ISSN 0975-6299 Vol 3/Issue 2/April – June2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIO CHEMISTRY

INFLUENCE OF COPPER AND ZINC STRESS ON PROTEIN METABOLISM IN *VIGNA MUNGO* (L.) HEPPER

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ABSTRACT

The study was performed to assess the effect of copper and zinc ions on *Vigna mungo* (L.) Hepper grown hydroponically for seven days. A significant reduction was observed in free amino acid and protein levels of seedlings. Moreover, copper and zinc exposure resulted in apparent increase in specific activity of protease enzyme which was manifolds in comparison to control. The molecular response of the plant was studied by comparing the protein pattern of treated seedlings with control seedlings. SDS-PAGE of crude protein extract showed absence of some protein band in the region between 31.0 KD – 66.2 KD due to copper and zinc stress in comparison to control.

KEYWORDS

Heavy metal stress; seedling growth; hydrolysis; translocation; protein banding pattern.

INTRODUCTION

All living organisms have to survive in the available conditions. There are many records that agricultural land adjacent to industrial areas are polluted to varied extent by many toxic heavy metals. Different plant absorbs toxic and non-toxic metals from soil and water to varied extent and accumulates in different body parts. So, heavy metal stress is a serious intimidation to agriculture. Copper and zinc metals are essential for normal plant growth and development as they serve as structural and functional components of specific proteins. The plants are endowed with an inherent capability of tolerating toxic metals to some extent. Metal ions turn toxic as soon as their concentration exceeds a metal specific threshold which varies strongly among plant species and ecotypes and also with metal properties[26]. Seed germination is the first interface of material exchange between plant development cycle and environment. The subsequent growth of embryonic axis is a key step of the plant life, which is highly sensitive to the surrounding medium fluctuation[5]. Thus, alterations in this system would impair seed germination as well as seedling growth. Metal induced changes in the development of plants are the result of either a direct and immediate impairment of metabolism or signaling processes that initiate adaptive or toxicity responses that need to be considered as active processes of the organism[11,9]. Hydrolyzing enzymes play a major role in the mobilization of food reserves by hydrolyzing complex biomolecules. The elevated level of heavy metals in plants may suppress the metabolism and translocation of reserve material to the growing regions and their subsequent utilization. Thus, heavy metals at supra-optimal concentrations affect the agronomic traits of plants[28]. It is very important to know which heavy metal and in what concentration they will be toxic to the plants in order to assess optimal growth on more or less contaminated soils. This can be achieved by comparative investigations of the effect of heavy metals at the biochemical, physiological and molecular levels. Recently, the molecular and physiological basis for plant interaction with heavy metals has attracted considerable interest. So, the response of heavy metal stress was assessed by performing a detailed examination of the effect of copper and zinc on the expression of low molecular weight proteins. Thus, the present study is an attempt to explore a possible relationship between copper and zinc metal induced biochemical and physiological changes in *Vigna mungo* (L.) Hepper.

MATERIALS AND METHODS

Source – Seeds of *Vigna mungo* (L.) Hepper cv. T-9 (Black Gram) was obtained from National Seed Corporation Unit, I.A.R.I., New Delhi.

Growth Conditions- Healthy seeds of uniform size were sorted and sterilized with 0.1% $HgCl_2$ solution for 5 min. and washed with distilled water. Then, seeds were germinated in petriplates containing Whatman filter paper No. 1, moistened with Arnon and Hoagland media (Control). Copper metal was added to the nutrient solution at concentration 0.05, 0.1 and 0.2 mM as $CuSO_4.5H_2O$. Zinc metal was added as 0.25, 0.50, 1.00 and 1.50 mM of $ZnSO_4.7H_2O$. And, a mixture of both salts was added to study the interaction of both metals at the concentrations mentioned above. Sterile conditions were maintained by adding 20µg/ml of streptomycin sulphate in the medium to

suppress microbial growth. All experiments were carried out for 7 days at 28±2^{0}C in dark. At regular interval of time required number of seeds were withdrawn and used for analysis of various parameters.

Heavy metal assessment - Heavy metals concentration was determined by using atomic absorption spectrophotometer (Z-6100, Hitachi) by the method (EPA method 3050) as outlined by Gupta[6] .

Extraction and estimation of protein and free amino acid- Protein was estimated by the Bradford method[4]. And, free amino acids were assayed by the procedure described by Moore and Stein[19].

Enzymatic Assay- On the indicated days, seedlings were taken and washed thoroughly with Distilled water and then used. The extraction media and assay procedure for protease enzyme was as described by Beever[3].

SDS-PAGE- It was performed by the method as described by Laemmli[15].

Statistical Analysis- Statistical analysis was done by using Microsoft excel and Graphpad prism 5.0 software. All the experiments were conducted in triplicates. The obtained data were statistically analysed for the mean ±SD and difference between the control and treated plants were analyzed by one way ANOVA taking p≤ 0.05 as significant level according to Dunnett's multiple comparison test. Percent Variance was calculated by Two-way ANOVA to determine the extent up to which treatment conditions and time period are responsible for the occurrence of variations in values for different parameters.

RESULTS AND DISCUSSION

Uptake and accumulation of zinc and copper metal ions in cotyledons and embryonic axis of Vigna mungo (L.)

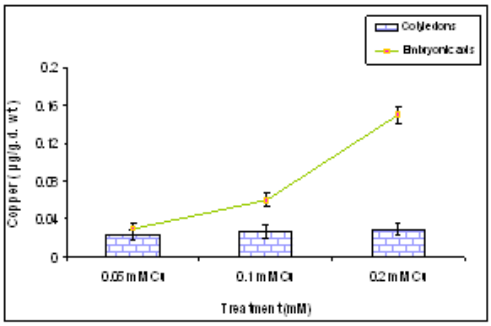
Seedlings grown at 0.05mM, 0.1mM and 0.2mM Cu have shown 0.03, 0.06 and 0.15 µg/g DW increase in copper content in embryonic axis over control as shown in **figure-1a**. Whereas, the accumulation of zinc content in embryonic axis at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM was 83.19, 90.12, 124.79 and 231.14 µg/g DW over control as given in **figure-1b**. With the increase in concentration from 0.25 to 1.50 mM zinc, a 2.7 folds increase in zinc uptake has occurred in embryonic axis of *Vigna mungo* (L.).

