academicJournals

Vol. 8(19), pp. 2297-2306, 23 May, 2013
DOI: 10.5897/AJAR2012.6686
ISSN 1991-637X ©2013 Academic Journals
http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Modulation of antioxidant responses of *Medicago sativa* under cadmium and copper stress

Sabrine HATTAB[1]*, Sara HATTAB[2], Mohamed BANNI[2], Luis Eduardo HERNÁNDEZ[3] and Hamadi BOUSSETTA[2]

[1]Regional Research Centre in Horticulture and Organic Agriculture, Chott-Mariem, 4042 Sousse, Tunisia.
[2]Laboratory of Biochemistry and Environmental Toxicology, ISA Chott-Mariem, Sousse, Tunisia.
[3]Laboratory of Plant Physiology, Department of Biology, Universidad Autónoma de Madrid, 28049 Madrid, Spain.

Accepted 7 May, 2013

Alfalfa (*Medicago sativa*) plants exposed for 8 weeks in pots to increasing concentrations of cadmium (Cd; 0.35 and 7 mg·kg^{-1}dry soil) and copper (Cu; 3.5 and 70 mg.kg^{-1}dry soil) were tested for metal accumulation, growth inhibition and oxidative stress response in roots and leaves. The investigated parameters were biometric measurements (root and shoot length), malondialdehyde accumulation (MDA; index of lipid peroxidation), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) activities, and reduced glutathione (GSH) content. Analysis of Cd uptake showed that roots accumulated almost 40 fold more Cd than above-ground parts of the plant. Cu was more mobile, as it exhibited only 2 to 6-fold higher concentration in roots than in leaves. Both metals rendered a pronounced MDA accumulation in roots, especially at the highest doses and in parallel a decrease in the activities of the antioxidant enzymes CAT, GR and SOD, as well as a reduction in GSH content. The different stress signature observed in Cd- and Cu-treated plants could help to understand the mechanisms of toxicity triggered by each metal.

Key words: Alfalfa, heavy metals, growth, oxidative stress.

INTRODUCTION

Biochemical and physiological processes in plants are known to be affected by exposure to environmental pollutants such as heavy metals. Contaminants, such as cadmium (Cd), copper (Cu), lead and zinc, enter the environment through industrial waste, mill tailings, and landfill runoff. Cd is a toxic trace pollutant for humans, animals, and plants which enters to the environment mainly from industrial processes and phosphate fertilizers and then is transferred to the food chain (Wagner, 1993). Cd is one of the major industrial pollutants that show phytotoxicity even at low doses (Chakravarty and Srivastava, 1992; Das et al., 1997). However, Cu is an essential metal for plants at low concentrations, but can become toxic at higher concentrations (Hattab et al., 2009a).

Among many other physiological alterations, heavy metals trigger oxidative stress in plants leading to the oxidation of proteins and membrane lipids (Schützendübel and Polle, 2002), even after a short-term exposure (Ortega-Villasante et al., 2005). Copper is a redox active metal, known to intervene through Fenton reactions in the accumulation of oxygen free radicals, leading to the alteration of cell membranes by peroxidative degradation of polyunsaturated fatty acids

*Corresponding author. E-mail: sabrine_hattab1@yahoo.fr. Fax: 00 216 73 327 591.

(Weckx and Clijsters, 1996; Fry et al., 2002, Schützendübel and Polle, 2002).

Oxidative stress is a disturbance of the cellular redox status that is often observed in stress situations. Reactive oxygen species (ROS) are produced constantly under normal aerobic metabolism, and plant cells posses a well- equipped anti- oxidative defense system to maintain a redox homeostasis (Bhattacharjee, 2005; Foyer and Noctor 2005). Indeed, enzymes such as superoxide dismutases (SOD) are able to scavenge superoxide ($O_2^{\cdot -}$) radicals, whereas catalases (CAT) and peroxidases (POXs) have a role in quenching hydrogen peroxide (H_2O_2). Among the latter, ascorbate peroxidases (APX) are ubiquitous to several cellular parameters, are known to be a major component of the antioxidant network in several plant species (Davletova et al., 2005). Many of the indicated antioxidant enzymes use the soluble antioxidants ascorbate and glutathione (GSH) (Noctor and Foyer, 1998). In particular, 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 in a constant level (Noctor, 2006).

