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Membrane Damage and Activity of Antioxidant Enzymes in Response to Zinc and High Irradiance Stress in Cowpea Plant

Prabhu Inbaraj Michael and Muthuchelian Krishnaswamy*

Department of Bioenergy, School of Energy, Environment and Natural resources, Madurai Kamaraj University, Madurai, India

*Corresponding author

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A B S T R A C T

The effect of the zinc (Zn) and high irradiance (HI) on growth, lipid peroxidation (MDA), membrane permeability (EC), hydrogen peroxide (H_2O_2), proline and ascorbic acid (AsA) and activities of antioxidant enzymes (superoxide dismutase, SOD; peroxidase, POX; polyphenol oxidase, PPO) have been investigated in cowpea plants grown in half strength Hoagland solution. A significant decrease in root and shoot length and fresh and dry weight of leaf, shoot and root was detected in Zn-excess plants. Under Zn and HI stress, significant increase in EC, MDA and H_2O_2 were observed under Zn-deficient (0 ppm) and Zn-excess (50 ppm). The SOD activity significantly increased both in Zn-deficient and Zn-excess leaves under Zn and HI stress. Under Zn and HI stress, the antioxidant enzyme activities, POX and PPO and AsA and proline accumulation were found to be significantly decreased in the Zn-excess leaves than in the Zn-sufficient (5 ppm) which showed the susceptibility and inability of the cowpea plant to tolerate the excess level of Zn and HI stress. Further, a significant increase in MDA, H_2O_2 , and EC with a simultaneous decrease in the antioxidant enzyme activities under Zn-deficiency showed the inefficiency of the cowpea plant in response to Zn deficiency.

Introduction

Plants responses to heavy metals are drawing growing attention, as metal pollution is still increasing worldwide and threatens the stability of our ecosystems (Menon et al., 2007). Metals exist naturally in the environment and take part in the normal metabolism of plants. Yet beyond a certain threshold, that is variable according

to the metals and plant species, phytotoxicity can occur (Markovska et al., 2009). Zinc is an essential micronutrient for plants, but at supra optimal concentrations, which are occurring in some industrially-polluted soils, it can become phytotoxic (Cuypers et al., 1999). This effect may be due to Zn induced (1) decrease in the

contents of essential nutrients as Fe, Cu and Mn (Siedleska, 1995; Ebbs and Kochian, 1997), (2) oxidative damage to membranes (Weckx and Clijsters, 1997; Cuypers et al., 2001), despite of its known effect to protect membrane integrity when present at normal concentrations (Cakmak, 2000) and (3) disturbances in photosynthetic performance (Vangronsveld and Clijsters, 1994). Zn induces oxidative stress by promoting the generation of reactive oxygen species (ROS) and lipid peroxidation (Madhava Rao and Sresty, 2000).

Among various stresses encountered by plants in tropical environments, high intensity of irradiance is the most significant that account for remarkable alterations in plant metabolism (Dubey, 1999). Light is essential for plant growth and development, but when plants are subjected to excessive light, active oxygen generation is increased (Asada, 2006), often resulting in photo-oxidative damages; thus light can also be one of the most deleterious environmental factor. Acclimation to different light environments is crucial for photosynthetic organisms to grow and survive. Low light limits cell growth, whereas excess light or high irradiance (HI) causes oxidative damage to proteins, lipids, and nucleic acids, ultimately leading to a loss of cell viability (Halliwell and Gutteridge, 1999; Mittler, 2002).

In plants, reactive oxygen species (ROS) are continuously generated as byproducts of photosynthesis and other cellular metabolic processes (Foyer and Noctor, 2000). ROS can also be actively induced by stresses such as pathogen infection, drought, nutrition imbalance, heat and heavy metals (Marschner, 1995). High ROS levels can damage lipids and proteins and thus ROS concentrations are normally well controlled by complex mechanisms in plants (Apel and

Hirt, 2004). To counter ROS, plants produce antioxidants and antioxidant enzymes such as peroxidase (POX; EC 1.11.1.7) and superoxide dismutase (SOD; EC 1.15.1.1) (Zou et al., 2011) and many non-enzymatic solutes like proline and ascorbic acid (AsA). Ascorbic acid (AsA) is an ubiquitous soluble antioxidant in photosynthetic organisms, and the most important reducing substrate for H₂O₂ detoxification and plays an important role in detoxification of toxic metal ions in combination with other antioxidants like non-protein thiols, cysteine and proline (Singh and Sinha, 2005).

Cowpea is one of the most ancient crops known to man, with its center of origin and subsequent domestication being closely associated with pearl millet and sorghum. Cowpea is one of the important kharif pulses grown in India. Cowpea is tolerant to heat and dry conditions, but is intolerant to frost (Davis et al., 2000). The crop is grown from March to April and is harvested between June and July depending upon its end use. So far, we have not encountered any report on the response of cowpea plants to Zn together with HI stress. The objective of this study was to investigate the influence of different levels of zinc and HI stress on the membrane damage and antioxidant enzymes activities in cowpea plants.

