Mycopath (2013) 11(2): 59-64 **Research Article**

Correlation between copper induced changes in peroxidase activities and its accumulation in seedling roots of *Brassica juncea*

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Abstract

Plants have an excellent ability to take up and gather heavy metals from their ambient surrounding. It is a well known fact that high level of heavy metals affects different physiological and metabolic processes in plants. In the present study, changes in soluble peroxidase and ionically bound cell wall peroxidase activity along with accumulation of copper (Cu) in seedlings of *Brassica juncea* (L.) Czern. were investigated. It was found that Cu inhibited root growth and reduced fresh biomass, while increased soluble peroxidase, ionically bound cell wall peroxidase activity and metal accumulation in roots. Through present study, it is concluded that root growth inhibition by Cu is associated with increment in metal accumulation in seedlings that induced metabolic adaptations to heavy-metal stress.

Key words: *Brassica juncea*, cell wall peroxidase, copper, soluble peroxidase.

Introduction

Copper is an essential trace element, considered necessary for the normal growth and development of plants. Cu participates in membrane structure, metalloproteinase enzymes, and plastocyanin in chloroplasts membrane (Marschner, 2005). Both deficiency and overabundance of Cu inhibit the plant growth and spoil important cellular processes (Fariduddin *et al*., 2009; Jain *et al*., 2009). At cellular level, Cu plays an indispensable role in cell wall metabolism, signalling to the transcription and protein trafficking machinery, iron mobilization, oxidative phosphorylation and the biogenesis of molybdenum cofactor (Gratao *et al*., 2005; Yruela, 2005; Krämer and Clemens, 2006; Pilon *et al.*, 2006; Puig *et al*., 2007). The sensitivity of plants to heavy metals depends on an interconnected network of molecular and physiological mechanisms (Kashirad, 1970; Hall, 2002; Cho *et al*., 2003; Kundu *et al*., 2011). The extremes of physiological responses of plants to heavy metals are accumulators and excluders. Excluders have large amounts of metal in their roots and check metal from entering to their aerial parts over a broad range of metal concentration in the soil. Hyper-accumulators concentrate heavy metals in above-ground tissues in levels exceeding those in the soil with concentration ratio more than six (Baker, 1981).

Reactive oxygen species (ROS) are considered as the chief source of damage to cells undergoing biotic and abiotic stresses (Mittler, 2002; Vaidyanathan *et al*., 2003; Candan and Tarhan 2003; Gara *et al*., 2003). ROS are extremely cytotoxic and can seriously react with vital bio-molecules such as proteins, lipids, nucleic acid; these can cause protein denaturation, lipid peroxidation and DNA mutation (Mittler, 2002; Quiles and Lopez, 2004). Plants have developed various protective antioxidant mechanisms to remove or reduce deleterious effects of ROS (Mittler, 2002; Beak and Skinner, 2003; Candan and Tarhan, 2003). In response to reduced growth rate an increased rate of peroxidase activity has been reported in many plant systems (Chen and Kao, 1995; Lee and Lin, 1995; Cosgrove, 1997). Monomeric precursors of lignin are enzymatically dehydrogenated in the cell wall to phenoxy radicals (Lee *et al*., 2007). These radicals polymerize impulsively, yielding a complex net of cross-linking among proteins, polysaccharides and monolignols (Iiyama *et al*., 1994). Peroxidases have been concerned in these cross-linking reactions (Lewis and Yamamoto, 1990; Polle *et al*., 1994).

