Morphology and physiology of zinc-stressed mulberry plants

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Abstract

The aim of this study was to induce symptoms of zinc deficiency and Zn excess and to relate the generation of reactive oxygen species (ROS) and the altered cellular redox environment to the effects of Zn stress in mulberry (*Morus alba* L.) cv. Kanva-2 plants. The antioxidative responses of Zn-stressed mulberry plants were studied by determining malondialdehyde content (MDA, a measure of lipid peroxidation) as indicator of oxidative damage and the ratio of dehydroascorbate (DHA) to ascorbic acid (AsA) as an index of the cellular redox state. The Zn-deficiency effects appeared as faint paling and upward cupping of the young emerging leaves. The paling intensified with time, and affected leaves finally developed necrotic spots. At advanced stage of Zn deficiency, newly emerged leaves were spindle-shaped, pale, and small in size. Apart from their stunted appearance, the plants supplied excess Zn did not show any specific visible symptom. Leaf water status of mulberry plant was affected in Zn-stressed plants. Deficient leaves had decreased water potential (Ψ) and specific water content (SWC), contained less tissue Zn, less chloroplastic pigments, and high tissue Fe and Mn concentrations. However, excess supply of Zn was found to increase Ψ and decrease tissue Fe. Both hydrogen peroxide and MDA accumulated in leaves of Zn-stressed plants. While the concentration of DHA did not vary in Zn-deficient leaves, it was increased in leaves of plants supplied excess Zn. The ratio of the redox couple (DHA to AsA) was increased both in Zn-deficient or Zn-excess plants. The activities of superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), and ascorbate peroxidase (EC 1.11.1.11) increased in Zn-stressed plants. The results suggest that deficiency or excess of Zn aggravates oxidative stress through enhanced generation of ROS and a disturbed redox homeostasis in mulberry plants.

Key words: antioxidants / *Morus alba* / reactive oxygen / superoxide dismutase / Zn-deficiency / Zn-toxicity

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1 Introduction

Zinc is a transition metal with atomic number 30 and is the 23rd most abundant element on earth (*Broadley* et al., 2007). In solution, Zn exists in the +II oxidation state and, unlike Fe^{2+} and Cu^{2+}, is redox-stable under physiological conditions as a result of a complete d-shell of electrons (*Barak* and *Helmke*, 1993; *Auld*, 2001). It is an essential element for living organism and an integral component of thousands of proteins, although it is toxic in excess (*Broadley* et al., 2007). The total Zn content of soils is reported to vary between 10 and 300 $\mu g\ g^{-1}$. Zinc deficiency is a widespread micronutrient disorder in different agroclimatic regions of the world. Zinc deficiency particularly occurs in plants growing in alkaline soils of arid and semiarid regions of the world (*White* and *Zasoski*, 1999). Average concentration of Zn in plant material is reported to be 0.3 μmol (g dry matter [DM])$^{-1}$ corresponding to 20 μg (g DM)$^{-1}$ (*Marschner*, 1995).

Cakmak (2000) has attributed much of the damage due to Zn deficiency of plants at the cellular level to the attack by reactive oxygen species (ROS). Loss of membrane integrity resulting from the attack of ROS is among the primary effects of Zn deficiency (*Cakmak* and *Marschner*, 1988). The redox reactions, particularly those involving electron transfer, lead to production of ROS like superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$). Under normal conditions of growth, plants take care of the generated ROS (*Apel* and *Hirt*, 2004). However, damages due to environmental stresses, including nutrient disorders (*Tewari* et al., 2004), are believed to result when the production of ROS is enhanced to a level in excess of the capacity of the cell to detoxify them (*Apel* and *Hirt*, 2004). Zinc deficiency is reported to accentuate photo-oxidative damage under high light intensities (*Cakmak*, 2000). It has also been reported that Zn deficiency enhances $O_2^{\cdot-}$ generation in two ways: (1) by enhancing NADPH-dependent oxidase activity, as Zn is reported to inhibit NADPH oxidase (*Pinton* et al., 1994), and (2) by decreasing NADP-to-NADPH ratio as a result of decreased uptake (*Cakmak*, 2000) and photosynthetic fixation of CO_2 (*Sharma* et al., 1994, 1995). Moreover, being an integral constituent of Cu/Zn-superoxide dismutase (Cu/Zn-

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SOD), Zn plays an important role in the detoxification of the $O_2^{\cdot-}$ as it dismutates $O_2^{\cdot-}$ to H_2O_2 (*Apel* and *Hirt*, 2004). Indeed, deficiency of Zn has been reported to decrease activities of superoxide dismutases (SODs) (*Cakmak* et al., 1997; *Yu* et al., 1998; *Yu* and *Rengel*, 1999), particularly Cu/Zn-SOD that has Zn as its constituent (*Sharma* et al., 2004). Deficiency of Zn is also reported to decrease activities of some other antioxidative enzymes like catalase (CAT) (*Cakmak* and *Marschner*, 1988), ascorbate peroxidase (APX) (*Cakmak* and *Marschner*, 1993; *Yu* et al., 1998), and glutathione reductase (GR) (*Cakmak* and *Marschner*, 1993). In contrast to these reports, our previous studies have shown an up-regulation in the activities of peroxidase (POD), APX, and GR in Zn-deficient wheat. Restoring the normal supply of Zn to these Zn-deficient wheat plants resulted in the down-regulation of these enzymes activities (*Sharma* et al., 2004).

