Copper and zinc metal accumulation and response of antioxidative enzyme systems in *Vigna mungo* (L.)

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Abstract- The present study was conducted to investigate the influence of copper and /or zinc metal on growth parameters and antioxidants. Both metal led to decreased and delayed seed germination and production of seedling biomass in comparison to control seedlings. The combined effect of copper and zinc was proved antagonistic for seedling growth at early stage, however the situation was reverse at high metal ion concentration. The study also emphasize on the response of antioxidative enzymes to copper and/or zinc stress. The result showed active involvement of peroxidase enzyme in combating the metal stress rather than catalase enzyme.

Keywords- Antioxidative enzymes, heavy metal accumulation, seed germination rate, seedling biomass

Introduction- Due to rapid industrialization, urbanization and intensive agriculture, increasing contamination of heavy metals in soil has become a major problem. Environmental deterioration has generated an increase of stress in all forms of life. With the development of industries, mining activities, application of waste water and sewage sludge on land, heavy metal pollution of soils is becoming a serious environmental problem [8]. Heavy metals are of great interest for research purpose with respect to toxicological importance to human health, plants and animals [3], [5], [14]. Currently, environmental pollution and plant exposure to heavy metals is a matter of great concern at the global level.

Toxic heavy metals are normally present as soil constituents or can also be spread out in the environment by human activity and agricultural techniques. Anthropogenic activities like mining, combustion of fossil fuels, metal industries, phosphate fertilizers, sewage sludge or municipal waste, emission from municipal waste incinerators, car exhaust, residues from mining and smelting industries, etc., lead to the emission of heavy metals and accumulation of these compounds in the ecosystem.

There is much evidence that agricultural land adjacent to industrial areas is polluted to a varied extent by many toxic heavy metals. Metal-contaminated wastes in various parts of the world usually contain more than one metal and these may occur at toxic concentrations e.g., metalliferous mines spoil (copper, lead, zinc), smelter wastes (copper, lead, zinc), coal spoils (aluminium, copper, manganese, nickel, zinc, iron), sewage sludge and refuse compost (copper, zinc, lead, etc., depending on the source). Hence, metal pollution is a multielement problem. Environmental effects of combined heavy metals may be quite different from those of individual metal due to interactions between heavy metals [22], [23].

Further action of heavy metals is due to generation of reactive oxygen species and induction of oxidative stress [20]. When plants are subjected to any biotic or abiotic stress, it results in production of reactive oxygen species such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen [11]. To counter the deleterious effect of reactive oxygen species plants have evolved various enzymatic (CAT, APX, GPX, SOD, GR, etc.) and non-enzymatic (ascorbate, glutathione, α-tocopherol) antioxidant systems which protect the plants from their toxic action [2]. 2014). Antioxidant pathways are usually sufficient to protect them from oxidative stress during periods of normal growth and moderate stress, but when severely stressed the production of reactive oxygen species (ROS) can exceed the capacity of the antioxidant system to neutralize them and oxidative damage can occur.

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. Thus, the present study is an attempt to explore a possible relationship between zinc and copper metal induced changes at the biochemical and physiologcal level in *Vigna mungo* (L.).

Material and Methods

Source- Seeds of *Vigna mungo* (L.) Hepper cv. T-9 (Black Gram) was obtained from National Seed Corporation Unit, Indian Agricultural Research Institute, PUSA, 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 $CuSO_4.5H_2O$. 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 different concentrations. Sterile conditions were maintained by adding 20µg/ml of streptomycin sulphate in the medium to suppress microbial growth. All experiments were carried out for seven days at 28 ± 2^0C in dark. At regular interval of time required number of seeds were withdrawn and used for analysis of various growth indices and enzymatic activities.

Heavy metal assessment - Heavy metals were determined by the method (EPA method 3050) as outlined by Gupta (2000).

Enzymatic assay- Catalase activity was measured by following the method of Abei (1984) and peroxidase activity was assayed as described by Pundir *et al.* (1999).