It has been well recognized that abiotic and biotic stresses are accountable for the boost in cell wall lignification (Chazen and Neumann, 1994; Katerji *et al*., 1997) which would be associated with decreased nutrient content and subsequent plant growth (Guenni *et al*., 2002). For the reason that cell wall peroxidases (CWP) are associated with the generation of hydroxyl radicals in cell walls mediating extension growth (Liszkay *et al.*,

2003). Lin and Kao (1999, 2001) have reported an increase in ionically bound cell wall peroxidase activity is linked with growth inhibition of rice seedling roots caused by NaCl. Simultaneously, Chen *et al*. (2000) reported an increase in cell wall peroxidase activity in response to Cu stress. However, not much has been known about changes in ionically bound CWP activity related to growth responses under heavy metal stress conditions. Accordingly, to test the hypothesis that a rise in ionically bound CWP activity is associated with growth inhibition and plays some role in tolerance of plants against heavy metal stress in plants. The objective of the present work was to investigate changes in soluble peroxidase and CWP activity along with Cu accumulation in seedlings of *B. juncea.*

Materials and Methods

Plant growth and culture

B. juncea seeds were procured from the Krishi Vigyan Kendra, Banasthali University, Rajasthan, India. These seeds were stored in airtight desiccators containing fused $CaCl_2$ for further use. Before use, seeds were surface sterilized with 5% sodium hypochlorite solution for 15 minutes and washed thoroughly 4-5 times with distilled water. These seeds were then germinated in glass Petriplates (10-cm diameter) lined with two sheets of autoclaved blotting paper soaked with 10 mL of distilled water at 30 ºC in the dark. Each Petriplate contained 30 seeds. The covers of Petri plates were removed on 3rd day and germinated seeds were transferred to light in thermo statistically controlled culture room maintained at 25 ± 2 ºC and at less than 50% relative humidity. Water of the Petriplates was replaced with Hoagland's nutrient solution. pH of the Hoagland's nutrient solution was adjusted to 6.4. Seedlings were placed in light (500 µmol m^{-2} s^{-1}) for 10/14 hours day/night daily.

After 3 days growth, seedlings of uniform size were sorted out and transferred to hydroponic culture medium in plastic containers of 10×10 cm (height × width). The culture medium and conditions were maintained same as described above. The nutrient solution was bubbled with glass rod to provide sufficient oxygen and mixing of nutrients. Furthermore, medium was changed on every 3rd day to avoid any nutrient deficiencies to seedlings.

Cu treatment

When seedlings attained 2-3 leaf stage, Cu treatments (0, 10, 50 and 100 µmol) was given in the growth medium containing Hoagland's nutrient solution for various experimental setups accordingly. Each pot contains 10 plants in 800 mL of Hoagland's nutrient solution. Duplicate of each concentration was taken so as to arrange the experiment in randomized design.

Plant growth parameters

Cu toxicity was determined by measuring the root elongation and plant biomass. Root length was measured before and after 5 days of Cu stress. Measurements of three plants per concentration per replicate were taken and each experiment was performed in triplicate.

Soluble peroxidase and ionically bound cell wall peroxidase (IBCWP)

Cell walls were prepared by homogenizing 30 mg tissue in ice cold sodium acetate buffer (10 mM, pH 6.0), using 100 µL of buffer per miligram of fresh weight of tissue (fresh wt.). Homogenate was mixed and centrifuged at 3000 g for 15 min at 3 ºC. The supernatant was collected and used for assay of soluble peroxidase. Pellet was re-suspended in the same buffer and centrifuged again. This washing procedure was repeated up to six times to ensure that all the soluble peroxidase had been washed out. Supernatant from final wash was assayed for peroxidase activity to confirm their reduction to a negligible level. Finally, washed pellet was used as cell wall fraction to extract ionically bound cell wall peroxidase.

From cell walls, ionically bound cell wall peroxidase was extracted with 1 M NaCl. The washed pellet was re-suspended in 100 µL mg^{-1} (fresh weight) in 100 mM sodium acetate buffer, pH 6, containing 1 M NaCl. The suspension was mixed carefully and incubated on ice bath for 60 min with intermittent shaking. After incubation this sample was centrifuged at 3 ºC for 15 min at 3000 g. The supernatant holds the salt extractable cell wall peroxidase; this fraction was believed to signify that fraction of peroxidase that is ionically bound to the cell wall *in vivo* (Bacon *et al*., 1997).

