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NITRIC OXIDE (NO)-DEPENDENT ALLEVIATION OF COPPER- AND ZINC-INDUCED TOXIC EFFECTS ON *HORDEUM VULGARE* L. SEEDLINGS

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The aim of present study was to examine whether SNP (sodium nitroprusside, an NO donor) could alleviate the Cu and Zn-induced suppression of seedling growth of *Hordeum vulgare*. Both Cu (0-100 µM) and Zn (0-1000 µM) inhibited the seedling growth in terms of root length, shoot length and seedling fresh weight in a concentration-dependent manner. Metal- and organ-specific differences were evident. Thus, the magnitude of Cu-induced suppression was more as compared to Zn and root elongation was affected invariably more than the shoot length. α-amylase activity in the seeds was reduced due to treatment with higher Cu or Zn- concentrations. The activity of two antioxidative enzymes namely, ascorbate peroxidase (APX) and catalase (CAT) was enhanced in the Cu and Zn-stressed seedlings implying the involvement of redox metabolism. SNP pretreatment of seeds prior to the exposure to Cu or Zn led to a substantial alleviation of the HM-induced toxic effects on seedling growth. The SNP-dependent alleviation was accompanied by specifically altered activities of α-amylase, APX and CAT. The findings suggest a role of NO in alleviation of toxic effects of surplus Cu and Zn on *H. vulgare* seedling growth via altered hydrolytic and redox metabolism.

Keywords : Antioxidative enzymes, Heavy metals, *Hordeum vulgare*, Nitric oxide, Seedling growth.

The phytotoxic effects of heavy metals (HMs) are well documented (Clemens 2006). Some HMs e.g. Zn, Cu, Ni etc. play an indispensable role in diverse plant metabolic processes. However, beyond certain specific concentrations, they adversely affect the plant growth and productivity (Marschner 1995, 2012). For example, copper constitutes an integral component of plastocyanin which has a regulatory function in the photosynthetic electron transport between PSII and PSI (Yruela 2005). Cu also plays an essential role in signaling of transcription and protein trafficking, oxidative phosphorylation and iron mobilization. Likewise, Zn is imperative for the biological activity of all six enzyme classes namely, oxido-reductases, transferases, hydrolases, lyases, isomerases and ligases (Marschner 2012). The carbonic anhydrase enzyme, involved in carbonate mediated pH buffering in cell, requires Zn^{2+} as a cofactor (Sandmann and Böger 1983). Also, Cu/Zn superoxide dismutase (SOD), a part of cellular antioxidative enzyme system, is an example of Cu and Zn protein. At elevated concentrations, both Cu and Zn are known to impose toxic effects in plants often causing oxidative damage via enhanced generation of ROS (Schutzendubel and Polle 2002, Sharma and Dietz 2009). Upon exposure to toxic HM concentrations, plants activate several mechanisms including chelation of metal ions via various ligands (phytochelatins, glutathione and amino acids etc.), subcellular compartmentation and antioxidative defence system (Sharma and Dietz 2006). Despite the progress in understanding of various HM tolerance strategies in plants, there is a dearth of information concerning the signaling of HM stress in plants. Nitric oxide (NO) has emerged as a bioactive signaling gaseous molecule. It regulates a variety of physiological processes and co-ordinates the plant responses to different biotic and abiotic stresses including HM stress (Besson-Bard *et al.* 2008, Xiong *et al.* 2010, Fernández-Marcos *et al.* 2012, Abat and Deswal 2013). In present study, it was of interest to determine whether nitric oxide (NO) could influence Cu- and Zn-induced changes in seedling growth and certain associated metabolic parameters in *Hordeum vulgare* L.

MATERIALS AND METHODS

Seed source

Hordeum vulgare caryopses were procured from a substation of Indian Agricultural

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Research Institute (IARI) at Tutikandi (Shimla).