In cotyledons, there was only 1.2 fold increase in copper content with the increase in copper ions concentration from 0.05 to 0.2mM in the nutrient medium whereas zinc ions have caused 1.63 fold increase in zinc uptake in cotyledons from 0.25mM to 1.50mM concentration. In case of combined treatment, copper and zinc ions accumulation was less in 0.05mM Cu + 0.25 mM Zn and 0.1 mM Cu + 0.25 mM Zn treated seedlings in comparison to individual metal treatment. However, at the high concentration of combined metal ions stress, accumulation of both metals were very high in cotyledons as well as in embryonic axis as shown in **figure- 2a and 2b**.

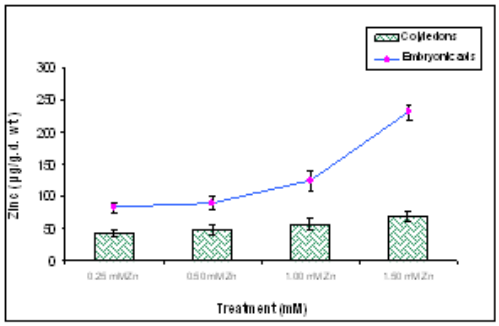
Reduced zinc and copper content in cotyledons in comparison to embryonic axis could be attributed to the differential permeability of the seed coat during water imbibition. It can avoid over-accumulation of contaminants in the cotyledons which resulted in lowered toxicity. Therefore, the behavior of seed germination should not be considered with respect to heavy metal doses in the nutrient medium, but with respect to the accumulation and compartmentation of heavy metals at the cellular and sub-cellular levels of *Vigna mungo* (L.) seedlings.

Figure-1(a and b)

Accumulation of copper metal ions (a) and zinc metal ions (b) inVigna mungo (L.) seedlings



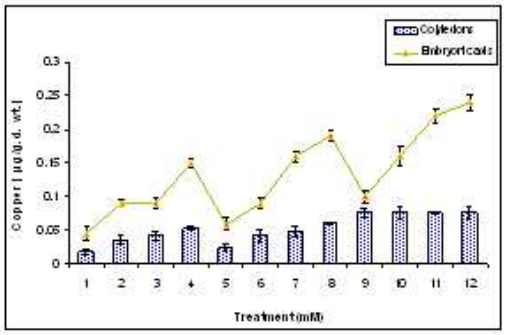
(1a)



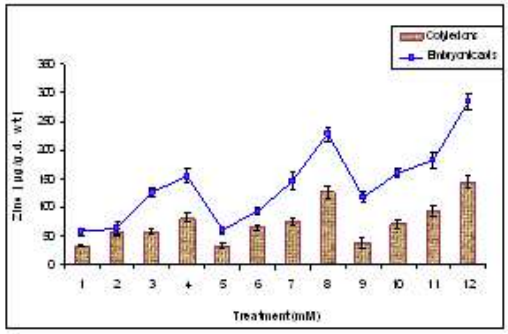
(1b)

Figure-2(a,b)

Effect of copper and zinc metal ions combined stress on copper and zinc content in Vigna mungo (L.) seedlings



(2a)



(2b)

Treatment conditions

(1) 0.05 mM $CuSO_4$+ 0.25 mM$ZnSO_4$ (2) 0.05 mM $CuSO_4$+ 0.50 mM$ZnSO_4$ (3) 0.05 mM $CuSO_4$ + 1.00 mM$ZnSO_4$ 4) 0.05 mM $CuSO_4$ +1.50 mM$ZnSO_4$ (5) 0.1 mM$CuSO_4$ + 0.25mM $ZnSO_4$ (6) 0.1 mM$CuSO_4$ + 0.50mM $ZnSO_4$ (7) 0.1 mM$CuSO_4$ + 1.00mM $ZnSO_4$ (8) 0.1 mM$CuSO_4$ + 1.50mM $ZnSO_4$ (9) 0.2 mM$CuSO_4$ + 0.25mM $ZnSO_4$ (10) 0.2 mMCuSO4 + 0.50mM ZnSO4 (11) 0.2 mMCuSO4 + 1.00mM ZnSO4 (12) 0.2 mMCuSO4 + 1.50mM ZnSO4

Effect of zinc and copper metal ions on protein metabolism in *Vigna mungo* (L.) seedlings

Protein content - The protein content declined in cotyledons and increased in embryonic axis with the onset of germination (1-7th day). The protein content was greater in cotyledons of seedlings exposed to copper and zinc stress in comparison to control seedlings as shown in **table-1**. In one day old seedlings, protein content was found 20.86%, 40.48% and 65.95% greater in the presence of 0.05mM, 0.1mM and 0.2mM concentration of copper ions as compared to control. Whereas, zinc ions had caused 7.02%, 17.85%, 22.83% and 37.47% accumulation of proteins in cotyledons at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM respectively.

At 0.05mM Cu + 0.25mM Zn, 0.1mM Cu + 0.25mM Zn and 0.2mM Cu + 0.25mM Zn the protein content was 30.40, 34.56 and 34.93 mg/g FW respectively. It was 5.19%, 19.58% and 20.86% more than that which was observed under control conditions. In case of 0.05mM Cu + 1.50mM Zn, 0.1mM Cu + 1.50mM Zn and 0.2mM Cu + 1.50mM Zn, 40.00%, 53.97% and 57.09% accumulation in protein content was noticed respectively. This trend showed that protein accumulation was more at high metal concentrations. In seven day old seedlings the protein content was 10.70 mg/g FW under control conditions. The protein content was 13.50, 17.30 and 20.33 mg/g FW at 0.05mM,

0.1mM and 0.2mM copper ion concentration. Zinc ions stress also led to accumulation of protein in cotyledons. In case of combined metal ions treatment, the accumulation of proteins in cotyledons was more as compared to individual copper and zinc ions treatment conditions. It reached up to 197.19% in case of 0.2mM Cu + 1.50mM Zn followed by 0.1mM Cu + 1.50mM Zn which resulted in 192.14% protein accumulation in cotyledons of *Vigna mungo*(L.).

