Academic Journal of Plant Sciences 3 (1): 01-10, 2010
ISSN 1995-8986

Response of Non-Enzymatic Antioxidants to Zinc Induced Stress at Different pH in *Glycine max* L. cv. Merrill

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Abstract: Zinc induced non-enzymatic antioxidative reactions in the shoots and roots of *Glycine max* L. cv. Merrill were investigated in both pH and concentration-dependent manners. The cytotoxic effects of Zn^{2+} on hydroponically grown seedlings were elucidated at various H^+ concentration. The rapid uptake of Zn^{2+} was observed immediately after the start of treatment and it seems to clearly depend on the pH and the accumulation becomes faster at a higher pH. Application of Zn^{2+} at 2000 μmol L^{-1} caused 67.27 and 72.04 percent reduction in dry mass at pH 8.0 in shoot and root cells as compared with control. Zn^{2+}-induced shoots showed regular inhibition in chlorophyll contents as increasing concentration 500 to 2000 μmol L^{-1} and pH levels. The major change was seen in the protein content at higher pH in presence of 2000 μmol L^{-1} Zn^{2+}. Seedlings posses to a variable extent non-enzymatic antioxidant that have the ability to detoxify various reactive oxygen species against oxidative stress caused by Zn^{2+} stress. We found transient increment of non-enzymatic components of the antioxidative systems viz. ascorbic acid, carotenoids, non-protein thiols, total phenol and proline in the fraction isolated from shoot and root cells in the presence of Zn^{2+} at stress condition.

Key words: *Glycine max* L. · Non-enzymatic antioxidants · Non-protein thiols · Total phenol

INTRODUCTION

Over the past few decades, heavy metal contamination of terrestrial system has attracted the attention of several investigators both in the developed and developing countries of the world. Zinc (Zn^{2+}) is essential for cell physiological processes and in most living organisms it is the second most abundant transition metal after iron. Zn^{2+} has no redox activity but plays structural and / or catalytic roles in many processes and is the only metal present in all enzyme classes [1]. When present at high concentrations, Zn^{2+} can be toxic and plants affected may show symptoms similar to those found in other heavy metal toxicities, such as those of Cd^{2+} or Pb^{2+}. In most cases, excess Zn^{2+} generates reactive oxygen species (ROS) and displaces other metals from active sites in proteins. Zinc toxicity also induces chlorosis in young leaves and this has been suggested to result from a Zn^{2+}-induced Fe^{2+} or Mg^{2+} deficiency, based on the fact that the three metals have similar ion radii [2].

The mechanisms controlling Zn^{2+} homeostasis in plants are still not fully known [3-5]. Plant roots acquire zinc predominantly as the Zn^{2+} and the metal is then distributed throughout the whole plant in a complex series of processes. The transport of metals is significantly influenced by pH levels, metal accumulation increase as pH increase from 3.5 to 8.0 [6]. Several families of plant metal transporters have been identified in recent years with at least three being involved in Zn^{2+} transport through membranes: ZIP (IRT-like proteins) [7] CDF (Cation Diffusion Facilitator proteins) [8] and P1B-type ATPases (HMAs, metal transporting ATPases) [9].

After uptake, Zn^{2+} can be transported in the xylem where it is chelated by different small molecules [10] including organic acids such as malate and citrate His [11] and nicotianamine [12]. Under high Zn^{2+} supply, a large part in the cell is also chelated by organic acids such as malate and citrate [13] amino acids such as His and NADH, phytate and metallothioneins [14] and is most likely stored in vacuoles.

The increased production of toxic oxygen derivatives can be caused by both natural and stress situations [15]. These highly cytotoxic species of oxygen can seriously disrupt normal metabolism through oxidative damage to

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cellular components. One of the most damaging effects of these molecular species and their products in cells is the peroxidation of membrane lipids. An excess of redox active metals, for example, Cu^{2+} and Fe^{2+}, as well as metal ions unable to perform univalent oxidoreduction reactions, such as Zn^{2+}, induces oxidative damage and are also capable of affecting lipid peroxidation and antioxidative protection [16]. To counteract this metabolic dysfunction caused by abiotic stress, higher plants employ defense strategies and developed different enzymatic and non-enzymatic defense mechanisms against oxidative stress induced by ROS. Non-enzymatic antioxidants like ascorbic acid, carotenoids, non-protein thiols, total phenol and proline etc are the most important defense metabolites of plants against active oxygen species. We were therefore interested in identifying the involvement of antioxidative mechanisms responsible for the Zn^{2+} induced oxidative stress in shoot and root cells of *Glycine max* L. The purpose of the study was to examine antioxidative metabolites like ascorbic acid, carotenoids, non-protein thiols, total phenol and proline exposed to different level of Zn^{2+} at different pH levels. We also measured growth and physiological parameters in the Zn^{2+} induced shoots and roots.

MATERIALS AND METHODS

Plant Material and Treatment: 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-day old seedlings were grown hydroponically on 1/4 strength modified Hoagland nutrient solution [17]. Seedlings were grown at 24°C, with a light intensity of 300 μmol.m^{-2}. s^{-1} and a 14-h photoperiod. After growing for 10 days (four true leaves), they were treated with 0, 500, 1000 and 2000 μmol l^{-1} 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 mmol $CaCl_2$ to remove Zn accumulated on their surface and stored in an-80°C freezer.