Materials and methods

Plant material and zinc treatments

Cowpea seeds (*Vigna unguiculata* L. Walp. P152) were rinsed in distilled water and surface sterilized with 1% sodium hypochlorite for 20 min, rinsed again and germinated on moistened filter paper in darkness at 23 °C. After 3 days, uniformly germinated seedlings were transferred to plastic cups containing half-strength Hoagland's nutrient solution (Hoagland and

Arnon, 1950). After 7 days of growth, Zn treatments were given in the concentrations of 0 ppm (Zn-deficient), 5 ppm (Zn-sufficient) and 50 ppm (Zn-excess) as ZnSO₄·7H₂O. The growth solutions were adjusted to pH 5.6 ± 0.2 and were replaced every two days.

Plants were grown in a growth chamber with the following conditions: day/night temperature, 22 ± 2 °C/18 ± 2 °C; relative humidity, 60-70%; 16-h light: 8-h dark photo-cycle; light intensity, 150 µmol m⁻² s⁻¹. Two weeks after germination, the plants were collected and analyzed.

Photoinhibition and recovery under controlled conditions

Detached leaves which were already subjected to Zn stress were placed in a controlled environment chamber equipped with a 24 V/250 W metal-halide lamp. The leaf surface was exposed to a photosynthetic photon flux density (PPFD) of 1900 µmol m⁻² s⁻¹ for up to 60 min. After this period, some leaves exposed to HI were returned to normal condition (Rec-recovery) by adapting dark recovery for 60 min before sampling and analyzed.

Plant growth

After 2 weeks of Zn treatment, 10 plants from each group (Zn-deficient, Zn-sufficient and Zn-excess) were taken at random and divided into shoot and root fractions. Shoot and root lengths were measured. Weight measurements were conducted from base to the tip of primary leaves, shoot and entire roots of the seedlings.

After measuring the fresh weights of seedlings the same tissues were dried in an oven at 70 °C for 48 h, and the dry weights were measured.

Determination of zinc content

The Zn content was determined by atomic absorption spectrophotometer (Piper, 1942) after wet digestion of 1 g of dried and powdered leaf material in 5 mL of ternary mixture of HNO₃: H₂SO₄: HClO₄ in the ratio of 10:1:4 (v:v:v). The total Zn concentration was expressed as µg g⁻¹ DW.

Membrane permeability

Electrolyte leakage was used to assess membrane permeability. This procedure was based on Lutts et al. (1996). Leaf discs (1 cm in diameter) from two randomly chosen plants per replicate were taken from the middle portion of fully developed leaf and were then placed in individual stoppered vials containing 10 mL of deionized water after three washes with deionized water to remove surface contamination. These samples were incubated at room temperature (ca. 25 °C) on a shaker (100 rpm) for 24 h. Electrical conductivity (EC) of bathing solution (EC₁) was read after incubation. The same samples were then placed in an autoclave at 120 °C for 20 minutes and the second reading (EC₂) was determined after cooling to room temperature. The electrolyte leakage (EC) was expressed following the formula $EC = (EC_1/EC_2) \times 100$.

Hydrogen peroxide and lipid peroxidation assay

H₂O₂ concentration from leaf samples was measured spectrophotometrically according to the procedure of Velikova et al. (2000). Fresh leaf tissue (0.5 g) was homogenized with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) in a pre-chilled mortar and pestle and the homogenate was then centrifuged at 12,000 × g for 15 min. To 0.5 mL of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of

potassium iodide (1 M) were added. The mixture was vortexed and its absorbance was read spectrophotometrically at 390 nm.

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) content according to the procedure of Davenport et al. (2003). Fresh leaf (0.2 g) was homogenized with 2 mL of 5% (w/v) trichloroacetic acid in an ice bath, and centrifuged at $10,000 \times g$ for 10 min at 4 °C. 2 ml of the supernatant was mixed with 2 mL of 0.67% (w/v) thiobarbituric acid and incubated in boiling water bath for 30 min, then cooled and centrifuged. The absorbance of the supernatant was read spectrophotometrically at 450, 532, and 600 nm. The MDA content was calculated based on the following formula,

$$\text{MDA } (\mu\text{mol g}^{-1}) = [6.45 \times (A_{532} - A_{600}) - (0.56 \times A_{450})] \times Vt/W,$$

where $Vt = 0.0021$; $W = 0.2 \text{ g}$

Proline and ascorbic acid

The amount of proline was measured according to the procedure of Bates et al. (1973). 0.5 g of fresh leaf was homogenized in 10 mL of 3% aqueous sulfosalicylic acid and filtered through Whatman #2 paper. 2 mL of the filtrate was then mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid and heated at 100 °C for 60 min. The reaction was terminated in an ice bath and 4 mL of toluene was added to the mixture and contents of tubes were stirred for 20 s. Absorbance of the pink red upper phase was recorded at 520 nm against toluene blank. A standard curve for proline was constructed to determine the proline concentration in each sample.

Ascorbic acid (AsA) content was estimated by following the procedure of Omaye et al. (1979). Fresh leaf (1 g) was grounded with 5 mL of 10% trichloroacetic acid (TCA),

centrifuged at $1,235 \times g$ for 20 min, re-extracted twice and the supernatant was made up to 10 mL. To 0.5 mL of extract, 1 mL of 6 mM 2,4-dinitrophenylhydrazine-thiourea-CuSO₄ (DTC) reagent was added, incubated at 37 °C for 3 h and then 0.75 mL of ice-cold 65% H₂SO₄ was added, allowed to stand at 30 °C for 30 min and the resulting colour was read at 520 nm. The ascorbic acid content was determined using a standard curve prepared with ascorbic acid.