Seed germination and seedling growth

Uniform sized caryopses were surface sterilized with 0.1% $HgCl_2$ for 2-3 min. After thorough washing with distilled water, they were imbibed in 1 mM SNP (sodium nitroprusside, an NO donor) or distilled water for 15 h. Following this pretreatment, the caryopses were shifted to the solutions containing Cu and Zn in the form of $CuSO_4$ (0-100 µM) and $ZnSO_4$ (0-1000 µM) for 5 h. Thereafter, they were shifted to Petri plates lined with Whatman filter paper made wet with the solutions (5 ml) of respective Cu or Zn concentrations or water (control). Subsequently, distilled water was used to maintain the substratum wet. The Petri plates were placed in a plant growth chamber (Sanyo, Japan) for seed germination and seedling growth for 5 d at 25±2ºC (16 h photoperiod; PAR : 90 µmol m^{-2} s^{-1}). Seed germination was observed daily for 3 d. The growth of 5-d-old seedlings was measured in terms of root length, shoot length and seedling fresh weight. Besides, the activity of α-amylase a hydrolytic enzyme and two antioxidative enzymes (ascorbate peroxidase and catalase) was monitored. Whereas the α-amylase was assayed in seeds after 20 h (15 h +5 h HM) of treatment, antioxidative enzymes were assayed in 5- d- old whole seedlings.

α-amylase assay

α-amylase activity was determined in the caryposes pretreated with SNP or H_2O for 15 h followed by treatment with Cu/Zn (5 h) according to the method given by Filner and Varner (1967). The caryopses (approx. 100 mg) were homogenized with chilled 0.05 M Tris-HCl buffer, pH-7.2. The homogenate was centrifuged at 10,000 rpm for 10 min (4˚C); supernatant served as enzyme extract. The assay mixture containing 1 ml of enzyme extract and 1 ml of substrate (0.15% starch dissolved in 0.2 mM $CaCl_2$) was incubated for 10 min. Thereafter, 3 ml of IKI (0.6% iodine in 6% KI; 1 ml diluted to 50 ml with 0.05 N HCl) reagent was added. In the blank (control), enzyme extract was added after IKI addition. Absorbance was read at 620 nm. The α-amylase activity was determined from the calibration curve prepared with starch.

Ascorbate peroxidase assay

Ascorbate peroxidase (APX) activity in the seedlings was estimated according to Hossain and Asada (1984). The tissue (approx. 100 mg) was homogenized with 1 ml 100 mM HEPES-NaOH buffer (pH 7.6) containing 5 mM ascorbate. The homogenate was centrifuged at 10,000 rpm for 5 min (4°C). The supernatant served as enzyme extract. The reaction mixture contained 50 µl 5 mM ascorbate in water, 1 ml 50 mM HEPES-NaOH (pH 7.6) buffer, 50 µl enzyme extract and 100 µl 3 mM H_2O_2. Following the addition of substrate, change in A_{290} was monitored. The enzyme activity was determined using the extinction coefficient 2.8 mM^{-1} cm^{-1} for ascorbate.

Catalase assay

The catalase (CAT) in the seedlings was assayed polarographically using a Clarke-type O_2 electrode (Hansatech, U.K.). In brief, the tissue (approx. 100 mg) was homogenized with 1 ml (100 mM) HEPES-KOH buffer (pH 7.6). The homogenate was centrifuged at 10,000 rpm for 5 min. The supernatant served as enzyme extract. O_2 electrode was calibrated using sodium dithionite. The reaction mixture contained 890 µl HEPES-KOH (100 mM), 10 µl of enzyme extract and 100 µl H_2O_2 (100 mM). The reaction was initiated by adding the enzyme extract at last. The enzyme activity was calculated from the slopes recorded on chart paper using the value of dissolved O_2 at 25°C.

Determination of protein contents

Protein estimation was done by using Bradford reagent (Bradford 1976). The reaction mixture contained 790 µl H_2O, 10 µl sample extract and 200 µl Bradford reagent. The reaction mixture was kept for 5 min at

room temperature and absorbance read at 595 nm. Protein contents were determined with the help of a calibration curve prepared using BSA.

