Antioxidant enzymes and their role in mitigating oxidative stress

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Abstract

Heavy metals are known to cause oxidative damage in plants by producing reactive oxygen species (ROS). Zinc ions are also known to exhibit oxidative stress by triggering the production of free radicals. To counter the harmful effects of ROS, plant cells possess an efficient antioxidant defense system comprising enzymes. Soil experiment was carried out on Sorghum bicolor plants treated with different concentrations of zinc taken as zinc sulphate solution. various antioxidant enzymes were analyzed to understand their role in mitigating oxidative stress caused by ROS under excess zinc. In the present study, the increased activity of SOD under zinc stress indicates better scavenging of superoxide radicals which results in the generation of the reactive intermediate H_2O_2. CAT, which showed decreased activity at latter stages of plant growth, might not have efficiently participated in detoxifying H_2O_2. . A continuous oxidative assault on plants due to zinc stress might have led to generation of enzymatic defenses to counter the phenomenon of oxidative stress.

Keywords: Heavy metal, Reactive oxygen species (ROS), Antioxidants, Enzymes

Introduction

Oxidative stress can be defined as the physiological changes resulting from the formation of excess quantities of reactive oxygen species (ROS) . Although higher plants produce ROS during different metabolic processes in cellular organelles, their rate of production is dramatically elevated under heavy metal stress.

Heavy metals are known to cause oxidative toxicity in plants. They have the ability to produce ROS, resulting in lipid peroxidation, DNA damage, depletion of sulphydryl and altered calcium homeostasis (Maksymiec *et al*., 2008). Several studies suggest that in plants, toxicities associated with heavy metals may be at least in part due to oxidative damage (Shaw and Rout, 1998; Gill and Tuteja, 2010).

Heavy metal induced oxidative damage may be due to two reasons: (1.) As a result of enhancement in production of ROS and (2.) as a result of slowing down or inhibition of the removal / scavenging of ROS (Shaw *et al*., 2004)

In plants heavy metals may enhance production of ROS by interfering with the respiratory and photosynthetic process (Schwartz *et al*., 2003).

Zinc ions are known to exhibit oxidative stress (Madhava rao and Sresty, 2000) by triggering the production of free radicals (Wecks and Clijsters, 1997) and ROS such as H_2O_2, superoxide and hydroxyl radical, formed by the leakage of electrons on to molecular oxygen from the electron transport chain of mitochondria and chloroplast during normal metabolism (Cakmak, 2000). To counter the harmful effects of ROS, plant cells possess an efficient antioxidant defense system comprising enzymes; namely catalase (CAT), guaiacol peroxidase (Gu – POD) and glutathione reductase (GR), ascorbate peroxidase (APx) as well as non – enzymatic antioxidant such as glutathione (GSH), ascorbate (ASA), tocopherol, Carotenoids, proline, phenols and polyamines (Kleckerova *et al*., 2011; Ivanov *et al*., 2011).

Ultrastructural, biochemical, and molecular changes in plant tissues and cells are brought about by the presence of heavy metals (Gamalero *et al*., 2009). There have been extensive studies on the activities of the enzymes involved in ROS defense in plants subjected to heavy metal stress (Sharma and Dietz, 2009). Although different metals can cause oxidative stress, their mode of action is different depending on plant species, plant organ, type of metal, duration of the treatment, plant age, and growing media (Gratao *et al*., 2005).
Zn excess increases lipid peroxidation and membrane permeability diminishing the sulphahydryl content (Tripathi and Gaur, 2004). Oxidative damage is observed as malondialdehyde (MDA) content, which is a product of lipid peroxidation. The level of MDA in tissue is considered as a measure of lipid peroxidation status, or, in other words, the oxidative stress an organism in experiencing (Chakraborty and Pradhan, 2012). ROS induce deleterious effect and damage plasma membrane permeability by triggering strong lipid peroxidation (Ramos, 2002) leading to electrolytic leakage from the cells (Lin YX *et al*., 2022). Increase in lipid peroxidation and electrolytic leakage under zinc stress was reported by a number of investigators (Sinha *et al*., 2013).

The present study is focused on the role of enzymatic antioxidants in mitigating oxidative stress caused by excess Zn in *Sorghum bicolor* plants

MATERIALS AND METHODS

The experimental plant, *Sorghum bicolor* (L.) Moench cultivar CSH 14 belongs to the family Poaceae (graminae). Sorghum is one of the important crops for food security in semi-arid and arid regions of the world due to high nutritional quality. Certified seeds of *Sorghum bicolor* L. Moench cultivar CSH 14 were obtained from the National seed corporation, Hyderabad.
The soil was collected from a local nursery. The soil was air dried and sieved through 2mm sieve to discard the non soil particles. Earthen pots of 20cm diameter and 25cm height were selected for growing the plants. Each pot was filled with 3kg of air dried soil.

Seeds were surface sterilized with 0.001M mercuric chloride. Ten sterilized seeds were sown in each pot. All the pots were watered to field capacity daily. Plants were thinned to a maximum of three seedlings per pot after a week of germination.

The seven day old plants were treated with five different concentrations of zinc solution viz: 1.5, 3.5, 5.5, 7.5 and 9.5mM taken as zinc sulphate. Different concentrations of zinc solution (300 ml) were given once in two days to the field capacity, total ten such doses were given during the experimental period. Plants treated with water served as control. The plants were grown under natural photoperiod. During the growth period plants were regularly monitored for any morphological changes and phytotoxicity symptoms. Each treatment including the control was replicated six times.

Sample collection

The plant samples were collected three time at fifteen days interval approximately viz: 15, 30 and 50 days. The plants were first removed from the soil, the entire plant with roots and shoots were put under constant flow of water to remove the soil particles and exogenous contaminants adhered to the plants. The water droplets were blotted dry with help of blotting paper. Sampling was done in the early hours for the measurement of various morphological, growth and biochemical

parameters. Fresh material was used to estimate lipid peroxidation, electrical conductivity, enzymatic antioxidants, non enzymatic antioxidants and for proline estimation fresh leaf material was used.

