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Involvement of Antioxidant Defense Capacity of Rice and Sunflower in Their Response to Cu and Mn Toxicity under Different Light Intensities

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Abstract

Effects of toxic concentrations (up to 100 µM) of copper (Cu) and manganese (Mn) on growth and oxidative defense system of rice (*Oryza sativa* L.) and sunflower (*Helianthus annuus* L.) were studied under three light intensities in hydroponic medium. Rice expressed higher susceptibility to both Cu and Mn toxicity than sunflower. Higher light intensity strengthened the effect of toxicity of Cu while ameliorated that of Mn. Activity of ascorbate peroxidase and glutathione reductase responded to Cu and Mn differently in rice. Cu toxicity increased their activity but Mn did not change or rather decreased it. Activity of catalase was induced in response to both Cu and Mn particularly under higher light intensities. Activity of peroxidase, in contrast to other tested enzymes, was correlated with growth response of plants. Copper was not effective in induction of malondialdehyde accumulation. Mn, in concentrations causing no growth inhibition (100 µM), resulted in a significant increase of malondialdehyde. Changes in the tissue content of proline, similar with peroxidase activity, was mainly associated with stress conditions as judged by growth data. On the other word, activity of peroxidase and accumulation of proline monitored the stress conditions without any protecting role and any effect on adaptation of plants. Our results suggested that the antioxidant capacity of plants is not independently correlated with the growth response of plants without regarding the other physiological responses to metal exposure.

Keywords: Antioxidant capacity; Cu toxicity; light intensity; Mn toxicity; Rice; Sunflower

Introduction

Toxic heavy metals find their ways into environment through variety of sources such as metal smelters, industrial effluents including uses of fertilizers and pesticides, *etc.* [47]. Excess amounts of heavy metals induce a wide range of biochemical effects and physiological processes and affect photosynthesis, pigment synthesis, protein metabolism and membrane integrity [31].

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One of the underlying causes of tissues injury following exposure of plants to Cu [19] and Mn [25] is the increased accumulation of reactive oxygen species (ROS) mediated-oxidative stress. Highly cytotoxic species of oxygen can seriously disrupt metabolism through oxidative damage to cellular components [32]. One of the primary effects of these molecular species and their products in cells is the peroxidation of membranes [37,54]. Plants have evolved various protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration [22,48,49]. Enzymatic antioxidant system, which is one of the protective mechanisms including superoxide dismutase (SOD) are located in various cell compartments and catalyse the disproportion of two O_2^- radicals to H_2O_2 and O_2 [48]. H_2O_2 is eliminated by various antioxidant enzymes such as catalase (CAT) and peroxidases (POD) converting H_2O_2 to water [48]. Ascorbate peroxidase (APX) eliminates peroxides by converting ascorbic acid to dehydroascorbate [4]. Ascorbate peroxidase and glutathione reductase (GR) are important components of the ascorbate-glutathione cycle responsible for the removal of H_2O_2 in different cellular compartments [36].

Involvement of proline in tolerance to water deficiency and salt stress is well documented [6,33]. Exposure to heavy metals, for example Cu [16] is known to deteriorate the plant water balance. The functional significance of proline accumulation would lie in its contribution to water balance maintenance [17] scavenging of hydroxyl radicals [52] and metal chelation in the cytoplasm [20].

Antioxidant capacity of leaves is not only affected by heavy metal toxicity [22], but also modified by long-term light acclimation [26]. High light acclimated plants with a more efficient antioxidant and photoprotective system are better protected against heavy metal toxicity as compared to low light grown plants [3]. However, a contradictory result was obtained in other work, in that a higher susceptibility to heavy metal toxicity was observed in high-light grown plants though having a higher concentration of antioxidants [3]. Therefore, it is important to explore how antioxidant capacity of plants is modified by light intensity in plants with different heavy metal tolerance.

Because of highly different physicochemical properties of Mn and Cu, as well as different behavior regarding chelation by organic molecules and binding to the cell wall [41], it is necessary to conduct a comparative study of Mn and Cu toxicity on biochemical processes particularly antioxidant defense system. In this work we studied the response of two important crop species differing in tolerance to Cu [29] and Mn [30] toxicity as influenced by dual effect of metal toxicity and high light intensity. The main objective of this work was the evaluation of the importance of inducible or constitutive antioxidant defense capacity of plants in growth and biomass production under heavy metal toxicity.

Materials and Methods

Two crop species were used in this study including rice (*Oryza sativa* L. cv. Amol) and sunflower (*Helianthus annuus* L. cv. Azarghol). Seeds were provided by the Rice Research Institute (Guilan, Iran) and Seed and Plant Improvement Institute (SPII) (Karaj, Iran) respectively.

Plants Growth and Treatments

Experiments were conducted in a growth chamber with a temperature regime of 25°/18°C day/night, 14/10 h light/dark period and relative humidity of 70/80%. Surface-sterilized seeds were germinated in the dark on sand, moistened with distilled water and 0.05 mM $CaSO_4$. The 7-day-old seedlings with uniform size were transferred to hydroponic culture [29] in plastic container with 2 L of nutrient solution (50%) and pre-cultured for 3 days. Copper and Mn treatments were started in 10-day-old plants. Seedlings were treated with three levels of $CuSO_4$ or $MnSO_4$, 0 (control) 50 and 100 µM. Nutrient solutions were completely changed every 3 days, pH of the medium was adjusted at 6.5 and controlled every day.