RESULTS

Seedling growth

Germination of *H. vulgare* seeds, monitored for 3 d, was not affected by either of the effectors (Cu, Zn, SNP; data not shown). However, both Zn and Cu suppressed the seedling (5-d-old) growth measured in terms of root length, shoot length and seedling fresh weight in a concentration dependent manner. The root length reduction due to Cu and Zn was much stronger than that of shoot length and seedling fresh weight. For example, at 100 µM Cu, the root length, shoot length and seedling fresh weight were 20, 46 and 31% of control, respectively (Fig. 1). These values for 500 µM Zn-treated seedlings were 53, 70 and 75%, respectively (Fig. 2). Apparently, Cu was more toxic than Zn.

SNP pretreatment alone had no effect on seedling growth performance. However, due to SNP pretreatment, the extent of Cu- or Zn-induced suppression of seedling growth was substantially reduced particularly in terms of

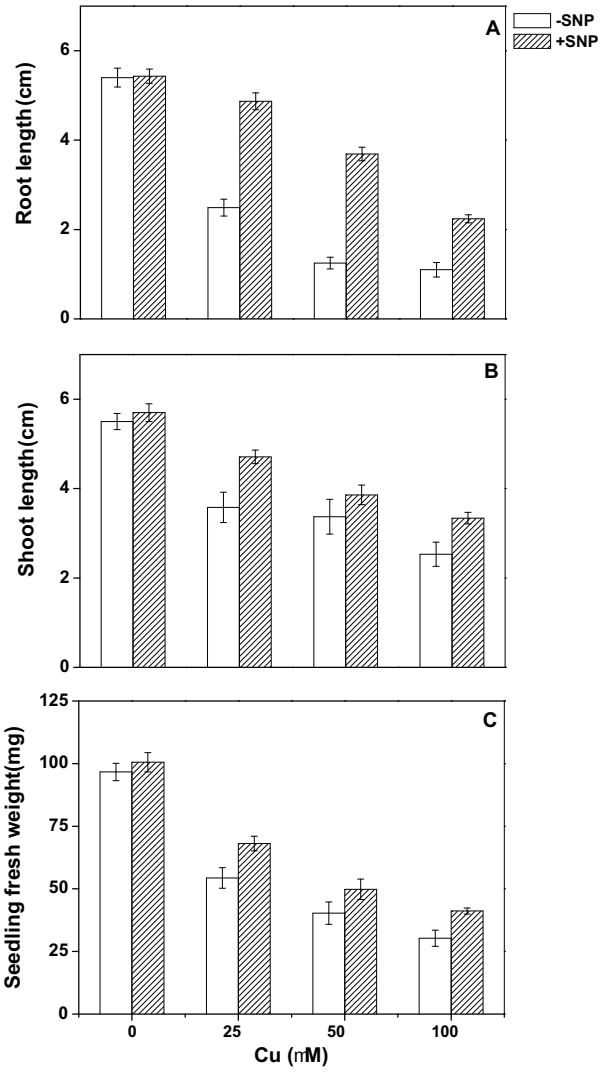

Fig. 1 (A-C) : Influence of sodium nitroprusside (SNP, 1 mM) pretreatment on copper- induced suppression of *Hordeum vulgare* seedling growth: root length **(A)**, shoot length **(B)** and seedling fresh weight **(C)**. Data are arithmetic means ± SE. n = 45.

