

Interaction of Mg with heavy metals (Cu, Cd) in *T. aestivum* with special reference to oxidative and proline metabolism

Vijeta Singh¹ · Bhumi Nath Tripathi² · Vinay Sharma³

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Abstract Little effort has been made to understand the influence of Mg on cellular processes of plant cell during Cu and Cd toxicities. The present work demonstrates the influence of magnesium (Mg) on copper (Cu) and cadmium (Cd) toxicity on *Triticum aestivum* (Wheat). We measured a range of parameters related to oxidative stress in wheat exposed to Cu or Cd toxicity in media with different concentrations of Mg. Decreasing Mg concentration significantly exacerbated Cu and Cd toxicity and optimum supply of Mg improved the growth and decreased the toxicity-induced oxidative stress (a substantial decline in the amount of hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) in root and shoot tissues). Activity of antioxidant enzymes—superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) was restored upon optimum Mg concentration in the presence of Cu and Cd toxicity. An increase in proline concentration in roots and shoots that was triggered by Cu and Cd exposure was partly reversed. This was due to decline in pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) activity and enhanced proline dehydrogenase (PDH) activity. In conclusion, decreasing supply of Mg effectively exacerbated the toxicities of Cu and Cd in wheat.

Keywords Cadmium · Copper · Heavy metals · Oxidative stress · Proline metabolism · *Triticum aestivum*

Introduction

Anthropogenic activities, such as mining, smelting, metal-based manufacturing and agricultural practices might contaminate the environment with heavy metals (Nriagu and Pacyna 1988). Some of these heavy metals including Cu, Zn, and Ni are required in trace amount for various metabolic activities of plants. However, an excess of any heavy metal, including essential nutrients, has deleterious impacts on plant metabolism (Hall 2002; Reichman 2002). Retarded growth, reduced photosynthesis, altered nutrient uptake, disturbed membrane permeability, and chlorosis are the major consequences of the heavy metal toxicity in plants (Sarvari et al. 1999).

The toxic effects of heavy metals are mostly attributed to (1) their affinity to bind with thionyl, histidyl and carboxyl groups in proteins, and (2) displacement/substitution of essential metal ions from enzymes (Hall 2002; Sharma and Dietz 2009). In addition, heavy metals are known to interfere with the oxidative metabolism of the plant cells as evident from several studies showing enhanced oxidation of lipids, proteins, and nucleic acids (Dat et al. 2000), thereby leading to modification (stimulation or inhibition) in the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) (Schutzen-dubel and Polle 2002). Besides altering oxidative metabolism, metals often enhance the intracellular concentrations of certain metabolites including proline and histidine. (Sharma and Dietz 2006). A metal-induced increase in the intracellular proline concentration has been well documented in various photosynthetic organisms, like cyanobacteria

✉ Vijeta Singh
vijetasingh1@gmail.com; vsingh9@amity.edu

¹ Amity Institute of Biotechnology, Amity University, Gautam Budha Nagar, Noida 201303, India

² Department of Botany, Guru Ghasidas University, Bilaspur 495009, Chhattisgarh, India

³ Department of Bioscience and Biotechnology, Banasthali University, Banasthali, Rajasthan, India

(Choudhary et al. 2007); algae (Mehta and Gaur 1999; Tripathi and Gaur 2004), and plants (Alia et al. 1995; Bassi and Sharma 1993; Chen et al. 2001; Costa and Morel 1994; Dhir et al. 2004; Farago and Mullens 1979; Schat et al. 1997). The proline accumulation has a protective role against metal toxicity as well as against other stresses (Sharma and Dietz 2006). Therefore, proline metabolism can be considered important for developing plant varieties resistant to multiple stresses.

Heavy metal toxicity in the environment depends on a variety of factors, especially availability of Ca^{2+} , Mg^{2+} , and Na^+ in the metal-contaminated soil. Magnesium, being the structural constituent of chlorophyll molecules, is an important macronutrient (Beale 1999; Marschner 1995). It acts as an activator or a regulator of several kinases, ATPases, ribulose-1, 5-bisphosphate (RuBP) carboxylase/oxygenase and several other enzymes of carbohydrate metabolism (Marschner 1995). A few studies (Deng et al. 2006; Keltjens and Dijkstra 1991; Keltjens and Tan 1993; Kinraide et al. 2004; Lock et al. 2007; Pedler et al. 2004; Saleh et al. 1999; Silva et al. 2001) have been published on the influence of Mg on metal toxicity, but consensus on the precise effect of Mg on metal toxicity is yet to emerge. Kinraide et al. (2004) reported the alleviation of rhizotoxicity of Cu and Zn by Mg in wheat. Improvement of Al tolerance in *Nicotiana benthamiana* was noticed after the over-expression of magnesium transport protein (AtMGT1) (Deng et al. 2006). In contrast, Saleh et al. (1999) did not notice any influence of Mg on Cu toxicity in sugarbeet.

The alleviation of metal toxicity in plants by Mg is linked to decreased bioavailability of metal ions (Kinraide 2003; Parker et al. 1998; Pedler et al. 2004) due to extracellular competition with Mg^{2+} and other cations. However, limited efforts (Kinraide 2003; Parker et al. 1998) have been made so far to understand the influence of Mg on Cu toxicity, warranting further research. But, the influence of Mg on cellular processes of plant cells during the toxicities of Cu and Cd is less known.

The present study was designed to understand the influence of varying concentrations of Mg on oxidative and proline metabolism of wheat (*Triticum aestivum*) exposed to excess Cu or Cd and to investigate the extent to which Mg can mitigate Cu or Cd toxicity.