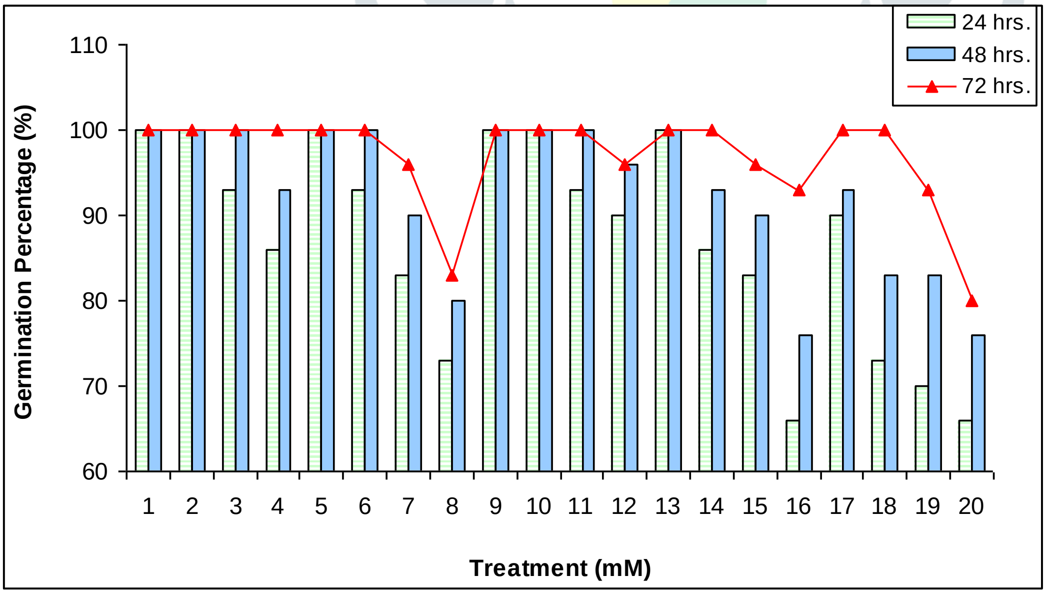
3.16 Statistical Analysis- Statistical analysis was done by using Microsoft excel. All the experiments were conducted in triplicates. The obtained data were statistically analysed for the **mean ±S.D.**

Result and Discussion

1. Effect of zinc and copper metal ions on *Vigna mungo* (L.) germination behavior

In the present study, the rate of germination decreased with the increase in zinc and copper metal ion concentrations. The inhibitory effect of zinc and copper was more apparent to the concentration of 0.50mM and 0.1 mM respectively. The combined effect of zinc and copper ions at the low concentrations was found to be negligible as it resulted in 100% germination rate. Whereas the high concentration of zinc and copper ions have caused a decline in the rate of seed germination as shown in “Fig.” -I. The results have shown that copper and zinc ions have induced delayed response in case of germination because the number of seed germinated after 24 hrs. were less in comparison to those which were noticed after 48 hrs. and 72 hrs. Maximum reduction has been noticed at the highest level of combined stress (0.2 mM Cu + 1.50 mM Zn) which have caused 20% reduction in rate of *Vigna mungo* (L.) seed germination.

“Fig.”-I) Effect of zinc and copper metal ions on germination behaviour of *Vigna mungo* (L.) seedlings



Treatment conditions

(1) Control **(2)** 0.05 mM $CuSO_4$ **(3)** 0.1 mM $CuSO_4$ **(4)** 0.2 mM $CuSO_4$
(5) 0.25 mM $ZnSO_4$ **(6)** 0.50 mM $ZnSO_4$ **(7)** 1.00 mM $ZnSO_4$ **(8)** 1.50 mM $ZnSO_4$
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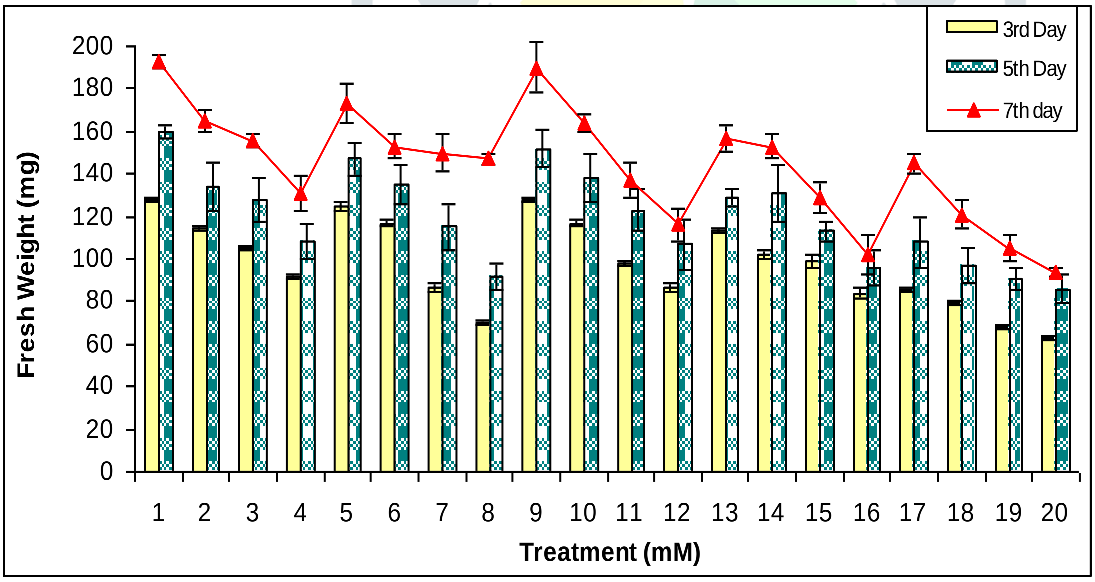
(15) 0.1 mM$CuSO_4$ + 1.00mM $ZnSO_4$ **(16)** 0.1 mM$CuSO_4$ + 1.50mM $ZnSO_4$ **(17)** 0.2 mM$CuSO_4$ + 0.25mM $ZnSO_4$
(18) 0.2 mM$CuSO_4$ + 0.50mM $ZnSO_4$ **(19)** 0.2 mM$CuSO_4$ + 1.00mM $ZnSO_4$ **(20)** 0.2 mM$CuSO_4$ + 1.50mM $ZnSO_4$