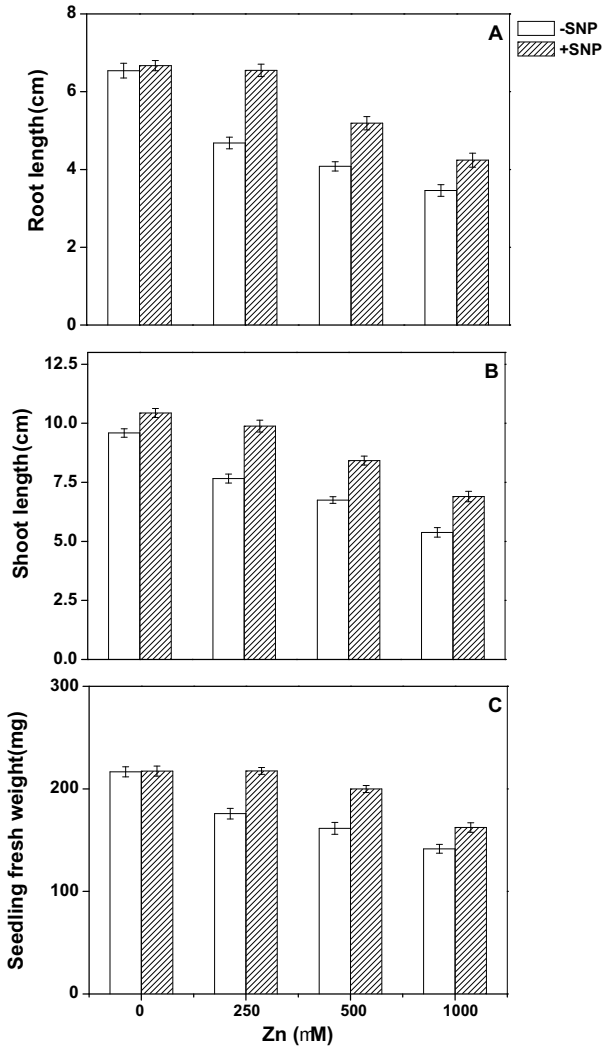

Fig. 2 (A-C) : Influence of sodium nitroprusside (SNP, 1 mM) pretreatment on zinc- induced suppression of *Hordeum vulgare* seedling growth: root length **(A)**, shoot length **(B)** and seedling fresh weight **(C)**. Data are arithmetic means ± SE. n = 45.

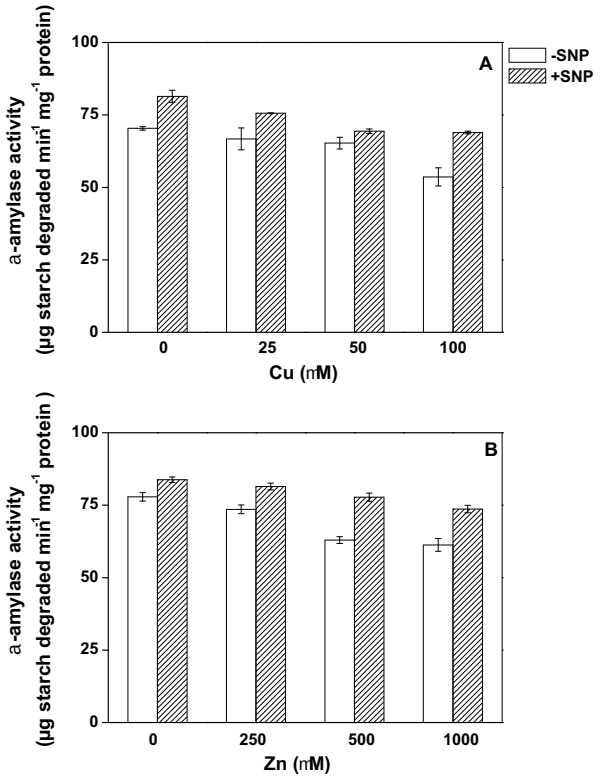

Fig. 3 (A-B) : Influence of SNP on Cu **(A)** and Zn **(B)**-induced suppression of α-amylase activity in seeds of *Hordeum vulgare*. Seeds were pretreated with SNP (1 mM) for 15 h prior to Cu /or Zn treatment (5 h). Data are arithmetic means ± SE. n=3.