Enzyme extraction and protein determination

Fresh leaves (0.3 g) were homogenized with 3 mL of ice-cold 0.05 M potassium phosphate buffer (pH 7.0) containing 1% (w/v) PVP in an ice bath. The homogenized slurry was centrifuged at $10,000 \times g$ for 15 min at 4 °C and the supernatant was collected. Protein concentration in the supernatant was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Antioxidant enzyme assays

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT) at 560 nm (Beauchamp and Fridovich, 1971). The assay was carried out at 25 °C in a reaction mixture (3 mL) containing 33 μM NBT, 10 mM L-methionine, 0.66 mM EDTA and 0.0033 mM riboflavin in 50 mM sodium phosphate buffer (pH 7.8). Riboflavin was added last and the test tubes containing the reaction mixture were incubated at 25 °C for 10 min under $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ irradiance. The reaction mixture with no enzyme developed maximum colour due to the maximum rate of reduction of NBT. The non-irradiated reaction mixture did not develop colour and was used as the control.

One unit of SOD activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT, and the specific enzyme activity was expressed as Units mg^{-1} protein.

Peroxidase (POX; EC 1.11.1.7) activity was assayed by the method of Kumar and Khan (1982). Assay mixture of POX contained 2 mL of 0.1 M sodium phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H_2O_2 and 0.5 mL of enzyme extract. The solution was incubated at 25 °C for 5 min and the reaction was terminated by adding 1 mL of 2.5 N H_2SO_4 . The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The POX activity was expressed as Units mg^{-1} protein. One unit is defined as the change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Polyphenol oxidase (PPO; EC 1.10.3.1) activity was assayed by the method of Kumar and Khan (1982). Assay mixture for PPO contained 2 mL of 0.1 M sodium phosphate buffer (pH 6.0), 1 mL of 0.1 M catechol and 0.5 mL of enzyme extract. The assay mixture was incubated at 25 °C for 5 min and the reaction was stopped by adding 1 mL of 2.5 N H_2SO_4 . The absorbance of the purpurogallin formed was read at 495 nm. To the blank, 2.5 N H_2SO_4 was added at the zero time of the same assay mixture. PPO activity is expressed in Units mg^{-1} protein. One unit is defined as the change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Statistical analysis

The data compiled were submitted to one-way analysis of variance (ANOVA) by using SigmaPlot 11.0. Each data point was the mean of five replicates ($n = 5$) and

comparisons with P-values < 0.01 were considered significantly different by Tukey's test.

Result and Discussion

Plant growth

Plant growth was assessed using differences in fresh weight (FW), dry weight (DW), shoot and root length between Zn treatments (Table 1). There were significant reductions in the FW and DW of leaf, shoot and root in plants grown in Zn-excess whereas the Zn-deficient plants showed an insignificant decrease in FW of shoot and root. The Zn-excess plants showed a significant decrease in shoot and root length while Zn-deficient ones showed an insignificant decrease when compared to Zn-sufficient plants. Visual Zn deficiency symptoms such as inhibition of shoot elongation and development of chlorotic areas appeared on plants grown at Zn-deficient condition. Zinc toxicity symptoms appeared on all plants grown at excess Zn concentration. These included inhibition of shoot and root growth, reduced leaf size, and general chlorosis.

Zinc accumulation

Increased Zn concentration in the nutrient solution led to a corresponding increase in the leaves (Figure 1). Seedlings without Zn treatment contained some Zn, which may be related to the prior growth of cowpea plants in the half strength nutrient solution for 7 days without any stress.

Membrane permeability

Both Zn-deficiency and Zn-excess conditions impaired membrane permeability by increasing electrolyte leakage (Figure 2a). There was a significant increase of EC in Zn-deficient and Zn-excess leaves when

compared to the Zn-sufficient ones under Zn and HI stress. Even after recovery from the HI stress imposed on them, the Zn-deficient and Zn-excess leaves showed a significant rise in EC when compared to Zn-sufficient ones. These results indicate that normal membrane permeability characteristics are seriously impaired in cowpea leaves when Zn is either deficient or excess.

Hydrogen peroxide and lipid peroxidation

Zn-deficient and Zn-excess conditions induced significant increase in H₂O₂ and MDA content in leaves compared to Zn-sufficient leaves under Zn stress (Figure 2b and 2c). Further HI stress lead to a significant increase in H₂O₂ and MDA in Zn-deficient and Zn-excess leaves. Both Zn-deficient and Zn-excess conditions showed a lower degree of recovery from HI stress. Our results suggest that Zn and HI stress directly or indirectly lead to the production of oxygen radicals, which results in increased lipid peroxidation and oxidative stress under Zn-deficient and Zn-excess conditions in the cowpea plants.

Proline and Ascorbic acid

Under Zn stress, the proline accumulation and AsA content were significantly low in Zn-deficient and Zn-excess leaves (Figure 3a and 3b) when compared to Zn-sufficient ones. Upon further HI stress and thereafter recovery from HI stress, the Zn-deficient and Zn-excess leaves showed a meager increase in proline accumulation and AsA contents when compared to their respective levels under Zn stress. Though there was an increase in the accumulation of proline and AsA in Zn-deficient and Zn-excess conditions under HI stress and recovery, both the parameters were kept at similar level in Zn-deficient conditions while the

Zn-excess leaves showed marked and significant decrease when compared to the level of Zn-sufficient leaves under Zn stress alone. The Zn-sufficient leaves had a significant increase in both proline and AsA under HI stress and recovery.

