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COMBINED EFFECTS OF ZINC AND COPPER ON SEED GERMINATION AND SEEDLING GROWTH OF *BRASSICA JUNCEA* L.

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The aim of present study was to determine the combined effects of the elevated concentrations of Zn and Cu on seed germination and seedling growth of *Brassica juncea* L. Also, the TTC reduction ability (a measure of the activity of dehydrogenases) of the excised embryos and α-amylase activity in the seeds were monitored. Seed germination and seedling growth were differentially influenced by Zn and Cu, applied individually or in combinations. Higher Zn ($\geq$ 500 µM) and Cu ($\geq$ 50 µM) concentrations, applied individually, did not markedly influence the seed germination whereas the lower ones were promotory at 1 d; certain Zn-Cu mixtures also proved promotory. However, the promotion was completely abolished at 3 d. In contrast, the seedling growth was suppressed by Zn and Cu, applied individually or in combinations; magnitude of inhibition of root elongation was much stronger than that of shoot. Based on the analysis of root elongation data according to response additivity, the joint effects of Zn and Cu were found to be antagonistic except in a couple of combinations i.e. 250 µM Zn + 50 µM Cu and 500 µM Zn + 100 µM Cu. The TTC reduction ability of excised embryos was strongly promoted by most of the tested binary combinations of Zn and Cu whereas the same was only marginally promoted by certain Zn or Cu concentrations applied individually. The lower Zn and Cu concentrations were promotory for α-amylase activity when applied individually as well as in combination. In contrast, the higher ones suppressed the α-amylase activity individually but not in combination. The measured metabolic parameters seem to be among the early metabolic targets of heavy metals (HMs) in influencing the seed germination and subsequent growth.

Keywords : Copper, Combined effects, Seed germination, Seedling growth, Zinc.

Heavy metals (HMs), among serious environmental contaminants, impose phytotoxic effects and in turn reduce the plant productivity (Woolhouse 1983). HMs often find their way into food chain via uptake and accumulation in plants. Some HMs such as Zn, Cu, Ni etc. are essential in small quantities for plant growth and development as they are required in specific cellular processes and functions (Marschner 2012). However, the essential HMs also become phytotoxic as and when their concentrations exceed certain threshold values. On the other hand, HMs such as Cd, Pb, Hg etc. have no known function in plants and are toxic even at low concentrations. Although phytotoxic effects of HMs are well documented, most of these are concerned with plant responses to single HMs. However, in an environmental context, HMs generally exist in combinations (Keltjens and Van Beusichem 1998, Sharma *et al.* 1999). Indeed, the single metal situations are rare. Obviously, the co-occurring HMs are likely to interact in imposing their phytotoxic effects. For example, different HMs applied in combinations have been reported to produce antagonistic, additive as well as synergistic effects in *Silene vulgaris* depending upon the HMs as well as their concentrations in different combinations (Sharma *et al.* 1999, Sneller *et al.* 2000). Plant responses to HM mixtures need to be characterized involving different plant species, particularly the ones that are potentially important in the context of phytoremediation. The latter offers a plant-based means of toxic metal clean-up of the environment (Kramer 2010). We have assessed the combined effects of elevated Zn and Cu copper concentrations on seed germination and seedling growth of Indian mustard (*Brassica juncea*) that is known for its ability to accumulate certain HMs in reasonably high concentrations. Both Zn and Cu are essential for plant growth and development but turn toxic at elevated concentrations (Marschner 2012). Besides, with a view to get an idea about the early metabolic targets of HMs within the seeds, the effects of Zn and/or Cu on TTC reduction ability of excised embryos and α-amylase activity in the seeds have been monitored.

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MATERIALS AND METHODS

Plant Material : Seeds of Indian mustard (*Brassica juncea* L.) cultivar Pusa Jai Kisan were procured from Indian Agricultural Research Institute, New Delhi and stored in plastic jars under ambient conditions until they were used.

