

Heavy Metal Perception in a Microscale Environment: A Model System Using High Doses of Pollutants

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Abstract The characterization of the mechanisms of heavy metal detoxification has been undertaken through several experimental approaches, where high metal concentrations have been frequently used. A *microscale* hydroponic system was used to discriminate between the direct and indirect phytotoxic effects that may occur under heavy metal stress at short exposure times. Induction of oxidative stress and generation of stress signaling molecules are some of the physiological responses triggered soon after the exposure of plant cells to heavy metals, which might be part of stress perception mechanisms. The generation of reactive oxygen species, in particular H₂O₂, ethylene or jasmonate are envisaged as messengers in signaling pathways that may result ultimately in cell senescence and growth inhibition.

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Abbreviations

ACC	Amino-cyclopropane-1-carboxylic acid
APX	Ascorbate peroxidase
ECS	Gamma glutamylcysteine synthetase
GR	Glutathione reductase
GS	Glutathione synthase
JA	Jasmonic acid
PCS	Phytochelatine synthase
ROS	Reactive oxygen species
SA	Salicylic acid, SOD, superoxide dismutase

1 Introduction

The accumulation of heavy metals in some ecosystems as a consequence of several contaminating human activities, such as mining or melting, poses a relevant environmental risk (Patra et al. 2004). Heavy metals that accumulate in soils are taken up by plants, and through a biomagnification process in the trophic chain, they can constitute a serious health problem for animals and humans. Due to their sessile nature, terrestrial plants have restricted mechanisms for stress avoidance, but during the course of evolution some plant species have developed mechanisms to cope with environmental stresses (Pastori and Foyer 2002). These tolerance mechanisms rely on the activation of complex processes of perception, transduction and transmission of stress stimuli (Mittler et al. 2004). In recent years, the understanding of physiological responses to heavy metal stress has been the subject of many studies, with the aim of discovering mechanisms of tolerance. The contribution of signaling molecules like ethylene, jasmonate or reactive oxygen species (ROS) has recently been described (Maksymiec 2007). However, to identify the signaling components a plethora of experiments have been carried out, using different plant species, doses of metals and exposure times, as thoroughly reviewed by Schützendübel and Polle (2002). In most studies, treatments are long enough to provoke substantial metabolic changes, such as the onset of oxidative stress symptoms (Gratão et al. 2005). Upon a stress situation, an unbalance of the cellular redox status occurs due to the accumulation of ROS and alterations in the antioxidant defences. Superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are scavenged by the action of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) enzymes, which use the soluble antioxidants ascorbate and glutathione (GSH; Noctor and Foyer 1998). The cellular thiol status apparently plays a central role in redox homeostasis and cell function, in which the concentration of GSH and the balance with its oxidized counterpart (GSSG) is kept at a constant level (Noctor 2006). It is expected that components of the antioxidant system may play a relevant role in heavy metal stress perception, as alterations in the redox cellular

homeostasis is well documented (Hall 2002; Schützendübel and Polle 2002; Grátão et al. 2005; Sharma and Dietz 2009).

The responses might differ as a function of doses, plant species, growing conditions and phenology status (Sanità di Toppi and Gabbrielli 1999). Therefore, in many experiments it is extremely difficult to distinguish between direct and indirect responses if metal concentrations or treatment interval are too high or excessively prolonged. In such experiments, the metabolic alterations observed might reflect general failure of plant metabolism; but little is known about the earlier stages. Therefore, the characterization of heavy metal stress perception mechanisms should be undertaken in adequate experimental conditions, where we could learn about the primary cellular components involved. Little is known about the early effects of metal treatments, mainly because of the difficulty of detecting ROS accumulation or oxidative damage at the cellular level. Such a task could be accomplished by using highly sensitive fluorescent probes that react with ROS (Olmos et al. 2003; Garnier et al. 2006), or which are capable of tracing cellular integrity (Ortega-Villasante et al. 2005). This kind of experiments is paving the way to understanding in detail the dynamic aspects of plant cellular responses to heavy metals.

