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ANTIOXIDATIVE RESPONSES IN RADISH (*RAPHANUS SATIVUS* L.) PLANTS STRESSED BY COPPER AND LEAD IN NUTRIENT SOLUTION AND SOIL

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Radish (*Raphanus sativus* L.) is commonly grown in urban and suburban areas where the soil may be polluted with heavy metals such as Cu or Pb. In this study, short exposure of radish plantlets to 0.5 mM Cu or Pb in nutrient solution (two days) in growth chamber conditions elicited an antioxidative response, measured in terms of lipid peroxidation, protein and proline accumulation, and peroxidase and catalase activity. Longer exposure to Cu or Pb when radish was grown outdoors for 50 days in pots filled with field soil with different Cu and Pb content also resulted in higher lipid peroxidation and proline accumulation, and altered protein content and enzyme activity. The tested parameters of radish antioxidative responses to heavy metal stress differed depending on plant part (leaf or hypocotyl) and stress intensity (heavy metal content in growth medium, exposure duration). The reported data show that plants grown in soil from sites where this crop could be cultivated do show an oxidative stress response similar but not identical to that seen under laboratory treatment with heavy metals.

Key words: Catalase, copper, hypocotyl, guaiacol peroxidase, heavy metals, lead, leaf, oxidative stress, proline, *Raphanus sativus* L., radish.

INTRODUCTION

Amongst many abiotic stresses influencing plant growth and development, heavy metal toxicity is very important, especially if crop species are grown in the vicinity of sites of heavy industry, particularly in developing countries (Bi et al., 2006; Ona et al., 2006). The term "heavy metal" is generally used to refer to metals and semi-metals associated with pollution and toxicity, but the term also includes some elements which in low concentrations are essential nutrients for cells (Gratão et al., 2005).

Copper is a major contaminant which is released into the environment by human activity (Dučić and Polle, 2005). Although it is known to be an essential micronutrient for the growth and development of plants, playing a key role in many metabolic mechanisms, it can be toxic when the copper content in tissues is higher than optimal (Chen et al., 2002). Because of its electron configuration, with one electron transferred from the fourth orbital in order to fill the third orbital, copper reacts easily with reactive oxygen species (ROS). ROS are generated from the normal metabolic activity of mitochondria and chloroplasts, but are also produced during abiotic and biotic stress responses, for example from NADPH oxidase-like enzymes and peroxidases (for review: Neill et al., 2002). Once formed, ROS can undergo further reactions, often catalyzed by metal ions as in the Fenton reaction, and so generate the much more reactive hydroxyl radical, which may be responsible for alterations of macromolecules and ultimately may contribute to cell death (Briat and Lebrun, 1999).

Although lead is not an essential element for plants, it is easily absorbed and accumulated in different parts of the plant, and its phytotoxicity can lead to inhibition of enzyme activity, disturbed mineral nutrition, water imbalance, changes in hormonal status and alteration of membrane permeability (Sharma and Dubey, 2005). Lead also acts as a ROS-promoting heavy metal, inducing antioxidative

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responses in plant roots where it is mostly accumulated, as well as in leaves.

Plants growing on contaminated soils will reflect elevated concentrations of heavy metals in the soils to varying extents, depending on the total concentrations in soil, soil physico-chemical conditions (especially pH) and the genotype of the plant (Alexander et al., 2006). The uptake of heavy metals in plants disturbs growth and metabolism by triggering secondary responses such as oxidative damage (Choudhury and Panda, 2004), perhaps shifting the balance of ROS metabolism towards accumulation of H_2O_2 (Mithöfer et al., 2004). Removal of H_2O_2 is therefore a protective mechanism for the preservation of biological membranes when lead and other metals accumulate in the symplast of the cell (Singh et al., 1997). Enzymatic degradation of superoxide is ensured by superoxide dismutases, while that of hydroperoxides is ensured by catalase, glutathione peroxidase or ascorbate peroxidase (Chaudière and Ferrari-Iliou, 1999; Foyer and Noctor, 2005). Increased peroxidase activity is also reported as a defensive response to most if not all metals, which may cause damage or disturb the normal functions of plant cells (Fang and Kao, 2000). However, H_2O_2 has been shown to induce cell-protection genes, and has been shown to act as a diffusible element which mediates the regulation of gene expression (Desikan et al., 2001; Vanderauwera et al., 2005). Because ROS are toxic but also participate in signalling events, plant cells require at least two different mechanisms to regulate their intracellular concentration (Mittler, 2002). Besides activating enzymatic defence mechanisms, adverse environmental conditions induce the accumulation of specific antioxidants and various stress metabolites in plants. Amongst those metabolites, proline is probably the most widespread, and is considered to be an indicator of environmental stress (Chen et al., 2003). Accumulation of proline has been shown to protect plants against damage by ROS (Matysik et al., 2002), acting as a very effective singlet-oxygen quencher, binding to redox-active metal ions and also activating and protecting enzymes such catalase, peroxidase and polyphenol oxidase (Özturk and Demir, 2002).

