Zinc stress induces physiological, ultra-structural and biochemical changes in mandarin orange (*Citrus reticulata* Blanco) seedlings

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Abstract Zinc (Zn) is an essential micronutrient for higher plants; yet, at higher concentrations it is toxic. In order to explore the effect of Zn stress on growth, biochemical, physiological and ultra-structural changes, 1 year old mandarin plants were grown under various Zn concentrations (1, 2, 3, 4, 5, 10 15 and 20 mM) for 14 weeks. The biomass of the plants increased with increasing Zn concentrations and finally declined under excess Zn concentration but the prime increase was observed at 4 and 5 mM Zn. Zn stress reduced the photosynthetic rate, stomatal conductance, and transpiration along with reduction of chlorophyll a, chlorophyll b, and carotenoids content in leaf. Superoxide anion, malondialdehyde, hydrogen peroxide and electrolyte leakage were elevated in Zn stressed plants. The activities of ascorbate peroxidase (EC 1.11.1.11), catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1) and peroxidase (EC 1.11.1.7) enzymes were increased in both Zn-deficient and Zn-excess plants. Therefore it is suggested that antioxidant defense system did not sufficiently protect the plants under rigorous Zn stress which was also corroborated by the alteration in cell ultrastructure as revealed by transmission electron microscopy.

Keywords Antioxidative enzymes · *Citrus reticulata* · Pigments · Reactive oxygen species · Transmission electron microscopy · Zinc stress

Introduction

Zinc (Zn), an essential micronutrient, is required for growth and development of plants (Kochian 1993). Crop response to Zn application has widely been reported (Alloway 2004) which is crucial for a number of physiological and biochemical processes such as plasma membrane functions, oxidative stress tolerance etc. (Kochian 1993). Zn has stabilizing and protective effects against oxidative, peroxidative damages, loss of integrity and alteration of membrane permeability (Aravind and Prasad 2003). It is involved in the biosynthesis of chlorophyll (chl), carotenoids (car) and in scores of metabolic reactions (Aravind and Prasad 2005a, b). Most significantly Zn acts as cofactor of number of enzymes such as dehydrogenases, oxidases, peroxidases (Vallee and Auld 1990) as well as anhydrases (Aravind and Prasad 2004, 2005c) and performs significant role in the defense system. Further Zn performs very critical role in the defense system, composed of metabolites like ascorbic acid (AsA), glutathione and enzymatic scavengers (Asada 1999), and prevents oxidation of vital components (Mukhopadhyay et al. 2013a).

The genus of Citrus plant consisting of 17 species belongs to the sub-family Auarantioideae, under the family of Rutaceae. It is one of the major commercially grown fruit crops of the world (Pal et al. 2013), distributed throughout the tropical and temperate regions (Chutia et al. 2009). The fruit is precious as it is rich in various nutrients such as vitamins and antioxidant compounds (Rivas et al. 2007). It is cultivated in more than 135 countries with about 102.64 million tones of production worldwide (Sajid et al. 2010). In India, more than 52 varieties of citrus are found in the North-Eastern hilly states

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up to an altitude of 1,200 m. Some common species noticed are; *C. indica*, *C. ichangensis*, *C. macroptera*, *C. latipes*, *C. aurantium*, *C. megaloxycarpa*, *C. jambhiri* and *C. reticulata* (Chutia et al. 2009). Among them, mandarin (*C. reticulata*) occupies nearly 40 % of the total area under citrus cultivation with a share of about 41 % of all citrus fruits produced in India.

Zn deficiency is a widespread phenomenon of many agroclimatic zones worldwide including India (Mukhopadhyay et al. 2013a). Zn availability is inversely related to soil pH and its deficiency in variety of plant species is frequently noted on calcareous soils with pH>8.0 (Swietlik 1989). It has been accounted that 48 % of Indian soils are Zn deficient (Mukhopadhyay et al. 2013a). Deficiency of Zn, which comes next to nitrogen, is considered as the most widely seen nutrient deficiency (Tariq et al. 2007), and physiological disorders of fruit trees, for instance, ‘little leaf disease’ is attributable to Zn deficiency (Ahmed et al. 2012). Hence, Zn is the most widely used micronutrients for the cultivation of fruit crops; attributable to its effect on biosynthesis of tryptophan, a precursor of indole-3-acetic acid (Tariq et al. 2007). Rosetting is the other characteristic symptom of Zn deficiency in fruit trees (Swietlik 2002). Zn deficiency, in citrus, is described by a number of names such as ‘frenched leaf’, ‘frenching’, ‘mottle leaf’ etc. Zn is undoubtedly the single most desired nutrient, reported to be deficient across the various citrus belts of India (Srivastava and Singh 2005). Citrus apparently has difficulty in absorbing sufficient Zn from many soils, and anything that affects the root system adversely, is likely to reduce the Zn-intake strikingly (Srivastava and Singh 2005). Several studies had been undertaken to understand the effect of Zn on the growth of Citrus plants, but none of them have intended to detect the alterations of physiological, ultrastructural and biochemical parameters under deficiency and excess taken together. Hence, the objective of this work was to study the effect of Zn deficiency on structural, physiological and biochemical changes of *C. reticulata*. Additionally, we have incorporated the effect of Zn excess as a reference, because soil pollution by toxic metals is a critical environmental stress that surfaces due to agricultural activities, urbanization and industrialization, especially. Metal excess is correlated to bioavailability of metal in the soil, rather than the total concentration and may decrease both quality and productivity of plant products (Kösesakal et al. 2011).

