



## ALLEVIATION OF COPPER- AND CADMIUM-INDUCED SUPPRESSION OF BARLEY (*HORDEUM VULGARE* L.) SEEDLING GROWTH BY PROLINE

NAMARTA AND SHANTI S. SHARMA

Department of Biosciences, Himachal Pradesh University, Shimla 171005

E-Mail : shantisharma@hotmail.com

In the present study, it was examined whether proline could alleviate the suppression of copper- or cadmium-induced suppression of seedling growth in barley (*Hordeum vulgare* L.). Both Cu and Cd, applied in a range of 0-500  $\mu$ M, inhibited the seedling growth of *H. vulgare* in terms of root length, shoot length and seedling fresh weight. Distinct organ- as well as metal-specific differences in the response were evident. The pretreatment of caryopses with proline (1, 10 mM) led to partial alleviation of Cu- or Cd-induced suppression of seedling growth; the magnitude of alleviation was greater at 10 mM proline. Furthermore, proline was found to be more effective in reverting the inhibitory effects of Cu as compared to those of Cd. Such difference could inter alia be ascribed to the fact that Cu, a redox element, imposes oxidative stress more strongly that could be countered by proline. The findings are consistent with a role of proline in HM detoxification.

**Keywords :** Copper, Cadmium, Heavy metals, *Hordeum vulgare*, seedling growth.

The environmental contamination with heavy metals (HMs), owing to a range of anthropogenic reasons, is an issue of great concern. HMs are taken up and accumulate in plants imposing deleterious phytotoxic effects that leads to considerable yield losses. In fact, the plants often comprise the entry points for HMs into food chain with potential adverse effects on human health. Plants possess several strategies to minimize the heavy metal toxicity (Hall 2002, Sharma and Dietz 2006). These include the chelation of toxic HM ions by certain specific ligands like phytochelatin (PCs) and metallothioneins (MTs). The PC-HM complexes are sequestered into the vacuole thus reducing the HM contents in the cytoplasm (Song *et al.*, 2010). The vacuolar compartmentalization thus plays an important role in HM detoxification (Martinoia *et al.* 2012, Sharma *et al.* 2016). PCs and MTs, however, have limitations and could not explain the detoxification of all HMs. Obviously; additional mechanisms are involved in HM detoxification.

In response to the exposure to different HMs, plants have been reported to accumulate free proline e.g. in *Lemna minor* (Bassi and Sharma 1993a), *Triticum aestivum* (Bassi and Sharma 1993b) and *Silene vulgaris* (Schat *et al.* 1997). Proline is likely to perform multiple functions that might help plants adapt to HM

stress. These include osmoregulation, enzyme protection possibly through HM binding (Sharma *et al.* 1998) and free radical scavenger (Kaul *et al.* 2008). The protective role of proline against HM-induced toxic effects needs to be evaluated at different growth stages of diverse plant species. In the present study, we have examined whether pretreatment of barley (*Hordeum vulgare*) caryopses could alleviate the suppression of seedling growth due to Cu and Cd, representing a redox and a non- redox HM, respectively.

### MATERIALS AND METHODS

The caryopses of barley (*Hordeum vulgare* L.) cultivar BHS-169 were procured from Indian Agricultural Research Institute substation Tutikandi, Shimla. They were stored in plastic containers under ambient conditions in the laboratory.

Barley caryopses selected for uniformity of size were imbibed with proline (1 and 10 mM) for 15 h. The control caryopses were simultaneously imbibed in distilled water. Thereafter, they were treated with different Cu/Cd (applied as sulphates) concentrations (0-500  $\mu$ M) for 5 h. The caryopses imbibed simultaneously with distilled water constituted the control. The caryopses treated in this manner were transferred to petriplates

lined with Whatman filter paper made wet with 5 ml solution containing the respective Cu or Cd concentrations for germination and seedling growth. Subsequently, water was used to maintain the substratum wet. Each treatment comprised 20 caryopses per petriplate in triplicate. After 4 d, seedling growth was measured in terms of root length, shoot length and seedling fresh weight.

The experiments were performed in triplicate and repeated at least once. Data are presented as arithmetic means  $\pm$  S.D.