2. Effect of zinc and copper metal ions on *Vigna mungo* (L.) Seedling biomass

The effect of zinc and copper ions on biomass produced has been expressed in terms of fresh weight and dry weight of *Vigna mungo* (L.) seedlings. Under control conditions, the increase in fresh weight and dry weight of embryonic axis was 65.0mg and 20.0mg respectively from 3rd to 7th day of the experiment. Zinc and copper treated seedlings have been observed to cause a decline in seedling biomass. The fresh weight and dry weight of zinc and/or copper treated seedlings was very less relative to control seedlings. Though seedling biomass has increased with time but there was a progressive decrease in seedling biomass with respect to the concentration of zinc as well as copper metal ions as shown in "Fig."-II and III.

John *et al.* (2009) reported that the reduction in seedling growth during copper and/or zinc stress may be due to low water potential, hampered nutrient uptake and secondary stress such as oxidative stress. Reduced seedling growth under heavy metal treatment could be due to the reduction in meristematic cells present in this region and inhibition in activities of some enzymes present in cotyledons and embryonic axis. During seedling growth hydrolysis of food reserves take place, which is carried out by hydrolytic enzymes.

"Fig."- II) Effect of zinc and copper metal ions on fresh weight of *Vigna mungo* (L.) seedlings



Treatment conditions

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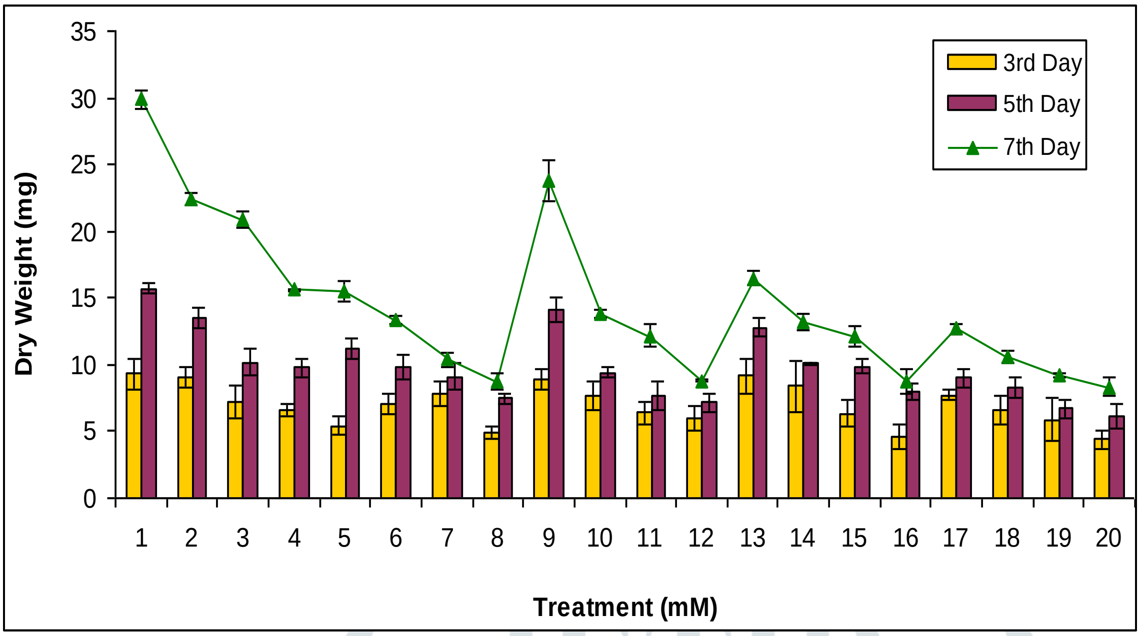
"Fig."-III) Effect of zinc and copper metal ions on dry weight of *Vigna mungo* (L.) seedlings



