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BIOTIC AND ABIOTIC STRESS

Modulation of copper toxicity-induced oxidative damage by excess supply of iron in maize plants

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Abstract In this study, we examined the modulation of Cu toxicity-induced oxidative stress by excess supply of iron in *Zea mays* L. plants. Plants receiving excess of Cu (100 μM) showed decreased water potential and simultaneously showed wilting in the leaves. Later, the young leaves exhibited chlorosis and necrotic scorching of lamina. Excess of Cu suppressed growth, decreased concentration of chloroplastic pigments and fresh and dry weight of plants. The activities of peroxidase (EC 1.11.1.7; POD), ascorbate peroxidase (EC 1.11.1.11; APX) and superoxide dismutase (EC 1.15.1.1; SOD) were increased in plants supplied excess of Cu. However, activity of catalase (EC 1.11.1.6; CAT), was depressed in these plants. In gel activities of isoforms of POD, APX and SOD also revealed upregulation of these enzymes. Excess (500 μM)-Fe-supplemented Cu-stressed plants, however, looked better in their phenotypic appearance, had increased concentration of chloroplastic pigments, dry weight, and improved leaf tissue water status in comparison to the plants supplied excess of Cu. Moreover, activities of antioxidant enzymes including CAT were further enhanced and thiobarbituric acid reactive substance (TBARS) and H_2O_2 concentrations decreased in excess-Fe-supplemented Cu-stressed plants. In situ accumulation of H_2O_2, contrary to that of $O_2^{\cdot-}$ radical, increased in both leaf and roots of excess-Cu-stressed plants, but Cu-excess plants supplied with excess-Fe showed reduced accumulation H_2O_2 and little higher of $O_2^{\cdot-}$ in comparison to excess-Cu plants. It is, therefore, concluded that excess-Cu (100 μM) induces oxidative stress by increasing production of H_2O_2 despite of increased antioxidant protection and that the excess-Cu-induced oxidative damage is minimized by excess supply of Fe.

Keywords Antioxidants · Copper toxicity · Hydrogen peroxide · Lipid peroxidation · Oxidative stress · Superoxide dismutase · *Zea mays*

Abbreviations

APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
DAT	Days after treatments
EDTA	Ethylenediamine tetraacetic acid
MDA	Malondialdehyde
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCA	Trichloroacetic acid

Introduction

Copper is an unparalleled redox active agent in biological systems. It is an integral component of many electron transport carriers and involved in reductive and oxidative

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electron transport pathways in chloroplasts and mitochondria, respectively. Apart from this, it also participates in defense against pathogens by involving itself in the cell wall lignification and ascorbic acid metabolism (Shingles et al. 2004). Moreover, being an integral constituent of Cu/Zn-superoxide dismutase (Cu/Zn-SOD), copper plays a critical role in the detoxification of superoxide radical ($O_2^{\cdot-}$) as it dismutates $O_2^{\cdot-}$ to H_2O_2 (Marschner 1995). The $O_2^{\cdot-}$ and H_2O_2 can generate potentially dangerous hydroxyl radical ($OH^{\cdot}$) by undergoing Haber–Weiss reaction in the presence of metallic ions like Cu^{2+} and Fe^{2+} (Apel and Hirt 2004; Halliwell 2006). The reactivity of Cu not only makes it highly suitable for redox activities but also renders it more toxic causing destruction of thylakoid structure of chloroplasts, and considerable modification of the lipid and protein composition of thylakoid membrane (Maksymiec 1997), even at mild excess. Free copper ions can readily oxidize the thiol bond present in the proteins, causing disruption of their structure and functions. The destruction of membrane lipids and leakage of electrolytes from root membranes was reported to result from enhanced generation of $O_2^{\cdot-}$ in both Cu-deficient and Cu-excess plants (Quartacci et al. 2001). Several reports concerning excess-Cu-induced oxidative stress and antioxidant responses are available (Raeymaekers et al. 2003; Morelli and Scarano 2004; Lombardi and Sebastiani 2005; Maksymiec and Krupa 2006; Doncheva et al. 2006; Tewari et al. 2006). However, we did not find any significant change in the accumulation of $O_2^{\cdot-}$ and H_2O_2 in the leaves of mulberry plants supplied excess Cu, though, there were increases in the accumulation of thiobarbituric acid reactive substance (TBARS) and activities of antioxidant enzymes in these plants (Tewari et al. 2006).

Apart from copper, excess of iron also induces oxidative damage (Becana et al. 1998; Kampfenkel et al. 1995). It is also an active ingredient of redox centers of various functional proteins and enzymes (Motta et al. 2001). Iron overload is toxic due to the iron-dependent conversion of $O_2^{\cdot-}$ and H_2O_2 (produced in the chloroplasts when light is in excess of its capacity to use the photochemical products) into the extremely reactive $OH^{\cdot}$ in the Haber–Weiss reaction (Motta et al. 2001; Halliwell 2006). Moreover, Fe^{2+} also participates in antioxidant defense, as it is an integral component of various antioxidant enzymes responsible for H_2O_2 (CAT, POD, APX) and $O_2^{\cdot-}$ (Fe-SOD) scavenging (Apel and Hirt 2004). Therefore, excess of Fe on one hand is lethal and on other hand, is known to protect plants from oxidative damage by scavenging reactive oxygen species (ROS) from the cellular environment. Earlier studies suggested that chlorosis caused by toxicities of several heavy metals, including that of Cu, were identical to that caused by deficiency of Fe (Hewitt 1963; Taylor and Foy 1985) and that the chlorosis induced by Cu toxicity could be ameliorated by supply of Fe (Mehrotra et al. 1976). Hauck et al. (2003) reported that excess supply of Mn and Cu caused intracellular Fe deficiency in the lichen *Hypogymnia physodes* and that the effects of Mn and Cu toxicity could be alleviated by supply of $FeCl_3$ in moderate (500 μM) concentrations, though higher (1 mM) supply of $FeCl_3$ caused toxicity more severe than that of Mn or Cu. Studies made by Chen et al. (2004), Ylivainio et al. (2004) and Cornu et al. (2007) have also indicated that status of Fe in rooting medium has a reciprocal relationship towards Cu uptake by the plants. Hewitt (1963) and Mehrotra et al. (1976) discussed at length the various mechanisms of interaction of Fe with heavy metals including Cu and concluded that, although toxicities of heavy metals induced chlorosis similar to that due to Fe deficiency, they also produced effects other than chlorosis that could be specific to the metal toxicity. It has been shown that chlorosis in plants with high concentrations of Cu may result from membrane damage due to lipid peroxidation (Pätsikkä et al. 2002). However, no satisfactory explanation of amelioration of toxicities of heavy metals by Fe is available. The work presented here was, therefore, aimed to study if amelioration of Cu toxicity effects by excess supply of Fe involves mitigation of Cu toxicity-induced oxidative stress by induction of antioxidative activity in the plants.

