

Antioxidant Modulation in Response to Zinc Induced Oxidative Stress at Different pH in *Glycine max* L. cv. Merrill

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Abstract: Oxidative damage and antioxidant properties have been studied in shoot and root cells of hydroponically grown *Glycine max* L. subjected to short term exposure of Zn (0, 500, 1000 and 2000 μ M) as $ZnSO_4 \cdot 7H_2O$ along with different pH (3.0, 6.0 and 8.0). An increased level of superoxide anion radical ($O_2^{\cdot -}$) and H_2O_2 evidenced oxidative stress conditions in seedlings. Zn-treated seedlings showed elevated levels of lipid peroxides with a concomitant increase in the activities of the antioxidative enzymes viz. ascorbate peroxidase (APX), guaiacol peroxidase (GPOD), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) at different given pH values. Though Zn was readily absorbed by growing seedlings, its localization was greater in roots than shoots. Seedlings grown for 3-14 days in presence of 2000 μ M $ZnSO_4 \cdot 7H_2O$ showed 65.94% to 84.52% increase in the level of malondialdehyde (MDA) in shoots and roots, respectively indicating enhanced lipid peroxidation compared to control at maximum pH. Under controls as well as Zn treated experimental tissues maintained higher activities of enzymes at pH 8.0. Results suggest that Zn induced oxidative stress in seedlings and peroxidases, SOD, CAT and GR could serve as important components of antioxidative defense mechanism were highly influenced by different pH levels.

Key words: Catalase % Glutathione reductase % Lipid peroxides % Peroxidase % Superoxide dismutase % Zinc

INTRODUCTION

Plants respond to heavy metal ion stress in different ways including exclusion, chelation, compartmentalization and expression of stress protein genes. Zn is essential for cell physiological processes and in most living organisms it is the second most abundant transition metal after Fe. Zn has no redox activity but plays structural and catalytic roles in many processes and is the only metal present in all enzyme classes. The mechanisms controlling Zn homeostasis in plants are still not fully known [1-3]. However, like other heavy metals when Zn is accumulated in excess in plant tissues, it causes alterations in vital growth processes such as photosynthesis and chlorophyll biosynthesis and membrane integrity [4].

In particular, Zn has been reported to have a negative effect on mineral nutrition and enzyme activities related to oxidative metabolism [5]. The increased production of toxic oxygen derivatives can be caused by Zn stress situations [6]. These highly cytotoxic species of oxygen can seriously disrupt normal metabolism through

oxidative damage to cellular components. One of the most damaging effects of these molecular species and their products in cells is the peroxidation of membrane lipids [7]. An excess of redox active metals, for example, Cu and Fe, as well as metal ions unable to perform univalent oxidoreduction reactions, such as Zn, induces oxidative damage and are also capable of affecting lipid peroxidation and antioxidative protection.

To combat the oxidative damage plants have the antioxidant defense system comprising of enzymes catalase (EC 1.11.1.6), peroxidases (EC 1.11.1.7), superoxide dismutases (EC 1.15.1.1) and the nonenzymic constituents α -tocopherol, ascorbate and reduced glutathione which remove, neutralize and scavenge the ROS [8]. The enzymes of Halliwell-Asada pathway or ascorbate-glutathione cycle such as ascorbate peroxidase (EC 1.11.1.11), monodehydro ascorbate reductase (MDAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) also play a significant role in scavenging H_2O_2 mainly in chloroplasts and in maintaining the redox status

of the cell [9]. The possible interference to Zn contaminated soils with Zn phytoextraction should be the first step of soil remediation followed by rising soil pH to minimize any residual phytotoxic effect of Zn to *Glycine max* L.

The objective of the study was to assess the effect of pH on the Zn solubility and uptake by *G. max* from Hoagland nutrient solution. The pH values were adjusted to cover a broad pH range from acid to alkaline. The objective of present study is also directed to investigate metal uptake and translocation, O_2^- and H_2O_2 production and response of some antioxidant enzymes such as SOD, CAT, APX and GR are involved in scavenging active oxygen species.