Dry Weight Determination: One gram fresh weight of shoot and root cells from various samples were taken, wrapped in aluminum foil and oven dried at 70°C in hot air oven until a constant weight was recorded [18].

Chlorophyll Determination: The chlorophyll content in shoot samples was determined in 80% acetone extract as described by Arnon and Stout [19].

Protein Assay: Protein content in the extracts was determined according to Lowry *et al.* [20]. The plant material was homogenized with a china homogenizer in a boron buffer (pH 8.7), in a refrigerated centrifuge at 5000 g for 15 min. The solution absorbency was determined at the presence of Folin reagent, at wavelength of 660 nm. The protein amount was determined following a standard curve obtained with bovine serum albumin.

Non-enzymatic Antioxidants Measurements

Ascorbic Acid: Ascorbic acid (AsA) was estimated as described by AOAC [21]. One gram dried and finely powdered shoot and root sample was dissolved with 10 ml of 0.4% oxalic acid in water and centrifuged at 8000 g. Supernatant was used to test AsA content in samples. The 500 μl and 1000 μl aliquots of the supernatant in triplicate were maintained to 3 ml by 0.4% oxalic acid followed by the addition 7 ml of DCPIP dye solution. The test mixture was properly mixed and its absorbance was recorded immediately at ëmax 518 nm. The amount of AsA was calculated by comparison with standard curve drawn under identical experimental conditions.

Carotenoids: Total carotenoids in the plant tissues were estimated according to the method by Jensen [22]. One gram of each sample were extracted with 80% methanol and centrifuged. The supernatants were concentrated to dryness. The residues thus obtained were dissolved in 15 ml of diethyl ether and after addition of 15 ml of 10% methanolic KOH the mixture was washed with 5% ice-cold saline water to remove alkali. The collective saline washings were extracted with ether (3:15 v/v). The ether extract from both were mixed together followed by washing with cold water till alkali free. The alkali free ether extract was dried over anhydrous Na_2SO4 for two hours in the dark. The ether extracts were filtered and its absorbance was measured at ëmax 450 nm by using ether as blank.

Non-protein Thiols: Non-protein thiols (NPT) were extracted by grinding 0.5 gram shoot and root sample in 1.0 ml ice-cold 5% (w/v) sulfosalicylic acid solution. After centrifugation at 10000 g at 4°C for 30 min, the supernatants were collected and immediately assayed. NPT was measured with Ellman's reagent [23]. Briefly, 300 μl of the supernatant was mixed with 1.2 ml of 0.1 M PBS (pH 7.6). After a stable absorbance reading of 412 nm was obtained, 25 μmol DTNB solution (6 mol DTNB

dissolved in 5 mmol EDTA, 0.1 mol PBS, pH 7.6) was added and the increase in absorbance at 412 nm was monitored.

Total Phenol: Total phenolic contents in different extracts were measured by the method of Ragazzi and Veronese [24]. Ten mg plant extract was dissolved in 10 ml of 50% MeOH: H_2O (1:1, v/v), overnight at room temperature, 1.0 ml of Folin's reagent (1N) and 2.0 ml of Na_2CO_3 (20%) were added subsequently. The test mixture was mixed properly on cyclomixer, left at room temperature for 30 min and maintained to 25 ml with water. The absorbance of test mixture was measured at $\ddot{e}_{max}$ 725 nm.

Proline: Proline concentration in tissue fraction of soybean was determined spectrophotometrically by the method of Bates *et al.* [25]. The sample was homogenized in 2.0 ml of sulphosalicylic acid, centrifuged at 15000 g for 30 min. The reaction mixture contained 2.0 ml of supernatant, 2.0 ml of glacial acetic acid and 2.0 ml of ninhydrin. It was then heated up to 1 h and change in color was seen. After cooling, 4.0 ml of acetone was added and shaken vigorously for 30 sec and absorbance was measured at 520 nm.

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 [26].

Statistical Analysis: Each result shown tables and figures was the mean of at least five replicated treatments. The significance of differences ($p<0.05$) between treatments and pH levels was statistically evaluated by standard deviation and three way classification of "Analysis of Variance" technique were based on the appropriate *F*-tests.

RESULTS

The significant change in dry weight in shoot and root cells of *Glycine max* were observed during course of present study. The results were shown at the pH 3.0, 6.0 and 8.0 in the presence of different concentration of Zn^{2+}. Significant ($p<0.05$) reduction was noted at all doses of given metal compared with over control. The maximum reduction was reported at 2000 μmol l^{-1} on pH 8.0 in shoot cells of seedlings with the value 0.018 g $plant^{-1}$. In the root cells of *G. max* the content of dry weight showed upper relationships at various pH levels. The value at 2000 μmol l^{-1} at pH 8.0 was reported 0.013 g $plant^{-1}$ (Table 1). The percent decrement was two fold in 1000 μmol l^{-1} at all given pH in root cells as compared with the 500 μmol l^{-1} treatment; this trend has not been reported in 2000 μmol l^{-1} concentration (Figure 1 A).

The chlorophyll content in shoot cells of *G. max* showed sensitive influence to Zn^{2+} stress at all concentration. The chlorophyll content decreased with increasing pH of the solution from 3.0 to 8.0 but not showed in regular trend with metals concentrations gradually. At 500 μmol l^{-1} Zn^{2+} value of chlorophyll content at pH 3.0 and 6.0 was 1.745 and 1.422 mg g^{-1} FW but it did not increase as significant rate on 8.0 pH (Table 1). The maximum percent decrement ($p<0.05$) values were to be found at 2000 μmol l^{-1} and pH 8.0 given 55.33% against control shown in Figure 1 B.

