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ALTERATION OF AMYLASE AND PHOSPHATASE IN *Macrotyloma uniflorum* UNDER INFLUENCE OF CADMIUM, MERCURY AND ZINC STRESSES

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ABSTRACT

Physical weathering of parent rocks, mining, and other anthropogenic activities lead to impermissible levels of heavy metals in plant tissues. Effect of Cd²⁺, Hg²⁺, and Zn²⁺ on metabolic enzymes, Amylase (EC; 3.2.1.1), and AP (EC; 3.1.3.2), were studied to assess horse gram response to these heavy metal stress. Enzyme activity and TC were evaluated. Amylase decreased in response to increasing Cd²⁺ concentration, with great impact at 48-72h. It rose under 5 ppm Hg²⁺, while dropped under 10 ppm beyond 24h reaching ~25% of control, and recovered with time to reach 80-90% under 20 ppm. Under Zn²⁺ stress, amylase rose in concentration- and time-dependent manner. AP declined under metal stress in concentration, time-dependent manner, except at 100 ppm Zn²⁺. TC fluctuated in metal-specific manner with positive influence by Zn²⁺ (300-600 ppm) and Cd²⁺ (100-200 ppm) at 24 h. TC diminished under Cd²⁺ stress beyond 24 h, and over entire duration under Hg²⁺ stress. It can deduce that Hg²⁺ and Cd²⁺ exhibited toxic effects on horse gram while Zn²⁺ had positive effects. Amylase responded negatively to Hg²⁺, positively to Zn²⁺, and showed mixed response to Cd²⁺. AP levels decreased under metal stresses, except Zn²⁺.

INTRODUCTION

Mining, fossil fuels, industrial wastes, and phosphate fertilizers are important environmental pollutants, particularly in areas of high anthropogenic pressure. Their presence in the atmosphere and soil can cause serious problems to all organisms, with alarming heavy metal bioaccumulation in the food chain [1]. Among these heavy metals, Fe, Mn, and Mo are important microelements. Cu, Cr, V, Zn, Ni, and W are toxic elements with high or low importance as trace elements [2]. Heavy metals have inhibitory effect on germination [3]. However, at moderate levels, they are known to play important role as activators or co-factors in all vital processes of plant growth and productivity, but, beyond permissive levels, induce harmful effects on plant physiology [4, 5].

Cd and Hg are nonessential metals in higher plants. Whereas, Zn is required as a trace element for plants, but toxic when present in excess. Of the three heavy metals chosen for study, Cd is more phytotoxic due to longer biological half-life and mutagenicity. Plants grown on Cd-enriched soil show visible symptoms of injury reflected by chlorosis, growth inhibition,

browning of root tips and death [1, 6, 7]. Inhibition of root Fe (III) reductase induced by Cd is known to cause Fe (II) deficiency that will affect photosynthesis [8]. In general, Cd is known to interfere with uptake, transport and use of several elements (Ca, Mg, P and K) and water by plants [9].

As one of the six most harmful pollutants, Hg accumulates in agricultural soil via sludge, fertilizers, herbicides, and disinfectants of seeds. High level of Hg²⁺ is highly phytotoxic to plant cells. Toxic level of Hg²⁺ can induce visible injuries and physiological disorders in plants [10]. The large input of mercury (Hg) into the arable lands has resulted in the widespread occurrence of mercury contamination in the entire food chain. Mercury is a unique metal due to its existence in different forms like HgS, Hg²⁺ and methyl-Hg. However, in agricultural soil, ionic form (Hg²⁺) is predominant [11]. Hg released to the soil, mainly remains in solid phase through adsorption onto sulfides, clay particles and organic matters. Increasing evidence has shown that Hg²⁺ can readily accumulate in higher and aquatic plants [12-14]. Mercurials can disturb almost any function in which critical or non-protected proteins are involved, including processes like light and dark reactions of photosynthesis, electron transport and Hill reaction.

