of Cu in root than in leaf tissue. Quantum efficiency of photosystem II measured as F_v/F_m ratio and photochemical quenching (qP) decreased significantly with increase in non-photochemical quenching (qNP) as the Cu treatment increased. Accumulation of proline an indicator of osmotic stress increased considerably in Cu treated plants. Inverse relationship between increasing Cu treatment and photosynthetic rate, transpiration rate and stomatal conductance was observed. Photosynthetic pigments such as Chlorophyll *a*, chlorophyll *b*, Neoxanthine, Violaxanthine, Antheraxanthine, β - Carotene and Lutein showed significant linear decrease with the increasing Cu treatment. Progressive enhancement in the H_2O_2 level at 1, 2 and 3 mmol l^{-1} Cu treatments was observed which was coupled with greater level of lipid peroxidation measured as MDA. Superoxide dismutase (SOD), an antioxidant enzyme exhibited significant activity only at highest Cu treatment.

INTRODUCTION

Cu having atomic density of 8 gm cm^3 is referred as heavy metals (HMs) (Kidd *et al,* 2009). HMs are non-biodegradable and non-thermally degradable thus easily gets accumulated into the soil and translocated into the plants system and as a result readily enters the food chain which can harm the human and animal health (Yruela, 2009). Due to unrelenting industrialization and urbanization there has been escalating stipulate for minerals leading to a surge in mining activities involving crushing, grinding, washing and smelting which generate waste products such as mine overburden and mine tailings (waste soil) contracting the useful land for cultivation, forestry and grazing, leading to a decline in overall productivity (Sheoran *et al,* 2011). World refined Cu production has increased from 18,987 to 22,842 thousand metric tonnes from 2010 to 2015 (http://www.icsg.org/index.php/statistics/selected-data). In addition to mining activities repeated application of bordeaux mixture [$CuSO_4.5H_2O$+$Ca(OH)_2$] as fungicide, hogmanure and sewage sludge amendments further leads to Cu toxicity in soil (Sharma *et al,* 2007).

As a mineral nutrient Cu is an indispensable metallic elements ordinarily found in the range of 4-20 ppm in plant tissues (Maksymiec, 1997) and perform various metabolic activities. Presence of Cu is essential in cytosol, endoplasmic reticulum, inner membrane, chloroplast stroma, the thylakoid lumen and the apoplast for their normal function (Marschner, 1995). Additionally, Cu at 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 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 state *i.e.* Cu^{2+} and Cu^{+} which may catalyzes the formation of hydroxyl radicals ($OH^{\cdot}$) from the non- enzymatic chemical reaction between superoxide ($O_2^{\cdot}$) and H_2O_2 (Hydrogen peroxide) through Haber-Weiss reaction (Halliwell and Gutteridge, 1984) which is known to damage cell membrane by binding to a SH groups of membrane proteins and induces lipid peroxidation.

Supraoptimal concentration of Cu proves to be phytotoxic (Dewez *et al,* 2005) by causing electrolyte leakages, affecting 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,*

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1988). Excess Cu also interferes with the uptake of other elements like N, P, S, Ca and Mg (Cambrolle *et al,* 2011).

The present report aims at gaining understanding about the copper effect on photosynthesis and growth, its accumulation and the relationship between ROS generation and induction of oxidative stress and antioxidant responses in rice.

MATERIALS AND METHODS

Plant material and growth condition

Seeds of *Oryza sativa.* cv. Jaya, 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 growth chamber for 20 days under control conditions of temperature (25 ± 2°C) and light (200 µmol m^{-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 0, 0.1, 0.25, 0.5, 1, 2 and 3 mmol l^{-1}.

Growth and copper content analysis

Growth of the plant was determined by measuring the length and fresh and dry mass of shoot and root. Copper content was estimated using Atomic Absorption spectrophotometry (Analyst 200 version 30, Perklin Elmer 3110, Germany). Briefly 1g of dried tissue of leaf was subjected to ash formation at 450°C for 5 hours and acid digested the ash mass with 15 ml of 1% HNO_3 for 24 h and filtered.