Plants challenged with harmful environmental conditions lose the ability to effectively scavenge ROS, leading to an oxidative burst. Exposure to heavy metals lead to remarkable alterations in the activities of the antioxidant enzymes and also in the cellular pools of GSH and ascorbate (Schützendübel and Polle, 2002; Gratão et al., 2005). Loss of cellular redox homeostatic have been demonstrated in alfalfa seedlings treated with high doses of Cd and Hg, even after very short-term treatments, preceding cell death (Ortega-Villasante et al., 2007). However, under milder stress conditions, metabolism can effectively cope with heavy metal driven stress, and some detoxification physiological responses might be triggered (Sobrino-Plata et al., 2009). In addition to the antioxidant protective role of GSH under heavy metal stress, GHS is required *via* the synthesis of phytochelatins (PCs) (Sharma and Dietz, 2009; Beladi et al., 2011). These family of biothiols ($(\gamma$-Glu-Cys$)_n$-Gly) are synthesised by phytochelatin synthase, enzyme that catalyses the condensation of the γ-Glu-Cys moiety of GSH with the Glu residue of a second GSH or PC, releasing Gly (Vatamaniuk et al., 2004; Clemens, 2006).

Recently, an increasing interest has risen on the use of legume plants for bioremediation of both, metals and organic pollutants. In this study, alfalfa plants were selected based on previous research showing their phytoremediation potential and their ability to germinate and grow in heavy metal (Carrasco et al., 2005; Pastor et al., 2003; Sriprang et al., 2002) and organic (Barac et al., 2004; Martí et al., 2009) contaminated soils.

The aim of this study was to examine the phytotoxic effects of increasing realistic Cd and Cu concentrations on oxidative stress parameters of alfalfa plants grown in an artificial substrate for 8 weeks. The accumulation of metals in roots and leaves, lipid peroxidation, and the effects on the antioxidant defence system (CAT, SOD, GR activities and GSH content), was investigated to reveal stress specific signature triggered by each metal.

MATERIALS AND METHODS

Plant material and growing conditions

Alfalfa (*Medicago sativa*) seeds were obtained from the Tunisian Seed Control Agency. Plants were grown in the greenhouse (mean T°: 20.5 ± 1.5°C, with 12 h light- period) in plastic pots (1 plant/pot) filled with 1kg dry mixture of peat, coarse granite sand and quartz sand (4:3:3 w/w) for 60 days (Peralta-Videa et al., 2004). 30 control plants were maintained under optimum growing conditions not-supplemented with heavy metals. A second set of plants was supplemented with 0.35 and 7 mg·kg^{-1} $CdCl_2$ (30 plants each treatment), which constituted treatments CD1 and CD2 respectively. A third set was treated with 35 and 700 mg·kg^{-1} $CuCl_2$ was of plants (30 plants each level), which constituted treatments CU1 and CU2 respectively. Heavy metals were dissolved in distilled water were added as a single application to the pots at sowing. The roots and shoots of control, Cd-exposed and Cu-exposed plants were collected after 60 days of exposure, the length measured prior desiccation or freezing until further analysis.

Cadmium and copper accumulation

The concentrations of Cd and Cu in roots and leaves samples were determined in the Marine Biology Laboratory of the National Institute of Sea Sciences and Technologies, Tunisia. The alfalfa tissues were carefully washed with deionized water and oven-dried at 105°C for 60 min and 60°C for 24 h, then ground into fine powder, and sieved through 1 mm nylon sieve. 0.5 gram samples were then digested by HNO_3 : $HClO_4$ (3 : 1 v/v) in the microwave system. The concentrations of Cd and Cu were determined by a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer 3300, Perkin-Elmer, Wellesley, MA). Standard materials consisting of known concentrations of $CdCl_2$ and $CuCl_2$ were included for assurance control. Means of Cd and Cu concentrations were calculated from 3 replicates. The limit of detection (LOD) of Cd and Cu was 0.05 µg·g^{-1} dry weight.

Analysis of antioxidant enzymatic activities

0.2 g of frozen leaf and root were homogenized in 2 cm^3 of an ice-cold 0.1M Tris–HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrolidone. The homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 15,000 × g and 4°C. The enzymatic activities were measured spectrophotometrically in the supernatant at 25°C. Analysis of SOD activity was based on the inhibition of cytochrome C at 550 nm (McCord and Fridovich, 1969). Analysis of GR and CAT activities were determined by measuring the disappearance of NADPH and H_2O_2 over time at 340 and 240 nm respectively, as described by Bergmeyer et al. (1974).