Zinc is a redox-inactive micronutrient, however, excess of it induces toxicity and impairs growth of plants. Zinc is a major industrial pollutant of the terrestrial and aquatic environment (*Broadley* et al., 2007). Plants have developed partial exclusion or specific compartmentation to alleviate metal stress (*Vazquez* et al., 1992; *Wollgiehn* and *Neumann*, 1999). However, because of the similarities in ion radii of bivalent cations (Mn, Fe, Cu, and Mg), excess Zn can shift certain physiological equilibria by local competition at various sites (*Singh* and *Singh*, 1987). Divalent cations such as Zn^{2+} were found to displace Mg^{2+} in ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBISCO) and resulted in a loss of its activity (*van Assche* and *Clijsters*, 1986). This displacement of Mg^{2+} from RuBISCO may cause the underutilization of reductive power (NADPH) in CO_2 fixation, resulting into spill-over of electrons from oversaturated electron-transport systems to O_2 and consequently generation of the $O_2^{\cdot-}$ radicals. Moreover, metals like Zn and Cd induce toxicity by binding strongly with O, N, and S atoms (*Nieboer* and *Richardson*, 1980) of enzymes. Zinc has been shown to inactivate enzymes by binding to cysteine residues (*Schützendübel* and *Polle*, 2002).

Although these studies suggest that both deficiency and excess of Zn induces oxidative stress, information regarding responses of mulberry plants to Zn deficiency could not be found in literature. Mulberry leaves serve as feed for silkworm, whose cocoons yield silk—a commercially valuable fibre of India and China. Since 50% of the cultivated soil in India and Turkey, a third of cultivated soil in China and most soils in Western Australia, are deficient in Zn (*Broadley* et al., 2007), aims of the present study were: (1) to describe the symptoms of a Zn-deficient phenotype of mulberry for diagnostic purpose under field condition and (2) whether Zn deficiency and toxicity is related with oxidative stress or not, in mulberry plants exhibiting characteristic symptoms of Zn stress.

2 Materials and methods

2.1 Plant material and growth conditions

Mulberry (*Morus alba* L.) cv. Kanva-2 plants were grown in solution culture under glasshouse conditions. Mulberry plants were grown from cuttings obtained from a single plant. Initially, the cuttings were rooted in acid-washed sand and were supplied glass-distilled water (GDW). After 25 d, when roots in the cuttings were sufficiently induced, plantlets were transplanted to 20 L plastic buckets containing aerated nutrient solution of the following composition (*Hewitt*, 1966): 2.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.67 mM NaH_2PO_4, 0.05 mM NaCl, 0.05 mM FeK_2EDTA, 5.0 μM $MnSO_4$, 0.5 μM $CuSO_4$, 1.0 μM $ZnSO_4$, 16.5 μM H_3BO_3, 0.1 μM Na_2MoO_4, 0.05 μM $CoSO_4$, and 0.05 μM $NiSO_4$. The salt solutions providing macronutrients were purified by calcium carbonate-phosphate adsorption and, more specifically against Zn, by dithizone extraction (*Hewitt*, 1966). The pH of the nutrient solution was maintained at 6.7 ± 0.2. One week after transplantation, pots were grouped into three lots having four pots each. The lot 2 continued to receive 1.0 μM Zn and is considered to serve as control. Supply of Zn to the plants in lots 1 and 3 was 0.0 μM and 250 μM, respectively. The volume of the nutrient solution in the pots was made-up daily by GDW. The nutrient solution was refreshed every fourth day. The glasshouse conditions during the experiment were: maximum photosynthetic photon-flux density (12:00 noon) 1168–1348 μmol m^{-2} s^{-1}, daily maximum and minimum temperatures 33.7°C–41.0°C and 24.5–31.2°C, respectively, and relative humidity (9:00 AM) 63%–79%. The average photoperiod was 12:00 ± 0:20 h. For water relations, enzymes, metabolite, and metal analyses, the fourth fully expanded young leaf (from the apex) was sampled at 20 d after treatment (DAT). For dry-matter determination, samples were taken at the time of final harvesting (*i.e.*, 40 DAT).

2.2 Visual observation, dry-matter yield, and metal concentration

The visual symptoms were recorded day to day. Finally, 40 DAT the plants were harvested, separated into roots and shoots and then dried in an oven at 80°C for 48 h and weighed. Iron, Mn, Cu, and Zn were analyzed in HNO_3 : $HClO_4$ (10:1 [v/v]) digests of young leaves sampled at 20 DAT using atomic-absorption spectrometer.