For study the effect of different light intensities, plants were grown simultaneously under three photosynthetic photon flux densities (PPFD): low light (LL=100 µmol $m^{-2}s^{-1}$ PPFD), intermediate light (IL=500 µmol $m^{-2}s^{-1}$ PPFD) and high light (HL=800 µmol $m^{-2}s^{-1}$ PPFD) intensities supplied by fluorescent lamps. Light intensity was measured using light sensor accessory of gas exchange analyzer (LCA-4, ADC Company, UK).

Harvest

Twelve days after treatment, plants were harvested. For removing of the apoplasmic Cu and Mn from roots, plants were placed for 1 h in 5 mM $CaCl_2$+25% nutrient solution. For determination of Cu and Mn content, oven-dried samples were ashed in a muffle furnace at 550°C for 8 h, resolved in HCl and made up to volume by distilled water. Metal concentration was determined by atomic absorption spectrophotometry (Shimadzu, AA 6500). For determination of chlorophyll concentration, third leaves (pair of leaves in sunflower)

immediately after harvest were used for extraction of chlorophyll by N,N dimethylformamide according to Moran [44].

Enzyme Assays

Fresh leaf samples were used for enzyme extraction and measurement of protein and metabolites. Samples were ground in extraction buffer using pre-chilled mortar and pestle. Each enzyme assay was tested for linearity between the volume of crude extract and the measured activity.

Ascorbate Peroxidase

The enzyme was extracted in 50 mM phosphate buffer (pH=7.0). The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured using modified method of Boominathan and Doran [9]. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH=7.0) containing 0.2 mM EDTA, 0.5 mM ascorbic acid (Sigma), 50 mg of BSA (Sigma), and crude enzyme extract. The reaction was started by addition of H_2O_2 at final concentration of 0.1 mM. Oxidation of ascorbic acid as a decrease in absorbance at 290 nm was followed 2 min after starting the reaction. The enzyme activity was calculated using an absorbance coefficient for ascorbic acid of 2.8 $mM^{-1}cm^{-1}$. One unit of APX oxidizes ascorbic acid at a rate of 1 µmol min^{-1} at 25°C.

Catalase

Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease in absorbance of H_2O_2 at 240 nm [40]. The enzyme was extracted in 50 mM phosphate buffer (pH=7.0). The assay solution contained 50 mM phosphate buffer and 10 mM H_2O_2. The reaction was started by addition of enzyme aliquot to the reaction mixture and the change in absorbance was followed 2 min after starting the reaction. Unit activity was taken as the amount of enzyme, which decomposes 1 M of H_2O_2 in one min.

Peroxidase

Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test [13]. The tetraguaiacol formed in the reaction has an absorption maximum at 470 mm, and thus the reaction can be readily followed photometrically. The enzyme was extracted by 10 mM phosphate buffer (pH=7.0), and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. The reaction was started by addition of the enzyme extract at 25°C and was followed 2 min after starting the reaction. The enzyme unit was calculated as enzyme protein required for the formation of 1 µM tetraguaiacol for 1 min.

Superoxide Dismutase

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries [24]. The enzyme was extracted in 25 mM HEPES (pH=7.8) and 0.1 mM EDTA, the homogenate was centrifuged at 15,000 *g* for 15 min. Test tubes containing 25 µl of enzyme extract, 25 µl extraction buffer and 450 µl of the reaction mixture were incubated in a growth chamber at 22°C and at a light intensity of 400 µmol $m^{-2}s^{-1}$. The reaction buffer contained 25 mM HEPES (pH=7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH=10.2), 12 mM L-methionine, 75 µM NBT and 1 µM riboflavin. The reaction was started by removing a dark plastic foil from the surface of samples and continued for 10 min. One unit of SOD was defined as the amount of enzyme required to induce a 50 % inhibition of NBT reduction as measured at 560 nm, compared with control samples without enzyme aliquot.

Glutathione Reductase

The enzyme was extracted in 50 mM phosphate buffer (pH=7.0) containing 5 mM EDTA and 2% (W/V) of insoluble polyvinylpolypyrrolidone (PVPP). The extract was centrifuged at 15,000 *g* in 4°C for 20 min. The activity of Glutathione reductase (GR, EC 1.6.4.2) was assayed by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{-1}) as described by Foyer and Halliwell [21]. The reaction mixture contained 100 mM *Tris*-HCl (pH=7.8), 2.0 mM EDTA, 0.05 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 50 µl of enzyme extract at 25°C. One unit of enzyme activity was calculated as enzyme protein required for oxidation of one µM NADPH in 1 min.

Other Assays

Total Protein Concentration

Soluble proteins were determined as described by Bradford [10] using a commercial reagent (Sigma) and BSA (Merck) as standard.

