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ARTICLE

Responses of antioxidant defense capacity and photosynthesis of bean (*Phaseolus vulgaris* L.) plants to copper and manganese toxicity under different light intensities

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ABSTRACT Effects of toxic concentrations of Cu and Mn were studied in bean (*Phaseolus vulgaris*) plants under different light intensities. Exposure of plants to 100 µM Cu, inhibited their growth up to 79%, while similar concentrations of Mn caused only a slight (21%) reduction of plants dry weight. Net assimilation rate (*A*) was inhibited with similar extent by both heavy metals and F_v/F_0 ratio decreased particularly by Mn treatment. With the exception of ascorbate peroxidase in Cu treated plants, activity of catalase, superoxide dismutase and glutathione reductase were stimulated by both heavy metals with similar extent. Change in guaiacol peroxidase activity was correlated with growth response, increased by Cu but was not affected by Mn toxicity. Similar to peroxidase activity, accumulation of proline monitored the stress conditions but did not cause more protection against Cu toxicity. On the other hand, concentration of H_2O_2 and malondialdehyde indicated a higher oxidative stress simultaneous with higher growth in the Mn than Cu treated plants. Results imply that, growth response to Cu and Mn could be reflected neither by the chlorophyll fluorescence nor the net assimilation rate. Additionally, antioxidant defense capacity did not involve in different response to Cu and Mn in bean plants.
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KEY WORDS

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gas exchange
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Mn toxicity

Copper and manganese are essential micronutrients for plants. However, excess amounts of these heavy metals induce a wide range of biochemical effects and physiological processes and alter photosynthesis, pigment synthesis, protein metabolism and membrane integrity (Hall 1994). Usual causes of high levels of Cu in the soil are mining activities or the prolonged application of Cu-based fungicides. In contrast, manganese (Mn) is not a common pollutant in soils, but various soil conditions often present in acid and volcanic soils or submergence can lead to Mn reduction and create Mn toxicity in many natural and agricultural systems (Foy et al. 1978).

Toxic trace pollutants can induce many alterations in plant cells (Woolhouse 1983), but it is difficult to draw a general mechanism about the physiology of stress, since metal toxicity results from complex interaction of metal ions with several metabolic pathways. However, one of the underlying causes of tissues injury following exposure of plants to Cu (Chen et al. 2000) and Mn (González et al. 1998) is the increased accumulation of reactive oxygen species mediated-oxidative stress (De Vos and Schat 1991).

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 usually correlated with an improved tolerance (Gressel and Galun, 1994). Accordingly, 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 (Schöner and Krause 1990; Bridger et al. 1994).

The deleterious effects resulting from cellular oxidative state may be alleviated by detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT), and enzymes of ascorbate-glutathione cycle, ascorbate peroxidase (APX) and glutathione reductase (GR). Peroxidases are considered to be heavy metal stress-related enzymes (Karataglis et al. 1991) and can be used as stress markers in metal poisoning situations (Mocquot et al. 1996; Chaoui et al. 2004). However, increase in their activity is thought to be a common response and can protect plants to various stress factors (Gaspar et al. 1985; Castillo 1992).

Proline accumulation accepted as an indicator of environmental stresses, is also considered to have important protective roles (Alia and Saradhi 1991). Accumulation of free proline in response to heavy metal exposure seems to be wide-spread among plants (Schat et al. 1997). The

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functional significance of proline accumulation would lie in its contribution to water balance maintenance (Costa and Morel 1994) scavenging of hydroxyl radicals (Smirnoff and Cumbes 1989) and metal chelation in the cytoplasm (Farago and Mullen 1979).

One of the most important effects of reactive oxygen species is the loss of membrane integrity (Vichnevetskaia and Roy 1999). Thylakoid lipids are susceptible to oxidation due to predominance of polyunsaturated fatty acids (Gounaries et al. 1986) and the availability of oxygen producing free radicals around functional PSII (Kyle 1987). Therefore, photosynthetic membranes in chloroplasts are the most susceptible structures in plants grown under conditions of oxidative stresses. Reactive oxygen species not only are produced in response to heavy metal stress in shoot and root, but also higher light intensity alone or in combination with heavy metal toxicity could led to a severe oxidative damage of leaves in stressed plants.

It was reported that, response of plants to excess Mn is affected by light intensity (González et al. 1998). However, reports on the effect of high light intensity on Mn toxicity are contradictory, including increasing (Horiguchi 1988; Nable et al.1988) or lessening toxicity symptoms (Wissemeier and Horst 1992). A factor that complicates the interpretation of previous studies of the effect of light intensity on Mn-toxicity symptoms is the fact that plants grown in low light usually accumulate less foliar Mn than those grown at a higher light intensity (Mc Cain and Markley 1989). In contrast to Mn, there is no report on the effect of growth under various light intensities on Cu tolerance of plants.

Copper is a potent inhibitor of photosynthesis and chlorophyll synthesis (Fernandes and Henriques 1991) in some plants such as barley and spinach. However, no significant effect of Cu toxicity on chlorophyll synthesis in maize was reported (Chaoui et al. 1997). Similarly, reports on the effect of Mn toxicity on chlorophyll content of plants, is contradictory, from no change (Wissemeier and Horst 1992) to severe chlorosis (Horiguchi 1988; Nable et al.1988) depending on plant species.

Copper has been shown to increase susceptibility to photo-inhibition particularly in intact leaves (Pätsikkä et al. 1998) but the underlying mechanism has remained unclear. It has been known that high concentrations of copper when added to the incubation medium of isolated thylakoids, inhibit PSII electron transfer activity on the acceptor side (Yruela et al. 1996) and finally cause the release of the external polypeptides of the oxygen-evolving complex on the donor side of PSII (Pätsikkä et al. 2001). Some authors suggested that excess copper in the growth medium did not cause loss of photoprotection, but reduced chlorophyll content causes the high photosensitivity of PSII in copper treated plants (Pätsikkä et al. 2002). Very limited data are available concerning photochemistry of leaves under Mn toxicity.

Changes in chlorophyll fluorescence emissions are indications of changes in photosynthetic activity (Kautsky et al. 1960). Chlorophyll fluorescence gives information about the state of Photosystem II and about the extent to which PSII is using the energy absorbed by chlorophyll and the extent to which it is being damaged by excess light (Maxwell and Johnson 2000). The flow of electrons through PSII is indicative of the overall rate of photosynthesis and is an estimation of photosynthetic performance. To our knowledge, this is the first study of the potential impact of Cu and Mn toxicity in combination with high light intensity on photosynthesis.