## RESULTS

Due to Cu or Cd treatment, the seedling growth of *H. vulgare* was suppressed in a concentration dependent manner. The organ- as well as HM-specific differences in response were evident. Thus, at higher Cu concentrations, the shoot growth was suppressed to a greater extent as compared to the root growth. Cu inhibited the root growth by 22 and 26% at 200 and 500  $\mu$ M Cu, respectively, whereas these values were 27 and 35% in case of shoot growth. However, at lower Cu concentration (100  $\mu$ M), the root growth inhibition (20%) was more than that of shoot growth (12%) (Table 1, Plate 1A). In case of Cd, root growth was invariably inhibited by a greater extent as compared to that of shoot. Thus, the magnitude of Cd-induced root growth inhibition was 42, 62 and 68% at 100, 200 and 500  $\mu$ M, respectively whereas these values were 12, 29 and 42% in case of shoot growth (Table 2, Plate 1B). Seedling fresh weight was suppressed by 15, 20 and 25% at 100, 200 and 500  $\mu$ M Cu, respectively whereas the suppression was 8, 23 and 36% in case of Cd (Table 1, 2).

Proline pretreatment of caryopses led to alleviation of the Cu-or Cd-induced inhibition of *H. vulgare* seedling growth. The proline effect was generally greater at the higher concentration. Proline-dependent reversal was stronger in case of Cu. Thus, the Cu-induced suppression of root length was almost completely reverted by both the proline concentrations. In fact, the root length in case of Cu, 100  $\mu$ M + Pro (1 or 10 mM) was slightly greater than that in case of control.

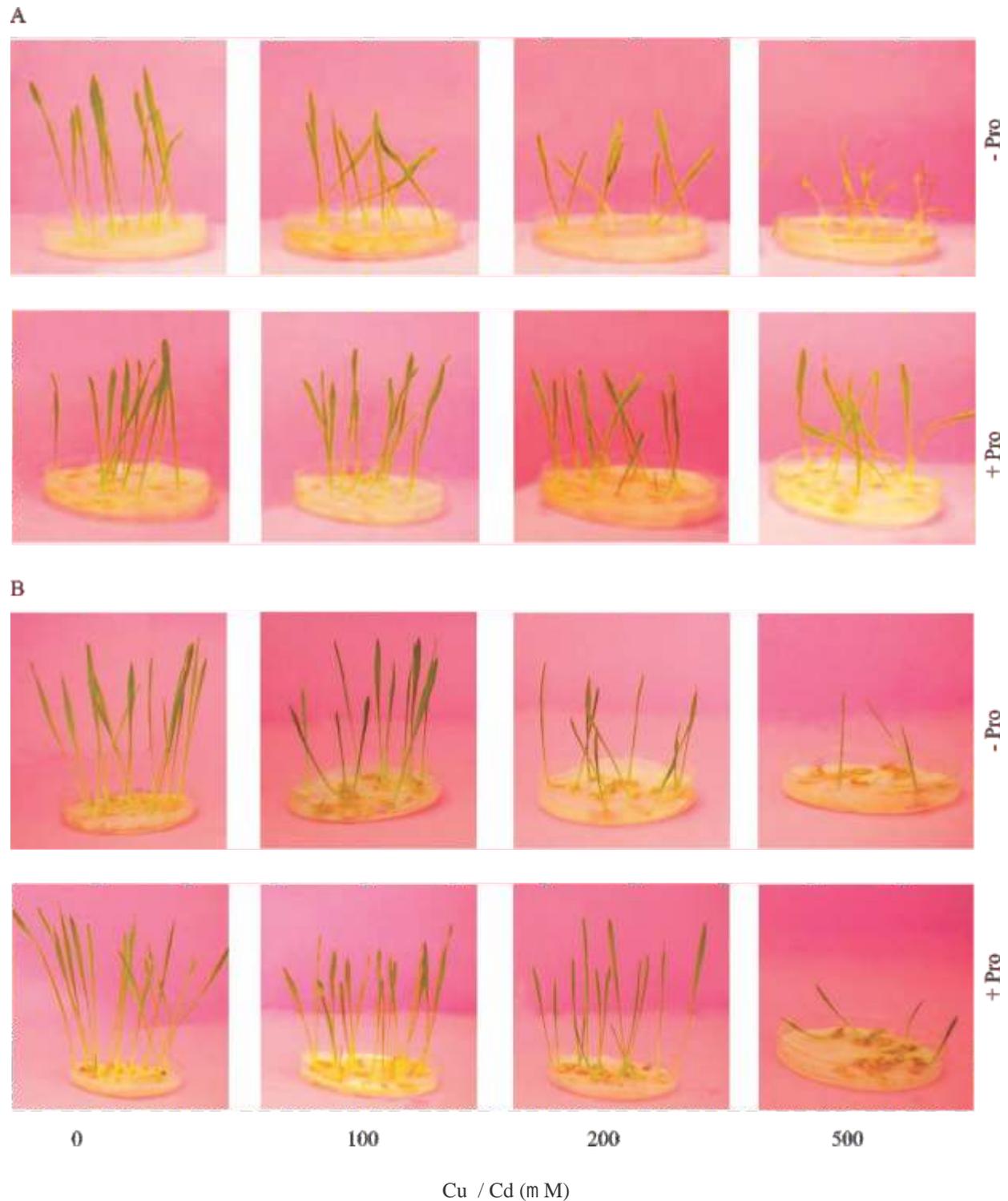
Likewise, the Cu-induced shoot length reduction was also reverted due to the Pro pretreatment. Thus, in case of Pro (1 mM) pretreated seedlings, Cu inhibited the shoot length by 11, 5 and 20% at 100, 200 and 500  $\mu$ M, respectively. In contrast, these values were 12, 27 and 35%, respectively in case of Pro untreated seedlings. Similar findings were obtained with 10 mM Pro. Cd induced suppression of root length was only marginally reverted by Pro. For example, Cd suppressed the root length by 31, 51 and 61% in case of Pro (10 mM) pretreated seedlings against 42, 62 and 68 % in case of seedlings not pretreated with proline. Pro (1 mM) was not much effective in reverting the root growth inhibition due to Cd. Similar findings were obtained with the shoot length. For example, 100, 200 and 500  $\mu$ M Cd suppressed the shoot length by 12, 5 and 26%, respectively, in the presence of 10 mM Pro and by 12, 29 and 42% respectively, in the absence of 10 mM Pro (Table 2). Seedling fresh weight changes due to Pro and/or HM (Cu/Cd) treatment generally paralleled those in the root and shoot length with some quantitative variations.