Glutathione content

The concentration of GSH was determined by fluorimetry (Hissin and Hilf, 1976; Zhou et al., 2009). One-gram of fresh tissues were ground in 1cm^3 of 25% H_3PO_3 and 3 cm^3 of 0.1 M sodium phosphate-EDTA buffer (pH 8). The homogenate was centrifuged at 10,000 × g for 20 min. The reaction mixture (4 cm^3) contained

Table 1. Root and shoot size and mass of alfalfa plants exposed to Cd (CD1: 0.35 mg.kg^{-1} dry soil; CD2: 7 mg.kg^{-1}dry soil) and Cu (CU1: 3.5 mg.kg^{-1} dry soil; CU2: 70 mg.kg^{-1}dry soil) at the growth stage of 60 days. Root length was measured from the main apex to the crown whereas shoot length was measured from the crown to the main apex.

Metal	Root length (cm)	Shoot length (cm)	Root mass (g/plant)	Shoot mass (g/plant)
Control	41.42 ± 4.33	46.57 ± 4.19	19.85 ± 1.41	22.93 ± 2.41
CD1	33.64* ± 4.11	39.88 ± 5.01	15.55* ± 1.83	17.38* ± 1.75
CD2	22.47* ± 3.76	34.85* ± 4.69	11.16* ± 2.14	14.37* ± 1.86
CU1	37.47 ± 4.66	42.77 ± 4.19	17.67 ± 2.04	21.29 ± 2.74
CU2	32.76* ± 3.21	35.76* ± 4.12	14.22* ± 2.76	15.35* ± 2.88

*Denotes significant differences in comparison with control (n = 20; P < 0.01).

3.88 cm^3 of 0.1 M sodium phosphate-EDTA buffer (pH 8.0), 20 µl of the supernatant and 100 µl of O-phthalaldehyde (1 mg cm^{-3}). After thorough mixing and incubation for 15 min, the solution was transferred to a quartz cuvette and the fluorescence at 420 nm was measured after excitation at 350 nm.

Lipid peroxidation

Lipid peroxidation in roots and leaves was determined in terms of malondialdehyde (MDA) concentration, one of the major thiobarbituric acid reactive substances (TBARS; Heath and Packer, 1968). Fresh samples (0.3 g) were ground in 5 cm^3 of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × g for 10 min and 0.5 cm^3 of the supernatant fraction was mixed with 2 cm^3 of 0.5% TBA in 20% TCA. The mixture was heated at 95°C for 30 min, chilled on ice, and centrifuged at 10,000 × g for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of TBARS was calculated by using the extinction coefficient of 155 Mm^{-1} cm^{-1}.

Statistical analyses

All investigated parameters were expressed as mean values ± standard deviations. Differences among treatments were assessed by one-way analysis of variance followed by protected least significant difference Fisher's exact test. Values were considered statistically significant when $p < 0.01$.

RESULTS

Biometric parameters

The size and mass of alfalfa plants was affected by the exposure to Cd and Cu, decreasing in a metal-concentration manner after a 60 days treatment (Table 1). The inhibitory effects were more pronounced with the highest dose of Cd, where length was reduced by 46% in roots and 25% in shoots (Table 1). The fresh weight of roots and shoots followed a similar trend in Cd-treated plants (Table 1). On the other hand, plants exposed to the lowest Cu concentration were not affected, and no significant change in root and shoot length or fresh weight was recorded. However, alfalfa plants grown with the highest dose of Cu, a significant decrease in roots and shoots length and fresh weight was detected, but to a lower extent as found with Cd doses.

Accumulation of Cd and Cu

Cadmium tissue concentration increased linearly over 60 days exposure reaching values ranging between 6.47 µg·g^{-1} DW to 19.95 µg·g^{-1} DW, respectively for plants exposed to CD1 and CD2 concentrations (Figure 1A). The same trend of Cd accumulation was observed in shoots (Figure 1B), but at a much lower extend. Therefore, alfalfa showed a typical excluder behaviour, as Cd accumulated approximately in shoots two orders of magnitude less than in roots. On the other hand, Cu accumulated at much higher concentrations in roots (Figure 1C) and shoots (Figure 1D), approximately 10-fold the level found for Cd. The concentration in roots was only 2 to 6 fold higher than in leaves respectively for CU1 and CU2 doses, suggesting that Cu was readily more mobile than Cd, distributing homogenously throughout plant organs.