There are only limited data available concerning response of photosynthesis capacity of plants to heavy metal toxicity. It is still an open question as to whether stomatal closure is the main factor inhibiting photosynthesis and biomass production under heavy metal stress. It is also plausible that heavy metals toxicity affects the photochemistry of leaves and inhibits biophysical processes of photosynthesis.

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 (Marschner 1995), it is necessary to under taken a comparative study of Mn and Cu toxicity on biochemical processes of plants particularly antioxidant defense system and photosynthesis processes. In this work we studied the response of bean plants to Cu and Mn 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 and various physiological traits of photosynthesis in growth and biomass production of bean plant under heavy metal toxicity.

Materials and Methods

Seeds of one cultivar of bean (*Phaseolus vulgaris* cv. Naz) was used in this study were provided by Seed and Plant Improvement Institute (SPII) (Karaj, Iran).

Plant cultures and treatments

The 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 $CaSO_4$ at 0.05 mM. The 7-day-old seedlings with uniform size were transferred to hydroponic culture in plastic container with 2L of nutrient solution (50%) and pre-cultured for 3 days. Copper and Mn treatments were started for 10-days-old plants, consisted of three levels of $CuSO_4$ or $MnSO_4$ at 0 (control), 50 and 100 µM. Plants were treated for 14 days and nutrient solutions were completely changed every 3 days. Composition of the nutrient solutions were used according to Neumann et al. (1999), pH was 6.5 and adjusted every day.

For study of the effect of different light intensities, plants were grown simultaneously under three light intensities: 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. The incident photosynthetic photon flux density (PPFD) was measured by a quantum sensor attached to the leaf chamber of the gas exchange unit.

Harvest

After 14 days treatment, plants were harvested. For removing of the apoplasmic Cu and Mn from roots, plants were placed for 20 min in 5 mM $CaCl_2$+25% nutrient solution. Thereafter, plants were divided into shoots and roots, roots were washed with distilled water, weighed and blotted dry on filter paper and dried at 70°C for 2 days to determine plant dry weight.

For the determination of Cu and Mn content, oven-dried samples were ashed in a muffle furnace at 550°C for 8h, thereafter samples were resuspended in 2 ml 10% HCl and made up to volume by double-distilled water. Copper and Mn concentration was determined by atomic absorption spectrophotometry (Shimadzu, AA 6500).

Chlorophyll concentration was measured spectrophotometrically in the third youngest leaf after weighing and a 48 h extraction in N,N-dimethylformamide. The absorbance of chlorophyll was measured at 644, 647 and 603 nm using spectrophotometer (Specord 200, Analytical Jena, Germany) and chlorophyll concentration was calculated using following formula (Moran 1982). Thereafter, values were recalculated on the fresh weight basis of leaves.

Chlorophyll a (µg ml^{-1}) = 12.91 A_{644} – 2.12 A_{647} – 3.85 A_{603}

Chlorophyll b (µg ml^{-1}) = -4.67 A_{644} + 26.09 A_{647} – 12.79 A_{603}

Another group of plants were used for measurement of gas exchange parameters and chlorophyll fluorescence.

Determination of gas exchange parameters

CO_2 assimilation and transpiration rates of attached leaves were measured with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) always between 9:00 A.M. and 15:00 P.M. Measurements were carried out on one mature, fully expanded and attached leaf from 4 plants per treatment illuminated with the treatment specific light intensity (LL, IL or HL). With the exception of PPFD, no microenvironmental variable inside the chamber was controlled.

The net photosynthesis rate by unit of leaf area (*A*) and the stomatal conductance to water vapor (g_s) were calculated using the values of CO_2 and humidity variation inside the chamber, both measured by the infrared gas analyzer of the portable photosynthesis system. Other parameters calculated were the ratio of intercellular air space and atmospheric CO_2 molar fraction (*Ci/Ca*) and photosynthetic water use efficiency (*WUE*) was determined by the ratio of net photosynthesis rate (*A*) to transpiration rate (*E*) (*WUE=A/E*).

Determination of chlorophyll fluorescence

Chlorophyll fluorescence parameters were recorded in parallel for gas exchange measurements in the same leaf, using a portable fluorometer (FIM, ADC Bioscientific Ltd., UK). Leaves were acclimated to dark for 30 min before measurements were taken. Initial (F_0), maximum (F_m), variable ($F_v=F_m-F_0$) as well as $F_v:F_m$ and $F_v:F_0$ ratios were recorded.

Experiments were under taken in complete randomized block design with 4 replications. Statistical analyses were carried out using Sigma stat (3.02) with Tukey test ($p<0.05$).

Enzyme assays

Fresh leaf samples were used for enzyme extraction and measurement of protein and metabolites. Whole shoot was 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. Change in the absorbance of substrates or products (depending on assay protocol) was measured using spectrophotometer (Specord 200, Analytical Jena, Germany). Activity of enzymes were calculated on the protein concentration basis (specific 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 (2002). 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 (Simon et al. 1974). 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 mol of H_2O_2 in one min.

Peroxidase: Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test (Chance and Maehly 1955). 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