Total Amino Aacids

Content of total free α-amino acids was assayed using ninhydrin colorimetric method [35]. Leaf tissues were homogenized using ice cold 50 mM phosphate buffer (pH=6.8). The homogenate was centrifuged at 18,000 *g* for 20 min. Ninhydrin reagent (1:5 diluted solution of 350 mg in 100 ml ethanol) was added to sample solution and after gentle stirring was incubated for 4-7 min at 80-100°C in a water bath. After cooling to room temperature in a water bath, the absorbance was recorded at 570 nm. Glycine was used for production of standard curve.

Proline Concentration

Proline was extracted and its concentration determined by the method of Bates *et al.* [8]. Leaf tissues were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 g for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined. Proline (Sigma) was used for production of standard curve.

Hydrogen Peroxide

The concentration of H_2O_2 was determined using methods described by Patterson et al [46]. 1-1.5 g of leaf was homogenized with 0.2 g activated charcoal (Sigma) and 5 ml of 5% w/v trichloroacetic acid (TCA) in an ice bath using a prechilled mortar and pestle. The homogenates was filtered through four layers of cheesecloth and centrifuged at 14,000 g for 15 min at 4°C. The supernatant was then filtered through a 0.45 µM filter (Millipore). The colorimetric reagent was a 1:1 v/v mixture of 0.6 mM 4-(2-pyridylazo) resorcinol (disodium salt) and 0.6 mM potassium titanium-oxalate. To a known volume of supernatant, 1 ml of colorimetric reagent was added and the mixture was incubated at 45°C on a heating plate for 60 min. The absorbance was measured at 508 nm against a reference solution containing 50 µl of 50% w/v TCA and 1.95 ml of 100 mM potassium phosphate buffer (pH=8.4). The concentration of H_2O_2 was determined from a standard curve.

Malondialdehyde Assay

Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture [34]. Leaf tissues were homogenized (1:5) in 0.1% w/v TCA. The homogenate was centrifuged at 10,000 g for 5 min. To 1 ml of the supernatant, 4 ml of 20% w/v TCA containing 0.5% w/v thiobarbituric acid (Sigma) was added. The solution was heated at 95°C for 30 min and then quickly cooled on ice. The mixture was centrifuged 10,000 g for 15 min and the absorbance measured at 532 nm. MDA levels were calculated from a 1,1,3,3-tetraethoxypropane (Sigma) standard curve [9].

Results

Growth and Dry Matter Production

Cu Toxicity

Shoot and root growth was inhibited in response to Cu toxicity. Shoot growth of rice was inhibited 60-73%, while growth of sunflower was inhibited only 11-49%. Reduction of root growth was also different in Cu-treated rice and sunflower plants, in rice the reduction was 58-82% and in sunflower was 35-64%. A clear effect of light conditions on the extent of growth inhibition by Cu toxicity was observed. Growth under higher light intensity caused an increased susceptibility of plants to Cu toxicity. This effect was more prominent in sunflower, in which both of shoot and root growth responded much more negatively to Cu toxicity when grown under higher light intensity e.g. up to 49% reduction in HL compared to only 11% reduction in LL plants. However, dry matter of shoot and root in plants grown at higher light intensity was higher than plant at lower light conditions at similar Cu treatments (Fig. 1).

Mn Toxicity

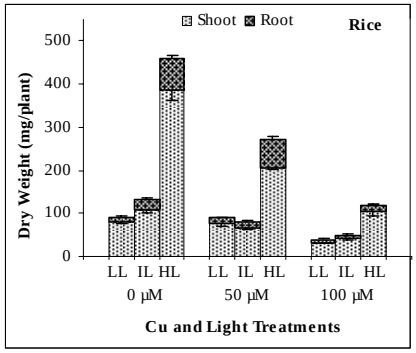
Mn toxicity inhibited shoot and root growth with different extent than Cu toxicity, and the reverse effect of light intensity on the expression of Mn toxicity was observed. The response of shoot growth was different, from 63% reduction for LL plants to 10% inhibition in HL plants for rice and from 57% reduction in LL to 79% increase in HL sunflower plants. Similar trend was observed for root growth, with the exception of no growth improvement in response to Mn toxicity in HL plants. Such as Cu, susceptibility to Mn toxicity was higher in rice than sunflower, regarding both shoot and root growth (Fig. 1).

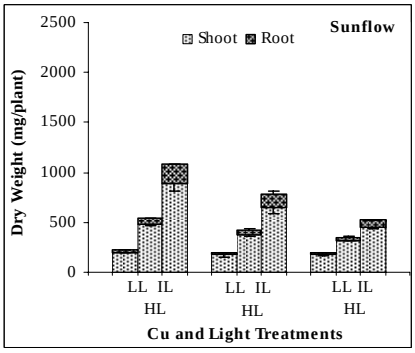
Shoot Content of Cu and Mn

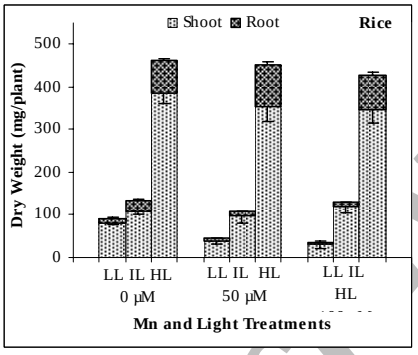
Copper accumulation was much higher in sunflower than rice at similar treatments. Concentration of Cu increased in response to higher light intensity in rice, but decreased in sunflower. Effect of higher light intensity on the concentration of Mn was similar between rice and sunflower, it caused reduction of Mn accumulation (Table 1).