Materials and methods

Plant material and culture conditions

The seeds of the test plant, wheat (*Triticum aestivum*) were collected from Agricultural Science Centre, Banasthali University. Seeds were surface sterilized in 0.10 % (v/v) sodium hypochlorite for 20 min, rinsed many times with

distilled water, and germinated on moist filter paper in Petri plates. For germination, the seeds were kept in the dark for 2–3 days at 25 ± 2 °C. The 3-days-old seedlings were transplanted to 1-L plastic pots in Hoagland nutrient solution: KNO_3 , 1.50 (mM); $\text{Ca}(\text{NO}_3)_2$, 0.70 (mM); $(\text{NH}_4)_2\text{HPO}_4$, 0.25 (mM); H_3PO_3 , 0.011 (mM); EDTA, 0.012 (mM); MnSO_4 , 1.30(μM); ZnSO_4 , 0.2 (μM); Na_2MoO_4 , 0.3 (μM); CuSO_4 , 0.8 (μM) (Hoagland and Arnon 1941) of pH 6.8. The hydroponic cultures of wheat were maintained in a growth chamber (16 h light/8 h dark) providing white fluorescent light of $30 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ light intensity, and 55 ± 5 % relative humidity. The seedlings were acclimatized in hydroponics culture for 24 h before treatment.

Treatment with various concentrations of Mg and heavy metal (Cu or Cd)

The acclimatized wheat seedlings were dosed with the stock solution of Mg prepared from the analytical-grade salt of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to obtain the final concentration of 0, 5, 50, 100, 250, 500, 750, 1000 μM Mg in different pots. 500 μM is the optimum Mg concentration required for growth, which was selected after screening (Fig. 1). Altered supply of sulfur caused by various Mg treatments was compensated for by supplying the appropriate amounts of Na_2SO_4 . A separate set of pots with different Mg concentrations (0, 5, 50, 100, 250, 500, 750, 1,000 μM) was also supplemented with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ or CdCl_2 to get final concentration of 50 μM of Cu or Cd. Thus, the pots amended with 0, 5, 50 and 500 μM Mg are 500, 100, 10 and 0 times deficient in Mg respectively. For all the analysis other than screening 0, 5, 50, 500 μM Mg and 50 μM Cu or Cd was selected for study.

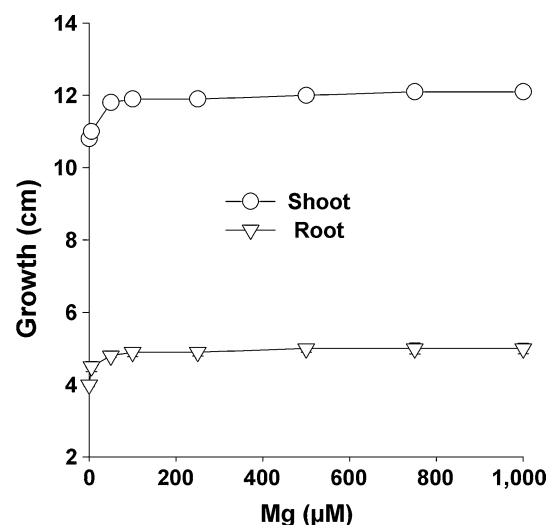


Fig. 1 Shoot (a) and root (b) growth of *T. aestivum* on exposure to various concentrations of Mg. Each data points are mean value \pm SE of three replicates

The nutrient solution in the plastic pots was aerated manually thrice a day till the end of the experiment. Ionic speciation of Cu and Cd was calculated using GEOCHEM-PC as described elsewhere (Parker et al. 1995).

After metal treatment, the wheat seedlings were separated into roots and shoot for further analysis. The growth was recorded by measuring root and shoot length.

Pigment estimation

The photosynthetic pigments were extracted by grinding 0.05 g of leaves in 5 mL of 80 % (v/v) acetone. The homogenate was centrifuged at 600g for 10 min. The supernatant was collected and the absorbance was measured at 645, 663 and 480 nm. Total chlorophyll, chlorophyll *a* and *b* and carotenoids were calculated according to Arnon (1949).

Determination of oxidative metabolism

The oxidative metabolism was characterized by measuring lipid peroxidation, H₂O₂ content (as indicators of oxidative stress) and activities of antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT).

Lipid peroxidation was recorded as the amount of malondialdehyde (MDA) produced by 2-thiobarbituric acid (TBA) as per method given by De Vos et al. (1989). Shoot and root samples (1.0 g) were homogenized in 5.0 mL of 10 % (w/v) trichloroacetic acid (TCA) containing 0.25 % (w/v) 2-thiobarbituric acid (TBA). The homogenates were heated at 95 °C for 30 min and subsequently cooled in an ice bath. After cooling on ice, the homogenate was centrifuged at 1,700g for 20 min, and MDA content was determined by subtracting the absorbance at 600 nm from 532 nm and using an absorbance coefficient of 155 mM cm⁻¹ (Kwon et al. 1965).

Hydrogen peroxide (H₂O₂) content in shoots and roots was determined according to Velikova et al. (2000). A sample of 0.05 g of plant material was homogenized in 5.0 mL of 0.10 % (w/v) trichloroacetic acid (TCA) in an ice bath. The homogenate was centrifuged at 12,000g for 15 min, and 0.50 mL of supernatant was added to 0.50 mL potassium phosphate buffer (50.0 mM pH 7.0) and 1.0 mL potassium iodide (1.0 M). The absorbance of the supernatant was measured at 390 nm.

For determination of the activities of antioxidant enzymes, 1.0 g of shoot and root samples were homogenized in 5.0 mL extraction solution comprising 50.0 M phosphate buffer (pH 7.0), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.05 % (v/v) Triton X-100, 2.0 % (w/v) polyvinylpyrrolidone (PVP) and 1.0 mM ascorbic acid. The homogenate was centrifuged at 10,000g for 25 min at 4 °C;

the resulting supernatant was kept at -20 °C for measurement of SOD, APX and CAT activities.

