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Zinc and copper induced changes in physiological characteristics of *Vigna mungo* (L.)

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Abstract

The effect of deleterious concentration of zinc and copper provided either individually or in combination in the nutrient media was investigated in order to assess the effect of metal interaction in *Vigna mungo* (L.). Both metals showed negative effect and led to a marked decrease in seed germination (20%), seedling growth (91.7%) and nitrate reductase activity (85.7%) with the increase in metal concentrations. The present study also emphasizes on the response of catalase and peroxidase enzyme under zinc and copper stress. Both antioxidant enzymes exhibited an increasing trend under different treatment conditions but it was reverse at highly toxic metal concentration. The results showed active involvement of peroxidase enzyme in regulating oxidative stress rather than catalase enzyme, as the specific activity of peroxidase enzyme got increased by 8.94% under the combined metals stress whereas catalase activity got declined by 60.97% in comparison to control due to excessive stress. The combined effect of copper and zinc metal was more pronounced in comparison to their individual effects.

Key words

Heavy metal stress, Seedling growth, Nitrate reductase, Catalase, Peroxidase

Introduction

Heavy metals are of great interest for research purpose with respect to toxicological importance to human health, plants and animals (Azevedo and Lea, 2005; Jarup, 2003; Almeida *et al.*, 2007). Due to rapid industrialization, urbanization and intensive agriculture increasing contamination of heavy metals in soil has become a major concern. Excessive level of heavy metals in the soil environment adversely affect the germination of seeds, plant growth, alter the level of biomolecules in the cells and interfere with the activities of many key enzymes related to normal metabolic and developmental processes (Ahsan *et al*., 2007; Kuriakosa and Prasad, 2008; Kachout *et al.*, 2009; Zhang *et al.*, 2009; Rahoui *et al.*, 2010). Seed is a stage in life cycle of plant that is well protected against various stresses. However, after imbibition and subsequent vegetative developmental processes, they become stress sensitive (Li *et al*., 2005) especially if the crop is grown in the vicinity of heavy industries particularly in developing countries (Bi *et al*., 2006; Ona *et al.*, 2006). Heavy metals have high affinity to sulfydryl groups and disulfide bonds which cause damage to secondary structure of proteins and alter the enzymatic activities (Siediecka and Krupa, 2002). The mechanism of action lies in their ability to form strong bonds with bases and phosphates of nucleic acids. They compete with other divalent cations and replace them in their physiological roles (Tabaldi *et al.*, 2007). Further action of heavy metals is due to generation of reactive oxygen species and induction of oxidative stress (Schutzendubel and Polle, 2002). 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 (Jung, 2004; Malekzadeh *et al*., 2007). To counter the deleterious effect of reactive oxygen species plants have evolved various enzymatic and non-enzymatic antioxidant systems which protect the plants from their toxic action (Foyer *et al*., 1994; Boscolo *et al*., 2003; Shreedevi *et al*., 2008). Metal pollution is a multielement problem, in many cases, it is more appropriate to study the combined effect of sub-toxic and toxic concentration of heavy metals. The effect of combined heavy metals on plants may be quite different from those of individual pollutants due to interaction between heavy metals. Both, copper and zinc metal are essential mineral elements for plant growth but at high concentration these are highly toxic to plants. Distribution of heavy metals in plants depends upon availability and concentration of heavy metal as well as particular plant species. India has been the largest producer, consumer and importer of pulses because pulses dominantly constitute the staple diet of the people in India. In India, the production of black gram hovers around 1.3 to 1.5 million tons annually. Therefore, in this study attempt has been made to investigate the individual and combined effect of copper sulphate and zinc sulphate on seed germination, seedling growth, nitrate reductase and antioxidant enzymes activity in black gram, *Vigna mungo*.

Materials and Methods

Seeds of *Vigna mungo* (L.) Hepper Cv. T-9 (black gram) was obtained from National Seed Corporation Unit, I.A.R.I., PUSA, New Delhi. 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 was added to the nutrient solution at concentration 0.05, 0.1 and 0.2 mM as $CuSO_4.5H_2O$, Zinc as 0.25, 0.50, 1.00 and 1.50 mM of $ZnSO_4.7H_2O$ and a mixture of $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$ at different concentrations (Table 1).