Materials and methods

Plant material, growth conditions and treatments

Experiment 1:

Maize (*Zea mays* L. cv. 777) plants were grown in solution culture under greenhouse conditions. Initially, the plantlets were grown in acid-washed sand and were supplied glass-distilled water. After 15 days, when roots in the plants were sufficiently induced, plantlets were transplanted to 3.0 l plastic buckets containing aerated nutrient solution having composition (Hewitt 1966): 2.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.67 mM NaH_2PO_4, 0.05 mM NaCl, 0.05 mM Fe-K_2EDTA, 5.0 μM $MnSO_4$, 0.5 μM $CuSO_4$, 1.0 μM $ZnSO_4$, 16.5 μM H_3BO_3, 0.1 μM Na_2MoO_4, 0.05 μM $CoSO_4$ and 0.05 μM $NiSO_4$. The pH of the nutrient solution was maintained at 6.7 ± 0.2. After a week of transplantation, pots were grouped into 2 lots having 4 pots each. The lot 1 continued to receive above basal nutrient medium and considered it to be the control. Plants in lot 2 received an excess (100 μM) supply of $CuSO_4$ superimposed on the basal nutrient medium. The volume of nutrient solution in the pots was made-up daily by de-ionized water. The nutrient solution was refreshed every alternate day. Plants were analyzed for various

parameters at different time interval after Cu supply as indicated in the tables and figures. The greenhouse conditions during the experiment were: maximum photosynthetic photon flux density (12:00 noon) 1155–1315 μmol m^{-2} s^{-1}, daily maximum and minimum temperatures 33.3–36.4°C and 24.4–28.6°C, respectively and R.H. (9:00 AM) 70–80%. The average photoperiod was 12:00 ± 0:20 h.

Experiment 2:

For this experiment maize plants were grown and maintained strictly as described in experiment 1. Here plants were grouped in 4 lots. Plants in lot 1 received only basal nutrient medium as described elsewhere and considered as control. Plants in lots 2 and 3 were respectively supplied 500 μM of Fe-K_2EDTA and 100 μM $CuSO_4$ and in lot 4 along with 100 μM of $CuSO_4$, 500 μM Fe-K_2EDTA also was supplemented. Analysis for various parameters was performed 15 days after differential treatments. In another set of plants we supplied 500 μM of K_2-EDTA but we observed them to be absolutely similar to that of control and did not induce any ill effects, therefore data of these plants have not been provided.

Visual observation, dry matter yield, water potential and copper concentration

The visual symptoms were recorded day to day. Finally, 37 day after initiating treatment (DAT) the plants were harvested, separated into roots and shoots and then dried in an oven at 80°C for 48 h and weighed. Samples for water potential (Ψ) measurement were taken from the fourth expanded leaf. The measurements were made hygrometrically on five leaf discs (11 mm diameter) cut from three leaves taken from randomly selected plants, using a Wescor (Logan, UT) microvoltmeter (model HR 33T) and C-52 leaf chambers. Copper was estimated in HNO_3: $HClO_4$ (10:1 [v/v]) digest of young leaves by atomic absorption spectrophotometer.

Chloroplastic pigments, hydrogen peroxide and lipid peroxidation

For measuring the concentrations of chlorophylls and total carotenoids, 100 mg fresh leaf tissue was extracted in 25 ml 80% (v/v) acetone by the method of Lichtenthaler (1987). H_2O_2 concentration was determined as H_2O_2–titanium complex formed by reaction of tissue-H_2O_2 with titanium tetrachloride by the method of Brennan and Frenkel (1977). Lipid peroxidation was determined by method of Heath and Packer (1968) in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction. The amount of TBARS was calculated from the difference in absorbance at 532 and 600 nm using extinction coefficient of 155 mM^{-1} cm^{-1}.

In situ detection of superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide

Histochemical staining of tissues for $O_2^{\cdot-}$ and H_2O_2 in the fourth youngest expanded leaves and small segments derived from newly emerging roots was performed as described by Hernández et al. (2001) with minor modification. Detection of $O_2^{\cdot-}$ was made by vacuum infiltrating leaf quarters/discs and small segments of roots with 0.1 mg ml^{-1} solution of *p*-nitroblue tetrazolium (NBT) in 0.2 M sodium phosphate buffer (pH 7.6) for 15 min and then incubating at 25°C in the dark for 2 h. Controls or blanks in presence of SOD + $MnCl_2$ (scavengers of $O_2^{\cdot-}$) were also performed simultaneously by adding 100 unit of SOD and 10 mM of $MnCl_2$ into the infiltration buffer.

For in situ detection of H_2O_2 an endogenous peroxidase dependent histochemical staining was used. The leaf quarters/discs and small segments of roots were vacuum infiltrated with 0.1 mg ml^{-1} 3, 3′-diaminobenzidine in 50 mM Tris-acetate buffer (pH 5.0) for 15 min and then incubated at 25°C in dark for 24 h on an orbital shaker (200 rpm). Control or blanks for H_2O_2 was performed in presence of 10 mM AsA in the infiltration buffer. Pigments present in the leaf discs were cleared in 80% (v/v) ethanol for 20 min at 70°C and then mounted in lactic acid, phenol and water mixture (1:1:1, v/v). The mounted leaf discs were photographed using Nikon advanced research microscope (Model E-400) with photographic system (H-III) and roots were photographed using Nikon camera.

Enzyme extraction and protein determination

Fresh tissue (2.5 g) of fourth expanded youngest leaves was homogenized in 10.0 ml chilled 50 mM potassium phosphate buffer (pH 7.0) containing 0.5% (w/v) insoluble polyvinylpolypyrrolidone and 1.0 mM phenylmethylsulfonylfluoride in a chilled pestle and mortar kept in ice bath. The homogenate was filtered through two fold muslin cloth and centrifuged at 20,000×*g* for 10 min at 2°C. The supernatant was stored at 2°C and used for enzyme assays within 4 h. For the assay of APX activities, 5.0 mM AsA was also included in the extraction medium. The protein concentration in the homogenate was determined in the TCA precipitate according to Lowry et al. (1951).