The SOD activity was measured according to Beauchamp and Fridovich (1971) by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture contained 0.10 mM EDTA, 13.0 mM methionine and 75.0 μM NBT. An aliquot of 2.5 mL of this reaction mixture was supplemented with 0.10 mL of the enzyme extract and 0.40 mL of riboflavin (2.0 mM) in the dark; the mixture was then incubated under light for 30 min. An illuminated blank without enzyme extract gave the maximum reduction of NBT, and therefore the maximum absorbance at 560 nm. SOD activity was calculated as absorbance of the blank minus absorbance of the sample.

The method given by Clairbone (1985) was followed for the determination of CAT activity. The assay mixture contained 0.10 mL of 3.125 mM H₂O₂ in 2.8 mL of 50.0 mM phosphate buffer (pH 7.0) and 0.10 mL of enzyme extract. The extinction coefficient for CAT was 0.039 mmol cm⁻¹ (Aebi 1984).

The APX activity was determined according to the method given by Asada (1984). An aliquot of 0.1 mL of enzyme extract was added to 2.8 mL of reaction mixture containing 0.5 mM ascorbic acid in 50.0 mM phosphate buffer (pH 7.2) in a cuvette. Absorbance of the mixture was recorded at 290 nm. The peroxidase reaction was started by the addition of 0.1 mL of H₂O₂ (5.0 mM), and a decrease in the absorbance was recorded at 290 nm after 15–20 s. Under these conditions, a decrease of 0.01 absorbance units corresponded to 3.6 mM ascorbate oxidized.

Proline metabolism

Proline metabolism was characterized by measuring proline accumulation (intracellular concentration) and activities of enzymes involved in proline synthesis [pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR)] and degradation [proline dehydrogenase (PDH)].

The intracellular concentration of proline was determined according to Bates et al. (1973). Root or shoot samples (0.05 g) were homogenized in 5.0 mL of 3.0 % (w/v) sulphosalicylic acid with a mortar and pestle. The extract was separated from tissue debris by centrifugation at 17,000g for 20 min. Free proline in the supernatant was treated with acid-ninhydrin and glacial acetic acid at 80 °C for 1 h. The reaction was terminated in an ice bath and the colored complex was separated into toluene and was recorded at 520 nm. The standard curve for proline (covering the range 0.10–5.00 μg mL⁻¹) was prepared by dissolving proline in 3.0 % (w/v) sulfosalicylic acid.

For determining activity of proline-synthesizing enzymes, the wheat samples were ground in a cold mortar

and pestle with 5.00 mL of 50.0 mM Tris–HCl buffer (pH 7.4) containing 7.0 mM MgCl₂, 0.6 mM KCl, 3.0 mM EDTA, 1.0 mM dithiothreitol (DDT) and 5.0 % (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged for 20 min at 15,000g at 4 °C. The supernatant was stored at –20 °C and used for enzyme assays.

P5CS activity was assayed as per the method described by Garcia-Rios et al. (1997). The 3.0 mL reaction mixture contained: 100 mM Tris–HCl buffer (pH 7.2), 25.0 mM MgCl₂, 75.0 mM sodium glutamate, 5.0 mM ATP and 0.20 mL of enzyme extract. The reaction was initiated by the addition of 0.20 mL of 0.4 mM NADPH. The enzyme activity was measured as the rate of consumption of NADPH monitored by a decrease in absorbance at 340 nm.

P5CR activity was estimated by a NADH-dependent pyrroline-5-carboxylate reductase reaction (Madan et al. 1995). The assay mixture comprised 0.4 mM NADH, 1.5 mM pyrroline-5-carboxylate, 50.0 mM potassium phosphate buffer, 0.8 mM dithiothreitol, and the enzyme extract. The reaction was initiated by the addition of pyrroline-5-carboxylate, and a decrease in absorbance was followed at 340 nm.

PDH activity was assayed by the method given by Reno and Splittstoesser (1975). The reaction mixture was prepared by supplementing 2.8 mL of reaction mixture containing 100 mM Na₂CO₃–NaHCO₃ (pH 10.30) and

20 mM L-proline with 0.5 mL of enzyme extract. To initiate the reaction 0.20 mL of 10.0 mM NAD was added, and a change in absorbance at 340 nm was recorded.

Statistical analysis

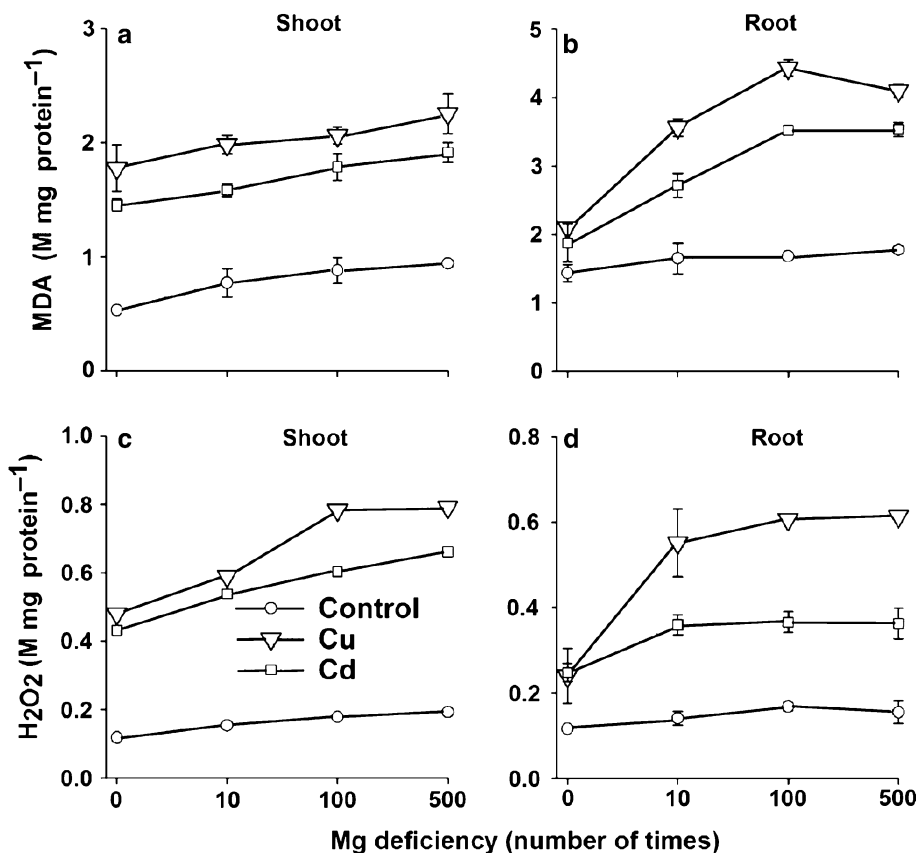
The experiments were conducted in triplicate. Data were analyzed by two way ANOVA and the means separated by Tukey's HSD test at the significance level of 95 %.