Antioxidants

Enzymatic Antioxidants

The antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidases like (guaiacol peroxidase (Gu-POD), Ascorbate peroxidase (APx), Glutathione peroxidase (GPx) and Polyphenol oxidase (PPO) were analysed.

Enzyme Extraction

0.2 grams leaf tissue was homogenized in pre chilled mortar and pestle with 10 ml of 100 mM potassium phosphate buffer (pH 7.0) under ice-cold conditions. The homogenate was centrifuged at 4°C for 20 min at 18,000 rpm and the supernatants were used for determining the activities of SOD, CAT, Gu-POD, APx, GPx, GR and PPO.

Assay of Superoxide dismutase (SOD) EC 1.15.1.1

Assayed by the method of Sen Gupta *et al*., (1993). Superoxide dismutase (SOD), a metal containing enzyme plays a vital role in scavenging superoxide (0_2^-) radical.

$$0_2^- + 2H^+ \longrightarrow H_2O_2$$

SOD activity was estimated by recording the decrease in absorbance of superoxide nitroblue tetrazolium complex by the enzyme. About 3 ml of reaction mixture, containing 0.1ml of 200mM methionine, 0.01 ml of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml of 60 µM riboflavin and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal colour. A non-irradiated complete reaction mixture which did not develop colour served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

Assay of catalase (CAT) EC 1.11.1.6

Catalase activity was assayed by the method of Barber, (1980).

To 0.5 ml enzyme extract 2.0 ml of hydrogen peroxide and 3.5 ml of phosphate buffer (pH 7.0) was added and was incubated for 1 min. The reaction was stopped by adding 10.0 ml of 2% (v/v) concentrated sulphuric acid, and the residual hydrogen peroxide was titrated against 0.01M $KMnO_4$ until a faint purple colour that persisted for at least 15 sec. The activity of the enzyme was expressed as mg of H_2O_2 decomposed min^{-1} gr^{-1} fr. wt. of tissue.

Peroxidases

Plant peroxidases are important in diverse cellular functions such as lignin biosynthesis, hormone generation and detoxification of hydrogen peroxide. Each plant has numerous peroxidase isoenzymes that differ in substrate specificity and localization within the plant.

Guaiacol peroxidase (Gu-POD) EC 1.11.1.7

Guaiacol peroxidase activity was assayed by the method of Putter, (1974). Guaiacol is used as a substrate in the assay of peroxidase.

$$\text{Guaiacol} + H_2O_2 \longrightarrow \text{Oxidised guaiacol} + 2H_2O$$

The resulting oxidized guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the POD activity and can be assayed spectrometrically at 436 nm.

Pipette out 3ml 0.1 M, pH 7 buffer solution, 0.05ml of 20 mM guaiacol solution, 0.1 ml enzyme extract and 0.03 ml 12.3 mM hydrogen peroxide solution in a cuvette (bring the buffer to 25°C before assay). Mix well and place in the spectrophotometer and note the time required in minutes (Δt) to increase the absorbance by 0.1. A water blank is used in the assay. The extinction coefficient of guaiacol dehydrogenation product at 436 nm is 6.39 per micromole.

The POD activity was expressed as the amount of enzyme catalyzing the formation of 1 µmol guaiacol dehydrogenation products (GDHP)/ min /gfw.

Ascorbate peroxidase (APx) EC 1.11.1.11

APx activity was measured according to Yoshimura *et al*., (2000).The reaction mixture contained 25mM phosphate buffer (pH 7), 0.1 mM EDTA, 1 mM H_2O_2, 0.25 mM ascorbic acid (AsA) and the enzyme sample (APx). No change in absorption found in the absence of AsA in the test medium. The rate of ascorbate oxidation was monitored at 290 nm (Extinction coefficient = $2.8 mM^{-1}cm^{-1}$). One unit of enzyme activity was that which catalyzed the oxidation of 1µM ascorbate min^{-1}. Activity expressed as IU/ gfw.

Glutathione peroxidase (GPx) EC 1.11.1.9

Assayed by the method of Rotruck *et al*., (1973). Free radicals are continuously produced in *vivo* under stress and there are a number of protective antioxidant enzymes for dealing with these toxic substances. Glutathione peroxidase is one of the free radical scavenging enzymes, which is reduced progressively due to exposure to heavy metals. Therefore this enzyme level is an indicator of such contamination.

The procedure is based on the reaction between left over glutathione in the following reaction with 5, 5' –dithiobis 2–nitrobenzoic acid (DTNB) to form a compound, which absorbs maximally at 412 nm.

$$2GSH + H_2O_2 \longrightarrow GPx \longrightarrow GSSG + 2H_2O$$

Pipette out 0.4ml of buffer 0.1 ml sodium azide, 0.2 ml of reduced glutathione, 0.5 ml enzyme extract and 0.1 ml H_2O into a test tube and make up the volume to 2 ml with distilled water and incubate the tubes at 37°C for 20 min. Add 0.5ml of 10% TCA to stop the reaction. The contents are centrifuged and to the supernatant 0.3 ml of disodium hydrogen phosphate and 0.1 ml of DTNB reagent are added. The colour developed is read at 412 nm. The blank is set with disodium hydrogen phosphate and 0.1 ml of DTNB reagent. The activity is expressed as µg of glutathione consumed $min^{-1}gr^{-1}$. fr.wt.

Assay of Glutathione Reductase (GR) EC 1.6.4.2

Glutathione reductase was assayed by the procedure adopted by David and Richard, (1983). Glutathione reductase catalyzes the conversion of oxidized glutathione to reduced glutathione employing NADPH as substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

The assay system contained 1ml (0.12 M, pH 7.2) of phosphate buffer, 0.1 ml (15 mM) of EDTA, 0.1 ml (10 mM) of sodium azide, 0.1 ml (6.3 mM) of oxidized glutathione and 0.1 ml of enzyme source and the volume was made up to 2 ml with distilled water. The tubes were incubated for 3 minutes and 0.1 ml (9.6 mM) of NADPH was added. The absorbance was read at 340 nm in a spectrophotometer at every 15 seconds interval for 3 minutes. For each series of measurement, controls were set up that contained water instead of oxidized glutathione. The enzyme activity was expressed as µM of NADPH oxidized/minute/gfw.