Zinc (Zn) is an essential micronutrient required for several metabolic processes of plants [15], and has a long biological half-life. Zinc is second most abundant transition metal after iron in biological systems, is a component of thousands of proteins in plants. Zn toxicity in plants is far less widespread than its deficiency. The metal exhibits toxicity between 100-300 mg kg⁻¹ of leaf DW and the symptoms include reduced yield, stunted growth, and Fe-deficiency induced chlorosis through reduction in chlorophyll synthesis, chloroplast degranulation and interference with Mn, P, and Mg uptake. The phytotoxicity of Zn involves decrease in growth and development; and metabolism [16–18]. Photo oxidative damage caused by Zn in various plant species such as *Phaseolus vulgaris*[15] and *Brassica juncea* [19] have been documented.

Amylases are enzymes involved in starch breakdown in cotyledons of legume seeds [20]. Recent studies have shown that they play a major role in transitory breakdown of starch [21]. In addition, sugar availability plays an important role in plant growth and tolerance to abiotic stresses [22]. Decline in amylase activity levels with increasing concentration of cyanide and heavy metals such as Pb has been demonstrated in a number of plants [23, 24]. There are also reports of increased activity of amylase in the presence of heavy metal stress, resulting in elevated levels of total soluble sugars [25]. Acid phosphatases are ubiquitous in plants, animals, fungi, and bacteria. They catalyze the hydrolysis of Pi from a broad range of phosphate monoesters, and anhydrides. They act in the production, transport, and recycling of Pi which is crucial for cellular metabolism and bioenergetics. Induction of acid phosphatase under osmotic and salt stresses is known to maintain inorganic phosphate (Pi), which can be co-transported with proton. Reports have shown increased acid phosphatase activity under salt stress in *P. sativum* [26, 27].

Heavy metals like Cd are known to be toxic to photosynthesis, causing a decrease in the chlorophyll content. Cd and Al have been shown to inhibit chlorophyll biosynthesis and photosynthetic electron transport via water splitting enzyme in photosystem-II [28, 29]. Several reports have also indicated constancy of soluble sugars owing to inhibition of photosynthesis and stimulation of respiration rate by heavy metals, which seem to interact with reactive center in Rubisco [30].

In the present study, seedlings of horse gram were subjected to heavy metal stress with 3 different concentrations each of Cd, Hg and Zn ions, independently for 72 h to assess the toxicity of these heavy metals on horse gram. Effect of heavy metal stress on activities of metabolic enzymes; amylase and acid phosphatase were monitored by quantitative estimation and qualitative in-gel assays. Also, the effect of heavy metal stress on total soluble sugar and chlorophyll was monitored.

MATERIALS AND METHODOLOGY

Plant material

Seeds of *Macrotyloma uniflorum* (Horse gram) were surface sterilized with 0.1 % sodium hypochlorite for 20 s, and immediately washed with distilled water five times. The seeds were then sown in trays containing vermiculite, and irrigated daily with half strength Hoagland medium.

Heavy metals Stress treatment

Root samples of five days old horse gram seedlings were washed repeatedly with distilled water, divided into four groups and transferred to vessels containing half strength Hoagland media [31]. One of these four groups served as control and the remaining three groups were further subdivided into three groups each treated with three different concentrations of heavy metals ions independently as follows: 50, 100 and 500 ppm CdCl₂; 5, 10 and 20 ppm HgCl₂; and 100, 300 and 600 ppm ZnSO₄. Shoot samples were collected at 24, 48 and 72 h after application of stress and frozen at -20 °C until further analysis.

Preparation of crude extract

Frozen shoots were homogenized with 50 mM sodium phosphate buffer pH 7.5 containing 1mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM β-mercaptoethanol at 4°C. The homogenates were centrifuged at 5600 x g for 20 min and the supernatant was used as a crude extract of enzymes.

Assay of enzymes

AP activity was assayed according to the method described by Hojring & Svensmark [32] using p-nitrophenyl phosphate (pNPP) as a substrate. The assay mixture contained 50 mM sodium acetate buffer (pH 5.5), 5 mM pNPP and 100 µl of the homogenate, in a final volume of 0.5 ml. After 10 min incubation at 37 °C, the reaction was stopped by adding 1 ml of 0.2 M NaOH. Each unit of enzyme activity was expressed as micromoles of p-nitrophenol (pNP) released per minute. Amylase activity was assayed in vitro by estimating reducing sugar liberated using 3,5-DNS method [33]. The reaction mixture consisted 0.5 ml of 2% starch solution in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme extract. One unit of activity was expressed as the amount of enzyme that produced one micromole of maltose per minute. Soluble protein content was determined according to Lowry et al.,[34] using BSA as standard.