MATERIALS AND METHODS

Plant Material and Growth Conditions: Seeds of *Glycine max* L. cv. Merrill (Soybean) were sterilized with 1% NaClO for 5 min, then washed twice with distilled water and germinated for 4 days in the dark in petri dishes. Four-days old seedlings were grown hydroponically on 1/4 strength modified Hoagland nutrient solution [10]. Seedlings were grown at 24°C, with a light intensity of $300 \mu M.m^{-2}.sG^1$ and a 14-h photoperiod. After growing for 10 days (four true leaves), they were treated with 0, 500, 1000 and 2000 $\mu M Zn^{2+}$ as $ZnSO_4.7H_2O$ for 5 days. The pH was adjusted 3.0, 6.0 and 8.0 for both culture and treatment solution and renewed everyday. The shoots and roots of seedlings were collected and washed in 10 mM $CaCl_2$ to remove Zn accumulated on their surface and stored in an -80°C freezer.

Determination of Superoxide Anion Radical (O_2^-)

Production Rate: Shoots and roots were placed in a test tube and poured over with a solution containing 0.05 M PBS (pH 7.8), 0.05% nitroblue tetrazolium (NBT) and 10 mM NaN_3 . After 5 min incubation in the dark, 2 ml of the solution was taken up from the tubes and heated at 85°C for 15 min. Then, the samples were cooled and absorbance was measured at 580 nm [11].

H_2O_2 determination: The content of H_2O_2 was measured according to the modified method of Patterson [12]. Harvested tissues were ground in 6 ml ice-cold acetone. The homogenate was centrifuged at $8,000 \times g$ at 4°C for 30 min. The supernatant was collected. 0.5 ml of supernatant was mixed with 1.5 ml of mixture of $CHCl_3$ and

CCl_4 (1:3, v:v). Then, 2.5 ml of distilled water was added. The mixture was centrifuged at $1,000 \times g$ for 1 min and the water phase was collected for H_2O_2 determination. To set controls, 0.1 ml of CAT (0.3 unit) was added to the 1 ml of supernatant to remove the H_2O_2 . For the treatments, CAT solution was replaced by 0.1 ml MQ (QTUM 0001 X; Millipore Co.) water. The mixtures were incubated at 37°C for 10 min. 1 ml of phosphate buffer solution (0.2 M, pH 7.8) and 1 ml of 4-(2-pyridylazo) resorcinol (200 mM) solution were added to samples. The reaction mixtures were incubated at 45 °C for 20 min and the absorbance at 508 nm was read.

Determination of Lipid Peroxidation: Lipid peroxidation was measured as described by Hedges *et al.* [13]. Approximately 0.5 gram plant tissues was homogenized in 80% ethanol and centrifuged at $3000 \times g$. Afterwards, the extract obtained was analysed in two steps. At the first step, 1 volume of 20% (w/v) (TCA) and 1 volume of 0.01% BHT were added to 1 volume of supernatant. At the second step, 1 volume of 20% TCA that contained 1 volume of 0.65% TBA and 1 volume 0.01% BHT were added to 1 volume extract taken from the supernatant. After vortexing the sample for 10 sec, they were incubated in a hot water bath adjusted to 95°C for 25 min followed immediately by a shock treatment in an ice bath. The cooled samples were centrifuged at $3000 \times g$ and absorbance values of supernatants were measured in spectrophotometer. First step samples were measured at 532 and 600 nm, whereas second samples at 440, 532 and 600 nm.

Results were obtained using the following formulas (Ab: absorbance, MDA: malondialdehyde)

$$\begin{aligned} & [(Ab_{532} + TBA) - (Ab_{600} + TBA) - (Ab_{532} - TBA) - (Ab_{600} - TBA)] = A \\ & [(Ab_{440} + TBA - Ab_{600} + TBA) \times 0.0571] = B \\ & \text{nmol MDA/ml} = (A-B/157000) \times 10^6 \end{aligned}$$

Assays of Antioxidative Enzymes: Approximately 0.5 gram fresh samples were homogenized in 50 mM PBS (pH 7.6) including 0.1 mM Na-EDTA. Samples were generally homogenized in 8 ml and then centrifuge for 15 min at $20,000 \times g$ and 4°C.