Peroxidase assay

Cell wall peroxidase was analyzed using substrate 3, 3', 5, 5'-tetramethyl benzidine (TMB) following Bos *et al*. (1981). TMB was made up at 20 mg mL^{-1} in DMSO (dimethylsulphoxide), and stored in aliquots at -20 ºC. sample (5 µL) was added to each assay tube followed by 100 µL of 100 mM sodium acetate buffer, pH-6.0 containing

0.1 mg mL^{-1} TMB and 0.5µL mL^{-1} of 6% (w/v) H_2O_2 (hydrogen peroxide). Blend was incubated for 60 min and reaction was stopped by 100 µL of 0.6 M sulphuric acid (H_2SO_4) and absorbance was recorded at 450 nm. There was no reaction in the lack of hydrogen peroxide. Enzyme activity was calculated by using extinction coefficient of a terminal oxidation product that absorb light at 450 nm (Extinction coefficient = 5.9×10^4 M^{-1} cm^{-1}).

Intracellular Cu content estimation

Intracellular copper accumulation was estimated by the method of Bates *et al*. (1982). Plants were harvested and washed systematically with 20 mL of 2 mM EDTA solution and roots were separated. After oven drying at 80 ºC for overnight, dry weight was determined and 100 mg of dried plant material was digested in 5 mL of digestion mixture containing HNO_3 (70 %) + H_2O_2 (30 %) + purified water in 1:1:3 ratio until the solution become colourless. Residues were dissolved in 2% (v/v) nitric acid to a final volume of 5 mL and Cu concentration was determined by atomic absorption spectrophotometer [AAS (Varian, Model No. 240 FS)]. The calculation of intracellular Cu was done on weight by weight (w/w) basis and results were reported in mg g^{-1} of sample. Merck Cu standard was used for quantification of intracellular Cu content.

Statistical analysis

Every treatment was run in triplicate for statistical validity. Data from this investigation were analyzed with standard statistical software (Sigma Plot 12.5) using means ± SE. The mean ± SE and exact number of experiments are given in figures. For equality of averages the Student's *t*-test was applied. Results were considered statistically significant at $P \leq 0.05$.

Results and Discussion

Growth parameters of *B. juncea* seedlings were analyzed by measuring fresh weight (Fig. 1A) and root length (Fig. 1B), ionically bounded cell wall peroxidase (IBCWP; Fig. 1C), soluble peroxidase (Fig. 1D) and accumulation of Cu content (Fig. 1E) after 5 days of metal treatment. Decrease in fresh weight, root length and increase in ionically bounded CWP and soluble peroxidase activity can be correlated with increasing metal stress and its accumulation in root (Saathoff *et al*., 2013). Augmentation in ionically bound CWP actions in roots of seedlings could reveal the alteration of mechanical properties of the cell wall, which in turn, can be interconnected with metal stress. Analogous to our results, activities of lignifying peroxidase were stimulated in response to copper-induced oxidative stress (Chen *et al*., 2000; Jouili and El Ferjani, 2003). Likewise, the growth decline caused by Cu (Chen *et al*., 2002) was closely related with the improved activity of cell wall bound peroxidase. Like NAD(P)H oxidase and xanthine oxidase, cell wall bound peroxidases could also activate the oxidative burst in plants (Bolwell *et al*., 1998). The production of ROS in the apoplast can originate the oxidative cross-linking of cell wall components, such as hydroxyl proline-rich glycoproteins. The cross-linking of structural proteins in the wall has been anticipated as a metabolism to restrict cell growth (Iiyama *et al*., 1994) and to limit cell elongation (De Cnodder *et al*., 2005).

Plant metabolism must be extremely synchronized in order to permit successful combination of a different spectrum of biosynthetic pathways that are reductive in character. This regulation does not fully pass up reductive or photodynamic activation of molecular oxygen to generate reactive oxygen species (ROS) predominantly superoxide, H_2O_2 and singlet oxygen. Plant cells generate ROS, particularly superoxide and H_2O_2, as second messengers in several processes related with plant growth and development. Metal ions may trigger the generation of ROS either by direct transfer of electrons in single electron reactions concerning metal cations or as a result to metal inactivated metabolic reactions (Dietz *et al*. 1999; Foyer and Noctor, 2005).