Results

Figure 1 shows the effect of various Mg concentrations (0, 5, 50, 100, 250, 500, 750 and 1000 μM) on shoot and root growth. 500 μM Mg has been set as a control. The increase in shoot and root growth was 2 and 5 %, respectively with the increase in Mg concentration from 50 to 500 μM. Shoot and root growth was almost constant and did not increase with the further enhancement of Mg concentration from 500 to 1000 μM.

Like decline in growth, Mg deficiency enhanced the Cu and Cd induced oxidative damages (lipid peroxidation and formation of hydrogen peroxide, see Fig. 2) in *T. aestivum* seedling (shoot and root both part). Mg deficiency significantly ($P < 0.05$) increased the amount of MDA,

Fig. 2 Malondialdehyde (a, b) and hydrogen peroxide (c, d) content in control, 50 μM Cu and 50 μM Cd treated *T. aestivum* shoot and root in the deficiency of Mg. Each data points are mean value ± SE of three replicates

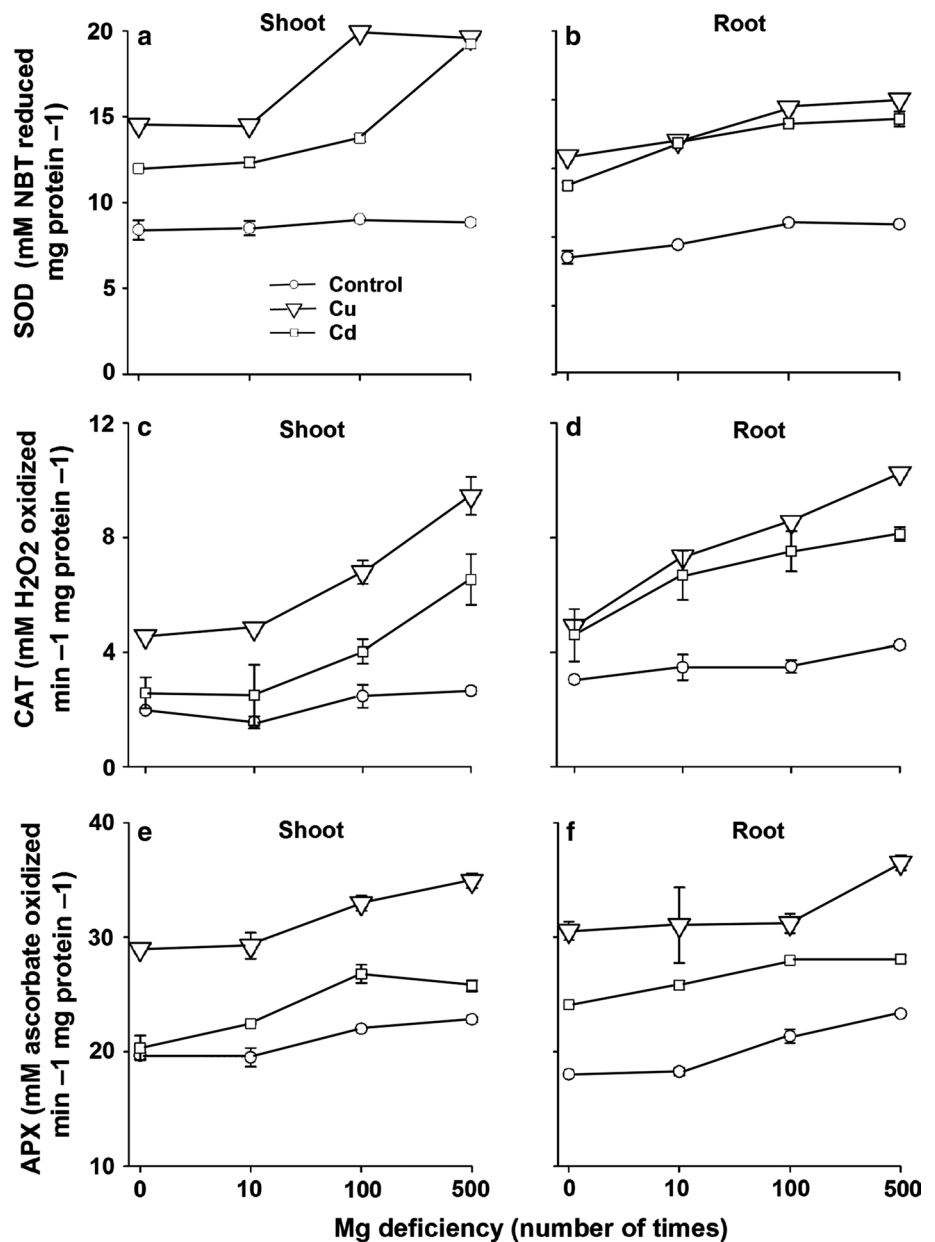


the product of lipid peroxidation. The enhancement of lipid peroxidation by Mg deficiency was higher in shoot in comparison to root of *T. aestivum* seedlings (Fig. 2a, b), as Mg deficiency enhanced the lipid peroxidation by approximately 3.3 times of control in case of shoot whereas nearly 2.6 times of control in root tissues. Figure 2c, d shows the Mg deficiency dependent induction in the level of H₂O₂ produced by Cu or Cd. Mg deficiency increased the amount of H₂O₂ maximally 5.2 fold in shoot and 3.9 fold of control in root. An almost similar trend was also observed for Cd treated shoot and root tissues. Particularly, in Cd treated shoot part, the Mg deficiency mediated enhancement of H₂O₂ content was lower than that of Cu treated. However,

in root, Mg deficiency was equally effective to increase the amount of H₂O₂ produced by Cu and Cd.