Activity of Antioxidant Enzymes

Increase of light intensity influenced activity of all studied enzymes. The most prominent effect of light intensity was observed in the activity of APX. This increase was 3.8 times and 7.4 times in rice and sunflower respectively. Activity of CAT decreased in response to higher light intensity in both rice and sunflower with the exception of intermediate light intensity in rice. Activity of POD decreased slightly or significantly in response to higher light intensity in rice but increased in sunflower. Activity of SOD and GR increased only under HL conditions in both rice and sunflower but remained unchanged in IL compared to LL plants (Table 2).







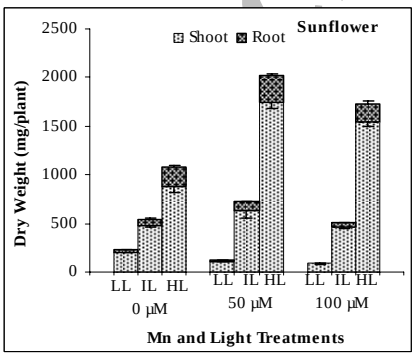


Figure 1. Effect of Cu or Mn treatment on dry matter production of rice (*Oryza sative* L. cv. Amol) and sunflower (*Helianthus annuus* L. cv. Azarghol) grown under different light conditions including low (LL=100 µmol $m^{-2}s^{-1}$ PPFD), intermediate (IL=500 µmol $m^{-2}s^{-1}$ PPFD) or high (HL=800 µmol $m^{-2}s^{-1}$ PPFD) light intensity.

Activity of APX increased in response to Cu treatment in both rice and sunflower. Effect of Cu toxicity on the activity of CAT and POD was different between rice and sunflower with the exception of HL plants. POD activity decreased slightly or significantly in rice but increased up to 109% in sunflower. SOD and GR activity increased in both rice and sunflower in response to exposure of plants to toxic concentrations of Cu. Similar extent of changes was observed for rice and sunflower (Table 2).

In response to toxic concentration of Mn, APX activity was inhibited only in HL plants, in LL and IL plants it remained unchanged or increased, the latter change was observed only in sunflower. In contrast to APX, CAT activity increased in Mn treated HL plants, but reduced in IL plants. For LL rice plants, CAT activity increased but decreased in sunflower. Activity of POD in rice was generally decreased in response to Mn treatment, slightly or significant. In contrast to rice, POD activity increased (statistically non significant) in response to Mn treatment with the exception of HL plants. SOD activity increased in response to Mn treatment in both rice and sunflower, for GR changes were not significant either in rice or in sunflower (Table 2).

Concentration of Protein, Amino Acids and Metabolites

Protein concentration increased with increasing light intensity of growth environment. This increase was higher in sunflower than in rice. Concentration of total amino acids was higher in plants grown under higher light intensity. Proline concentration of leaves increased with increasing light intensity, however, this change was observed only in Cu treated but not in control plants. In response to higher light intensities, H_2O_2 accumulated in leaves, this change was significant only in HL plants. Application of the highest light intensity (HL) increased drastically the concentration of MDA in both control and heavy metal treated plants (Table 3).

Application of 100 µM Cu increased protein concentration in IL rice and HL sunflower plants, total amino acids did not change significantly in response to Cu treatment, but proline concentration increased significantly in response to Cu treatment under all three light conditions. Although H_2O_2 concentration increased in response to Cu treatment, these changes were mainly non significant with the exception of HL sunflower plants. In contrast to the effect of light, Cu treatment did not change MDA concentration either in rice or sunflower (Table 3).

In contrast to Cu, Mn treatment did not influenced protein concentration of leaves either in rice or

Table 1. Effect of Cu or Mn treatment on the shoot concentration of Cu and Mn (μg g^{-1} DW) in rice (*Oryza sative* L. cv. Amol) and sunflower (*Helianthus annuus* L. cv. Azarghol) grown under different light conditions including low (LL=100 μmol $m^{-2}s^{-1}$ PPFD), intermediate (IL=500 μmol $m^{-2}s^{-1}$ PPFD) or high (HL=800 μmol $m^{-2}s^{-1}$ PPFD) light intensity. Values are mean±SD from 4 replicates. Data in each column followed by the same letter are not significantly different (P<0.05)

Light	Cu (μM)	Cu Concentration		Mn (μM)	Mn Concentration	
		Rice	*Sunflower*		*Rice*	*Sunflower*
LL	0	51±2 ^{e}	1797±41 ^{d}	0	446±20 ^{d}	130±10 ^{d}
	100	696±19 ^{c}	16327±18 ^{a}	100	4650±20 ^{a}	6193±34 ^{a}
IL	0	78±6 ^{de}	1254±32 ^{e}	0	357±17 ^{e}	100±15 ^{de}
	100	871±15 ^{b}	12358±93 ^{b}	100	3812±46 ^{b}	4758±13 ^{b}
HL	0	83±4 ^{d}	846±19 ^{f}	0	301±10 ^{e}	80±6 ^{e}
	100	1258±21 ^{a}	10256±53 ^{c}	100	3273±54 ^{c}	2549±21 ^{c}