Absorption of heavy metals can seriously affect the quality and safety of harvested crop material (Reid and Yermiahu, 2005; Hu et al., 2005), hence an understanding of the biochemical detoxification strategies that plants adopt against oxidative stress induced by accumulated metal ions is key to the manipulation of heavy metal tolerance in plants (Dixit et al., 2001). The present study examines the antioxidative response to copper and lead toxicity in hypocotyls and leaves of radish exposed to short-term heavy metal stress in nutrient solution or to long-term stress in heavy metal-enriched soils. Radish is a good species to study, as it has economic and nutritional value and also is a rich source of two important medicinal compounds – peroxidases and isothiocyanates (Curtis, 2003). The radish hypocotyls are the edible plant parts, which are in direct contact with soil that can be polluted with heavy metals, especially in urban and suburban areas and along roadsides where soil lead content can be increased as a consequence of long-term use of fossil fuels. In agricultural areas, especially vine-producing areas, soils may be enriched with copper by the frequent use of copper-based pesticides. This anthropogenic pollution of soil brings into focus the risk of heavy metals entering the food chain through plants grown in such environments. On the other hand, hypocotyl growth and yield depend on the physiological processes in leaves, and from that point of view it is important to understand the responses to heavy metals in the different parts of the plant in controlled and realistic conditions (nutrient solution vs. soil). In this study, plants were grown under defined conditions in the laboratory, and their oxidative stress responses were compared to those in plants grown in soil from field sites where crops such as radish would commonly be grown for agricultural purposes.

MATERIALS AND METHODS

Seeds of radish (*Raphanus sativus* L., cv. Non Plus Ultra) were sown in plug plates filled with commercial substrate and grown for 3 weeks in glasshouse conditions, until the 4–5-leaf stage. The uniform plantlets were selected and uprooted from the substrate with a fine jet of water, causing minimal damage to the roots. After washing thoroughly with running deionized water they were planted on perforated polystyrene fasteners containing Hoagland nutrient solution (Experiment 1). The nutrient medium was renewed every 3 days and aerated for 1 h each day. The experiment was carried out in three replicates of four plants each. The pots were kept for 3 weeks in a growth chamber and rotated there every day. Temperature was maintained at 20°C, with 70% relative humidity and a 12 h photoperiod. Light was supplied by cool white fluorescent lamps providing photosynthetic photon flux density of 120 μmol m^{-2} s^{-1} at leaf level. Subsequently, the plantlets were treated with 31.77 mg L^{-1} Cu [0.5 mM $Cu(SO_4)$ or 103.6 mg L^{-1} Pb (0.5 mM $Pb(NO_3)_2$ in nutrient solution for 2 days. Control plants had 0.05 mg L^{-1} Cu (as essential) with no additional copper or lead in the growth media.

In the experiment with radish grown in soil (Experiment 2), young plants developed in substrate were planted in soil at the same growth stage as in Experiment 1 and grown outdoors. For Cu toxicity

TABLE 1. Agrochemical properties of soils used in pot experiment with radish response to heavy metal content (HM; Cu, Pb) in the soil