Material and methods

One year-old uniform and well-rooted plants (45 cm height) of mandarin orange (raised in sand bed nursery), were transplanted in earthen pots (12" diameter) filled with well-sieved acid-washed sand. Plants were supplied with 1/10 strength of MS solution (pH 5.6) (Murashige and Skoog

1962) every alternative day. Seven weeks after transplanting, the stress treatment was applied for 14 weeks until pronounced visual structural indications of stress (e.g., chlorosis of leaves, growth impairment etc.) appeared. On every alternate day, each pot was fed with 500 ml of 1/10 strength MS solution (pH 5.6) together with ZnSO₄ solutions containing 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 10 mM, 15 mM and 20 mM of Zn. Without Zn (0 mM Zn) was served as the control. At the end of experiment (after 14 weeks) main shoots and roots from different replications and treatments were used for measurements of all the parameters. The stress treatment was accomplished in controlled greenhouse conditions at a light intensity of 300 $\mu\text{M m}^{-2} \text{s}^{-1}$ with, 25 ± 2 °C temperature as well as 70 % of relative humidity. For each treatment, five pots (each with 1 plant) were arranged in a completely randomized block design.

Determination of fresh weight (FW) and dry weight (DW)

At the end of the experiment i.e after 14 weeks of the treatment, six plants per treatment from various pots were collected. The plants were sliced into leaves, stems and roots and weighed (FW). The plant materials were then dried at 80 °C for 48 h for measuring DW. There were 3 replicates per treatment. Then the ratio between the root DW and shoot DW were determined.

Determination of physiological parameters

After 14 weeks of treatment, photosynthetic rate (P_n), transpiration (E) and stomatal conductance (g_s) were recorded in physiologically matured, fully expanded 3rd leaf from the top of the main shoot. Measurements were made with a portable photosynthesis system (LCpro+, ADC, UK) with the specifications, such as leaf surface area 6.25 cm², ambient CO₂ concentration 371 $\mu\text{mol mol}^{-1}$, temperature of leaf chamber 25–28 °C, leaf chamber molar gas flow rate (U) 400 $\mu\text{mol s}^{-1}$, ambient pressure (P) 97.95 kPa, PAR (Q_{leaf}) at leaf surface maximum up to 770 $\mu\text{mol m}^{-2} \text{s}^{-1}$. There were 3 replicates per treatment and for every replicate, an average of 5 records from different parts of individual leaf was considered.

Light microscopic study and Transmission electron microscopy (TEM)

In order to study stomatal apertures, intercellular spaces and the accessory cells, thin sections and peels of young leaves of all the plants were taken out, stained with methylene blue and observed under microscope (DM1000, Model-Leica).

Samples from leaf and root were fixed in 2.5 % (m/v) glutaraldehyde solution in 50 mM potassium phosphate (pH 6.8). The samples were kept overnight at room temperature and washed 3 times for 15 min each with 50 mM sodium

cacodylate buffer (pH 6.9). After that, the samples were diluted (1:1) with 50 mM sodium cacodylate buffer (pH 6.9) for TEM study (Sandalio et al. 2001).

Determination of chlorophyll and carotenoid

Chl, chl *a*, chl *b*, and car were assayed (Mukhopadhyay et al. 2013b) using the 3rd leaf as explained in the previous section. Approximately 1 g leaf tissue was ground in 100 % acetone, centrifuged at 5,000 rpm for 10 min at 4 °C before recording the absorbance at 470, 663, and 645 nm in a spectrophotometer (Lambda 25, Perkin-Elmer, USA). There were 3 replicates per treatment (5 disks from the same leaf per replicate).