2 Microscale Versus Macroscale Analysis: Time Resolved Responses

To identify the specific physiological responses to metal stress it is important to fix the time of exposure and dose, avoiding acute metabolic alterations. The dose–response relationship is a complex phenomenon in which toxic metals produce several events at molecular, physiological and morphological levels. Moreover, the dose that is considered toxic depends on the specific heavy metal and is characteristic for the different plant species (Schützendübel and Polle 2002). In fact, the dose–response curves of essential elements have three phases: deficiency, tolerance and toxicity, while non-essential elements do not present a deficiency phase (Hagemeyer 2004). Early and direct phytotoxic symptoms of heavy metals could be followed by analyzing several physiological parameters. Thus, the reduction in cell proliferation and inhibition of growth correlates well with metal intoxication, and is frequently used as a phytotoxic index (Schützendübel et al. 2001). Another parameter is the rapid alteration of nutrient uptake and accumulation, as heavy metals provoke damages in the plasma membrane of exposed cells (Hernández and Cooke 1997), affecting water uptake and solute permeability (Hernández et al. 1997). A number of different studies have also shown that such early alterations could be caused by the induction of oxidative stress, which leads to lipid peroxidation (Lozano-Rodríguez et al. 1997; Chaoui et al. 1997) and cell senescence (Ortega-Villasante et al. 2005, 2007).

An extensive array of experiments, described in the literature, aimed to identify the direct effects of heavy metals on plant cell metabolism. In the vast majority of such experiments, plants under study were treated in a pure hydroponic culture for a certain interval to several doses of heavy metals. As an example of the experiments carried out to analyze the short-term effects of heavy metals, we show in Fig. 1a the *macroscale* system used to test the influence of Cd on nitrate, potassium and manganese uptake in *Pisum sativum* plantlets during 24–96 h (Hernández et al. 1997, 1998). A similar approach can be followed by treating the plants in a semi-hydroponic culture system, where plants are grown on an almost inert substrate (perlite) moistened by the nutrient solution (Fig. 1b). In these conditions, the growth and development of roots resembles the pattern typical of plants grown in soil, where nutrient and metal availability is more restricted than in pure hydroponics (Vázquez and Carpena-Ruiz 2005). As a consequence, remarkable differences in the responses to Cd and Hg were found with plants treated in pure hydroponic systems, inferring that each kind of growing condition affects the perception of heavy metals (Sobrino-Plata et al. 2009).

In spite of the valuable information that is provided by both kinds of macroscale experimental settings described above, they are unsuitable when very short and direct effects are to be characterized, since the dynamic responses might be poor with particular toxic concentrations of heavy metals. For example, alfalfa plants treated with 30 μM Hg showed a saturated toxic response of growth inhibition and lipid peroxidation after just 24 h exposure, whereas both parameters increased linearly with time in plants treated with 30 μM Cd (Ortega-Villasante et al. 2005). To overcome the saturation phase under certain experimental conditions, and to monitor minute and rapid changes in plant metabolism, alternative plant materials and experimental conditions have been used. A viable option is the use of plant cell cultures or protoplasts isolated from leaves (see protoplasts from alfalfa in Fig. 1c), which can be kept in a suspension culture to which the metals can be added directly (Sobkowiak and Deckert 2003). DNA damage was observed in protoplasts exposed to heavy metals (Zhang et al. 2009). Similar negative effects on DNA stability and cell apoptosis induction were found in tobacco BY2 cells treated with Cd, and, interestingly, a DNA repair mechanism was induced when Cd was removed from the culture medium (Fojtová et al. 2002). In this experimental design, tobacco BY2 cells suffered an abrupt accumulation of H_2O_2 when exposed to extreme high concentration of Cd (1–5 mM) after just 15 min (Olmos et al. 2003), or after 24 h (Garnier et al. 2006). Such treatments also caused a severe depletion of GSH in *Arabidopsis thaliana* cultured cells after 24 h under 50 and 200 μM Cd (Sarry et al. 2006). Therefore, it is possible to gain some information about the early responses of plant cells to heavy metals, albeit the behavior might be completely different from root cells, since this organ accumulates higher concentrations of pollutants in the excluder plants. This problem was solved by using the *microscale* hydroponic system proposed by Ortega-Villasante et al. (2005, 2007) shown in Fig. 1d. We have successfully combined the *microscale* system with fluorescent probes to detect in vivo parameters related with heavy metal stress in intact alfalfa seedlings with a moderate supply of Cd or Hg, in the range 3–30 μM :