Soil parameter	Cu		Pb	
	Vineyard soil	Field soil	By road	100 m away from road
pH in H_2O	8.59	8.51	8.50	7.81
pH in 1 M KCl	7.65	7.72	7.94	7.42
AL – extractable P_2O_5 (mg kg^{-1} dry soil)	156.0	506.0	752.0	599.0
AL – extractable K_2O (mg kg^{-1} dry soil)	294.3	816.5	528.8	525.6
Organic matter content (%)	2.76	2.81	2.59	2.47
HM total content (aqua regia) (mg kg^{-1} dry soil; ppm)	180.0	120.0	36.0	22.0
HM exchangeable (EDTA) (mg kg^{-1} dry soil; ppm)	41.4	25.7	7.5	2.9

assay, plants were grown in plastic pots (10 L volume) filled with vineyard soil or field soil taken in the vicinity of the vineyard, to ensure that both variants had the same soil type but different Cu levels (Tab. 1). For lead toxicity assay, plants were grown in pots filled with soil taken from a field near one of the most frequently used roads in the Osijek (eastern Croatia) suburban area, assuming the lead content of the soil along the road to be higher than soil taken from the same locality but 100 m away from the road. Soil pH was determined in 1:5 suspensions of soil in 1 M KCl solution and deionized water. Soil organic matter (humus content) was determined by sulfochromic oxidation as prescribed by ISO 14235. P_2O_5 and K_2O content of soils was determined from ammonium lactate-acetic acid extractions (AL) and measured by VIS spectrophotometry and atomic absorption spectrophotometry (AAS), respectively. Total copper content in soil was determined using aqua regia extraction, while the Cu exchangeable fraction was determined using EDTA extraction (Brun et al., 1998), with some modifications. Briefly, 10 g soil was extracted with 20 ml 0.01 M Na^2-EDTA + 1 M CH_3COONH_4 for 30 min with stirring, prior to filtering. The copper concentration in soil extract was measured by AAS, and lead by ICP-OES, and expressed as mg kg^{-1} soil (ppm in figures). Plants (20 plants per pot-replicate, three replicates) were grown for 50 days (May 22 – July 12, 2007) outdoors (mean air temperature 21.8 °C), watered regularly with tap water, and harvested when they reached consumable size of hypocotyls.

Plants from both experiments were divided into leaves and hypocotyls, and washed with tap water followed by deionized water. For determination of lipid peroxidation, protein content, guaiacol peroxidase and catalase activity, fresh leaves or hypocotyls were ground in liquid nitrogen and the frozen powder was used for subsequent analysis. Lipid peroxidation was measured as the amount of thiobarbituric acid (TBA) reactive substances (TBARS-l, leaf; TBARS-h, hypocotyl) as described by Heath and Packer (1968). Protein content (PROT-l, leaf; PROT-h, hypocotyl) was estimated using the method of Bradford (1976), with bovine serum albumin (BSA) as the standard. Free proline content (PRO-l, leaf; PRO-h, hypocotyl) was determined using the method described by Bates et al. (1973). Peroxidase (EC 1.11.1.7) activity in leaves and hypocotyls was determined using guaiacol as substrate, by following the formation of tetraguaiacol at 470 nm (Siegel and Galston, 1967). Total activity of peroxidase is expressed as U g^{-1} tissue fresh weight (GTA-l, leaf; GTA-h, hypocotyls). Peroxidase specific activity was calculated taking into account the protein content in leaf tissue, and expressed as U mg^{-1} protein (GSA-l, leaf; GSA-h, hypocotyl). Catalase (EC 1.11.1.6) activity was measured according to Aebi (1984). Catalase total activity in hypocotyls (CTA-h) and leaves (CTA-l) was expressed as U g^{-1} fresh weight, and specific activity as U mg^{-1} protein (CSA-h, hypocotyls; CSA-l, leaves).

The experiments were performed and statistically analyzed using a split-plot design. Data obtained from the measurements and analyses were evaluated statistically using ANOVA, and least significant difference (LSD) was calculated at $p \leq 0.05$.

RESULTS

The results of both experiments are shown together in Figures 1–4, showing the means of each determined parameter in different experimental conditions.