Poly phenol oxidase (PPO) EC 1.10.1.3

Assayed by the method proposed by Kar and Mishra, (1976). The same assay mixture for polyphenol oxidase activity consisted of 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 7.0) and 1.0 ml of 0.01 M pyrogallol. After incubating for 5 min at 25°C, the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The absorbance of the purpurogallin formed was taken at 420 nm. Polyphenoloxidase activities were expressed in absorbance units. Assay mixture without pyrogallol served as blank. Units expressed in absorbance units (ΔA at 420 nm).

RESULTS

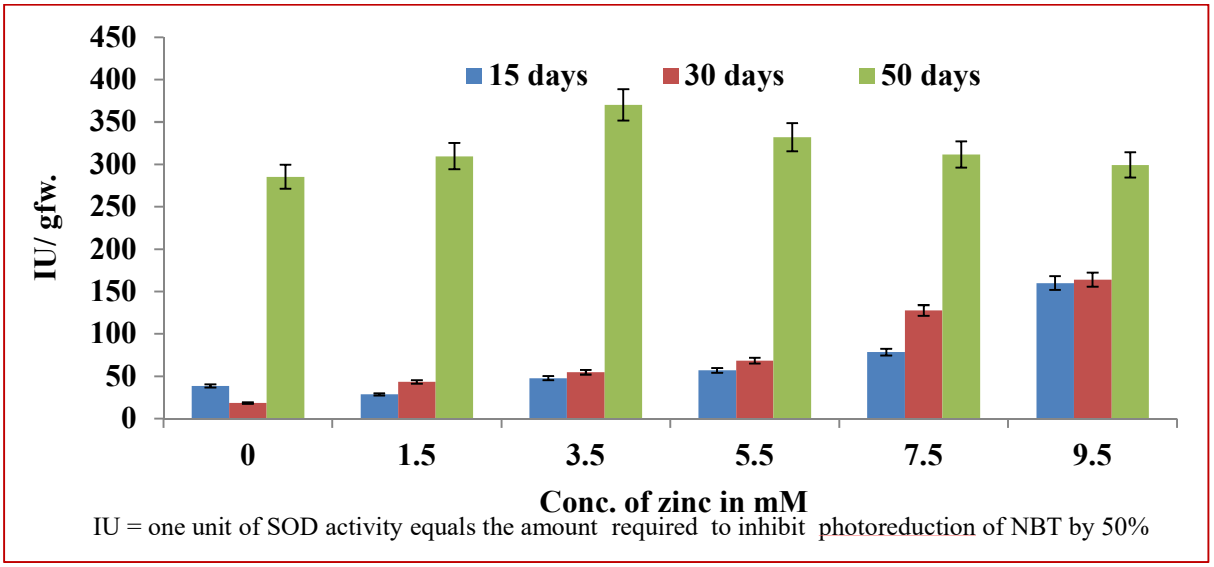
Superoxide dismutase (SOD) EC 1.15.1.1

The effect of zinc on SOD activity in the leaves of *Sorghum bicolor* at different stages of plant growth is shown in Fig. 1.

The application of zinc significantly (at $P < 0.05$) increased the activity of SOD at different stages of growth but not significant ($P < 0.05$) with increase in zinc concentration. SOD plays an important role in detoxification process by catalyzing the conversion of O_2^- and H_2O. In 15 days of the treatment period, the SOD activity increased with increase in zinc treatment. A maximum increase of 316% (4 folds) was recorded at 9.5mM of zinc as compared to control. There was a linear increase in SOD activity with increase in zinc concentration in 30 days old plants. The observed values of SOD activity indicate that at 1.5mM of zinc the percent increase was 136% (2.3 folds) and at 9.5mM the percent increase was 792.8%(8.9 folds) as compared to the control.

In 50 day old plants, the zinc treated plants recorded higher value than the untreated control plants. The SOD activity at 3.5, 5.5 and 7.5 mM of zinc recorded higher values as compared to the control.

Fig.1. Effect of zinc on Superoxide dismutase activity (IU/ gfw) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.

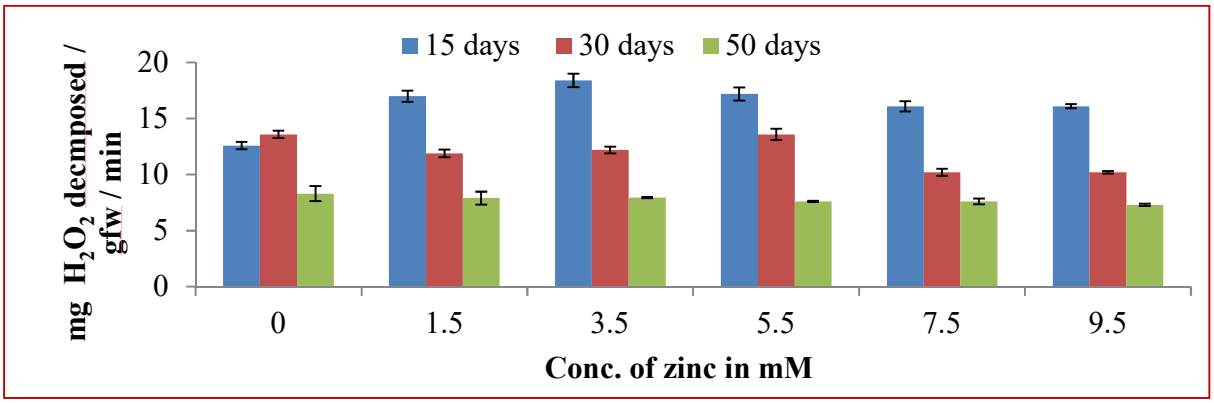


Vertical bars represent ± SE). Sampling days** (** Significant at P < 0.05).