Assays of enzymes

SOD (EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT at 560 nm. The reaction mixture (5.0 ml) contained 25 mM phosphate buffer pH 7.8, 65 μM NBT, 2.0 μM riboflavin, enzyme extract [equivalent to 20 mg fresh matter (80 μl) with water (220 μl)], and 15 μl *N,N,N′,N′*-tetramethylethylenediamine (TEMED; modified from Beauchamp and Fridovich, 1971). Reaction mixture was exposed to light (350 μmol m^{-2} s^{-1}) for 15 min. Fresh matter equivalent of enzyme extract corresponding to 50% inhibition of reaction was considered as one enzyme unit. The activity is expressed as units min^{-1} mg^{-1} protein.

APX (EC 1.11.1.11) was measured in 3.0 ml reaction mixture containing 50 mM phosphate buffer pH 7.0, 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA and suitable quantity (40 μl) of enzyme extract (equivalent to 10 mg fresh matter); both minus tissue-extract and minus H_2O_2 blanks were run and the changes in absorbance every 15 s were read at 290 nm (Nakano and Asada 1981). The activity of APX was calculated in terms of μmol ascorbate oxidized min^{-1} mg^{-1} protein.

CAT (EC 1.11.1.6) activity was estimated in a reaction mixture containing 500 μmoles H_2O_2 in 10 ml 100 mM phosphate buffer (pH 7.0) and tissue extract [equivalent to 20 mg fresh matter (80 μl) with water (920 μl)]. H_2O_2 decomposed after 5 min reaction was assayed by titrating the reaction mixture with 0.1 N $KMnO_4$ [as described earlier by Bisht et al. (1989)]. CAT activity is expressed as μmol H_2O_2 decomposed min^{-1} mg^{-1} protein.

POD (EC 1.11.1.7) activity was estimated after Bisht et al. (1989) in a reaction mixture containing 5.0 ml 100 mM phosphate buffer pH 6.5, 1.0 ml 0.5% (w/v) *p*-phenylenediamine, 1.0 ml 0.01% (v/v) H_2O_2 and tissue extract [equivalent to 10 mg fresh matter (40 μl) with water (960 μl)]; change in absorbance after 5.0 min was measured at 485 nm. The enzyme activity has been expressed as units min^{-1} mg^{-1} protein. The enzyme unit is defined as ΔA_{485} of 0.01 between the blank and the sample min^{-1} of reaction time.

Native PAGE and localization of enzymes on polyacrylamide gels

Polyacrylamide gel electrophoresis was carried out for separation of different enzyme isoforms using discontinuous gel under non-denaturing condition essentially as described by Hames (1990). Ten per cent or 7.0% native gels were prepared and run at constant potential (200 V) and current (30 mA) at 4.0°C using a discontinuous buffer system as described by Hames (1990).

SOD was localized on 10% native gel using photochemical method of Beauchamp and Fridovich (1971). SOD activity was localized by soaking the gels in 2.45 mM NBT for 20 min followed by immersion in a solution containing 28.0 mM TEMED, 0.028 mM riboflavin, and 36 mM potassium phosphate buffer (pH 7.8). The gels were then placed on a clean glass plate and illuminated by cool florescent light for 2–3 min. Different species of SOD isoforms was identified by incubating gels either in 2.0 mM KCN (an inhibitor of Cu/Zn-SOD) or in the 5.0 mM H_2O_2 (an inhibitor of Cu/Zn-SOD and Fe-SOD).

Method of Mittler and Zelinska (1993) was used for localizing APX activity on the 10% native gels. Gel was equilibrated for 20 min in the solution containing 50 mM potassium phosphate buffer (pH 7.0), 4 mM AsA and 2 mM H_2O_2. The gel was washed in buffer with gentle agitation for 1 min and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT with gentle agitation. The APX activity was observed as achromatic bands on a purple blue background. The reaction was allowed to continue for approximately 10 min more and stopped by a brief wash with glass-distilled water.

Activity staining of CAT was performed as per description of Anderson et al. (1995). Isoforms of CAT were separated on the 7.0% native gel and the gel was equilibrated with 3.27 mM H_2O_2 for 20 min. After rinsing with water gel was stained for 3 min in a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) $FeCl_3$.

Electrophoretic separation of POD was performed on 10% native gel. POD was localized by soaking the gel in the reaction mixture (Luck 1963) containing 20 ml of 0.1 M phosphate buffer (pH 7.0) and 4 ml of 0.01% (v/v) H_2O_2 and 4.0 ml of 0.5% (w/v) *p*-phenylenediamine for 20 min. Then it was rinsed with glass-distilled water and scanned immediately (within 10 min.). Native gels stained for activities of SOD, APX, CAT and POD were scanned on a Bio-Red gel imaging densitometer model GS-690 using the "Multi-Analyst" software from Bio-Rad.

Statistical analysis

All results are the mean of three independent experimental replicates ($n = 6$). The data were analyzed by analysis of variance (ANOVA) and tested for significance by Bonferroni *t*-test using Sigma-stat software.

Table 1 Effects of copper supply (100 μM) on growth attributes and Cu concentration in the fourth expanded leaves of maize (*Zea mays* L.) cv. 777, plants grown in solution culture [Data at 37 days of growth and 14 days after treatment (DAT)]

Parameters	DAT	Control	Copper (100 μM)
Fresh wt (g plants^{-1})	0	38.97 ± 2.2 c	
	14	148.82 ± 1.0 a	75.16 ± 5.47 (–49.50)b
Shoot/roots (Fresh wt)	0	6.27 ± 0.95 a	
	14	6.59 ± 1.5 a	2.71 ± 0.12 (–58.88)b
Dry wt (g plant^{-1})	0	6.24 ± 1.1 c	
	14	23.31 ± 1.2 a	11.66 ± 1.14 (–49.98)b
Shoot/roots (Dry wt)	0	11.23 ± 1.5 a	
	14	10.15 ± 3.3 a	3.57 ± 0.45 (–64.83)b
Copper (μg g^{-1} leaf dry wt)	0	10.02 ± 0.01 a	
	14	10.01 ± 0.01 a	82.52 ± 0.01 (+724.37)b

Data represent the means ± SE of three experimental replicates; values with different letters in the same row are significantly different ($P \leq 0.05$). Values given in parentheses denote percent decrease (–) or increase (+) over the control

Results

Growth, visual effects, water potential and metal concentration

Both fresh and dry weight of Cu-excess plants was decreased. Copper excess suppressed stem growth and plants had stunted appearance. Root growth was also inhibited by excess supply of Cu. However, a low shoot/root ratio in plants supplied Cu-excess suggests that the effect of Cu toxicity was more pronounced in the shoot despite of the fact that roots were continuously bathed in toxic concentration of Cu (Table 1). The visible effect of Cu toxicity became perceptible in the form of wilting in the young leaves on the very first day of excess supply of Cu. Later, these leaves showed shrinkage of tissues in the apical region of leaf laminae, which finally turned brown and necrotic. Apart from this, Cu-excess also induced accelerated senescence of older leaves. Decreased water potential (Fig. 1a) and consequent wilting of leaves of Cu-excess plants suggests malfunctioning of water uptake system of the roots. The plants supplied Cu-excess also showed increased accumulation of Cu in the leaf tissue (Table 1). Maize plants supplied Fe-excess also produced dark bronzed leaf and showed significant decline in water potential (Fig. 1b). However, maize plants supplied excess-Cu along with excess (500 μM) Fe, showed an improvement in their phenotypic appearance and in the water potential of leaf (Fig. 1b). These plants also showed increased dry matter (Fig. 2a), lowered the concentration of Cu and improved tissue Fe status compared to excess-Cu supplied plants (Fig. 2b).