Plants exposed to heavy metal in nutrient solution showed significantly higher TBARS levels both

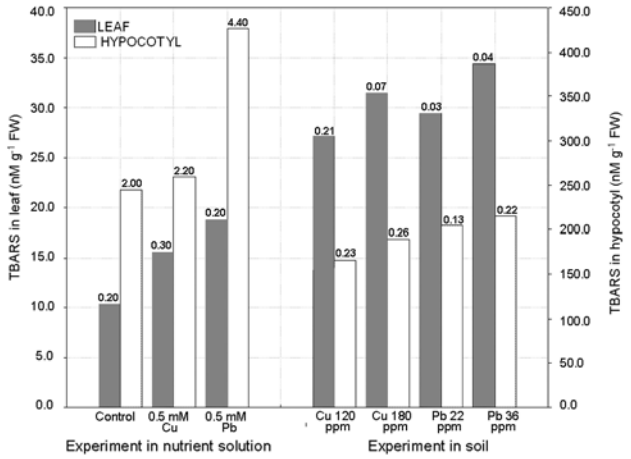

Fig. 1. Lipid peroxidation levels in leaves and hypocotyls of radish grown in nutrient solutions and soil, under the influence of copper or lead. Nutrient solutions: control – standard Hoagland solution; soils: Cu content 120 and 180 ppm, Pb content 22 and 36 ppm; bars are means of three replicates, with SE as bar labels.

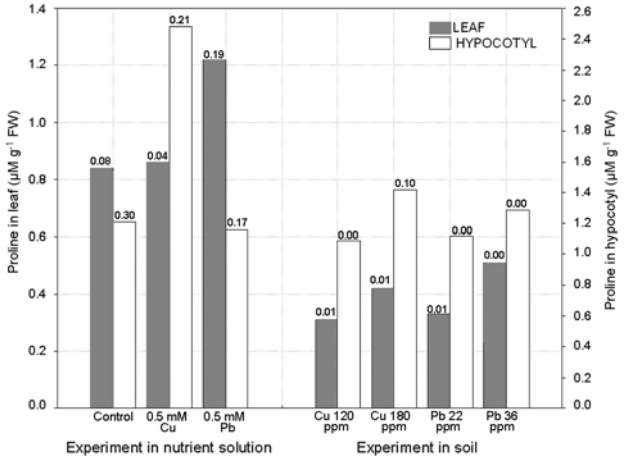

Fig. 2. Free proline content in leaves and hypocotyls of radish grown in nutrient solutions and soil, under the influence of copper or lead. Nutrient solutions: control – standard Hoagland solution; soils: Cu content 120 and 180 ppm, Pb content 22 and 36 ppm; bars are means of three replicates, with SE as bar labels.

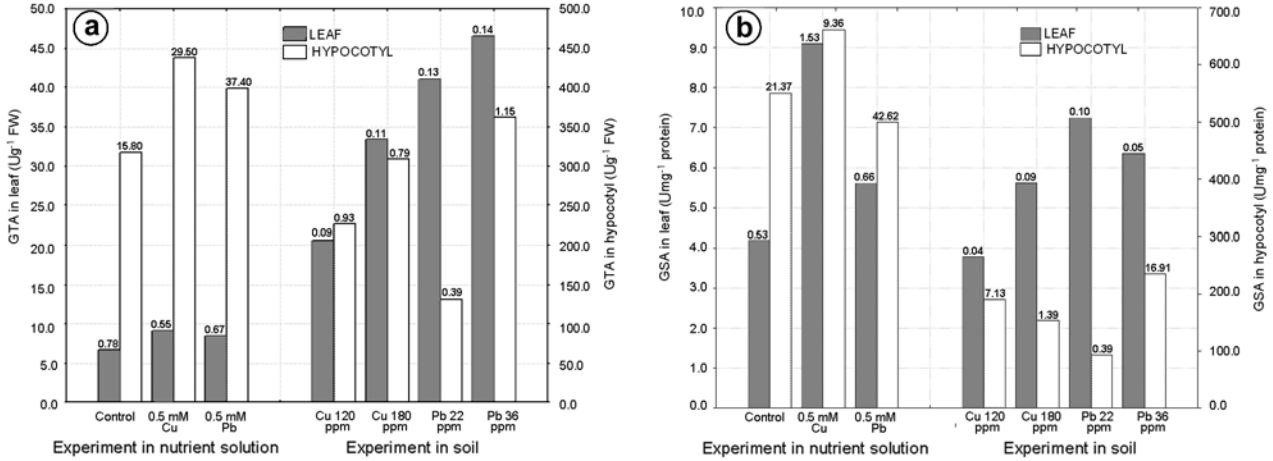

Fig. 3. (**a**) Guaiacol peroxidase total activity in leaves and hypocotyls of radish grown in nutrient solutions and soil, under the influence of copper or lead, (**b**) Guaiacol peroxidase specific activity in leaves and hypocotyls of radish grown in nutrient solutions and soil, under the influence of copper or lead. Nutrient solutions: control – standard Hoagland solution; soils: Cu content 120 and 180 ppm, Pb content 22 and 36 ppm; bars are means of three replicates, with SE as bar labels.