Table 2. Effect of Cu and Mn toxicity on the specific activity of ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR) in rice (*Oryza sative* L. cv. Amol) and sunflower (*Helianthus annuus* L. cv. Azarghol) grown under different light conditions including low (LL=100 μmol $m^{-2}s^{-1}$ PPFD), intermediate (IL=500 μmol $m^{-2}s^{-1}$ PPFD) or high (HL=800 μmol $m^{-2}s^{-1}$ PPFD) light intensity. Values are mean±SD from 4 replicates. Data in each column followed by the same letter are not significantly different (P<0.05)

Treatments			APX	CAT	POD	SOD	GR
			μmol H_2O_2 mg^{-1} protein min^{-1}	μmol H_2O_2 mg^{-1} protein min^{-1}	μmol Guaiacol mg^{-1} protein min^{-1}	Unit mg^{-1} protein	nmol NADPH mg^{-1} protein min^{-1}
Cu (μM)							
Rice	LL	0	2.2±1.5 ^{b}	891±26 ^{e}	23.7±1.2 ^{a}	658±34 ^{d}	36±10 ^{d}
		100	8.3±2.3 ^{a}	3442±56 ^{a}	11.4±0.6 ^{c}	742±62 ^{dc}	80±8 ^{d}
	IL	0	3.2±0.5 ^{b}	1724±67 ^{c}	15.7±2.3 ^{b}	674±53 ^{d}	82±7 ^{d}
		100	10.3±2.6 ^{a}	2312±13 ^{b}	12.1±1.5 ^{b}	815±33 ^{c}	245±33 ^{c}
	HL	0	8.4±1.2 ^{a}	879±26 ^{e}	14.1±2.4 ^{b}	958±85 ^{b}	226±26 ^{b}
		100	12.6±3.7 ^{a}	1399±67 ^{d}	11.3±0.8 ^{cb}	1369±65 ^{a}	319±20 ^{a}
Sunflower	LL	0	5.0±2.2 ^{d}	8799±66 ^{b}	13.0±2.6 ^{b}	640±58 ^{dc}	47±6 ^{c}
		100	25.5±4.3 ^{b}	1424±15 ^{d}	22.2±6.5 ^{b}	650±74 ^{d}	72±4 ^{c}
	IL	0	14.5±2.2 ^{b}	7201±52 ^{c}	19.5±9.4 ^{b}	500±65 ^{d}	64±7 ^{c}
		100	37.0±4.4 ^{a}	678±98 ^{e}	27.6±9.4 ^{b}	721±42 ^{c}	223±58 ^{b}
	HL	0	36.9±5.3 ^{a}	1507±67 ^{d}	32.1±9.2 ^{b}	963±87 ^{b}	199±9 ^{b}
		100	20.6±4.8 ^{b}	9214±46 ^{a}	67.1±12.2 ^{a}	1269±76 ^{a}	401±23 ^{a}
Mn (μM)							
Rice	LL	0	2.2±1.5 ^{b}	891±26 ^{c}	23.7±1.2 ^{a}	658±34 ^{c}	36±10 ^{c}
		100	2.2±0.5 ^{b}	1200±252 ^{b}	19.8±4.8 ^{ab}	841±66 ^{b}	65±28 ^{bc}
	IL	0	3.2±2.2 ^{b}	1724±67 ^{a}	15.7±2.3 ^{b}	674±53 ^{c}	82±7 ^{b}
		100	3.2±2.3 ^{b}	950±150 ^{c}	4.9±2.8 ^{c}	921±48 ^{b}	93±35 ^{b}
	HL	0	8.4±4.5 ^{a}	879±26 ^{c}	14.1±2.4 ^{b}	958±85 ^{b}	226±26 ^{a}
		100	2.8±0.4 ^{b}	1600±245 ^{a}	7.9±3.5 ^{bc}	1148±76 ^{a}	181±19 ^{a}
Sunflower							
	LL	0	5.0±1.5 ^{c}	8799±66 ^{a}	13.0±2.6 ^{b}	640±58 ^{ab}	47±6 ^{b}
		100	32.7±12.5 ^{a}	2612±146 ^{e}	25.7±6.8 ^{ab}	821±268 ^{a}	45±18 ^{b}
	IL	0	14.5±4.6 ^{b}	7201±52 ^{b}	19.5±9.8 ^{ab}	500±65 ^{b}	64±7 ^{b}
		100	14.0±3.8 ^{b}	6288±96 ^{c}	23.1±8.2 ^{ab}	912±98 ^{a}	68±8 ^{b}
	HL	0	36.9±16.6 ^{b}	1507±67 ^{f}	32.1±9.4 ^{a}	963±87 ^{a}	99±9 ^{a}
		100	7.3±2.4 ^{a}	4234±220 ^{d}	14.8±6.8 ^{b}	1136±89 ^{a}	85±15 ^{a}