Determination of proline and phenol

Proline content in the leaves was measured by acidic ninhydrin method according to Bates et al. (1973). Phenol in tissue was determined on the basis of the reaction with phosphomolybdate in Folin-Ciocalteu reagent under alkaline conditions. It resulted in the formation of a blue colored complex absorbance which was measured at 650 nm (Mukhopadhyay et al. 2013b). There were 3 replicates per treatment.

Determination of ascorbic acid, starch and total sugar

Approximately 100 mg fresh leaf tissues were used for determination of AsA, starch and total sugar. AsA was estimated as illustrated by Mukherjee and Choudhari (1983) and starch content was quantified as described by Mukhopadhyay et al. (2013b). Determination of soluble sugar content was carried out by the addition of anthrone reagent that formed a blue-green colored complex upon condensation with furfural. Final spectrophotometric absorbance was recorded at 630 nm (Hedge and Hofreiter 1962). There were 3 replicates per treatment.

Determination of superoxide anion (O_2^-), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2)

Approximately 100 mg fresh leaf tissues from newly growing shoots were taken for all the assays. Determination of O_2^- assay was done following the protocol of Jordan and DeVay (1990). Lipid peroxidation (MDA) was determined by measuring the amount of MDA (Ohkawa et al. 1979), while generation of H_2O_2 was measured according to the standard protocol of Sagisaka (1976). There were 3 replicates per treatment.

Extraction and assay of enzymes

Approximately 100 mg leaf tissues were taken for determination of enzyme activity. The samples were ground with liquid

nitrogen and resuspended in 1 ml buffer solution containing 50 mM Tris-HCl (pH 7.8) fortified with 1 % PVP (polyvinylpyrrolidone). Homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was used to measure the activities of APX, CAT, SOD, and POX. However, for APX, the extraction buffer was consisting of 50 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA (Sigma), 1 mM AsA (Hi-Media) and 1 % polyvinyl pyrrolidone (PVP, Hi-Media). APX activity was determined by monitoring the decrease in absorbance at 290 nm (Nakano and Asada 1981). Conversely, CAT and SOD activity were determined by measuring the absorbance at 240 nm and 560 nm, respectively (Mukhopadhyay et al. 2013b). POX activity was determined following the protocol of Chance and Maehly (1955). There were 3 replicates per treatment.

Statistical analysis

Experiment was led out in CRD (complete randomized design). The data were analyzed by Fisher's Analysis of Variance (ANOVA) technique. The significance of different sources of variance was tested by error mean square of Fisher-Snedecor's 'F' test at 0.05 % of probability levels and differences among the various treatments were determined by using least significant difference (LSD) test at 0.05 % probability level (Steel and Torrie 1984). For determination of LSD (critical difference) at 0.05 % level of significance, Fisher and Yates table was consulted.

Results

Effect of Zn on plant morphology and biomass

Plant growth was comprehensively influenced by Zn deprivation. Young leaves were principally affected, compared to their mature counterparts. Symptoms of Zn-deficiency were characterized by irregular green spots along the midrib and under prolonged deficiency, leaves turned small sized coupled with very thin twigs that died back later. Height of the plants increased with an increase in Zn concentration from 3 to 5 mM but afterward the plant height was reduced with additional Zn concentration. Plants supplied with 4 and 5 mM Zn were superior in growth than the plants that received higher doses (10, 15 and 20 mM) of Zn. Growth retardation, defoliation and sluggish root growth were the prime features in the plants supplied with 10, 15 and 20 mM Zn. However, among all the Zn supplemented treatments, 5 mM Zn induced prolific growth and sprouted abundantly. Based on these morphological features; up to 4 mM of Zn treated plants are termed Zn-deficient whereas 10 mM and above treatments are called Zn-toxic, and the remaining plants (5 mM) are considered Zn-sufficient. Both deficiency and excess supply decreased

shoot and root biomass and Zn-deficient plants were more affected. The FW and DW of leaf, stem and root of mandarin orange plants increased up to the level of 5 mM and thereafter decreased with further increase in concentration (Fig. 1).

Effect of Zn on physiological parameters

All parameters related to exchange of gases revealed a steady decline under both Zn deficient and Zn excess treatment. Zn-optimum (5 mM) plants however, showed remarkable increment of P_n , g_s and E , compared to the deficient as well as Zn-excess treatments. Plants supplied with 5 mM Zn (optimum dose) demonstrated 86 % and 35 % increase of g_s and E respectively than the Zn starved plants, and 77 % and 42 % more increase than the 20 mM Zn treatment (Table 1).