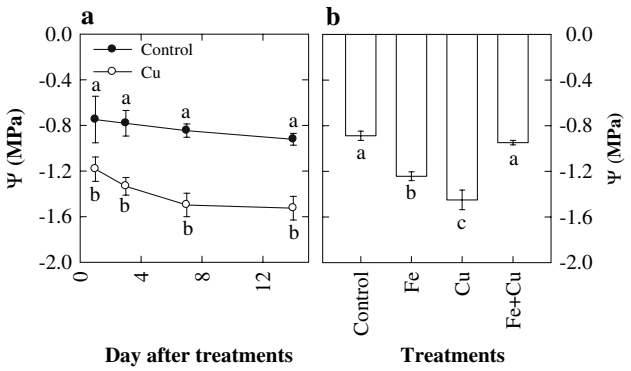

Fig. 1 Temporal effect of excess (100 μM) supply of Cu (**a**) at different days after treatment (DAT) and modulation by excess Fe (500 μM) supply at 15 DAT (**b**) on the water potential of young expanded leaves of maize plants grown in solution culture. The data plotted represent mean ± SE of replicates ($n = 6$). Symbol carrying different letters are significantly different on the same day at $P \leq 5\%$ by Bonferroni *t*-test

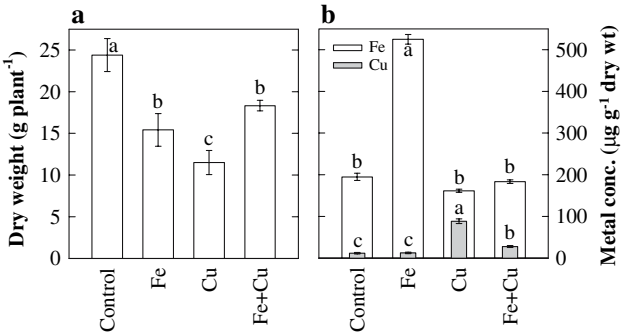

Fig. 2 Effect of excess (100 μM) Cu and modulation of Cu-toxicity by supplementing excess Fe (500 μM) to excess Cu (100 μM) plants on the dry weight (**a**), and Fe (**b** *vacant bar*) and Cu (**b** *gray bar*) concentrations in the young expanded leaves of maize plants grown in solution culture. The data plotted represent mean ± SE of replicates ($n = 6$) at 15 DAT. Bars carrying different letters are significantly different for same parameter at $P \leq 5\%$ by Bonferroni *t*-test

Chloroplastic pigments

Supply of excess-Cu decreased the concentrations of chlorophylls and carotenoids. Though changes in carotenoids or chlorophylls concentrations were not statistically significant at 7 days of excess-Cu supply, both chlorophyll *a* and chlorophyll *b* were significantly decreased at 14 DAT but chlorophyll *a/b* ratio was not affected in Cu-excess plants (Table 2). Also, ratio of car/ chl also did not change in plants receiving excess-Cu supply (Table 2). However, excess supply of Fe was not found to cause any major change in chloroplastic pigments (Fig. 3). Moreover, an improvement in the concentrations chloroplastic pigments

Table 2 Effects of copper supply (100 μM) on the concentrations of chlorophylls and carotenoids in the fourth expanded young leaf tissue of maize (*Zea mays* L.) cv. 777 plants grown in solution culture [Data at 30 or 37 days of growth and 7 or 14 days after treatment (DAT)]

Pigments	DAT	Control	Copper (100 μM)
Chlorophyll *a*	7	2.17 ± 0.040 a	1.97 ± 0.056 (–9.21%)a
	14	2.66 ± 0.024 a	2.12 ± 0.026 (–20.3%)b
Chlorophyll *b*	7	0.80 ± 0.034 a	0.75 ± 0.043 (–6.3%)a
	14	1.18 ± 0.011 a	0.97 ± 0.013 (–17.8%)b
Carotenoids	7	0.41 ± 0.005 a	0.39 ± 0.022 (–9.8%)a
	14	0.38 ± 0.007 a	0.32 ± 0.003 (–15.7%)a
Total chl	7	2.96 ± 0.073 a	2.72 ± 0.167 (–8.11%)a
	14	3.84 ± 0.035 a	3.10 ± 0.031 (–19.27%)b
Chl *a*/*b*	7	2.71 ± 0.064 a	2.62 ± 0.073 (–3.32%)a
	14	2.25 ± 0.011 a	2.19 ± 0.005 (–19.18%)a
Car/chl	7	0.140 ± 0.008 a	0.142 ± 0.006 (+7.14%)a
	14	0.099 ± 0.001 a	0.103 ± 0.003 (+4.04%)a

Data represent the means ± SE of three experimental replicates; values with different letters in the same row are significantly different ($P \leq 0.05$). Values given in parentheses are percent decrease (–)/increase (+) over the control

was observed on supply of excess-Fe (500 μM) to Cu-excess plants (Fig. 3).

Hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substance (TBARS)

The concentration of H_2O_2 increased in the leaves of plants supplied excess of Cu at 7 DAT, and the magnitude of the

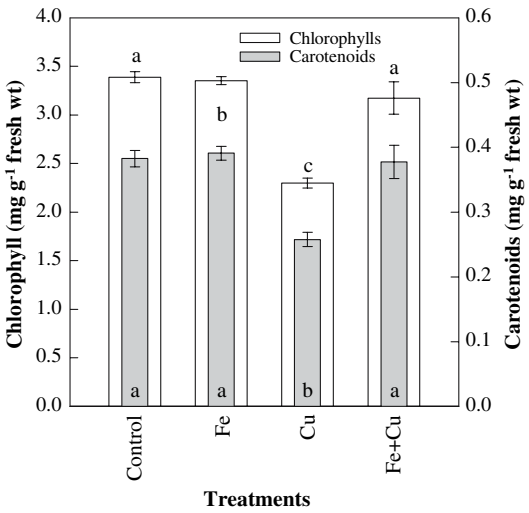

Fig. 3 Effect of excess (100 μM) Cu and modulation of Cu-toxicity by supplementing excess Fe (500 μM) to excess Cu (100 μM) plants on the chlorophylls (*vacant bars*), carotenoids (*gray bars*) in the young expanded leaves of maize plants grown in solution culture. The data plotted represent mean ± SE of replicates ($n = 6$) at 15 DAT. Bars carrying different letters are significantly different for same parameter at $P \leq 5\%$ by Bonferroni *t*-test

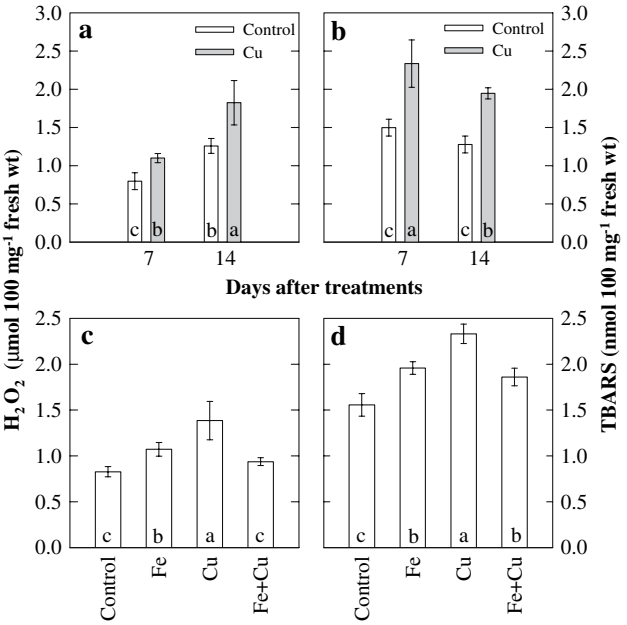

Fig. 4 Effect of excess (100 μM) supply of Cu (**a**, **b**) at 7 and 14 DAT, and modulation of Cu-toxicity by supplementing excess Fe (500 μM) to excess Cu (100 μM; **c**, **d**) plants at 15 DAT on the concentrations of H_2O_2 (**a**, **c**) and TBARS (**b**, **d**) in the expanded young leaves of maize plants grown in solution culture. The vertical bars represent mean ± SE of replicates ($n = 6$). Bars carrying different letters are significantly different at $P \leq 5\%$ by Bonferroni *t*-test

effect was increased further after 14 days of Cu treatment (Fig. 4a). TBARS an equivalent of MDA and an index of lipid peroxidation, was also increased significantly and the effect was more marked at 7 DAT (Fig. 4b). An excess supply of Fe also increased H_2O_2 concentration and TBARS but extents of their accumulation were less compared to Cu-excess plants (Fig. 4a, b). Concentrations of H_2O_2 and TBARS decreased in excess-Fe supplemented Cu-excess plants compared to Cu-excess plants and became almost similar to non-stressed (control) plants (Fig. 4c, d).

In situ localization of superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide

Superoxide anion radical as indicated by purplish blue color of formazan formed from the reduction of NBT, was declined in the leaf (Fig. 5c) and root segments (Fig. 5g) of excess-Cu supplied plants. Contrary to $O_2^{\cdot-}$, accumulation of H_2O_2 was increased as indicated by intensified H_2O_2 specific dark brown precipitate formed from diaminobenzidine (DAB) in the leaf (Fig. 5k) and root segments (Fig. 5o) of Cu-excess plants. Plants supplied excess-Fe enhanced intensity of purplish blue formazan, particularly in the root segments (Fig. 5f), and dark brown precipitate

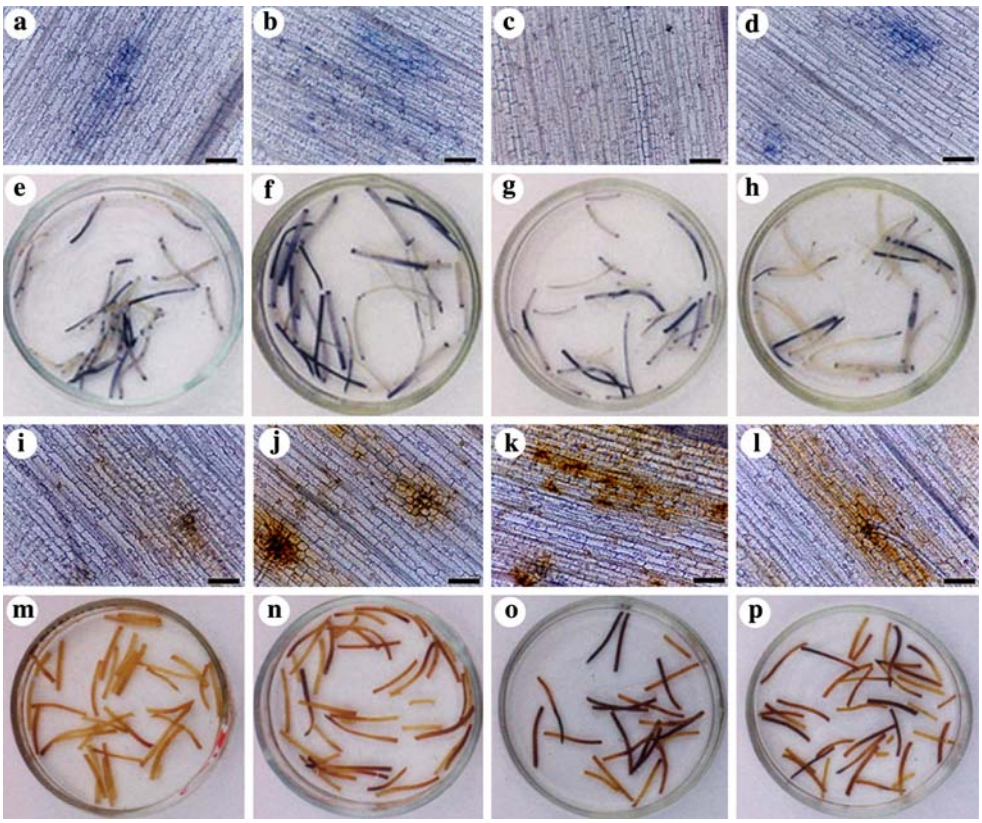

Fig. 5 Histochemical localization of $O_2^{\cdot-}$ (**a**–**h**) and H_2O_2 (**i**–**p**) in the expanded young leaves or roots of maize plants grown in solution culture. Accumulation of $O_2^{\cdot-}$ (**a**–**h**) in the leaf (**a**–**d**) or root (**e**–**h**) segment of control (**a**, **e**), excess Fe (**b**, **f**) excess Cu (**c**, **g**), and excess Fe-supplemented Cu-excess (**d**, **h**) plants. Accumulation of H_2O_2 (**i**–**p**) in the leaf (**i**–**l**) or root (**m**–**p**) segments of control (**i**, **m**) excess Fe (**j**, **n**), excess Cu (**k**, **o**) and excess Fe-supplemented Cu-excess (**l**, **p**) maize plants. Bars = 100 μm