Responses of antioxidant enzymes to Cd and Cu

Our results showed that after 8-weeks exposure to the highest Cd dose (CD2; 7 mg·kg^{-1}), CAT and SOD activities decreased significantly in roots, whereas this negative effect was less pronounced in shoots (Figures 2 and 3). However, GR activity augmented in the root of plants treated with 7 mg Cd·kg$^{-1,}$ while the increase observed in shoots was much milder in both CD1 and CD2 treatments (Figure 4). With regards to Cu exposure, CAT activity augmented in the lowest treatment (CU1; 35 mg·kg^{-1}), whereas, it was inhibited in the highest Cu concentration (CU2; 700 mg·kg^{-1}). A similar pattern was detected in shoots, where CAT activity decreased but to

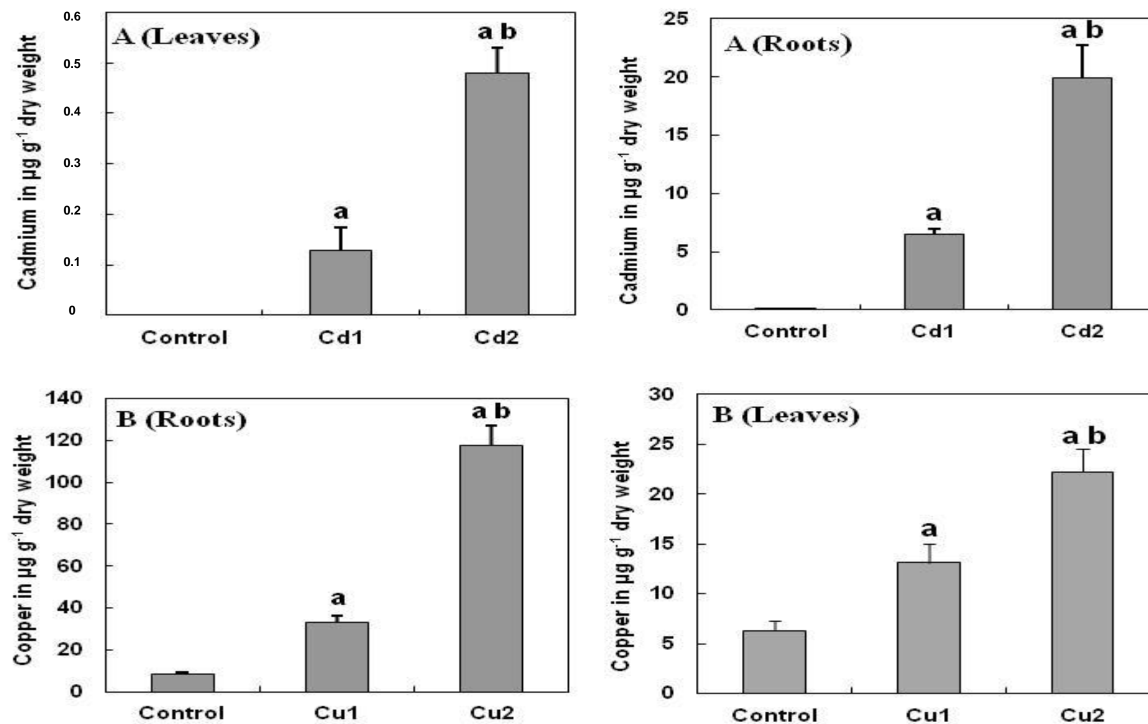

Figure 1. Cadmium (A) and Copper (B) uptake (mean± SD) in alfalfa roots and leaves after exposure of the plant to increased $CdCl_2$ (CD1: 0.35 mg·Kg^{-1} and CD2: 7 mg·Kg^{-1}) and $CuCl_2$ concentrations (CU1: 3.5 mg·Kg^{-1} and CU2: 70 mg·Kg^{-1}) for 60 days. (a): Statistically significant differences at $p < 0.01$ relaive to control plants. (b): Statistically significant differences at $p < 0.01$ in comparison with CD1 or CU1.