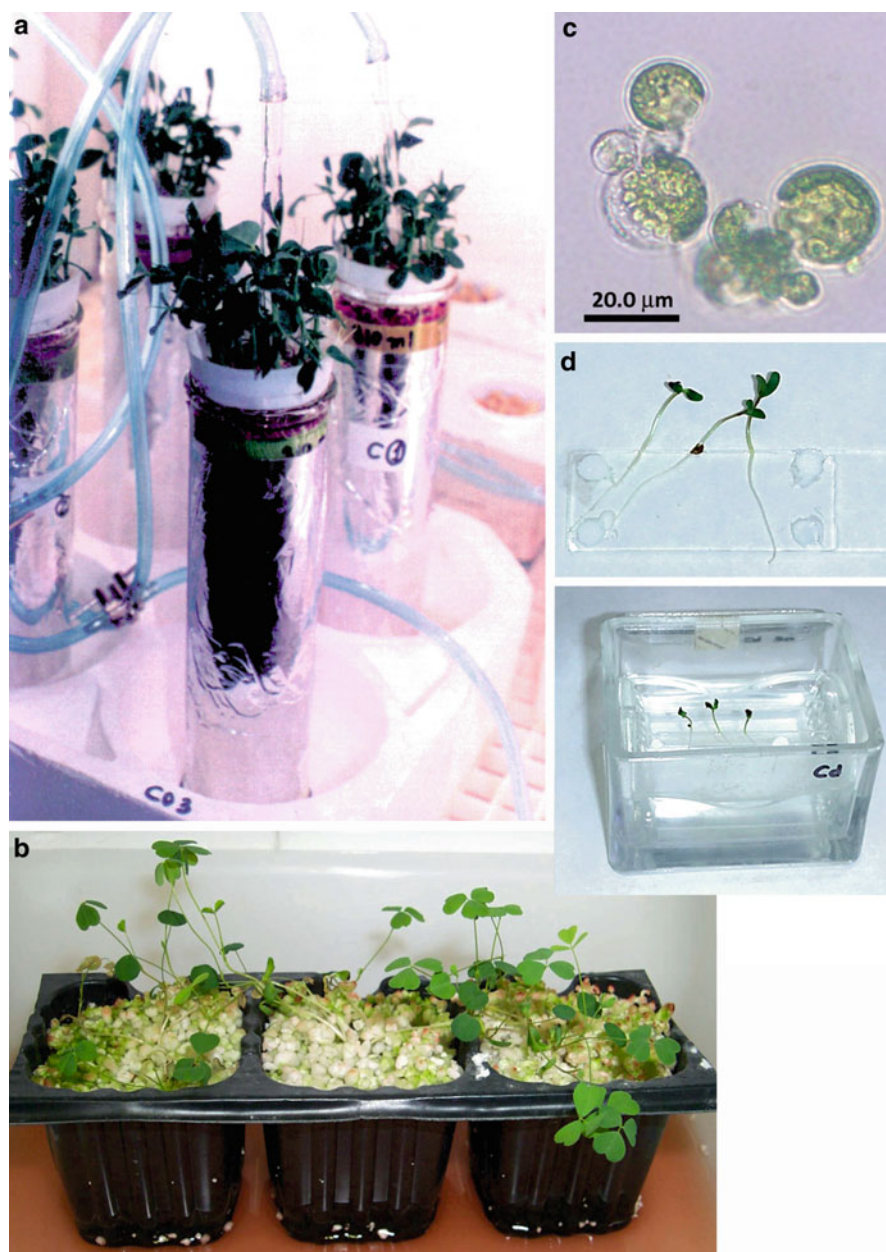


Fig. 1 Different plant materials used to study the direct effects of heavy metals. (a) Pure hydroponic culture using a nutrient solution with reduced volume, used to study short-term responses of pea plants. (b) Semi-hydroponic system where alfalfa plants are grown in an inert substrate. (c) Protoplast isolated from alfalfa leaves after digestion with cell wall-degrading enzymes. (d) Microscale hydroponic system, which allows very precise control of exposure times and in vivo visualization of heavy metal stress in alfalfa seedlings

(a) 2',7'-dichlorofluorescein diacetate (H₂DCFDA) to visualize oxidative stress; (b) monochlorobimane (MCB) to detect the cellular concentrations of GSH and homogluthathione (hGSH; homologous tripeptide to GSH that accumulates in alfalfa); and (c) propidium iodide (PI) to estimate the amount of cellular death. In addition, we used Amplex Red to monitor in situ the secretion of H₂O₂ from alfalfa root tips using a fluorescence titer-plate reader (Ortega-Villasante et al. 2007).