Table 3. Effect of Cu and Mn toxicity on the concentration of protein (mg g^{-1} FW), total amino acids (TAA), proline, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in rice (*Oryza sative* L. cv. Amol) and sunflower (*Helianthus annuus* L. cv. Azarghol)) grown under different light conditions including low (LL=100 µmol $m^{-2}s^{-1}$ PPFD), intermediate (IL=500 µmol $m^{-2}s^{-1}$ PPFD) or high (HL=800 µmol $m^{-2}s^{-1}$ PPFD) light intensity. Values are mean±SD from 4 replicates. Data in each column followed by the same letter are not significantly different (P<0.05)

Treatments			Protein	TAA	Proline	H_2O_2	MDA
			mg g^{-1} FW	µg g^{-1} FW	µmol g^{-1} FW	µmol H_2O_2 g^{-1} FW	nmol g^{-1} FW
Cu (µM)							
Rice	LL	0	120±4 ^{e}	85±22 ^{a}	0.6±0.05 ^{d}	1.3±0.5 ^{b}	6.4±3.2 ^{b}
		100	150±6 ^{e}	103±12 ^{a}	1.3±0.07 ^{c}	3.4±2.2 ^{ab}	8.2±4.5 ^{b}
	IL	0	320±2 ^{d}	110±36 ^{a}	0.7±0.06 ^{d}	1.8±0.8 ^{b}	12.3±2.2 ^{ab}
		100	450±30 ^{c}	81±25 ^{a}	1.6±0.08 ^{b}	3.8±2.4 ^{ab}	11.7±5.5 ^{ab}
	HL	0	720±42 ^{a}	101±30 ^{a}	0.8±0.09 ^{d}	2.7±1.3 ^{ab}	20.3±8.5 ^{a}
		100	620±31 ^{b}	94±14 ^{a}	2.8±0.10 ^{a}	6.4±3.4 ^{a}	22.4±9.7 ^{a}
Sunflower							
	LL	0	130±7 ^{e}	112±20 ^{a}	0.4±0.05 ^{e}	1.0±0.4 ^{b}	10.2±5.4 ^{c}
		100	180±5 ^{de}	75±16 ^{ab}	2.0±0.07 ^{c}	3.0±2.2 ^{ab}	11.4±6.2 ^{c}
	IL	0	280±48 ^{cd}	85±22 ^{a}	0.5±0.06 ^{e}	1.3±0.4 ^{b}	14.5±7.4 ^{b}
		100	340±97 ^{c}	83±18 ^{ab}	2.2±0.08 ^{b}	2.9±1.6 ^{ab}	13.3±2.2 ^{b}
	HL	0	996±81 ^{b}	50±24 ^{b}	1.1±0.09 ^{d}	1.2±0.4 ^{b}	32.8±9.5 ^{a}
		100	1317±70 ^{a}	42±11 ^{b}	2.5±0.10 ^{a}	4.9±2.3 ^{a}	35.8±6.6 ^{a}
Mn (µM)							
Rice	LL	0	120±4 ^{c}	85±22 ^{b}	0.6±0.08 ^{d}	1.5±0.4 ^{a}	10.3±4.2 ^{b}
		100	118±8 ^{c}	98±12 ^{b}	1.1±0.07 ^{c}	2.6±1.3 ^{a}	25.2±8.6 ^{b}
	IL	0	320±21 ^{b}	110±36 ^{b}	0.7±0.04 ^{d}	2.2±1.5 ^{a}	20.8±9.7 ^{b}
		100	346±16 ^{b}	121±22 ^{b}	1.6±0.10 ^{b}	2.3±2.4 ^{a}	27.8±5.4 ^{b}
	HL	0	720±42 ^{a}	101±30 ^{b}	0.8±0.07 ^{d}	2.5±0.6 ^{a}	22.4±4.3 ^{b}
		100	695±31 ^{a}	192±21 ^{a}	1.8±0.09 ^{a}	3.4±1.6 ^{a}	56.2±12.6 ^{a}
Sunflower							
	LL	0	130±7 ^{c}	112±20 ^{a}	0.4±0.09 ^{e}	1.3±0.6 ^{b}	12.4±5.4 ^{b}
		100	99±14 ^{c}	123±17 ^{a}	0.9±0.03 ^{d}	2.5±1.2 ^{ab}	30.8±8.2 ^{ab}
	IL	0	280±48 ^{b}	85±22 ^{ab}	0.5±0.07 ^{e}	1.8±0.4 ^{ab}	20.2±9.4 ^{b}
		100	278±31 ^{b}	132±33 ^{a}	1.4±0.08 ^{b}	3.3±2.2 ^{ab}	32.8±9.6 ^{ab}
	HL	0	996±81 ^{a}	50±24 ^{b}	1.1±0.05 ^{c}	2.1±1.3 ^{ab}	28.7±9.7 ^{b}
		100	895±91 ^{a}	124±14 ^{a}	1.8±0.09 ^{a}	5.2±2.5 ^{a}	52.7±16.6 ^{a}

sunflower. However, the concentration of total amino acids was affected by Mn treatment significantly only in HL plants. Concentration of total amino acids increased 90% and 148% in HL rice and sunflower treated by 100 µM Mn respectively. Similar with Cu, proline concentration increased in response to Mn treatment, similar extent of proline was accumulated in rice and sunflower. Mn treatment caused a slight increase of H_2O_2 concentration, however, these increases were not statistically significant. In contrast to Cu, Mn caused a significant increase in MDA accumulation particularly in HL plants and similar amounts of MDA was accumulated in HL rice and sunflower treated by 100 µM Mn (Table 3).