Ascorbate Peroxidase (APX): Activity of APX was measured according to Cakmak [14] by monitoring the rate of H_2O_2 -dependent oxidation of AsA at 290 nm. The reaction mixture (1 ml) contained 50 mM PBS (pH 7.6), 0.1 mM EDTA, 12 mM H_2O_2 , 0.25 mM AsA and enzyme aliquot.

Guaiacol peroxidase (GPOD): Activity of GPOD was measured according to Hammerschmidt *et al.* [15]. To assay the activity of GPOD 3 ml of guaiacol solution, 3 ml of PBS, 0.4 ml of reaction mixture and 0.1 ml H₂O₂ solution was added and assayed spectrophotometrically at 436 nm. The time (Ät) was recorded in which the reading increased by 0.1.

Catalase (CAT): Catalase (CAT) activity was measured as described by Cakmak and Marschner [16]. The assay was based on the degradation of H₂O₂ at 240 nm and the reaction medium (1.0 ml) contains 50 mM PBS (pH 7.6), 0.1 mM EDTA, 0.1 ml 100 mM H₂O₂ and the enzyme aliquot. The decrease in H₂O₂ was monitored at 240 nm.

Superoxide dismutase (SOD): The superoxide dismutase (SOD) activity was estimated by recording the decrease in optical density of formazone made by O₂^{•-} and nitroblue tetrazolium chloride (NBT) dye by the enzyme [16]. For the assay of SOD, the reaction medium (5.0 ml) containing 50 mM phosphate buffer, pH 7.6, 0.1 mM Na-EDTA, enzyme aliquots (50-150 µM), 50 mM Na₂CO₃ (pH 10.2), 12 mM L-methionine, 75 µM NBT and 2 µM riboflavin was maintained in glass vials. Riboflavin was the last compound to be added. Reactions were carried out at room temperature and under a light intensity of about 400 µM m⁻² sG¹. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction measured at 560 nm.

Glutathione reductase (GR): Glutathione reductase (GR) was assayed using the method described by Cakmak and Marschner [16] and Cakmak [14] by following the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction mixture (1 ml) contained 50 mM PBS (pH 7.6), 0.1 mM EDTA, 0.5 mM GSSG, 0.12 mM NADPH (0.1 ml) and the enzyme aliquot.

Measurement of Zinc accumulation: Roots and shoots of intact plants were thoroughly rinsed with deionized water and blotted dry. Samples were dried at 70°C in a forced-air oven, weighted and digested with 1:1 nitric to perchloric acid. Zn was determined by atomic absorption spectrometry [17].

RESULTS

The obtained results revealed that the O₂^{•-} production in shoots was concentration dependent to

heavy metals and different pH levels. At pH 3.0 in shoot cells of *G. max* the radical produced two fold at 1000 µM and approx five fold at 2000 µM concentration with the recorded values 29.054 and 64.541 mol minG¹ per mg protein FW as compared to 500 µM concentration to Zn²⁺. At the physiological pH the O₂^{•-} produced not insignificantly different manner but at pH 3.0 it represented remarkably percent difference (Table 1). In root cells of *G. max* the pH difference possesses similar impact on radical production rate at all metal concentrations except pH 8.0.

H₂O₂ was measured to determine whether application of excessive Zn²⁺ caused oxidative stress in shoots and roots of *G. max*. In metal concentrations and pH dependent manner experiment, the H₂O₂ content increased markedly during the 2000 µM exposure. This stimulation reached at peak on pH 8.0. The dose response changes in H₂O₂ levels also examined during the course of study and observed that at 500 to 2000 µM significantly increased the production of H₂O₂ compared with over control (Table 1). These results suggested a close relation between the GSH content and the H₂O₂ content in the roots of *G. max*. The results also imply that H₂O₂ may not have toxic effect, but acts as a positive signal for the hyperaccumulation of Zn²⁺ in *G. max*.

Lipid peroxidation is a free radical mediated process. The level of lipid peroxidation was measured in terms of MDA (malondialdehyde) concentration. The striking increase in lipid peroxidation in Zn treated seedlings of *G. max* demonstrated in shoot and root cells at various pH levels. The observed value at pH 8.0 and 2000 µM concentration for Zn²⁺ was 85.323 and 174.901 n mol gG¹ FW in shoot and root cells of *G. max* (Table 2). The percent increment were to be found two or three fold maximum in all tissues at pH 3.0, 6.0 and 8.0 with 2000 µM concentration as compared to 1000 µM with the respect of 500 µM metal supply.