in leaves and in hypocotyls; the effect of Pb was more pronounced than that of Cu, especially in hypocotyls (Fig. 1). The proline level in leaf was almost the same in the control and Cu-treated plants, as well as in hypocotyls of control and Pb-treated plants (Fig. 2). Proline content was significantly higher in leaves of Pb-treated plants and in hypocotyls of Cu-treated plants than in control plants. To gauge the effect of heavy metal treatment on the antioxidant defenses in radish plants, the activity of guaiacol peroxidase and catalase was measured, expressed as both total and specific activity. Total peroxidase activity was much higher in radish hypocotyls than in leaves; the increase was significant ($p \leq 0.05$) with both heavy metals (Fig. 3a). The specific activity of peroxidase appeared to be dependent on the plant part and less related to the heavy metal applied, with higher values in the Cu treatment (Fig. 3b). Catalase activity was higher in leaves than in hypocotyls (Figs. 4a,b). Pb treatment

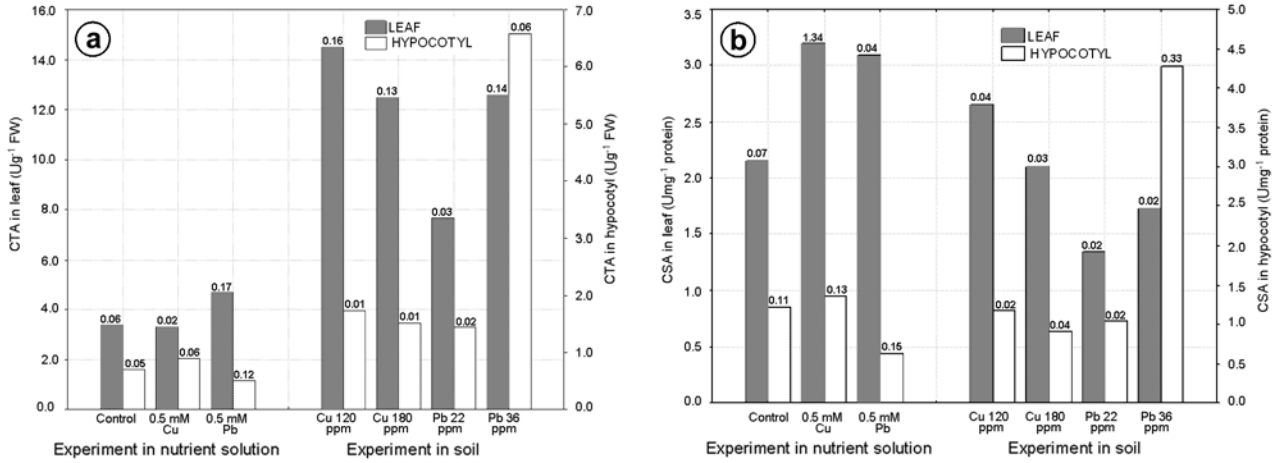

Fig. 4. (**a**) Catalase total activity in leaves and hypocotyls of radish grown in nutrient solutions and soil, under the influence of copper or lead, (**b**) Catalase specific activity in leaves and hypocotyls of radish grown in nutrient solutions and soil, under the influence of copper or lead. Nutrient solutions: control – standard Hoagland solution; soils: Cu content 120 and 180 ppm, Pb content 22 and 36 ppm; bars are means of three replicates, with SE as bar labels.

significantly increased leaf total catalase activity ($p \leq 0.01$), and Cu had a similar effect on total catalase activity in hypocotyls ($p \leq 0.05$). The specific activity of catalase in leaf was increased by both heavy metals; Pb treatment inhibited catalase activity in hypocotyls (Fig. 4b).