Antioxidant enzymes

Figure 4 shows the changes in the activities of SOD, POX and PPO enzymes in cowpea plants subjected to Zn, HI stress and Rec. Under Zn and HI stress, SOD activity (Figure 4a) increased significantly in Zn-deficient and Zn-excess leaves when compared to Zn-sufficient leaves. Upon recovery from HI stress, the Zn-sufficient leaves showed a maximum rate of recovery while after recovery too, the SDO activity was found to be significantly high in Zn-deficient and Zn-excess leaves than the Zn-sufficient ones.

Under Zn stress, POX and PPO enzyme activities were significantly low in Zn-deficient and Zn-excess leaves when compared to Zn-sufficient leaves (Figure 4b and 4c). After further HI stress and upon Rec from HI stress, though there was a slight increase in POX and PPO activities in Zn-deficient and Zn-excess leaves, both the parameters were kept at lower level in Zn-deficient and Zn-excess conditions when compared to the level of POX and PPO activities in Zn-sufficient conditions under Zn stress alone.

As expected, adding high Zn to nutrient solution increased leaf Zn in the cowpea leaves. These results are in good agreement with those for black gram (Gupta et al., 2011). Inhibition in growth because of metal toxicity leads to a reduction in biomass production (Quariti et al., 1997).

Table.1 Growth parameters of Zn-stressed cowpea plant (*Vigna unguiculata* L. Walp. P152). Values are means \pm SE of 10 replicates. Different letters indicate that the mean value is significantly different between treatments within each column ($P \leq 0.01$)

Zn treatment	Fresh weight (g plant ⁻¹)			Dry weight (g plant ⁻¹)			Root length (mm)	Shoot length (mm)
	Leaf	Shoot	Root	Leaf	Shoot	Root		
0 ppm	0.22 \pm 0.013 ^a	1.03 \pm 0.038 ^a	0.66 \pm 0.021 ^a	0.03 \pm 0.002 ^a	0.08 \pm 0.004 ^a	0.02 \pm 0.002 ^a	93 \pm 0.716 ^a	229 \pm 1.426 ^a
5 ppm	0.39 \pm 0.032 ^b	1.12 \pm 0.057 ^a	0.74 \pm 0.028 ^a	0.04 \pm 0.003 ^b	0.10 \pm 0.005 ^b	0.03 \pm 0.002 ^b	147 \pm 0.870 ^b	279 \pm 1.567 ^a
50 ppm	0.17 \pm 0.022 ^c	0.73 \pm 0.029 ^c	0.31 \pm 0.024 ^c	0.02 \pm 0.002 ^c	0.06 \pm 0.003 ^c	0.01 \pm 0.001 ^c	82 \pm 0.663 ^c	208 \pm 1.519 ^b

Figure.1 Zn concentrations of the leaves of cowpea plants grown with 0, 5 and 50 ppm of Zn. Values are means \pm S.E. (n = 5). Bars carrying different letters are significantly different compared to the sufficient level (5 ppm Zn) at $P \leq 0.01$ as determined by Tukey's test

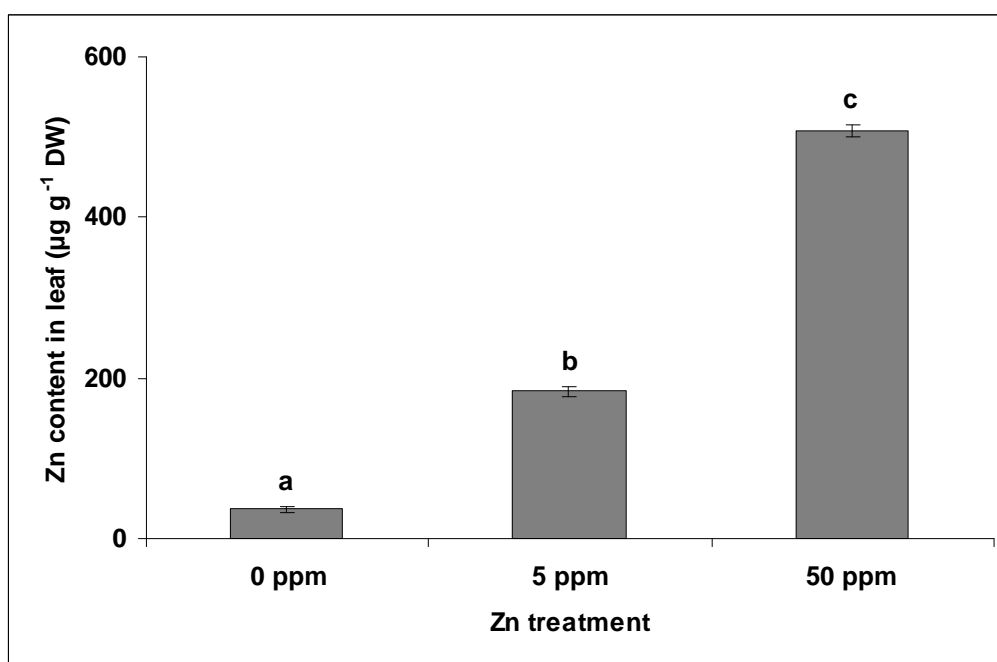


Figure.2 Changes in EC (a), H₂O₂ (b) and MDA (c) contents in the leaves of *Vigna unguiculata* L. Walp. P152 subjected to Zn, Zn + HI stress and recovery. Values are means ± S.E. (n = 5). Bars carrying different letters are significantly different compared to the sufficient level (5 ppm Zn) at P ≤ 0.01 as determined by Tukey's test