Catalase (CAT) EC 1.11.1.6

The effect of zinc on catalase activity in the leaves of *Sorghum bicolor* is depicted in Fig. 2. The Catalase activity in the leaves of *Sorghum bicolor* was assayed at different stages of plant growth. CAT activity showed a highly significant (P < 0.05) decrease with the age of the plants whereas not significant with increase in zinc treatment. CAT is one of the major antioxidant enzymes that plays very important role in the protection against oxidative damage by breaking down hydrogen peroxide. In 15 day old plants, the CAT activity recorded higher values at all levels of zinc treatment. At 1.5mM the percent increase was 36.5% and at 9.5mM the percent increase was recorded to be 27.7% as compared to the control. In 30 day old plants, a decrease in CAT activity was observed in the zinc treated plants as compared to the control. The percent decrease was 12.5% at 1.5mM of zinc and it decreased to 25% at 9.5mM. Low CAT activity was observed in 50 days old plants. As the zinc concentration increased the CAT activity decreased. At 1.5mM of zinc 4.8% decrease was observed and at 9.5mm the decrease was 12%.

Fig.2. Effect of zinc on Catalase activity (mg H_2O_2 decomposed/ gfw / min) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.



Vertical bars represent ± SE). Sampling days** (** Significant at P < 0.05).

Peroxidases

Guaiacol peroxidase (Gu-POD) EC: 1.11.1.7

The effect of zinc on the activity of Guaiacol peroxidase in the leaves of *Sorghum bicolor* at different stages of plant growth is represented in Fig. 3.

Guaiacol peroxidase activity was assayed at different stages of plant growth in the leaves of *Sorghum bicolor*.

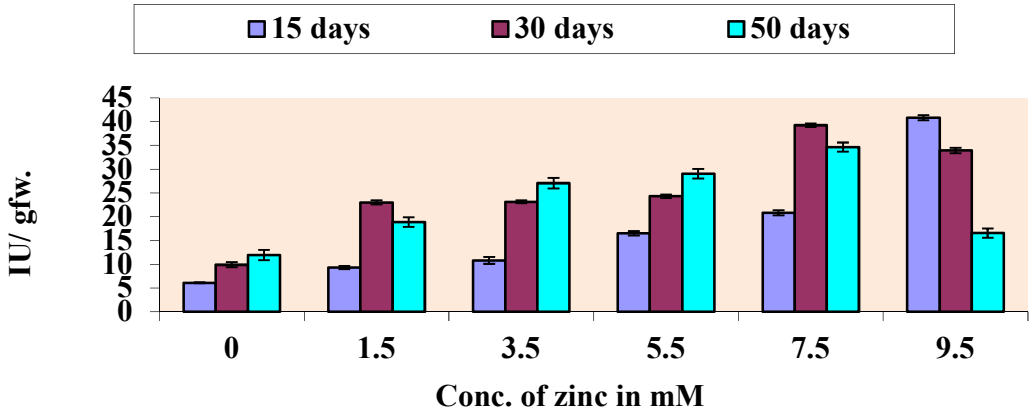
In 15 day old plants Guaiacol peroxidase increased concomitantly with increase in zinc concentration. The percent increase was 1.5 folds at 1.5mM of Zn and it increased to 6.6 folds at 9.5mM of zinc as compared to the control.

In 30 day old plants, gradual increase in the activity of guaiacol peroxidase was noted with increase in zinc treatment as compared to the control. At 1.5mM of zinc a 2.3 fold increase was recorded and at 9.5mM an increase of 3.4 folds was observed.

In 50 days old plants same trend was observed guaiacol peroxidase activity increased with increase in zinc concentration in the soil. The increase was 1.58 folds at 1.5mM and 1.38 folds at 9.5 mM of zinc.

Guaiacol peroxidase activity increased significantly (P < 0.05) with increase in zinc treatment at all stages of plant growth in the leaves of *Sorghum bicolor*.

Fig.3. Effect of zinc on Guaiacol peroxidase activity(IU/ gfw) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.



IU represents the amount of enzyme catalysing the conversion of 1 µm of guaiacol per minute

Vertical bars represent ± SE). Zinc treatment **, Sampling days** (** Significant at P < 0.05).

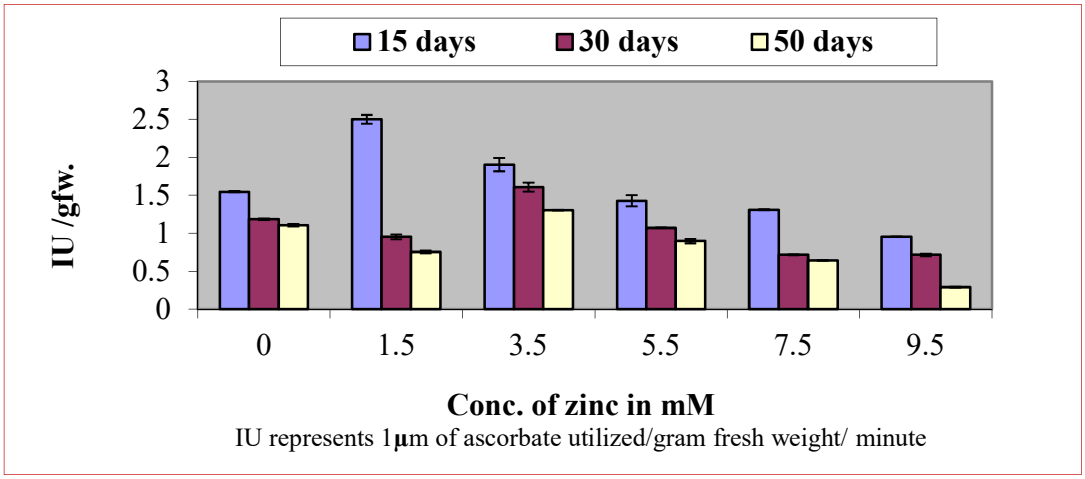
Ascorbate peroxidase (APx) E.C 1.11.1.11

The effect of zinc on the activity of ascorbate peroxidase is represented in Fig.4. Ascorbate peroxidase activity was assayed in the leaves of *Sorghum bicolor* at different stages of plant growth. Ascorbate peroxidase is an antioxidant enzyme that participates in the ascorbate – glutathione cycle and acts in chloroplast and the cytosol. It reduces H_2O_2 by using ascorbate as reducer agent, thus protecting the plants. The activity of ascorbate peroxidase in fifteen day old

plants increased at lower concentration of zinc and decreased at elevated levels of zinc treatment. Maximum increase of 61.6% was recorded at 1.5mM of zinc which declined gradually to 38% at 9.5mM.