Sterile conditions were maintained by adding 20 µg ml^{-1} of streptomycin sulphate in the medium to suppress microbial growth. All experiments were carried out for 8 days at 28±2°C in alternate light and dark period of 8 and 16 hr respectively under the light intensity of 120 µmol$m^{-2}s^{-1}$. Seed germination (24, 48 and 72 hr) and seedling growth activities (plumule and radicle length) were observed at 4,6 and 8th day.

Enzyme assay: Seedlings were taken on 2,4,6 and 8th day and washed thoroughly with distilled water. The extraction media and assay procedure for various enzymes were as described by Srivastava (1975) for nitrate reductase, by Abei (1984) for catalase and by Pundir *et al*. (1999) for peroxidase.

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 $p \leq 0.05$ as significant level according to Dunnett's multiple comparison test.

Results and Discussion

Effect on seed germination: The inhibitory effect of copper and zinc is apparent at the concentration of 0.1 and 0.50 mM respectively. But, the effect is more severe when copper and zinc were combined together as shown in Fig. 1. Maximum reduction has been noticed at 0.2 mM $CuSO_4$ + 1.50 mM $ZnSO_4$ causing 20% decrease in rate of seed germination. It was followed by 1.50 mM $ZnSO_4$ which resulted in 17% reduction in seed germination. It is obvious from the result of present investigation that seed germination under different treatment conditions were affected by the presence of both copper and zinc ions in such a way that the rate of germination was retarded with increasing concentration. This decrease was significant at all the concentrations when compared to the control condition. Moreover, results showed that heavy metals have induced delayed response in case of germination because the number of seeds germinated after 24 hr is less in comparison to those which were noticed after 48 and 72 hr. These results are in agreement with the findings of Burhan *et al.,* 2001; Munzuroglu and Geckil, 2002;

Table - 1: Effect of copper and zinc on plumule and radicle length (mm) in *Vigna mungo* (L.)

Concentration (mM)		Plumule length			Radicle length		
$CuSO_4.5H_2O$	$ZnSO_4.7H_2O$	4th d	6th d	8th d	4th d	6th d	8th d
0.0	0.0	15.56 ± 0.73	33.23 ± 1.60	60.60 ± 2.21	32.60 ± 3.20	35.80 ± 0.56	39.06 ± 0.30
0.05	0.0	13.23 ± 0.60**	20.50± 0.75***	27.30± 0.90***	17.30± 1.40***	30.36± 0.70***	37.60 ± 2.30
0.1	0.0	11.10± 0.36***	17.93± 0.90***	21.10± 1.60***	12.60± 1.00***	22.80± 0.20***	25.76± 0.90***
0.2	0.0	7.90 ± 1.73***	10.10± 0.26***	11.13± 2.65***	12.00± 1.30***	12.23± 0.35***	14.06± 0.20***
0.0	0.25	14.20 ± 0.60	17.50± 2.05***	24.00± 2.60***	26.30± 2.10***	37.06 ± 1.10	38.00 ± 0.60
0.0	0.50	9.60 ± 0.62***	14.60± 1.20***	18.40± 0.91***	15.30± 1.40***	29.96± 1.28***	30.40 ± 0.75*
0.0	1.00	7.26 ± 0.45***	9.43 ± 0.73***	13.30± 1.67***	14.60± 0.10***	22.50± 1.15***	24.40± 0.96***
0.0	1.50	6.30 ± 0.36***	8.10 ± 0.20***	10.60± 0.35***	13.30± 0.90***	14.33± 1.61***	15.63± 0.77***
0.2	0.25	6.30 ± 0.55***	7.46 ± 0.15***	8.36 ± 0.25***	7.03± 0.15***	7.40 ± 0.26***	7.60 ± 0.90***
0.2	0.50	5.30 ± 0.40***	5.96 ± 0.25***	7.23 ± 0.25***	6.60 ± 1.20***	6.43 ± 0.47***	7.06 ± 0.20***
0.2	1.00	3.60 ± 0.30***	3.96 ± 0.15***	5.03 ± 0.25***	4.43 ± 0.56***	5.10 ± 0.45***	5.23 ± 0.20***
0.2	1.50	3.03 ± 0.25***	3.46 ± 0.15***	3.56 ± 0.15***	4.30 ± 0.20***	4.63 ± 0.35***	4.63 ± 0.45***

Values are mean of three replicates ± SD, *, ** and *** indicate significant difference from the control at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$

Table - 2: Effect of copper and zinc on specific activity of nitrate reductase enzyme (µmol NO_2. g^{-1}.f.wt..hr^{-1} mg^{-1} protein) in *Vigna mungo* (L.)