2.3 Plant water relations

Water status was evaluated in the leaf tissue of plants by measuring water potential (Ψ), relative water content (RWC), specific water content (SWC), and degree of succulence (DS). Samples for Ψ were taken between 9:00 and 9:30 AM from the fourth fully expanded leaf. Measurements were made hygrometrically on five leaf discs (11 mm diameter) punched from three leaves of randomly selected plants, using Wescor (Logan, UT) microvoltmeter (model HR 33T), and C-52 leaf chambers**.** Determination of RWC was made by measuring 40 fresh-leaf discs from the same leaves used for Ψ measurement, weighing them again after rehydration for 3 h at 11°C in the dark on GDW and determining the dry weights after oven-drying (*Slavík*, 1974). The RWC was calculated by following formula:

$$\mathrm{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Saturated weight} - \text{Dry weight}} \cdot 100.$$

The determination of SWC and DS were made by measuring water contents of the leaves per unit dry weight and per unit area of leaves. The leaf area of the entire leaves was measured with a LI-COR portable area meter model LI-3000A.

2.4 Chloroplastic pigments, hydrogen peroxide, and lipid peroxidation

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

2.5 Ascorbate and dehydroascorbate

Fresh leaf tissue (250 mg) was homogenized in 2.0 mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged for 5 min at 10,000 *g*. Total ascorbate (after reducing dehydroascorbate [DHA] to ascorbic acid [AsA] by dithiothreitol [DTT] and then neutralizing the excess of DTT by N-ethylmaleimide) and AsA in the supernatant were measured as Fe^{2+}-bipyridyl complex (absorbance max at 525 nm) formed from the reduction of Fe^{3+} by AsA by the method of *Law* et al. (1983). Dehydroascorbate (DHA) content was calculated from the difference between total ascorbate and AsA.

2.6 Enzyme extraction and protein determination

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

2.7 Assays of enzymes

Activity of SOD (EC 1.15.1.1) was assayed in 25 mM phosphate buffer pH 7.8, containing 65 μM NBT, 2.0 μM riboflavin, enzyme extract (equivalent to 20 mg fresh matter [80 μL] with water [220 μL]), and 15 μL *N,N,N',N'*-tetramethylethylenediamine (TEMED) in a total volume of 5 mL. The reaction mixture was exposed to light of 350 μmol m^{-2} s^{-1} for 15 min, and SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT. The change in absorbance was measured at 560 nm (modified from *Beauchamp* and *Fridovich*, 1971). Activities of different species of SOD were measured by including either 2.0 mM KCN (an inhibitor of Cu/Zn-SOD) or 5.0 mM H_2O_2 (an inhibitor of Cu/Zn-SOD and Fe-SOD) in the reaction mixture. The activity is expressed as units (mg protein)$^{-1}$. Fresh-matter equivalent of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

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

Activity of POD (EC 1.11.1.7) was measured in 100 mM phosphate buffer (pH 6.5), 1.0 mL 0.5% (w/v) *p*-phenylenediamine, 1.0 mL 0.01% H_2O_2, and tissue extract (equivalent to 10 mg fresh matter [40 μL] with water [960 μL]) in a total volume of 5.0 mL. The change in absorbance after 5.0 min was measured at 485 nm (*Bisht* et al., 1989). The enzyme activity is expressed as units (mg protein)$^{-1}$. The enzyme unit is defined as A_{485} of 0.01 between the blank and the sample per minute of reaction time, calculated using purified horse radish peroxidase.

Activity of APX (EC 1.11.1.11) was assayed in 50 mM phosphate buffer (pH 7.0), containing 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and a suitable quantity of enzyme extract (equivalent to 10 mg fresh matter) in a total volume of 3.0 mL. Both minus tissue extract and minus H_2O_2 blanks were run, and the changes in absorbance every 15 s were read at 290 nm (*Nakano* and *Asada*, 1981). The activity of APX was calculated in terms of units (μmol ascorbate oxidized min^{-1}) (mg protein)$^{-1}$.

2.8 Statistical analysis

All results are the mean of replicates (n = 6). The data were analyzed by analysis of variance (ANOVA) and tested for significance by Bonferroni t-test using Sigma-stat 2.1 software.

3 Results

3.1 Visible effects

Both deficient and excessive Zn supply suppressed plant growth. Growth depression in Zn-deficient plants became perceptible 10 d after the treatment initiation. The effects of Zn deficiency appeared as faint paling and upward cupping of the young emerging leaves 12 d after treatment initiation. The paling was intensified in plants supplied nil Zn. The affected leaves finally developed necrotic spots (Fig. 1). These plants were short, thin, had fewer lateral branches and smaller leaves. Plants supplied 250 μM Zn did not exhibit any specific toxicity effects except for a decrease in plant growth.