formed from DAB (Fig. 5j, n), representing $O_2^{\cdot-}$ and H_2O_2 accumulation respectively. Though supplementation of excess-Fe to excess-Cu plants, improved generation of $O_2^{\cdot-}$ (became comparable to those of control plants) in the leaf (Fig. 5d) and the root segments (Fig. 5h) as indicated by intense purplish blue coloration in the analysed segments. Moreover, accumulation of H_2O_2 was declined in both tissues i.e. the leaf (Fig. 5l) and root (Fig. 5p) segments on supplementation of excess-Fe to excess-Cu plants.

Antioxidant enzymes

Activities of antioxidant enzymes were found to be altered by excess supply of Cu. Activity of SOD was increased in Cu-excess plants. The effect was much higher during initial stages, up to 7 DAT, which later on became steady but increase remained significant even at 14 DAT (Fig. 6a). Activity of APX (Fig. 6b) was increased enormously at all the stages under study, after excess-Cu treatment. Isoforms of antioxidant enzymes—SOD (Fig. 6c), APX (Fig. 6d), and POD (Fig. 7d), were qualitatively increased in their bands intensity and in none of these enzymes any additional isoforms of enzyme was induced on exposure of the plants to excess-Cu. Activity of CAT was not altered significantly by excess supply of Cu (Fig. 7a). Similarly, isoforms of CAT also showed little variation in the bands between control and Cu-excess plants (Fig. 7c). Apart from the marginal decline in magnitude of increase on third day after excess-Cu supply, activity of POD (Fig. 7b) remained consistently higher throughout period of investigation (Fig. 7b) in these plants. In Fe-excess plants though activity of SOD did not change (Fig. 8a) but activities of H_2O_2 scavenging enzymes (POD, CAT and APX) were increased (Fig. 8b–d). Activities of these antioxidant enzymes (CAT, POD, APX and SOD) were further upregulated (Fig. 8) in the Fe-supplemented Cu-excess plants. Higher activities of H_2O_2-scavenging enzymes—CAT, POD and APX, have probably provided sufficient antioxidant protection and reduced accumulation of H_2O_2 in the excess-Fe supplemented Cu-excess plants.

Discussion

Excess supply of Cu suppressed plant growth, reduced biomass production (Table 1, Fig. 2a) and produced visible

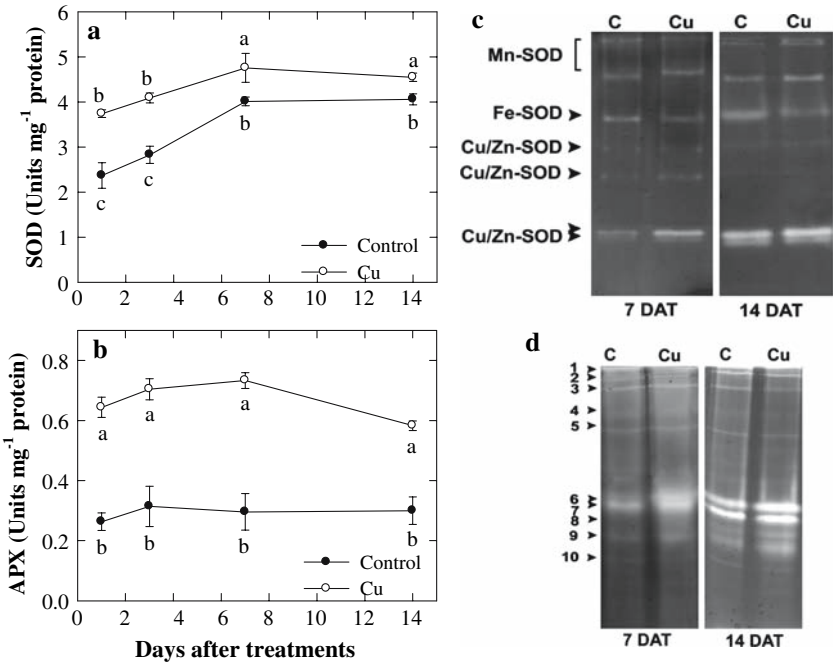

Fig. 6 Temporal effect of excess (100 μM) supply of Cu on the activity of SOD (**a**), APX (**b**) and activity stained gel for SOD (**c**) and APX (**d**) isoforms at different DAT in the expanded young leaves of maize plants grown in solution culture. For in gel activities of SOD and APX 80 μg (7 DAT) and 100 μg (14 DAT) protein was loaded in the lanes representing control and excess-Cu. The data plotted represent mean ± SE of replicates (n = 6). *Symbol* carrying *different letters* are significantly different on the same day at $P \leq 5\%$ by Bonferroni t-test

effects of Cu toxicity implying that the concentration of Cu (100 μM) provided in the growth medium of plants was able to exert its toxic effects. This assumption is further supported by nearly seven folds increase in Cu concentration in the leaves of maize plants supplied Cu-excess. Considering 5–20 μg g^{-1} Cu in plant tissues as normal (Reuther and Labanauskas 1966), excessive accumulation of Cu in Cu-excess plants might have ultimately caused oxidative damage. It has previously been reported that excess-Cu induces oxidative stress (Pätsikkä et al. 2002; Babu et al. 2003; Raeymaekers et al. 2003; Morelli and Scarano 2004; Lombardi and Sebastiani 2005; Tewari et al. 2006; Doncheva et al. 2006).The observed necrosis in the affected foliage may be attributed to the enhanced accumulation of H_2O_2. Accelerated rate of senescence in Cu-excess plants also appears to be a resultant of enhanced generation of H_2O_2. This enhanced senescence in Cu-excess plants appears to be a mechanism of getting rid of excess-Cu by shedding off Cu-rich older leaves. Furthermore, excess Cu also induced water deficit in Cu stressed