Chlorophyll fluorescence

Chlorophyll fluorescence recorded using a fluorescence monitoring system (FMS, Hansatech) according to Sharma *et al,* 1997. The Fv/Fm ratio, which is an indicator of photosynthetic efficiency, qP, an indicator of use of harvested light in photochemical event and qNP an indicator of dissipation of harvested light in the form of heat was measured. The leaves were dark adapted for 10 min. The dark-adapted leaf was exposed to weak modulated measuring beam with an intensity of 3 µmol m^{-2} s^{-1} to measure initial fluorescence (Fo). This was followed by an exposure to a saturating pulse of white light of 4000 µmol m^{-2} s^{-1} to provide the maximum fluorescence (Fm). After measurement of Fm leaves were allowed to reach steady state fluorescence (Fs) while exposed to actinic light intensity of 330 µmol m^{-2} s^{-1}. Another burst of saturating light at Fs state was used to measure F'm. After reaching the steady state again, leaves were exposed to far-red radiation light to measure F'o. Variable fluorescence (Fv) was determined by subtracting Fo from Fm (Fv = Fm - Fo) and the Fv/Fm and Fv/Fo ratio was calculated. Photochemical quenching coefficients were calculated as (qP) = (Fm'-Fs)/(Fm'-Fo') and non photochemical quenching coefficients were calculated as (qNP) = 1- (F'm-F'o)/(Fm-Fo). The values were calculated according to Schreiber *et al,* 1985.

Gas exchange measurements

Photosynthetic rate (A), stomatal conductance (Gs), transpiration rate (E) and internal CO_2 concentration (C*i*) was measured using portable infrared gas analyzer (IRGA, LCi - SD, ADC Bioscientific, U.K.) in a closed system using 2 cm^2 leaf chambers according to Sharma and Hall, 1996. Measurements were made at ambient temperature and CO_2 conditions at 1200 PAR using dichoric white halogen lamp at leaf level.

Extraction and separation of photosynthetic pigment using HPLC

Pigment extraction and separation was carried out using HPLC (High Performance Liquid Chromatography, Waters) according to Sharma and Hall, 1996. Fresh 0.2 g of leaf tissue cut into fine pieces was homogenized in 100% acetone and the total volume was made to 2 ml. Few crystals of B.H.T (Butyl hydroxy toluene) were added as an antioxidant and incubated overnight at 4°C. Samples were centrifuged at 4°C for 10 min at 6000 rpm. Supernatant was collected in 2 ml eppendorf tubes and filtered through 0.2 µm-nylon filter (Pall Pharmalabs) and it was used for injecting into the HPLC system. Separation of pigments was done on reverse phase C18 column (Waters Spherisorb ODS .5µm, 4.6 mm x 250 mm) using a phase diode array detector (Waters 2996). Using a glass syringe 20 µl of filtered samples was injected into the HPLC. The gradient for separation was 0 - 100 % ethyl acetate in acetonitrile/water (9:1) over 34 min with flow rate of 1.2 ml/min. Temperature was maintained at 25°C and pressure was 1200 psi/min. The pigments were detected at 450 nm. Quantity of the molecules was calculated using β-carotene as external standard on the peak area basis. Pigment content was expressed as µg g^{-1} f. w.

Determination of H_2O_2 radical

H_2O_2 was determined according to the method of Thounajan *et al,* 2012. Leaf tissue (0.5 g) homogenised in 3.5 ml of 0.1% TCA (Trichloroacetic acid) and centrifuged at 7000 rpm for 10 min at 4°C. To 0.5 ml of supernatant 0.5 ml of 100 mM potassium phosphate buffer (pH = 7.0) and 2 ml of 1M of KI solution were added. Reaction mixture was incubated at room temperature for 1 hour in darkness. O.D was taken at 390 nm.