Table 1: Contents of dry weight (g $plant^{-1}$) and chlorophyll (mg g^{-1}) at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn^{2+}

Treatments (μmol l^{-1})		Dry weight			Chlorophyll		
In shoots		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
	Control	0.092±0.0	0.087±0.0	0.085±0.0	1.801±0.50	1.628±0.66	1.612±0.33
Zn^{2+}	500	0.073±0.0	0.070±0.0	0.061±0.0	0.041±0.0	0.040±0.0	0.036±0.0
	1000	0.038±0.0	0.022±0.0	0.018±0.0	1.745±0.36	1.422±0.49	1.422±0.58
	2000	1.584±0.38	1.080±0.81	1.215±0.58	1.396±0.50	0.940±0.55	0.521±0.17
In roots							
	Control	0.085±0.0	0.083±0.0	0.080±0.0	……..	……..	……..
Zn^{2+}	500	0.072±0.0	0.068±0.0	0.062±0.0	……	……..	……..
	1000	0.023±0.0	0.016±0.0	0.013±0.0	…….	……..	……..
	2000	0.041±0.0	0.036±0.0	0.028±0.0	……	……..	……..

Results are the means of five replicate determinations on fresh weight basis with ± standard deviation

Table 2: Protein (mg g^{-1}) and ascorbic acid (mg $100g^{-1}$) contents at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn^{2+}

Treatments (μmol l^{-1})		Protein			Ascorbic acid		
In shoots		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
	Control	0.267±0.03	0.280±0.06	0.288±0.07	0.124±0.01	0.128±0.03	0.131±0.06
Zn^{2+}	500	0.324±0.04	0.340±0.01	0.351±0.0	0.410±0.03	0.434±0.03	0.475±0.05
	1000	0.524±0.03	0.542±0.04	0.569±0.04	0.146±0.07	0.152±0.01	0.156±0.08
	2000	0.189±0.08	0.197±0.05	0.210±0.07	0.210±0.13	0.219±0.03	0.253±0.08
In roots							
	Control	0.304±0.04	0.311±0.05	0.317±0.07	0.118±0.01	0.124±0.03	0.132±0.01
Zn^{2+}	500	0.344±0.07	0.389±0.07	0.399±0.04	0.424±0.00	0.457±0.01	0.475±0.03
	1000	0.510±0.05	0.560±0.03	0.591±0.00	0.148±0.03	0.152±0.05	0.154±0.06
	2000	0.173±0.05	0.181±0.06	0.189±0.07	0.210±0.09	0.244±0.07	0.259±0.05

Results are the means of five replicate determinations on fresh weight basis with ± standard deviation

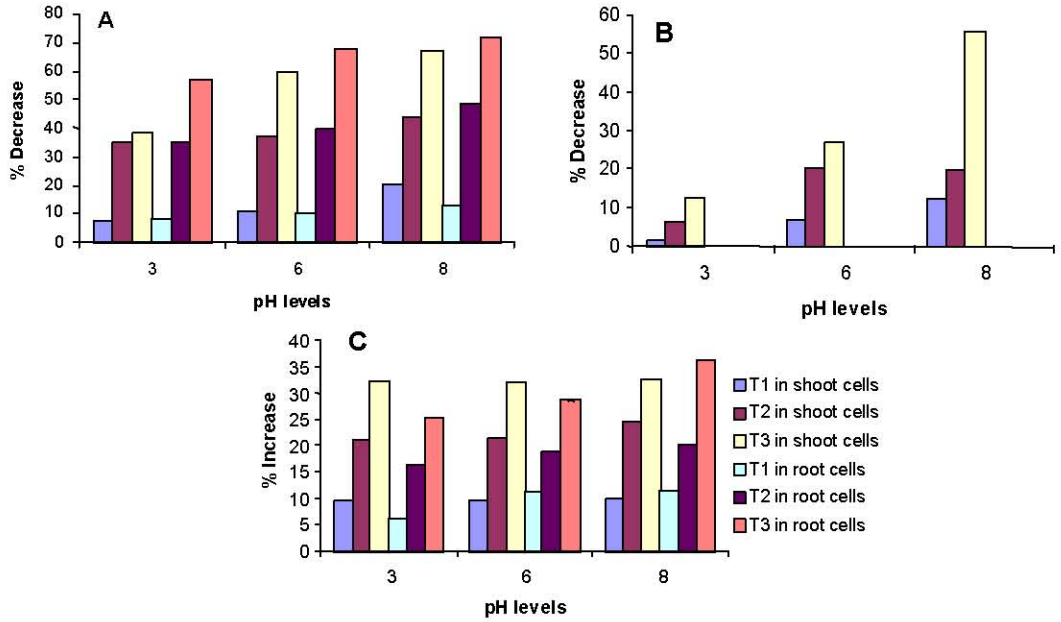

Fig. 1: Comparative effect of Zn^{2+} on dry weight, chlorophyll and protein in shoot and root cells of *Glycine max* L. Data represent for one control and three treatments each of five replicates. (A) Dry weight: percent decrement over control. (B) Chlorophyll: percent decrement over control. (C) Protein: percent increment over control. (T1= 500 μmol L^{-1}, T2= 1000 μmol L^{-1}, T3= 2000 μmol L^{-1})

Zn^{2+} showed enhancement in the level of protein at pH range between 3.0 and 8.0 in the shoot and root cells of seedlings (Table 2). The percent increment ($p<0.05$) value of protein content at 500 μmol l^{-1} for shoot cells at pH 6.0 and 8.0 were 9.67%, 9.85%. Similar kinds of findings were observed in root cells of plants. The dose dependent impact of metal was higher on protein content as compared to vary pH levels from 3.0 to 8.0 (Figure 1 C).