Treatment conditions

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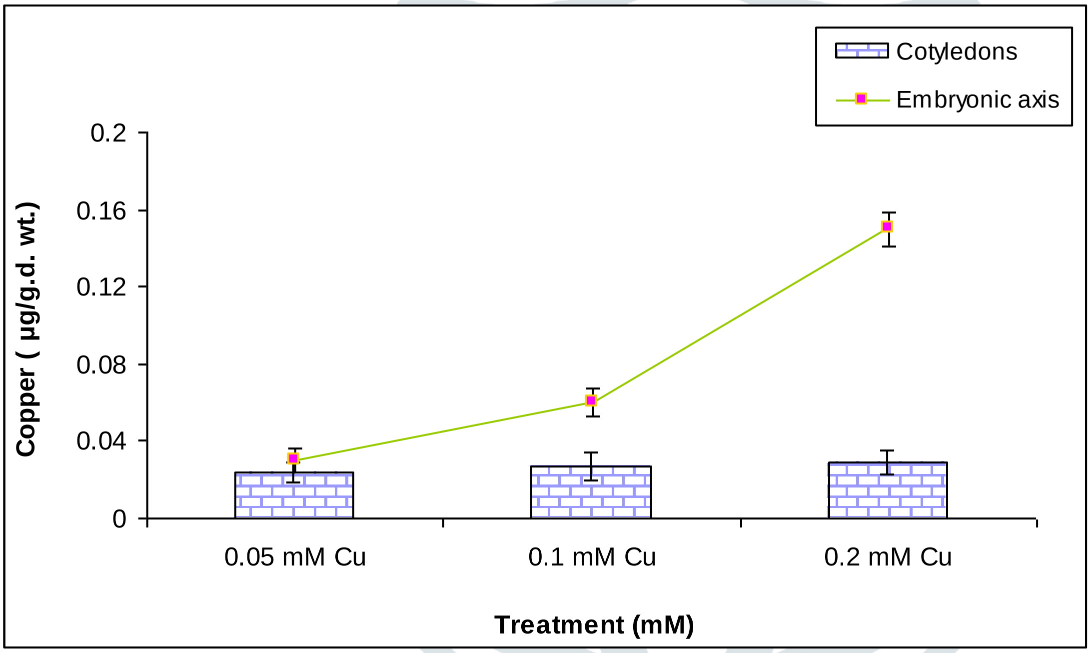
3. Uptake and accumulation of zinc and copper metal ions in cotyledons and embryonic axis of *Vigna mungo* (L.)

Zinc and copper content in cotyledons and embryonic axis of *Vigna mungo* (L.) seedlings were observed to get increased with the increase in metal ion concentration in the germination medium. The content of zinc and copper metals was greater in embryonic axis in comparison to cotyledons at the same concentration. Seedlings grown at 0.05mM, 0.1mM and 0.2mM Cu have shown 0.03, 0.06 and 0.15 µg/g.d.wt. Increase in copper content in embryonic axis over control as shown in "Fig."-IV. 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.d.wt. over control as given in "Fig."-V. 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.).

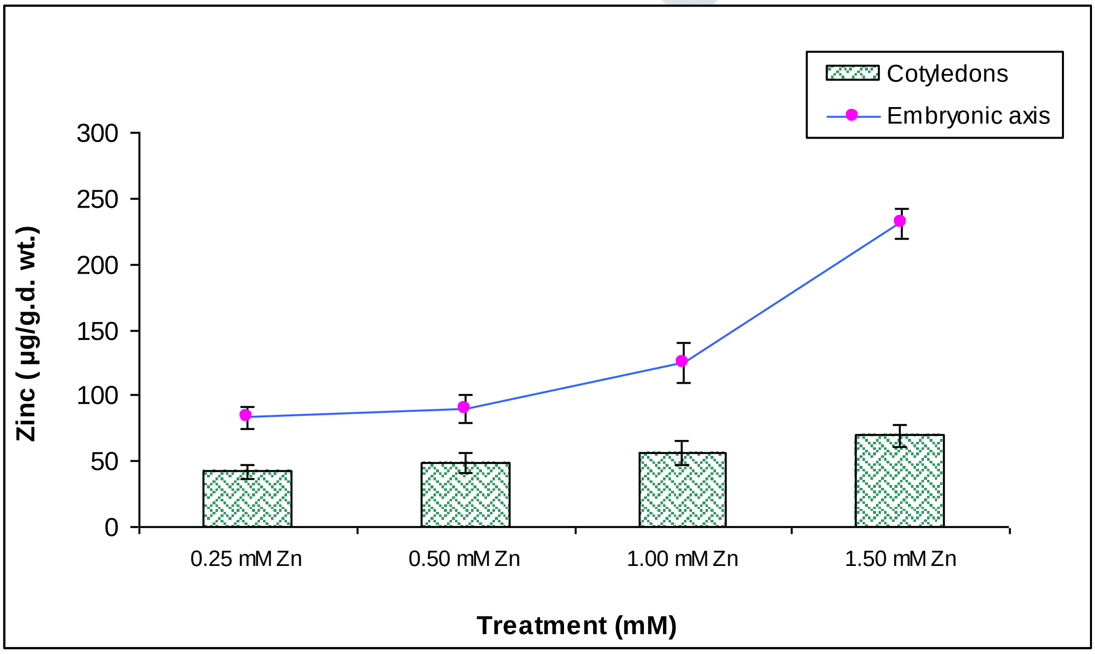
In cotyledons, there was only 1.2 fold increase in copper content with the increase in copper ions concentration from 0.05 to 0.2mM in the nutrient medium whereas zinc ions have caused 1.63 fold increase in zinc uptake in cotyledons from 0.25mM to 1.50mM concentration. In case of combined treatment, copper and zinc ions accumulation was less in case of 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 "Fig."- VI and VII.