Seed germination and seedling growth

B. juncea seeds of uniform size were surface sterilized with 0.1% $HgCl_2$ for 2-3 min and then washed thoroughly with distilled water. Thereafter, the seeds were imbibed with solutions containing the stated concentrations of Zn and Cu (sulphates), individually or in the desired binary combinations, for 24 h. Seeds imbibed simultaneously with distilled water constituted the control. Thereafter, the seeds were transferred to the petriplates lined with three layers of filter paper made wet with the respective concentrations of HMs applied individually and in combinations. In case of control, filter papers were made wet with an equal volume of distilled water. HM solutions were added only once at the start of treatment. Subsequently, distilled water was used to wet the substratum for next 7 d at regular intervals. The petriplates were placed in a plant growth chamber (Saveer, India) for seed germination and seedling growth for 7 d at 25 ± 2°C and relative humidity of 60% (16 h photoperiod, PAR: 90 µmol m^{-2} s^{-1}). Seed germination (%) was recorded at completion of 1, 2 and 3 d of treatment. After 7 d treatment, the seedling growth was measured in terms of root length, shoot length and fresh weight of seedlings. The nature of interaction between Zn and Cu was determined using the response addition model according to Sharma *et al.* (1999). Briefly, the expected root elongation inhibition due to the binary mixtures was determined by the following formula: $E = 1- (1- E_A) (1- E_B)$ where E represents the expected root inhibition; and E_A and E_B are the relative root inhibition (as a fraction of control) in the samples treated with HM A and B (i.e. Zn and Cu), respectively. Afterwards, student's t test was performed in order to confirm that E was within the 95% confidence interval of the observed mean root elongation inhibition caused by the binary mixture. Additive and antagonistic effects have been designated by 0 and -, respectively.

Determination of TTC reduction ability (a measure of dehydrogenase activity) of excised embryos

B. juncea seeds were soaked in solutions containing the stated concentrations of Zn and Cu (individually or in combinations) for 24 h. From the Zn/Cu treated seeds, embryos were excised and incubated with 0.1% TTC (triphenyl tetrazolium chloride) for 24 h in dark. The stained embryos (due to formazan formation) were homogenized with MetOH and centrifuged at 5000 rpm for 5 min at 4°C. Absorbance of supernatant was read at 485 nm and TTC reduction ability was expressed in terms of A_{485} per 5 embryos.

α–amylase assay

α-amylase activity was determined in the *B. juncea* seeds imbibed with the stated concentrations of Zn and Cu (individually or in combinations) for 24 h according to the method given by Filner and Varner (1967). Ten seeds were homogenized with 0.05 M Tris- HCl buffer, pH 7.2 (chilled). The homogenate was centrifuged at 10,000 rpm for 10 min (4°C) and the collected supernatant was used as enzyme extract. The reaction mixture contained 1 ml enzyme extract and 1 ml of substrate (0.15% starch containing 0.2 mM $CaCl_2$), incubated for 10 min (25°C). Thereafter, 3 ml IKI (0.6% iodine in 6% potassium iodide; 1 ml diluted to 50 ml with 0.05 N HCl) reagent was added. In control set, enzyme extract was added after addition of IKI. Absorbance was read at 620 nm. The α-amylase activity was determined with the help of calibration curve made with the help of starch.

Determination of protein contents

Protein contents were determined with the Bradford reagent (Bradford 1976). The reaction mixture contained 790 µl distilled water, 10 µl enzyme extract (as above) and 200 µl Bradford reagent. The mixture was allowed to react for 5 min at room temperature. The absorbance was read at 595 nm. The protein content was measured using calibration curve made with the help of BSA.