An effective significant rise in AsA content was observed in shoots of *G. max* at pH level increases as per Zn^{2+} dose. The percent increment ($p<0.05$) values at 2000 μmol l^{-1} were recorded 25.74% at pH 3.0; 26.22% at pH 6.0 and 37.77% at pH 8.0 over control. In root cells of experimental seedlings content of AsA given randomly results but in increasing order with increasing concentrations at different levels of pH (Table 2). In the case of roots at pH 3.0 the percent increment values were recorded 11.27%, 18.90% and 28.04% with 500, 1000 and 2000 μmol l^{-1} supply respectively as compared to control (Figure 2 A).

Treatments with metal caused significant increased in carotenoids content in shoot cells of *G. max* with the mean values at 500 μmol l^{-1} was 240.345, 254.334, 269.118 μg g^{-1} FW at pH 3.0, 6.0 and 8.0 respectively

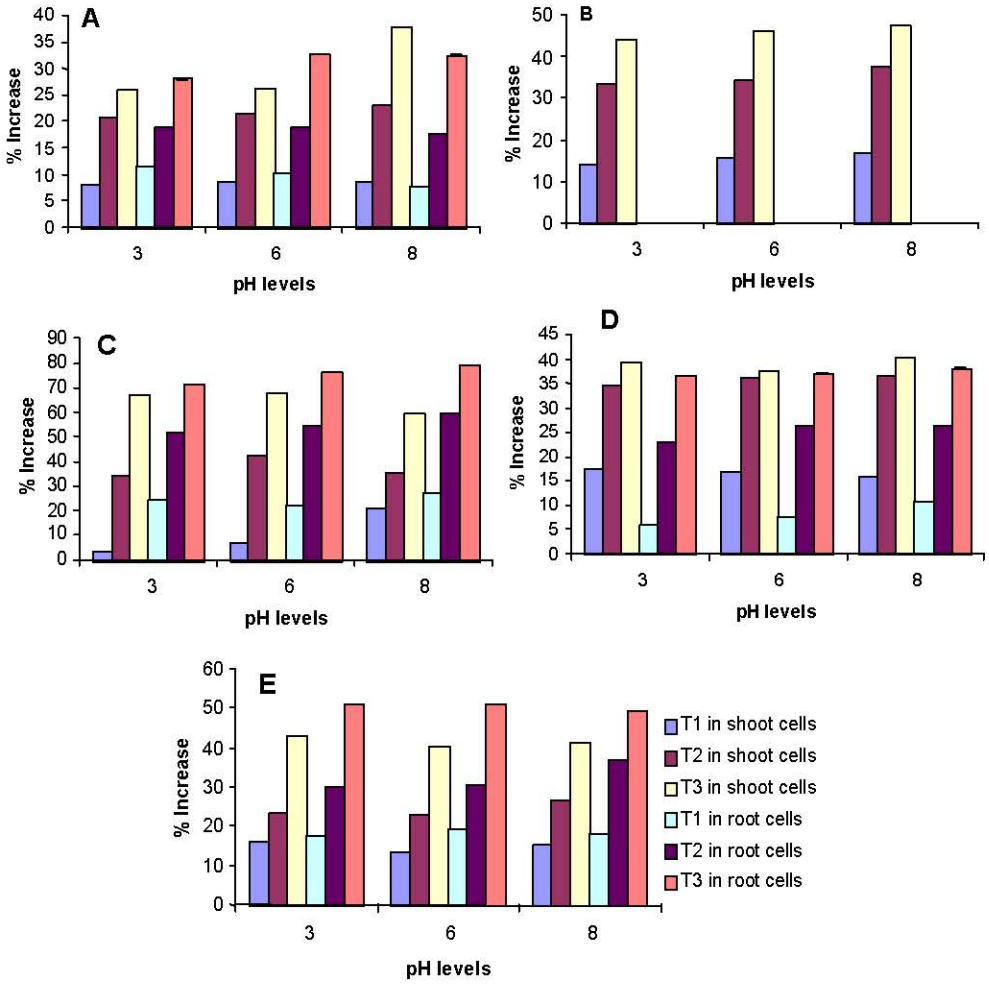

Fig. 2: Comparative effect of Zn^{2+} on ascorbic acid, carotenoids, non-protein thiols, total phenol and proline in shoot and root cells of *Glycine max* L. Data represent for one control and three treatments each of five replicates. (A) Ascorbic acid: percent increment over control. (B) Carotenoids: percent increment over control. (C) Non-protein thiols: percent increment over control. (D) Total phenol: percent increment over control. (E) Proline: percent increment over control. (T1=500 μmol L^{-1}, T2= 1000 μmol L^{-1}, T3= 2000 μmol L^{-1})