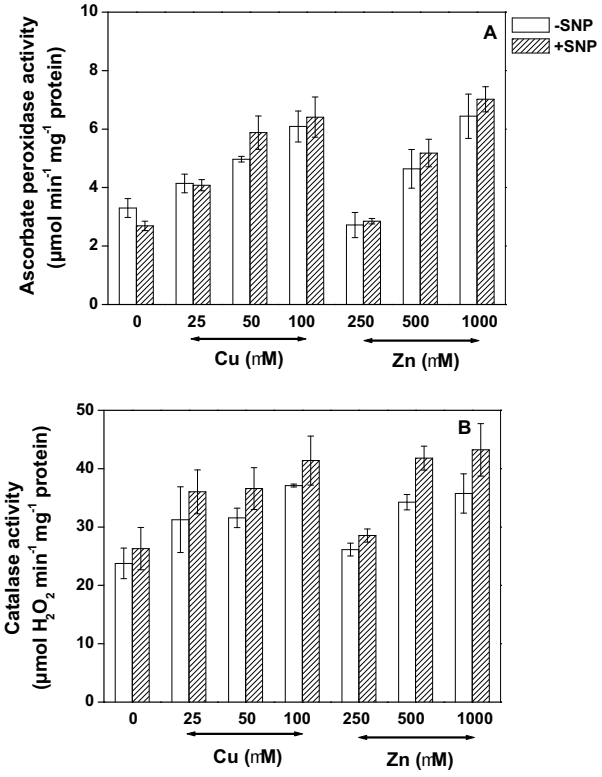

Fig. 4 (A-B) : Interactive effects of SNP and HMs (Cu, Zn) on ascorbate peroxidase **(A)** and catalase **(B)** activity in 5-d old seedlings of *Hordeum vulgare*. Seeds were pretreated with SNP (1 mM, 15 h) prior to HM treatment. Data are arithmetic means ± SE. n=3.

root length. For example, root length suppression due to 100 µM Cu was observed to be 80 and 59% without and with SNP pretreatment, respectively (Fig. 1). In case of 500 µM Zn treated seedlings, these values were 38 and 22%, respectively (Fig. 2). The recovery due to SNP pretreatment in case of shoot length and seedling fresh weight was of lower magnitude (Fig. 1, 2).

α-amylase activity

α-amylase activity in *H. vulgare* seeds was not affected by lower concentrations of Cu (25 µM) and Zn (250 µM). A slight inhibition in the activity was observed beyond these concentrations. For example, α-amylase activity was 95 and 76% of control at 25 and 100 µM Cu, respectively (Fig. 3A) and 94 and 79 % of control at 250 and 1000 µM Zn, respectively (Fig. 3B). Due to SNP pretreatment of seeds, Cu/ Zn-induced inhibition of α-amylase activity was completely abolished. SNP alone marginally increased the α -amylase activity.

Ascorbate peroxidase (APX) and catalase (CAT) activity

In response to Cu and Zn treatment, the activity of APX in *H. vulgare* seedlings increased in a concentration dependent manner. At 25, 50 and 100 µM Cu, APX activity was 1.25-, 1.51- and 1.85- fold that of control, respectively whereas it was 0.82, 1.41- and 1.95- fold that of control at 250, 500 and 1000 µM Zn, respectively (Fig. 4A). SNP pretreatment further enhanced the Cu and Zn effect to some extent. Thus, APX activity was 1.24-, 1.78- and 1.94- fold of control at 25, 50 and 100 µM Cu and 0.86-, 1.57- and 2.13- fold of control at 250, 500 and 1000 µM Zn, respectively (Fig. 4 A).

As in case of APX activity, CAT activity in *H. vulgare* seedlings also increased in response to Cu and Zn treatment. It was 1.32-, 1.33-

and 1.56- fold that of control at 25, 50 and 100 µM Cu, respectively and 1.10-, 1.44- and 1.50- fold that of control at 250, 500 and 1000 µM Zn, respectively. SNP pretreatment further enhanced the HM-induced increase in CAT activity. A marginal enhancement in activity was recorded in case of SNP pretreatment alone (Fig. 4B).