Conclusion

We observed analogous results that exposure of *B. juncea* seedlings to Cu resulted decline in fresh biomass and root length while increase in ionically bound cell wall peroxidase (CWP) and soluble peroxidase activity with enhanced Cu accumulation in roots. This substantial heave in both CWP and soluble peroxidase activity in roots reveal the alteration of mechanical properties of cell. From present study, it can be suggested that CWP and soluble peroxidase play protective role against the metal stress.

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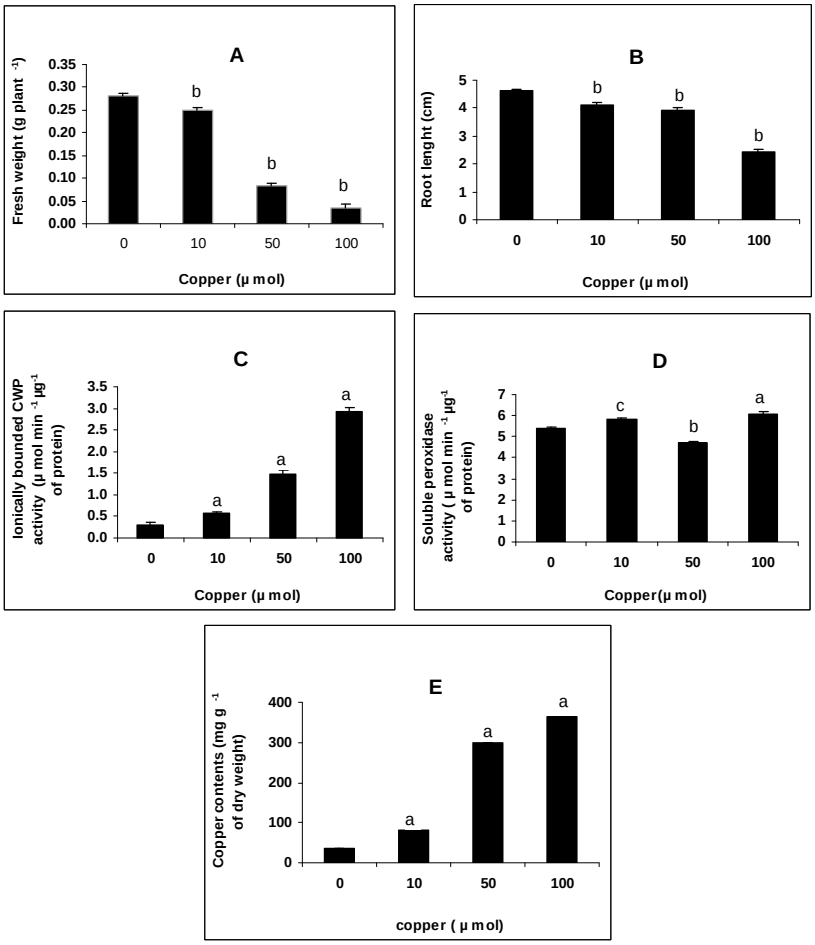

Fig. 1 (A-E): Effects of copper on (A) Fresh weight (B) Root length (C) Ionically bound cell wall peroxidase (D) Soluble peroxidase and (E) Copper accumulation in roots of *Brassica juncea* seedlings on day 5 of the treatment. Vertical bars represent mean ± SE (n = 3). Vertical bar points marked with the letter *a* show significant increase, *b* significant decrease and *c* insignificant differences ($P \leq 0.05$) as determined by Student *t*-test.

References

Bacon MA, Thompson DS, Ho LC, Burton KS, 1997. Can cell wall peroxidase activity explains the leaf growth response of *Lolium temulentum* L. during drought*? J. Exp. Bot.,* **48**: 2075-2085.

Baker AJM, 1981. Accumulators and excluders – strategies in the response of plants to heavy metals. *J. Plant Nutr.*, **3**: 1-4.

Bates SS, Tessier A, Campbell PGC, Buffle L, 1982. Zinc adsorption and transport by *Chlamydomonas variabilis* and *Scenedesmus subspicatus* (Chlorophyceae) grown in semi-continuous culture. *J. Phycol.,* **18**: 521-529.