Epifluorescence or laser confocal microscopy can be used to visualize the changes in the above mentioned parameters in *Medicago sativa* root epidermal cells (Fig. 2). Cadmium and Hg caused a depletion of the GSH/hGSH fluorescence signal in the epidermal cells of the alfalfa root (Fig. 2c, d). Buthionine sulfoximine (BSO) is a potent inhibitor of GSH/hGSH synthesis, and was used as a negative control to discriminate auto-fluorescence cell epidermis (Fig. 2b), which in turn permits to visualize the hazardous effect of GSH/hGSH depletion in cellular redox homeostasis. In parallel, oxidative stress was detected in BSO and Cd treated seedlings using H₂DCFDA (Fig. 2f, g). Interestingly, oxidative stress appeared scattered under Cd stress, implying that different epidermal cells accumulated the metal depending on their particular physiological status, as we could confirm in a kinetic experiment after very short exposure times (Ortega-Villasante et al. 2007). Remarkably, Hg caused an extremely high rate of cell death at the same dose of

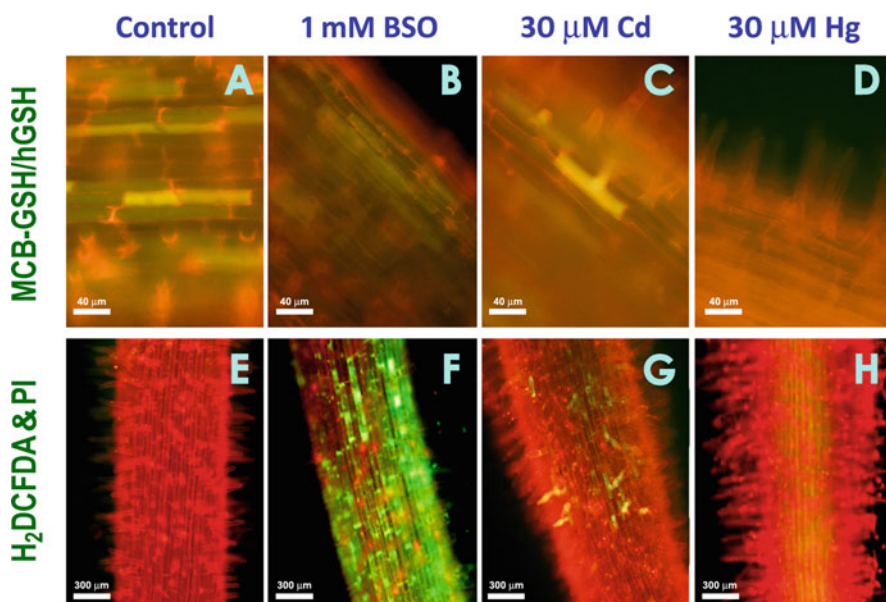


Fig. 2 Fluorescence probes that can be used to trace alteration in the pool of glutathione (monochlorobimane-MCB), oxidative stress (2',7'-dichlorofluorescein diacetate-H₂DCFDA), and cell death (propidium iodide-PI). MCB (green) was visualized with epifluorescence microscopy (a–d), and H₂DCFDA (green) and IP (red) with confocal microscopy (e–h). Alfalfa seedlings were treated with Cd, Hg and buthionine sulfoximine (BSO) for 24 h prior to staining with the different probes

30 μM (observed as red fluorescence of PI in condensed nuclei; Fig. 2h), suggesting that oxidative stress only occurs in still viable cells with functional metabolism (Ortega-Villasante et al. 2005). A similar experiment with barley root tips showed that after 3–6 h exposure to 1 mM Cd or 0.5 mM Hg caused the overexpression of several genes encoding aquaporins and dehydrins, suggesting the onset of dehydration stress by heavy metal. These responses were accompanied by a significant inhibition of root growth and induction of oxidative stress (Tamás et al. 2010).

Another experimental alternative used frequently to study the direct effects of heavy metals on particular cellular components is the extraction of such materials from untreated plants, which are then tested with different treatments of metals *in vitro*. For example, it has been observed that direct exposure of thylakoid membranes caused the release of their components, especially proteins of the splitting water system and galactolipids probably connected with photosystem I (PSI) inhibition after heavy metals exposure (Skórzyńska and Baszyński 1993; Nouari et al. 2006). These experiments showed that heavy metals bind to membranes through oxygen atoms or aminoacids such as histidine, tryptophan or tyrosine in proteins, leading to electron flow disturbance in photosystem II after illumination (Maksymiec 1997). Other studies indicated that high heavy metal concentration lead to substitution of the Mg in the chlorophyll molecules (Kowalewska et al. 1987). Similarly, Hg substitutes Cu in plastocyanin molecule, blocking electron passage to PSI (Radmer and Kok 1974), and Cd, Hg, and Pb may also bind to LHCII producing conformational changes. Inhibition of enzymes involved in chlorophyll production also produces a decrease in its synthesis (Böddi et al. 1995). In spite of these findings, it is not clear that alterations in the photosynthetic electron transport and dark phase reactions observed *in vivo* are the direct effect of metals, as these effects appear only after several days of exposure (Wang et al. 2009).