Further work was done to determine the effects of heavy metals on plants grown under more realistic conditions, to compare the responses to those of plants grown under laboratory conditions. Plants were grown outdoors in pots of soil collected in the field from sites where crops such as radish would be grown for agricultural purposes, but with different, and determined, Cu and Pb content, until their hypocotyls developed to consumable size. Total Cu content was 180 mg kg^{-1} (or ppm) in vineyard soil and 120 mg kg^{-1} in soil from a field near the vineyard (Tab. 1). The exchangeable amounts were 23% and 21%, respectively. The soil used for the assay of Pb effects had total Pb content of 36 mg kg^{-1} (roadside soil) and 22 mg Pb kg^{-1} (soil 100 m away from the road). The exchangeable amounts were 21% of total Pb for soil near the road and 13% for the soil 100 m away.

In the soil Cu analyses, plant part and soil Cu level significantly influenced TBARS levels, which were higher in hypocotyls grown in vineyard soil (180 ppm Cu) than in those grown in field soil (120 ppm Cu; Fig. 1). Proline content was higher in hypocotyls, with Cu-induced accumulation found in both the hypocotyls and the leaves of plants grown in vineyard soil (Fig. 2). Peroxidase total activity was mostly higher in leaves, except in plants grown in field soil with lower Cu content (Fig. 3a). Enzyme specific activity was also higher in leaves but lower than in hypocotyls (Fig. 3b). Catalase total activity was much lower in hypocotyls, and the higher Cu content of vineyard soil had a negative but non-significant impact (Fig. 4a). Catalase specific activity was higher overall in leaves, with a decline seen in both parts of plants grown on vineyard soil having higher Cu content (Fig. 4b).

DISCUSSION

In this work, radish plants were grown in agricultural soils from actual sites where Cu and Pb levels in the soil differ. The oxidative stress responses of the plants were compared to those of plants grown with Cu- or Pb-spiked nutrient solutions. As seen in Table 1, the levels of Cu and Pb in the soils used from the field were not the only variable parameters, and the noted stress responses could be due to other factors. In terms of microelement availability, which is mostly lower in soils with higher pH, the estimated soil pH could be considered higher than optimal, but there were no visible deficiency symptoms. Indirectly, differences in soil fertility (potassium and phosphorus supply) may have affected plant growth and development in interaction with other environmental conditions. However, it is important to determine the stresses plants undergo in the conditions that prevail where the crops are grown and harvested commercially, and not to assume that laboratory conditions duplicate those of the farm. In this study, oxidative stress responses did increase when Cu and Pb were increased, in both soil-grown plants and plants treated with spiked nutrient solutions. Radish plants clearly show an oxidative stress

response to heavy metals, one which can account for the responses seen with the soil-grown plants.

For example, the TBARS levels in tissues of plants treated with nutrient solution or grown on soil with higher heavy metal content (Fig. 1) indicate lipid peroxidation as a consequence of oxidative stress caused by those heavy metals. In the data presented here, lipid peroxidation is more pronounced in hypocotyls, and Pb excess has a stronger impact. Chen et al. (2004) reported increased proline content and lipid peroxidation in rice shoots after copper exposure, but in rice roots both were decreased. On the other hand, Yurekli and Porgali (2006) found that the increase of lipid peroxidation after 10-day treatment of bean plants with 0.1 mM copper was significant in root tissue but not in stem and leaf tissue. In lead-treated rice seedlings, Verma and Dubey (2003) observed increased lipid peroxides in shoots, indicating elevated oxidative stress. The rationale for increased proline in a plant is linked with the ability of proline to quench singlet oxygen (Özturk and Demir, 2002), which may arise as a byproduct of lipoxygenase in the presence of Cu^{2+} (Arora et al., 2002). Proline can also react directly with the hydroxyl radicals that might result from metal-catalyzed Fenton chemistry, and therefore increased proline would mitigate the damage from free radicals and a produce a more reducing cellular environment (Siripornadulsil et al., 2002). Claussen (2005) reported that average proline concentrations in tomato leaves increased with increasing nutrient concentrations in hydroponics during reproductive growth, and suggested that proline content is a measure of the stress experienced by plants in this period. According to Shetty (2004), during development and stress response, when phenolic biosynthesis is stimulated, an alternative mode of oxidative phosphorylation linked to proline metabolism may be more efficient and suitable; therefore the increased proline content seen in this study (Fig. 2) may be significant for the overall response of the plant to heavy metal exposure.