Beak KH, Skinner DZ, 2003. Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. *Plant Sci*., **165**: 1221-1227.

Bolwell GP, Davis DR, Gerrish C, Auh CK, Murphy TM, 1998. Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiol*., **116**: 1379-1385.

Bos ES, van der Doelen AA, Van Rooy N, Schuurs AHWM, 1981. Tetra methyl benzidine as an ames-test negative

chromogen for horse-redish peroxidase enzyme immunoassay. *J. Immunoassay*., **2**: 187-204.

Candan N, Tarhan L, 2003. The correlation between antioxidant enzyme activities and lipid peroxidation levels in *Mentha pulegium* organs grown in Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} stress conditions. *Plant Sci*., **163**: 769-779.

Chazen O, Neumann PM, 1994. Hydraulic signals from roots and rapid cell-wall hardening in growing maize (*Zea mays* L.) leaves are primary responses to polyethylene glycol-induced water deficits. *Plant Physiol*., **104**: 1385-1392.

Chen EL, Chen YA, Chen LM, Liu ZH, 2002. Effect of copper on peroxidase activity and lignin content in *Raphanus sativus*. *Plant Physiol. Biochem*., **40**: 439-444.

Chen LM, Lin CC, Kao CH, 2000. Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H_2O_2 level and cell wall peroxidase activity in roots. *Bot. Bull. Acad. Sin*., **41**: 99-103.

Chen SL, Kao CH, 1995. Cd induced changes in proline level and peroxidase activity in roots of rice seedlings. *Plant Growth Regul*., **17**:67-71.

Cho M, Chardonnens AN, Dietz KJ, 2003. Differential heavy metal tolerance of *Arabidopsis halleri* and *Arabidopsis thaliana*: a leaf slice test. *New Phytol*., **158**: 287-293.

Cosgrove DJ, 1997. Assembly and enlargement of the primary cell wall in plants. *Annu. Rev. Cell Dev. Biol.,* **13**: 171-201.

De Cnodder T, Vissenberg K, Van Der Straeten D, Verbelen JP, 2005. Regulation of cell length in the *Arabidopsis thaliana* root by the ethylene precursor 1-aminocyclopropane–1-carboxylic acid: A matter of apoplastic reaction. *New Phytol*., **168**: 541-550.

Dietz KJ, Baier M, Kramer U, 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: Prasad MNV, Hagemeyer J (eds.) Heavy metal stress in plants. Springer, Berlin, Heidelberg New York, pp 73-97.

Fariduddin Q, Yusuf M, Hayat S Ahmad A, 2009. Effect of 28-homobrassinolide on antioxidant capacity and photosynthesis in *Brassica juncea* plants exposed to different levels of copper. Environ. *Exp. Bot*., **66**: 418-424.

Foyer CH, Noctor G, 2005. Oxidant and antioxidant signaling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environ.,* **28**: 1056-1071.

Gara LD, Pinto MC, Tommasi F, 2003. The antioxidant systems and reactive oxygen species during plant-pathogen interaction. *Plant Physiol. Biol*., **41**: 863-870

Gratao PL, Polle A, Lea PJ, Azevedo RA, 2005. Making the life of heavy metal stressed plants a little easier, *Funct. Plant Biol.*, **32**: 481- 494.

Guenni O, Douglas M, Baruch Z, 2002. Responses to drought of five *Brachiaria* species. I. Biomass production, leaf growth, root distribution, water use and forage quality. *Plant Soil*, **243**: 229-241.

Hall JL, 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot*., **53**: 1-11.

Iiyama K, Lam TBT, Stone BA, 1994. Covalent cross-links in the cell wall. *Plant Physiol*., **104**: 315-320.

Jain A, Poling MD, Smith AP, Nagarajan VK, Lahner B, Meagher RB, Raghothama K, 2009. Variations in the composition of gelling agents affect morphophysiological and molecular responses to deficiencies of phosphate and other nutrients. *Plant Physiol*., **150**: 1033-1049.