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 (50mM Tris HCl + 150mM NaCl, pH = 8.0) and freshly prepared 0.5% TBA in 20% TCA. The absorbance was recorded at 532 nm and non-specific turbidity was corrected by subtracting absorbance at 600 nm. Concentration of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}.

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. Supernatant

was incubated with glacial acetic acid and acid ninhydrine 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. Proline concentration was calculated using proline standard graph.

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. Supernatant used to carry out antioxidant activity. Reaction one consisted of 10 mM Na_2CO_3, 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 as blank. Enzyme kinetics was carried out at 480 nm. Protein concentration of enzyme extract was measured using Bradford method at 595 nm. SOD activity was calculated as SOD activity (A) mg^{-1} protein min^{-1}.

Statistical analysis

Each treatment was conducted with three replicates and the results were presented as mean ± SD (standard deviation). The statistical analysis of experimental data utilized the student's *t*-test. Each of the experimental values was compared to its corresponding control. Statistical significance was accepted when the probability of the result assuming the null hypothesis (*p*) is less than 0.05.

RESULTS

Growth, Biomass and Cu content analysis

The data showed a significant decrease in the shoot and the root length (Fig. 1a) and their biomass (Fig. 1b) when grown over Cu treatment of 1 mmol l^{-1} and above as compared to control.

Fig. 1 a

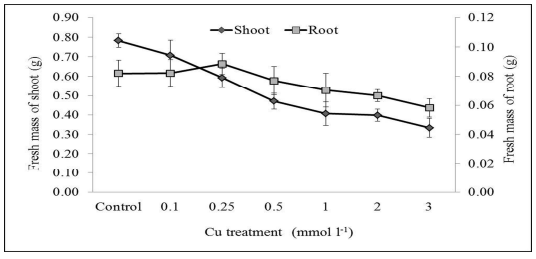

Fig. 1b

Fig. 1 Effect of Cu treatment on (a) growth (b) leaf and root fresh mass production in *Oryza sativa* cv. Jaya in 20 days old plants. The values are given as mean ± SD are determined with 3 independent experiments (n = 3). Significant differences are marked with "asterisk" ($p < 0.05$) assessed by student *t* test.

Fresh biomass of shoot and root decreased by 57% and 28% respectively at 3 mmol l^{-1} Cu treatment as compared to control (Fig. 1b).

Cu accumulation study carried out using AAS showed a non-linear accumulation of Cu in leaf and root (Fig. 2). A slight initial increase of 325% in accumulation of Cu was observed in leaves grown at 0.1 mmol l^{-1} Cu treatment with no further significant increase till the treatment of 1 mmol l^{-1} Cu. Further increase in the Cu treatment to 2 and 3 mmol l^{-1} increased the Cu content in leaf to 664 and 718% respectively as compared to its control. Similar results were observed with the Cu accumulation in the root. An initial increase of 227% in Cu was observed in roots of plants grown at 0.25 mmol l^{-1} with an increase of 292% Cu in plants treated with 1 mmol l^{-1} Cu, however, further increase in the Cu treatment to 2 and 3 mmol l^{-1} increased the Cu content in the root to 559 and 916% respectively as compared to its control. When accumulation of Cu in leaf and root was compared at highest Cu treatment (3 mmol l^{-1}) it was observed that Cu accumulation in root tissue (176.3 µg g^{-1} D.W.) was 69 fold greater than observed in leaf tissue (2.55 µg g^{-1} D.W.).