Table 3: Carotenoids (μg g^{-1}) and non-protein thiols (n mol g^{-1}) content at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn^{2+}

Treatments (μmol l^{-1})		Carotenoids			Non-protein thiols		
In shoots		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
	Control	180.060±5.05	184.532±8.44	190.214±11.53	12.083±1.47	13.151±1.32	19.341±1.79
Zn^{2+}	500	240.345±7.53	254.334±9.05	269.118±6.05	360.213±8.01	376.110±11.05	421.224±8.31
	1000	465.134±11.03	503.223±7.34	532.443±11.51	12.912±1.49	15.107±1.53	29.471±1.78
	2000	24.521±2.50	32.301±2.13	40.310±2.72	61.371±4.20	69.100±4.31	75.121±4.12
In roots							
	Control	…….	……..	……	7.00±1.10	8.642±1.52	9.452±0.34
Zn^{2+}	500	…….	……..	……	11.342±0.31	13.597±1.02	16.517±1.02
	1000	…….	……..	……	22.045±0.19	29.625±2.14	37.091±2.51
	2000	…….	……..	……	41.489±1.73	64.571±1.74	79.590±2.30

Results are the means of five replicate determinations on fresh weight basis with ± standard deviation.

Table 4: Total phenol (mg $100g^{-1}$) and proline (μ mol g^{-1}) contents at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations of Zn^{2+}.

Treatments (μmol l^{-1})		Total phenol			Proline		
In shoots		pH 3.0	pH 6.0	pH 8.0	pH 3.0	pH 6.0	pH 8.0
	Control	0.303±0.01	0.324±0.03	0.350±0.03	0.181±0.01	0.196±0.06	0.199±0.02
Zn^{2+}	500	0.434±0.03	0.455±0.07	0.482±0.05	0.623±0.04	0.690±0.04	0.750±0.08
	1000	0.694±0.03	0.715±0.09	0.820±0.09	0.252±0.03	0.259±0.08	0.268±0.04
	2000	0.292±0.04	0.312±0.07	0.342±0.03	0.452±0.01	0.461±0.03	0.482±0.01
In roots							
	Control	0.278±0.01	0.284±0.07	0.291±0.06	0.184±0.01	0.189±0.04	0.210±0.10
Zn^{2+}	500	0.314±0.07	0.332±0.08	0.360±0.01	0.443±0.08	0.486±0.06	0.499±0.03
	1000	0.598±0.03	0.620±0.05	0.648±0.09	0.262±0.03	0.279±0.04	0.301±0.06
	2000	0.341±0.06	0.358±0.07	0.454±0.07	0.571±0.08	0.581±0.01	0.620±0.09

Results are the means of five replicate determinations on fresh weight basis with ± standard deviation

Table 5: Accumulation of zinc (mg kg^{-1}) at various pH levels in shoot and root cells of *Glycine max* L. exposed to different concentrations.

Treatments (μmol l^{-1})		Shoots			Roots		
In shoots		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
Zn^{2+}	500	68.532±4.51	74.365±6.59	82.229±5.34	118.346±5.42	125.806±5.03	136.440±7.21
	1000	198.547±6.52	210.204±7.45	249.333±5.42	87.346±2.59	98.252±5.01	98.947±4.34
	2000	138.545±4.34	148.110±6.02	162.947±7.03	210.343±7.45	254.101±4.03	238.424±9.34

Results are the means of five replicate determinations on dry weight basis with ± standard deviation

(Table 3). Similar patterns were followed at other supplied level of metals with various pH. Carotenoids showed remarkable ($p<0.05$) percent enhancement with rising stress as compared to control (Figure 2 B).

With increasing supply of Zn^{2+} the content of NPT were increased significantly in shoots and roots of seedlings and the amount rises as with progression of pH levels. The maximum mean values recorded in shoot and root cells was 75.121 and 79.590 nmol g^{-1} FW at 2000 μmol l^{-1} with pH 8.0 (Table 3). The percent increment amounts were also reported significantly ($p<0.05$) higher at extreme metal concentration compared with control but did not give any correlations with pH (Figure 2 C).

Total phenols were high in treated seedlings under control conditions at various pH. The result showed significant positive relationships with phenol content and metal accumulation rates. The total phenol content at 2000 μmol l^{-1} showed highest values ranged from 0.694 to 0.820 mg 100 g^{-1} FW for shoot cells of *G. max* at the pH 3.0 and 8.0 The gradual increments have been determined in root cells of seedlings parallel with the contamination of metals (Table 4). The elevation of total phenol content ($p<0.05$) in the percent reported marginally significant at all metal stress conditions but the value at various pH did not give moderate relationship against control (Figure 2 D).