RESULTS

Seed germination and seedling growth responses of *B. juncea* to Zn (0-1500 µM) and Cu (0-200 µM), applied either individually or in desired combinations, were monitored. Seed germination was observed at completion of 1, 2 and 3 d of treatment (data presented for 1 and 3 d; Table 1). At 1 d, higher Zn (≥ 500 µM) and Cu (≥ 50 µM) concentrations, applied individually, did not markedly influence the seed germination. However, the lower concentrations were promotory. For example, 25 µM Cu and 250 µM Zn promoted the seed germination by 26 and 21%, respectively. At 3 d, the described promotory effect was completely abolished while the higher concentrations remained without effect (Table 1). The promotory effect of Zn (250 µM) and Cu (25 µM) on seed germination at 1 d was not evident when these two concentrations were combined. Occasionally, the combined effects of Zn and Cu appeared to be promotory e.g., seed germination was enhanced by 27% due to the seed treatment with 500 µM Zn + 100 µM Cu. These concentrations did not alter the seed germination much when applied individually (Table 1). Combinations of other concentrations did not substantially affect the seed germination except some quantitative fluctuations. At 3 d, none of the treatments was much effective. Seedling growth, measured after 7 d of treatment in terms of root length shoot length and seedling fresh weight, was suppressed by Zn and/or Cu. Root length suppression was invariably much stronger than that of shoot. For example, 200 µM Cu and 1500 µM Zn, applied individually inhibited the root length by 59 and 78%, respectively whereas these figures were 20 and 9% only in case of shoot length and 15 and 25% in case of seedling fresh weight (Fig. 1).

The nature of interaction between Zn and Cu in affecting the *B. juncea* seedling growth was determined by analyzing the joint effects of Zn and Cu on root length, the most affected parameter, on the basis of response additivity (Table 2). This analysis revealed the effects of most of the combinations of Zn and Cu to be antagonistic except 250 µM Zn + 50 µM Cu and 500 µM Zn + 100 µM Cu, where the joint effects were found to be additive (Fig. 1A; Table 2). In contrast to root elongation, the effect of mixtures of Zn and Cu concentrations on shoot length was not prominent (Fig. 1B). Likewise, the seedling fresh weight was not affected much due to the binary combinations of Zn and Cu in lower range of concentrations (Zn ≤ 500 µM; Cu ≤ 100 µM). At higher concentrations, the combined effects were stronger as compared to the individual effects. Thus, at 1500 µM Zn + 200 µM Cu, a 32% inhibition of seedling fresh weight was observed (Fig. 1C).

TTC reduction ability of excised embryos and α-amylase activity

Activity of dehydrogenases was measured in terms of TTC reduction ability of embryos excised from *B. juncea* seeds treated with stated HM concentrations, applied individually or in combinations, for 24 h. Due to the treatment of seeds with Zn and Cu alone, dehydrogenases activity of embryos was moderately enhanced at certain concentrations. For example, an increase of 8 and 17% in TTC reduction ability was observed at 25 and 200 µM Cu, respectively; but no change was observed at 50 and 100 µM Cu. Similarly, an increase of 12 and 38% was observed at 500 and 1500 µM Zn, respectively (Fig. 2A). Irrespective of the effects of Zn and Cu alone, most of the tested binary combinations of Zn and Cu proved strongly promotory for TTC reduction ability of the embryos. For example, a 49% promotion was evident with a combination of 1000 µM Zn and 100 µM Cu. Combinations involving 1500 µM Zn were the exception. A distinct concentration dependent pattern of change, however, was not observed (Fig. 2A). α-amylase activity was assayed in seeds of *B. juncea* treated with HMs for 24 h. Both Zn and Cu, applied alone, promoted the α-amylase activity at their lower concentrations and inhibited the same at higher ones. The effects of Cu were more pronounced as compared to those of Zn. Thus, Cu promoted