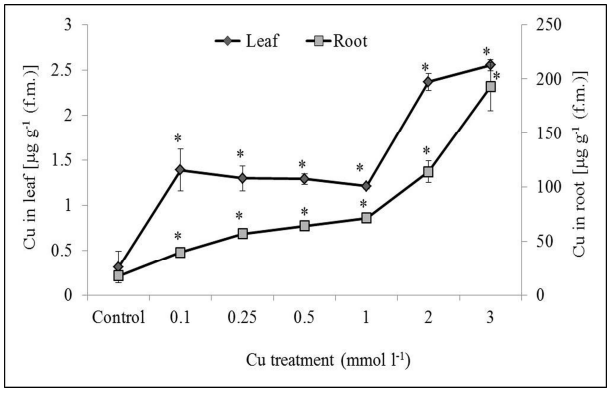

Fig. 2 Bioaccumulation of Cu in the leaf and the root tissue of the rice seedlings measured after 20 days of different Cu treatment. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

Chlorophyll fluorescence

PS II light reactions measured in terms of fluorescence are presented in Fig. 3. The value of Fo was increased by 40% and Fm value decreased by 22% at 3 mmol l^{-1} Cu treatment as compared to control (Fig. 3a). The ratio of the variable fluorescence to maximal fluorescence (Fv/Fm) was reduced by 12 and 17% at 1 and 3 mmol l^{-1} Cu treatment respectively as compared to its control (Fig. 3b). Non photochemical quenching (qNP) showed significant increase of 8% only at 3 mmol l^{-1} Cu treatment as compared to its control whereas photochemical quenching (qP) reduced significantly by 16, 58 and 66% at 0.1, 1 and 3 mmol l^{-1}Cu treatment respectively with respect to control (Fig. 3c).

Gas exchange measurements

Increasing Cu treatment was negatively correlated with net Photosynthetic rate (A), Transpiration rate (E), Stomatal conductance (Fig. 4). Cu treatment drastically reduced A followed by parallel trend of reduction exhibited by E and gs.

The result showed linear decline of 24, 77 and 89% in A at 0.1, 1 and 3 mmol l^{-1} Cu treatment respectively as compared to control (Fig. 4a). Similarly, E and gs showed decrease of 18 and 17% at 0.1 mmol l^{-1} of Cu, 70 and 69% at 1 mmol l^{-1} of Cu, and 82 and 85% at 3 mmol l^{-1} Cu treatment respectively as compared to their control (Fig. 4a and Fig 4b) but no significant change was observed in C*i* value as compared to its control (Fig. 4b).

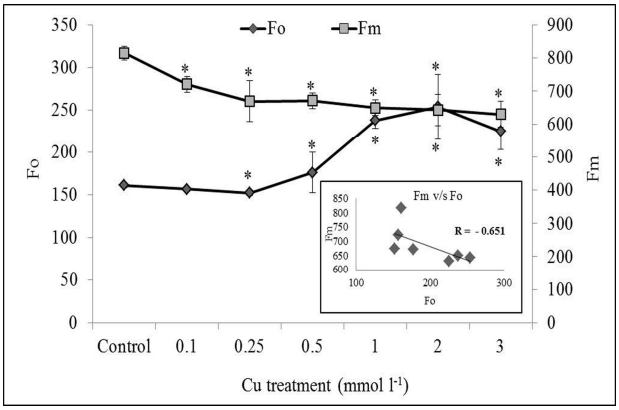

Fig. 3a

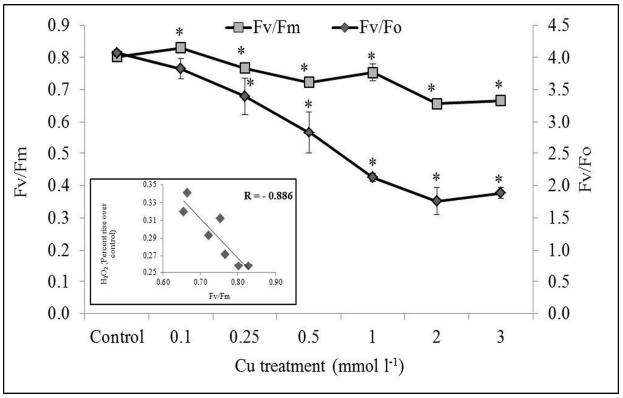

Fig. 3b

Fig. 3 Effect of Cu treatment on (a) Initial fluorescence (Fo) and Maximum fluorescence (Fm) (b) Maximum quantum efficiency of PSII photochemistry (Fv/Fm) and Oxygen evolving complex (Fv/Fo) (c) photochemical quenching (qP) and Non photochemical quenching (qNP) in *Oryza sativa* cv. Jaya grown for 20 days. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