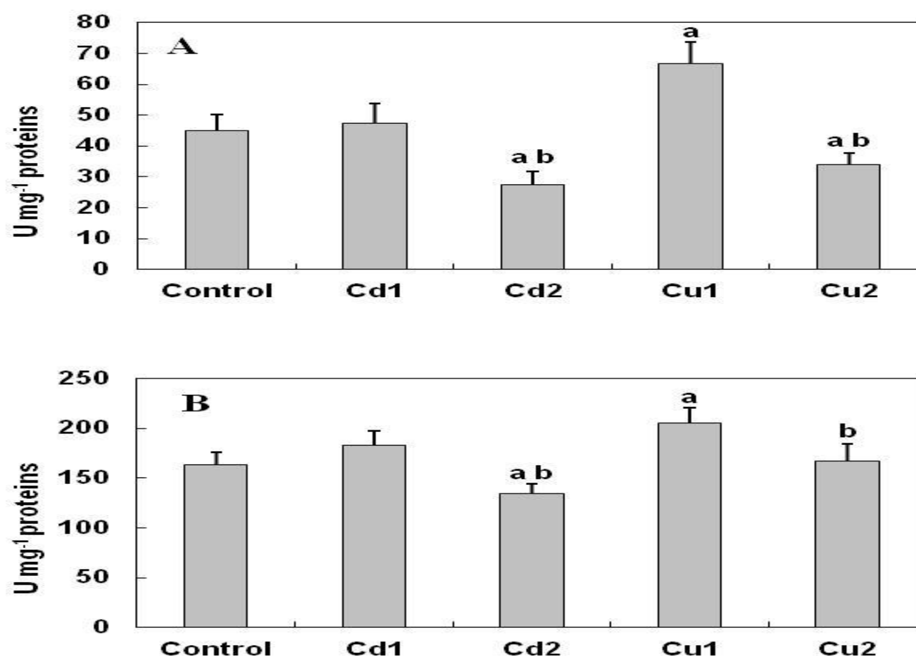

Figure 2. The effect of metals exposure on CAT activity (U/mg protein) in roots (A) and leaves (B) of alfalfa plants exposed to $CdCl_2$ (CD1 and CD2) and $CuCl_2$ concentrations (CU1 and CU2) or grown under control conditions for 60 days. (a) Statistically significant differences at $p < 0.01$ in comparison with control. (b) Statistically significant differences at $p < 0.01$ in comparison with CD1 or CU1.