3 ROS Signaling and Antioxidant Responses

An increase in H_2O_2 production has been reported in plant cells treated with several heavy metals, even those that have no direct or very little redox activity such as Cd or Hg. Cadmium is one of the heavy metals most widely studied, and relevant information has been provided by Olmos et al. (2003), Garnier et al. (2006), Cho and Seo (2005), and Romero-Puertas et al. (2002, 2004). Mercury is also a potent H_2O_2 inducer, as shown by Cho and Park (2000) and Ortega-Villasante et al. (2007). Similar responses were found for Cu (Xiang and Oliver 1998; Maksymiec and Krupa 2006) and Mn (Demirevska-Kepova et al. 2004), although at much higher doses due to their essential nature, which depends on the threshold of toxicity for each plant species. This production of ROS usually leads to damage in several cellular components, causing membrane lipid peroxidation (Lozano-Rodríguez et al. 1997), alteration of nucleic acids structure (Fojtová et al. 2002), or oxidation of proteins (Romero-Puertas et al. 2002; Rellán-Álvarez et al. 2006) and photosynthetic pigments (Somashekaraiah et al. 1992; Weckx and Clijsters

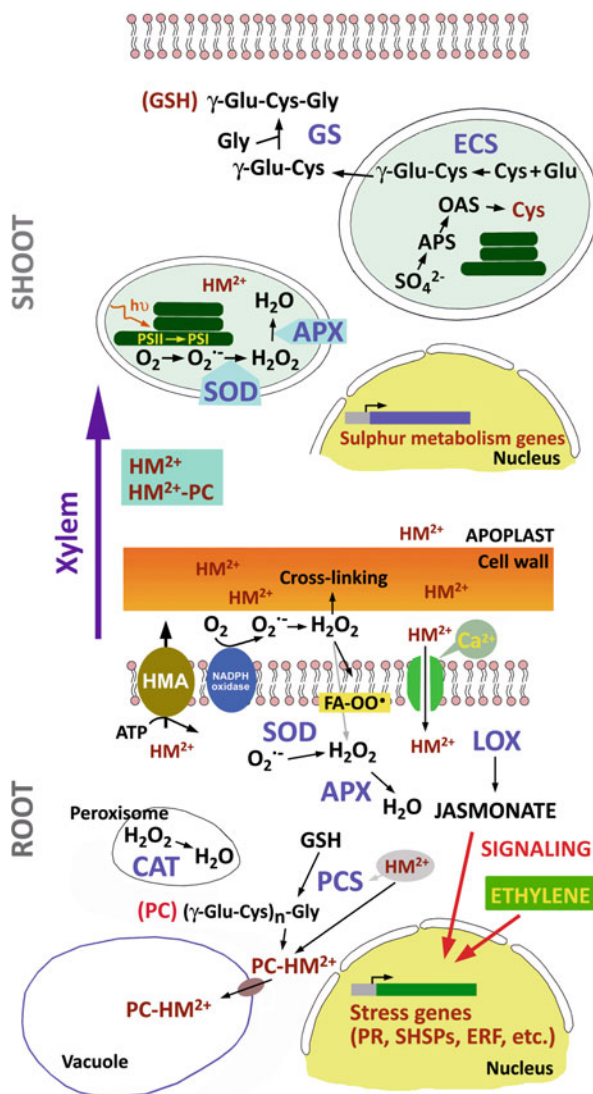


Fig. 3 Major physiological changes described in plants treated with heavy metals (HM^{2+}). In excluder plants metals accumulate in the root, where most of the alterations occur. The cell wall can act as a sink, but when this barrier is overridden, metals enter the protoplast probably through ion channels (for example Ca^{2+} -channel). Part of the uptaken metal can be expelled by transporters of the HMA, possibly loading them in the xylem. Plasma membrane NADPH-oxidases are activated to generate $O_2^{\cdot-}$, which is converted to H_2O_2 in the apoplast to serve as substrate of cell wall peroxidases to stiffen cell wall polymers. H_2O_2 can also oxidize membrane lipids, generating peroxide lipid radicals ($FA-OO^{\cdot}$), or permeate to the cytoplasm where antioxidant enzymes must control cellular levels of ROS (SOD , CAT and APX). On the other hand, metals would activate phytochelatin synthase (PCS) triggering the synthesis of phytochelatin (PC). These biothiols would form complexes that are thought to be transported to the vacuoles.