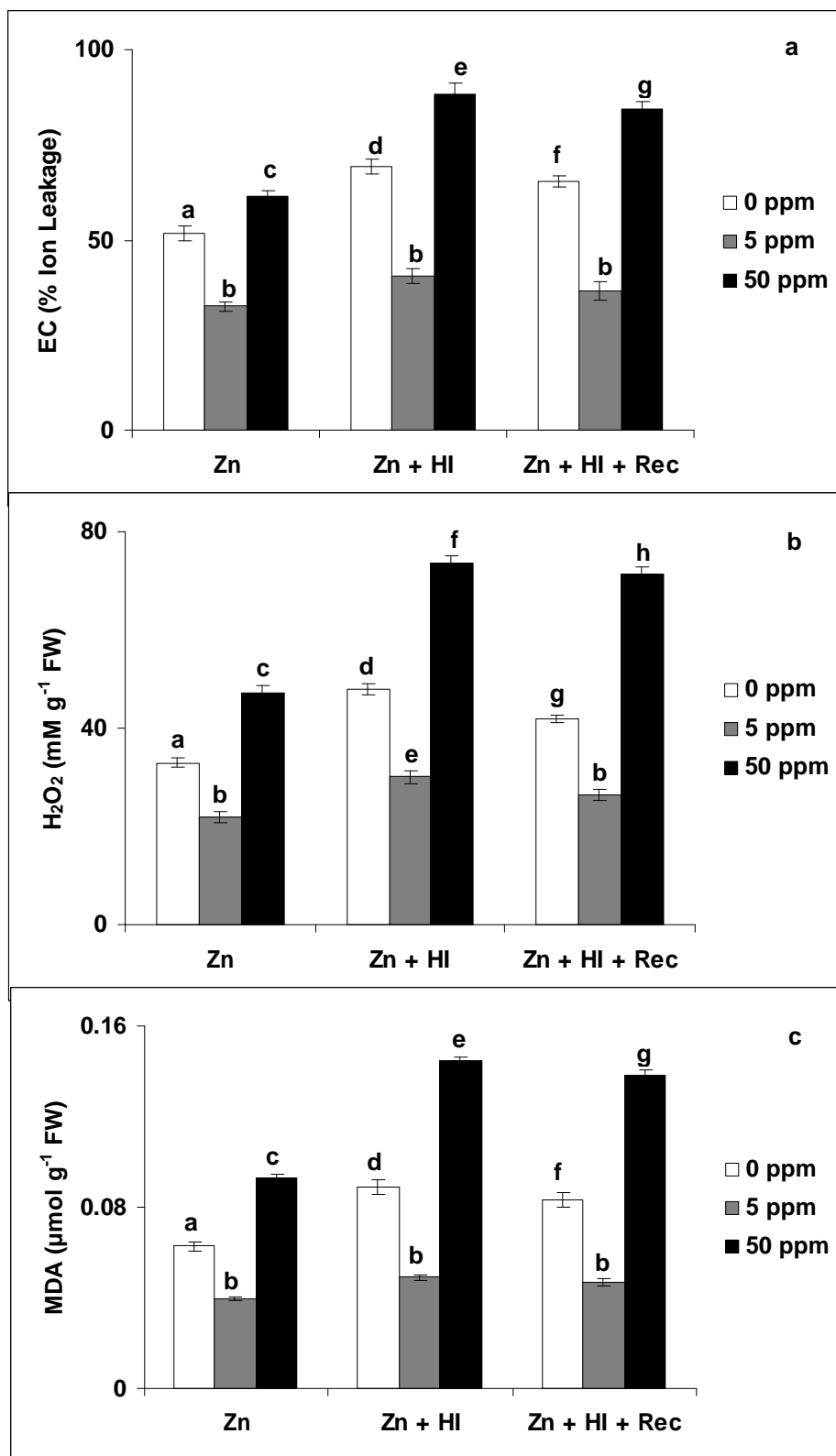


Figure.3 Changes in proline accumulation (a) and ascorbic acid (AsA) (b) content in the leaves of *Vigna unguiculata* L. Walp. P152 subjected to Zn, Zn + HI stress and recovery. Values are means \pm S.E. (n = 5). Bars carrying different letters are significantly different compared to the sufficient level (5 ppm Zn) at $P \leq 0.01$ as determined by Tukey's test