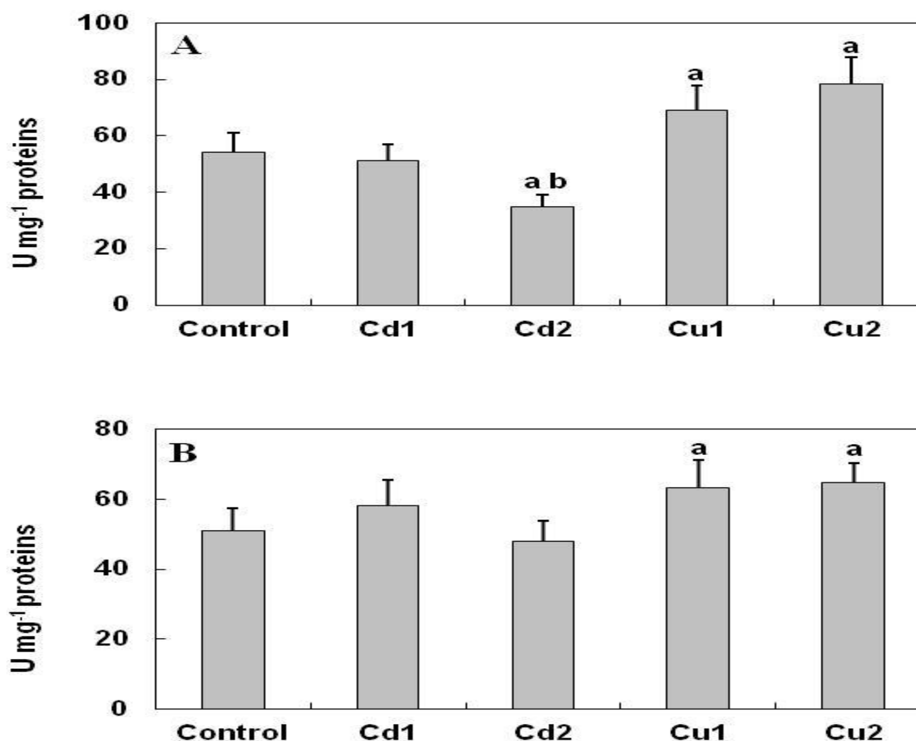

Figure 3. The effect of metals exposure on SOD activity (U/mg protein) in roots (A) and leaves (B) of alfalfa plants exposed to $CdCl_2$ (CD1 and CD2) and $CuCl_2$ concentrations (CU1 and CU2) or grown under control conditions for 60 days. (a) Statistically significant differences at $p < 0.01$ in comparison with control. (b) Statistically significant differences at $p < 0.01$ in comparison with CD1 or CU1.

a lesser extent that the level found in roots (Figure 2). A different behaviour was followed by SOD activity, which increased in roots and shoots in parallel to Cu accumulation in the plants (Figure 3). Finally, GR activity in roots and shoot of alfalfa was not affected by Cu.

Glutathione concentration and lipid peroxidation

There was a notable alteration in GSH contents in roots and leaves of plants treated with CD1, CD2 and CU2 (Figure 5). The minimum concentration was reach in CD2 exposed plants with values down to 61.43 ± 10.89 nmol g^{-1} FW and 843.83 ± 59.23 nmol g^{-1} FW in roots and leaves, respectively. This represented a remarkable depletion of 17.6% in roots, whereas in shoots this diminution was less pronounced (67.5% compared to the control values). The concentration of GSH in plants treated with CD1 decreased more moderately, representing a 40% and 88% of the controls in roots and shoots respectively. In respect to Cu, GSH only decreased in plants treated with the highest dose in CU2, whereas reached 68.6% in roots and 80% in shoots of control plants (Figure 5A and B).

Root lipid peroxidation, measured as MDA concentration, augmented significantly in most treatments, but in plants exposed to the lowest dose of Cu (Figure 6A). At the highest doses of Cd and Cu, MDA content increased to 300.2 % and 249.4 %, respectively. Although the degree of lipid peroxidation in leaves followed a similar pattern as observed in roots (Figure 6B), increasing significantly by 163.7% and 152.3% in plants treated with CD2 and CU2, respectively.

DISCUSSION

Alfalfa plants accumulated Cd and Cu in a close to linear manner both in roots (R^2 = 0.92 and R^2 = 0.97) and shoots (R^2 = 0.93 and R^2 = 0.84), respectively. Alfalfa behaved also as a typical Cd and Cu excluder, since Cd accumulated 40-fold more in roots than in shoots, and 6-fold in the case of Cu, in agreement with previous studies of alfalfa plants grown hydroponically in perlite with Cd for 7 days (Sobrino-Plata et al., 2009). Other plants with relevant agronomic importance, such as tobacco or pea (Valverde et al., 2001; Gichner et al. 2004; Hattab et al., 2009a), showed a similar Cd and Cu excluder pattern. Cadmium and Cu caused clear diminution in root and shoot growth and biomass in alfalfa plants, although at a much higher concentration of Cu. One of the characteristic responses of plants to metals is the