Concentration (mM)		Specific activity of nitrate reductase			
$CuSO_4.5H_2O$	$ZnSO_4.7H_2O$	2nd d	4th d	6th d	8th d
0.0	0.0	6.108 ± 0.22	8.406 ± 0.52	16.295 ± 1.99	21.717 ± 0.67
0.05	0.0	4.402 ± 0.05	6.593 ± 1.31	10.376 ± 1.11	13.987 ± 0.09
0.1	0.0	4.040 ± 0.23	6.371 ± 0.59	9.110 ± 0.73	12.468 ± 0.10
0.2	0.0	1.975 ± 0.05	4.725 ± 0.34	3.530 ± 0.36	10.749 ± 0.17
0.0	0.25	5.911 ± 0.27	6.201 ± 0.90	10.124 ± 2.13	14.802 ± 1.55
0.0	0.50	4.986 ± 0.55	6.008 ± 0.20	9.747 ± 0.94	14.073 ± 1.85
0.0	1.00	4.925 ± 1.25	5.998 ± 0.96	9.094 ± 0.57	13.027 ± 2.58
0.0	1.50	3.615 ± 0.14	5.681 ± 0.92	8.754 ± 2.50	10.067 ± 1.73
0.2	0.25	3.002 ± 0.28	4.418 ± 0.14	5.507 ± 0.09	5.889 ± 0.61*
0.2	0.50	2.540 ± 0.36	3.912 ± 0.04	5.359 ± 0.53	4.177 ± 0.85*
0.2	1.00	2.310 ± 0.09	3.878 ± 0.56	4.741 ± 1.15	4.040 ± 0.34*
0.2	1.50	2.018 ± 0.87	3.532 ± 0.10	4.020 ± 0.74	3.606 ± 1.01**

Values are mean of three replicates ± SD, *, ** and *** indicate significant difference from the control at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$

Table - 3: Effect of copper and zinc on specific activity of catalase and peroxidase enzyme (mm H_2O_2 decomposed $min^{-1}.g^{-1}$.f.wt. mg^{-1} protein) in *Vigna mungo* (L.)

Concentration (mM)		Specific activity of catalase				Specific activity of peroxidase			
$CuSO_4.5H_2O$	$ZnSO_4.7H_2O$	2nd d	4th d	6th d	8th d	2nd d	4th d	6th d	8th d
0.0	0.0	0.505 ± 0.01	0.945 ± 0.06	1.278 ± 0.09	0.674 ± 0.05	7.945 ± 0.53	9.688 ± 0.40	12.310 ± 2.61	15.748± 2.98
0.05	0.0	0.560 ± 0.01	0.639 ± 0.04	0.546 ± 0.01	0.365 ±0.04*	14.141 ± 0.24	19.564 ± 1.44	21.244 ± 1.19	15.363 ± 2.28
0.1	0.0	0.617 ± 0.12	0.819 ± 0.05	0.679 ± 0.03	0.411 ±0.03	18.202 ± 0.92	23.813 ± 0.58	18.181 ± 1.67	11.918 ± 0.52
0.2	0.0	0.601 ± 0.24	0.742 ± 0.04	0.461 ± 0.03	0.417 ±0.02*	7.981 ± 0.51	18.852 ± 0.94	9.245 ± 0.35	8.838 ± 1.45
0.0	0.25	0.571 ± 0.03	0.438 ± 0.03	0.372 ± 0.02	0.268 ±0.05*	14.254 ± 0.15	14.485 ± 2.04	18.015 ± 0.93	13.613 ± 1.51
0.0	0.50	0.509 ± 0.09	0.434 ± 0.06	0.324 ± 0.02	0.179±0.02**	19.706 ± 0.35	19.310 ± 0.64	20.302 ± 1.06	17.580 ± 1.22
0.0	1.00	0.273 ± 0.03	0.394 ± 0.11	0.304 ± 0.06	0.084±0.00**	25.695 ± 2.87	26.716 ± 2.93	22.451 ± 1.23	24.412 ± 2.61
0.0	1.50	0.263 ± 0.02	0.247 ± 0.00	0.292 ± 0.00	0.073±0.01**	17.693 ± 1.29	24.967 ± 2.17	32.745 ± 3.47	17.979 ± 2.00
0.2	0.25	0.469 ± 0.02	0.513 ± 0.01	0.441 ± 0.01	0.348 ±0.03*	17.076 ± 1.191	17.124 ± 0.71	15.631 ± 0.74	18.666 ± 1.66
0.2	0.50	0.460 ± 0.03*	0.528 ± 0.02	0.291 ± 0.02	0.306±0.03**	2.110 ± 0.60	13.749 ± 0.70	16.489 ± 0.56	18.212 ± 1.05
0.2	1.00	0.407 ± 0.02*	0.335 ± 0.00	0.279 ± 0.03	0.298±0.03**	11.165 ± 0.33	10.706 ± 0.86	14.849 ± 0.74	17.564 ± 1.05
0.2	1.50	0.253 ± 0.01*	0.286 ±0.01*	0.271 ± 0.02	0.263±0.02**	9.657 ± 3.08	10.129 ± 0.53	14.999 ± 4.40	17.157 ± 2.25