A marked decrease in the protein content was observed in embryonic axis with an increase in zinc and/or copper concentration. In one day old seedlings, copper treatment at the concentration of 0.05mM, 0.1mM and 0.2mM resulted in 16.93%, 37.68% and 50.14% decrease in protein content. Whereas zinc ions have caused 14.51%, 31.08% 55.05% and 68.28% reduction in protein level of embryonic axis at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM respectively. The trend was continued up—to seven days. Among individual metal treatment conditions the effect of copper ions was more severe in comparison to zinc ions. On 7th day, the combined treatment of copper and zinc has caused 30.96%, 42.96% and 48.90% reduction in total proteins at the concentration of 0.05mM Cu + 0.25mM Zn, 0.1mM Cu + 0.25mM Zn and 0.2mM Cu + 0.25mM Zn. Further increase in metal concentration caused a consistent reduction in protein content in embryonic axis. There were 55.55%, 64.57% and 67.28% decrease in protein content at the concentration of 0.05mM Cu + 1.50mM Zn, 0.1mM + 1.50mM Zn and 0.2mM Cu + 1.50mM Zn in comparison to control.

Table-1

Effect of zinc and copper metal ions on protein content (mg/ g.f.wt.$^{-1}$)in Vigna mungo (L.) seedlings

Concentration		In cotyledons				In embryonic axis			
		Days after imbibition				Days after imbibition			
$CuSO_4.5H_2O$ (mM)	$ZnSO_4.7H_2O$ (mM)	1	3	5	7	1	3	5	7
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
0.0	0.0	28.29 ± 0.45	25.46 ± 0.65	17.36 ± 1.05	10.70 ± 0.50	13.64 ± 1.73	18.00 ± 0.57	22.96 ± 0.11	29.16 ± 0.57
0.05	0.0	34.93 ± .85[a]	28.63 ± 0.55[a]	21.50 ± 0.60[a]	13.50 ± 0.65[a]	11.33 ± 0.40[b]	14.86 ± 0.72[a]	15.16 ± 0.36[a]	20.40 ± 0.36[a]
0.1	0.0	40.60 ±0.55[a]	32.23 ± 0.65[a]	28.50 ± 0.50[a]	17.30 ± 0.65[a]	8.50 ± 0.60[a]	9.80 ± 0.65[a]	11.70± 0.50[a]	15.46 ± 0.55[a]
0.2	0.0	47.96 ± 0.61[a]	38.96 ± 0.70[a]	31.46 ± 0.85[a]	20.33 ± 0.85[a]	6.80 ± 0.65[a]	7.40 ± 0.55[a]	9.33 ± 0.75[a]	12.40 ± 0.75[a]
0.0	0.25	30.93 ± 0.75[a]	27.00 ± 0.80	20.63 ± 0.55[a]	13.03 ± 0.35[a]	11.66 ± 0.65[c]	15.83 ± 0.70[a]	17.90 ± 0.70[a]	22.66 ± 0.55[a]
0.0	0.50	34.06 ± 0.70[a]	31.36 ± 0.66[a]	24.86 ± 0.66[a]	17.46 ± 0.35[a]	9.40 ± 0.56[a]	8.26 ± 0.50[a]	12.36 ± 0.47[a]	18.00 ± 0.25[a]
0.0	1.00	35.50 ±0.81[a]	32.46 ± 0.90[a]	28.93 ± 0.61[a]	20.83 ± 0.32[a]	6.13 ± 0.65[a]	6.83± 0.30[a]	9.73 ± 0.25[a]	13.00 ± 0.25[a]
0.0	1.50	39.73 ± 0.90[c]	33.13 ± 1.00[a]	30.43 ± 0.55[a]	23.63 ± .035[a]	4.33 ± 0.55[a]	5.40 ± 0.45[a]	7.83 ± 0.60[a]	10.56 ± 0.45[a]
0.05	0.25	30.40 ± 0.70[a]	25.86 ± 0.55	24.56 ± 0.50[a]	18.13 ± 0.55[a]	8.70 ± 0.55[a]	13.20 ± 0.65[a]	14.46± 0.45[a]	20.13 ± 0.50[a]
0.05	0.50	33.53 ± 0.70[a]	31.70 ± 0.70[a]	27.63 ± 0.55[a]	22.63 ± 0.45[a]	8.36 ± 0.41[a]	9.50± 0.65[a]	12.76 ± 0.50[a]	15.53 ± 0.70[a]
0.05	1.00	37.70 ±0.80[a]	35.26 ± 0.60[a]	29.23 ± 0.40[a]	25.83 ± 0.50[a]	8.03 ± 0.60[a]	8.53 ± 0.55[a]	11.56 ± 0.45[a]	14.50 ± 0.45[a]
0.05	1.50	40.46 ± 0.65[a]	37.33 ± 0.70[a]	34.13 ± 0.55[a]	29.80 ± 0.35[a]	6.76 ± 0.70[a]	7.63 ± 0.55[a]	10.40 ± 0.55[a]	12.96 ± 0.65[a]
0.1	0.25	34.56 ± 0.85[a]	28.80 ± 0.75[a]	25.36 ± 0.55[a]	19.13 ± 0.30[a]	8.63 ± 0.75[a]	10.86 ± 0.50[a]	14.16 ± 0.50[a]	16.63 ± 0.50[a]
0.1	0.50	38.53 ± 0.55[a]	33.50 ± 0.60[a]	29.86 ± 0.45[a]	24.93 ± 0.30[a]	7.73± 0.65[a]	8.91± 0.60[a]	10.53 ± 0.65[a]	13.33 ± 0.50[a]
0.1	1.00	41.06 ±0.90[a]	36.80 ± 0.47[a]	32.23 ± 0.60[a]	27.53 ± 0.45[a]	6.87 ± 0.55[a]	8.01± 0.65[a]	8.70 ± 0.45[a]	11.23 ± 0.55[a]
0.1	1.50	44.50 ± 0.65[a]	38.83 ± 0.70[a]	35.53 ± 0.70[a]	31.26 ± 0.55[a]	6.48 ± 0.60[a]	7.76 ± 0.60[a]	8.33± 0.75[a]	10.33 ± 0.85[a]
0.2	0.25	34.93 ± 0.60[a]	29.53 ± 0.70[a]	25.70 ± 0.86[a]	20.76 ± 0.70[a]	8.30 ± 0.70[a]	9.50 ± 0.55[a]	13.90 ± 0.70[a]	14.90 ± 0.75[a]
0.2	0.50	38.66 ± 0.70[a]	35.80 ± 0.91[a]	30.40 ± 0.55[a]	25.41 ± 0.50[a]	7.03 ± 0.65[a]	8.36 ± 0.51[a]	9.28 ± 0.70[a]	12.50 ± 0.75[a]
0.2	1.00	43.40 ±0.65[a]	38.46 ± 0.70[a]	33.13 ± 0.56[a]	28.46 ± 0.35[a]	6.83 ± 0.55[a]	7.20 ± 0.70[a]	8.12 ± 0.32[a]	10.30 ± 0.65[a]
0.2	1.50	45.40 ± 0.80[a]	41.83 ± 1.10[a]	36.10 ± 0.66[a]	31.80 ± 0.35[a]	6.13 ± 0.20[a]	6.86 ± 0.55[a]	7.63 ± 0.60[a]	9.54 ± 0.45[a]