1997; Drażkiewicz et al. 2004). Such accumulation of H_2O_2 is usually connected with changes in the cellular redox status, which is considered as a component of stress signaling processes (Lamb and Dixon 1997; Pastori and Foyer 2002; De Gara et al. 2010), being part of a complex redox sensing network between several cellular compartments (Foyer and Noctor 2003). Recent evidence also indicates that H_2O_2 accumulation interacts with other relevant signaling molecules like Ca^{2+} (Rentel and Knight 2004). Indeed, Rivetta et al. (1997) described the involvement of Ca^{2+} -calmodulin in Cd toxicity during the early phases of radish seed germination. The relationship between heavy metals and Ca could also be explained by the possible permeation of Cd^{2+} through Ca^{2+} channels (Fig. 3), as was observed in protoplasts prepared from *Vicia faba* stomata cells (Perfus-Barbeoch et al. 2002).

NADPH oxidase is probably the major source of H_2O_2 under heavy metal stress, which accumulated principally in the apoplast after the generation of $\text{O}_2^{\bullet-}$ (Romero-Puertas et al. 2004; Rodriguez-Serrano et al. 2009; Fig. 3). Similarly, H_2O_2 was exudated in alfalfa seedling roots shortly after Cd and Hg exposure (90 min), which was partially inhibited by the addition of diphenyleneiodonium chloride (DPI), a NADPH-oxidase inhibitor (Ortega-Villasante et al. 2007). Using the microscale system, we have also observed that NADPH-oxidase activity augmented in alfalfa roots in particular under Cd stress (3 and 10 μM) after 6 h of treatment, confirming the involvement of this plasma membrane enzyme in ROS generation under heavy metal stress (Hernández et al. personal communication). The accumulation of H_2O_2 in the apoplast could also increase the activity of cell wall peroxidases, which would be followed by cell wall stiffening due to cross-linking reactions among cell wall polysaccharides and proteins, as described under Cu stress (Lin et al. 2005). Extensins (hydroxyproline-rich glycoprotein), a group of cell wall structural protein prone to suffer cross-linking reactions, are induced under heavy metal stress (Chai et al. 1998). It has been proposed that, under stress conditions, basic peroxidases could render the cell wall more rigid, which could be correlated with root growth inhibition under Cd stress (Tamás et al. 2007; Sobrino-Plata et al. 2009). In addition, H_2O_2 can be a diffusible signal to trigger at least partially some of the physiological responses to heavy metals (Miller et al. 2008). Tamás et al. (2010) showed in barley root tips that the supply of 10 mM H_2O_2 mimicked the transcriptional response of Cd and Hg, such as the overexpression of stress genes like a cell wall peroxidase.

To counteract the accumulation in ROS caused by heavy metal treatments the plant cells possess versatile antioxidant defence mechanisms in which GSH plays a central role (Schützendübel and Polle 2002; Sharma and Dietz 2009). In addition to

Fig. 3 (continued) Alternatively, some HM^{2+} –PC complexes could be transferred to the shoot via xylem long transport. Signaling molecules like ethylene or jasmonate accumulate, which would cause the overexpression of stress related genes (PRs, SHSPs, ERF, etc.). In shoots, the most significant changes would be the modification of important metabolic processes, such as photochemical reactions and electron transport in the tylakoid. In addition, the overexpression of sulfur metabolism genes and activation of GSH synthesis occur, which are key metabolites in heavy metal detoxification and tolerance, for example ECS