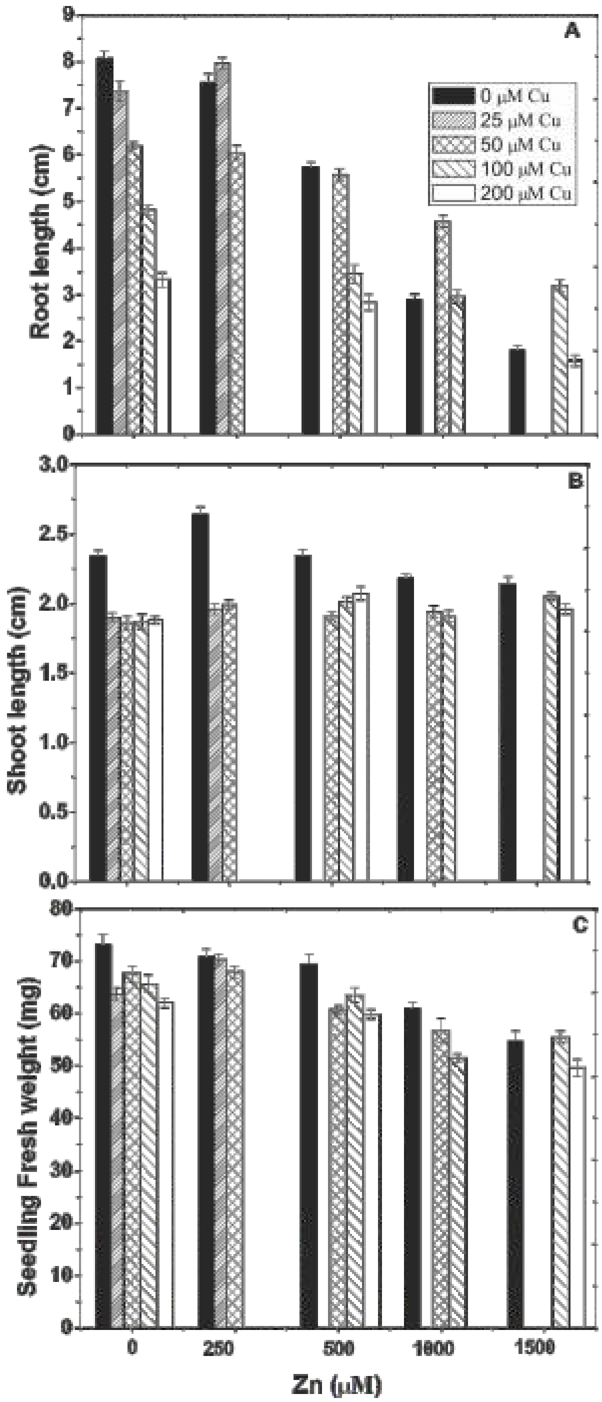

Fig. 1 : Effects of Zn and Cu, applied individually or in combinations, on seedling growth (7-d) of *Brassica juncea* in terms of root length A), shoot length B) and seedling fresh weight C). Data are arithmetic means ± SE, n=20.