Source of variation		% of total variation	Source of variation		% of total variation
Interaction	-	04.53	Interaction	-	06.77
Time	-	39.38	Time	-	57.08
Treatment	-	55.61	Treatment	-	34.97

Mature Vigna mungo seed was found to contain 44.29µg of proteins
1) Above values are averages of three replicates ± SD
2) a, b and c indicates the values that differ significantly from the control at p≤ 0.001, p≤ 0.01 and p≤ 0.05 level respectively.

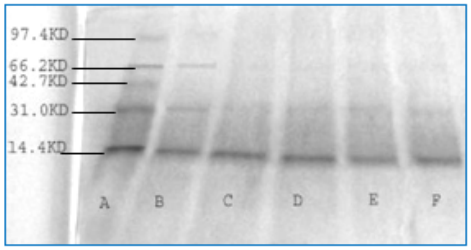
Using a proteomic approach the effect of zinc and/or copper ions on the protein profile of *Vigna mungo* (L.) seedlings was also investigated. Proteins were extracted from embryonic axis and separated by SDS-PAGE. Protein profiling yielded some visible differences in the protein banding pattern of the control versus copper and/or zinc treated seedlings as shown in **plates: 1-3**. The electrophoretic patterns of buffer soluble proteins showed that the number of bands corresponding to zinc and/or copper treatment was less than those of control. There were three bands having molecular weight - 14.4 KD, 31.0 KD and 66.2 KD in control. Furthermore, in case of copper and/or zinc pronounced variations were found in the region between 31.0 KD – 66.2 KD. Copper stress at various studied concentrations has caused inhibition of 31.0 KD and 66.2KD protein bands whereas zinc stress led to the inhibition of 66.2 KD protein band.

Moreover, it was also noticed that the 31.0 KD protein band appeared less intense under zinc treatment at all studied concentrations in comparison to control. However, the combined effect of copper and zinc has shown the absence of 31.0 KD protein band and bands of 14.4 KD, and 66.2 KD had no appreciable change in the color intensity. 14.4 KD band is present in all samples, whether it has been grown under individual or combined treatment of copper and zinc metal. So, protein banding pattern of copper and/or zinc treated seedlings suggested that low molecular weight proteins of about 14.4 KD are involved in the adaptive tolerance mechanism that respond to copper and/or zinc toxicity in *Vigna mungo* (L.) seedlings.

These results are in general agreement with John et al. 2009[10]; Hamid et al. 2010[7], who found a decrease in total protein content due to heavy metal stress. However, these results are contradictory to the findings of Zhang et al. (2009)[30] and Maheshwari and Dubey (2008)[16] as they have recorded an increase in total protein content in seedlings of *Oryza sativa* (L.) under copper and nickel stress respectively because heavy metal stress has been shown to induce a variety of stress proteins resulting in an overall increase in protein content[25].The decrease in total protein content may be attributed to the – (1) reduction in nitrogen content under severe heavy metal stress[2], which might have caused the decrease in *de novo* synthesis of amino acids. (2) Retardation in the assembly of amino acids into proteins which in turn affects the rate of protein synthesis[12]. (3) Enhanced protein degradation process as a result of increased protease activity that has been recorded to get increased under stressed conditions[20]. (4) It may be possible that heavy metal may have induced fragmentation of proteins due to the toxic effect of reactive oxygen species, which led to the reduction in total protein content[10].

Plate-1

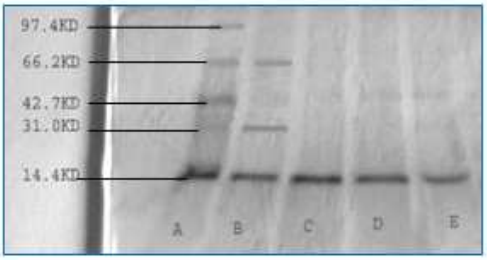
Changes in root protein banding pattern of Vigna mungo grown under zinc stress.



A - Marker
B- Control
C - 0.25mM Zn
D- 0.50mM Zn
E- 1.00mM Zn
F- 1.50mM Zn

Plate-2

Changes in root protein banding pattern of Vigna mungo grown under copper stress.