When applied individually, proline marginally suppressed the seedling growth at the lower concentration (1 mM); a 17 and 11% reduction of the root and shoot length, respectively was observed (Table 1). Higher proline concentration (10 mM) did not markedly affect the seedling growth.

## DISCUSSION

The present study aimed at examining whether proline pretreatment could alleviate the seedling growth suppression due to Cu and Cd in *H. vulgare* seedlings. In view of its diverse known functions, proline is likely involved in HM detoxification (Sharma and Dietz 2006).

In the present study, Cu and Cd proved to be strongly inhibitory for the growth of *H. vulgare* seedlings. These observations confirm to the earlier findings involving e.g., Cu in *Trifolium pratense* (Chu *et al.* 2004) and Cd in *Arabidopsis thaliana* (Li *et al.* 2005). Other HMs, like Ni and Hg, have been reported to



**Plate 1 :** Four-d-old *H. vulgare* seedlings grown in absence or presence of Cu (A) and Cd (B) concentrations. Caryopses were pretreated with proline (10 mM) or water for 15 h prior to the treatment with Cu or Cd concentrations.

**Table 1** : Influence of proline pretreatment on Cu-induced inhibition of barley (*H. vulgare*) seedling growth. Caryopses were pretreated or not with proline for 15 h prior to the exposure (5 h) to Cu. n = 15 ± S.D.

Treatment	Root length (cm)	Shoot length (cm)	Seedling fresh weight (g)
Control	7.20±0.96	9.38±0.69	0.187±0.43
+Pro, 1 mM	6.06±0.87	7.24±1.13	0.182±0.01
+Pro, 10 mM	7.19±0.51	8.26±0.99	0.195±0.01
Cu, 100 µM (-Pro)	5.72±0.86	8.24±0.89	0.160±0.01
Cu, 200µM (-Pro)	5.58±0.42	6.89±0.95	0.149±0.09
Cu, 500 µM (-Pro)	5.39±0.63	6.17±0.66	0.142±0.08
Cu, 100 µM (+Pro,1 mM)	8.14±0.97	8.34±0.78	0.195±0.17
Cu, 200 µM (+Pro,1 mM)	7.16±0.53	8.94±0.67	0.174±0.11
Cu, 500 µM (+Pro,1 mM)	6.84±0.74	7.51±1.78	0.171±0.11
Cu, 100 µM (+Pro,10 mM)	8.20±0.82	9.34±1.03	0.103±0.18
Cu, 200 µM (+Pro,10 mM)	6.90±0.80	7.90±1.00	0.176±0.13
Cu, 500 µM (+Pro,10 mM)	6.11±1.10	7.96±1.25	0.168±0.10

**Table 2** : Influence of proline pretreatment on Cd-induced inhibition of barley (*H. vulgare*) seedling growth. Caryopses were pretreated or not with proline for 15 h prior to the exposure (5 h) to Cd. n = 15 ± S.D.