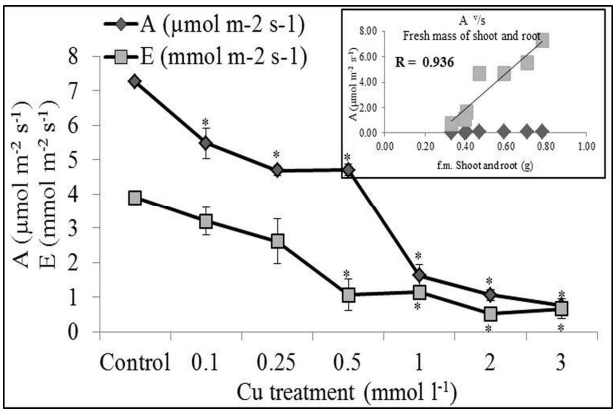

Fig. 4a

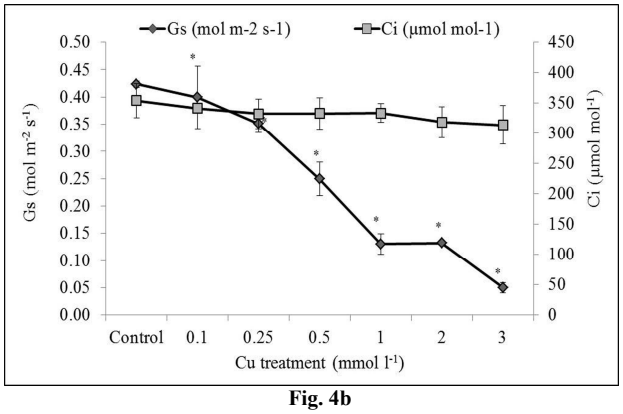

Fig. 4b

Fig. 4 Effect of Cu treatment on (a) Net photosynthetic rate (*A*) and Transpiration rate (E) (b) Stomatal conductance (Gs) and Internal CO_2 concentration (C*i*) in *Oryza sativa* cv. Jaya grown for 20 days. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

Determination of photosynthetic pigments

Neoxanthine and Antheraxanthine showed an initial increase of 58 and 47% at 0.1 mmol l^{-1} Cu treatment followed by linear decline of 22 and 31% at 1 mmol l^{-1} Cu and 31 and 57% at 3 mmol l^{-1} Cu treatment as compared to their control (Fig. 5a). Violaxanthine decreased by 40 and 69% at 1 and 3 mmol l^{-1} Cu treatment respectively as compared to its control (Fig. 5a). β carotene decreased by 35, 18 and 63% at 0.1, 1 and 3 mmol l^{-1} Cu concentration as compared to its control (Fig. 5a). Lutein showed linear decline of 7, 43 and 72% at 0.1, 1 and 3 mmol l^{-1} Cu treatment as compared to control (Fig. 5a).