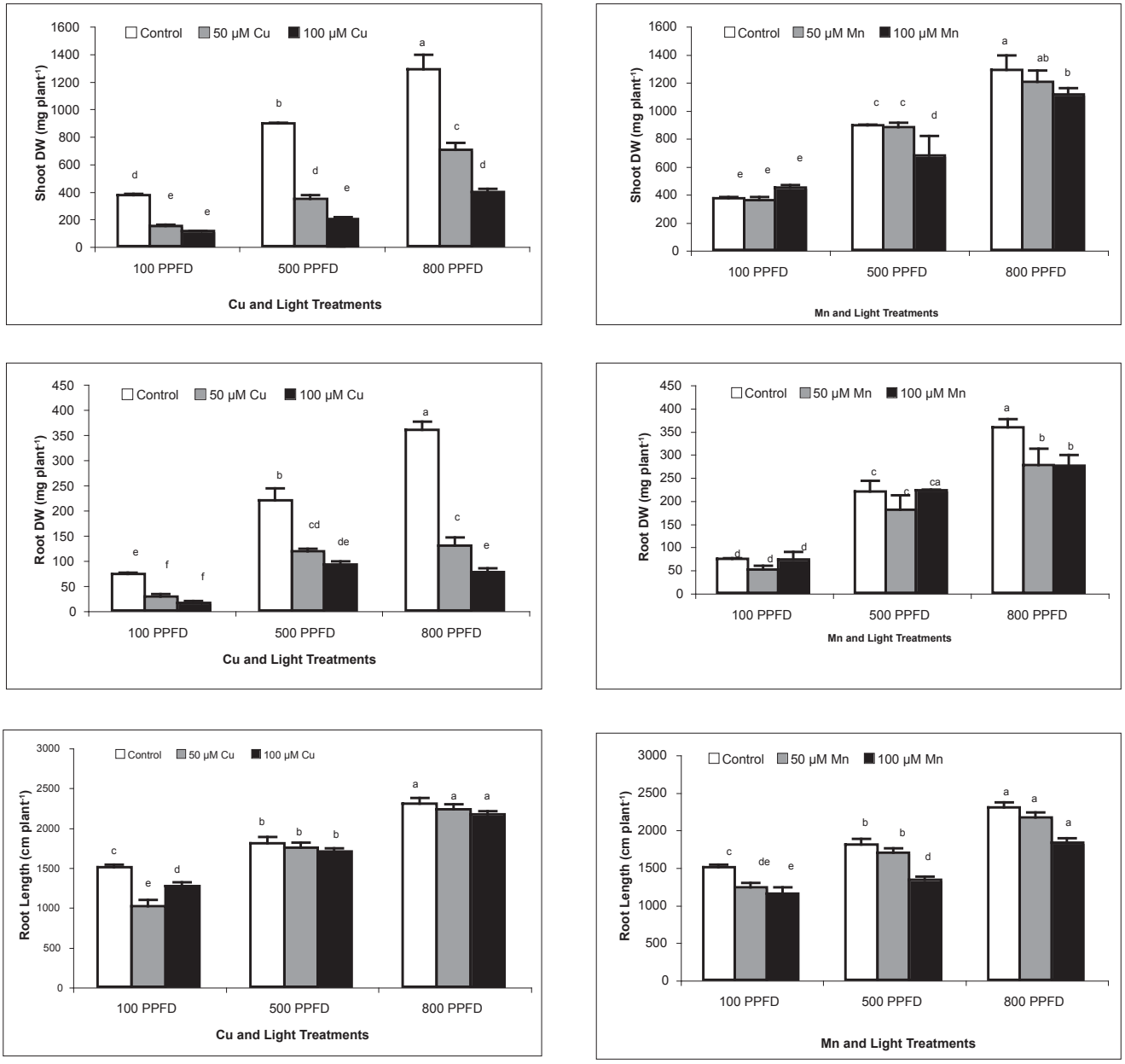

Figure 1. Effect of Cu and Mn treatments on shoot and root dry weight and root length of bean (*Phaseolus vulgaris* L.) plants under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

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 µmol tetraguaiacol for 1 min.

Superoxide dismutase: Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). 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

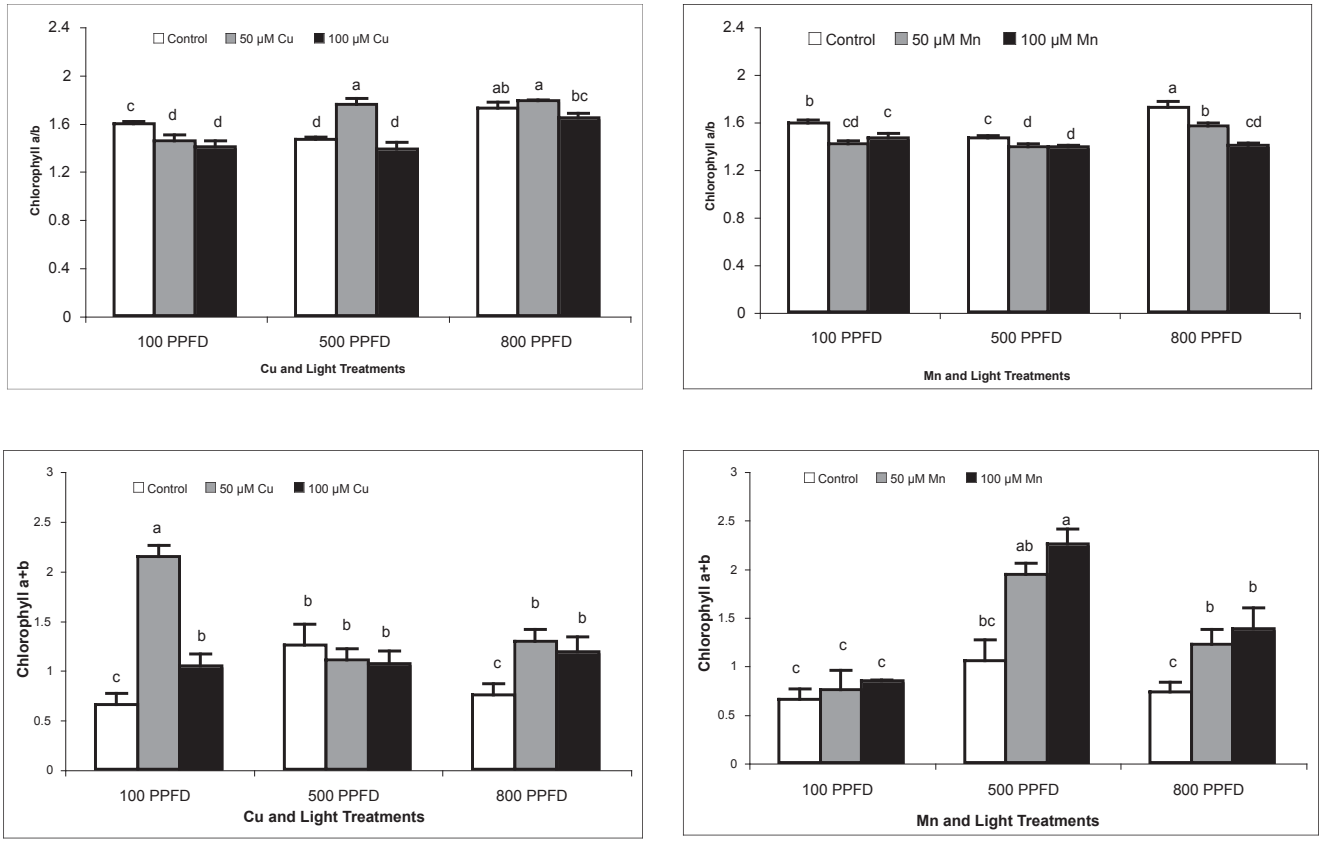

Figure 2. Changes in the leaf chlorophyll a/b ratio and chlorophyll a+b amounts in bean (*Phaseolus vulgaris* L.) plants treated by Cu or Mn and grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

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 4ºC 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 (1976). 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 25ºC. One unit of enzyme activity was calculated as enzyme protein required for oxidation of one µmol NADPH in 1 min.