this function, GSH is required to tolerate heavy metals via the synthesis of phytochelatins (PCs; Cobbett and Goldsbrough 2002), and it is also known to be conjugated with xenobiotics by the action of GSH S-transferases or to serve as the substrate of GSH peroxidases (Edwards et al. 2000). The redox couple formed by GSH and its oxidised form GSSG (GSH/GSSG ratio), along with ascorbate/dehydroascorbate (AA/DHA), is important to control the cellular redox homeostasis (Noctor 2006). Only under acute Hg-stress conditions characterised by high rates of cell death in alfalfa seedlings grown in the microscale hydroponic system, we could detect a displacement of the GSH/GSSG equilibrium towards the accumulation of GSSG (Ortega-Villasante et al. 2007). After just 24 h of treatment with 50 μM Cu there was an impairment of the GSH/GSSG and AA/DHA redox pairs in *Arabidopsis leaves* (Cuyppers et al. 2000). Similarly, *Arabidopsis* treated with 100 μM Cd or Cu for 24 h suffered a remarkable accumulation of GSSG under extensive oxidative stress (Xiang and Oliver 1998). Interestingly, there was in parallel an overexpression of GR gene, possibly as a mechanism exerted in highly stressed alfalfa seedlings to restore original GSH cellular levels (Ortega-Villasante et al. 2007). These results imply that the cellular level of GSH is tightly controlled relative to GSSG, by means of higher glutathione reductase (GR) activity or by increased synthesis of GSH. Indeed, the maintenance of a cellular GSH threshold is fundamental to direct many of the processes of heavy metal detoxification and plant defence against stress (Maughan et al. 2010), and for normal development of plant cells (Pasternak et al. 2008). *Arabidopsis thaliana* mutants defective in GSH accumulation, such as *rml1* or *cad2-1*, or plants incubated with BSO suffered a diminution in cell division at the root meristem, which results in abnormal root growth after germination under a low level of cytosolic GSH (Vernoux et al. 2000).

Glutathione is synthesized in a two-step reaction: the first catalyzed by the rate limiting glutamate-cysteine ligase, or γ -glutamyl cysteine synthetase (ECS), and the second catalyzed by glutathione synthase (GS). In *Arabidopsis*, ECS is located exclusively in the chloroplast, whereas GS is found in the chloroplast and in the cytosol (Wachter et al. 2005). Plants treated with BSO, which precisely inhibits ECS and causes a similar phenotypic response to stress than as *Arabidopsis thaliana rml1* or *cad2-1* mutants, suffered from redox cellular imbalance (Fig. 2f; Ortega-Villasante et al. 2005). One common response of plants to heavy metals is the activation of the sulphur metabolism, increasing the assimilation of sulfate and the production of Cys and GSH (Ernst et al. 2008). Indeed, the early overexpression of ECS has been found in a number of experiments, with *Arabidopsis thaliana* cultured cells upon Cd stress (Sarry et al. 2006), in the roots of Cd-treated rice (Aina et al. 2007) or in alfalfa seedlings exposed to Cd and Hg for up to 24 h (Ortega-Villasante et al. 2007). It is feasible that the accumulation of GSH could be regulated by the redox cellular status, as it has been reported recently that the genes encoding the enzymes involved in GSH synthesis are up-regulated by H_2O_2 , implying that the accumulation of ROS may trigger GSH accumulation (Queval et al. 2009). In addition, plant ECS responds to the redox cellular status, so under oxidizing conditions ECS forms a homodimer that enhances its activity three-fold, augmenting the level of GSH in the cells (Gromes et al. 2008). As commented,

phytochelatins are a large family of biothiols synthesized from GSH, known to bind Cd, Hg and other toxic elements by means of sulfhydryl residues (Cobbett and Goldsbrough 2002). A transient depletion of GSH has been observed under heavy metal stress due to the synthesis of PC, which might result in poorer cellular redox homeostasis, and may probably alter other metabolic processes driven by GSH (Saito 2004). The phytochelatin synthase enzyme (PCS) catalyses the condensation of the γ -Glu-Cys moiety of GSH with the Glu residue of a second GSH, releasing Gly and increasing the length of the PC molecule (Vatamaniuk et al. 2004; Clemens 2006). Interestingly, the PCS defective *Arabidopsis cad1-3* mutant suffered a remarkable and rapid increase in GSH concentration after short-term Cd and Hg treatments, which was not used eventually to synthesize PCs. Therefore, this implies that the GSH synthesis pathway was induced under heavy metal stress, and might be the limiting step in metal tolerance (Carrasco-Gil et al. 2011). The metal–PC complex formed is mainly transported to the vacuole where is safely stored, although it cannot be discounted that a portion of the metal might be secreted to the xylem, permitting the translocation of the metal to the shoots (Saathoff et al. 2011). However, *Arabidopsis cad1-3* mutants were capable of translocating more Cd to the shoot, indicating that transporters of the HMA family would have more free cytosolic Cd as transportable solute (Wong and Cobbett 2009).