Increasing Mg deficiency considerably induced antioxidant enzyme activity (Fig. 3). Mg deficiency mediated induction of SOD activity was significant ($P < 0.05$) in Cu and Cd treated *T. aestivum* shoot and root (Fig. 3a, b). The SOD activity was 70 % over control at 0 Mg deficiency and increased to 120 % over control at 500 fold Mg deficiency in Cu treated *T. aestivum* shoot. However, in *T. aestivum* root SOD activity increased about 80 % over control and was constant thereafter with the increased Mg deficiency. Likewise, enhanced Mg deficiency induced the SOD activity in Cd treated *T. aestivum* shoot and root. The induction of SOD activity was higher in Cd treated shoot and

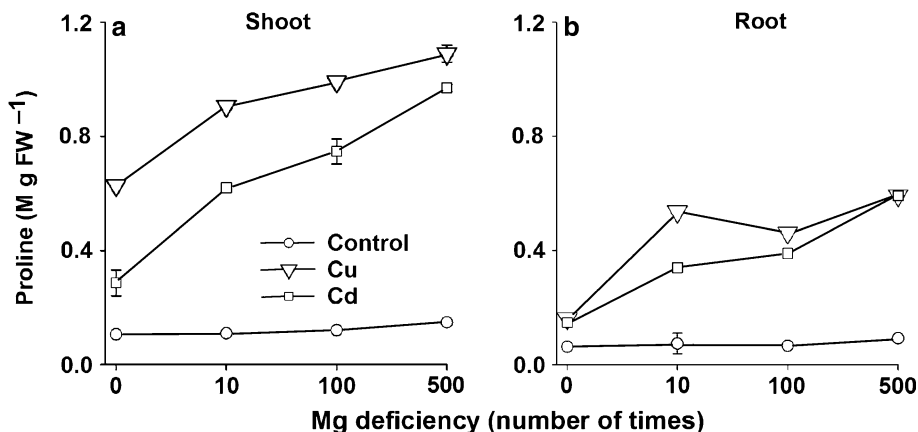
Fig. 3 Superoxide dismutase (a, b), catalase (c, d) and ascorbate peroxidase (e, f) activity in control, 50 μM Cu and 50 μM Cd treated *T. aestivum* shoot and root in the deficiency of Mg. Each data points are mean value ± SE of three replicates



increased with enhanced Mg deficiency. Whereas, SOD activity in Cd treated seedlings increased about 50 fold and 80 fold over control in shoot and root, respectively and remained almost the same thereafter with the increased Mg deficiency. Figure 3c,d presents the Cu and Cd induced CAT activity with the increased Mg deficiency. CAT activity in Cu treated *T. aestivum* shoot and root was enhanced considerably with Mg deficiency. However, the induction in CAT activity was higher in Cu treated shoot compared to root. At 500 fold, Mg deficiency CAT activity in Cu treated shoot and root was 260 and 140 % over control, respectively. However, the CAT activity in Cd treated shoot increased considerably and was 150 % over control at 500 fold Mg deficiency. CAT activity in Cd treated *T. aestivum* root increased with enhanced Mg deficiency. At 500 fold Mg deficiency CAT activity was 50 and 90 % over control in Cd treated root. The enhanced Mg deficiency induced the APX activity in Cu and Cd treated *T. aestivum* shoot and root (Fig. 3e, f). 500 fold Mg deficiency enhanced APX activity in Cu treated *T. aestivum* shoot was 53 % over control. Similarly, Cd treated *T. aestivum* shoot also showed enhanced APX activity with increased Mg deficiency. Induction in APX activity with increased Mg deficiency was much higher in Cu treated shoot than Cd treated. APX activity in Cu and Cd treated *T. aestivum* root was almost the same with enhanced Mg deficiency. However, in Cu treated *T. aestivum* root, induction of APX activity with enhanced Mg deficiency was higher than Cd treated.

Figure 4 shows the Mg deficiency dependent enhancement in the level of proline induced by Cu and Cd in *T. aestivum* seedlings (root and shoot both). At 0, 10, 100 and 500 fold Mg deficiency the proline level was 5.9, 8.2, 8.2, 7.4 fold of control in Cu treated *T. aestivum* shoot. Whereas, the level of proline was a approximately 1.9, 2.5, 7.1, 6.4 fold of control in Cu treated wheat root at 0, 10, 100, 500 times Mg deficiency, respectively. Proline accumulation increased 2.7–6.5 fold of control in Cd treated *T. aestivum* shoot with an increase in Mg deficiency from 0 to 500 fold.

Fig. 4 Proline accumulation (a, b) in control, 50 μM Cu and 50 μM Cd treated *T. aestivum* shoot and root in the deficiency of Mg. Each data points are mean value \pm SE of three replicates



Similarly, the change in proline level was 2.3–6.4 fold of control in Cd treated *T. aestivum* root with the increase of Mg deficiency from 0 to 500 fold.