In thirty day old plants, the APX activity decreased with increase in zinc concentration as compared to the control. The percent decrease was 19.5% at 1.5mM and 39.6% at 9.5mM of zinc. Maximum activity however was recorded at 3.5mM of zinc where 35.8% increase was observed as compared to the control. In fifty day old plants APX activity remained low with increase in zinc treatment. The decline was 31.9% at 1.5mM and 73.8% at 9.5mM zinc. A slight increase of 17.8% was noted at 3.5mM of zinc as compared to the control plants. Highly significant ($P < 0.05$) decrease was noted in APx activity with increase in zinc treatment and age of the plants.

Fig.4. Effect of zinc on Ascorbate peroxidase activity (IU/gfw/min) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.

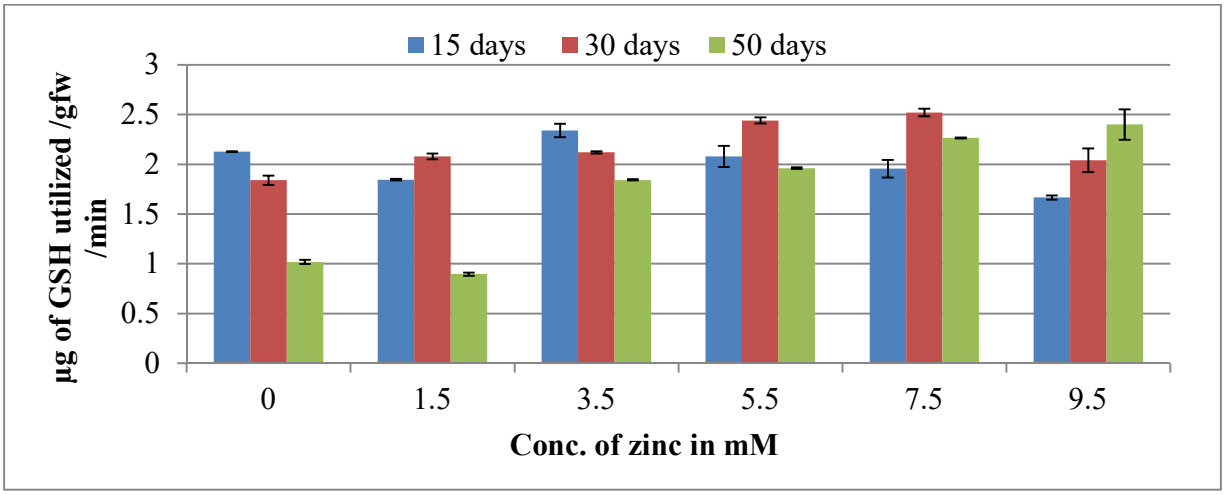


Vertical bars represent ± SE). Zinc treatment **, Sampling days** (** Significant at $P < 0.05$).

Glutathione peroxidase (GPx) E.C. 1.11.1.9

The effect of zinc on the activity of Glutathione peroxidase in the leaves of *Sorghum bicolor* is depicted in Fig. 5. Glutathione peroxidase is a family of isoenzymes seen in cytosol that uses glutathione to reduce H_2O_2 and organic and lipid hydro peroxides, thereby protecting cells against oxidative damage. In fifteen day old plants, the glutathione peroxidase activity decreased as the zinc concentration increased. The percent decrease at 1.5mM was 13.25% and at 9.5mM of zinc it was 21.9%. Glutathione peroxidase activity steadily increased with increase in zinc concentration in thirty day old plants. The percent increase at 1.5mM of zinc was recorded to be 13% and at 9.5mM the percent increase was 10.8%. In fifty day old plants, a steady increase in GPx activity was observed from 3.5mM zinc treatment. The glutathione peroxidase activity showed maximum increase of 135% at 9.5mM. Glutathione peroxidase activity in the leaves of *Sorghum bicolor* showed higher values compared to the control at all treatments in thirty and fifty day old plants but the increase was not significant at $P < 0.05$.

Fig. 5. Effect of zinc on glutathione peroxidase activity (µg of GSH utilized /gfw /min) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.

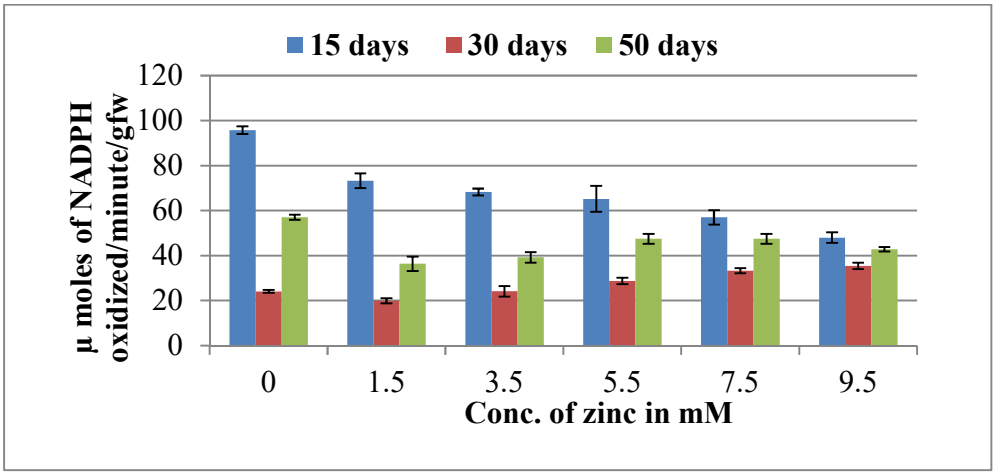


Vertical bars represent ± SE). Zinc treatment ^{ns}, Sampling days ^{ns} (ns - not significant at $P < 0.05$).