Discussion

Cu toxicity inhibited growth of plants much more than similar concentrations of Mn in the medium. A high susceptibility of plants to Cu toxicity compared to

other heavy metals such as Mn and Zn was reported by other authors for other plants species [41]. Rice expressed higher susceptibility to both Cu and Mn toxicity than sunflower independent from light conditions. At all levels of heavy metal treatments, light conditions of growth influenced drastically growth and response of plants to Cu and Mn toxicity. In both rice and sunflower, growth was significantly improved due to higher light intensity, indicated that even at HL intensity the light conditions could not be considered as a stress factor. Interestingly, though stimulation of growth by higher light intensity in both Cu and Mn treated plants, the inhibitory effect of Cu and Mn toxicity was affected differently. Higher light intensity strengthened the effect of toxicity of Cu while ameliorated that of Mn. Sunflower plants grown under HL conditions demonstrated even a significant growth stimulation of shoot and root by Mn concentrations as high as 100 µM in the medium which was reached for shoot to 94% at 50 µM and 74% at 100 µM Mn.

Sunflower plants accumulated up to 16 mg g^{-1} DW of Cu in leaf tissues while the highest Cu accumulation in rice was only 1.2 mg g^{-1} DW. A higher Cu accumulation in sunflower did not result in higher sensitivity to Cu toxicity than rice. The absence of any correlation of heavy metal accumulation on dry weight basis and growth response of plants was reported for other species and was attributed to a better compartmentation or detoxification of heavy metals in tolerant species [41].

Although growing under higher light intensities increased Cu concentration only in rice, strengthening effect of higher light intensity on the Cu toxicity-induced growth inhibition was observed in both species. As it was mentioned above, metal accumulation does not always involve in determination of plants tolerance to heavy metals [41]. In contrast, light intensity decreased Mn concentration of leaves in both rice and sunflower. The ameliorating effect of higher light intensity on the expression of Mn toxicity could be partly attributed to the dilution of Mn in leaves because of growth promotion under higher light intensities.

Effect of Cu and Mn Toxicity on Antioxidant Capacity of Plants

Higher light intensity induced activity of APX, such effect was not observed for other antioxidant enzymes studied, with the exception of GR, which was induced under the highest light intensity applied in this experiment. Light can produce reactive oxygen species (ROS) particularly in chloroplasts and induce enzymatic detoxification counteracting these effects [57]. It was shown that under photo-oxidative stress enhanced activity of thylakoid membrane-bound APX functions to maintain the ascorbate content and the redox status of ascorbate under stress conditions [57]. The effect of ascorbate-glutathione cycle in the detoxification of ROS was well documented [5]. Although in this work activity of chloroplastic and cytosolic APX was not individually determined, the main component of APX activity in leaves is localized in chloroplasts than cytosol [57]. In rice, APX activity responded to Cu and Mn differently, Cu toxicity increased APX activity but Mn did not change it or rather decreased. It could be the result of Mn exclusion from chloroplasts, while Cu enters chloroplasts and induces ROS production [58]. Similarly, activity of GR increased in Cu (but not Mn) treated leaves. This may support the above hypothesis on the compartment of the action of excess Cu in the cell. GR located in the chloroplast is responsible for scavenging of ROS in ascorbate-glutathione cycle [5].

Activity of CAT was differently affected in rice and sunflower but the effect of Cu and Mn within each plant species was similar. In rice, Cu and Mn induced the activity of CAT in both LL and HL plants, in sunflower, in LL plants reduced but increased it in HL plants in response to both Cu and Mn. This was in contrast to changes in APX activity. This difference could likely be attributable to the main compartment in which enzymes are localized as well as the different influence of heavy metals in producing ROS depending on light intensity. Activity of SOD and GR responded similarly in both rice and sunflower and in response to both Cu and Mn treatment. Values of APX activity were similar for LL and HL plants under Cu treatments but in HL plants treated by Mn were much lower than LL plants. Considering data for growth and Mn concentration of shoot, HL plants treated by Mn have greater shoot DW than control plants it could be concluded that, protecting effect of APX against ROS has no determining role in plants performance in the presence of Mn.

The amount of protein rose continuously with increasing light intensity. A higher protein concentration in leaf tissues was concomitant with growth improvement, indicating that it is the protein synthesis responsible for increase of protein concentration on fresh weight basis of plants and not a concentration effect. Copper toxicity increased protein concentration of leaves, but Mn had no effect. An increased amount of soluble proteins was also observed in response to Cd [45]. Increase in protein synthesis did not result in reduction of amino acids content. Therefore, a higher synthesis of amino acids in HL plants prevented substrate limitation. The content of total free amino acids did not respond to Cu and Mn treatment, only in HL plants Mn treatment caused a significant increase. Remind that under these treatments

growth of rice plants did not change compared to control and of sunflower rather stimulated.