Like proline accumulation 0, 10, 100 and 500 times Mg deficiency significantly ($P < 0.05$) enhanced the proline synthesizing enzymes (P5CS, P5CR) activities (Fig. 5) in *T. aestivum* seedlings (shoot and root both part). The induction of P5CS activity was higher in shoot in comparison to root of *T. aestivum* seedling (Fig. 5a, b). Mg deficiency dependent induction in the P5CS activity in Cu treated *T. aestivum* shoot was recorded, as induction of its activity ranged from 2.4 to 4.1 fold of control, by lower to higher Mg deficiency. Whereas, in Cu treated root induction of P5CS activity ranged from 1.8 to 2.9 fold of control as the Mg deficiency increased. Similarly, the Mg deficiency dependent induction of P5CS activity was observed in Cd treated wheat shoot and root. Particularly, in Cd treated shoot and root parts the Mg deficiency mediated induction of P5CS activity was lower than that of Cu treated. Figure 5c, d shows the Mg deficiency mediated increase in P5CR activity induced by Cu or Cd. At 500 fold of Mg deficiency P5CR activity was 277 % over control in Cu treated *T. aestivum* shoot. Whereas, at 500 fold Mg deficiency induction of P5CR activity was 223 % over control in Cu treated *T. aestivum* root. Similar trends was also observed for Cd treated *T. aestivum* shoot and root.

Figure 6 shows the Mg deficiency mediated change in PDH activity in Cu and Cd treated *T. aestivum* shoot and root. Significant ($P < 0.05$) increase in PDH activity over control was observed both in Cu and Cd treated shoots and root parts. However, with the increased Mg deficiency, PDH activity upon Cu treatment declined from 6.2 to 4.0 fold of control in shoot. PDH activity remained almost constant in Cu and Cd treated *T. aestivum* root.

Several fold of Mg deficiency decreased the photosynthetic pigments in *T. aestivum* shoot exposed to 50 μM Cu and Cd (Table 1). Mg deficiency significantly ($P < 0.05$) decreased the amount of total chlorophyll, chl a, chl b, and

Fig. 5 Pyrroline-5-carboxylate synthase (a, b) and pyrroline-5-carboxylate reductase (c, d) activity in control, 50 μ M Cu and 50 μ M Cd treated *T. aestivum* shoot and root in the deficiency of Mg. Each data points are mean value \pm SE of three replicates

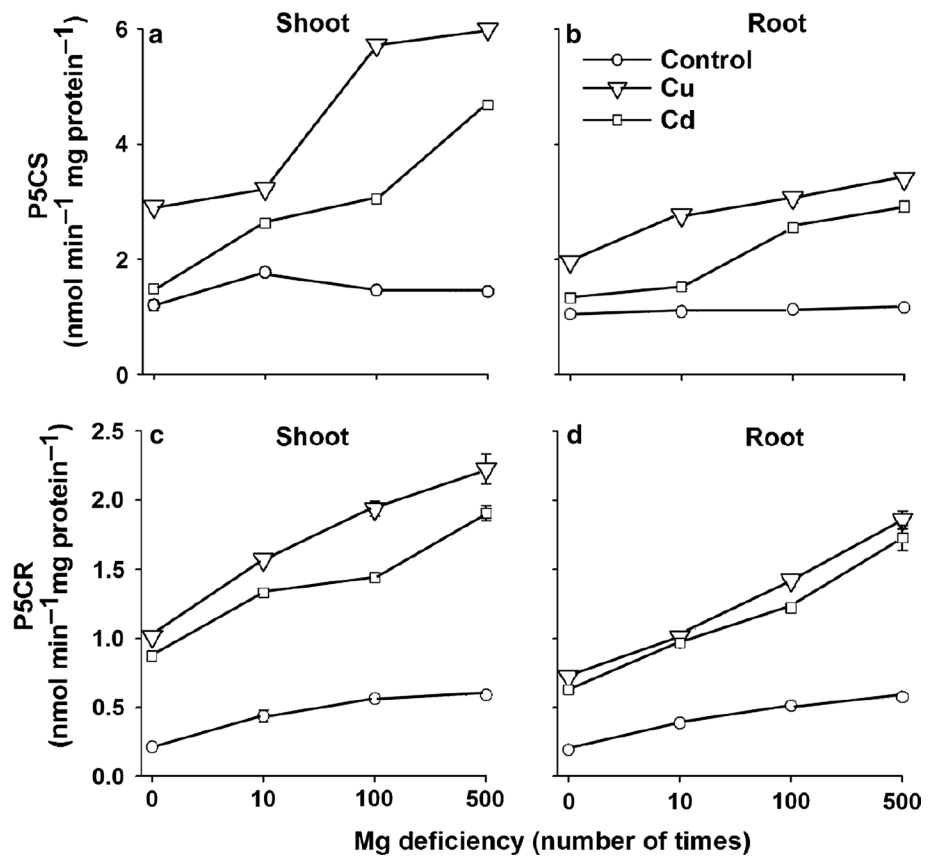
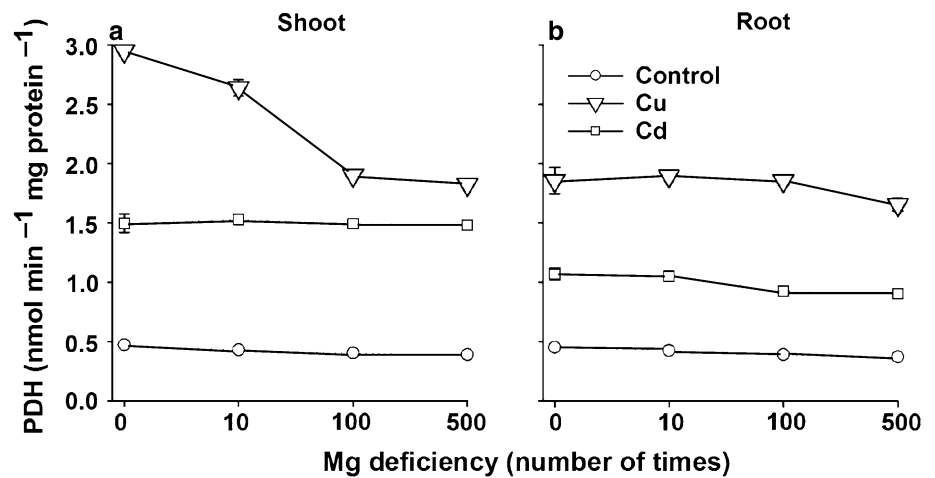