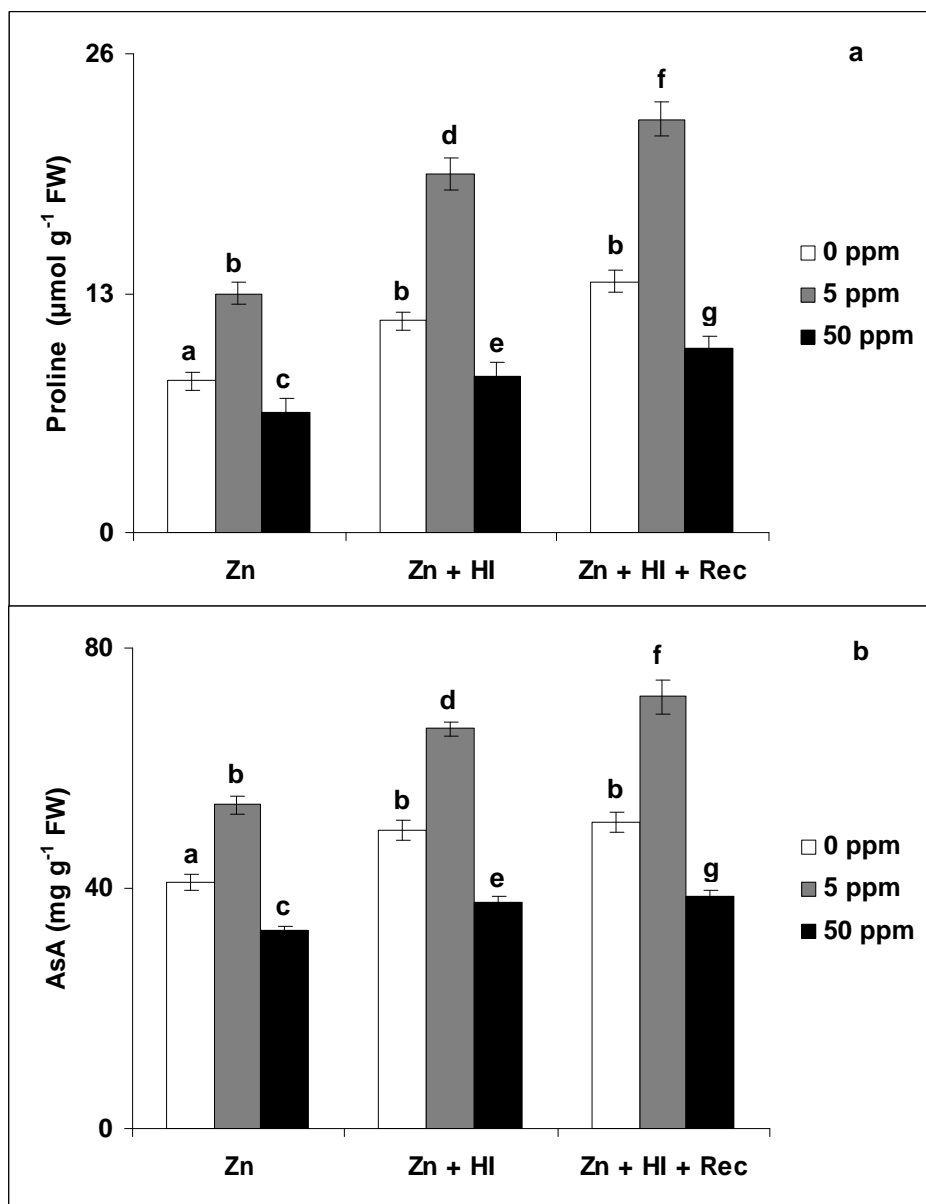
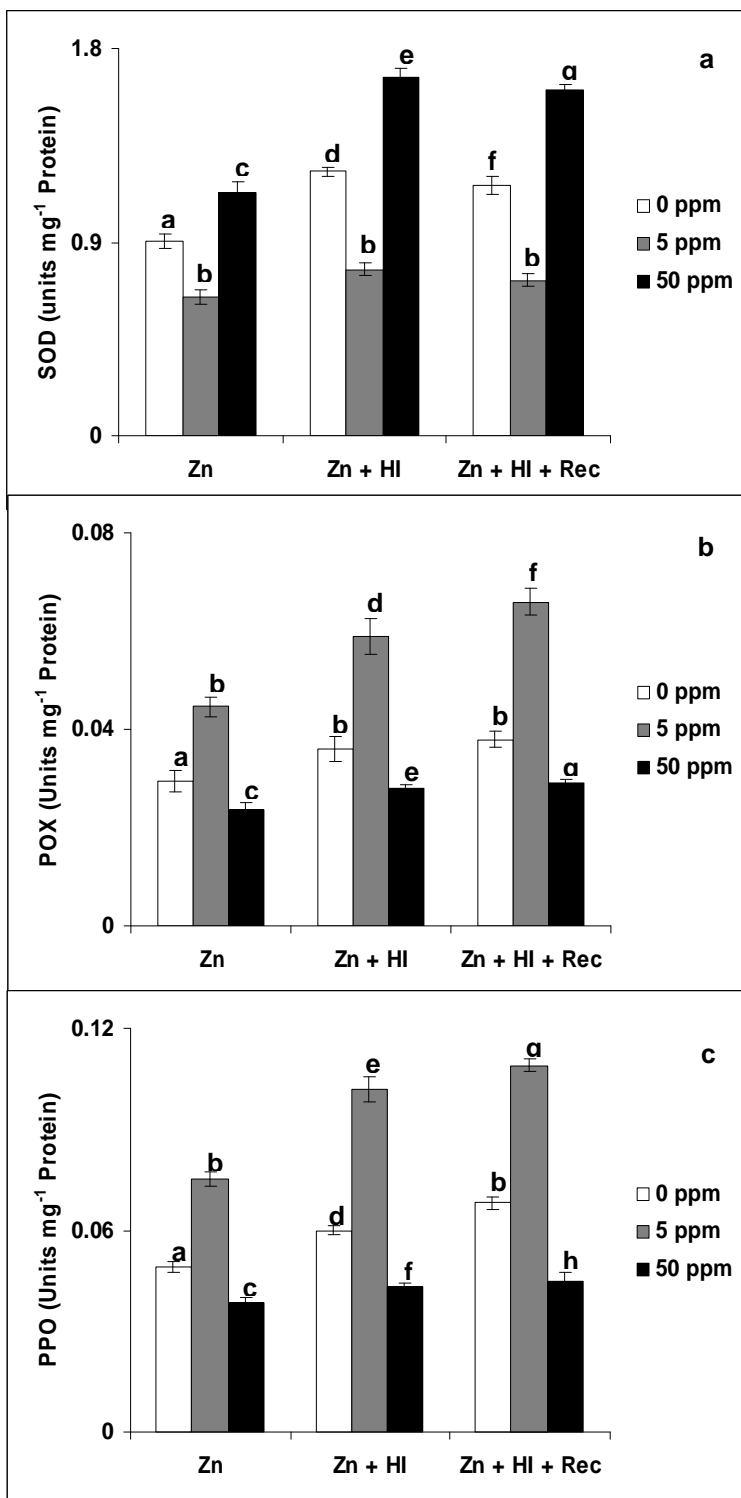