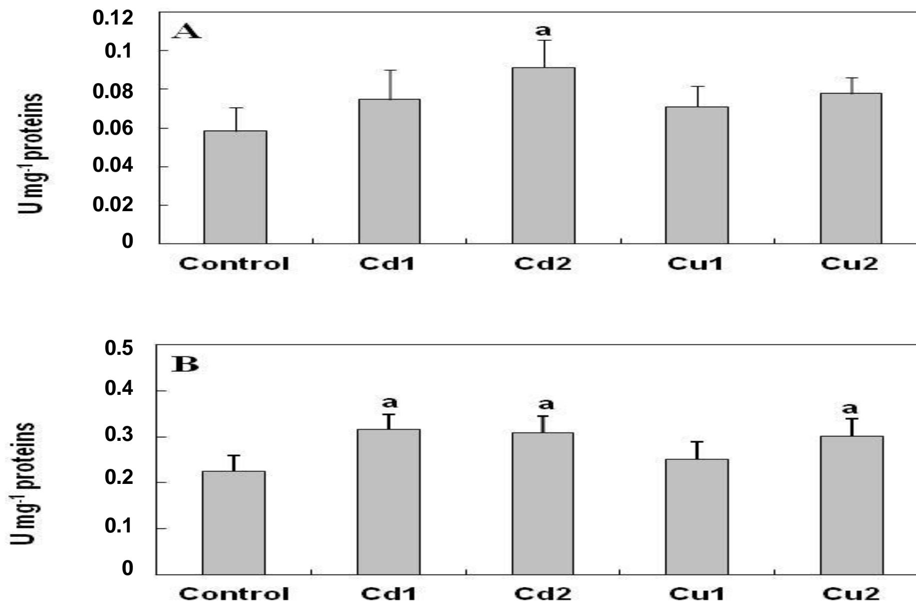

Figure 4. The effect of metal exposure on GR activity (U/mg proteins) in roots (A) and leaves (B) of alfalfa plants exposed to $CdCl_2$ (CD1 and CD2) and $CuCl_2$ concentrations (CU1 and CU2) or grown under control conditions for 60 days. (a) Statistically significant differences at $p < 0.01$ in comparison with control. (b) Statistically significant differences at $p < 0.01$ in comparison with CD1 or CU1.

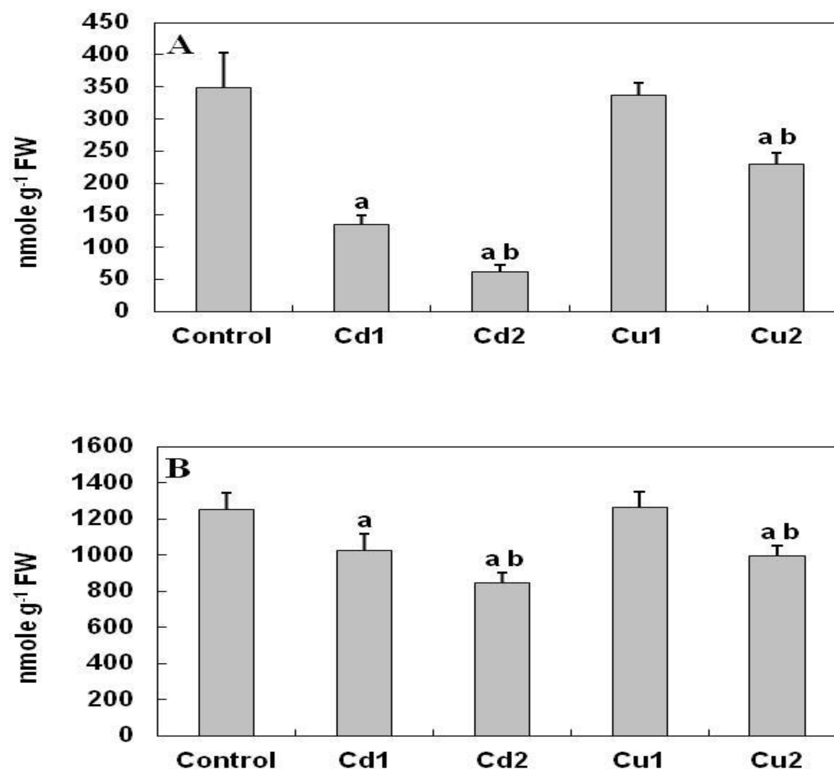

Figure 5. The effect of metal exposure on the content of glutathione (nmol.g^{-1}FW) in roots (A) and leaves (B) of alfalfa plants exposed to $CdCl_2$ (CD1 and CD2) and $CuCl_2$ concentrations (CU1 and CU2) or grown under control conditions for 60 days. (a) Statistically significant differences at $p < 0.01$ in comparison with control. (b) Statistically significant differences at $p < 0.01$ in comparison with CD1 or CU1.