DISCUSSION

The present study aimed at assessing the role of NO, if any, in alleviation of the inhibitory effects of surplus concentrations of Zn and Cu on seedling growth of *Hordeum vulgare.* Seed germination of *H. vulgare* was not affected by the tested Cu/Zn concentrations (data not shown). Similar observations involving Cu and Zn have been reported with *Zea mays* seed germination (Mahmood *et al.* 2005). The observed lack of effect on germination might be attributed to restricted uptake of HM ions due to seed coat or other structures. Alternatively, the processes associated with utilization of seed reserves during germination appear not to have been influenced by HMs (Stefani *et al.* 1991). In contrast to seed germination, the seedling growth was inhibited due to both Cu and Zn; the extent of inhibition being greater in case of Cu. In response to Cu and Zn, root and shoot length reduction was observed in tomato seedlings (Ashagre *et al.* 2013). Cu/Zn-induced inhibition of root length was invariably greater than that of shoot length. Higher than shoot sensitivity of roots to toxic HM concentrations is routinely observed in different plant species (Sharma *et al.* 2004, Thakur and Sharma 2015). Apparently, this is a consequence of greater retention of the HM burden within the root tissue. The pretreatment of *H. vulgare* seeds with SNP (an NO donor) prior to the exposure to Cu or Zn led to a substantial reduction in the magnitude of Cu/Zn-induced suppression of seedling growth; effects being more prominent in case of root than shoot. Similar SNP effects were observed in case of Cd-stressed rice seedlings (He *et al.* 2014). Root growth enhancement by nitric oxide might be due to the involvement of NO in auxin signalling pathway (Pagnussat *et al.* 2002). Primary root elongation, lateral root development or adventitious root formation have been shown to be regulated by the interaction of NO and auxin (Guo *et al.* 2008, Fernández-Marcos *et al.* 2012, He *et al.* 2012). However, SNP pretreatment alone had no effect on root elongation in our study.

We examined the possibility of NO influencing the hydrolytic and antioxidative enzymes in reverting the Cu or Zn-induced inhibition of seedling growth by assaying the activity of α-amylase, ascorbate peroxidase (APX) and catalase (CAT). α-amylase activity in *H. vulgare* seeds declined in response to HMs (Cu and Zn) which is similar to the observations in *B. campestris* due to Pb (Singh *et al.* 2011) and bean due to Cu (Sfaxi-Bousbih *et al.* 2010). SNP pre-treatment of seeds alleviated the HM (Cu and Zn)-induced decrease in amylase activity. SNP treatment significantly enhanced the amylase activity in wheat seeds under salt stress (Zheng *et al.* 2009). NO is likely to exert its influence via affecting the GA_3 levels (Beligni *et al.* 2002).

Redox metabolism is evidently involved in the regulation of seed germination and seedling growth processes (Bailly *et al.* 2002). Elevated HM concentrations impose cellular redox imbalance (Sharma and Dietz 2009) that might be overcome through development of antioxidant enzyme system comprising enzymes like APX, CAT etc. In present study, Cu- and Zn-stressed *H. vulgare* seedlings exhibited increased activity of APX and CAT, both involved in H_2O_2 degradation. HM-induced increase in APX (Zengin and Munzuroglu 2005, Thakur and Sharma 2015) and CAT activity (Singh *et al.* 2007) has been reported in different plant species. SNP pretreatment further enhanced the magnitude of HM-induced increase in the activities of APX and CAT in *H. vulgare* seedlings. Hasanuzzaman and Fujita (2013) reported the activity of APX and CAT in the presence of

SNP under As treatment to be much higher than those under As treatment without SNP in wheat seedlings. The observed changes in APX and CAT activities suggest the involvement of redox metabolism in HM detoxification. Taken together, the present findings reveal the alleviation of Cu- or Zn-induced inhibition of *H. vulgare* seedling growth through altered activities of amylase, APX and CAT.

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REFERENCES

Abat JK and Deswal R 2013 Nitric oxide modulates the expression of proteins and promotes epiphyllous bud differentiation in *Kalanchoe pinnata*. *J Plant Growth Regul* **32** 92–101.

Ashagre H, Almaw D and Feyisa T 2013 Effect of copper and zinc on seed germination, phytotoxicity, tolerance and seedling vigor of tomato (*Lycopersicon esculentum* L. cultivar Roma VF). *Int J Agr Sci Res* **2(11)** 312-317.

Bailly C, Bogatek-Leszcyncska R, Come D and Corbineau F 2002 Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Sci Res* **12** 45-47.

Beligni MV, Fath A, Bethke PC, Lamattina L and Jones RL 2002 Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol* **129** 1642–1650.

Besson-Bard A, Gravot A, Richaud P, Auroy P, Duc C, Gaymard F, Taconnat L, Renou JP, Pugin A and Wendehenne D 2009 Nitric oxide contributes to cadmium toxicity in *Arabidopsis* by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. *Plant Physiol* **149** 1302–1315.