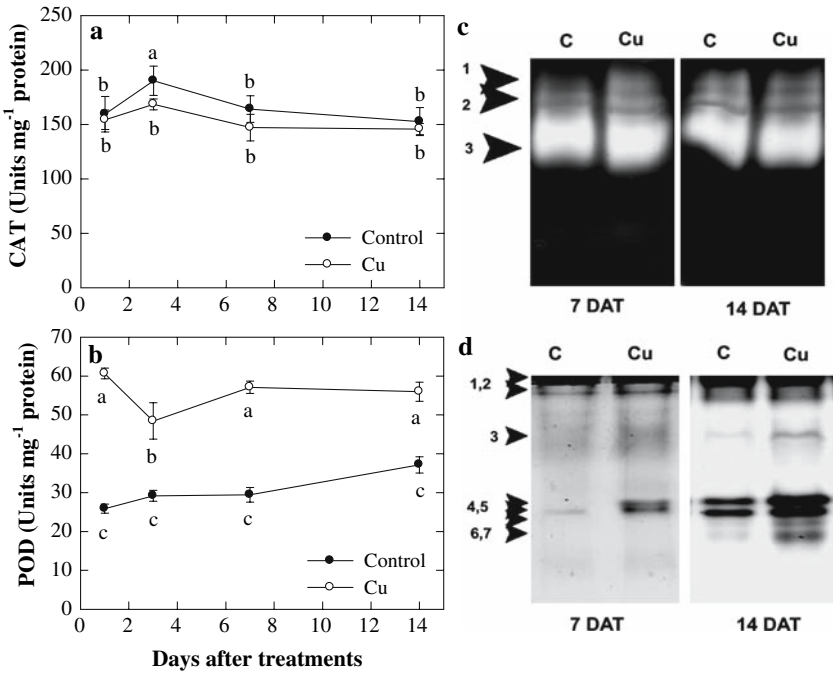

Fig. 7 Temporal effect of excess (100 μM) supply of Cu on the activity of CAT (**a**), POD (**b**) and activity stained gel for CAT (**c**) and POD (**d**) isoforms at different DAT in the young leaves of maize plants grown in solution culture. For in gel activity of CAT 80 μg protein was loaded in both control and Cu-excess at both time point (7 and 14 DAT). For in gel activity of POD 80 μg (7 DAT) and 100 μg (14 DAT) protein was loaded in lanes representing control and excess-Cu. The data plotted represent mean ± SE of three experimental replicates (n = 6). *Symbol* carrying *different letters* are significantly different on the same day at $P \leq 5\%$ by Bonferroni t-test

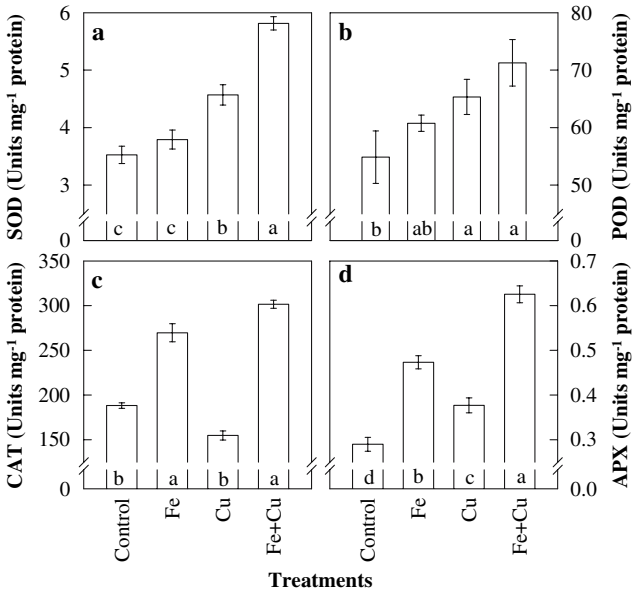

Fig. 8 Effect of excess (100 μM) Cu and modulation of Cu-toxicity by supplementing excess Fe (500μM) to excess Cu (100 μM) plants on the activities of SOD (**a**), POD (**b**), CAT (**c**), and APX (**d**) in the young expanded leaves of maize plants grown in solution culture. The data plotted represent mean ± SE of replicates ($n = 6$) at 15 DAT. Bars carrying different letters are significantly different for same parameter at $P \leq 5\%$ by Bonferroni t-test

plants, as indicated by decrease in Ψ (Fig. 1a, b) in the young foliage of plants (also observed in our earlier studies, Tewari et al. 2006), and also by curling of leaf apices and marginal scorching followed by drying of leaves. Excess Cu has been reported to decrease tissue water percentage of cucumber plants (Burzyński and Klobus 2004). Induction of water deficit might have also contributed to oxidative damage thereby aggravating Cu-toxicity. Presence of excess Fe and Cu in the growth medium retarded uptake of themselves competitively (Fig. 2b) and therefore Cu-excess plants supplemented with excess-Fe showed better growth and dry mass almost similar to that of control plants (Fig. 2a). Moreover, accumulation of a higher concentration of Fe in the Fe-excess plants corroborates a common uptake mechanism for both Fe and Cu (Fig. 2b). Increase in the concentration of Cu in Fe-deficient pea (Iturbe-Ormaetxe et al. 1995) further supports a common uptake mechanism for Cu^{2+} and Fe^{2+} ions and it has been shown that they compete with each other during uptake process (Schmidt et al. 1997; Patsikka et al. 2002; Chen et al. 2004). Apart from this, excess-Cu also declined the concentration of chloroplastic pigments, which recovered on supplementation of excess-Fe to Cu-excess plants. Copper toxicity is known to damage ultrastructure of chloroplasts, decrease PS II activity (Vassilev et al. 2003; Huang and Tao 2004; Perales-Vela et al. 2007) and induce photoinhibition (Pätsikkä et al. 2001). Since, Fe is well known to be involved in the syntheses of chloroplastic pigments and thylakoid proteins (Marschner 1995), supplementation of excess-Fe to Cu-excess plants provided a sort of protection by out-competing excess Cu (Fig. 2b) and therefore plants attained relatively better growth (Fig. 2a) and had increased concentration of chloroplastic pigments (Fig. 3) compared to the plants supplied excess-Cu only.