The effect of heavy metal toxicity on H_2O_2 concentration was mainly in tendency, however, higher light intensity increased H_2O_2 concentration in both control and heavy metal treated plants. As expected, concentration of MDA increased in response to higher light intensity. Copper was not effective in induction of MDA accumulation in leaves, but surprisingly, Mn caused a significant increase in HL rice and sunflower plants. Concentration of MDA increased in response to 100 µM Mn in rice and sunflower, this Mn concentration either did not reduce growth (rice) or rather stimulated it (sunflower). Because heavy metals are well known to induce accumulation of ROS [39], it is expected that plants with higher antioxidant capacity show higher tolerance to toxic concentrations of heavy metals in the medium. As it is obvious from changes in the activity of antioxidant enzymes as well as concentration of oxidant (H_2O_2) and the extent of lipid peroxidation (concentration of MDA), antioxidant capacity of plants could not explain either the different growth response to Cu and Mn or difference between rice and sunflower.

From enzymes studied, only activity of POD was correlated with growth response of plants. Activity of POD decreased in response to Cu and Mn in rice but in sunflower depending on light conditions, either remained unchanged or increased in response to Cu and decreased in the presence of Mn. Similarly, different effect of light intensity on the Cu and Mn responses was correlated with changes of activity of POD. Under LL conditions, activity of POD was lower in Cu treated (11.4 and 22.2 in rice and sunflower respectively) than Mn treated plants (19.8 and 25.7 in rice and sunflower respectively). But in HL plants, the reverse was observed (11.3 and 67.1 in Cu compared to 7.9 and 14.8 in Mn treated rice and sunflower respectively). It means that, changes in the activity of POD were coincided with the light intensity effect on Cu and Mn toxicity tolerance.

Peroxidases are considered to be heavy metal stress-related enzymes [38] and are used as stress markers in metal poisoning situations [15,43]. It was shown that, increase in their activity protect plants to various stress factors [12,23]. In many plant species, excessive uptake of heavy metals such as Ni, Pb and Cd induces a strong increase of peroxidase activities and qualitative changes to their isozyme patterns [7,14,42,53]. In the present work, the unspecific POD activity was assayed with guaiacol as a universal substrate and considered as total activity. Guaiacol POD can exhibit activity of APX (antioxidant enzyme), coniferyl alcohol peroxidase (lignifying enzyme), NADH oxidase and IAA oxidase (growth limiting peroxidases). The individual activity of these enzymes with the exception of APX, were not distinguished from the soluble pool in our extraction procedure. The effects of heavy metals on the activity of oxygen radical detoxifying peroxidases and their involvement in the defense mechanisms of plant tissues against metal-induced damages have been widely reported, but remain controversial [14,18,42,55,56].

Similar to the activity of POD, accumulation of proline was also mainly associated with stress conditions as judged by growth data. Higher light intensity did not affect proline concentration in control plants but caused an accumulation of proline in Cu and Mn treated plants. Differential growth response of rice and sunflower to Cu and Mn was reflected in the amounts of proline concentrations. Comparing percentage of proline concentration between control and heavy metal treated plants, it can be concluded that Cu was most effective in induction of proline accumulation than Mn in both species and under all light conditions. Similarly, the highest accumulation of proline (2.5 times higher than control) was observed in HL rice plants treated with 100 µM Cu and showed the lowest tolerance e.g. up to 73% growth reduction. The lowest change of proline concentration was observed in HL sunflower treated by 100 µM Mn with only 63% increase compared to control, which was associated by 79% growth improvement. Therefore, the concentration of proline monitored the actual performance of plants.

Proline accumulation as an accepted indicator of environmental stresses, is also considered to have important protective roles [1,50]. According to anti-oxidant activity and chelating properties, accumulation of proline in this work should results in higher tolerance of plants. Although there was a correlation between growth response and extent of the proline concentration, it did not cause higher tolerance to heavy metals. In other word, similar to POD activity, accumulation of proline monitored the stress conditions without any protecting role and effect on adaptation of plants.

Exposure to various photooxidative stress factors can stimulate the plant free radical scavenging systems. The activity of one or more antioxidative enzymes can increase and the concentration of low molecular weight antioxidants can be elevated in response to oxidative stress. Such changes are correlated with an improved tolerance [27]. Pre-exposure to sub-lethal levels of one kind of oxidative stress may provide a better acclimation to other kinds of oxidative stress (cross-resistance) due to the activation of the protection system [11,59]. Accordingly, it was expected that induction of GR and APX activity and increase in the concentration

of proline under higher light intensity alleviate the heavy metal stress. However, ameliorating effect of light was observed only in Mn- but not in Cu-treated plants.

Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress. These all appear to be involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell and thus preventing the damaging effects, rather than, developing proteins that can resist the heavy metal effects. Accordingly, tolerant species or ecotypes show mainly an enhanced avoidance and homeostatic mechanisms to prevent the onset of stress. In this work, differential chelation or compartmentation may have a major role in the differential response of plant species studied. Proline accumulation of leaves in Cu treated plants was much higher in sunflower than rice. Therefore, that is likely the cause of different response of these two plant species to Cu toxicity.

Results of present work imply that the antioxidant capacity of plants could not be independently correlated with the growth response of plants without regarding the other physiological responses to metal exposure. It has been suggested that the tolerance to metal toxicity is more dependent on the availability of reduced cell metabolites, than on antioxidative enzymes capacity of plant tissues alone [15,18,39].

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