4 Phytohormone Signaling Pathways

The accumulation of H_2O_2 driven by plasma membrane NADPH-oxidases are thought to induce the formation of lipid peroxides (i.e., fatty acid radicals or FA-OO; Neil et al. 2002). This oxidative process, together with the activity of several classes of lipoxigenases, generate oxylipins, which are precursors of jasmonate (JA; Turner et al. 2002; Foreman et al. 2003). Several short-term experiments have shown that heavy metals can induce the overexpression of several genes encoding LOXs. Thus, the exposure to μM levels of Cd and Cu for 24 h caused the overexpression of LOX in *Arabidopsis* (Remans et al. 2010). Similar results were observed by Maksymiec et al. (2005) in *Arabidopsis* treated with excess of Cd or Cu, seedlings that accumulated JA after just 6–7 h of incubation. In addition, Rakwal et al. (1996) showed that Cu induced a rapid increase of JA content in *Oryza sativa* excised leaves. A biphasic JA accumulation was found in Cu or Cd stressed plants, e.g., a rapid one within a few hours, and a slow one after several days (Maksymiec et al. 2005) may be the reason of the usually observed rapid decrease in growth processes and senescence intensification of plants after prolonged exposure to the heavy metals.

Ethylene could also be part of the signaling process triggered by the exposure of plants to heavy metals (Sanit  di Toppi and Gabbrielli 1999). The first evidence came from the studies of Sandmann and B ger (1980), which showed that Cu induced the synthesis of ethylene, leading to cell senescence. It is feasible that the growth inhibition observed in dicotyledonous plants treated with Cu may be mediated by ethylene (Maksymiec and Krupa 2006). Cu stimulated ethylene

production possibly via an augmentation of ACC synthase enzymatic activity in *Arabidopsis* plants (Arteca and Arteca 2007). Similarly, pea plants exposed to 50 μM Cd for 14 days accumulated a remarkable amount of ethylene in leaves (Rodríguez-Serrano et al. 2009), which would be part of the signaling process involved in the defence responses to heavy metals (Guo and Ecker 2004). We have concluded a transcriptomic analysis of the early responses of alfalfa seedlings to 3 μM Hg using the microscale hydroponic system. Our results support the idea that ethylene might be part of the signalling molecules that are induced by heavy metals, and we found that transcription factors responding to ethylene (such as ERF1) are up-regulated after just 3 h of treatment (Montero-Palmero et al., personal communication). Ethylene acts by the activation of the mitogen-activated protein kinase (MAPK) cascade (Guo and Ecker 2004), signal transduction process that is also activated by Cd and Cu (Yeh et al. 2003; Jonak et al. 2004), suggesting the ethylene-mediated signaling processes might be induced in plant cells exposed to heavy metals. There might be interactions between different phytohormones, as has been found that there was a cross-talk between JA, ethylene and other phytohormones to mediate the expression of genes like cytochrome P450 under several biotic and abiotic stresses (Narusaka et al. 2004). On the other hand, Metwally et al. (2003) observed that Cd toxicity might be alleviated by salicylic acid, which is a known as a signaling factor that often blocks the JA pathway (Gupta et al. 2000). Some parallels have been found in the responses in plants exposed to heavy metals and to JA, for example, at the gene expression pattern. Xiang and Oliver (1998) found common responses between plants treated with Cd or Cu and JA in the transcription profile of genes involved in the GSH metabolism. A similar effect was observed in the induction of VSP2 (vegetative-storage protein) and MAPK transcription (Mira et al. 2002; Agrawal et al. 2003a, b; Kim et al. 2003). Secondary metabolites and pathogenesis-related proteins have been described in heavy metal-stressed plants (Cruz-Ortega and Ownby 1993; Rakwal et al. 1996; Pitta-Alvarez et al. 2000; Schützendübel and Polle 2002), in which transcription is mediated by JA and/or ethylene, implying that biotic and abiotic stresses may share some signaling processes (Mithöfer et al. 2004). Interestingly, Ghoshroy et al. (1998) and Mittra et al. (2004) showed that a pre-exposure to a mild dose of Cd increased plant resistance to both viral and fungus infection. Poschenrieder et al. (2006) suggested that some plants may increase tolerance to pathogens by taking advantage of the induction of defences like glucanases, chitinases or proteinases; which genes were overexpressed under heavy metal stress (Przymusiński and Gwóźdz 1999).

5 Conclusion

The information provided by short-term experiments using appropriate experimental settings indicates that several signalling processes are activated, in which H_2O_2 , jasmonate and ethylene play a central role. The microscale hydroponic system

might provide new data, as it is possible to study the changes that occur at the cellular level. Future work should be directed to describe in detail the components that permit the perception of the stress induced by heavy metals, and how different phytohormones and signaling components interact. Functional tools available (i.e. mutants) from the studies of biotic interactions could be used to the characterization of metal responsive components, due to the common responses found.

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