Figure.4 Changes in the enzyme activities of SOD (a), POX (b) and PPO (c) in the leaves of *Vigna unguiculata* L. Walp. P152 subjected to Zn, Zn + HI stress and recovery. Values are means \pm S.E. (n = 5). Bars carrying different letters are significantly different compared to the sufficient level (5 ppm Zn) at $P \leq 0.01$ as determined by Tukey's test



In our study, Zn-deficiency resulted in decreased shoot and total dry matter yields as has been reported in potato (Pandey et al., 2012). Similar to our study, the inhibition of root growth due to Zn-deficiency has been reported in oilseed rape (Grewal et al., 1997). Inhibition of growth and biomass reduction in black gram in response to Zn stress has been recently reported by (Gupta et al., 2011). In our study, the root and shoot length, FW and DW of Zn-excess cowpea plant is much affected when compared to the plants grown under Zn-deficiency. Earlier studies have already reported that Zn toxicity in plants results in reduced growth of both root and shoot which is concurrence with our results (Fontes and Cox, 1998; Kaya et al., 2009). This decrease could be due to their interference with metabolic processes associated with normal development (Lidon and Henriques, 1992). Welch et al. (1982) suggested that external supply of Zn in the root environment is required because Zn may be important in structural and functional integrity of the root-cell plasma membrane. This role was confirmed by Welch and Norvell (1993) and thus a sufficient supply of Zn in the external medium favored good growth in our study.

Despite of their positive role in several metabolic processes, heavy metals cause severe cellular damage (Kyunghee and Junghoon, 2001). Though Zn is an essential element for plant growth, it's deficiency and toxicity have caused a significant increase in the membrane permeability in our study. Moreover, when the Zn stressed cowpea leaves were exposed to HI stress, there was a drastic increase in EC which revealed that Zn-deficient and Zn-excess leaves underwent a severe oxidative damage. In Zn-deficient plants, loss of membrane integrity and increase in membrane permeability are very common in different crop species (Cakmak, 2000). Thus, the

increase in EC under Zn-deficiency in our study is in agreement with that of the recent report by Chen et al. (2009). Weckx and Clijsters (1997) observed that Zn toxicity caused membrane damage of *Phaseolus vulgaris* leaves. Moreover, the increase in membrane permeability under HI stress is in concurrence with that of the results reported by Xu et al. (2010).

H₂O₂, an important ROS, is induced in plants following exposure to a wide variety of abiotic and biotic stress (Karpinski et al., 1999). MDA is the final product of peroxidation of membrane lipid and is an indicator of lipid peroxidation. Hence, the occurrence of MDA in Zn-deficient and Zn-excess cowpea leaves is considered as a useful index of general lipid peroxidation vis-à-vis status of the cowpea leaves under Zn and HI stress (Hodges et al., 1999). Similar to our results an increase in MDA and H₂O₂ content under excess zinc has been reported in *Alternanthera philoxeroides* (Yuan et al., 2009) and under Zn deficiency in rice (Chen et al., 2009). The absorption of excess light can be deleterious since it can potentially result in the production of singlet oxygen and reduced reactive oxygen species such as superoxide and H₂O₂. In our study, a significant increase in lipid peroxidation and H₂O₂ was observed in Zn-deficiency and Zn-excess under Zn stress. Moreover, the increase in MDA and H₂O₂ was drastic and significant under both the conditions when the leaves were subjected to further HI stress, which suggest synergetic action of the two stresses (Behera and Choudhury, 2002) and thus Zn-deficiency and Zn-excess leaves underwent a severe oxidative damage under Zn and HI stress.