The effect of Zn²⁺ stress on specific activity of antioxidant enzyme APX in shoot and root cells of *G. max* at pH 3.0, 6.0 and 8.0 is depicted in Table 2. The stress led to a continuous increase in the activity of APX but the highest results were reported at 2000 µM concentration in seedlings. The increase specific activity of APX also measured in percent numeric, in shoot cells of *G. max* activity of APX as compared to 500 µM were recorded approximately two and three fold increment at 1000 and 2000 µM with the respect of control. In root cells of seedlings APX activity was followed at same aspect but in remarkable concern than shoot cells. The results showed that with increasing metal contamination the rate of H₂O₂ degradation was also enhanced and peak values recorded at 2000 µM with pH 8.0.

Table 1: Effect of different concentrations of Zn on endogenous levels of superoxide anion radicals (O₂⁻) and hydrogen peroxide (H₂O₂) at various pH levels in shoot and root cells of *Glycine max* L

Treatments (μM)		O ₂ ⁻ (MminG ¹ mgG ¹ protein)			H ₂ O ₂ (μM gG ¹)		
		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
In shoots							
Control		8.114±0.35	11.250±0.70	6.549±1.03	0.143±0.01	0.151±0.01	0.158±0.01
Zn	500	14.116±1.05	19.546±1.25	30.395±2.510	0.267±0.02	0.289±0.02	0.299±0.03
		(+26.99)	(+26.93)	(+29.49)	(+31)	(+31.36)	(+30.85)
	1000	29.054±1.89	45.317±2.35	78.256±4.35	0.447±0.03	0.521±0.03	0.571±0.03
		(+56.33)	(+60.22)	(+65.08)	(+51.52)	(+55.05)	(+56.65)
	2000	64.541±4.03	72.090±5.04	131.153±8.59	0.324±0.03	0.597±0.04	0.599±0.04
		(+77.66)	(+73)	(+77.59)	(+38.75)	(+58.23)	(+57.90)
In roots							
Control		7.115±0.25	9.512±0.34	10.532±0.75	0.124±0.01	0.128±0.01	0.132±0.02
Zn	500	14.118±0.35	19.032±1.49	30.942±1.94	0.135±0.03	0.139±0.02	0.142±0.03
		(+32.98)	(+33.35)	(+49.21)	(+4.24)	(+4.11)	(+3.64)
	1000	32.896±1.04	50.359±2.56	52.452±2.84	0.165±0.04	0.174±0.05	0.178±0.04
		(+64.43)	(+68.22)	(+66.55)	(+14.18)	(+15.23)	(+14.83)
	2000	64.356±2.56	83.452±3.04	125.620±5.94	0.235±0.07	0.256±0.08	0.284±0.66
		(+80.08)	(+79.53)	(+84.52)	(+30.91)	(+33.33)	(+36.53)

Values are means ± SE (n=5). Means within the column with the same letter are not significantly different (p<0.05). Values in parenthesis were expressed as percentage of the respective parameters over control.

Table 2: Lipid peroxidation (LP) and specific activity of ascorbate peroxidase (APX) at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn

Treatments (μM)		LP (nM gG ¹)			APX (μM minG ¹ mgG ¹ protein)		
		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
In shoots							
Control		11.821±2.26	15.551±1.07	17.510±2.53	190.538±7.18	195.345±6.34	200.347±4.34
Zn	500	20.082±0.16	29.412±1.89	36.310±2.94	240.331±4.34	249.006±9.44	264.014±7.94
		(+25.89)	(+30.82)	(+24.93)	(+11.55)	(+12.07)	(+13.71)
	1000	34.531±3.10	49.942±2.75	68.110±3.43	320.430±7.94	340.730±11.53	357.114±8.00
		(+48.99)	(+52.51)	(+59.09)	(+25.42)	(+27.12)	(+28.12)
	2000	68.139±2.15	78.179±3.06	85.323±2.10	389.031±9.34	395.442±9.15	411.394±9.34
		(+70.43)	(+66.81)	(+65.94)	(+34.24)	(+33.86)	(+34.49)
In roots							
Control		68.041±2.03	69.594±3.04	72.112±2.94	183.054±9.51	187.543±11.59	192.045±7.34
Zn	500	79.594±3.42	84.036±2.01	87.247±1.09	224.437±5.03	238.941±8.94	254.594±8.54
		(+7.80)	(+9.40)	(+9.49)	(+10.15)	(+12.05)	(+14)
	1000	99.244±1.05	124.941±4.97	132.243±5.07	284.597±8.03	299.014±9.93	327.904±7.34
		(+18.65)	(+28.45)	(+29.44)	(+21.71)	(+22.91)	(+26.12)
	2000	143.420±5.94	159.047±6.90	174.901±8.90	350.394±3.05	364.530±4.94	378.112±9.41
		(+35.64)	(+39.12)	(+41.62)	(+31.36)	(+32.05)	(+32.64)

Values are means ± SE (n=5). Means within the column with the same letter are not significantly different (p<0.05). Values in parenthesis were expressed as percentage of the respective parameters over control.

The activity of GPOD assessed in shoots and roots of *G. max* with Zn²⁺ at pH level 3.0, 6.0 and 8.0. Heavy metal treated plants showed statistically significant increase activity of GPOD compared with control. At 2000 μM the highest value of GPOD activity recorded for

G. max, with the mean values 285.623, 301.923, 315.057 unit mgG¹ protein FW at pH 3.0, 6.0 and 8.0 respectively (Table 3). Plants exposed to Zn²⁺ in the nutrient solution showed significantly higher GPOD activity in root cells with all given pH as compared over control.

Table 3: Specific activity of guaiacol peroxidase (GPOD) and catalase (CAT) at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn

Treatments (μM)		GPOD (Unit mgG ¹ protein)			CAT (Unit mgG ¹ protein)		
		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
In shoots							
Control		172.346±8.34	181.590±3.34	185.018±4.78	125.304±4.50	131.054±8.53	135.511±9.34
Zn	500	201.442±6.43 (+7.78)	211.513±6.48 (+7.61)	219.054±9.34 (+8.42)	141.330±7.05 (+6.01)	152.394±6.94 (+7.54)	162.730±8.34 (+9.12)
	1000	225.301±11.12 (+13.31)	257.114±8.93 (+17.21)	268.513±11.51 (+18.40)	191.340±4.13 (+20.85)	218.434±8.84 (+25)	230.143±6.43 (+25.88)
	2000	285.623±6.51 (+24.73)	301.932±4.93 (+24.88)	315.057±6.53 (+26)	230.630±8.01 (+29.59)	250.773±9.01 (+31.35)	274.345±9.31 (+33.87)
In roots							
Control		173.624±14.51	180.424±9.34	184.431±3.41	132.410±6.04	139.042±11.09	140.594±7.77
Zn	500	213.413±8.91 (+10.28)	220.741±2.53 (+10.04)	227.509±8.59 (+10.45)	158.730±8.01 (+9.04)	162.943±4.51 (+7.91)	180.334±2.44 (+12.38)
	1000	268.510±7.93 (+20.31)	275.137±8.34 (+20.79)	314.409±9.43 (+26.05)	182.114±9.53 (+15.80)	197.256±9.43 (+17.31)	230.447±9.54 (+24.41)
	2000	324.620±11.25 (+30.30)	364.349±4.04 (+33.76)	413.517±8.68 (+46)	230.994±11.40 (+27.12)	258.339±4.33 (+30.02)	288.339±8.44 (+34.44)

Values are means ± SE (n=5). Means within the column with the same letter are not significantly different (p<0.05). Values in parenthesis were expressed as percentage of the respective parameters over control.