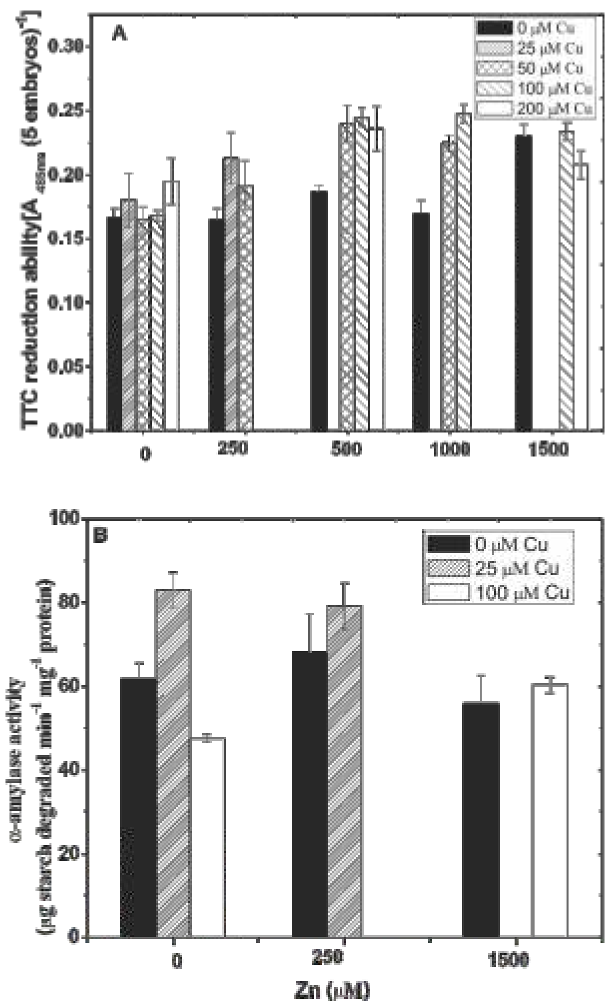

Fig.2 : Effects of Zn and Cu, applied alone or in combinations, on activity of dehydrogenases in embryos excised from treated seeds (A) and α-amylase acitivity (B) in seeds of *B. juncea.* Data are arithmetic means ± SE, n=3 (A), 4 (B).

the α-amylase activity by 34% at 25 μM and inhibited the same by 23% at 100 μM (Fig. 2 B). Zn promoted and suppressed the activity by 10% at 250 μM and 1500 μM Zn, respectively. The combined effects of Zn and Cu on α-amylase activity were concentration dependent. Whereas a combination of lower HM concentrations (250 μM Zn + 25 μM Cu) promoted the activity by 28% that of higher concentrations (1500 μM Zn + 100 μM Cu) had no effect (Fig. 2 B).

DISCUSSION

The present study aimed to determine the seed germination and seedling growth responses of *Brassica juncea* cv. Pusa Jai Kisan to the binary mixtures of Zn and Cu. The idea was

Table 1 : Effects of Zn and Cu, applied individually or in combinations, on seed germination (%) of *Brassica juncea*. Data at completion of 1 and 3 d of treatment. Values are arithmetic means ± SE, n=4 (10 seeds per replicate). NT = not tested.

Zn (µM)		Cu (µM)				
		0	25	50	100	200
0	1 d	58.75 ± 5.41	73.75 ± 6.47	55.00 ± 6.85	60.00 ± 6.12	65.00 ± 3.95
	3 d	78.75 ± 4.80	81.25 ± 3.17	70.00 ± 7.50	81.25 ± 5.12	83.75 ± 4.46
250	1 d	71.25 ± 7.15	63.75 ± 4.80	53.75 ± 3.70		
	3 d	85.00 ± 1.77	81.25 ± 2.07	81.25 ± 5.69	NT	NT
500	1 d	51.25 ± 8.90		62.50 ± 7.40	75.00 ± 3.54	61.25 ± 6.70
	3 d	81.25 ± 1.77	NT	82.50 ± 6.25	86.25 ± 4.10	81.25 ± 3.25
1000	1 d	56.25 ± 2.07		65.00 ± 6.12	62.50 ± 4.51	
	3 d	83.75 ± 1.08	NT	82.50 ± 5.45	90.00 ± 1.77	NT
1500	1 d	56.25 ± 5.69			55.00 ± 6.37	53.75 ± 2.07
	3 d	80.00 ± 2.50	NT	NT	86.25 ± 5.41	88.75 ± 2.07

Table 2 : Combined effects of binary mixtures of Zn and Cu on root growth elongation of 7-d-old seedlings of *Brassica juncea* based on response addition model where 0 = join effect additive, - = joint effect antagonistic (α = 0.05) (no combination was synergistic). NT = not tested.