Values are mean of three replicates ± SD, *, ** and *** indicate significant difference from the control at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$

EL-Ghamery *et al.,* 2003; Shri *et al*., 2009. Ionic toxicity may be the cause of drastic effects of heavy metal salts on seed germination or it could be due to osmotic effect (Shaukat *et al*., 1999). Reduction of seed germination can also be attributed to the alterations of selection permeability properties of cell membrane (Muhammad *et al*., 2008).

Effect on seedling growth: Copper and zinc had showed inhibitory effect on seedling growth. Both, root and shoot length were significantly reduced. Combined effect of both copper and zinc was synergistic on seedling growth and caused severe reduction as shown in Table 1. The longest plumule and radicle length was observed under control conditions. There was a marked difference in radicle and plumule length at varying concentration of $CuSO_4$ and $ZnSO_4$ and reduction increased with increase in concentration of heavy metal stress. The shortest plumule and radicle length was observed in case of 0.2 mM $CuSO_4$ + 1.50 mM $ZnSO_4$. There was 94.06 and 84.14% reduction in plumule and radical length, respectively. The inhibition was stronger in roots than in the shoot because the plant roots are first point of contact with these toxic heavy metal species in the nutrient media. Reduced root and shoot length in response to heavy metal has been reported by a number of investigators (Nag *et al.,* 1989; Zhenguo *et al*., 1998; Tomulescu *et al*., 2004; Zhang *et al*., 2009; John *et al*., 2009). The reduction in seedling growth during stress may be due to low water potential, hampered nutrient uptake and secondary stress such as oxidative stress (John *et al*., 2009). The reason for reduced seedling growth under metal treatment could be the reduction in meristematic cells present in this region and some enzymes present in cotyledons and endosperm. During seedling growth hydrolysis of food reserves takes place which is carried out by hydrolytic enzymes. So, the