Fig. 6 Proline dehydrogenase (a, b) activity in control, 50 μ M Cu and 50 μ M Cd treated *T. aestivum* shoot and root in the deficiency of Mg. Each data points are mean value \pm SE of three replicates



carotenoids in *T. aestivum* shoot treated with Cu or Cd. At maximum Mg deficiency (500 times) the amount of chl a decreased by nearly 7.0 and 1.2 fold in Cu and Cd treated *T. aestivum* shoot respectively. Whereas, at 500 times of Mg deficiency decline in chl b was 1.8 and 2.0 fold of control in Cu treated *T. aestivum*. Similar decline was noticed in Cd treated *T. aestivum* shoot. Likewise, the level of carotenoids also decreased with the Mg deficiency in Cu and Cd treated *T. aestivum* shoot.

Table 2 depicts the free Cu and Cd ion activity in Hoagland’s nutrient solution containing various Mg concentrations and 50 μ M Cu or Cd. Free Cu and Cd ion activity in Hoagland nutrient solution was the same at 0 and 500 μ M Mg i.e. 1.44 % of Cu and 1.99 % of Cd. However, free Cu and Cd ion activity slightly increased with subsequent increase of Mg concentration in the nutrient solution. Free Cu ion activity was 1.15 and 1.22 % and free Cd ion activity was 2.00 and 2.12 % at 5 and 500 μ M Mg, respectively.

Table 1 Chlorophyll *a*, chlorophyll *b* and carotenoids in 50 μM Cu or Cd treated wheat exposed to various concentrations of MgSO_4

Parameter	Treatment	Mg SO_4 (μM)			
		0	5	50	500
Chlorophyll <i>a</i> ($\mu\text{g mg protein}^{-1}$) ^a	Control	14.6 \pm 0.6	15.2 \pm 0.8	15.5 \pm 1.2	17.0 \pm 0.2
	Cu	2.4 \pm 0.2	2.8 \pm 0.1	2.5 \pm 0.2	2.6 \pm 0.2
	Cd	13.9 \pm 0.2	13.2 \pm 0.3	14.2 \pm 1.5	15.8 \pm 1.5
Chlorophyll <i>b</i> ($\mu\text{g mg protein}^{-1}$) ^a	Control	3.3 \pm 0.1	4.5 \pm 0.2	4.9 \pm 0.2	4.4 \pm 0.6
	Cu	2.1 \pm 0.1	2.7 \pm 2.1	2.3 \pm 0.1	2.3 \pm 0.1
	Cd	4.6 \pm 0.2	4.1 \pm 1.1	4.4 \pm 0.4	4.9 \pm 0.1
Carotenoids ($\mu\text{g mg protein}^{-1}$) ^a	Control	152 \pm 3.5	158 \pm 7.1	178 \pm 3.3	178 \pm 8.3
	Cu	108 \pm 7.1	99.7 \pm 2.7	103 \pm 1.7	107 \pm 4.5
	Cd	148 \pm 4.2	147 \pm 9.6	163 \pm 7.8	177 \pm 1.1

^a Mean \pm SE ($n = 3$)**Table 2** Distribution of Cu and Cd in Hoagland nutrient solution with various concentrations of Mg

	MgSO_4 concentration (μM)			
	0	5	50	500
Distribution of Cu (%)				
As free metal	1.14	1.14	1.15	1.22
Complexed with NH_3	0.02	0.02	0.02	0.02
Complexed with PO_4^{3-}	0.90	0.90	0.90	0.89
Complexed with SO_4^{2-}	0.07	0.07	0.07	0.07
Complexed with OH^-	0.64	0.64	0.64	0.68
Complexed with EDTA	23.57	23.57	23.57	23.54
In solid form with PO_4^{3-}	73.64	73.63	73.62	73.54
Distribution of Cd (%)				
As a free metal	1.99	1.99	2.00	2.12
Complexed with PO_4^{3-}	0.13	0.13	0.13	0.13
Complexed with SO_4^{2-}	0.16	0.16	0.16	0.16
Complexed with Cl^-	0.01	0.01	0.01	0.02
In solid form with PO_4^{3-}	75.74	75.74	75.73	75.62
Complexed with EDTA	21.94	21.94	21.94	21.95

Discussion

The inhibition of plant root growth is a quite obvious phenomenon of metal toxicity as the consequence of disturbed metabolism. The decreasing concentrations of Mg significantly increase the toxic effect of Cu and Cd on root elongation of *T. aestivum*. The Mg-mediated improvement in root growth of metal-treated wheat as noticed in the present study is in accordance with earlier studies (Kinraide et al. 1992, Parker et al. 1998, Pedler et al. 2004). Possibly the optimum concentrations of Mg in the present study may have relieved at least part the root of wheat from metal-induced arrest of cell division and eventually led to the improvement in root growth. The importance of Mg in regulating cell division has also been documented earlier

(Webb 1951). Apart from this possibility, Mg being the important constituent of enzymes such as several kinases, ATPase, and RuBP carboxylase/oxygenase, chelatase (Marchner 1995; Shaul 2002) might also have improved these enzymes and relative metabolic activities that collectively resulted into improved root growth of wheat under metal stress when provided in optimum concentration. However, earlier studies (Kinraide et al. 1992, 2004, Kinraide 1998) related the alleviation of metal toxicity on root growth with Mg-mediated reduction in free metal ion activity. But, it is not valid for the present case because free metal ion activity was increased with optimum Mg concentration. Kinraide et al. (2004) also suggested that ameliorative effect of Mg from Zn toxicity on growth of wheat seedling do not associated with diminished metal uptake. Thus, the exacerbation of Cu and Cd stress on the root growth of wheat seedling in the present study possibly depend on ineffective internal detoxification of Cu/Cd or on decreased metabolic activities or on modification of metal sensitive internal binding sites with decreasing Mg concentration.