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.

Clemens S 2006 Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochim* **88** 1707–1719.

Fernández-Marcos M, Sanz L and Lorenzo O 2012 Nitric oxide: an emerging regulator of cell elongation during primary root growth. *Plant Signal Behav* **7** 196–200.

Filner P and Varner JE 1967 A test for *de novo* synthesis of enzymes: density labeling with H_2O^{18} of barley alpha-amylase induced by gibberellic acid. *Proc Natl Acad Sci USA* **58(4)** 1520-1526.

Guo K, Xia K and Yang ZM 2008 Regulation of tomato lateral root development by carbon monoxide and involvement in auxin and nitric oxide. *J Exp Bot* **59** 3443–3452.

Hasanuzzaman M and Fujita M 2013 Exogenous sodium nitroprusside alleviates arsenic-induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by enhancing antioxidant defense and glyoxalase system. *Ecotoxicol* **22** 584–596.

He HY, He LF, Gu MH and Li XF 2012 Nitric oxide improves aluminum tolerance by regulating hormonal equilibrium in the root apices of rye and wheat. *Plant Sci* **183** 123–130.

He J, Ren Y, Chen X and Chen H 2014 Protective role of nitric oxide on seed germination and seedling growth of rice (*Oryza sativa* L.) under cadmium stress. *Ecotoxicol Env Saf* **108** 114-119.

Hossain MA and Asada K 1984 Inactivation of ascorbate peroxidase in spinach chloroplasts on dark addition of hydrogen peroxide : its protection by ascorbate. *Plant Cell Physiol* **25(7)** 1285-1295.

Mahmood S, Hussain A , Saeed Z and Athar M 2005 Germination and seedling growth of corn (*Zea mays* L.) under varying levels of copper and zinc. *Int J Environ Sci* **2** 269–274.

Marschner, H 1995 *Mineral nutrition of higher plants*. Academic : San Diego, CA.

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

Pagnussat GC, Simontacchi M, Puntarulo S and Lamattina L 2002 Nitric oxide is required for root organogenesis. *Plant Physiol* **129** 954–956.

Sandmann G and Böger P 1983 The enzymatological function of heavy metals and their role in electron transfer processes of plants. In *Encyclopedia of Plant Physiology, New Series*. eds. A. Lävchli and R.L. Bieleski, Springer Verlong. **15** 563-596.

Schützendübel A and Polle A 2002 Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J Exp Bot* **53** 1351–1365.

Sfaxi-Bousbih A, Chaoui A and Ferjani EE 2010 Copper affects the cotyledonary carbohydrate status during the germination of bean seeds. *Biol Trace Elem Res* **137** 110–116.

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, 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)** 1287-1295.

Singh D, Nath K and Sharma YK 2007 Response of wheat seed germination and seedling growth under copper stress. *J Environ Biol* **28(2)** 409-414.

Singh HP, Kaur G, Batish DR and Kohli RK 2011 Lead (Pb)-inhibited radicle emergence in *Brassica campestris* involves alterations in starch-metabolizing enzymes. *Biol Trace Elem Res* **144** 1295–1301.

Stefani A, Arduini I and Onnis A 1991 *Juncus acutus* : Germination and initial growth in presence of heavy metals. *Ann Bot Fenn* **28** 37-43.

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/s 00709-0-15-0835-0.

Xiong J, Fu G, Tao L and Zhu C 2010 Roles of nitric oxide in alleviating heavy metal toxicity in plants. *Arch Biochem Biophys* **497** 13−20.

Yruela L 2005 Copper in plants. *Braz J Plant Physiol* **17** 145-156.

Zengin FK and Munzuroglu O 2005 Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (*Phaseolus vulgaris* L.) seedlings. *Acta Biol Cracov Bot* **47** 157–164.

Zheng C, Jiang D, Liu F, Dai T, Liu W, Jing Q and Cao W 2009 Exogenous nitric oxide improves seed germination in wheat against mitochondrial oxidative damage induced by high salinity. *Env Exp Bot* **67** 222–227.