Other assays

Total protein concentration: Soluble proteins were determined as described by Bradford (1976) using a commercial reagent (Sigma) and BSA (Merck) as standard.

Total amino acids: Content of total free α-amino acids was assayed using ninhydrin colorimetric method (Hwang and Ederer 1975). 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. (1973). 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. (1984). 1-1.5 g of leaf was homogenized with 0.2 g activated charcoal

Table 1. Effect of Cu and Mn treatment on the shoot concentration of Cu and Mn (μg g^{-1} DW) in bean (*Phaseolus vulgaris* L. cv. Naz) plants grown under three light conditions including low (LL), intermediate (IL) or high (HL) for 14 days in hydroponic medium. The means refer to 4 repetitions ± SD. Data in each column followed by the same letter are not significantly different (P<0.05).

Light Treatment	Cu/Mn (μM)	Cu Concentration	Mn Concentration
LL	0	794±34 ^{f}	120±25 ^{e}
	100	7239±52 ^{c}	854±12 ^{c}
IL	0	1100±43 ^{e}	152±12 ^{e}
	100	8456±82 ^{b}	1017±26 ^{b}
HL	0	1289±63 ^{d}	239±33 ^{d}
	100	12300±125 ^{a}	1321±45 ^{a}

(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 (Heath and Packer 1968). 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 (Boominathan and Doran 2002).

All experiments were conducted using 4 independent replications. Statistical analyses were carried out using Sigma stat (3.02).

Results

Dry matter production and heavy metal accumulation

In general, plants grown under higher light intensity have higher dry matter of shoot and root and root length. Toxic concentrations of Cu decreased shoot and root growth drastically, this reduction was 71% and 69% for shoot, 79% and 79% for roots dry weight of LL and HL plants respectively. Root length was affected much less than shoot and root dry weight and was only reduced 16% and 6% for LL and HL plants respectively. Tolerance of plants to toxic concentrations of Mn, was much higher compared to Cu. Shoot dry weight was reduced by 100 μM Mn only 21% and 14% in LL and HL plants respectively. Root dry weight was not affected in LL plants treated by Mn concentration as high as 100 μM, and in HL plants rather an increase up to 23% was observed. As it is obvious from growth data, light intensity did not influence Cu and Mn tolerance of bean plants, so that the extent of inhibitory effect of toxic concentrations of Cu and Mn in the medium was similar between LL, IL and HL plants (Fig. 1). Growing under higher light intensity increased accumulation of both Cu and Mn in shoots. Copper accumulated in shoots up to 12 mg g^{-1} DW, the corresponding amount for Mn was only 1.3 mg g^{-1} DW (Table 1).

Chlorophyll concentration

In the absence of Cu and Mn, chlorophyll (a+b) concentration increased under light intensity of 500 PPFD (IL) but decreased with further increasing of light intensity. Chlorophyll (a+b) concentration was influenced by both Cu and Mn treatment. Moderately toxic levels of Cu (50 μM) increased chlorophyll (a+b) concentration in LL plants, resulting from higher reduction of leaf growth than chlorophyll concentration. Cu treatment at 50 μM, caused an increase of the ratio of chlorophyll a/b in IL and HL plants, which was the result of increased values of chlorophyll a in Cu treated leaves (data of individual values of chlorophyll a and b were not shown). Manganese treatment caused a significant increase of chlorophyll a+b concentration in plants grown under higher light conditions. In contrast to Cu, Mn caused a reduction of the ratio of chlorophyll a/b, which was the result of increasing values of chlorophyll b values of Mn treated leaves (Fig. 2).

Gas exchange parameters

As expected, net assimilation rate (*A*) was higher in plants grown under higher light intensity. Cu and Mn treatment of plants affected negatively the net assimilation rate, the reduction were 40% and 71% in response to Cu treatment, 50% and 76% in response to Mn treatment for LL and HL plants respectively. Therefore, plants were much sensitive to inhibitory effect of heavy metals treatments when grown under higher compared to lower light intensities. Water loss via transpiration was not affected by Cu and Mn treatment significantly, however, increase in transpiration due to growth under higher light intensities was lower in heavy metal treated plants. Transpiration was 3.5 times higher in HL compared to LL in control plants, while the corresponding value for Cu treated plants was only 2.5 and for Mn treated ones was 2.0.

Table 2. Gas exchange parameters including net photosynthetic rate (*A*), transpiration (*E*), the ratio of intercellular air space and atmospheric CO_2 molar fractions (*Ci/Ca*), stomatal conductance to water vapor (*gs*) and instantaneous water use efficiency (*WUE*) and chlorophyll fluorescence parameters including F_v/F_m and F_v/F_0 of bean (*Phaseolus vulgaris* L.) treated with Cu or Mn under three light conditions. The means refer to 4 repetitions ± SD. Data in each column followed by the same letter are not significantly different (P<0.05).