The induction of oxidative stress as indicated by increased MDA content (product of lipid peroxidation) and H_2O_2 levels in root and shoot parts of wheat by Cu and Cd is quite expected during metal toxicity. The test metals, Cu and Cd are well known to induce oxidative stress in plants by directly catalyzing the formation and by indirectly increasing the levels of reactive oxygen species, respectively (Brait 2002; Stohs and Bagchi 1995). Low level of Mg impairs the photosynthetic CO_2 fixation and thereby over reduces the photosynthetic electron transport that potentiates the formation of reactive oxygen species (Cakmak and Kirkby 2008) and subsequently causes oxidative stress in plants. Thus the increased MDA content and H_2O_2 level in the root and shoot of test plants in the present study must be due to the above mention reasons. The alleviation of metal-induced oxidative stress by increasing concentrations of Mg could be due to the maintenance of

optimum cellular status of Mg of the test plant. The maintenance of high or optimum level of Mg in plant cells may decrease the excess load of reactive oxygen species possibly by overcoming the excess reduction of photosynthetic electron transport (Cakmak and Kirkby 2008) or by some other unknown cellular process. The improved efficiency of the enzymes e.g. SOD, CAT and APX for the detoxification of reactive oxygen species by optimum Mg would be the reason in the present case, as decreasing concentrations of Mg have declined these enzyme activities.

In wheat seedlings, 50 μM Cu or Cd treatments resulted in a significant rise in antioxidant enzymes (SOD, CAT, APX) activities, which can be considered as circumstantial response against increased ROS levels. Various ROS act as substrate for these enzymes, therefore increased availability of the substrates might cause stimulation in the activities of SOD, CAT and APX. Stimulated activities of antioxidant enzymes, namely, SOD, CAT and APX of various plants due to heavy metals including, Cu and Cd have already been reported by several authors (Van Assche and Clijsters 1990; Mylona and Polidoros Scandalios 1998; Ruzsa and Scandalios 2003; Weckx and Clijsters 1996). The results of the present study demonstrate the possible role of Mg in minimizing the formation of ROS (as evident from decreased MDA and H_2O_2 content) and hence lessen the substrates for antioxidant enzymes. Therefore, the stimulated activities of SOD, CAT and APX reversed back towards the level of control due to decreased substrate (reactive oxygen species-ROS) availability in the presence of optimum concentration of Mg.

Occurrence of proline hyper accumulation during metal stress and deficiency of nutrients is a quite common phenomenon of plants (Alia and Saradhi 1991; Bassi and Sharma 1993; Heuer 1994). The proline hyper accumulation has been considered a tolerance strategy of plants exposed to abiotic stresses, including metals and nutrient limitation (Claussen 2002; Hare and Cress 1997). As noted earlier, excess of the test metals and Mg deficiency increase the concentration of H_2O_2 . However, plants activate few enzymes including SOD, CAT, and APX as also noticed in the present study to effectively destroy the toxic H_2O_2 . But due to easy diffusibility H_2O_2 may escape from these detoxification processes and convert into highly reactive hydroxyl radicals with the help of transition metals (Brait 2002). Thus, test plants triggered the accumulation of proline as an additional strategy to detoxify extremely toxic hydroxyl radicals. The ability of proline for scavenging hydroxyl radicals has already been confirmed for other organisms (Tripathi and Gaur 2004, 2006). Cu and Cd-induced proline hyper accumulation in wheat was possibly achieved via increased proline biosynthesis as well as decreased catabolism. Because the activities of proline synthesizing enzymes e.g. P5CS, P5CR was increased

along with decrease in proline degrading enzyme (PDH) of wheat exposed to Cu and Cd. However, the contribution of both, increased synthesis or decreased catabolism in proline hyper accumulation still needs to be studied. Since, decreased concentration of Mg substantially increased Cu and Cd stress in wheat seedlings, the withdrawal of proline level as well P5CS and P5CR and also the restoration of PDH activities of metal-treated wheat in presence of optimum Mg concentrations are quite understandable.

The present study concluded that decreasing supply of Mg exacerbated toxicities of Cu and Cd in wheat, a common crop in India. However, based on the available results it is difficult to speculate the mechanism of Mg-dependent amelioration of Cu and Cd toxicity in wheat. But possibly decreased supply of Mg would provide fewer sites for metal chelation inside the cell or inactivate efficient sequestration of metals into vacuoles thereby exacerbating the toxicities of Cu or Cd. Salt and Rauser (1995) have already reported the transport of phytochelatin-Cd complex across the oat root tonoplast by an ABC transporter, which was energized by Mg-ATP. It has also been demonstrated that the over expression of an *Arabidopsis* magnesium transport gene, AtMGT1 with capability to transport other divalent cations including Cu^{2+} , Ni^{2+} , and Co^{2+} (Li et al. 2001) confers Al tolerance in *Nicotiana benthamiana*. But predicting such possibility for Cu and Cd is not feasible with available data. Reports suggesting the substitution of Mg from the chlorophyll by heavy metals such as Cu, Cd, Ni, Zn, and Pb leading to impairment of photosynthesis, the prime cause of metal toxicity are also available (Kupper et al. 1996, 1998). Perhaps, sufficient concentrations of Mg could prevent the metal-induced displacement of Mg from chlorophylls and therefore substantially avoid metal toxicity in wheat. In all prospects, the decreasing concentrations of Mg have significantly exacerbated the toxicities of Cu and Cd in wheat.

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