Jouili H, El Ferjani E, 2003. Changes in antioxidant and lignifying enzyme activities in sunflower roots (*Helianthus annuus* L.) stressed with copper excess. *Crit. Rev. Biol*., **326**: 639-644.

Kashirad A, 1970. 'Effect of nitrogen, zinc, copper and manganese on yield and chemical composition of irrigated winter wheat in Iran. *Isr. J. Agric. Res.,* **20**: 179-182.

Katerji N, van Hoorn JW, Hamdy A, Mastrorilli M, Mou Karzel E, 1997. Osmotic adjustment of sugar beets in response to soil salinity and its influence on stomatal conductance, growth and yields. *Agric. Water Manage*., **34**: 57-69.

Krämer U, Clemens S, 2006. Functions and homeostasis of zinc, copper, and nickel in plants. In: Tamás M, Martinoia E (eds) Molecular Biology of Metal Homeostasis and Detoxification from Microbes to Man. Berlin, Springer. pp. 214-272.

Kundu S, Chakraborty D, Pal A, 2011. Proteomic analysis of salicylic acid induced resistance to Mungbean Yellow Mosaic India Virus in *Vigna mungo*. *J. Proteomics*, **74**: 337-349

Lee RB, Kim KY, Jung WJ, Avice JC, Ourry A, Kim TH, 2007. Peroxidases and lignification

in relation to the intensity of water deficit stress in white clover (*Trifolium repens* L.). *J. Exp. Bot*., **58**: 1271-1279.

Lee TM, Lin YH, 1995. Changes in soluble and cell wall bound peroxidase activities with growth in anoxia- treated rice (*Oryza sativa* L.) coleoptiles and roots. *Plant Sci*., **106**: 1-7.

Lewis NG, Yamamoto E, 1990. Lignin: occurrence, biogenesis and biodegradation. *Ann. Rev. Plant Physiol. Plant Mol. Biol*., **41**: 455-496.

Lin CC, Kao HC, 1999. NaCl induced changes in ionically bound peroxidase activity in roots of rice seedlings. *Plant Soil*, **216**: 147-153.

Lin CC, Kao HC, 2001. Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings. *Plant Soil*, **230**:135-143.

Liszkay A, Kenk B, Schopfer P, 2003. Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. *Planta*, **217**: 658-667.

Marschner H, 2005. Mineral Nutrition of Higher Plants. Academic Press: London. pp. 313-323.

Mittler R, 2002. Oxidative stress, antioxidants, and stress tolerance. *Trends Plant Sci*., **7**: 405-410.

Pilon M, Abdel-Ghany SE, Cohu CM, Gogolin KA, Ye H, 2006. Copper cofactor delivery in plant cells. *Curr. Opin. Plant Biol.* **9**: 1-8.

Polle A, Otter T, Seifert F, 1994. Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L.). *Plant Physiol*., **106**: 53-60.

Puig S, Andrés-Colás N, García-Molina A, Peñarrubia L, 2007. Copper and iron homeostasis in *Arabidopsis*: responses to metal deficiencies, interactions and biotechnological applications. *Plant Cell Environ.*, **30:** 271-290.

Quiles MJ, Lopez NI, 2004. Photoinhibition of photosystems I and II induced by exposure to high light intensity during oat plant grown effects on the chloroplastic NADH dehydrogenase complex. *Plant Sci*., **166**: 815-823.

Saathoff AJ, Donze T, Palmer NA, Bradshaw J, Heng-Moss T, Twigg P, Tobias CM, Lagrimini M, Sarath G, 2013. Towards uncovering the roles of switchgrass peroxidases in plant processes. *Front Plant Sci*., **4**: 202.

Vaidyanathan H, Sivakumar P, Chakrabarty R, Thomas G, 2003. Scavenging of reactive oxygen species in NaCl stressed rice (*Oryza sativa* L.)-differential response in salt tolerant and sensitive varieties. *Plant Sci.*, **165**: 1411-1418.

Yruela I, 2005. Copper in plants. *Braz. J. Plant Physiol.*, **17:** 145-146.