These results are in agreement with Rahoui *et al.* (2008) and Kuriakosa and Prasad (2008). 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.

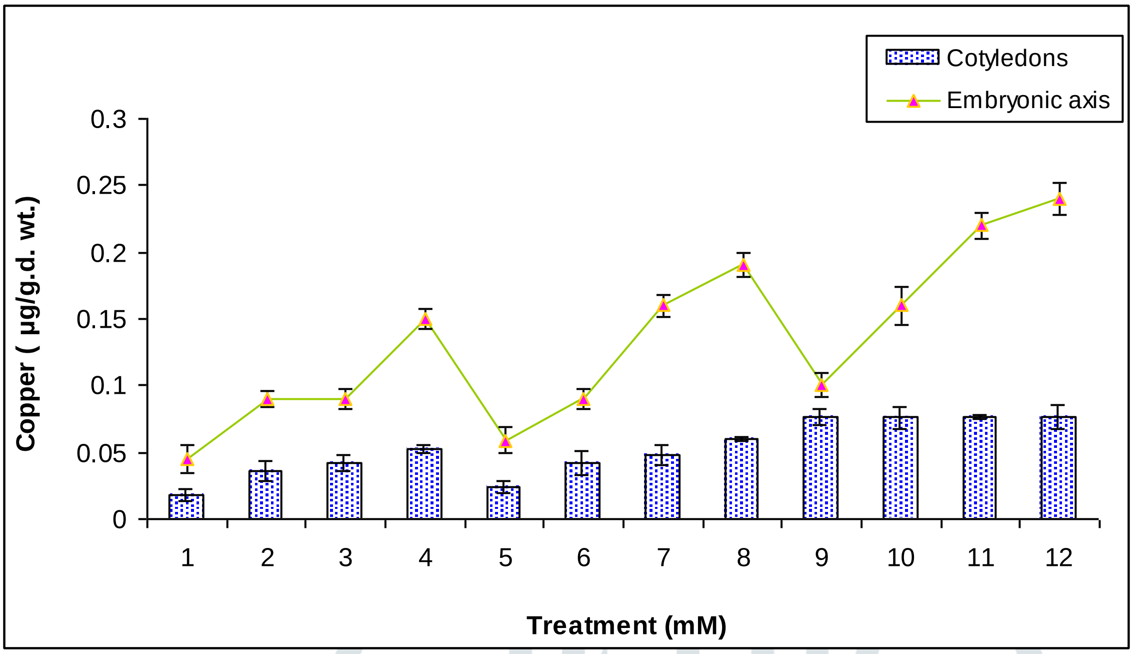
"Fig."-IV) Effect of copper metal ions stress on copper content in *Vigna mungo* (L.) seedlings



"Fig."-V: Effect of zinc metal ions stress on zinc content in *Vigna mungo* (L.) seedlings