Stomatal structure and TEM

In mandarin orange leaves, it was observed that under Zn deficient condition (Fig. 2a), the guard cells were marginally opened while at optimum level; the guard cells were open fully (Fig. 2b). In addition, excess Zn treatment induced a decrease in the size of stomata with smaller stomatal slits along with the distortion of guard cells (Fig. 2c). Zn deficient leaves of mandarin orange plants had smaller stomatal size and reduction in stomatal number. In order to detect the transformation in ultrastructure, we undertook TEM analysis which demonstrated electron dense vacuole of Zn-deficient plants (Fig. 3a) but Zn-sufficient plants possessed no deformity in vacuole ultrastructure (Fig. 3b). In contrast, aberrations in the vacuolar structures were noted when plants were treated with 15 mM Zn (Fig. 3c). The ultrastructure of mitochondria in the leaf cells of plants treated with Zn optimal dose (Fig. 3e) did not display any abnormality. In these plants, mitochondria demonstrated no disorder in the structures of cristae. The

Table 1 Effect of Zn stress on gas exchange parameters leaf of citrus plants

Zn (mM)	P_n ($\mu\text{mol. m}^{-2}.\text{s}^{-1}$)	g_s ($\text{mmol.m}^{-2}.\text{s}^{-1}$)	E ($\text{mmol.m}^{-2}.\text{s}^{-1}$)
0	6.25±0.03 g	28.31±0.01	1.110±0.01ef
1	6.11±0.01 g	29.48±0.02	1.245±0.03de
2	8.85±0.03d	29.93±0.02	1.820±0.14c
3	8.60±0.05d	35.64±0.01	1.400±0.16d
4	11.91±0.07c	41.98±0.33	2.230±0.11b
5	15.34±0.22a	46.09±0.14	2.885±0.04a
10	13.21±0.01b	44.10±0.02	2.450±0.03b
15	7.06±0.09e	35.23±0.01	1.345±0.03de
20	6.65±0.20f	25.89±0.01	0.880±0.01f

Data are mean±standard error ($n=3$). Within a column, values followed by different letters are significantly different at $P<0.05$. (P_n photosynthetic rate, g_s stomatal conductance, E transpiration, Zn zinc)

mitochondrial membranes of excess Zn treated plants were disorganized and the cristae were randomly distributed (Fig. 3f). Excess level of Zn treatment caused a collapse of the cristae and localized condensations in the mitochondrial matrix.

Effect of Zn stress on pigments

The content of total chl as well as chl a , chl b and their ratio (chl a/b) decreased either in Zn deficiency or in Zn excess treatment. Similarly car also increased in Zn sufficiency compared with rest of the treatments (Table 2). At Zn-optimum dose (5 mM), an increase of 27 % and 19 % chl a content were recorded, compared to without Zn i.e 0 mM and 20 mM Zn treatment respectively; whereas, chl b content showed augmentation by 100 % and 60 % in 5 mM plants compared to 0 mM and 20 mM Zn treated plants respectively. A noticeable increase in the content of total chl and car were observed in the Zn optimum plants than the remaining treatments.

Fig. 1 Effect of Zn treatments on biomass of citrus. Bars represent the mean±SE. Different letters above standard error bars of the same parameter indicate significant difference at $P>0.05$ (FW fresh weight, DW dry weight)

Fig. 2 Zn deficient guard cells closed whereas optimum plant had their stomata opened. Under excess the structure of the guard cells got distorted (Scale=1 mm)

Effect of Zn on proline and phenol

Proline content in both leaf and root of mandarin orange plants increased with an increase in Zn stress. The Zn-optimum plants manifested very low level of proline content compared with the rest of the treatments (Fig. 4a). Proline content decreased by 64 % and 51 % in the leaf and root tissues of the plants treated with Zn-optimum dose compared to the control. The phenol content in both leaf and root of mandarin orange plants increased with an increase of Zn concentration up to 5 mM Zn and thereafter the phenol content in both leaf and root decreased with the further increase in concentration of Zn up to 20 mM Zn treatment (Fig. 4b).

Effect of Zn on ascorbic acid, starch and total sugar

Doses of Zn application influenced AsA content in the leaf and root significantly. As the levels of Zn were increased, a significant increase of AsA content in both leaf and root tissues were observed (Fig. 5a). The root tissues of Zn optimally treated plants showed 38 % increment compared to the 20 mM Zn treated plants. Zn deficiency and excess supply influenced the plants detrimentally with regard to their starch and sugar concentrations because both of them decreased gradually in the stressed plants compared to optimally treated plants (Fig. 5b and c). The starch content in mandarin orange plants increased progressively with an increase in Zn concentration and

Fig. 3 Ultrastructural changes of the mandarin leaf under Zn-deficiency and Zn-excess: **a** vacuole of a palisade parenchyma cell filled with electron dense phenolic compounds without (0 mM) Zn; **b** normal

vacuole at 5.0 mM Zn; **c** ruptured vacuole at 15 mM Zn; **d** mitochondria without (0 mM) Zn; **e** mitochondria of Zn (5.0 mM) sufficient plants and **f** distorted mitochondria at 15 μ M Zn

thereafter decreased with the further increase in Zn concentration. Apart from the total sugar content, leaf tissues contained higher quantity of the biochemicals, i.e. AsA and starch.