Table 4: Specific activity of supermodels dismutase (SOD) and glutathione reductase (GR) at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn

Treatments (μM)		SOD (Unit mgG ¹ protein)			GR (Unit mgG ¹ protein)		
		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
In shoots							
Control		72.401±2.38	75.092±4.22	79.010±8.54	164.345±8.53	170.399±8.03	175.134±11.34
Zn	500	84.934±6.34 (+7.96)	92.514±6.11 (+10.39)	106.914±7.54 (+15)	187.114±3.33 (+6.47)	190.443±6.34 (+5.55)	198.113±12.34 (+6.15)
	1000	120.104±7.30 (+24.78)	138.039±11.51 (+29.53)	143.346±8.09 (+28.93)	201.553±6.34 (+10.16)	240.663±8.44 (+17.09)	259.140±7.43 (+19.34)
	2000	190.309±9.04 (+44.88)	210.343±9.53 (+47.38)	224.940±4.03 (+48.01)	264.339±9.91 (+23.32)	280.413±9.01 (+24.40)	291.336±8 (+24.91)
In roots							
Control		81.530±6.34	85.940±8.93	89.014±3.05	172.430±8.43	180.341±6.44	182.124±3.40
Zn	500	97.341±4.30 (+8.83)	98.104±6.35 (+6.63)	102.054±1.01 (+6.82)	191.340±11.43 (+5.19)	195.062±8.43 (+3.92)	210.643±8.06 (+7.26)
	1000	148.301±3.51 (+29.05)	152.094±3.21 (+27.71)	167.344±9.01 (+30.55)	230.450±3.64 (+14.18)	251.420±3.43 (+16.46)	264.340±5.32 (+18.41)
	2000	197.401±3.13 (+41.54)	210.310±7.54 (+41.98)	219.342±6.34 (+42.26)	420.630±8.46 (+41.85)	450.342±8.32 (+42.81)	478.927±10.63 (+44.89)

Values are means ± SE (n=5). Means within the column with the same letter are not significantly different (p<0.05). Values in parenthesis were expressed as percentage of the respective parameters over control.

Table 5: Accumulation of zinc (mg kgG¹) at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations

Treatments (μM)		Shoots			Roots		
		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
Control		40.034±2.37	42.567±3.55	45.340±3.94	64.341±2.35	68.534±4.94	69.249±3.49
Zn2+	500	68.532±4.51 (+26.24)	74.365±6.59 (+27.19)	82.229±5.34 (+28.91)	87.346±2.59 (+15.61)	98.252±5.01 (+17.81)	98.947±4.34 (+17.65)
	1000	118.346±5.42 (+49.44)	125.806±5.03 (+49.43)	136.440±7.21 (+50.11)	138.545±4.34 (+36.57)	148.110±6.02 (+36.73)	162.947±7.03 (+40.35)
	2000	198.547±6.52 (+66.43)	210.204±7.45 (+66.31)	249.333±5.42 (+69.22)	210.343±7.45 (+53.15)	254.101±4.03 (+57.51)	238.424±9.34 (+54.98)

Values are means ± SE (n=5). Means within the column with the same letter are not significantly different (p<0.05). Values in parenthesis were expressed as percentage of the respective parameters over control.

Changes of the activity of enzyme CAT were determined in extracts obtained from shoots and roots of *G. max* with various doses of metals at pH 3.0, 6.0 and 8.0. With the manner of concentration rises, a progressive increase in CAT activity was observed and then the highest activity of enzyme was monitored at 2000 μM in shoot cells of hydroponically grown seedlings. At the same pH, the percent activity of CAT remarkable enhanced nearly three fold at 1000 μM compared with 500 μM for both metal contaminations as noted 20.85%, 25%, 25.88% at pH 3.0, 6.0 and 8.0 (Table 3). CAT activity in roots was increased by increasing the concentration of Zn^{2+} at pH increases.

In order to investigate the role of SOD in shoots and roots of *G. max* under short term heavy metal treatments, the maximum activity of SOD were recorded at the maximum concentration of metals at pH 8.0. As compared to control significant percent increment of SOD activity in shoot cells also were recorded gradually increasing concentration of metals but not in dose dependent manner. In root cells of soybean the activity of SOD measured at 500, 1000 and 2000 μM were 97.341, 148.301, 197.401 unit mgG^{-1} protein FW at pH 3.0; 98.104, 152.094, 210.310 unit mgG^{-1} protein FW at pH 6.0; 102.054, 167.344, 219.342 unit mgG^{-1} protein FW at pH 8.0 to Zn^{2+} (Table 4).