A - Marker
B- Control
C- 0.05 mM Cu
D- 0.1 mM Cu
E- 0.2 mM Cu

Plate-3 (a,b,c)

Changes in root protein banding pattern of Vigna mungo grown under copper and zinc combined stre

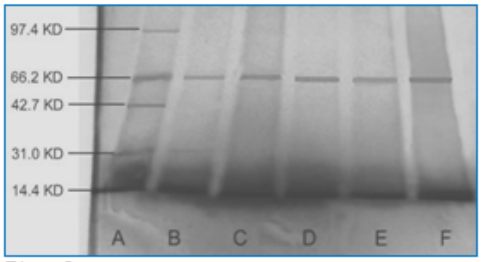


Plate-3a

A – Marker
B- Control
C- 0.05mM Cu + 0.25mM Zn
D- 0.05mM Cu + 0.50mM Zn
E- 0.05mM Cu + 1.00mM Zn
F- 0.05mM Cu + 1.50mM Zn

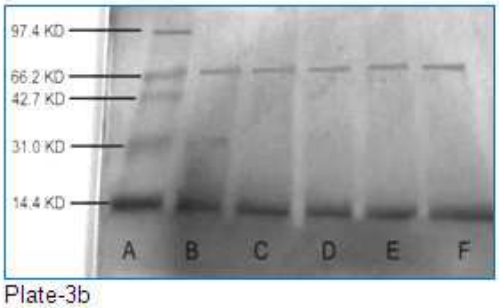
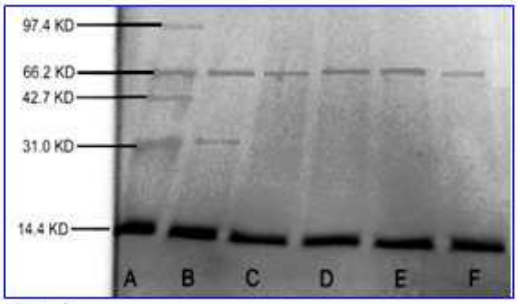


Plate-3b

A – Marker
B- Control
C- 0.1 mM Cu + 0.25mM Zn
D- 0.1 mM Cu + 0.50mM Zn
E- 0.1 mM Cu + 1.00mM Zn
F- 0.1 mM Cu + 1.50mM Zn



A – Marker
B- Control
C- 0.2 mM Cu + 0.25mM Zn
D- 0.2 mM Cu + 0.50mM Zn
E- 0.2 mM Cu + 1.00mM Zn
F- 0.2mM Cu + 1.50mM Zn

Plate-3c

Amino acid content- The level of free amino acids in cotyledons of *Vigna mungo* seeds increased with the start of germination during 1-7 days and decrease with respect to copper and zinc concentration as compared to control. On 1st day, cotyledons of seeds grown under control conditions were having 2.56mg amino acid/ g FW. At initial stage (up to three days) the amino acid content was increased slowly, but during 5-7 days the rate of amino acid formation was more rapid and reached up to 50.56 mg/ g FW under control conditions. On contrary, as the concentration of zinc and copper ions increased the level of free amino acids decreased with respect to control as shown in **table-2**. In one day old seedlings, copper at the concentration of 0.05mM, 0.1mM and 0.2mM lowered the amino acid content by 5.07%, 10.15% and 20.70% in cotyledons. Furthermore, zinc stress resulted in decrease in free amino acid content by 3.90%, 12.10%, 17.18% and 21.87% at 0.25mM, 0.50, 1.00 and 1.50mM concentration respectively.

This increase in the reduction of free amino acid content was continued with the increase in metal concentrations and reached to 46.87% decrease in free amino acid content in case of 0.2mM Cu + 1.50mM Zn. Similar trend was followed up to seven days. The reduction of amino acid content in *Vigna mungo* (L.) cotyledons was more during 5-7 days of germination. In seven day old seedling copper ions lowered the amount of free amino acids in cotyledons of *Vigna mungo* by 7.63%, 24.24% and 60.18% at the concentration of 0.05 mM, 0.1 mM and 0.2 mM. Whereas zinc at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM led to 50.83%, 63.35%, 67.70% and 72.54% decline in free amino acid content. However, the combination of zinc and copper metal ions at the high concentration was very harmful and maximum reduction (78%) in amino acid content was recorded in cotyledons of seedlings grown with 0.2mM Cu + 1.50mM Zn treatment.

The level of free amino acids in embryonic axis of *Vigna mungo* increased as a function of time and concentration of copper and zinc ions in the medium up to three days. Later, during 5-7 days there was a decrease in amino acid content with respect to copper and/ or zinc concentration in comparison to control. In one day old seedlings, there was 1.66%, 15.14% and 35% reduction in amino acid content at the concentration of 0.05mM, 0.1mM and 0.2mM copper respectively. Whereas, zinc stress has resulted in the increase in amino acid content below 1.00 mM concentration and at the concentration of 1.50mM it led to decline in amino acid content by 1.66% with respect to seedlings grown under control conditions. At the concentration of 0.05mM Cu +0.25mM Zn, 0.1mM Cu + 0.25mM Zn and 0.2mM Cu + 0.25mM Zn amino acid content got increased by 102.50%, 50% and 2.50% as compared to control. Further increase in copper and zinc concentration lowered the free amino acid content in embryonic axis. The amino acid content started increasing with time, but after three days the concentration of amino acid got decreased with the increase in zinc and/ or copper metal concentration with respect to

control. Maximum reduction was recorded at 1.50mM Zn (86.34%) followed by 0.2mM Cu + 1.50mM Zn which have caused 75.52% decrease in amino acid content.