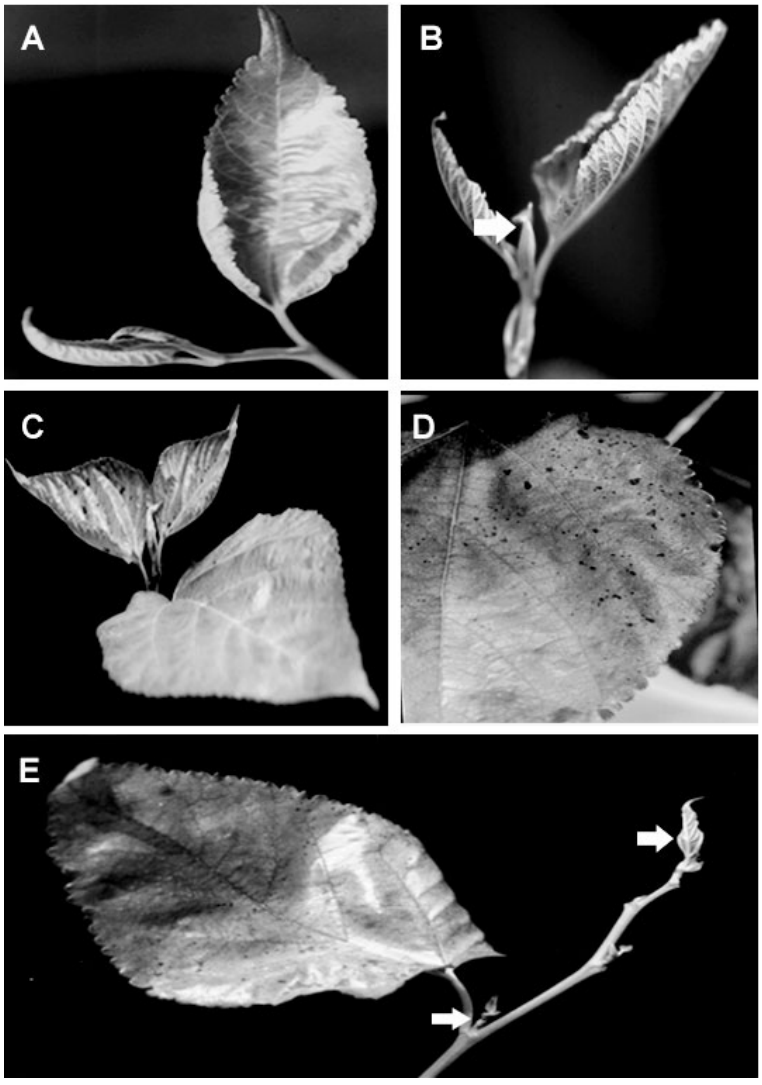

Figure 1: Visible effects of deficiency of Zn in mulberry (*Morus alba* L.) cv. Kanva-2 plants grown in solution culture. A), B), and C): Apical parts of deficient plants exhibiting distorted expansion of young leaves, retarded apical growth (arrows), chlorosis, and necrotic black spots in a young leaf; D) Zn-deficient fully expanded leaves showing necrotic spots; E) branch of a Zn-deficient plant showing distorted necrotic leaf, retarded growth in the axillary and apical buds (arrows), and premature shedding of leaves.

3.2 Plant growth and mineral concentration

Deficient supply of Zn decreased plant growth, plant height, leaf-area expansion rate, and dry-matter yield. Excessive (250 μM) supply of Zn also caused retardation in plant growth and a decrease in the leaf-area expansion rate (Tab. 1). The plants grown at deficient supply of Zn had the lowest shoot-to-root ratio, indicating a greater susceptibility of the shoot to Zn deficiency (Tab. 1). More than 80% decrease in the yield was well marked at deficient supply level of Zn. The excess supply of Zn also suppressed growth and decreased dry-matter yield. The concentrations of Fe, Mn, and Cu decreased

Table 1: Effects of deficient or excess supply of Zn on plant height, leaf-area expansion rate, and dry-matter yield of mulberry (*Morus alba* L.) cv. Kanva-2 plants grown in solution culture. Plant-height and dry matter–yield data at 40 DAT. Leaf area–expansion rate data are based on measurements made between 5 and 20 DAT (means ± SE, $n = 6$). Means not assigned by the same letters are significantly different at $p < 0.05$ by Bonferroni t-test.

Parameter	**Zn supply** (μM)		
	0	1	250
Plant height (cm)	61.4 ± 0.1 c	200.0 ± 1.6 a	155.5 ± 0.9 b
Leaf-area expansion rate (cm^2 day^{-1})	1.33 ± 0.03 c	3.47 ± 0.02 a	3.00 ± 0.02 b
Dry-matter yield (g $plant^{-1}$)	25.4 ± 0.0 c	132.5 ± 7.8 a	109.0 ± 4.2 b
Shoots	21.1 ± 0.2 c	118.5 ± 5.7 a	95.3 ± 4.4 a
Roots	4.3 ± 0.2 b	14.1 ± 2.0 a	13.8 ± 0.2 a
Shoot-to-root ratio	4.95 ± 0.24 c	8.81 ± 0.87 a	6.94 ± 0.39 a

with increasing supply of Zn in the young leaves. Maximum accumulation of these metals (Fe, Mn, and Cu) was observed in Zn-deficient leaves (Tab. 2). Zinc concentration was decreased below threshold level (approx. 18–20 μg Zn [g DM]$^{-1}$) in the plants receiving deficient supply of Zn. Zinc concentration in plants progressively increased with supply of Zn (Tab. 2).