Effect of Zn on ROS and lipid peroxidation

O_2^- generation, MDA and H_2O_2 were higher in Zn deficient and Zn-excess treated plants than in the Zn-sufficient plants. O_2^- accumulation was higher in leaves and roots of Zn-deficient plants compared to the leaves and roots of control, deficient and the excess Zn treated plants (Fig. 6a). Similarly, under deficiency, MDA content increased in leaf and root tissues, compared to Zn sufficient plants (Fig. 6b). Concentration of H_2O_2 increased in response to both Zn deficiency and excess, in shoot and root. In shoot, Zn deficiency caused an abrupt increase in concentration of H_2O_2 , but in roots, the increment was higher in the Zn excess plants (Fig. 6c) compared to Zn-optimum treatment (5 mM). On the other hand, Zn-deficient and -excess treatment posted an increment of 55 % and 58 % respectively, than the root tissues of the plants received sufficient quantity of Zn (Fig. 6).

Effect of Zn on antioxidative enzymes

Zn-deficient and Zn-excess treated plants had higher APX, CAT, SOD and POD enzyme activities and in all the treatments, the leaf tissues contained higher activities compared to the root, with SOD being the sole exception (Fig. 7a) which shows their vulnerability to stress. Noticeably, antioxidant enzyme activities were steadily lower under the Zn-optimum treatment. A significant increase in the specific activity of APX was observed in roots of Zn-deprived (0 mM) and

20 mM plants that increased up to 77 % and 75 % respectively, compared to 5 mM Zn application (Fig. 7a). The CAT activity increased by 327 % and 88 % in the leaf and root tissues of Zn-deficient plants respectively (Fig. 7b), compared to optimum Zn supply (5 mM). Zn deficiency and excess supply induced the activity of SOD enzyme compared to the Zn-optimum supply in both leaf and root tissues similarly (Fig. 7c) and in the Zn-optimum plants (5 mM) the specific activity was lower considerably. Zn deficiency enhanced POD activity in both shoot and root tissues simultaneously (Fig. 7d). Compared to the leaf tissues of control (0 mM) and 20 mM Zn treated plants, the Zn-sufficient plants showed 24 % and 13 % lower activities of POD.

Discussion

Nutrient requirements of plants are specific to the species and growth inhibition is a general phenomenon associated with most of the heavy metals. Zn, to be particular, promotes growth at optimal concentration but at higher or lower levels restrain growth by interfering with the common metabolic activities of the plant (Mukhopadhyay et al. 2013a). Deficient supply of Zn, in mandarin plants, suppressed growth, and produced typical Zn deficiency symptoms (Fig. 1). Development of chlorosis and necrosis, under Zn deficiency, had been reported in citrus (Srivastava 2013) which corroborates with our observation. Toxic effect of Zn, in this study (Fig. 1), was evident from truncated growth and reduced FW and DW that is in consonance with the same phenomenon observed in *Jatropha* seedlings under Zn excess. Enhanced ROS production and oxidative damage inhibited growth by interfering with normal cellular events and physiological disorder (Luo et al. 2010). Similarly, excess Zn, in *Camellia sinensis*, reduced biomass production and slowed