Electrophoretic analysis of enzymes

Non-denaturing discontinuous slab gel electrophoresis was carried out according to the method of Davis [35]. Crude extracts of enzyme in sample buffer containing 0.05% EDTA. Na was loaded to the gel and electrophoresed at 4°C.

Amylase isozyme

Amylase isozyme bands were visualized by soaking gels in 2% of soluble starch in 50 mM phosphate buffer, pH 7.0 for 30 min at room temperature. The gels were washed twice with distilled water and immersed in 0.025% iodine solution for 2 min to visualize clear bands against blue back-ground.

AP isozymes

After electrophoresis on 9% native gel, the gels were washed with distilled water, and equilibrated with 0.05 M acetate buffer, pH 5.5 for 2 min. The gel was then transferred to a mixture containing 1% 1-Sodium naphthyl phosphate in 0.05 M acetate buffer, pH 5.5 and coupling dye solution (0.2% fast blue-RR). After

development of activity bands (10-15 min), gels were washed with a mixture of 5% acetic acid and 10% methanol.

Estimation of TSS

Total soluble sugars were estimated using anthrone method [36]. 200 mg of anthrone was dissolved in 100 ml of 95 % ice-cold H₂SO₄. 100 mg of plant sample was ground in 5 ml of 2.5 N HCl and hydrolyzed by boiling for 3 h. The contents were neutralized by adding sodium bicarbonate powder until no more effervescence, made up to 100 ml with distilled water and centrifuged. 0.5 ml of the supernatant was heated with 4 ml of the anthrone solution for 10 min and was allowed to cool at room temperature. The intensity of blue-green color was measured at 625 nm. The total sugar contents were calculated using glucose as reference standard.

Estimation of TC

TC was determined according to the method described by Arnon [37]. 1.0 g of sample was used for extraction in 25 ml of 80% acetone, centrifuged at 5000 x g for 5 min. Supernatant was transferred to a 100 ml volumetric flask. The extraction was repeated many times until the residue became colorless. Volume was made up to 100 ml with 80% acetone. Absorbance of the extract was read at 645 and 663 nm against 80% acetone as blank. The total chlorophyll in the sample was calculated using the formula:

$$\text{Total chlorophyll} = 20.2 (A_{645}) + 8.02 (A_{663}) \times V / 1000 \times W$$

Where; V= final volume of the extract, W = fresh weight of the leaves. The values were expressed as mg chlorophyll/g of sample fresh weight.

Statistical analysis

All data expressed as means of triplicate experiments. Two-way ANOVA analysis performed using GraphPad Prism software ver. 6. (Differences in mean values were considered significant if at least, significant-difference value calculated from the pooled variance exceeded $p < 0.05$., accepted as significant).

RESULTS AND DISCUSSIONS

Enzyme activity

Amylase activity in horse gram stressed with Cd showed a concentration dependent decrement (Fig. 1 A). During the initial 24 h of exposure, 50 ppm of Cd had no effect on amylase, while 100 and 500 ppm caused significant ($p < 0.0001$) and ($p < 0.001$) reduction in concentration dependent manner. With extended exposure of 72 h, 50 ppm Cd caused ~ 70% reduction in enzyme levels, while 100 and 500 ppm caused > 90% reduction. The observed reduction in amylase level is in agreement with those obtained for sorghum under Cd stress [38].

Hg, the most toxic heavy metal to horse gram caused anomalous variation in amylase activity. At lower level of 5ppm, the metal caused a slight decrease in amylase activity at 24 h of exposure. However, at extended period of 48 and 72 h (Fig. 1B), the same concentration of metal caused a considerable increase in amylase levels. 10 and 20 ppm Hg had conspicuous effect on

amylase activity, wherein the former had better inhibitory effect than the latter. As Zn ion is considered as a micronutrient for plants, elevation in amylase levels are on the expected lines (Fig. 1C). Although the positive effect on amylase was concentration and time dependent, the raise in enzyme levels was not commensurate with increment in metal concentration or time of exposure. While similar enhancement in amylase activity was observed in horse gram seedlings subjected to salinity stress [39], they declined under drought [40].