3.3 Leaf water status

Both deficient and excess supply of Zn mildly increased RWC and succulence of leaves, but effects were not significant (Tab. 3). Water potential (Ψ) and SWC of leaves of Zn-deficient plants decreased. The plants supplied excess of Zn had increased Ψ and SWC (Tab. 3).

3.4 Chloroplastic pigments

Total chlorophyll as well as chlorophyll *a* and chlorophyll *b* and carotenoids decreased significantly in Zn-deficient plants (Fig. 2A). While excess supply of Zn did not affect chlorophyll, it decreased carotenoid concentration significantly (Fig. A). These changes in the concentration of chlorophylls, however, did not affect the ratio of chlorophyll *a* to chlorophyll *b*, but a marked increase in the ratio carotenoids to chlorophyll in Zn-deficient plants was observed (Fig. 2B). Zn excess decreased the carotenoids-to-chlorophyll ratio (Fig. 2B).

3.5 Hydrogen peroxide and lipid peroxidation

The concentration of H_2O_2 increased in the leaves of plants exposed either to Zn deficiency or Zn excess (Fig. 2C). Malondialdehyde (MDA) equivalent of TBARS increased in

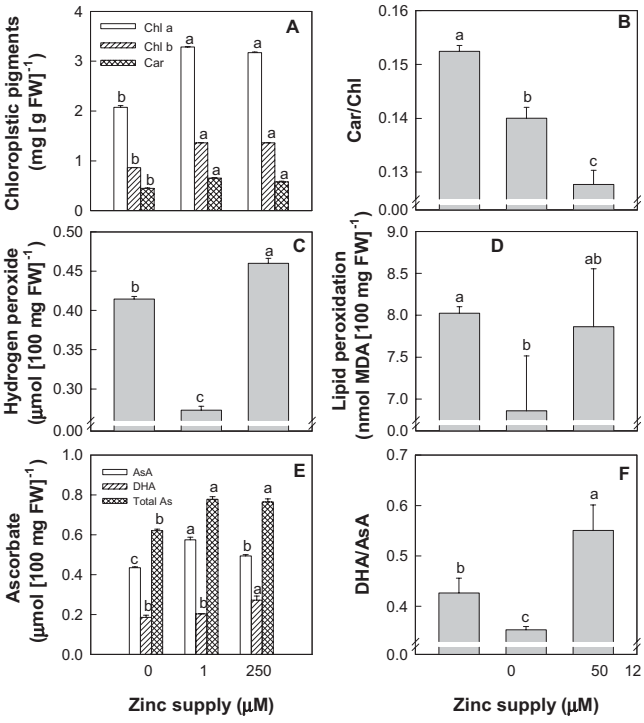

Figure 2: A) Chlorophyll *a*, chlorophyll *b*, and carotenoid concentrations; B) carotenoids-to-chlorophyll ratio; C) H_2O_2; D) MDA; E) ascorbic acid (reduced form, AsA), dehydroascorbate (DHA) and total ascorbate (As) concentrations; and F) DHA-to-AsA ratios in the fourth fully expanded young leaves of mulberry (*Morus alba* L.) cv. Kanva-2 plants grown at deficient (0 μM), optimum (1 μM, Control), and excess (250 μM) supply of Zn in solution culture. The vertical bars represent ±SE (n = 6) of replicates. Vertical bars not assigned the same letters are statistically different.

Table 2: Effects of deficient or excess supplies of Zn on micronutrient concentration in young leaves of mulberry (*Morus alba* L.) cv. Kanva-2 plants grown in solution culture. All values in μg (g dry wt.)$^{-1}$. All data refer to 20 DAT (means ± SE, n = 6). Means not assigned by the same letters are significantly different at $p < 0.05$ by Bonferroni t-test.

Element	**Zn supply** (μM)		
	0	1	250
Fe	218.7 ± 13.0 a	117.7 ± 2.8 b	72.5 ± 0.0 c
Mn	318.9 ± 36.0 a	70.9 ± 1.6 c	110.6 ± 4.9 b
Cu	4.54 ± 0.00 a	1.14 ± 0.00 b	1.14 ± 0.00 b
Zn	14.6 ± 0.1 c	19.9 ± 0.5 b	247.6 ± 1.1 a

Table 3: Effects of deficient or excess supply of Zn on relative water content (RWC), specific water content (SWC), degree of succulence (DS), and water potential (Ψ) of mulberry (*Morus alba* L.) cv. Kanva-2 plants grown in solution culture. All data refer to 20 DAT (means ± SE, n = 6). Means not assigned by the same letters are significantly different at $p < 0.05$ by Bonferroni t-test.