Applied Zn^{2+} have positive correlation with specific activity of GR in shoots and roots of experimental plant seedlings with consideration of pH values. The results in the attempt made in the current study are mentioned in Table 4 for shoot cells of *G. max*. The maximum activities were to be found out at 2000 μM concentration of metal; its level rises from 3.0 to 8.0 pH. In *G. max* the mean value were observed from range 264.339 to 291.336 unit mgG^{-1} protein FW unit mgG^{-1} protein FW at extreme concentration. Similar patterns for root cells have been observed in seedlings.

Table 5 showed the effect of pH on Zn^{2+} accumulation in shoots and roots of *G. max*. Metal accumulation was significantly increased under rising pH levels range from 3.0 to 8.0 as well as with the metal supply. The mean values recorded at 500 μM were 68.532, 74.365, 82.229 mg kg^{-1} DW at pH 3.0, 6.0 and 8.0 respectively in shoots, this trend was also followed in root cells but with higher content at each treatment compared to shoots. The accumulation rate of Zn^{2+} remarkably enhanced as concentration ranged from 500 μM to 2000 μM . The rate of percent accumulation was approximately two fold higher at 1000 μM level with the respect of 500 μM but highest contents were observed at 2000 μM concentration in shoot and root cells.

DISCUSSION

Above findings suggests that the toxic effect of heavy metals is probable exerted through free radical generation. In higher plants Zn^{2+} ions induce generation of O_2^- , H_2O_2 , HO^\cdot and $^1\text{O}_2$, collectively termed ROS and exert a variety of damaging effects, also called oxidative stress [18]. The O_2^- can rapidly attack all types of biomolecules such as nucleic acids, proteins, lipids and amino acids leading to irreparable metabolic dysfunction and cell death. Many redox-active and non-redox-reactive metals are known to cause oxidative stress, as indicated by lipid peroxidation and chlorophyll degradation in the cells [19].

Hydrogen peroxide (H_2O_2), a kind of natural ROS, is generated with various environmental and developmental stimuli. It has been approved to act as a new signal molecule and played important roles in many physiological processes such as cell expansion, development, stomatal closure and programmed cell death [20]. As a signal in plants, the H_2O_2 could be induced by many environmental stresses and was also an important reactor in the resistance of plants to environmental stresses [21]. The action of H_2O_2 in stress resistance of plants was involved in many regulation processes [22]. The accumulation of H_2O_2 could reflect the oxidative stress and the changes of antioxidants in different compartments of plant. Reactive oxygen species like O_2^- and H_2O_2 have been considered central components of signal transduction which triggers the defense genes responsible for antioxidant enzymes, including SOD [23].

Zinc (Zn^{2+}) induced lipid peroxidation in roots of seedlings may be due to membrane degeneration [24]. This result supports the possibility that the increase in peroxidation is due to the inhibition of ROS or by the enzyme lipoygenase that are activated under metal stress [25]. In studies with bean plants Somashekaraiyah *et al.* [26] showed that Cd causes an enhanced lipid peroxidation and consequently degradation of chlorophyll by lipid peroxides.

Recent studies have focused on the changes in activity of APX in higher plants subjected to several environmental stresses such as ozone, high lights, extremes of temperature, salts, heavy metals etc. [27]. APX activities generally increased along with activities of other antioxidative enzymes like CAT, SOD, GPOD and GR in response to heavy metal stress factors, suggesting that the component of ROS scavengers are co-regulated. APX is a component of ascorbate-glutathione pathway, which plays a role in scavenging H_2O_2 because it is a systemic

signal for the induction of APX [28]. (Morita *et al.* 1999). In well agreement with the results presented in this work, Hegedus *et al.* [29] found that activities of APX were increased in roots and leaves of barley and bean plants.