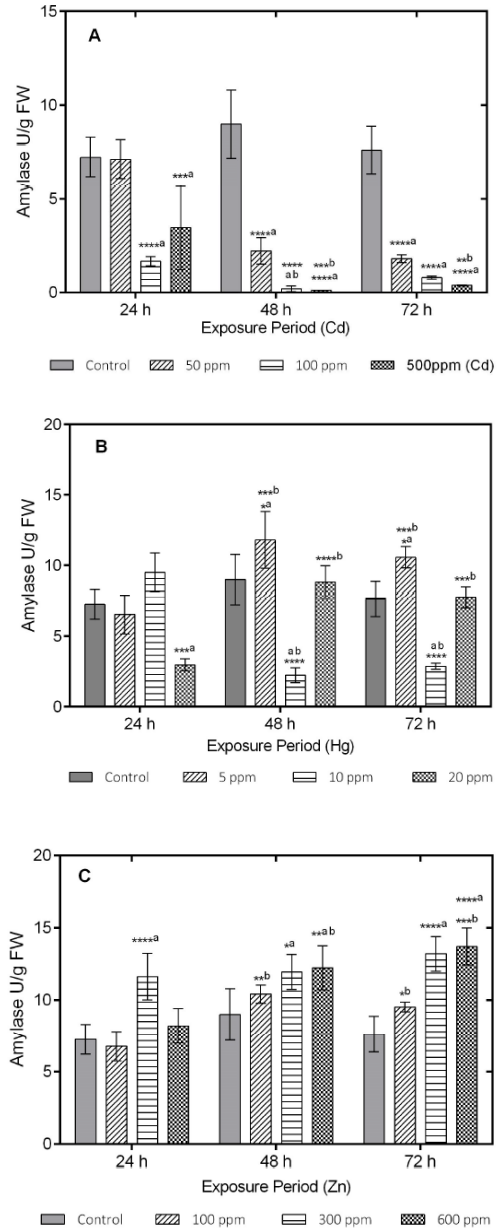


Figure 1. Levels of amylase activity in Horse gram seedlings exposed to different concentrations of Cd, Hg, and Zn ions for various durations of time. (A) Represent the activity after 24h; 48h and 72h subjected to Cd ions include (50, 100 and 500 ppm of CdCl₂). (B) Represent the activity of amylase after 24h; 48h and 72h subjected to Hg ions include (5, 10 and 20 ppm of HgCl₂). (C) Represent the activity after 24h; 48h and 72h subjected to Zn

ions include (100, 300 and 600 ppm of ZnSO₄). Data plotted are mean ± SD of duplicate of three separate replicates, means value were compared with control by using two-way ANOVA analysis. (Dunnett's multiple comparisons test) (p < 0.05 consider a significant). [* = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001. a: significant against control; b: against 24 h, c: against 48 h]

As major players in energy metabolism, acid phosphatases (AP) are widely distributed in plants, and are involved in hydrolysis of variety of phosphate esters (Duff et al., 1994). AP levels in horse gram treated with different heavy metal ions fluctuated in a metal-specific manner, with general progressive decrease with increasing concentration and time of exposure (Fig. 2). All three concentrations of Cd ions (50, 100, 500 ppm) caused significant reduction of AP activity over entire period of exposure (Fig. 2A), except 50 ppm Cd, at 24h, which did not cause any significant change. Similar inhibition of acid phosphatase by Cd was reported in *Pfaffia glomerata* [41]. There are also reports of AP activities unaffected by Cd stress [42]. All three different concentrations of Hg ions (5, 10 and 20 ppm) caused significant inhibitory effects on the activity of AP during entire exposure period (Fig. 2B). AP Activity showed different trend in response to Zn concentration. At 100 ppm, Zn positively affected AP activity with significant elevation at all exposure durations (Fig. 2C). Higher concentrations of 300 and 600 ppm of Zn caused a decrease in AP activity during all exposure time with the latter causing stronger effect. It can be suggested that at lower concentration, Zn facilitates induction of AP, and, exhibits toxic effects only at higher levels.