Parameter	**Zn supply** (μM)		
	0.0	1.0	250.0
RWC (%)	91.72 ± 0.63 a	88.08 ± 3.31 a	94.14 ± 0.14 a
SWC (g [g dry wt.]$^{-1}$	1.92 ± 0.05 b	3.41 ± 0.23 a	3.56 ± 0.04 a
DS (g dm^{-2})	1.16 ± 0.02 a	1.03 ± 0.05 a	1.11 ± 0.01 a
Ψ (MPa)	–1.32 ± 0.09 b	–1.22 ± 0.03 b	–0.72 ± 0.04 a

Zn-deficient and Zn-excess plant leaves, though the increase observed in Zn-excess plants was not significant (Fig. 2C, D).

3.6 Ascorbate and redox status

Plants subjected to either Zn deficiency or Zn excess exhibited significant decreases in the concentrations of AsA (Fig. 2E). While DHA level remained unaffected in Zn-deficient plants, its level increased in plants supplied with excess Zn (Fig. 2E). Total ascorbate concentration decreased in Zn-deficient plants. Significantly increased DHA-to-AsA ratios in both Zn-deficient and Zn-excess leaves suggest oxidative stress in these plants (Fig. 2F).

3.7 Antioxidative enzymes

Both deficient and excessive supply of Zn increased SOD activity. The increase in Mn- and Fe-SOD was higher compared to Cu/Zn-SOD in Zn-deficient plants resulting in the decreased ratio of Cu/Zn-SOD to non-Cu/Zn SOD activity (Fig. 3B). Activity of Fe-SOD declined in Zn-replenished plants (Fig. 3A). Activity of Cu/Zn-SOD increased in leaves of Zn-excess plant (Fig. 3A). An increase in Cu/Zn-SOD in Zn-excess plants resulted in an increased ratio of Cu/Zn-SOD to non-Cu/Zn-SOD (Fig. 3B). The activities of the other antioxidant enzymes CAT, POD, and APX increased significantly in both Zn-deficient and Zn-excess plants (Fig. 4).

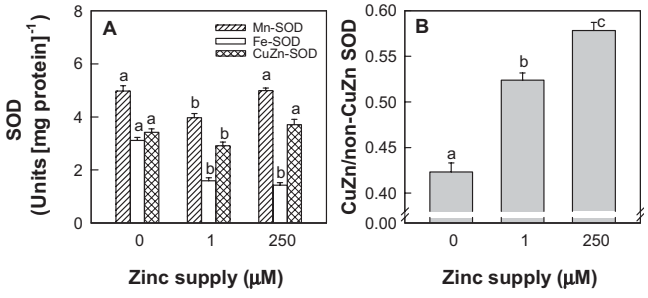

Figure 3: A) Mn-, Fe-, and Cu/Zn-superoxide dismutase (SOD) activity; B) Cu/Zn-SOD–to–Non-Cu/Zn-SOD ratios in the fourth young leaves of mulberry *Morus alba* L.) cv. Kanva-2 plants grown at deficient (0 μM), sufficient (1 μM, Control), and excess (250 μM) supply of Zn under solution culture. The vertical bars represent ±SE ($n = 6$) of replicates. Vertical bars with different letters are statistically different.

4 Discussion

Both deficient and excessive supply of Zn suppressed plant growth, reduced biomass production (Tab. 1) and produced effects of the respective Zn stresses. The effects of deficiency (Fig. 1) observed in the young emerging leaves and the apical meristem of Zn-deficient plants is attributed to the poor phloem mobility of Zn (*Marschner*, 1995). The observed symptoms like small spindle-shaped, distorted, pale, and necrotic young-leaf laminae in Zn-deficient plants (Fig. 1) were largely similar to those described earlier for other plant species (*Chapman*, 1966; *Bennett*, 1993; *O'Sullivan* and *Ernest*, 2007). The decreased Zn concentration in the leaf tissue of Zn-deficient plants (Tab. 2) further supports that the symptoms displayed by these plants were specific to Zn deficiency. The loss of photosynthetic pigments and development of symptoms like chlorosis and necrosis in plants receiving Zn at inadequate levels may possibly be due to enhanced production of ROS as suggested by *Cakmak* and *Marschner* (1992) and *Cakmak* (1994). The oxidizing environment created due to elevated generation of ROS may lead to senescence or tissue necrosis (*Schafer* and *Buettner*, 2001). The increased generation of $O_2^{\cdot-}$ in Zn-deprived plants is likely resulting from the decreased availability of reductants, leading to an accumulation of high-potential electrons and excited chlorophyll molecules as suggested earlier by *Grossman* and *Takahashi* (2001). Suppressed plant height and decrease in biomass production in plants deprived of Zn may largely be attributed to the loss of photosynthetic pigments (Fig. 2A) and the decrease in photosynthetic CO_2 fixation. Apart from affecting the light reaction as a consequence of decreased chloroplastic pigments, Zn deficiency has also been shown to decrease intercellular CO_2 concentration that results from the decreased activity of the Zn-dependent enzyme carbonic anhydrase and stomatal conductance (*Sharma* et al., 1994, 1995). The shoot-to-root dry weight ratio was decreased in plants provided with Zn at inadequate levels (Tab. 1). The decreased shoot-to-root ratio under Zn deficiency stems from