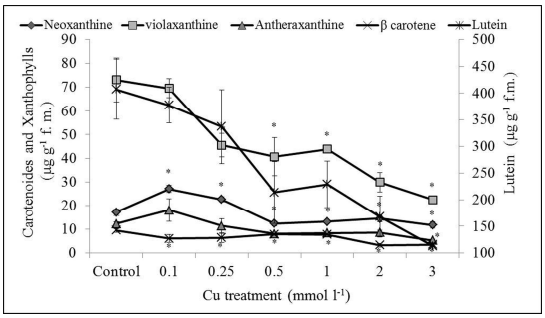

Fig. 5a

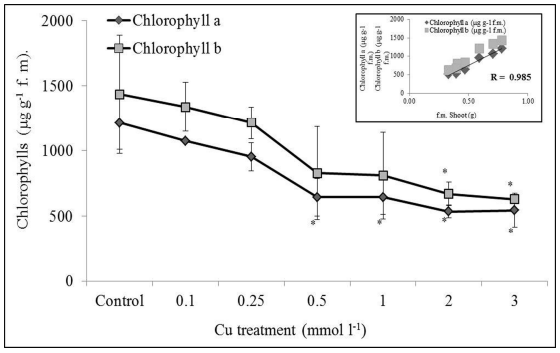

Fig. 5b

Fig. 5 Effect of Cu treatment on photosynthetic pigments (a) Carotenoides and Xanthophylls (b) Chlorophylls in *Oryza sativa* cv. Jaya grown for 20 days. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

Chlorophyll *a* and chlorophyll *b* decreased linearly by 11 and 6% at 0.1 mmol l^{-1} Cu, 46 and 43% at 1 mmol l^{-1} Cu, and 56% at 3 mmol l^{-1} Cu treatment respectively as compared to their control (Fig. 5b).

Determination of H_2O_2 content, lipid peroxidation and proline estimation and Antioxidant activity

The level of H_2O_2 increased in leaf tissue with the increasing Cu treatment. It showed an increase of 18% and 47% at 1 and 3 mmol l^{-1} Cu treatment respectively as compared to control (Fig. 6).

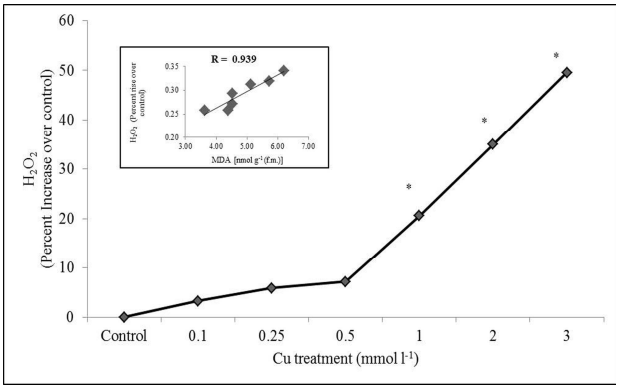

Fig. 6. Effect of Cu treatment on H_2O_2 level in *Oryza sativa* cv. Jaya grown for 20 days. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

Increase in MDA content was directly proportional to increase in Cu treatment in leaf tissue. MDA level increased linearly with 20, 41 and 71% in 0.1, 1 and 3 mmol l^{-1} Cu treatment respectively as compared to control (Fig. 7).

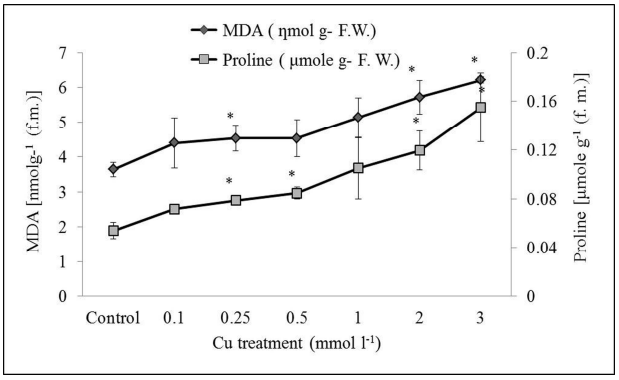

Fig. 7. Effect of Cu treatment on MDA (Malondialdehyde) and Proline content in *Oryza sativa* cv. Jaya grown for 20 days. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

Proline content increased by 32, 93 and 186% at 0.1, 1 and 3 mmol l^{-1} Cu treatment respectively as compared to its control (Fig. 7). Antioxidant role of SOD on detoxification of ROS radicals generated in response to oxidative stress caused due to Cu treatment is observed in the present study. The result showed significant increase of 53% in SOD activity at 3 mmol l^{-1} Cu treatment as compared to its control (Fig. 8).