Glutathione reductase (GR) E.C. 1.6.4.2

Glutathione reductase is an important component of the cell's scavenging system for reactive oxygen compounds in plants. GSH is a major reservoir of nonprotein reduced sulfur. GSH plays a crucial role in cellular defense, where it gets oxidized to glutathione disulfide (GSSG). GR mediates the reduction of GSSG to GSH by using NADPH as an electron donor, and thus a highly reduced state of GSH/GSSG ratios is maintained at the intracellular level during oxidative stress.
The effect of zinc on Glutathione reductase in the leaves of *Sorghum bicolor* is depicted in Fig.6.
The activity of Glutathione reductase was estimated at different stages of plant growth and the reduction in the activity was highly significant ($P < 0.05$) with the age of the plants.
In fifteen day old plants, the activity of Glutatione reductase reduced with increase in zinc treatment. 23.49% decrease was noted at 1.5mM whereas the decrease was maximum (49.86%) at 9.5 mM of Zn.
In thirty day old plants a linear increase in the activity was noted from 3.5mM of soil zinc treatment. Increase of 46.88% was noted at 9.5mM of Zn.
In fifty day old plants decrease in the activity of Glutathione reductase was noted. At 1.5mM a reduction of 36.28% reduction was noted and at elevated levels of soil applied zinc the activity of the enzyme remained almost same with a reduction of 25% at 9.5mM of Zn.

Fig.6. Effect of zinc on Glutathione reductase activity (µ moles of NADPH oxidized/minute/gr.fr.wt.) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.



Vertical bars represent ± SE). Zinc treatment ^{ns}, Sampling days**
(** Significant, ns – not significant at P < 0.05).

Polyphenol oxidase (PPO) E.C. 1.10.3.1

Plant polyphenol oxidases are enzymes that typically use molecular oxygen to oxidize ortho-diphenols to ortho-quinones. These commonly cause browning reactions following tissue damage, and are important in plant defense. The data is represented in Fig. 7.

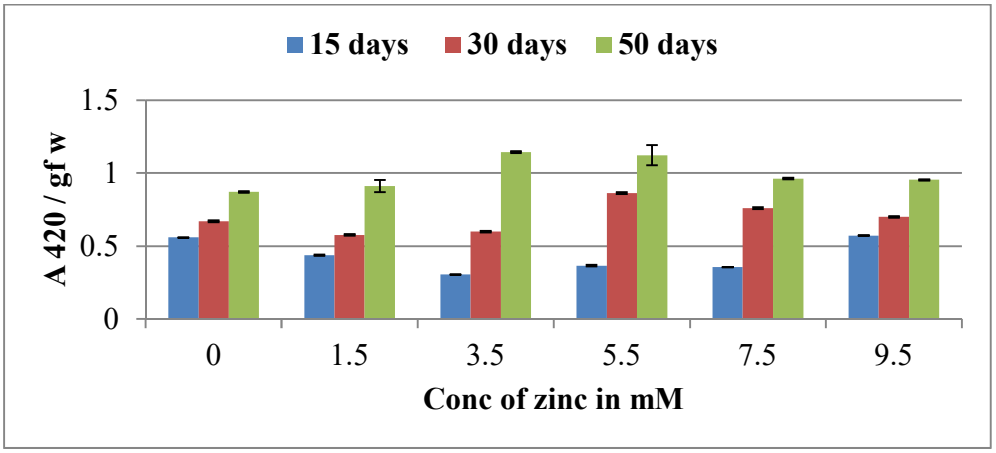
The activity of polyphenol oxidase was analysed at different stages of plant growth. Increase in the activity of polyphenol oxidase was highly significant (P < 0.05) with the age of the plants.

In fifteen day old plants, the activity of PPO decreased with increase in zinc concentration up to 7.5mM of zinc but recorded higher values at 9.5mM zinc as compared to control. The percent increase was 2.5% at 9.5mM zinc.

In thirty days old plants, increase in PPO activity was observed at higher levels of zinc treatment (from 5.5mM onwards).

In fifty day old plants, Increase in PPO activity was observed with increase in Zn treatment but higher values were recorded at 3.5mM and 5.5mM of zinc.

Fig. 7. Effect of zinc on Polyphenol oxidase activity (ΔA $_{420}$/ gfw) in the leaves of *Sorghum bicolor* (L.) Moench (CSH 14) at different stages of plant growth.



Vertical bars represent ± SE). Zinc treatment ^{ns}, Sampling days** (** Significant, ns – not significant at $P < 0.05$).

Discussion

Plants are known to develop different strategies to cope with heavy metal stress. Some use an avoidance strategy to reduce trace element assimilation while others use internal defense mechanisms to cope with the increasing levels of the toxic species and to allow them to grow despite the presence of variable concentrations of trace elements, but the threshold concentrations as well as the different response mechanisms strongly depend on plant species and on the type of metal. Metal toxicity can cause a redox imbalance and induce the increase of ROS concentration, activating the antioxidant defense mechanisms of plants (Jomova *et al*., 2023 2009). Involvement of the ascorbate-glutathione cycle enzymes is a major antioxidative defense mechanism, apart from other antioxidant enzymes like catalase, peroxidases and superoxide dismutase. Plants must adapt or perish to the conditions where they grow, the presence of heavy metals can induce oxidative stress and the activation of several defense factors in the plants (Maksymiec, 2007). It is important to understand how some plants can cope with high concentration of metals in order to grow crops on contaminated soils (Schroder *et al*., 2008), to help in environmental cleanup via phytoremediation and to breed plants with higher contents of essential nutrients. Tolerance to heavy metals in plants may be defined as the ability to survive in a soil that is toxic to other plants, and is manifested by an interaction between a genotype and its environment (Macnair *et al*., 2000).

Zn toxicity occurs in soils contaminated by mining and smelting activities, in agricultural soils treated with sewage sludge, and in urban and peri-urban soils enriched by anthropogenic inputs of Zn, especially in low-pH soils.

Enzymatic antioxidants

Free radical generation is one of the initial responses of plants to stress. Generation of free radicals and reactive oxygen species is stimulated in the presence of metals and this can seriously disrupt normal metabolism through oxidative damage of cellular components. To mitigate and repair the damage initiated by active oxygen, in plants complex antioxidant system is triggered. These antioxidants play an important role in the cellular defense strategy against oxidative stress by protecting labile macromolecules .