Table 2 Effect of Zn stress on pigment contents in the leaf of citrus plants

Zn (mM)	chl ($\mu\text{g g}^{-1}\text{FW}$)		chl <i>a/b</i> ratio	Total chl ($\mu\text{g g}^{-1}$)	car ($\mu\text{g g}^{-1}\text{FW}$)
	chl <i>a</i>	chl <i>b</i>			
0 mM	1369.15±10.06 h	531.81±0.15 h	2.58±0.01 b	1900.96±0.30 h	552.87±0.06 e
1.0 mM	1472.27±9.34 g	573.18±0.13 f	2.57±0.01 c	2045.49±0.31 g	532.82±0.08 f
2.0 mM	1569.63±15.52 e	623.23±8.08 e	2.52±0.01 e	2192.82±0.59 e	575.89±0.06 d
3.0 mM	1644.89±11.08 c	658.44±10.01 c	2.50±0.02 g	2303.47±14.31 c	613.33±3.05 b
4.0 mM	1721.03±11.31 b	699.58±6.07 b	2.47±0.01 h	2420.61±20.27 b	434.64±8.10 i
5.0 mM	1841.67±9.06 a	723.62±9.10 a	2.55±0.11 d	2565.27±9.27 a	685.43±11.08 a
10.0 mM	1630.54±10.23 d	650.25±11.11 d	2.51±0.02 f	2280.76±14.45 d	593.23±9.05 c
15.0 mM	1509.08±14.05 f	555.09±9.05 g	2.72±0.01 a	2064.22±16.18 f	504.06±5.06 g
20.0 mM	1069.78±21.27 i	452.25±7.11 i	2.37±0.01 i	1522.08±7.11 i	482.82±7.03 h

Data are mean±standard error ($n=3$). Within a column, values followed by different letters are significantly different at $P<0.05$. (chl chlorophyll, car carotenoids, FW fresh weight)

down growth severely (Mukhopadhyay et al. 2013a). Zn excess also acted upon root growth, probably by hampering cell division, a similar observation made in *Jatropha* sp (Luo et al. 2010).

Gas exchange parameters were appreciably lower in Zn-deficient mandarin plants (Table 1). Reduction of g_s under Zn deficiency was reported in maize and rice (Hajiboland and Amirzad 2010). Zn control K^+ uptake by the guard cells and Zn deficiency probably decreased accumulation of K^+ and other solutes in guard cells. Stomatal opening is controlled by carbonic anhydrase (CA), which maintain adequate HCO_3^- in the guard cells and here Zn plays a vital role (Sharma et al. 1995). The highest P_n , g_s and E were found in plants treated with 5 mM Zn implying that, optimal Zn application did not interfere with the gas exchange parameters in these plants (Table 1). However, the drastic reduction of P_n and concomitant reduction of g_s and E in Zn-toxic plants indicated severe

injuries to photosynthetic apparatus (Mukhopadhyay et al. 2013a). The decreased opening of the guard cells reduced the intake of CO_2 leading to lower Rubisco enzyme activity (Mukhopadhyay et al. 2013a). Rubisco and other enzyme activities decreased under Zn-excess, which probably inhibited P_n in the mandarin plants. In addition, g_s was significantly decreased in Zn-toxic plants that, in turn, reduced the P_n and E which indicate the effect of Zn stress on these plants.

Zn plays a greater role in stomatal opening by its structural involvement in CA, that is needed for maintaining adequate HCO_3^- in the guard cells and by controlling the K^+ uptake by the guard cells (Sharma et al. 1995). Due to absence of Zn, the activity of CA decreased, K^+ influx was low and thus the guard cells remained partially opened and the shape of the guard cells became almost round in the Zn deficient mandarin plants (Fig. 2a). In *Beta vulgaris*, stomatal size, density were smaller and stomatal slits were sealed in plants grown under

Fig. 4 Effect of Zn on **a** proline, and **b** phenol on tissue extracts of citrus. Bars represent means $\pm$ standard errors. Different letters above standard error bars of the same parameter indicate significant difference at $P > 0.05$ (FW fresh weight, L leaf, R root)

Fig. 5 Effect of Zn on **a** ASA **b** starch and **c** total sugar on tissue extracts of mandarin. Bars represent means $\pm$ standard errors. Different letters above standard error bars of the same parameter indicate significant difference at $P>0.05$ (ASA ascorbic acid, FW fresh weight, L leaf, R root)

high Zn (Sagardoy et al. 2010) and citrus plants when grown under 15 mM Zn manifested stress imposition by keeping pores slightly opened. In high Zn plants stomata were smaller and had a more rounded shape than those present in control plants (Sagardoy et al. 2009). In this study, when plants exposed to Zn-excess, stomata turned rounded and became comparatively smaller than the plants received Zn-optimum supply (Fig. 2b and c). In Zn-deprived leaves, electron dense material resembling phenolic compounds were deposited in the vacuoles (Fig. 3a) and biochemical assay endorsed elevated content of phenolic compounds (Fig. 4b). Phenol accumulation in response to Zn-deficiency was also reported in pecan and in tea (Kim and Wetzstein 2003; Mukhopadhyay et al. 2013a; Mukhopadhyay and Mondal 2014). Under Zn stress the ultrastructure of mitochondria changed, membrane disrupted and Zn-excess altered the structure, a similar change in structure was reported in tea (Li et al. 2011).