To better understand the responses seen to exposure to either Cu or Pb in this research, the amount of protein was measured (data not shown), but the response to heavy metal stress in terms of protein content was not consistent. On the other hand, peroxidase and catalase activity was strongly influenced by the heavy metal treatments in both experiments. The observed higher proline content (Fig. 2) and total peroxidase activity (Fig. 3a) in hypocotyls might be related to lignification as a means of suppressing heavy metal uptake by hypocotyls. In hypocotyls of Cu-stressed pepper seedlings, induction of peroxidase and shikimate dehydrogenase activity was associated with the accumulation of soluble phenolics and lignin (Díaz et al., 2001). In our work, total peroxidase activity was much higher in radish hypocotyls, with a significant increase in response to the two heavy metals in nutrient solution as well as in soil with higher Cu content (Fig. 3a). The specific activity of peroxidase seemed dependent on the plant part and less related to the heavy metal applied in nutrient solution or to soil heavy metal content (Fig. 3b). Willekens et al. (1997) suggested that the function of catalase in the cell is to remove the bulk of H_2O_2, whereas peroxidases would be involved mainly in scavenging the H_2O_2 not taken by catalase. Pandey and Sharma (2002) exposed cabbage plants to 0.5 mM Co, Ni or Cd, and found decreased activity of iron-containing enzymes, catalase and peroxidase, and enhanced proline accumulation in leaf. Peroxidase and catalase activity was also lowered by Cu toxicity in radish plants (Chatterjee et al., 2006). Wang et al. (2004) reported increased activity of guaiacol peroxidase and suppressed catalase activity in roots of *Brassica juncea* treated with 0.008 mM Cu. Verma and Dubey (2003) used 0.5 mM Pb in nutrient medium to simulate moderate soil pollution and observed an increase in guaiacol specific activity in both roots and shoots of two rice cultivars; roots showed higher guaiacol specific activity than shoots, but catalase specific activity declined in roots and increased in shoots. Here we also observed higher catalase specific activity in leaves than in hypocotyls, where it was inhibited by high Pb in nutrient solution (Fig. 4b). In the experiment with soil-grown radish, both CTA and CSA were lower in plants grown on soil with higher Cu, but higher in plants grown on soil with more Pb (Figs. 4a and 4b).

The observed increment in peroxidase and catalase activity in leaves after hypocotyls were exposed to excessive Cu or Pb for only two days (Experiment 1) might suggest that there is a signal of some kind from the hypocotyl to leaves. There is no evidence of the nature of the signal presented here, but Cuypers et al. (2000) reported a root-to-shoot signalling system that appears to be involved in copper-imposed oxidative stress as well as in the antioxidative defense response. They later stated that the early metabolic changes observed in leaves suggest that signal molecules are involved in induction of the defence against metal stress (Cuypers et al., 2002). Vitória et al. (2001) reported significant cadmium-induced increases in catalase, superoxide dismutase and glutathione reductase activity in both leaves and roots of radish seedlings, and also suggested that an oxidative stress signal is sent from roots to leaves. A possible component of a systemic signal is H_2O_2, which sets up an acclimatory response in unstressed regions of plants (Bhattacharjee, 2005). However, for H_2O_2 to act as a signalling molecule it must have regulated synthesis, specific responses and cellular targets, and there must be mechanisms

for its metabolism or removal subsequent to signalling events (Neill et al., 2002). The activity of peroxidase and catalase in our research suggests an increased level of H_2O_2 in radish leaves as a consequence of copper and lead toxicity. Was peroxide transported from hypocotyls as a signalling molecule, or generated in leaves as a consequence of heavy metal translocation? This remains to be elucidated by further work.

We found much higher stress levels in the treatments with nutrient solution, where the whole amount of spiked heavy metal is in plant-available form; there were no factors such as organic matter content or high pH to diminish their toxicity. The nutrient solution treatments used young plants, and their quick response to high metal concentrations were the object of evaluation; the mature plants grown in the soil were studied for their longer-term response, which could involve acclimatization to lower levels of heavy metal stress. Clearly the responses of plants grown in nutrient solution enriched with heavy metals did not match the responses seen in soil-grown plants, but this highlights the importance of determining how plants respond in the field, particularly at sites where relatively high levels of contamination are known.

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