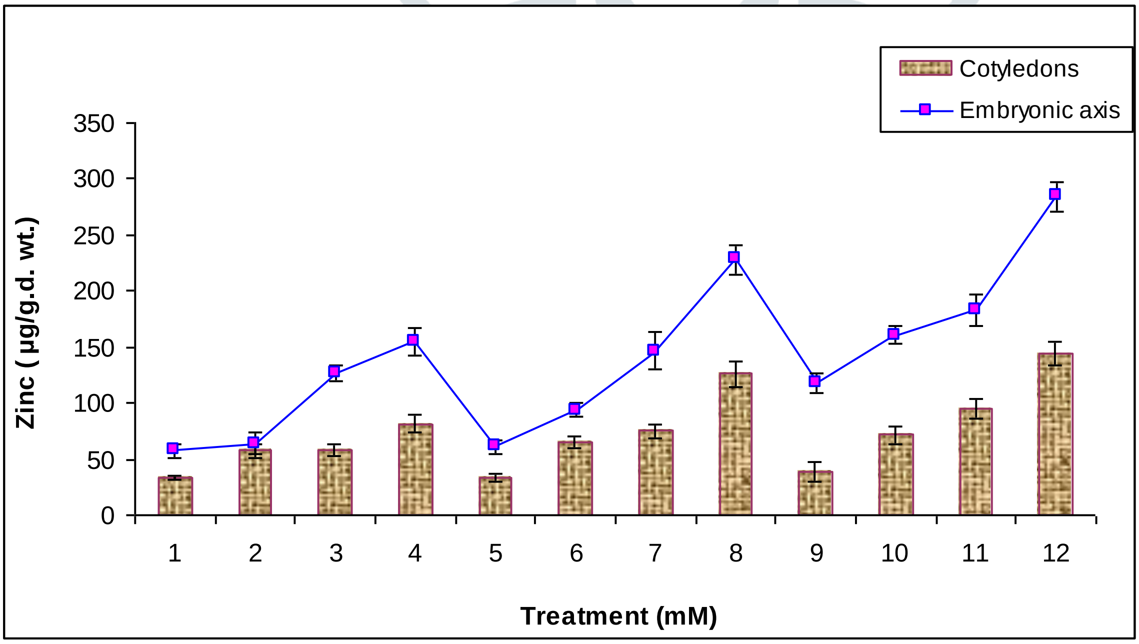
“Fig.”-VI) Effect of zinc and copper metal ions combined stress on copper content in *Vigna mungo* (L.) seedlings



Treatment conditions

(1) 0.05 mM CuSO$_4$+ 0.25 mMZnSO$_4$ **(2)** 0.05 mM CuSO$_4$+ 0.50 mMZnSO$_4$ **(3)** 0.05 mM CuSO$_4$ + 1.00 mMZnSO$_4$
(4) 0.05 mM CuSO$_4$ +1.50 mMZnSO$_4$ **(5)** 0.1 mMCuSO$_4$ + 0.25mM ZnSO$_4$ **(6)** 0.1 mMCuSO$_4$ + 0.50mM ZnSO$_4$
(7) 0.1 mMCuSO$_4$ + 1.00mM ZnSO$_4$ **(8)** 0.1 mMCuSO$_4$ + 1.50mM ZnSO$_4$ **(9)** 0.2 mMCuSO$_4$ + 0.25mM ZnSO$_4$
(10) 0.2 mMCuSO$_4$ + 0.50mM ZnSO$_4$ **(11)** 0.2 mMCuSO$_4$ + 1.00mM ZnSO$_4$ **(12)** 0.2 mMCuSO$_4$ + 1.50mM ZnSO$_4$

“Fig.”-VII) Effect of zinc and copper metal ions combined stress on zinc content in *Vigna mungo* (L.) seedlings



Treatment conditions

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4. Effect of zinc and copper ions on antioxidant enzymes activity in *Vigna mungo* (L.) seedlings

Catalase activity

Heavy metal induced reactive oxygen species in plants are quenched by a number of antioxidant enzymes. In one day old seedlings, it was observed that catalase activity in embryonic axis increased with the increasing copper and zinc ion concentration up to 0.2mM and 0.50mM respectively, representing 19.00% and 0.79% increment as compared to control

and declines later as shown in "Fig."- VIII. With the further increase in metal concentration a concomitant decline in catalase activity was noticed in embryonic axis of *Vigna mungo* (L.). Significant difference in enzymatic activity was observed between various treatment conditions and control. There were 10.89%, 22.17% and 19.00% increment in catalase activity at 0.05mM, 0.1mM and 0.2mM Cu treatment. The zinc metal ions treatment led to increase in catalase activity by 0.79% at 0.50mM whereas the further increase in zinc concentration lowered the specific activity of catalase enzyme by 47.92% at 1.50mM as compared to control.

The treatment of *Vigna mungo* seedlings with the combination of copper and zinc metal reduced catalase activity at all levels except at the lowest level of combination, i.e, 0.05mM Cu + 0.25mM Zn which have caused 2.77% increase in catalase activity. At 0.1mM Cu + 0.25mM Zn and 0.2mM Cu + 0.25mM Zn there were 0.99% and 7.12% decrease in enzymatic activity. After one day, the maximum stimulation in catalase enzyme activity was observed in seedlings exposed to 0.1mM Cu and maximum reduction was noticed at 0.1mM Cu + 1.50mM Zn (53.66%) followed by 0.2mM Cu + 1.50 mM Zn (49.90%). In seven days old seedlings the catalase activity was less than that which was noticed on 5th day of germination except in case of control where the catalase activity increased with the increase in time during the entire experimental study.