Copper toxicity though decreased $O_2^{\cdot-}$ accumulation (Fig. 5c, g), but it increased the activity of SOD (Figs. 6a, c, 8a) and accumulation of H_2O_2 (Figs. 4a, c, 5k, o)—a dismutation product of $O_2^{\cdot-}$, suggesting induction of oxidative stress in the Cu-stressed plants. The water stress condition in the Cu-excess plants as observed here could also have contributed to the increased H_2O_2 production (Figs. 4a, c) and induction of oxidative stress (Smirnoff 1993). Observed increase in TBARS (Fig. 4b, d) in the young leaves of Cu-stressed plants is suggestive of oxidative damage as a consequence of increased H_2O_2 production in these plants. High accumulation of H_2O_2 both in the leaves and roots segments of Cu-stressed plants, as visualized by histochemical localization (Fig. 5k, o), further corroborates oxidative stress in the Cu-excess maize plants. Nonetheless, over-accumulation of H_2O_2 in the leaves as well as in the roots appears to be a consequence of retarded H_2O_2 scavenging CAT activity in Cu-stressed plants (Figs. 7a, c, 8c) and CAT appears to be principally involved in H_2O_2 detoxification rather than other H_2O_2 scavenging POD and APX in the Cu-stressed maize plants. A reduction in H_2O_2 accumulation in excess-Fe supplemented Cu-excess plants (Fig. 4c) compared to Cu-excess plants, wherein recovery of CAT (Fig. 8c) activity was observed, further substantiates this supposition. Though, increased activity of SOD (Fig. 6a, c) is attributable to increased generation of $O_2^{\cdot-}$ in Cu-excess plants but increased $O_2^{\cdot-}$ accumulation was not observed in the leaves (Fig. 5c) as well as in the roots (Fig. 5g) of Cu-excess maize plants. However, accumulation of $O_2^{\cdot-}$ radical was higher in the roots and in the leaves of Fe-excess (Fig. 5b, f) and in the excess-Fe supplemented Cu-excess plants (Fig. 5d, h). Decline in the $O_2^{\cdot-}$ anion accumulation in Cu-excess plants is contrary to earlier observations (Quartacci et al. 2001; Babu et al. 2003; Maksymiec and Krupa 2006). A similar decline in the $O_2^{\cdot-}$ concentration has recently been observed in the Cu-stressed adventitious roots of mountain ginseng (Tewari et al. 2007). Decline in the accumulation of $O_2^{\cdot-}$ anion in the leaves and root segments of Cu-excess plants, is speculative of inhibition of NADPH-oxidase (one of the enzyme involved in $O_2^{\cdot-}$ generation) activity by high concentration of Cu in the leaves. Activity of this enzyme is probably recovered after supplementation of excess-Fe to Cu-excess plants

resulting into the accumulation of O_2^- (Fig. 5d, h) similar to that of control plants.

Observed increase in SOD activity in the plants supplied excess-Cu (Fig. 6a), mainly due to increase in Cu/Zn SOD (Fig. 6c), appears to be related with high Cu supply to these plants. The electrophoretic separations showed that all three forms of SOD viz., Mn-SOD, Fe-SOD and Cu/Zn-SOD, exist in the maize plants (Fig. 6c). There was marked induction in two isoforms of Cu/Zn SOD in plants supplied excess of Cu. Fe-SOD, on the other hand, showed little suppression in Cu-excess plants (Fig. 6c). The suppression of Fe-SOD in Cu-excess plants suggests a negative relationship between Cu supply and Fe-SOD induction. Decreased activity of Fe-SOD in Cu-excess plants appears to be compensated by increased induction of Cu/Zn-SOD. An upregulation of Fe-SOD in the Cu-deficient plant has been reported (Murao et al. 2004; Tewari et al. 2006). Induction of Cu/Zn-SOD and Mn-SOD for suppressed Fe-SOD has also been reported in the Cu-stressed (excess) *Lemna gibba* (Babu et al. 2003). In addition, the differences in the upstream regions of Fe-SOD genes with those of Cu/Zn-SOD and Mn-SOD suggest that there could be differential regulation of gene expression (Alscher et al. 2002). Oxidative stress in Cu-excess plants was also indicated, apart from high SOD, by increased activities of other antioxidant enzymes viz. APX (Fig. 6b, d) and POD (Fig. 7b, d). Increased accumulation of TBARS despite activation of protective mechanisms implies only partial effectivity of the antioxidative measures to cope with the oxidative challenge induced in Cu-excess plants. CAT activity was insignificantly decreased in plants supplied excess Cu (Figs. 7a, 8c). The isoforms of APX (Fig. 6d) and POD (Fig. 7d) were upregulated but isoform of CAT (Fig. 7c) again did not vary much by excess-Cu supply. Of the total 10 isoforms of APX induced in the maize plants, isoforms 4 and 10 were downregulated and rest others were upregulated by excess-Cu supply (Fig. 6d). POD isoforms were found to be dependent on growth stage of plants. A maximum 7 isoforms of POD was observed at 14 DAT and most of them were upregulated by excess Cu supply (Fig. 7d). This observation suggests that excess-Cu supply upregulated only pre-existing isoforms of POD but it might have some specificity in regard to isoforms of APX that is why it downregulated isoform 4 and 10 (Fig. 6d). Moreover, these results also indicate a differential regulation of genes involved for the induction of different isoforms of APX.

Upon supplementation of excess-Fe to Cu excess plants, while activity of CAT not only recovered but also was slightly higher than controls, activities of other antioxidant enzymes—POD, APX, and SOD were further increased resulting in the mitigation of excess-Cu induced oxidative challenge, which is reflected by a lower TBARS (Fig. 4d) and H_2O_2 (Fig. 4c) concentrations, higher chloroplastic pigments contents (Fig. 3), higher biomass (Fig. 2a) and overall improvement in plant health. Although both Fe^{2+} and Cu^{2+} are Fenton active metals and can produce extremely lethal $OH^{\cdot}$ radical in presence of O_2^- and H_2O_2 in Haber–Weiss reaction, however, excess-Fe together with excess-Cu did not cause any damage to the plants; Cu excess plants supplemented excess-Fe were fairly healthier than both excess-Fe or excess-Cu supplied plants (Figs. 2a, 4c, d). These observations suggest that excess-Fe mitigates excess-Cu induced oxidative challenge by outcompeting Cu uptake, improving Fe status, decreasing H_2O_2 concentration, and by increasing the activities of Fe-requiring H_2O_2 scavenging enzymes. So this Indian sayings appears to be true here that "*Vish vish ko marata hai aur loha lohe ko katata hai*". Translation of this saying is "Poison kills poison and iron cuts iron".

Conclusions

Excess of Cu induced oxidative stress by increasing generation of H_2O_2, decreasing statuses of water and Fe, and supplementation of excess Fe mitigated oxidative damage by out competing Cu uptake, improving water and Fe statuses, and activities of Fe-requiring antioxidant enzymes.

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