	Cu/Mn (µM)	Cu Treatment			Mn Treatment		
		LL	IL	HL	LL	IL	HL
A (µmol m^{-2} s^{-1})	0	20±9 [a]	23±14 [a]	42±25 [a]	20±9 [a]	23±14 [a]	42±25 [a]
	50	10±3 [a]	12±7 [a]	10±4 [b]	12±4 [a]	10±5 [a]	22±9 [ab]
	100	12±6 [a]	12±5 [a]	12±4 [b]	10±7 [a]	7±5 [a]	10±4 [b]
E (mmol m^{-2} s^{-1})	0	0.35±0.12 [a]	0.75±0.21 [a]	1.22±0.71 [a]	0.35±0.12 [a]	0.75±0.21 [a]	1.22±0.71 [a]
	50	0.35±0.01 [a]	0.73±0.51 [a]	0.93±0.51 [a]	0.38±0.11 [a]	0.74±0.20 [a]	0.95±0.50 [a]
	100	0.37±0.11 [a]	0.63±0.30 [a]	0.94±0.41 [a]	0.41±0.20 [a]	0.71±0. 01 [a]	0.83±0.30 [a]
Ci/Ca	0	1.01±0.45 [a]	0.89±0.31 [a]	0.95±0.50 [a]	1.01±0.45 [a]	0.89±0.31 [a]	0.95±0.50 [a]
	50	0.85±0.38 [a]	1.16±0.63 [a]	1.25±0.48 [a]	0.92±0.32 [a]	0.68±0.21 [a]	0.98±0.32 [a]
	100	0.85±0.32 [a]	1.10±0.88 [a]	0.97±0.34 [a]	0.86±0.42 [a]	0.63±0.33 [a]	1.08±0.45 [a]
g_s (mol m^{-2} s^{-1})	0	19±10 [a]	24±13 [a]	41±24 [a]	19±10 [a]	24±13 [a]	41±24 [a]
	50	11±5 [a]	16±10 [a]	32±14 [a]	16±8 [a]	20±13 [a]	31±12 [a]
	100	12±8 [a]	14±7 [a]	35±21 [a]	14±9 [a]	19±7 [a]	33±18 [a]
WUE (µmol mol^{-1})	0	57±8 [a]	31±5 [a]	35±8 [a]	57±8 [a]	31±5 [a]	35±8 [a]
	50	29±7 [b]	16±6 [b]	11±5 [b]	32±8 [b]	14±4 [b]	23±4 [b]
	100	32±6 [b]	19±5 [ab]	13±6 [b]	24±9 [b]	10±4 [b]	12±5 [b]
Fv/Fm	0	0.69±0.01 [a]	0.72±0.04 [a]	0.82±0.05 [a]	0.70±0.04 [a]	0.73±0.05 [a]	0.83±0.04 [a]
	50	0.66±0.02 [a]	0.70±0.03 [a]	0.80±0.02 [a]	0.67±0.04 [a]	0.71±0.05 [a]	0.81±0.05 [a]
	100	0.65±0.01 [a]	0.70±0.04 [a]	0.79±0.03 [a]	0.64±0.03 [a]	0.69±0.04 [a]	0.78±0.04 [a]
Fv/F_0	0	2.21±0.08 [a]	2.60±0.05 [a]	4.56±0.18 [a]	2.21±0.08 [a]	2.60±0.06 [a]	4.56±0.08 [a]
	50	1.96±0.04 [b]	2.37±0.14 [b]	4.33±0.11 [a]	1.89±0.09 [b]	2.25±0.11 [b]	4.13±0.13 [b]
	100	1.88±0.04 [b]	2.33±0.08 [b]	3.70±0.12 [b]	1.63±0.09 [c]	2.13±0.13 [b]	3.27±0.15 [c]

Stomatal conductance of leaves was only influenced by light intensity and a gradual reduction of g_s values in response to Cu and Mn treatment was mainly in tendency and not significant. Water use efficiency was lowered by higher light intensity, Cu and Mn treatment exerted also similar effect, but the changes were mainly in tendency and not significant (Table 2).

Chlorophyll fluorescence

In response to treatment with both of Cu and Mn, F_0 values increased which was observed under all three light conditions applied. Values of F_m were decreased in Cu and Mn treated plants, but only under the lowest light intensity applied in the experiment (PPFD=100 µmol $m^{-2}s^{-1}$). Under higher light intensities F_m values diminished in response to both of Cu and Mn treatment. Treatment by Cu and Mn caused reduction of F_v values, particularly under higher light conditions. Changes of F_0, F_m and F_v values were more pronounced in response to Cu than Mn treatment. T_m values were also decreased in heavy metal treated plants. The ratio of F_v/F_m remained mainly unchanged in response to Cu and Mn treatment. In contrast, the ratio of F_v/F_0 decreased significantly in response to both Cu and Mn treatment. This reduction was 15% and 19% for Cu- and 26% and 28% for Mn-treated plants (Table 2).

Activity of antioxidant enzymes

Activity of APX responded to light intensity and both heavy metals. Higher light intensity caused a reduction in the activity of APX in control as well as Cu treated plants. Cu treatment induced reduction of APX activity in tendency or significant. In contrast, Mn treatment not only did reduce APX activity, but also resulted in a significant increase of the activity under IL and HL intensity. Catalase, SOD and GR activity responded with similar manner, in which both of increasing light intensity and the presence of heavy metals induced an increase in their activity. Similar extent of increase was observed in response to Cu and Mn. Similar with APX, activity of POD responded differently depending on heavy metals, a significant increase of POD activity was observed in response to Cu, while Mn treatment did not affect it. In contrast to APX, CAT, SOD and GR, light intensity did not influence activity of POD either in Cu or Mn treated plants (Fig. 3).

Concentration of oxidant and antioxidant metabolites

Only the highest light intensity induced a significant accumulation of proline in control plants. Copper treatment of plants caused a drastic rise of proline concentration, however, the