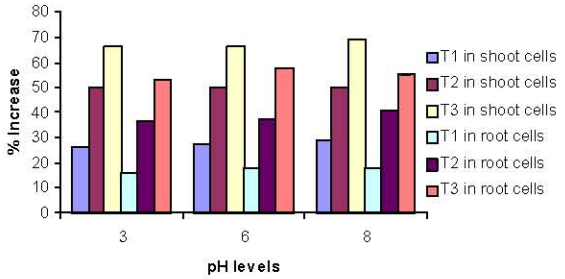

Fig. 3: Zinc accumulation in shoots and roots of *Glycine maxi* L. Data represent for one control and three treatments each of five replicates. Graph represent percent increment over control. (T1=500 μmol L^{-1}, T2= 1000 μmol L^{-1}, T3= 2000 μmol L^{-1})

The striking increase in proline content in $ZnSO_4.7H_2O$ treated seedlings demonstrated in shoot and root cells at various pH levels. The observed value at pH 8.0 and 2000 μmol l^{-1} concentration was 0.482 and 0.620 μmol g^{-1} FW in shoot and root cells respectively (Table 4). The percent increment were to be found significantly highest ($p<0.05$) in all experimental tissues at pH 3.0, 6.0 and 8.0 with 2000 μmol l^{-1} concentration as compared to control (Figure 2 E).

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 μmol l^{-1} 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 heavy metals remarkably enhanced as concentration ranged from 500 μmol l^{-1} to 2000 μmol l^{-1}. The rate of percent accumulation of Zn^{2+} was approximately two fold higher at 1000 μmol l^{-1} level with the respect of 500 μmol l^{-1} but highest contents were observed at 2000 μmol l^{-1} concentration in shoot and root cells of *G. max* (Figure 3).

DISCUSSION

In despite of their positive role in several metabolic processes, heavy metals cause severe cellular damage [27]. Zn^{2+} excess had different effects in soybean plants and extents of the effects were dependent on the concentration and pH in the nutrient solution. Zn^{2+} 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 metals between the root and shoot. The adverse effect of excess Zn^{2+} in the shoots and roots probably caused the decrease in dry mass content [28]. Present experiment results showed that as amount of metal stress increased the dry mass decrease at all pH levels with greater rates.

The decrease in chlorophyll content may be due to reduced chlorophyll biosynthesis by inhibiting δ-amino levulinic dehydrogenase and protochlorophyllide reductase activities and breakdown of pigment or their precursor as reported by Teramura and Sullivan [29]. Zn^{2+} leads to toxicity in shoots; it is now well known that these component, at high levels, can act as an efficient generator of toxic oxygen species, such as O_2-and H_2O_2, by inhibiting photosynthetic electron transport. The findings of this study are in agreement with the findings of Bonnet *et al.* [30] who reported a net decline in the capacities of maximal photochemical efficiency of photosystem II and in the quantum yield of electron flow throughout PS II in the leaves of rye grass.

The increase of protein or amino acid content in plants under the influence of heavy metals was reported by many authors [31]. Zn^{2+} and other heavy metals induce the synthesis of PCs in plants, which bind metals, keeping the intracellular availability of essential metals within certain limits and reducing the availability of nonessential metals. Also, heavy metals promote the synthesis of stress proteins, which might limit and repair the damages caused by metals to cell proteins and exert protective effects on membranes [32].

To understand the contribution of the non-enzymatic antioxidants in the response of soybean seedlings to Zn^{2+} toxicity, we examined their AsA, carotenoids, NPT, total phenols and proline concentrations. The AsA concentration was enhanced at three Zn^{2+} concentrations compared to the control, indicating that AsA is involved in antioxidant response. Similarly to present results other investigations have shown increased AsA for other plant species exposed to metals [33]. AsA is a key antioxidant and involved in protection of plant cells against oxidative damage catalyzed by ROS. It acts as a chain breaking antioxidant impairs with the formation of free radicals in the process of formation of intracellular substances throughout the plant body [34]. It is essentially required in i) scavenging of H_2O_2 by ascorbate-glutathione cycle, ii) elimination of ROS, iii) used in maintenance of α-tocopherol in reduced form and iv) utilized as a cofactor in xanthophylls cycle to protect chloroplast against photooxidative damage [35].

The carotenoids and their derivatives act as secondary photosynthetic pigment and as protectors against chloroplastic oxygen species dissipation processes [36]. Its ability of protection against photooxidative damage has been associated to the capacity of carotenoids to dissipate the excess of light acting as filter and to their antioxidant properties [37]. However, the role of carotenoids in protection of chloroplastic response against oxidative damage in one of four ways: by reacting with lipid peroxidation products to terminate the chain reaction; by scavenging singlet oxygen and dissipating the energy as heat; by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen, or by the dissipation or excess excitation energy through the xanthophylls cycle.

As presented in results NPT showed marked increase in shoot and root cells of hydroponically grown *G. max* with the severity of Zn^{2+} toxicity. The enhancement in NPT level might be considered as an indication for Zn^{2+} to promote H_2O_2 production in chloroplast and thus, activation of H_2O_2-scavenging ascorbate-glutathione cycle. NPT is also required for synthesis of metal-binding peptide such as phytochelatins (PCs), which bind and sequester metal in stable complexes in vacuoles [38]. The NPT represent a major pool of glutathione (GSH) [39]. GSH is a well known antioxidant playing a prominent role in the defense against ROS. Our findings suggested that Zn^{2+}-induced increase in the level of thiol compounds represented another defensive mechanism against oxidative stress.

Phenolics have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions. The induction of phenolic compound biosynthesis was observed in wheat in response to nickel toxicity [40]. Moreover it has been shown that phenolic compound can be involved in the H_2O_2 scavenging cascade in the plant cells [41]. The present findings were correlated with the Jallaluddin *et al.* [42] who reported the concentration of total phenolic compounds in hydroponically grown *Cicer arietinum* L.