Proline accumulation could be used as a physiological test for plant stress response to metal toxicity (Klimashevskii, 1983). In our study, the accumulation of proline was

significantly low under Zn-deficient and Zn-excess conditions than under Zn-sufficient condition. In accordance with our study, Zaifnejad et al. (1997) also reported that proline accumulation did not increase in shoots under increasing level of heavy metal. A significant decrease in the level of proline accumulation in Zn-deficient and Zn-excess cowpea plants in our investigation might be due to both down regulation of proline biosynthetic pathway enzymes and upregulation of proline degrading enzymes. Though the Zn-deficient and Zn-excess leaves too showed a meager increase in the proline accumulation under HI stress and recovery, still they were significantly lower than the Zn-sufficient leaves which shows that the cowpea plant doesn't have the capability to withstand the Zn and HI stress and it is not fully protecting the plants from the imposed oxidative damage.

As A plays a prominent role together with glutathione in scavenging free oxy-radicals (Smith et al., 1989). The reactive free oxygen radicals were assumed to be involved in the oxidation of AsA to dehydroascorbic acid, leading to reduction in the AsA content of plants (Fridovich and Handler, 1961). Sharma et al. (2004) reported a decline in AsA content under Zn deficiency which is similar to our results. Though there was an increase in the AsA content in the Zn-deficient and Zn-excess leaves under HI stress, the increase percent was still lower than that of the Zn-sufficient ones which indicated that the Zn-deficient and Zn-excess plants were under severe oxidative damage which had almost impaired the higher synthesis of AsA.

The lifetime of ROS within the cellular environment is determined by the antioxidant system, which provides crucial protection against oxidative damage. The

antioxidant system comprises numerous compounds of low molecular weights and enzymes (Noctor and Foyer, 1998). The levels of ROS are regulated by their rates of generation, their rate of reaction with target substances, such as proteins, lipids, and/or nucleic acids, their potential rate of degradation and their rate of scavenging/buffering by enzymatic and/or non-enzymatic antioxidants (Hodgs, 2003). Several enzymes are involved in the detoxification of ROS which result under different environmental stresses. SOD is the first defense enzyme which converts superoxide to H_2O_2 that can be scavenged by POX (Bowler et al., 1992).

Zn deficiency initiates production of superoxide radicals during photosynthetic electron transport (Cakmak and Engels, 1999), which, in turn, could give rise to other highly ROS that are even more aggressive to chloroplast constituents (Asada, 1999). SOD, an essential component of the plants' antioxidative defense mechanism, has been reported to increase in Zn deficient wheat (Hacisalihoglu et al., 2003) which is in good agreement with our study. Similar to our results, SOD activity was established to have increased under excess Zn (Wang et al., 2009). But the enhancement of SOD activity in Zn-deficient and Zn-excess leaves under Zn and HI stress alone cannot alleviate the burden of ROS. H_2O_2 is a highly toxic ROS and must be sequestered by the action of POX.

POX protects cells against harmful concentration of hydroperoxides (Sudhakar et al., 2001). In our study, POX activity was significantly low at severe Zn-deficiency and Zn-excess under Zn stress. These may be explained by the fact that the more peroxidative attacks and damage of the radicals brought by the Zn stress exceeded the maximum ability of antioxidant defense

system and diminished the activities of these enzymes (Chen et al., 2009). The proposed physiological roles of PPO in higher plants include pigment formation, molecular oxygen scavenging in the chloroplasts, and participation in the plant defense system (Constabel et al., 1995; Ho, 1999).

In the present investigation, under HI stress, the activities of PPO and POX were higher in Zn-sufficient leaves and it is likely that HI might have induced PPO and POX inactivation in Zn-deficient and Zn-excess ones. However, the drastic decrease in PPO and POX activity in Zn-excess indicated its lower ability to detoxify H₂O₂. The decline in POX activity combined with the rapid increase in SOD activity might have resulted in the rapid conversion of O₂⁻ to H₂O₂, and might have resulted in the rapid increase in cellular H₂O₂. H₂O₂ generated through SOD activity and photorespiration in plant cells (Hernandez et al., 1995) diffuses rapidly across the membranes and is toxic as it acts both as an oxidant as well as a reductant (Foyer et al., 1997).

In our study, a significant rise in the SOD activity under Zn-deficient and Zn-excess conditions occurred which can be considered a circumstantial evidence for enhanced production of free radicals under Zn and HI stress. Thus, the increase in the activities of SOD in Zn-deficient and Zn-excess leaves suggested an increased production of H₂O₂. However, the extent of decrease in PPO and POX activity induced by Zn-deficiency and Zn-excess and a meager increase after HI stress was lower than that of Zn-sufficient condition, which indicated that, at deficient and excess Zn concentrations, these enzymes might not have sufficiently protected the cowpea plants from oxidative damage.

In conclusion, our results demonstrate that Zn-deficiency and Zn-excess conditions mediate severe oxidative damage in the cowpea leaves under Zn and HI stress conditions. The decrease in the root length under Zn stress and decline in the activities of antioxidant enzymes in Zn-excess condition under Zn and HI stress indicates that the cowpea plant doesn't have the ability to withstand or tolerate the excess level of Zn and HI stress imposed on it. Moreover, a significant increase in MDA, H₂O₂, and membrane permeability with a simultaneous decrease in the antioxidant enzyme activities under Zn-deficient when compared to the Zn-sufficient condition shows the inefficiency of the cowpea plant in response to Zn deficiency. Since there is no information on the antioxidant response of cowpea plant under Zn and HI stress, we hope that this study provides a basis for understanding the role of antioxidant enzymes in response to Zn together with HI stress.

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