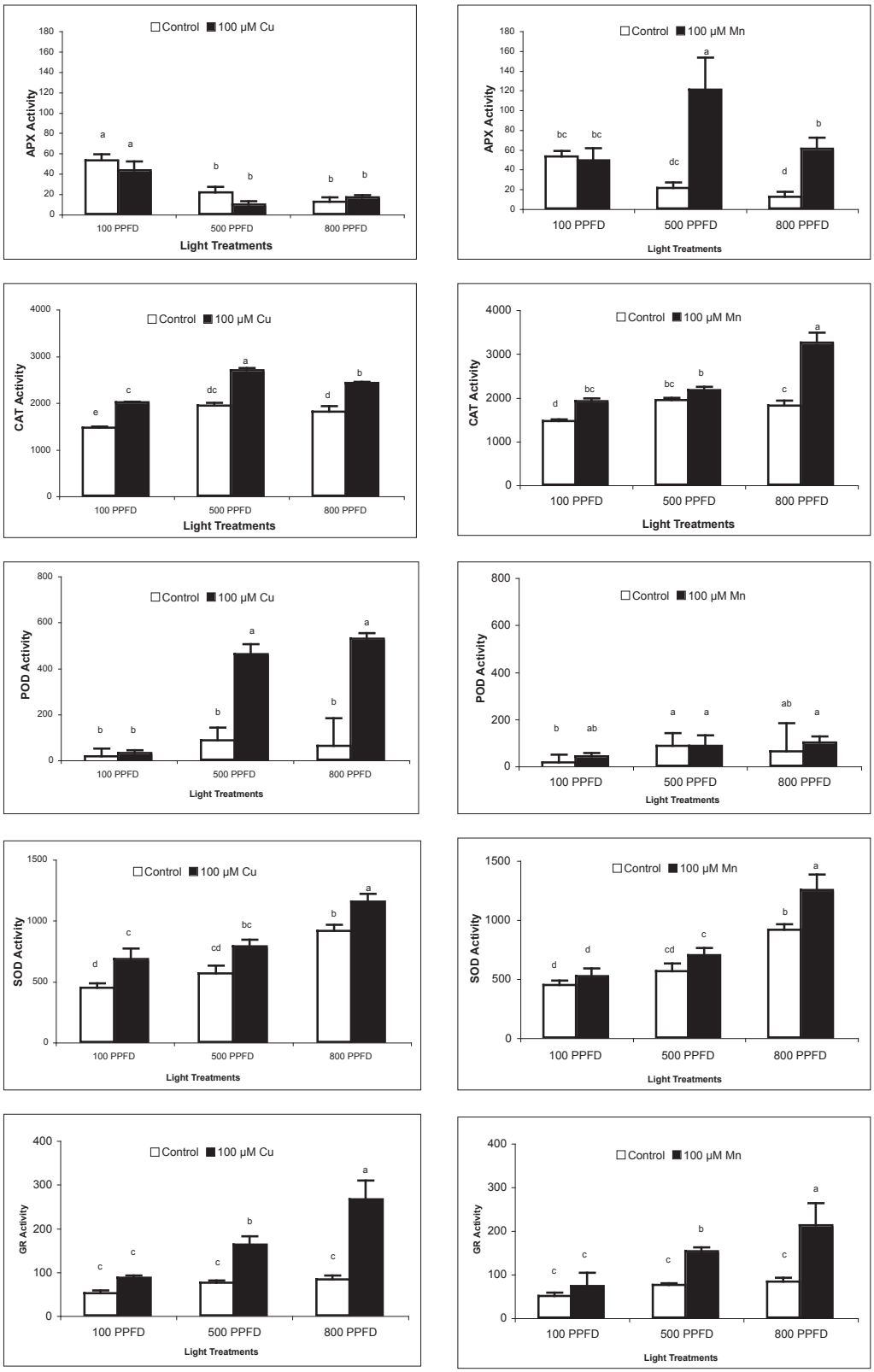

Figure 3. Effect of Cu and Mn treatment on the activity (Unit mg $^{-1}$ Pro. min^{-1}) of ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR) in leaves of bean (*Phaseolus vulgaris* L.) plants grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

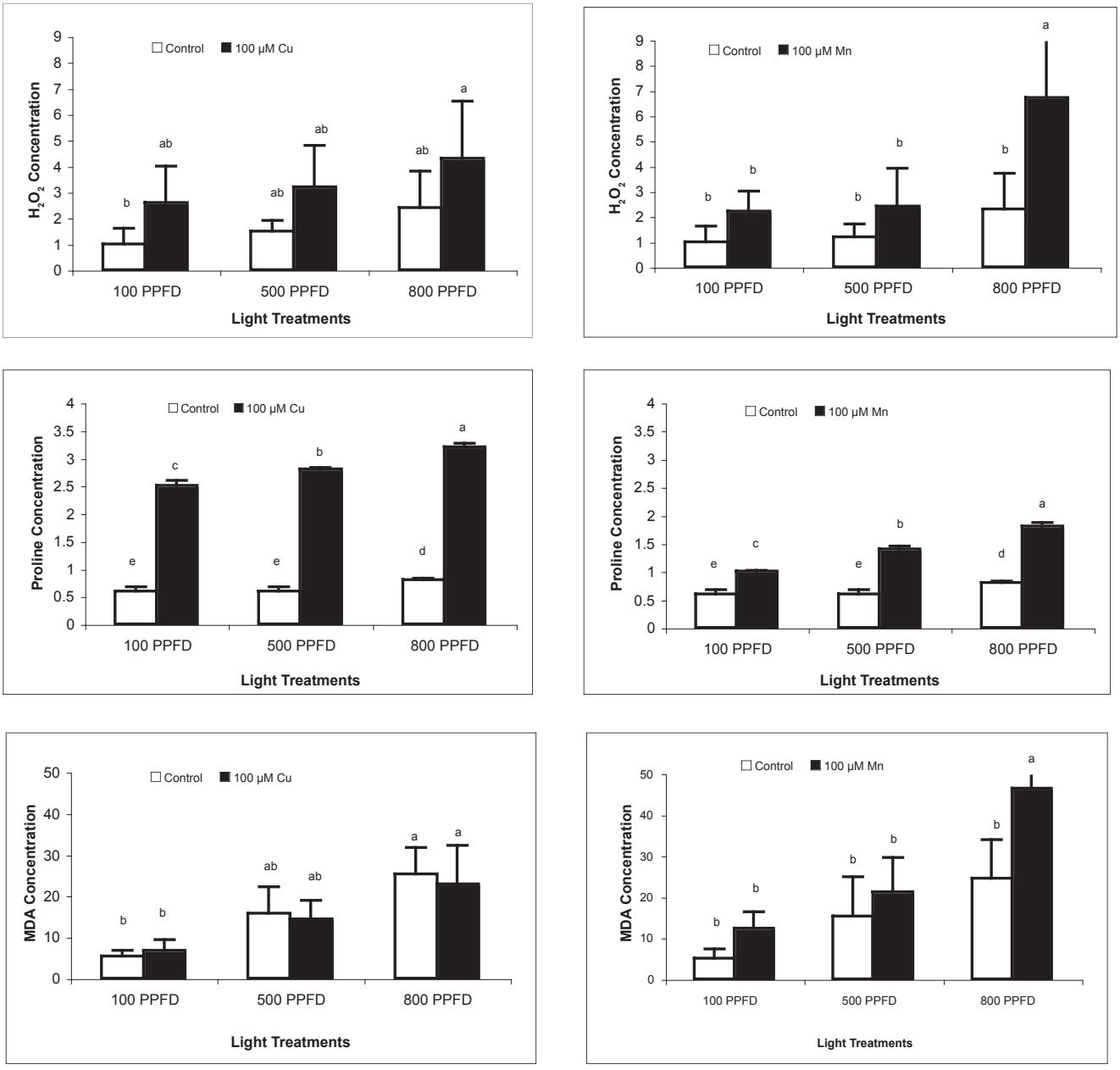

Figure 4. Effect of Cu and Mn treatment on the concentration of proline (µM g^{-1} FW), hydrogen peroxide (H_2O_2) (µM H_2O_2 g^{-1} FW) and malondialdehyde (MDA) (nM g^{-1} FW) in bean (*Phaseolus vulgaris* L.) plants grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

effect of Mn treatment was much less pronounced. Increasing light intensity resulted in an accumulation of H_2O_2 in leaves in tendency or significant, Cu and Mn treatment caused also further accumulation, Mn being effective than Cu. Similar changes were observed for MDA content of leaves. As expected, light intensity caused a significant increase in MDA content of leaves, however, in contrast to Mn, Cu did not affect MDA content of leaves (Fig. 4).

Total amino acids and protein concentration

Content of total amino acids of leaves increased in response to higher light intensity and both of studied heavy metals. Protein concentration, in contrast, remained unchanged in Cu and Mn treated plants compared to control. Growing under intermediate (500 PPFD) and high (800 PPFD) light intensities caused a rise of protein concentration compared to control (Fig. 5).