Zinc and copper ions have significantly reduced the amino acid content. This harmful effect might be due to one or more of several factors, namely, impairment of membrane integrity with loss of essential nutrients[22,23], Heavy metal mediated oxidative damage seems to alter the membrane structure or permeability due to lipid peroxidation process, which led to loss of amino acids[1,21]. Sharma and Dietz (2006)[27] reported that the protein degradation might have contributed to amino acid accumulation in copper, and zinc stressed plant during 0-3 days. Amino acids play an important role in osmoregulation, metal chelation and scavenging of free radicals. Osmoregulation appears to be a common element of plant reactions to various abiotic stresses. This implies that the plant population capable of efficient osmoregulation might better cope with the component of metal toxicity. So, increase in amino acid content in embryonic axis during an early stage may be an effort directed to get adapted in metal stressed condition.

Table-2

Effect of zinc and copper metal ions on free amino acid content (mg/ g.f.wt.$^{-1}$) in Vigna mungo (L.) seedlings

Concentration		In cotyledons				In embryonic axis			
		Days after imbibition				Days after imbibition			
$CuSO_4.5H_2O$ (mM)	$ZnSO_4.7H_2O$ (mM)	1	3	5	7	1	3	5	7
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
0.0	0.0	2.56 ± 0.17	8.60 ± 0.51	27.23 ± 0.25	50.56 ± 1.15	2.40 ± 0.36	15.23 ± 0.25	47.00 ± 2.00	93.00 ± 2.64
0.05	0.0	2.43 ± 0.25	8.13 ± 0.45	23.63 ± 0.15[a]	46.70 ± 1.04[a]	2.36 ± 0.25	14.43 ± 0.40[c]	37.33 ± 1.52[a]	64.33 ± 3.05[a]
0.1	0.0	2.30 ± 0.15	7.43 ± 0.15[b]	15.86 ± 0.17[a]	38.30 ± 1.11[a]	2.03 ± 0.20	14.36 ± 0.47[b]	33.33 ± 0.70[a]	62.36 ± 0.20[a]
0.2	0.0	2.03 ± 0.26	6.33 ± 0.70[a]	12.54 ± 0.30[a]	20.13 ± 0.11[a]	1.56 ± 0.25[c]	14.10 ± 0.10[a]	18.30 ± 0.43[a]	60.03 ± 0.21[a]
0.0	0.25	2.46 ± 0.02	8.56 ± 0.10	23.43 ± 0.47[a]	24.86 ± 0.85[a]	4.63 ± 0.26[a]	18.70 ± 0.30[a]	39.26 ± 0.30[a]	53.43 ± 0.40[a]
0.0	0.50	2.25 ± 0.20	8.40 ± 0.29	16.40 ± 0.94[a]	18.53 ± 0.41[a]	4.33 ± 0.30[a]	16.33 ± 0.10[a]	25.16 ± 0.30[a]	36.36 ± 0.37[a]
0.0	1.00	2.12 ± 0.25	7.62 ± 0.25[c]	15.26 ± 0.20[a]	16.33 ± 0.35[a]	3.63 ± 0.49[a]	14.10 ± 0.10[a]	21.03 ± 0.15[a]	28.43 ± 0.15[a]
0.0	1.50	2.00 ± 0.28	7.41 ± 0.52[b]	10.50 ± 0.40[a]	13.73 ± 0.30[a]	2.36 ± 0.20	12.60 ± 0.40[a]	18.56 ± 0.28[a]	22.70 ± 0.20[a]
0.05	0.25	2.35 ± 0.10	8.23 ± 0.20	25.60 ± 0.10[a]	35.15 ± 0.10[a]	4.86 ± 0.17[a]	20.30 ± 0.26[a]	39.50 ± 0.43[a]	80.86 ± 0.10[a]
0.05	0.50	2.26 ± 0.35	7.83 ± 0.15	21.30 ± 0.26[a]	26.86 ± 0.63[a]	3.23 ± 0.47[c]	18.43 ± 0.32[a]	32.30 ± 0.20[a]	70.00 ± 0.10[a]
0.05	1.00	2.14 ± 0.05	7.12 ± 0.04[a]	17.60 ± 0.26[a]	20.10 ± 0.10[a]	2.16 ± 0.55	16.80 ± 0.20[a]	25.43 ± 0.35[a]	58.40 ± 0.66[a]
0.05	1.50	2.03 ± 0.36	7.03 ± 0.80[a]	14.20 ± 0.45[a]	18.80 ± 0.20[a]	1.50 ± 0.20[c]	13.36 ± 0.35[a]	19.45 ± 0.35[a]	32.43 ± 0.11[a]
0.1	0.25	2.25 ± 0.36	8.06 ± 0.26	23.43 ± 0.25[a]	30.30 ± 0.34[a]	3.60 ± 0.36[a]	18.00 ± 0.20[a]	35.03 ± 0.11[a]	69.23 ± 0.73[a]
0.1	0.50	2.08 ± 0.60	7.10 ± 0.26[a]	18.60 ± 0.36[a]	22.23 ± 0.11[a]	3.10 ± 0.30	16.13 ± 0.15[b]	28.53 ± 0.35[a]	57.93 ± 0.15[a]
0.1	1.00	1.90 ± 0.36	6.92 ± 0.10[a]	14.40 ± 0.25[a]	16.73 ± 0.20[a]	1.80 ± 0.10	14.03 ± 0.15[a]	22.53 ± 0.35[a]	44.06 ± 0.30[a]
0.1	1.50	1.54 ± 0.45[b]	6.66 ± 0.15[a]	12.30 ± 0.15[a]	16.10 ± 0.10[a]	1.26 ± 0.15[a]	11.33 ± 0.10[a]	18.30 ± 0.15[a]	29.30 ± 0.92[a]
0.2	0.25	2.18 ± 0.72	7.70 ± 0.10[c]	19.20 ± 0.10[a]	25.20 ± 0.26[a]	2.46 ± 0.32	16.56 ± 0.35[a]	31.53 ± 0.25[a]	52.16 ± 0.47[a]
0.2	0.50	2.01 ± 0.05	7.20 ± 0.10[a]	13.46 ± 0.20[a]	17.12 ± 0.37[a]	2.13 ± 0.32	14.14 ± 0.15[a]	25.46 ± 0.30[a]	40.36 ± 0.15[a]
0.2	1.00	1.60 ± 0.28[c]	6.80 ± 0.25[a]	11.26 ± 0.94[a]	13.50 ± 0.26[a]	1.39 ± 0.26[b]	13.36 ± 0.40[a]	21.36 ± 0.90[a]	30.40 ± 0.36[a]
0.2	1.50	1.36 ± 0.45[b]	6.16 ± 0.18[a]	8.43 ± 0.10[a]	11.12 ± 0.17[a]	1.16 ± 0.09[a]	9.16 ± 0.20[a]	17.43 ± 0.04[a]	22.76 ± 0.15[a]