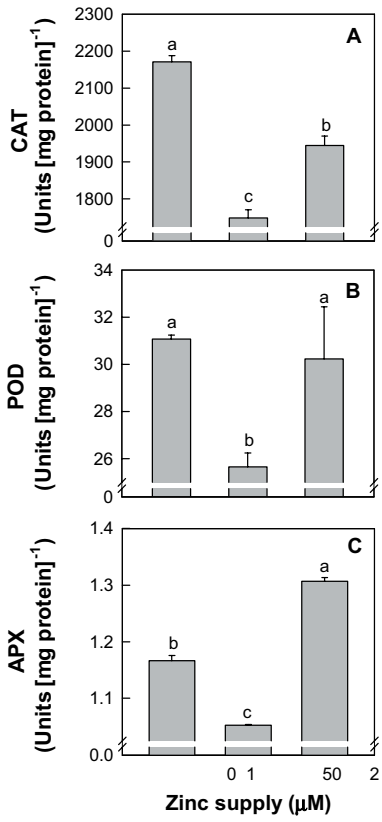

Figure 4: A) Activities of the antioxidative enzymes CAT; B) POD; and C) APX on in the fourth fully expanded young leaves of mulberry (*Morus alba* L.) cv. Kanva-2 plants grown at deficient (0.0 μM), sufficient (1.0 μM, Control), and excess (250 μM) supply of Zn in solution culture. The vertical bars represent ±SE ($n = 6$) of replicates. Vertical bars with different letters are statistically different.

the relatively greater decrease in shoot biomass (83%) than in root biomass (17%) in Zn-deficient plant compared to shoot (89.4%) and root dry mass (10.6%) of Zn-replenished mulberry plants (Tab. 1). *Mollier* and *Pellerin* (1999) have suggested preferential export of carbohydrates to roots under conditions of N and P deficiencies as a cause of decreased shoot-to-root dry weight ratios in bean plants. A similar mechanism is likely to decrease shoot-to-root ratio under conditions of Zn deficiency. Excess supply of Zn did not affect plant dry mass, however, it decreased plant height and leaf-area expansion rate significantly (Tab. 1).

Both deficient and excessive supply of Zn mildly increased RWC and succulence of leaves, but effects were not significant (Tab. 3). Water potential (Ψ) and SWC of leaves of Zn-deficient plants were decreased (Tab. 3). The plants supplied with excess of Zn increased Ψ. Decreased Ψ and specific water content (SWC) indicate that the Zn-deficient plants were water-stressed (*Sharma* and *Sharma*, 1987; *Sharma* et al., 1995). A mild improvement in water content per unit leaf area in the Zn-deficient plants suggests the induction of succulence (*Larcher*, 1980). A similar induction of succulence has also been reported in the leaves of Zn-deficient cauliflower plants (*Sharma* et al., 1995).

Concentrations of chlorophylls and carotenoids decreased by the deficient supply of Zn (Fig. 2A). Excess of Zn also mildly affected chloroplastic pigments (Fig. 2A). The car-to-chl ratio increased by Zn deficiency and decreased by Zn excess (Fig. 2B). A similar increase in the car-to-chl ratio has previously been reported in Zn-deficient wheat plants that were recovering from Zn deficiency (*Sharma* et al., 2004). The increased car-to-chl ratio has been suggested as an adaptive response to the increased ROS generation. Carotenoids are known to scavenge ROS, particularly singlet oxygen, and protect chlorophylls from photo-oxidative damage (*Pérez-Gálvez* and *Minguez-Mosquera*, 2002).

Induction of oxidative stress in leaves showing perceptible effects characteristic of Zn deficiency was indicated by an increased accumulation of H_2O_2 (Fig. 2C) and MDA (lipid peroxidation) (Fig. 2D) and an increase in the DHAs-to-AsA ratio (Fig. 2F). This suggests an unfavourable shift in the redox status. Though generation of $O_2^{\cdot-}$ by the NADPH-dependent oxidase system may be equally important (*Cakmak*, 2000), the observed antioxidative responses—high car-to-chl ratio (Fig. 2B), enhancements in the activities of H_2O_2-scavenging enzymes like APX (Fig. 4C), and increases in related antioxidant pools like ascorbate (Fig. 2E, F)—suggest an implication of the "Mehler-peroxidase reaction" (*Osmond* and *Grace*, 1995; *Cakmak*, 2000). Restricted uptake and photosynthetic fixation of CO_2 is reported to be among the early effects of Zn deficiency (*Sharma* et al., 1994, 1995). The resultant increase in the NADPH-to-NADP ratio may increase the generation of $O_2^{\cdot-}$ leading to induction of antioxidative responses in Zn-deficient plants. Deficiency of Zn, apart from increasing $O_2^{\cdot-}$ production, also increased the activity of SOD (Fig. 3) and the accumulation of H_2O_2 (Fig. 2C), the dismutation product of $O_2^{\cdot-}$, suggesting an induction of oxidative stress in the Zn-deficient mulberry. The high concentration of Fe (Tab. 2) in Zn-deficient plants is reported to increase generation of $OH^{\cdot}$ radicals (*Cakmak*, 2000), which may cause lipid peroxidation as reported by *Sharma* et al. (2004). Accumulation of H_2O_2 (Fig. 2C) and peroxidation of lipids (Fig. 2D) in plants with excess supply of Zn has already been reported (*Weckx* and *Clijsters*, 1997; *Prasad* et al., 1999) and supports the proposition that Zn toxicity induced oxidative stress in mulberry.