In many plants under various forms of heavy metal stress, the concentration of proline increases up to 80% of the amino acid pool. In addition to its role as an osmolyte and a reservoir of carbon and nitrogen, etc proline has been shown to protect plants against free radical-induced damage [43]. The possible role of proline under metal stress have been proposed with greater or lesser convictions, which include stabilization of protein, scavenging of hydroxyl radicals, quenching of singlet oxygen, regulation of the cytosolic pH and regulation of NAD/NADH ratio [44].

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 [45]. Zn^{2+} 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 [46]. The absorption of heavy metals in soil follows the Langmuir relation and increases with increasing pH between 3.0 to 8.5 [46]. However Blaylock *et al.* [47] 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^{2+} is available to plants even if they have the genetic capacity to accumulate it.

ACKNOWLEDGEMENT

We are thankful to Hon'ble Vice-chancellor of Allahabad Agricultural Institute-Deemed University, Allahabad, for providing all necessary facilities to conduct the experiment.

REFERENCES

1. Barak, P. and P. Helmke, 1993. The chemistry of zinc. *In:* A.D. Robson (eds.). Zinc in Soil and Plants. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp: 1-13.
2. Marschner, H., 1995. Mineral Nutrition of Higher Plants. Academic Press, London.
3. Hacisalihoglu, G., J.J. Hart, C.E. Vallejos and L.V. Kochian, 2004. The role of shoot-localized processes in the mechanism of Zn efficiency in common bean. Planta, 218: 704-711.
4. Broadley, M.R., P.J. White, J.P. Hammond, I. Zelko and A. Lux, 2007. Zinc in plants. New Phytologist, 173: 677-702.
5. Kramer, U., 2005. MTP1 mops up excess zinc in Arabidopsis cells. Trends in Plant Science, 10: 313-315.
6. Lee, S.Z., L. Chang, H.H. Yang, C.M. Chen and M.C. Liu, 1998. Absorption characteristics of lead onto soils. J. Hazardous Materials, 63: 37-49.
7. Wintz, H., T. Fox, Y.Y. Wu, V. Feng, W. Chen, H.S. Chang, T. Zhu and C. Vulpe, 2003. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. J. Biol. Chem., 278: 47644-47653.
8. Kim, D., J.L. Gustin, B. Lahner, M.W. Persans, D. Baek, D.J. Yun and D.E. Salt, 2004. The plant CDF family member TgMTP1 from the Ni / Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. The Plant Journal, 39: 237-251.
9. Mills, R.F., A. Francini, P.S.C. Ferreira da Rocha, P.J. Baccarini, M. Aylett, G.C. Krijger and L.E. Williams, 2005. The plant P1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. FEBS Letters, 579: 783-791.
10. Haydon, M.J. and C.S. Cobbett, 2007. Transporters of ligands for essential metal ions in plants. New Phytol., 174: 499-506.
11. Kupper, H., A. Mijovilovich, W. Meyer-Klaucke and P.M.H. Kroneck, 2004. Tissue-and age-dependent differences in the complexation of cadmium and zinc in the cadmium / zinc hyperaccumulator *Thlaspi caerulescens* (Ganges Ecotype) revealed by X-ray absorption spectroscopy. Plant Physiology, 134: 748-757.