Source of variation		% of total variation	Source of variation		% of total variation
Interaction	-	17.99	Interaction	-	14.07
Time	-	14.92	Time	-	12.68
Treatment	-	66.99	Treatment	-	73.19

1) Above values are averagesof three replicates ± SD

2) a, band c indicates the values that differ significantly from the control at p≤ 0.001, p≤ 0.01 and p≤ 0.05 level respectively.

Protease activity- There was a decline in protease activity in cotyledons of *Vigna mungo* (L.) as compared to control with increasing zinc and copper ions concentrations as shown in **table- 3**. On 1st day of germination the level of protease activity in 0.05mM, 0.1mM and 0.2mM copper was declined by 1.72%, 18.07 and 33.51%. Zinc at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM led to the reduction in protease activity by 1.99%, 2.90%, 16.62% and 25.15% respectively. This reduction in protease activity was continued for seven days.

According to the results, effect of copper ions has been proven more harmful than zinc metal ions. The combination of these two metals has shown a synergistic effect. At 0.05mM Cu + 0.25mM Zn, 0.1mM Cu +0.25mM Zn and 0.2mM Cu + 0.25mM Zn concentration there were 3.81%, 7.81% and 11.35% reduction in protease activity. However, the rate of reduction in specific activity of protease enzyme went on increasing with the increase in metal concentration. The decrease in protease activity in 0.05mM Cu +1.50mM Zn, 0.1mM Cu +1.50mM Zn and 0.2mM Cu + 1.50mM Zn was 42.30%, 36.96% and 70.20%. The protease activity seemed to be stimulated with respect to time, but decreased with respect to zinc and/or copper concentration in comparison to control.

Protease activity was found to be stimulated with time under all treated seedlings, but the rate of the increase in enzyme activity was very low in comparison to control. Protease activity got retarded with the increase in copper and zinc ion concentrations. Maximum reduction in protease activity was noted at 0.2mM Cu + 1.50mM Zn (72.94%) followed by 0.1mM Cu +1.50mM Zn (69.35%). The combination of copper and zinc ions in case of 0.05mM Cu + 0.25mM Zn resulted in 41.06% decrease in protease activity.

However, the trend was found reverse in embryonic axis of *Vigna mungo* (L.) seedlings. The specific activity of protease enzyme increased significantly with the start of germination with respect to time as well as zinc and copper ion concentration. In one day old seedling copper at the concentration of 0.05mM, 0.1mM and 0.2mM increased the protease activity by 174.95%. 131.73% and 49.24% whereas zinc resulted in 202.73%, 187.19%, 79.19% and 75.23% increase in protease activity at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM. The combined metal treatment has also increased the protease activity, but it was relatively less in comparison to copper and zinc individual treatment. Similar trend was followed for seven days. In seven day old seedlings, 0.05mM Cu + 0.25mM Zn, 0.1mM Cu + 0.25mM Zn and 0.2mM Cu + 1.50mM Zn accelerated the rate of protease activity by 203.36%, 139.41% and 93.81% in comparison to control. However, the percentage of the increase in protease activity decreases with the increase in copper and zinc concentration. There were 70.97%, 41.63% and 35.98% increase in protease activity in 0.05mM Cu + 1.50mM Zn, 0.1mM Cu +1.50mm Zn and 0.2mM Cu + 1.50mM Zn respectively.

Decline in protein content and the corresponding rise in the activity of hydrolytic enzymes such as protease due to heavy metal stress strongly suggest the catabolic activities. Similar results have been observed by Rastgoo and Alemzadeh (2011)[24]. In plant tissue, specific proteases are involved in the mobilization of reserve proteins, developmental processes and senescence[8]. According to Khudsar et al. (2004)[13] it is likely that heavy metal stress induces senescence through enhancement of catabolism of key metabolites such as chlorophyll, protein and RNA.

However, in cotyledons, the specific activity of protease was reported to be declined with the increase in metal concentration, which might have caused low hydrolysis and translocation of proteins from cotyledons to the embryonic axis at germination and early seedling stage. Protease activity is responsible for the hydrolysis of storage proteins and hydrolyzed products are mobilized to the

embryonic axis to support the growth of developing axis. Alterations in the activity of proteases have been noticed by various researchers Mishra and Dubey (2006)[18]; Maheshwari and Dubey (2007)[17]; Kuriakosa and Prasad (2008)[14]. Van Assche and Clijsters (1990)[29] suggested that decline in protease activity might be possibly due to the binding of heavy metals with the functional group of enzyme protein as general heavy metal specific response.