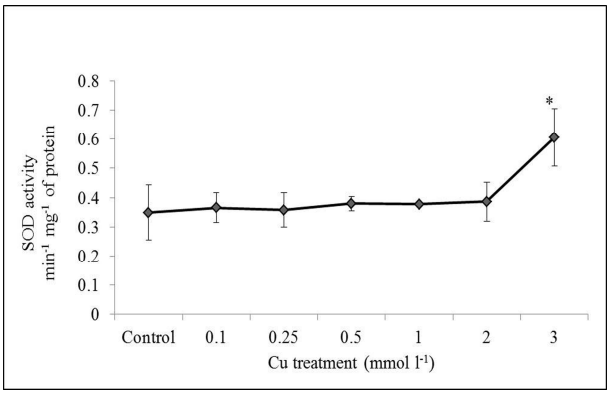

Fig. 8. Effect of Cu treatment on SOD activity in *Oryza sativa* cv. Jaya grown for 20 days. The values are given as mean ± SD determined with 3 independent experiments (n = 3). Significant differences are marked with * "asterisk" ($p < 0.05$) assessed by student *t* test.

DISCUSSION

Observed decrease in shoot and root length and biomass in this study (Fig. 1a and b) may be due to the toxic level of Cu accumulated within the leaf and the root tissue (Fig. 2). The Cu accumulation is reported to be inhibitory to cell division which may result in lower growth and biomass (Manivasagaperumal *et al,* 2011). As the inhibitory effect is found to be highest in higher treatment (Loneragan, 1988) indicating that mobility of Cu depends upon its availability. Initial enhanced uptake of Cu at lowest Cu treatment (0.1 mmol l^{-1}) suggests that this uptake was to suffix the optimum Cu concentration requirement by plants for better growth which was followed by steady state of Cu accumulation up to 1 mmol l^{-1} treatment suggesting a threshold point for Cu tolerance. Rapid Cu uptake observed over 1 mmol l^{-1} Cu treatment advocates for the ineffective metal exclusion mechanism at both soil-root level and root-shoot level and this observation can also be related to the phytotoxicity threshold value (PT50, tissue treatment of plant resulting in 50% biomass reduction (Cambrolle, *et al,* 2011).

Cu accumulation study also showed that root retained much greater amount of Cu than leaf tissue exhibiting the exclusion mechanism of Cu from root to leaf tissue. Greater accumulation of Cu in root can be due to the activation of Cu transporters at root level followed by its loading into xylem and exclusion of Cu from root to shoot via slow transport of Cu from roots to the shoots and also due to counter flow or recirculation of Cu from phloem back to roots by following source to sink route which has been observed previously (Kramen *et al,* 1996; Ducic and Polle, 2005; Liao *et al,* 2000). Our data of greater accumulation in root over shoot suggests similar process may be occurring in rice plants.

Plants also exhibited morphological adaptation in order to limit Cu uptake by decreasing root surface area (Fig. 1b) but reduced root surface area simultaneously compromised the absorption of water and nutrients resulting in osmotic stress as indicated by proline accumulation which acts as osmoproctectant (Fig. 7).

Water stress and nutrient deficiency further served as contributory factor for the reduced growth in addition to Cu toxicity. Accumulation of proline due to Cu stress is also observed in *Azolla* and wheat seedlings (Azooz *et al,* 2012; Mehta and Gaur, 1999).

Significant increase observed in Fo in the present study (Fig. 3a) indicate lower efficiency of absorption of light by light harvesting complex II (LHCII) which may be due to the disorganization of chlorophyll or carotenoids in LHC II. Observed decrease in Fm value (Fig. 3a) could be because of closing of reaction center (RC) attributable to nonfunctioning of RC, as a result of the Cu treatment suggesting reduced functional state of RC and therefore the small Fm and increased qNP (Fig. 3c). Decrease in Fm and increase in Fo resulted in significant decrease in Fv/Fm ratio (Fig. 3b) indicative of decreased quantum efficiency of photosystem II (PSII).