Chl *a* and *b* contents, in mandarin, decreased under both Zn-deficiency and -excess (Table 2). Similarly, reduction of pigment contents under Zn-stress were also observed in red cabbage and tea where chl *a*, *b* and *car* declined (Hajiboland and Amirazad 2010; Mukhopadhyay et al. 2013a). The decline in chl content in plants exposed to heavy metal stress is believed to be due to inhibition of enzymes of chl biosynthetic such as δ -aminolaevulinic acid dehydratase and protochlorophyllide reductase (Mukhopadhyay et al. 2013a). Reduction of pigments may be an integral result of disturbed biosynthesis or enhanced degradation of thylakoids (Vassilev et al. 2007) under Zn stress. Furthermore, the destruction of chl by Zn stress could be due to peroxidation processes in the chloroplast membrane lipids by the ROS (Sandalio et al. 2001) as our observation indicated. Conversely, *car* contents were recorded to be on the lower side in the Zn-deficient and Zn-toxic mandarin plants compared to the Zn-sufficiency. Increased *car* concentrations in the Zn-sufficient plants, perhaps offered additional fortification and paved the way for superior growth. Chl pigments are associated with the electron transport system and are the principal source of singlet oxygen ($^1O^2$) (Arora et al. 2002). *Car* is a key scavenger of $^1O^2$ and decreased level of *car* enhances the likelihood of ROS accumulation (Candan and Tarhan 2003), which probably happened in Zn deficient and toxic mandarin plants that led to lower rate of *Pn* and reduced growth.

Plants offer a number of counteractive mechanisms to withstand stresses. Apart from defensive enzymes, they accumulate osmotic solutes like proline, to neutralize the effect of stresses. In our study, proline contents increased in the Zn-deficient and toxic root and leaf tissues but the Zn-sufficient plants contained lesser quantity of proline comparatively (Fig. 4a). Accumulation of proline is an indication of protection for plants under environmental stresses (Chen et al. 2003). Increases in proline as a function of metal accumulation had been observed in response to heavy metals, including

Zn (Bassi and Sharma 1993). Zn deficiency and excess stimulated accumulation of proline, which apart from acting as a metal chelator and osmolyte, scavenges hydroxyl radical ($OH^\bullet$) and $^1O^2$ and thus provides protection against ROS induced cell damage (Radić et al. 2009) to some extent. Nevertheless, stresses beyond tolerance levels unavoidably culminate in oxidative damage attributable to intensive production of ROS (Smirnoff 1993). Polyphenols, the major alkaloids, present in plants, are known antioxidant compounds (Mukhopadhyay et al. 2012, 2013a, b) and offer strong antioxidant activity in plants growing under heavy metal stress. Principally, they are oxidized by peroxidase and contribute in scavenging H_2O_2 (Singh and Malik 2011).

AsA is the most abundant antioxidant in plant cells and found in sub-cellular compartments (Smirnoff 2000). Leaf and root tissues of the mandarin plants, subjected to Zn-deficiency and -excess, generated AsA in lower quantity (Fig. 5a) probably due to higher activity of APX, which along with AsA are the components of Halliwell-Asada pathway (Ozden and Aydin 2010). In our study, starch contents dwindled in the Zn-deficient and Zn-toxic plants (Fig. 5b), which could be the outcome of disturbed photosynthetic activity and modified sugar metabolism because Zn deficiency negatively influences the activity of aldolase and starch synthetase (Hajiboland and Amirazad 2010). Nevertheless, increased quantity of starch and sugar (Fig. 5c) could be involved in the superior growth of the Zn-optimum plants and these soluble carbohydrates probably provided energy and osmolytes necessary for growth (Mukhopadhyay et al. 2012).

In higher plants, heavy metals induce generation of O_2^- , H_2O_2 , $OH^\bullet$ and $^1O^2$ that exert oxidative stress (Devi and Prasad 1998). In addition, Zn is a redox-inactive metal, which disturbs the cellular antioxidant pool and disrupts the metabolic balance to develop ROS load (Stohs and Bagchi 1995). Stimulation of ROS production and oxidative stress in mandarin under Zn stress was indicated by increased accumulation of O_2^- , MDA, and H_2O_2 in the Zn-deficient and Zn-toxic plants (Fig. 6a, b and c). Similar results were reported in mulberry and tea under both deficiency and excess of Zn (Tewari et al. 2008; Mukhopadhyay et al. 2013a) and in citrus under B deficiency (Han et al. 2008). The level of MDA, an appraisal of lipid peroxidation, is associated to the production of O_2^- through the Fenton reaction (Choudhary et al. 2007). Thus, the increased level of MDA suggests that Zn stress stimulated free radical generation and consequent stress imposition in mandarin plants. Therefore, the increased lipid peroxidation was probably due to the harmful effects of excessive levels of ROS in the cellular compartments (Bowler et al. 1992). The concentration of H_2O_2 increased both in the leaves and roots of mandarin orange plants exposed either to Zn deficiency or excess (Fig. 6c). Similar findings had been reported in iron-starved mulberry and B stressed mulberry plants (Tewari et al. 2008, 2009). H_2O_2 is a constituent of