Table-3

Effect of zinc and copper metal ions on specific activity of protease (µg leucine liberated/min./mg of protein)in Vigna mungo (L.) seedlings

Concentration		In cotyledons				In embryonic axis			
		Days after imbibition				Days after imbibition			
$CuSO_4.5H_2O$ (mM)	$ZnSO_4.7H_2O$ (mM)	1	3	5	7	1	3	5	7
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
0.0	0.0	1.101 ± 0.06	1.634 ± 0.05	2.257 ± 0.08	3.071 ± 0.10	1.062 ± 0.06	1.145 ± 0.02	1.202 ± 0.08	1.309 ± 0.08
0.05	0.0	1.082 ± 0.08	1.392 ± 0.06[b]	1.501 ± 0.07[a]	2.821 ± 0.07[b]	2.920 ± 0.05[a]	3.047 ± 0.06[a]	3.264 ± 0.08[a]	3.337 ± 0.08[a]
0.1	0.0	0.902 ± 0.07[b]	0.996 ± 0.06[a]	1.485 ± 0.07[a]	1.856 ± 0.08[a]	2.461 ± 0.09[a]	2.851 ± 0.05[a]	3.038 ± 0.06[a]	3.122 ± 0.05[a]
0.2	0.0	0.732 ± 0.02[a]	0.876 ± 0.04[a]	0.895 ± 0.06[a]	1.111 ± 0.05[a]	1.585 ± 0.06[a]	1.889 ± 0.06[a]	1.906 ± 0.06[a]	2.798 ± 0.05[a]
0.0	0.25	1.079 ± 0.06	1.338 ± 0.05[a]	1.666 ± 0.05[a]	1.908 ± 0.05[a]	3.215 ± 0.05[a]	3.227 ± 0.06[a]	3.877 ± 0.07[a]	4.281 ± 0.06[a]
0.0	0.50	1.069 ± 0.05	1.218 ± 0.08[a]	1.319 ± 0.08[a]	1.497 ± 0.06[a]	3.050 ± 0.07[a]	3.096 ± 0.08[a]	3.461 ± 0.06[a]	4.066 ± 0.07[a]
0.0	1.00	0.918 ± 0.05[c]	1.008 ± 0.06[a]	1.129 ± 0.07[a]	1.263 ± 0.07[a]	1.903 ± 0.04[a]	2.782 ± 0.08[a]	3.004 ± 0.07[a]	3.285 ± 0.09[a]
0.0	1.50	0.824 ± 0.04[a]	0.869 ± 0.08[a]	0.985 ± 0.07[a]	1.146 ± 0.08[a]	1.861 ± 0.04[a]	2.177 ± 0.06[a]	2.445 ± 0.06[a]	2.983 ± 0.07[a]
0.05	0.25	1.059 ± 0.07	1.410 ± 0.07[b]	1.491 ± 0.04[a]	1.810 ± 0.09[a]	2.375 ± 0.07[a]	3.035 ± 0.06[a]	3.749 ± 0.06[a]	3.971 ± 0.08[a]
0.05	0.50	0.948 ± 0.05	0.959 ± 0.07[a]	1.214 ± 0.06[a]	1.183 ± 0.08[a]	2.070 ± 0.07[a]	2.522 ± 0.06[a]	2.855 ± 0.05[a]	3.096 ± 0.05[a]
0.05	1.00	0.749 ± 0.10[a]	0.877 ± 0.07[a]	0.975 ± 0.08[a]	1.004 ± 0.08[a]	2.057 ± 0.07[a]	2.244 ± 0.06[a]	2.510 ± 0.06[a]	2.573 ± 0.05[a]
0.05	1.50	0.633 ± 0.07[a]	0.836 ± 0.06[a]	0.892 ± 0.07[a]	0.944 ± 0.07[a]	1.876 ± 0.05[a]	2.218 ± 0.05[a]	2.478 ± 0.08[a]	2.238 ± 0.08[a]
0.1	0.25	1.009 ± 0.05	1.247 ± 0.06[a]	1.334 ± 0.05[a]	1.648 ± 0.05[a]	2.016 ± 0.04[a]	2.845 ± 0.06[a]	3.443 ± 0.05[a]	3.134 ± 0.08[a]
0.1	0.50	1.015 ± 0.05	1.081 ± 0.07[a]	1.191 ± 0.06[a]	1.269 ± 0.06[a]	1.992 ± 0.02[a]	2.228 ± 0.05[a]	2.460 ± 0.05[a]	2.706 ± 0.08[a]
0.1	1.00	0.859 ± 0.05[a]	0.902 ± 0.07[a]	0.975 ± 0.06[a]	0.958 ± 0.06[a]	1.569 ± 0.05[a]	1.908 ± 0.07[a]	2.023 ± 0.06[a]	2.168 ± 0.06[a]
0.1	1.50	0.694 ± 0.04[a]	0.856 ± 0.08[a]	0.879 ± 0.08[a]	0.941 ± 0.08[a]	1.541 ± 0.04[a]	1.657 ± 0.06[a]	1.691 ± 0.07[a]	1.854 ± 0.08[a]
0.2	0.25	0.976 ± 0.10	1.134 ± 0.09[a]	1.304 ± 0.05[a]	1.661 ± 0.05[a]	2.079 ± 0.05[a]	2.155 ± 0.06[a]	2.439 ± 0.08[a]	2.537 ± 0.08[a]
0.2	0.50	0.844 ± 0.06[a]	1.124 ± 0.08[a]	0.995 ± 0.06[a]	1.440 ± 0.06[a]	1.927 ± 0.05[a]	1.988 ± 0.06[a]	2.135 ± 0.07[a]	2.418 ± 0.08[a]
0.2	1.00	0.449 ± 0.08[a]	1.030 ± 0.07[a]	0.932 ± 0.04[a]	1.183 ± 0.04[a]	1.769 ± 0.07[a]	1.876 ± 0.08[a]	1.976 ± 0.07[a]	2.169 ± 0.08[a]
0.2	1.50	0.328 ± 0.06[a]	0.561 ± 0.06[a]	0.783 ± 0.07[a]	0.831 ± 0.07[a]	1.429 ± 0.05[a]	1.643 ± 0.06[a]	1.744 ± 0.06[a]	1.780 ± 0.07[a]
		Source of variation	% of total variation			Source of variation	% of total variation		
		Interaction -	18.50			Interaction -	06.70		
		Time -	54.39			Time -	76.89		
		Treatment -	25.61			Treatment -	15.87		

1) Above values are averagesof three replicates ± SD

2) a, b and c indicates the values that differ significantly from the control at p≤ 0.001, p≤ 0.01 and p≤ 0.05 level respectively.

ACKNOWLEDGEMENT

Authors are highly grateful to DST, New Delhi for their financial support.

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