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

SAPNA AND SHANTI S. SHARMA

Department of Biosciences
Himachal Pradesh University, Shimla 171005

E-Mail : shantisharma@hotmail.com

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 (≥ 500 μM) and Cu (≥ 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.
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% HgCl₂, 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 HMIs 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: \( E = 1 - \left(1 - E_A\right) \left(1 - E_B\right) \) 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 CaCl₂), 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 activity (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.

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

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.

<table>
<thead>
<tr>
<th>Cu (µM)</th>
<th>Zn (µM)</th>
<th>250</th>
<th>500</th>
<th>1000</th>
<th>1500</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>100</td>
<td>NT</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>NT</td>
<td>-</td>
<td>NT</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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 likely due to uptake antagonism. 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
s i g n i f i c a n c e i n t h e c o n t e x t o f 
phytoremediation since the seedling 
establishment in HM-rich conditions is a 
prerequisite for phytoremediation.

Award of NET-SRF by the University Grants 
Commission, New Delhi to Sapna is thankfully 
acknowledged.

REFERENCES

Beck E and Ziegler P 1989 Biosynthesis and 
degradation of starch in higher plants. Ann 
Rev Plant Biol 40(1) 95-117.

Bradford MM 1976 A rapid and sensitive 
method for the quantitation of microgram 
quantities of protein utilizing the principle of 
protein-dye binding. Anal Biochem 72(1) 248- 
254.

Filner P and Varner JE 1967 A test for de 
novo synthesis of enzymes: density labeling 
with H2O18 of barley alpha-amylase induced by 
hibberelic acid. Proc Natl Acad Sci USA 
58(4) 1520-1526.

Jadia CD and Fulekar MH 2008 Phytoremediation : 
the application of vermicompost to remove zinc, cadmium, 
copper, nickel and lead by sunflower plant. 
Environ Eng Manage J 7(5) 547-558.

Keltjens WG and Van Beusichem ML 1998 
Phytochelatins as biomarkers for heavy metal 
stress in maize (Zea mays L.) and wheat 
(Triticum aestivum L.) : combined effects of 
copper and cadmium. Plant soil 203(1) 119- 
126.

Kjaer C, Pedersen MB and Elmegaard N 1998 
Effects of soil copper on black bindweed 
(Fallopia convolvulus) in the laboratory and 
in the field. Arch Environ Contam Toxicol 
35(1) 14-19.

Krämer U 2010 Metal hyperaccumulation in 

Kuriakose SV and Prasad MNV 2008 
Cadmium stress affects seed germination and 
seedling growth in Sorghum bicolor (L.) 
Moench by changing the activities of 
hydrolyzing enzymes. Plant Grow Reg 54(2) 
143-156.

Lefèvre I, Marchal G, Corréal E, Zanuzzi A 
and Lutts S 2009 Variation in response to 
heavy metals during vegetative growth in 
Dorycnium pentaphyllum Scop. Plant Grow 
Reg 59(1) 1-11.

Marschner P 2012 Marschner's mineral 
nutrition of higher plants. Academic press, 
London.

Ozdener Y and Kutbay HG 2009 Toxicity of 
copper, cadmium, nickel, lead and zinc on 
seed germination and seedling growth in 

Sharma SS, Schat H, Vooijs R and Van 
Heerwaarden LM 1999 Combination 
toxicology of copper, zinc, and cadmium in 
binary mixtures: concentration-dependent 
antagonistic, nonadditive, and synergistic 
effects on root growth in Silene vulgaris. 
Environ Toxicol Chem 18(2) 348-355.

Sharma SS, Kaul S, Metwally A, Goyal KC, 
Finkemeier I and Dietz KJ 2004 Cadmium 
toxicity to barley (Hordeum vulgare) as 
affected by varying Fe nutritional status. 
Plant Sci 166(5) 287-1295.

Sneller FEC, van Heerwaarden LM, Schat H 
and Verkleij JAC 2000 Toxicity, metal uptake 
and accumulation of phytochelatins in Silene 
vulgaris exposed to mixtures of cadmium and 
arSENate. Environ Toxicol Chem 19(12) 2982- 
2986.

Street RA, Kulkarni MG, Stirk WA, 
Southway C and Van Staden J 2007 Toxicity 
of metal elements on germination and 
seedling growth of widely used medicinal 
plants belonging to Hyacinthaceae. Bull 
Environ Contam Toxicol 79(4) 371-376.

Thakur S and Sharma SS 2015 
Characterization of seed germination, seedling 
growth and associated metabolic responses of 
Brassica juncea L. cultivars to elevated nickel 
cent rations. Protoplasma (In press). DOI: 
10.1007/s 00709-0-15-0835-0.

Woolhouse HW 1983 Toxicity and tolerance 
in the responses of plants to metals. Encycl 
Plant Physiol NS 12 245-300.

Xiong ZT and Wang H 2005 Copper toxicity 
and bioaccumulation in Chinese cabbage 
(Brassica pekinensis Rupr.). Environ Toxicol 
20(2) 188-194.