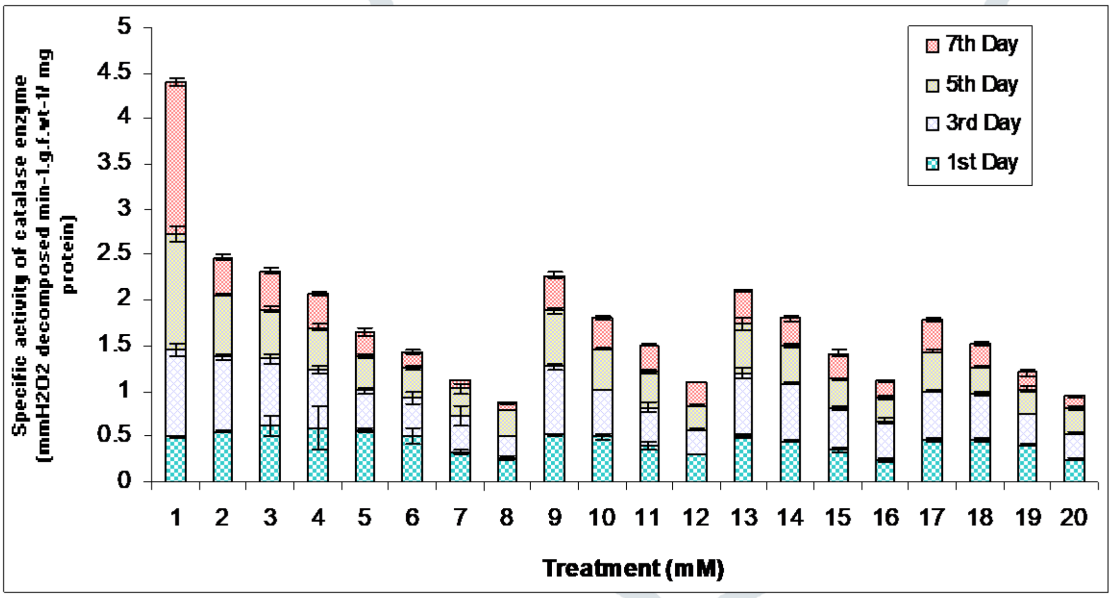
In seven days old seedlings, copper metal ions at 0.05mM, 0.1mM and 0.2mM concentration resulted in 75.08%, 75.44% and 79.02% decrease in catalase activity and zinc led to 83.99%, 89.30%, 94.98% and 95.57% reduction at 0.25mM, 0.50mM, 1.00mM and 1.50mM concentration respectively. Catalase activity got lowered by 76.76%, 79.27%, 82.19% and 84.28% at 0.05mM Cu + 0.25mM Zn, 0.05mM Cu + 0.50mM Zn, 0.05mM Cu +1.00mM Zn and 0.05mM Cu +1.50mM Zn. The rate of reduction was observed to be increased with the increase in metal concentration. At 0.2mM Cu + 0.25mM Zn, 0.2mM Cu + 0.50mM Zn, 0.2mM Cu + 1.00mM Zn and 0.2mM Cu + 1.50mM Zn the catalase activity was inhibited by 79.21%, 85.00%, 88.88% and 92.35% respectively as compared to control.

Catalase is the universally present oxido-reductase that decomposes H_2O_2 to water and molecular oxygen, and it is one of the key enzymes involved in removal of toxic peroxides[10]. In the present study, the increased catalase activity at germination stage may be an adaptive response because the roots emerge out in an environment already under stress. However, later the enzyme was not able to resist heavy metal stress.

Decline in catalase activity is regarded as general response to heavy metal stress due to their toxic effect on enzymes synthesis or change in the assembly of enzyme subunits. Moreover, catalase is a heme containing enzyme, hence decrease in catalase activity may be due to active competition between metal ion uptake and absorption by plant roots [6].

Verma and Dubey (2003) reported decreased intensity of two isoenzymic forms of catalase enzyme in shoots of lead (Pb) stressed seedlings of *Oryza sativa* (L.) which is in conformity with decreased activity of the enzyme under lead treatment. In our study also catalase enzyme did not appear to be an efficient scavenger of H_2O_2. Thus, inconsistent results regarded catalase activity might be due to a difference in concentration of zinc and copper metal ions taken up by *Vigna mungo* (L.) seedlings.

"Fig."- VIII) Effect of zinc and copper metal ions on specific activity of catalase enzyme in *Vigna mungo* (L.) seedlings.