Plants have a range of potential mechanisms at the cellular level which might be involved in the detoxification of heavy metals and thus tolerance to heavy metal stress (Hall, 2002).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is an essential component of plant antioxidative system that can be used as biomarker of environmental stress (Dazy *et al*., 2009). SOD is the first enzyme in ROS detoxifying process that converts O_2^- to H_2O_2 in cytosol, chloroplast and mitochondria and plays a crucial role in cellular defense mechanisms against the risk of OH^- formation (Gratao *et al*., 2005).

In the present study increase in SOD activity in all treatments at different stages of plant growth indicate high accumulation of ROS (H_2O_2) under zinc stress. The enhanced activities of SOD in zinc treated pigeon pea plants can be regarded as a consequence of metal induced oxidative stress (Madhava Rao and Sresty, 2000) it may be presumed that most of the oxidative stress result from decreased catalase activity. Increase in SOD activity induced by excess zinc in maize could be attributed to an increase of Cu/Zn SOD activity. Increase in SOD activity in rice seedlings under cadmium and lead stress may be due to superoxide radical accumulation, de-novo synthesis of

enzymatic protein under heavy metal stress (Shah *et al*., 2001;Verma and Dubey, 2003), in wheat seedling under lead stress (Dey *et al*., 2007).

Catalase activity (CAT)

Catalases are tetrameric heme containing enzymes with the potential to directly dismutate H_2O_2 in to H_2O and O_2 and are indispensable for ROS detoxification during stressed conditions. Increase in SOD activity leads to H_2O_2 generation which is detoxified by the enzyme CAT or peroxidase (POD) to maintain the cellular redox state. These pathways help in the ROS scavenging. CAT is one of the most important component of plant protective mechanism that exist in mitochondria and peroxisomes (Gupta *et al*., 2009) and has important role in scavenging free radicals specially H_2O_2 generated during stress condition Catalase catalyses H_2O_2 to H_2O and O_2 via two-electron transfer (Wang *et al*., 2008) and prevents the generation of OH^- and protect proteins, nucleic acids and lipids against ROS (Imlay and Linn, 1988). CAT activity increased in 15 day old plants in all treatments suggesting its role in detoxification of H_2O_2 produced under stress. Increased activity of CAT under excess zinc treatment was reported in maize by Cui and Zhao (2011). A decrease in CAT activity was observed in 30 and 50 day old plants. Decrease in CAT activity may be due to long term exposure to high concentration of metal. Zinc inhibited CAT activity in bean under long term exposure (Wink *et al*., 1993). Andrade *et al*., (2009) reported a decrease in CAT activity in jack bean grown in soil treated with excess zinc. Decrease in CAT activity might have resulted in increase in lipid peroxidation because of decrease in H_2O_2 detoxification . Heavy metal induced decrease in CAT activity could be attributed to inhibition of synthesis of catalase defense mechanism against zinc induced oxidative damage as suggested by Wecks and Clijsters, (1997) in the primary leaves of bean or changes in the assembly of enzyme subunits (Radic *et al*., 2010).

The variable response of CAT activity has been observed under metal stress. Its activity declined in Glycine max (Balestrasse *et al.,* 2005), *Phragmites australis* (Iannelli *et al.,* 2002), Capsicum annum (Leon *et al.,* 2002) and *Arabdiopsis thaliana* (Cho and Seo, 2005). Whereas, the activity increased in *Oryza sativa* (Hsu and Kao, 2004), *Brassica juncea* (Mobin and Khan, 2007), *Triticum aestivum* (Khan *et al*., 2007), *Cicer arietinum* (Hasan *et al*., 2008) and *Vigna mungo* roots (Singh *et al*., 2008) under Cd stress.

Peroxidases

Peroxidases, a class of enzymes in animals, plants and micro organisms tissues, catalaze oxidoreduction between hydrogen peroxide and various reductants. There are three classes of plant peroxidases, but ascorbate peroxidase (APX) and class III plant peroxidase (Gu POD and GPX) are considered to be the most important. Plant peroxidases are important part of antioxidative system (Sharma *et al*., 2014).

Guaiacol peroxidase (Gu POD)

Guaiacol peroxidase is an important form of peroxidases which oxidize guaiacol (O-methoxy-phenol) as a commonly used reducing substrate. They are found in cellular cytoplasm and apoplasm fractions and involved in a range of processes related to plant growth and development. The activity of guaiacol peroxidase in zinc treated *Sorghum bicolor* plants progressively increased with increasing zinc concentrations as compared to control at all stages of plant growth. Increase in guaiacol peroxidase might be possibly due to increased release of peroxidases localized in the cell walls as suggested by Verma and Dubey, (2003). An increase in guaiacol peroxidase activity was reported in the roots and shoots of two rice cultivars under Pb treatment (Verma and Dubey, 2003). It has been demonstrated that oxidative stress as measured by induced changes in guaiacol peroxidase activity is due to increased quantity of free radicals and reactive oxygen forms (Hippeli and Elstner, 1996).

Increase in peroxidase activity under metal polluted soils has been reported by several investigators (Cui and Zhoa, 2011; Morsy *et al*., 2012).

Ascorbate peroxide (APx)

Ascorbate peroxidase specifically uses ascorbic acid as a physiological reductant (Asada, 1992) and is considered a crucial component in the metabolic defense against oxidative stress. It acts by a series of coupled redox reactions, both in photosynthetic (Asada, 1994) and non- photosynthetic tissue (Arrigoni, 1994). APx is the most important peroxidase in H_2O_2 detoxification operating in cytosol, chloroplast, peroxisomes and glyoxisomes (Mittova *et al*., 2000; Shigeoka *et al*., 2002).

In the zinc treated *Sorghum* plants the APx activity in the leaves proportionally decreased with increase in zinc treatment on all sampling days suggesting a possible inactivation of the enzyme (Iturbe-ormaetxe *et al*., 1998), or could be ascribed to inhibition of enzyme biosynthesis and the denaturation of enzyme protein (Mohapatra, 1995). Diminished activity of APx as a result of metal toxicity was also observed in *Atriplex hortensis* and *Atriplex rosea* by Kachout *et al*., (2010).