12. Callahan, D.L., A.J.M. Baker, S.D. Kolev and A.G. Wedd, 2006. Metal ion ligands in hyperaccumulating plants. J. Biol. Inorganic Chem., 11: 2-12.
13. Sarret, G., P. Saumitou-Laprade, V. Bert, O. Proux, J.L. Hazemann, A. Traverse, M.A. Marcus and A. Manceau, 2002. Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiology, 130: 1815-1826.
14. Papoyan, A. and L.V. Kochian, 2004. Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. Plant Physiology, 136: 3814-3823.
15. Weckx, J.E.J. and H.M.M. Clijsters, 1997. Zn phytotoxicity induces oxidative stress in primary leaves of *Phaseolus vulgaris*. Plant Physiol. Biochem., 35: 405-410.
16. Kampfenkel, K.M. Van Montagu and D. Inzé, 1995. Effects of iron excess *on Nicotiana plumbaginifolia* plants. Implications to oxidative stress. Plant Physiology, 107: 725-735.
17. Pickering, I.J., R.C. Prince, M.J. George, R.D. Smith, G.N. George and D.E. Satt, 2000. Reduction and co-ordination of arsenic in Indian mustard. Plant Physiology, 122: 1171-1177.
18. Jallaluddin, M.A., P.K. Mishra, K. Umesh, M. Chaudhary and V. Prakash, 2009. Antioxidative defense to lead stress in root cells of *Cicer arietinum* L. Indian J. Plant Physiol., 14: 194-199.
19. Arnon, D.J. and P.R. Stout, 1949. The essentiality of certain elements in minute quantity for plants with special reference to copper. Plant Physiology, 14: 371-375.
20. Lowry, O., N. Rosenbough, A. Farr and R. Randall, 1951. Protein measurements with Folin phenol reagent. J. Biol. Chem., 193: 265-275.
21. AOAC., 1984. Official methods of analysis (14th edn.) association of official analytical chemists. pp: 23-27.
22. Jensen, A., 1978. Chlorophyll and carotenoids. In: *Handbook of physiological and biochemical methods*. Cambridge University Press, Cambridge, UK., pp: 5-70.
23. Ellman, G.L., 1959. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82: 70-77.
24. Ragazzi, E. and G. Veronese, 1973. Quantitative analysis of phenolic compounds after thin layer chromatographic separation. J. Chromatography, 77: 369-375.
25. Bates, L.S., R.P. Waldeen and I.D. Teare, 1978. Rapid determination of free proline for water stress studies. Plant Soil, 39: 205.
26. Yang, H., J.W.C. Wong, Z.M. Yang and L.X. Zhou, 2001. Ability of *Agrogyron elongatum* to accumulate the single metal of cadmium, copper, nickel and lead and root exudation of organic acids. J. Environ. Sci., 13: 368-375.
27. Kyunghee, L. and U. Junghoon, 2001. Protection of metal stress in *Saccharomyces cerevisae*: cadmium tolerance requires the presence of two ATP-Binding domains of HSP104 protein. Bulletin of Korean Chemical Society, 22: 514-517.
28. Jarvis, M.D. and D.W.M. Leung, 2002. Chelating lead transport in *Pinus radiate*: an ultrastructural study. Environment and Experimental Botany, 48: 21-32.
29. Taramura, A.H. and J.H. Sullivan, 1994. Effect of UV-B radiation on photosynthesis and growth of terrestrial plants. Photosynthesis Research, 39: 463-473.
30. Bonnet, M., O. Camares and P. Veisseire, 2000. Effects of zinc and influence of *Acremonium lolii* on growth parameters, chlorophyll a fluorescence and anti-oxidant enzyme activities of rye grass (*Lolium perenne* L. cv Apolli). J. Experimental Biol., 51: 945-953.
31. Lou, L., Z. Shen. and X. Li, 2004. The copper tolerance mechanisms of *Elsholtzia haichowensis*, a plant from copper-enriched soils. Environment and Experimental Botany, 51: 111-120.
32. Sanita di Toppi, L. and R. Gabbrielli, 1999. Response to cadmium in higher plants. Environment and Experimental Botany, 41: 105-130.
33. Liu, Y., X. Wang, G. Zeng, D. Qu, J. Gu, M. Zhou and L. Chai, 2007. Cadmium-induced oxidative stress and response of the ascorbate-glutathione cycle in *Bechmeria nivea* (L.) Gaud. Chemosphere, 69: 99-107.
34. Prakash, V., P.K. Mishra and M. Mishra, 2009. Screening of medicinal plants extract for antioxidant activity. J. Medicinal Plants Res., 3: 608-612.
35. Smirnoff, N., 2000. Ascorbic acid: metabolism and functions of a multifaceted molecule. Current Opinion in Plant Biology, 3: 229-235.
36. Havaux, M., J.P. Bonfils, C. Lutz and K.K. Niyogi, 2000. Photodamage of photosynthetic apparatus and its dependence on the leaf developmental stage in the *npq1 Arabidopsis* mutant deficient in the xanthophylls cycle enzyme violaxanthin de-epoxidase. Plant Physiology, 124: 273-284.

37. Niyogi, K.K., C. Shih, W.S. Chow, B.J. Pogson, D. DellaPenna and O. Bjorkman, 2001. Photoprotection in a xeaxanthin and lutein-deficient double mutant of *Arabidopsis*. Phptosynthesis Research, 67: 139-145.
38. Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta, 212: 475-486.
39. De Vas, C.H.R., M.J. Vonk, R.V. Vooijs and H. Schat, 1992. Glutathione depletion due to copper induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiology, 98: 853-858.
40. Dietz, K.J., M. Baier and U. Kramer, 1999. Free radicals and reactive oxygen species as mediaters of heavy metal toxicity in plants. *In:* M.N.V. Prasad, J. Hagemeyer, (eds), Heavy metal stress in plants: from molecules to ecosystems, pp: 73-97. Springer-Verlag, Berlin.
41. Takahama, U. and T. Oniki, 1997. A peroxide / phenolics / ascorbate system can scavenge hydrogen peroxide in plant cells. Physiologia Plantarum, 101: 845-852.
42. Jallaluddin, M.A., P.K. Mishra, U. Kumar, M. Chaudhary and V. Prakash, 2009. Antioxidative defense to lead stress in root cells of *Cicer arietinum* L. Indian J. Plant Physiol., 14: 194-199.
43. Alia, J. Matysik, B. Bholu and P. Mohanty, 2000. Molecular mechanism of quenching of ROS by proline under stress in plants. Biophysics and Organic Chem., 40: 203-230.
44. Matysik, J., Alia, B. Bholu and P. Mohanty, 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science, 82: 525-532.
45. Bibi, M. and M. Hussain, 2005. Effect of copper and lead on photosynthesis and plant pigmentsin black gram (*Vigna mungo* (L.) Hepper). Bulletin of Environ. Contamination Toxicol., 74: 1126-1133.
46. Verma, S. and R.S. Dubey, 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Science, 164: 645-655.
47. Lee, S.Z., L. Chang, H.H. Yang, C.M. Chen and M.C. Liu, 1998. Absorption characteristics of lead onto soils. Journal of Hazardous Materials, 63: 37-49.
48. Blaylock, M.J., D.E. Salt, S. Dushenkov, O. Zakarova, C. Gussman, Y. Kapulnik, B.D. Ensley and I. Raskin, 1997. Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ. Sci. Technol., 31: 860-865.