Treatment	Root length (cm)	Shoot length (cm)	Seedling fresh weight (g)
Control	6.84±0.45	12.75±0.97	0.173±0.55
+Pro, 1 mM	5.67±0.43	11.29±1.69	0.169±0.27
+Pro, 10 mM	6.47±0.38	11.70±0.49	0.164±0.15
Cd, 100 µM (-Pro)	3.94±0.47	11.21±0.75	0.160±0.20
Cd, 200µM (-Pro)	2.58±0.57	8.99±0.65	0.133±0.17
Cd, 500 µM (-Pro)	2.18±0.28	7.38±0.73	0.110±0.11
Cd, 100 µM (+Pro,1 mM)	4.01±0.42	10.90±1.01	0.153±0.17
Cd, 200 µM (+Pro,1 mM)	2.94±0.21	10.51±0.53	0.143±0.12
Cd, 500 µM (+Pro,1 mM)	2.45±0.45	10.29±0.65	0.161±0.79
Cd, 100 µM (+Pro,10 mM)	4.71±0.46	11.19±0.71	0.151±0.12
Cd, 200 µM (+Pro,10 mM)	3.38±0.47	12.12±0.75	0.169±0.14
Cd, 500 µM (+Pro,10 mM)	2.67±0.56	9.41±0.95	0.111±0.11

suppress the seedling growth in *Brassica juncea* (Thakur and Sharma 2015) and *Pisum sativum* (1985), respectively. A stronger inhibition of root growth particularly due to Cd is expected in view of the fact that the roots are first to react to the toxic HM ions (Sharma *et al.* 1999). Since the seedling growth relies upon the hydrolysis of reserves in the seeds/caryopses, it is quite likely that Cu/Cd suppressed the activities of hydrolytic enzymes restricting the availability of mobilizable substances. Diverse HMs could bind to the –SH groups in protein structure changing their conformation and thus causing the enzyme inhibition (Van Assche and Clijsters 1990). For example, Hg suppressed the activity of  $\alpha$ -amylase and protease in germinating seeds of *Pisum sativum* (Sharma 1985). Other reasons for Cu or Cd-induced inhibition of seedlings might include the interference of Cu/Cd with the redox metabolism causing oxidative stress (Sharma and Dietz 2009) and altered water relations (Barcelo and Poschenreider 1990).

Quite interestingly, proline pre-treatment of caryopses reversed the Cu or Cd-induced inhibition of *H. vulgare* seedling growth to varying extents. The stated proline effect was observed at 1 and 10 mM that appear to be physiologically relevant concentrations in view of the fact that proline could accumulate in plant tissues to high millimolar concentrations (Sharma and Dietz 2006). Proline dependent reversal of HM-induced seedling growth inhibition could be ascribed to multiple reasons. For example, proline is likely to maintain the water relations in the seed tissue through its well established role in osmoregulation (Kavikishor *et al.* 1995). Also, proline could scavenge the free radicals generated by the heavy metals that contribute to the toxic effects (Siripornadulsil *et al.* 2002, Kaul *et al.* 2008). Indeed, proline dependent reversal was more prominent in case of Cu as compared to Cd. Cu, being a redox active metal, could directly generate ROS; the proline dependent reversal of Cu effects could be linked to proline mediated efficient scavenging of free radicals as has been demonstrated *in vitro* (Kaul *et al.* 2008).

Proline was demonstrated to protect enzymes against HM-induced inhibition mainly through complexation of HM ions particularly Cd (Sharma *et al.* 1998). Hydrolytic enzymes relevant for seed germination and early growth processes might be similarly protected by proline. The proline dependent reversal of heavy metal toxicity has also been shown earlier in certain algae e.g. *Chlorella vulgaris* ((Mehta and Gaur 1999) and *Scenedesmus armatus* (El-Enany and Issa 2001) as well as higher plants such as *Solanum nigrum* (Xu *et al.* 2009). Present findings are consistent with a role of proline in HM detoxification. The characterization of HM effects on seedling growth is apparently of significance in the context of phytoremediation as the establishment of the seedlings on metal polluted soils is a prerequisite for the success of this technology (Kranter and Colville 2011).