A number of studies confirm the increase in antioxidant activity in the course of heavy metal. In treatment above certain concentration of the heavy metal the antioxidant enzymes were found to be inhibited (Stiborova *et al*., 2004). A stressed plant (Jing *et al*., 2003) is usually accompanied by a decrease in APx and glutathione reductase activities in parallel with an increase in lipid peroxidation.

Glutathione peroxidase

GPx is the key enzyme of the antioxidant network in plants, present in different subcellular organelles, their main activity is to catalyze the reduction of H_2O_2, organic hydroperoxides (ROOHS), and lipid hydroperoxides to H_2O and alcohol using GSH (reduced glutathione) and other reducing equivalents. GPx role in reponse to heavy metal is diverse (Hossain *et al*., 2011).

In the present investigation GPx activity showed a decrease in 15 day old *Sorghum bicolor* plants. Similar decrease in GPx activity in roots of pea plant under Cd stress was reported by Dixit *et al*., (2001) and in the leaves of pea plant under Cu stress by Edward, (1996). Reduction in GPx activity was also observed in water hyacinth under Cd stress (Vestena *et al*., 2011) and in *Sorghum bicolor* under Cr VI stress by Malmir, (2011).

A marked increase in GPx activity with increase in zinc treatment was observed in 30 and 50 day old plants. An increase in GPx activity prevents plants from toxic effects of H_2O_2. The hyperactivity of antioxidant enzyme GPx might be a strategy adapted by plants for its survival under heavy metal stress as suggested by Ali *et al*., (2003) and peroxidase activity can be used as a potential biomarker for heavy metal toxicity as shown by Cuypers *et al*., (2002).

Similar activity in GPx activity was observed in *Allium cepa* exposed to a variety of heavy metals (Hg, Pb, Cr, Cu, Zn and Cd) suggesting that elevated activity of GPx was the result of heavy metal induced free radical generations (Fatima and Ahmed, 2005) or might be an indication of protection against lipid peroxidation caused by heavy metal exposure (Semane *et al*., 2007).

Rastgoo and Alemzadeh, (2011) have also shown an increase in GPx activity in Cd treated *Aeluropus littoralis* plants. Van Assche and Clijster, (1990) suggested increase in GPx activity might be a result of increase in de novo protein synthesis or the activation of enzymes already present in plant cells to diminish the deleterious effect of ROS.

A significant increase in GPx activity in the leaf tissues of different plant species exposed to Se and Cu under both laboratory and field conditions Hartikainen *et al*., (2000) and in three plant species under copper stress Boojar and Goodarzi,(2000) and in mung bean seedling under Cd stress Hossain *et al*., (2011).

Glutathioine reductase

Glutathione reductase (GR) is a flavor-protein oxidoreductase which catalyses the reduction of glutathione disulphide (GSSG) to sulphydryl forms GSH. This enzyme employs NADPH as a reductant. GR is predominantly found in chloroplasts and a small amount is also found in mitochondria, cytosol and peroxisomes (Edwards *et al*., 1990). GR maintains a high GSH/GSSG ratio in cells (Alscher, 1989). It forms a salient part of ROS scavenging system along with SOD and the enzymes of ascorbate – glutathione cycle and involved in detoxification of H_2O_2 in the chloroplast and mitochondria. The reduced activity of GR with increase in soil applied zinc in the leaves of *Sorghum bicolor* in fifteen and fifty day old plants was noted. The observed inhibition of GR in *Sorghum bicolor* plants is correlated with a decrease in GSH concentration, lowering the amount of substrate available for GPx. Myrene and Devaraj, (2012) reported decline in GR activity in the roots with time in hyacinth bean under Zn stress. Heavy metal induced loss in GR activity has been observed in pea under Zn, Cu, and Fe (Bielawski and joy, 1986), in *Lemna minor* under Cu (Razinger *et al*., 2007). A heavy metal stressed plant (Jing *et al*., 2003) is usually accompanied by a decrease in APX and GR activities in parallel with an increase of lipid peroxidation

Polyphenol oxidase (PPO)

PPO is an oxidoreductase that catalyzes the oxidation of phenols to quinones and its activity has been shown to increase under heavy metal stress and has thus been associated to some form of defence mechanism (Martins and Mourato, 2006). Kovacik *et al*. (2009) observed an increase in root PPO activity with Cu and Cd and concluded that the formation of polymerized phenols could be used to complex free metal ions. On the other hand PPO has also been associated to a catalase-like activity (Gerdemann *et al*., 2001), and could thus have a role in direct H_2O_2 removal. The PPO activity in the leaves of *Sorghum bicolor* plants increased with increase in externally supplied zinc at all stages of plant growth. The results indicate that changes in PPO activity might be due to its participation in the defense mechanism against zinc toxicity.

The induction of PPO activity might be due to its role in phenolic compound synthesis, which plays an important role in detoxification of heavy metals in plants (Ruiz *et al*., 1999). PPO activity showed a significant increase under heavy metal stress as compared to control in *Matricaria chamomilla* (Kovacik and klejdus, 2008), in *Arabidopsis thaliana* (Saffar *et al*., 2009).

Conclusion

In the present study the increased activity of SOD under zinc stress indicates better scavenging of superoxide radicals which results in the generation of the reactive intermediate H_2O_2. CAT, which showed decreased activity at latter stages of plant growth, might not have efficiently participated in detoxifying H_2O_2. This accumulation of reactive intermediate H_2O_2, triggered the activity of other enzymatic and non enzymatic antioxidants as evident by the increased activity of Gu-POD, GPx in the leaves of *Sorghum bicolor.* They promoted detoxification of H_2O_2 and could protect the plant against oxidative damage induced by ROS. A continuous oxidative assault on plants during zinc stress might have led to generation of enzymatic defenses to counter the phenomenon of oxidative stress. These processes are strategies employed by plants, at least in part, to face the unavoidable stressful conditions.

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