Cu (µM)	Zn (µM)			
	250	500	1000	1500
25	-	NT	NT	NT
50	0	-	-	NT
100	NT	0	-	-
200	NT	-	NT	-

to get insight into the way two co-occurring HMs influence each other's effects. The plants growing in metal contaminated areas are often exposed to a mixture of metals rather than a single metal (Keltjens and Van Beusichem 1998, Sharma *et al.* 1999). However, the plant responses to the mixtures of HMs have not been worked out in detail in contrast to those due to single HMs. Both Zn and Cu are required by plants in limited amount for their normal growth and metabolism but become toxic at elevated concentrations. A time-course of seed germination revealed that some lower concentrations of Zn and Cu, applied either alone or in combination, proved promotory for germination initially i.e., at 1 d; the observed promotory effect was completely abolished at 3 d. A slight stimulation of seed germination due to low concentrations of HMs has been reported earlier also, e. g., in case of Cu (Kjaer *et al.* 1998) and Cd (Lefevre *et al.* 2009). The precise mechanism of HM-induced promotion of seed germination is not clear. Among other possibilities, low HM ion levels might influence the enzyme conformations stimulating their activity. In the present study also, the lower Zn and Cu concentrations were found to enhance the amylase activity in seeds. This could be expected to stimulate germination by enhanced utilization of seed reserves. Although different ROS are damaging for cellular processes, at low level they act as signaling molecules. The ROS and reactive nitrogen species (RNS) could stimulate germination (Lefevre *et al.* 2009). Since the initially induced promotory effect of HMs on *B. juncea* seed germination was eventually lost, the significance of the observations needs to be assessed. Some earlier reports also show no effect of HMs (Zn, Cu, Cd) on seed germination e.g., in *Merwilla natalensis* (Street *et al.* 2007) and *Eruca sativa* (Ozdener and Kutbay 2009).

Seedling growth of *B. juncea* was found to be much more affected due to HMs than seed germination. Stronger effect of HMs on root elongation is obvious due to the fact that the roots are the first to encounter toxic HMs retaining a larger part of HMs taken up by plant (Sharma *et al.* 1999, 2004). Zn- and Cu-induced root growth inhibition is comparable to that reported earlier in case of *Helianthus annuus* due to Zn (Jadia and Fulekar 2008), *Sorghum bicolor* due to Cd (Kuriakose and Prasad 2008) and *Brassica parkensis* due to Cu (Xiong and Wang 2005).

The combined effects of HMs, applied in binary mixtures, were analyzed using the root elongation data primarily because roots constitute the primary target of toxic HM ions. Of the several statistical models of analysis for the purpose (Sharma *et al.* 1999), response addition model was employed to determine the nature of interaction between Zn and Cu. The combination of Zn and Cu produced antagonistic effects on seedling root elongation except in a couple of cases. The observed antagonism could be a consequence of the mutual interference between Zn and Cu at the level of uptake by the roots although we did not measure the tissue HM contents. This was found to be the case with low Zn and Cu concentrations in *Silene vulgaris* (Sharma *et al.* 1999). In addition, Zn and Cu are likely to interact within the cell e.g., for binding to the target sites/molecules. In contrast to root length, the shoot length was much less affected, as has often been reported (Sharma *et al.* 2004, Thakur and Sharma 2015). This is apparently due to a restricted root-to-shoot translocation of HMs.

The TTC reduction ability, a measure of dehydrogenase activity, of embryos excised from metal treated seeds was observed to fluctuate in case of single metal treatments, but the same was promoted due to all binary combinations of Zn and Cu. Since dehydrogenases responsible for TTC reduction also include those from respiratory metabolism e.g., SDH, it seems that embryo respiration was not affected by the HMs applied individually. However, in a joint application of Zn and Cu, respiratory metabolism was stimulated in order to meet the energy demands of embryos under stress. α- amylase hydrolyzes the starch into metabolizable sugars, providing energy for the embryo growth (Beck and Ziegler 1989), which in turn affects germination. α-amylase activity was promoted by lower and suppressed by higher HM concentrations. Higher Cu concentration induced suppression of α-amylase activity was antagonized by higher Zn concentration most likey due to uptake antagonsim. HM ions might also influence the enzyme conformation in affecting the activity. The altered activity of dehydrogenases within the embryos themselves and α-amylase in the seeds likely contribute to the observed effects of Zn and/or Cu on seed germination and seedling growth of *B. juncea*. In brief, the findings are of significance in the context of phytoremedation since the seedling establishment in HM-rich conditions is a prerequisite for phytoremediation.

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