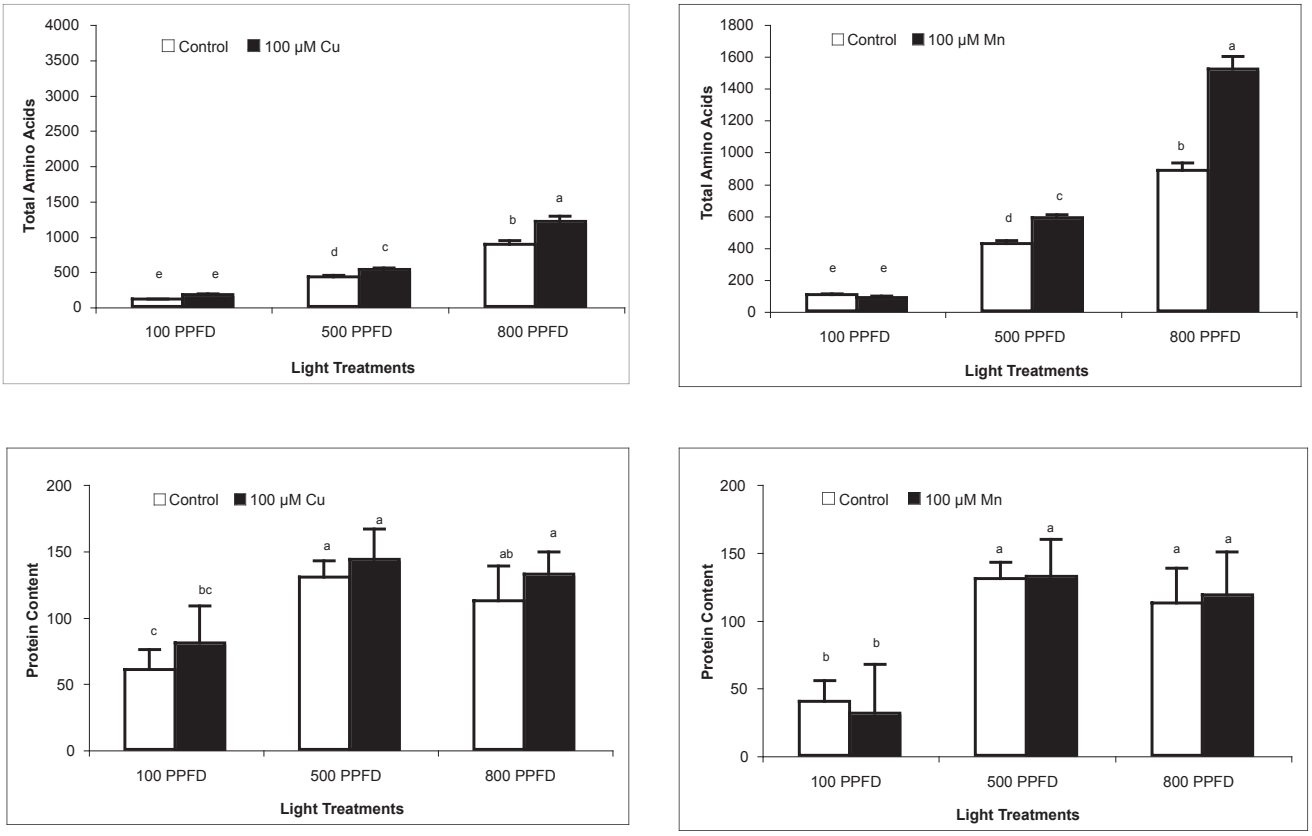

Figure 5. Effect of Cu and Mn treatment on the concentration of total amino acids (µg g^{-1} FW) and protein (mg g^{-1} FW) in bean (*Phaseolus vulgaris* L.) plants grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different ($P<0.05$).

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 (Marschner 1995). Growing under higher light intensity caused a significant rise of heavy metal accumulation likely due to higher transpiration. The rate of transpiration of HL leaves was 2.5 and 2.0 times higher than LL ones for Cu and Mn treated plants respectively.

Leaf chlorophyll a+b concentration, with the exception of an increase at 50 µM Cu in LL plants, did not affect significantly by excess Cu in the medium. In contrast, Mn treatment caused an increase of chlorophyll a+b at all light conditions, showing higher tolerance of leaf chlorophyll to Mn than leaf fresh weight and a concentration effect of chlorophyll. The ratio of chlorophyll a/b was changed by Cu and Mn differently. Considering fresh weight changes and individual values of chlorophyll a and b, it could be shown that degradation of chlorophyll b in response to Cu was greater than growth reduction of leaves, but degradation of chlorophyll a was less than growth impairment. The opposite was observed for Mn. The cause of a higher sensitivity of chlorophyll b to Cu toxicity and of chlorophyll a to Mn toxicity is not known. A higher chlorophyll b degradation than chlorophyll a under Cu stress was observed also in rice and sunflower (Hajiboland and Hasani 2007) and bean (Pätsikkä et al. 2002).

Reduction of assimilation rate in Cu treated plants under higher light intensity was only partly attributable to the reduction of stomatal conductance. Stomatal conductance diminished slightly (15%), while reduction of *A* was much higher and reached up to 71% compared to control in Cu treated HL plants. Greater reduction of *A* in response to Cu under higher light intensity could be attributed to the induction of oxidative stress. It seems most likely that, a combination of two factors i.e. Cu treatment (Luna et al. 1994) and higher light intensity (Behera and Choudhury 2002) both inducing oxidative damage to the cell and particularly photosynthetic membranes (Elefteriou and Karatagalis 1989), inhibit the net assimilation rate much more than that imposed by closure of stomata. Reduction of APX activity by increasing light intensity could be another causes of more reduction of photosynthesis in HL than LL plants in response to toxic concen-

trations of Cu. Limitation in antioxidant capacity due to Cu treatment prevents proper detoxification of reactive oxygen species, which were produced up to great amounts in plants treated by Cu and grown under high light intensity.

However, in Mn treated leaves under all three light conditions the extent of reduction of A was higher than that of g_s, indicating that other factors involve in the reduction of net assimilation rate than combination effect of light and heavy metal. In addition, changes of APX activity could not explain higher sensitivity of net assimilation rate than stomatal conductance to Mn treatment. Though activity of APX decreased in response to higher light intensity, increased in plants due to exposure to Mn. More inhibition of photochemistry of plants under Mn treatment could be of involving factors of decreased net assimilation rate (see below).