Award of Rajiv Gandhi Fellowship by UGC, New Delhi to Namarta is thankfully acknowledged.

## REFERENCES

- Bassi R and Sharma SS 1993a Changes in proline content accompanying the uptake of zinc and copper by *Lemna minor*. *Ann Bot* **72(2)** 151-154.
- Bassi R and Sharma SS 1993b Proline accumulation in wheat seedlings exposed to zinc and copper. *Phytochem* **33(6)** 1339-1342.
- Barcelo J and Poschenrieder C 1990 Plant water relations as affected by heavy metal stress: a review. *J. Plant Nutr* **13(1)** 1-37.
- Chu L, Liu D, Wang Y, Li Y and Liu H 2004 Effect of copper pollution on seedling growth and activate oxygen metabolism of *Trifolium pratense*. *J App Ecol* **15(1)** 119-122.
- El-Enany AE and Issa AA 2001 Proline alleviates heavy metal stress in *Scenedesmus armatus*. *Folia Microbiol* **46(3)** 227-230.
- Hall JL 2002 Cellular mechanisms for heavy metal detoxification and tolerance. *J Exp Bot* **53(366)** 1-11.

- Kaul S, Sharma SS and Mehta IK 2008 Free radical scavenging potential of L-proline: evidence from *in vitro* assays. *Amino Acids* **34(2)** 315-320.
- Kavi Kishor PB, Zonglie H, Miao GH, Hu CA and Verma DPS 1995 Overexpression of  $\gamma$ -pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol* **108(4)** 1387-1394.
- Li W, Khan M A, Yamaguchi S and Kamiya Y 2005 Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana*. *Plant Grow Reg* **46(1)** 45-50.
- Martinoia E, Meyer S, De Angeli A and Nagy R 2012 Vacuolar transporters in their physiological context. *Annu Rev Plant Biol* **63** 183-213.
- Mehta SK and Gaur JP 1999 Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris*. *New Phytol* **143(2)** 253-259.
- Kranner I and Colville L 2011 Metals and seeds: biochemical and molecular implications and their significance for seed germination. *Env Exp Bot* **72(1)** 93-105.
- Schat H, Sharma SS and Vooijs R 1997 Heavy metal-induced accumulation of free proline in a metal-tolerant and a nontolerant ecotype of *Silene vulgaris*. *Physiol Plant* **101(3)** 477-482.
- Sharma SS 1985 Effects of mercury on seedling growth, mobilization of food reserves and activity of hydrolytic enzymes in *Pisum sativum*. *Env Exp Bot* **25** 189-193.
- Sharma SS and Dietz KJ 2006 The significance of amino acids and amino acid derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot* **57(4)** 711-726.
- Sharma SS and Dietz KJ 2009 The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci* **14(1)** 43-50.
- Sharma SS, Dietz KJ and Mimura T 2016 Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. *Plant Cell Environ* (in press) doi: 10.1111/pce.12706.
- Sharma SS, Schat H. and Vooijs R 1998 *In vitro* alleviation of heavy metal induced enzyme inhibition by proline. *Phytochem* **49** 1531-1535.
- Siripornadulsil S, Traina S, Verma DPS and Sayre RT 2002 Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell* **14(11)** 2837-2847.
- Song WY, Park J, Mendoza-Cozatl DG, Suter-Grotemeyer M, Shim D, Hortensteiner S, Geisler M, Weder B, Rea PA, Rentsch D, Schroeder JI, Lee Y and Martinoia E 2010 Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *Proc Natl Acad Sci USA* **107** 21187-21192.
- Thakur S and Sharma SS 2015 Characterization of seed germination, seedling growth, and associated metabolic responses of *Brassica juncea* L. cultivars to elevated nickel concentrations. *Protoplasma* (In Press), DOI : 10.1007/s00709-0-15-0835-0.
- Van Assche F and Clijsters H 1990 A biological test system for the evaluation of the phytotoxicity of metal-contaminated soils. *Environ Pollut* **66(2)** 157-172.
- Xu J, Yin HX and Li X 2009 Protective effects of proline against cadmium toxicity in micropropagated hyperaccumulator, *Solanum nigrum* L. *Plant Cell Rep* **28** 325-333.