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$ZnSO_4$
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Peroxidase activity

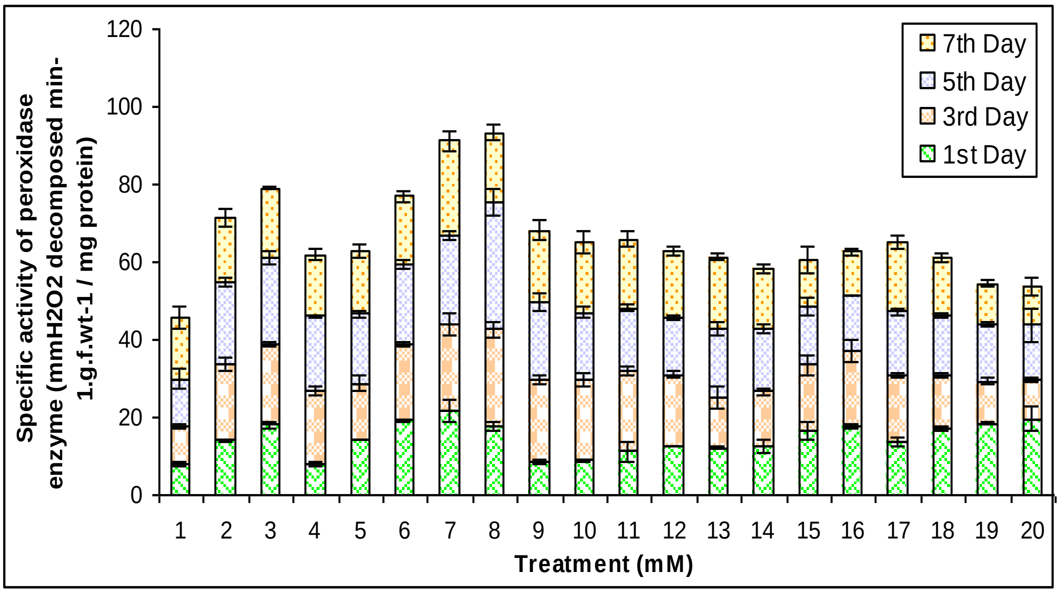
Inadequate response of catalase activity to heavy metal was compensated by the increased activity of peroxidase enzyme. It seemed to be important in resisting oxidative stress induced by zinc and copper in *Vigna mungo* (L.). Peroxidase activity in embryonic axis increased gradually with increasing copper and zinc concentration as compared to control as shown in "Fig."-IX. There was 77.98%, 129.10% and 0.45% increment in peroxidase activity at 0.05mM, 0.1mM and 0.2mM copper concentration and zinc at the concentration of 0.25mM, 0.50mM, 1.00mM and 1.50mM resulted in 79.40%, 148.03%, 173.06% and 122.69% increase in enzymatic activity in one day old seedlings.

The combined effect of zinc and copper ions in *Vigna mungo* (L.) seedlings has also led to increased specific activity of peroxidase enzyme. However, this increment in enzymatic activity was found to be intermediate as compared to individual zinc and copper treatment. The copper and zinc at 0.05mM Cu + 0.25mM Zn, 0.1mM Cu + 0.25mM Zn and 0.2mM Cu + 0.25mM Zn levels have upraised the peroxidase activity by 10.05%, 52.82% and 71.30%. Treatment of seedlings with 0.05mM Cu + 1.50mM Zn, 0.1mM Cu + 1.50mM Zn and 0.2mM Cu + 1.50mM Zn resulted in 57.58%, 122.15% and 146.06% increase in peroxidase activity respectively. These results suggest that this enzyme serves as an intrinsic defense tool to resist zinc and copper induced oxidative damage in *Vigna mungo* (L.).

The maximum up-regulation in peroxidase activity was noticed during early days of treatment up to five days of the experiment. On 7th day, the peroxidase activity at the low level of zinc and copper concentration was more than that which was recorded in case of control. However, the effect is opposite at high copper and zinc ion concentration. In case of 0.1mM Cu + 1.00mM Zn and 0.1mM Cu + 1.50mM Zn there were 22.42% and 28.25% reduction in enzymatic activity. Seedlings growing in the presence of 0.2mM Cu + 0.50mM Zn, 0.2mM Cu + 1.00mM Zn and 0.2mM Cu + 1.50mM Zn showed about 6.12%, 33.36% and 36.87% decrease in peroxidase activity in embryonic axis.

An increase in peroxidase activity is associated with strengthening of plant tissues against highly reactive free radicals, which are injurious to cellular entities [2], [12], [17]. However, it is difficult to counteract the stress above a certain limit. Therefore, decreased antioxidant enzymes activity under the highly stressed condition may be the result of imposition of oxidative stress, i.e, imbalance in generation and metabolism of reactive oxygen species [13], [15], [19].

"Fig."- IX) Effect of zinc and copper metal ions on specific activity of peroxidase enzyme in *Vigna mungo* (L.) seedlings



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Conclusion

Both antioxidant enzymes (catalase and peroxidase) exhibited an increasing trend under different treatment conditions thereby indicating the increase in oxidative stress. The results showed active involvement of peroxidase enzyme in regulating oxidative stress rather than the catalase enzyme, as the specific activity of peroxidase enzyme got increased up to seven days of treatment under different treatment conditions, whereas catalase activity in embryonic axis of *Vigna mungo* (L.) got declined at high metal ion concentration due to excessive stress.

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