A positive correlation between GPOD activities and heavy metals were recorded in present investigation. Such correlations are known from literature data, but pertain to Cd [30]. GPOD is an induced protein, its high activity showed that the plants can resist, tolerate and clear the oxidative stress caused by Zn^{2+} . The results indicate an enhancement in the activity of GPOD, suggesting that this enzyme serves as an intrinsic defense tool to resist Pb-induced oxidative damage in rice plants. Guaiacol peroxidases are widely accepted as 'stress enzymes'. Induction in GPOD activity has been documented under a variety of stressful conditions under toxic levels of Al, Cu, Cd, Zn [31]. As GPOD are located in cytosol, cell wall, vacuole and in extracellular spaces, increased peroxidase activity in Pb stressed seedlings might be possibly due to increased release of peroxidases localized in the cell walls. Under sublethal salinity and metal toxicity conditions, level of peroxidase activity has been used as potential biomarker to evaluate the intensity of stress [8].

Catalase eliminates H_2O_2 produced during the photorespiration in peroxisomes. The elevation of H_2O_2 content in shoots and roots of plants after 500 to 2000 μM Zn^{2+} treatment suggested that some H_2O_2 -scavenging enzymes would work effectively for the removal of H_2O_2 . For this reason the activity of CAT was examined as one of the major antioxidant enzyme that eliminates H_2O_2 by converting it into oxygen and water [32]. The present findings agree with the results of Moloi *et al* [21] in barley plants that showed marked increase in CAT activity by heavy metal stress. Activity of CAT in response to Cr has been studied in many crop plants like rice, wheat, green gram and even in lower plants like mosses [33]. In rice, Cr can either induce CAT activity or suppress it. Treatment of developing wheat seedlings to different concentrations of Cr showed varied responses.

Increase SOD activity in the shoot and root tissues of the seedling is due to the generation of active oxygen species under Zn^{2+} toxicity. Scandalios [34] explained obtained result assuming that oxidative stress could cause increased turnover and re-synthesis of SOD with no net change in its concentration [35]. Within a cell, SOD constitutes the first line of defense against ROS. O_2^- is produced at any location where an electron transport chain is present and hence oxygen activation may occur in different compartments of the cell. The phospholipids membranes are impermeable to charged O_2^- , so therefore, it is crucial that SOD are present for the removal of oxygen

in the compartments where O_2^- radicals are formed. The SOD activity of a plant is increased by the use of high concentration of heavy metal ions, by an increase in SO_2 concentration [36]. A general plant response to stress is activation of the system of antioxidative defense, which prevents each cell component from oxidative injury. One of the cell responses to the increased metal concentration in the environment is the increase in the capacity of antioxidative enzymes such as POD, SOD and CAT.

Glutathione reductase and glutathione (GSH, (-Glu-Cys-Gly)) are important components of the cell's scavenging system for reactive oxygen compounds in plants. GSH is a major reservoir of nonprotein reduced sulfur. In addition, 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 and ASA/DHA ratios is maintained at the intracellular level by this reaction during oxidative stress. GR activity has been shown to increase in various plant species under different types of stresses [37]. Di-Baccio *et al.* [38] found that total glutathione content (GSH+GSSG) was reduced with increasing heavy metal concentrations while the contribution of GSSG to total glutathione increased in *Populus spp.* Glutathione has also been described as a 'transport peptide' *in vivo* for NO through the formation of S-NO groups [39]. The glutathione redox couple, act as cellular redox buffer which maintains the given thiol/disulphide redox potential, has already been implicated in modulating the metal release form metallothionein in the absence of NO [40].

The translocation of metals from the roots into the shoot is a controversial issue. As roots remain completely immersed and fully exposed to higher metal concentrations in the growth medium, majority of the metals become sequestered in the roots [41]. Zinc moves predominantly into the root apoplast and thereby in a radial manner across the cortex and accumulates near the endodermis. The endodermis acts as a partial barrier to the movement of metal ions between the root and shoot. This may in part account for the reports of higher accumulation of Zn^{2+} in roots compared to shoots [7]. The absorption of heavy metals in soil follows the Langmuir relation and increases with increasing pH between 3.0 to 8.5 [42]. However Blaylock *et al.* [43] reported that in soil with a pH between 5.5 and 7.5 Pb^{2+} solubility is controlled by phosphate or carbonate precipitates and very little Pb is available to plants even if they have the genetic capacity to accumulate it. The heavy metal toxicity may be observed by changes in growth conditions.

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