Though Fe, Zn, or Mn form an integral constituent of the three major forms of SOD viz. Fe-SOD, Cu/Zn-SOD, or Mn-SOD, respectively, the deficiency of Zn did not decrease the activity of SODs (Fig. 3A). However, the Cu/Zn-SOD–to–non-Cu/Zn-SOD ratio was decreased, due to overactivation of Mn-SOD and Fe-SOD in particular, compared to an increase in Cu/Zn-SOD (Fig. 3B). The observed increase in the activity of SOD in Zn-deficient plants is in consonance with *Sharma* et al. (1999, 2004) who suggested an induction of SOD species that do not require Zn and which are structurally unrelated to Cu/Zn-SOD. Moreover, an increase in SOD activity has previously been reported under deficiency conditions of many macronutrients (*Tewari* et al., 2004, 2006a) and other micronutrients, like Fe (*Iturbe-Ormaetxe* et al., 1995; *Tewari* et al., 2005) and Cu (*Murao* et al., 2004; *Tewari* et al., 2006b).

The increase in the activity of APX (Fig. 4C) in Zn-deficient plants is indicative for the activation of the ascorbate-glutathione cycle in these plants. The decrease in the AsA content (Fig. 2E) and a significant increase in the ratio of the redox couple DHA/AsA (Fig. 2F) in these plants also suggests an activated operation of the ascorbate-glutathione pathway. Other H_2O_2 metabolizing enzymes—CAT and POD—were also up-regulated under Zn-deficient conditions (Fig. 4A, B). Despite of this increase in H_2O_2-scavenging enzyme activities, the H_2O_2 level in Zn-deficient plants was still higher (Fig. 2C). This higher H_2O_2 concentration might have triggered the Fenton/Haber-Weiss reaction (because of overaccumulation of Fe in the Zn-deficient leaves) and thus generation of $OH^{\cdot}$ radicals, resulting in the high lipid peroxidation and severe oxidative challenge in Zn-deficient leaves.

The observed increase in SOD activity in plants supplied with excess Zn, mainly due to the increase in Mn-SOD and Cu/Zn-SOD (Fig. 3A), appears to be associated with high Zn supply to these plants. However, oxidative stress and antioxidant defence in Zn-excess plants is indicated, apart from high SOD, by increased activities of other antioxidant enzymes (CAT, POD, and APX; Fig. 4) and an increase in the DHA-to-AsA ratio (Fig. 2F). Increased accumulation of H_2O_2 and MDA, the product of lipid peroxidation (Fig. 2C, D), despite activation of protective mechanisms implies only partial efficiency of the antioxidative measures to cope with the oxidative challenge in the Zn-excess plants. Potential toxicity of increased DHA concentrations (*Paciolla* et al., 2001) or disturbed redox status of the cellular environment due to increased DHA-to-AsA ratios could have impaired overall metabolism and thus growth, as indicated by suppressed dry-matter yield and leaf-area expansion rate in the Zn-excess plants.

5 Conclusion

Both deficiency and excess of Zn suppressed growth and decreased dry-matter yield of mulberry plants. Characteristic visible symptoms (faint paling, upward cupping, and reduction in lamina size) on young leaves developed only in plants receiving deficient supply of Zn. Plant-water relations were also affected in Zn-stressed plants; while low supply of Zn decreased both Ψ and SWC, excess supply of Zn was found to increase Ψ. Deficiency or excess of Zn induced oxidative stress by increased generation of ROS, disturbed status of redox couple as indicated by increased accumulation of TBARS. Zinc stress up-regulated the activities of the antioxidative enzymes SOD, CAT, POD, and APX. The redox couple, DHA-to-AsA ratio, is found to be a better index of oxidative stress compared to the concentration of total ascorbate.

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Abbreviations

AsA, ascorbic acid; APX, ascorbate peroxidase; CAT, catalase; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; MDA, malondialdehyde; POD, peroxidase; ROS, reactive oxygen species; RWC, relative water content; SOD, superoxide dismutase; SWC, specific water content; TCA, trichloroacetic acid; Ψ, water potential.

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