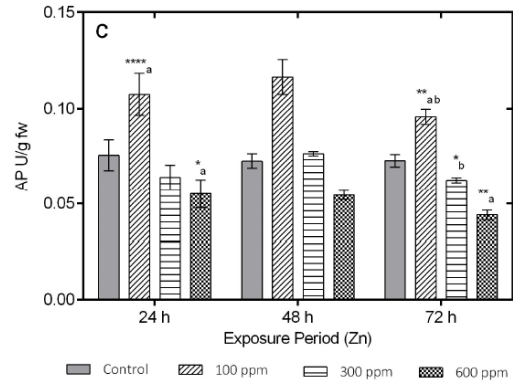
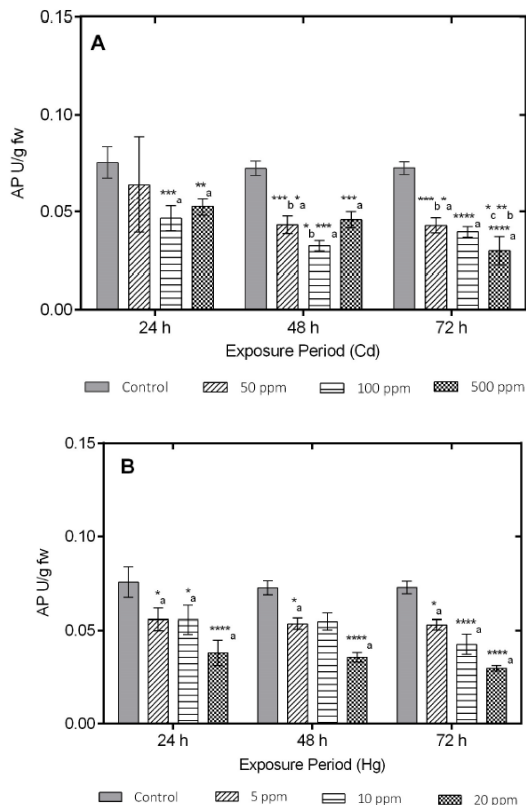


Figure 2. Levels of AP activity in Horse gram seedlings exposed to different concentrations of Cd, Hg, and Zn ions for various durations of time. (A) Represent the activity after 24h; 48h and 72h subjected to Cd ions include (50, 100 and 500 ppm of CdCl₂). (B) Represent the activity of amylase after 24h; 48h and 72h subjected to Hg ions include (5, 10 and 20 ppm of HgCl₂). (C) Represent the activity after 24h; 48h and 72h subjected to Zn ions include (100, 300 and 600 ppm of ZnSO₄). Data plotted are mean ± SD of duplicate of three separate replicates, means value were compared with control by using two-way ANOVA analysis. (Dunnett's multiple comparisons test) [* = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001. a: significant against control; b: against 24 h, c: against 48 h]

The activation of AP by Zn in horse gram is in agreement with observations of Blum and Schwedt [43]. Phosphatases are generally metalloenzymes, which depend on Ca²⁺ or Mg²⁺ for activity. Hence, it can be suggested that the toxicity of Cd, Hg and Zn at high concentrations might interfere with the Pi- binding site, which impair the mobilization of phosphate. Increased activity of AP under other abiotic stresses such as drought and salinity and its decline under heavy metal stress also indicated direct interference of metal ions with catalytic function.

In-gel assay patterns of amylase isozyme in control exhibited a major isozyme and four minor isoforms (Fig 3). In general, heavy metal stress did not alter the isozyme pattern, except altered intensities. Apart from weak inhibition at 24 h by 100 ppm, which correlated to in vitro units, Cd caused inhibition of most isozyme bands after 48, 72 h (Fig.3 B, C). The isozyme pattern of amylase in presence of Hg were not affected, intensities roughly matched in vitro assay units. About 20 ppm Hg reduced the intensity of isozyme bands after 24 h of exposure. However, 10 ppm Hg was most affective on amylase isozyme after 48 and 72h. Unlike Cd and Hg, Zn stress caused intensification of major constitutive form of enzyme and appearance of two minor bands in presence of 300 and 600 ppm, and extended time of exposure (Fig. 3 A, B, C). Thus, Zn caused not only increased intensity of the major isozyme, it also induced expression of new isoforms of amylase (Fig. 3 B, C). These variations of amylase patterns indicated that horse gram amylase is affected by different heavy metal stresses.