Rise in qNP at even growth light intensity of 200 PAR signified that the light absorption exceeded the capacity of ETC and non-functioning of RC led to over energization of PS electron transport leading to generation of ROS as a result of Mehler reaction. Decrease in Fv/Fm can also be attributed to the observed decrease in the activity of oxygen evolving complex (OEC) denoted by Fv/Fo ratio (Fig. 3b) since it serves as a source of electrons in the phosphorylation reaction and proved to be the limiting factor for Fv/Fm and this is further confirmed by the positive correlation exhibited by Fv/Fm and Fv/Fo ratio (R = 0.90).

In addition to structural changes in PS apparatus, PSII functioning was also deterioted as a result of generation of H_2O_2 (Fig. 6) due to Fenton because of excess accumulation of Cu leading to greater lipid peroxidation in the form of MDA (Fig. 7). Greater lipid peroxidation even at low concentration of H_2O_2 could be due to the rapid conversion of H_2O_2 into OH· (hydroxyl) radical by reacting with free Cu^{2+} ion and rapid diffusion of H_2O_2 reaction through phospholipid membrane as compared to charged $O_2^{\cdot-}$ (superoxide) across the membrane has been suggested earlier (Elstner *et al,* 1988; Wang *et al,* 2011). Superoxide ($O_2^{\cdot-}$) and OH· (hydroxyl) radical are known to cause more oxidation of lipid as compared to H_2O_2. Lipid peroxidation in response to Cu has been proposed as one of the mechanism causative in declining effectiveness of PSII (Upadhyay and Panda, 2009; Baron-Ayala *et al,* 1992; Camborelle *et al,* 2011). Our data suggests a similar underlying mechanism could be responsible for reduction of phosphorylation reaction.

Observed decrease in CO_2 fixation rate can be attributed to the observed decrease in the photosynthetic pigment in response to Cu (Fig. 5a and 5b). Decrease in chlorophyll pigment could be due to Cu's substitution of Mg atom in chlorophyll molecule suggested by (Kupper *et al,* 2002) and this could have resulted in chlorophyll degradation and inhibition of light dependent reaction which subsequently decreased the CO_2 fixation rate and declined the photo assimilate production. Interdependency of photosynthetic pigment to net assimilation rate is evident by the positive correlation (R = 0.910). Decrease in net assimilation rate is subsequently responsible for observed decrease in biomass production evident by their positive correlation (R = 0.93). Observed decrease in stomatal conductance denotes towards evading the water loss by significantly decreasing the transpiration rate without considering CO_2 intake as a limiting factor for CO_2 fixation since Ci remained unaltered as compared to control (Fig. 4b).

Besides, decrease in stomatal conductance could also be due to direct effect of metal ion on reduction of guard cell turgor by altering the K:Ca ratio which affect the movement of the guard cells and this may lead to the closure of the stomata (Cambrolle *et al,* 2011). Plants adopted defense against ROS by increased activity of SOD which plays a significant role in scavenging the ROS and rendering them less toxic. Significant increase in SOD activity was observed only at 3 mmol l^{-1} of Cu treatment and found to be insufficient for the complete detoxification of ROS as confirmed by level of lipid peroxidation at higher concentration of Cu (Fig. 7 and Fig. 8).

Our results clearly showed that Cu exhibited toxic effects and interfered with the basic physiological processes in plants due to massive accumulation of Cu in root and shoot resulting in reduced efficiency of PSII by facilitating the ROS generation causing the oxidative damage leading to greater level of lipid peroxidation and simultaneously caused reduction in chlorophyll biosynthesis which subsequently reduced the net assimilation and in order to combat the oxidative damage plant triggered the defense mechanism which found to be inefficient.

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