The biophysical basis underlying changes in the photosynthetic characteristics of bean plants was assessed using chlorophyll fluorescence. Measurement of chlorophyll a fluorescence is a non-invasive, powerful and reliable method, to assess the PSII function of Cu and Mn treated leaves in this work.

The initial chlorophyll fluorescence yield, F_0 reflects the minimal fluorescence yield when all Q_A are in the oxidized state. According to our results, the increase of F_0 recorded under Cu and Mn toxicity, can be interpreted as a reduction of the rate of energy trapping by PSII centers and this could be the result of a physical dissociation of light harvesting complex from PSII core, as it has been observed under other stress such as heat (Armond et al. 1980). The increase of F_0 value, was higher in Mn compared to Cu treated leaves, therefore, Mn was more effective in reduction of rate of energy trapping by PSII centers. In addition, Cu and Mn treated bean plants had a significant smaller F_m value compared to the control, which in relation to the slightly reduced T_m, indicates that an increasing fraction of reaction centers becomes inactivated. It is well known that when F_m is attained, all PQ molecules are reduced. Thus, we can suggest that F_m depression may reflect also a decreased size of antennas and/or a diminished pool of PQ (Ouzounidou et al. 1998). Moreover, reduction of F_m values in heavy metal treated plants was observed only in HL plants. These values remained unchanged in IL and increased in LL plants. Therefore, a decreased size of antennas and/or a diminished pool of PQ occurred only under the dual effect of heavy metal stress and high light intensity.

Manganese treated plants had higher reduction of F_v than Cu treated ones, indicating that Mn exerted higher inhibitory effect on photochemistry of leaves. Decrease in F_v indicates a reduction in the number of open PSII units and could be attributed to a structural and functional disorder of the photosynthetic apparatus and damage to the PSII (Osmond 1994; Pereira et al. 2000; Murkowski 2001).

The direct effect of increasing F_0, is the slight decrease of F_v/F_m ratio. The maximal quantum yield of PSII (F_v/F_m) ratio declined less than 10% between the control and heavy metal treated leaves. The preservation of F_v/F_m ratio under Cu and Mn toxicity, as indicated by very small changes, probably is the consequence of a modification of the Q_A to Q_B electron transfer (Ouzounidou et al. 1998). In contrast to F_v/F_m, F_v/F_0 ratio decreased significantly in response to heavy metals. The change in the ratio of F_v/F_0 was higher in Mn-compared to Cu-treated plants, nevertheless, it was not coincided on differential growth response of plants concerning Cu and Mn treatment.

A higher inhibitory effect of Mn on biophysical processes of photosynthesis could be attributed to the preferential entrance of Mn into chloroplasts and damaging photosynthesis membrane and apparatus. According to the concentration of H_2O_2 and MDA as an oxidant and a product of lipid peroxidation respectively, Mn produced more oxidative stress than Cu.

The increasing amount of F_v/F_m in response to increasing light intensity and the high amounts of 0.80-0.83 at HL conditions showed that an enough photochemistry occurred only in HL plants in growth chamber. Therefore, it is expected no photochemical damage occurred even at HL conditions in control plants.

Both of high leaf concentration of heavy metals (Dietz et al. 1999) and high light intensity (Asada 1999) induce an oxidative stress. In this work, activity of CAT, SOD and GR were similarly affected by Cu and Mn treatments. It indicated that, they could not explain different growth response to Cu and Mn. However, results imply that they play an important role in adaptation of plants under dual effect of high light intensity and heavy metal stress.

Activity of APX in Mn treated plants particularly under IL and HL conditions, was induced while in Cu treated ones rather inhibited. Considering that, this difference was coincided on different growth response of plants to Cu and Mn, it could be suggested that APX activity play an important role in Mn resistance of bean plants particularly under higher light intensities.

Activity of POD remained unchanged in Mn treated plants and increased in Cu treated ones. Therefore, activity of POD reflected plants performance under heavy metal stress and changes were coincided on the differential effect of Cu and Mn toxicity in bean plants. (Two lines were removed). 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 (Chaoui et al. 1997; Mazhoudi et al. 1997; Soudek et al. 1998; Baccouch et al. 2001). In the present work, the unspecific POD activity assayed with guaiacol as a universal substrate (Gross et al. 1977) was 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 (Van Assche and Clijsters 1990; Chaoui et al. 1997; Mazhoudi et al. 1997; Weckx and Clijsters 1997; Cuypers et al. 2000).

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. Copper was most effective in induction of proline accumulation than Mn under all light conditions. Proline accumulation accepted as an indicator of environmental stresses, is also considered to have important protective roles (Castillo 1992). According to antioxidant activity and chelating properties, accumulation of proline in this work should results in higher tolerance of bean plants to Cu toxicity compared to Mn. However, similar to POD activity, accumulation of proline monitored the stress conditions, but did not caused necessarily more protection against and higher adaptation to Cu compared to Mn in bean plants.

Conclusion

In this work, gas exchange parameters of photosynthesis were affected similarly by Mn and Cu. In contrast, biophysical processes of photosynthesis as judged by various chlorophyll fluorescence data, were more sensitive to Mn than Cu. Therefore, growth responses to Cu and Mn were reflected neither by the chlorophyll fluorescence parameters nor the net assimilation rate. However, the remarkable reduction of total plant leaf area could affect whole plant photosynthesis, contributing to the low biomass production of Cu compared to Mn treated plants.

From enzymes studied, only APX showed a role in the Mn tolerance of plants under higher light conditions. But unexpectedly, it was not associated with higher protection of photochemistry of Mn treated plants. On the other hand, concentration of H_2O_2 as an oxidant and MDA as the product of lipid peroxidation were higher in Mn than Cu treated plants, indicating a higher oxidative stress despite of a higher growth in the former than latter plants. Therefore, other biochemical and physiological processes should be involved in the plant performance under heavy metal stress as well as different response to Cu and Mn in bean 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 described above, rather than, developing proteins that can resist the heavy metal effects. Thus, for example, there is little evidence that tolerant species or ecotypes show an enhanced oxidative defense, rather tolerant plants show enhanced avoidance and homeostatic mechanisms to prevent the onset of stress. In this work, Cu induced accumulation of proline may have an important role in the chelation and detoxification of Cu, which resulted in lower H_2O_2 accumulation and lipid peroxidation, even though it did not results in higher resistance for bean plants comparing to the effect of Mn. It can be suggested 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 (Cuypers et al. 2000; Léon et al. 2002; Chaoui et al. 2004). On the other hand, deleterious effects of heavy metal stress in plants may be coupled to other physiological processes via the stimulation of some enzymatic activities that limit cell growth.

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