Similar variations in horse gram amylase isozymes have been observed under heat and salt stress [40], in addition drought stress in vivo and in vitro [39]. Electrophoretic patterns of AP

exhibited four isoforms in control plants (Fig 4). None of the metals caused any significant alteration in AP isozyme pattern in horse gram, except decrease in band intensities with increasing metal concentrations and time of exposure (Fig 4 A, B, C). Higher concentration of Cd (500 ppm) inhibited most AP isozymes during 48 and 72 h. On the other hand, 100 and 300 ppm of Zn caused enhancement of band intensity, especially, after 72 h, similar to in vitro assay units (Fig 4 B, C). These suggested that AP is highly sensitive to the amount of heavy metals in plant tissues. On the other hand, horse gram seedlings exhibited similarity in the number of AP isoforms that were reported under drought stress [39].

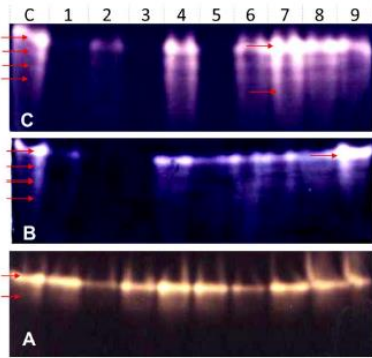


Figure 3. Native PAGE of Amylase isoenzymes; 150 µg protein of crude extract were loaded from each sample of different durations of exposure time were electrophoresed and stained. Panel (A) represent the activity after 24h, (B) represent activity after 48h and 18 (C); after 72h. For all panels (A), (B) and (C) the numbers assigns each lane defines as follows: C= Control; 1, 2, 3 represent samples stressed by Cd ions; 1= 50 ppm; 2= 100 ppm and 3=500 ppm of CdCl₂. The numbers 4, 5, & 6 represent samples stressed by different concentrations of Hg ions; 4= 5 ppm, 5= 10 ppm and 6= 20 ppm of HgCl₂. The numbers 7, 8 & 9 represents samples were subjected to Zn ions include; 7= 100 ppm, 8=300 ppm and 9=600 ppm.

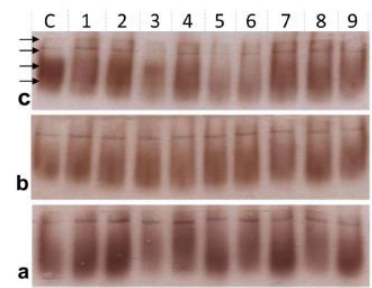


Figure 4. Native PAGE of AP isoenzymes. 100 µg protein of crude extract were loaded from each sample of different durations of exposure time were electrophoresed and stained. Panel (A) represent the activity after 24 h, (B) represent activity after 48h and (C); after 72h. For all panels (A), (B) and (C) the numbers assigns each lane defines as follows: C= Control; 1, 2, 3 represent samples stressed by Cd ions; 1= 50 ppm; 2= 100 ppm and 3=500 ppm of CdCl₂. The numbers 4, 5, & 6 represent samples stressed by different concentrations of Hg ions; 4= 5 ppm, 5= 10 ppm and

6= 20 ppm of HgCl₂. The numbers 7, 8 & 9 represents samples were subjected to Zn ions include; 7= 100 ppm, 8=300 ppm and 9=600 ppm.

Results of total chlorophyll content in horse gram seedling exposed to different concentrations of Cd ions did not show any significant variations during 24 h and 72 h of exposure. However, at 48 h there was a concentration dependent decrease in chlorophyll content with Cd and Hg, and increase with Zn (Table 1). Hg stress imposed on horse gram had a marginal inhibitory effect on total chlorophyll throughout the period of exposure (Table 1.) Zn stress on the other hand at 24 h of exposures exhibited positive influence on total chlorophyll ($p < 0.0001$). Extended periods of exposure however caused reduction in chlorophyll content (Table 1).