Fig. 6 Effect of Zn on generation of **a** superoxide anion, **b** MDA, and **c** hydrogen peroxide in mandarin plants. Bars represent means $\pm$ standard errors. Different letters above standard error bars of the same parameter indicate significant difference at $P > 0.05$ (FW fresh weight, MDA malondialdehyde)

oxidative plant metabolism and is a product of peroxisomal and chloroplasmic oxidative reactions (Del-Rio et al. 1991). H_2O_2 itself is an active oxygen species and it also reacts with O_2 to form more reactive $OH^\bullet$ in the presence of trace amount of Fe or Cu (Thompson et al. 1987). The $OH^\bullet$ initiate self-propagating reactions leading to peroxidation of membrane lipid and destruction of proteins (Asada and Takahashi 1987; Bowler et al. 1992; Halliwell 1987). H_2O_2 is a membrane permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Lavine et al. 1994).

Antioxaive enzymes are important defense system of plants against oxidative stresses caused by metals (Weckx and Clijsters 1996). The increased activity of APX in the root and leaf tissues of mandarin plants under Zn-deficiency vis-à-vis Zn-excess (Fig. 7a) indicates the activation of ascorbate-glutathione cycle that probably acted as a protective system in cells under stress. Heavy metals produce free radicals, which

induce enhanced catabolism of chl, proteins, RNA etc. In response to higher production of ROS under Zn stress, APX activity enhanced to counteract oxidative damage in stressed plants. Our finding corroborates with previous reports in mulberry under excess Zn (Tewari et al. 2008), in red cabbage under deficiency (Hajiboland and Amirzad 2010) and in *C. sinensis* under both deficiency and excess (Mukhopadhyay et al. 2013a). CAT, along with SOD and POX, are redox metalloenzymes involved in plant defense against oxidative stress (Luo et al. 2010). In the Zn stressed tissues (both root and shoot) of mandarin, CAT activity increased (Fig. 7b) indicating its active participation in the removal of ROS. Our observation is in consonance with the earlier studies in *Brassica*, *Jatropha* and tea where increased CAT activity was reported under Zn stress (Prasad et al. 1999; Luo et al. 2010; Mukhopadhyay et al. 2013a). SOD play an important role in detoxification processes by converting free O_2^- to O_2 and H_2O_2 under Zn stress (Bonnet et al. 2000) and several studies have proposed the vital role of SOD in plants under environmental adversity that often lead to increased generation of ROS (Mittler et al. 2004). Similar to our study (Fig. 7c) an increase in SOD activity has been reported in citrus under B stress (Han et al. 2008), in mulberry and tea under Zn stress (Tewari

Fig. 7 Enzyme activity in leaf and root extracts of citrus subjected to diverse Zn doses [**a**=APX activity, **b**=CAT activity, **c**= SOD activity and **d**= POX activity]. Bars represent means $\pm$ standard errors. Different

letters above standard error bars of the same parameter indicate significant difference at $P > 0.05$ (APX = ascorbate peroxidase, CAT = catalase, FW = fresh weight, POX = peroxidase, SOD = superoxide dismutase)

et al. 2008; Mukhopadhyay et al. 2013a). Hence, our experimental observations substantiate that CAT perhaps afforded additional fortification under Zn stress. POX is considered valuable marker due to their activity under heavy metal and other environment stresses (Luo et al. 2010). Our observation (Fig. 7d) is in consonance with other studies under Zn stress (Tewari et al. 2008; Mukhopadhyay et al. 2013a) where POX activity increased under deficient and excess supply. Enhancement of POX activity in mandarin plants revealed that it might play an important role in the defensive mechanisms under Zn stress.

Thus, the present study concludes that, exposure of mandarin plants to Zn-deficiency or Zn-excess reduces growth, biomass, *Pn*, *gs* and *E* but augments ROS accumulation, lipid peroxidation and membrane permeability. Although Zn stressed plants increased activities of antioxidant enzymes like APX, CAT, SOD and POX; but their defense system do not afford adequate reinforcement against ROS collectively, which terminates in visual symptoms of Zn- deficiency and Zn-excess stress and depressed growth.

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