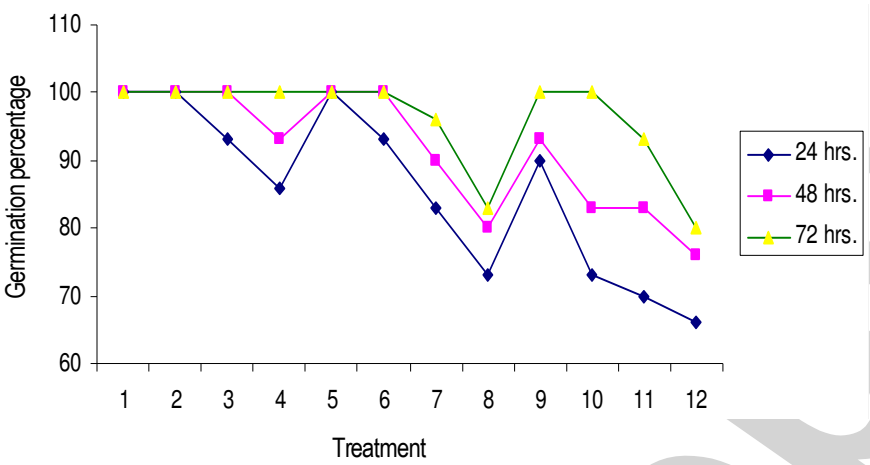

Fig. 1: Effect of copper and zinc on seed germination behaviour of seeds in *Vigna mungo* (L.)

activities of hydrolytic enzymes might be affected and the food did not reach to the radicle and plumule leading to the reduction in seedling growth. Similar observations have been made by several authors under various stressful conditions including metal toxicity (Mihoub *et al*., 2005; Ahsan *et al*., 2007; Kuriakosa and Prasad, 2008).

Effect on enzymes: Nitrate reductase activity was significantly reduced under treatment with copper and zinc. The inhibition was more pronounced with copper treatment as presented in Table 2. An inhibitory effect of $ZnSO_4$ and $CuSO_4$ on nitrate reductase activity began to be apparent at a concentration of 0.25 and 0.05 mM, respectively. Both copper and zinc showed negative effect on nitrate reductase activity and led to 85.47% reduction in specific nitrate reductase activity at the level 0.2 mM $CuSO_4$ + 1.50 mM $ZnSO_4$. These results agree with the findings of Luna *et al*., (1997 and 2000); Panda and Choudhury (2005). It indicates that even a low level of zinc and copper inactivate nitrate reductase enzyme. The toxic concentration of copper and zinc decrease the amount of protein-SH groups and induce membrane alterations as suggested by Luna *et al.,* (1997) and Brune *et al.,* (1995). It has been reported that NR activity depends upon active photosynthesis or production of photosynthates as it requires photosynthetically generated reductant and energy. Hence, reduction in NR activity could be due to reduced photosynthesis as a result of inhibition of chlorophyll biosynthesis (Rai *et al.,* 2004).

To mitigate and repair the damage initiated by reactive oxygen species the induction of the activities of a particular group of enzymes *i.e.*, antioxidant enzymes play an important role in the cellular defense strategy against oxidative stress caused by toxic heavy metal concentrations. The specific activity of catalase and peroxidase enzyme in *Vigna mungo* (L.) Hepper when subjected to copper and/or zinc stress are presented in Table 3. A variation in activity of both catalase and peroxidase enzyme has been noticed with respect to heavy metal concentration and time. The increased catalase activity at germination stage may be an adaptive response because the roots emerge out in an environment already under stress. But, later on the enzyme was not able to resist heavy metal toxicity. Catalase being photosensitive needs constant fresh synthesis (Feierabend *et al.,* 1992). Proper sequestration of heavy metals at germination stage probably prevented copper and zinc from inhibiting the enzymatic activity but at seedling stage there was decline in catalase activity by 60.97%. It might be due to inhibition of enzyme synthesis or change in enzymatic confirmation (Sreedevi *et al.,* 2008). Inadequate response of catalase activity to heavy metal was compensated by the increased activity of peroxidase enzyme. It seems to be important in resisting oxidative stress induced by zinc and copper in *Vigna mungo* (L.) Hepper. An increase in activity of peroxidase in response to zinc and/or copper suggests the active participation of the enzyme in scavenging reactive oxygen species under stressed conditions. On 8th day, there was 8.94% increase in specific activity of peroxidase enzyme at 0.2 mM Cu + 1.50 mM Zn treatment level. But, it is difficult to counteract the stress above a certain limit. Therefore, decreased antioxidant enzymes activity under highly stressed condition may be the result of imposition of oxidative stress *i.e.*, imbalance in generation and metabolism of reactive oxygen species (Luna *et al.,* 1994; Panda and Patra, 1998; Cakmak, 2000; Shah *et al.,* 2001; Rai *et al.,* 2004). The results showed the severity of metal induced oxidative stress. The study has concluded that copper and zinc in combination is more harmful in comparison to their individual effects.

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