Table 1. Levels of Total chlorophyll in horse gram seedlings exposed to different amounts of Cd, Hg and Zn ions

		Total Chlorophyll (mg g ⁻¹ FW)			
		24 h	48 h	72 h	
Control		2.32 ± 0.02	2.28 ± 0.019	2.37 ± 0.055	
	Cd ²⁺ (ppm)	50	2.87 ± 0.66	1.93 ± 0.335	2.02 ± 0.301
		100	2.75 ± 0.0398	1.80 ± 0.025	2.28 ± 0.023
500		2.68 ± 0.025	1.38 ± 0.019	2.14 ± 0.061	
Hg ²⁺ (ppm)	5	2.17 ± 0.46	1.82 ± 0.159	1.71 ± 0.246	
	10	1.12 ± 0.021****a	1.57 ± 0.025	2.16 ± 0.022	
	20	1.12 ± 0.023****a	1.57 ± 0.031	1.83 ± 0.022	
Zn ²⁺ (ppm)	100	3.51 ± 0.025****a	1.91 ± 0.225	1.38 ± 0.232	
	300	3.51 ± 0.025****a	2.89 ± 0.067	2.13 ± 0.024	
	600	3.74 ± 0.024****a	2.96 ± 0.028	2.63 ± 0.029	

ppm= part per million, h = hour, FW = fresh weight.

Values are means ± SD of three replicates from two separate set of treated seedlings, results were considered significant if they showed ($P < 0.05$) at least, by using Two ways ANOVA analysis. (Dunnett's multiple comparisons test) comparing to the control group. [*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. a: significant against control; b: against 24 h, c: against 48 h]

The TC levels observed under Hg stress in horse gram was similar to those reported for Phaseolus seedlings [44], wherein high concentrations of Hg caused a decrease in chlorophyll content. Reduction in chlorophyll contents under metal stress have also been reported in other plants [45], which have indicated differing effects of Hg and Zn. Deleterious influences of heavy metals on the export of carbohydrates from their source and their distribution in sink tissues have been reported in adult plants [19]. Heavy metal caused significant restriction in reserve mobilization such as soluble sugars and amino acids in pea seeds. Measurement of Total soluble sugar content in horse gram seedlings indicated no significant restriction in presence of any of the metal used, except a 47 % reduction at 600 ppm Zn after 72 h of exposure (Table 2), suggesting no interference of heavy metals in mobilization of sugars.

Table 2. Levels of Total soluble sugars in horse gram seedlings exposed to different amounts of Cadmium, Mercury and Zinc ions

		Total soluble sugars (mg g ⁻¹ FW)		
		24 h	48 h	72 h
Control		6.07 ± 0.1109	5.34 ± 0.0793	4.98 ± 0.0520
Cd ²⁺ (ppm)	50	6.47 ± 0.12****a	6.2 ± 0.05****a	4.33 ± 0.07****a
	100	6.49 ± 0.125****a	5.2 ± 0.04	6.38 ± 0.06****a
	500	6.54 ± 0.137****a	6.6 ± 0.042****a	6.92 ± 0.059****a
Hg ²⁺ (ppm)	5	6.63 ± 0.12 ^a	6.48 ± 0.0450****	5.03 ± 0.09
	10	6.65 ± 0.125 ^a	6.29 ± 0.485****	5.31 ± 0.08
	20	6.44 ± 0.175	6.29 ± 0.485****a	5.34 ± 0.05
Zn ²⁺ (ppm)	100	6.51 ± 0.109 ^a	5.64 ± 0.485	6.54 ± 0.06****a
	300	6.40 ± 0.105	5.44 ± 0.05	5.28 ± 0.06
	600	6.28 ± 0.184	5.31 ± 0.052	2.34 ± 0.06****a

ppm= part per million, h = hour, FW = fresh weight.

Values are means ± SD of three replicates from two separate set of treated seedlings, results were considered significant if they showed (P < 0.05) at least, by using Two ways ANOVA analysis. (Dunnett's multiple comparisons test) comparing to the control group. [*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001. a: significant against control; b: against 24 h, c: against 48 h]

From the above results, it is concluded that Hg caused the highest toxicity, while the least toxic effects were due to Zn ions. Amylase activity fluctuated in metal-specific manner with greater fall in presence of Cd, and enhancement in presence of Zn. Acid phosphatase levels showed declining trend in response to all three heavy metal stresses, albeit a marginal raise in presence of 100 ppm of Zn. The heavy metal stress did not exhibit any clear pattern of influence on total soluble sugars and chlorophyll, except slight negative impact by Cd and Hg, and a negligible increment due to Zn at early period of stress. The plant seems to possess metal-specific response/ detoxification mechanisms, can withstand up to 100 ppm Cd, 5ppm Hg and 100 ppm Zn, as evidenced by varying response exhibited against these metals.

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