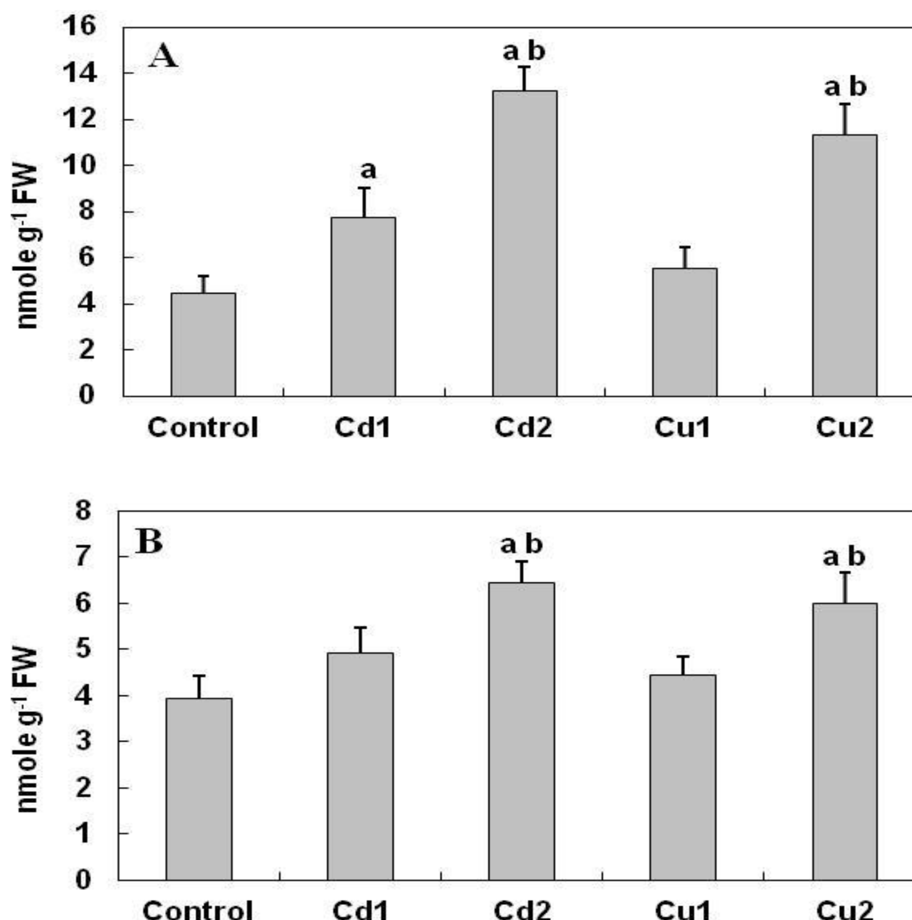

Figure 6. Lipid peroxidation measurement was based on the amount of TBA reactive metabolites (nmol.g^{-1}FW) and was analyzed in roots (A) and leaves (B) of alfalfa plants exposed to $CdCl_2$ (CD1 and CD2) and $CuCl_2$ concentrations (CU1 and CU2) or grown under control conditions for 60 day. (a) Statistically significant differences at $p < 0.01$ in comparison with control. (b) Statistically significant differences at $p < 0.01$ in comparison with CD1 or CU1.

inhibition of growth, and has been used frequently as an index of phytotoxicity (Schützendübel et al., 2001). Indeed, it is well reported that relatively low doses of Cd might lead to remarkable reductions in plant size and biomass (Sandalio et al., 2001; Ortega-Villasante et al., 2005; Hattab et al., 2009b; Zhang et al., 2010). In the other hand, Cu is an essential nutrient, and only at very high concentrations it may become toxic (Smeets et al., 2009), dose which might be different for a given 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 such as Cd do not present a deficiency phase (Hagemeyer, 2004).

The different biological role of Cd and Cu in plant cells also suggest that both metals would trigger different mechanisms of toxicity, provoking a differential oxidative stress signature. Although it is not clearly understood how plants accumulate ROS in response to Cd and Cu, both metals caused a clear oxidative stress detected by a remarkable increase in lipid peroxidation, slightly stronger in Cd-treated plants (Figure 6), which were in agreement with previous information available for both metals (De Vos et al., 1993; McCarthy et al., 2001, Quartacci et al., 2001; Bhattacharjee, 2005; Cho and Seo, 2005; Sobrino-Plata et al., 2009., Zhou et al., 2009). In particular, similar higher sensitivity to Cd was observed in *Lemna minor* plants, when exposed to similar doses of Cu (Hou et al., 2007).

The detected redox unbalance occurred along with remarkable changes in the activity of several antioxidant enzymes. This phytotoxic effect could be caused directly by redox active metals, such as Cu, by participating in the conversion of relatively stable oxidants like $O_2^{\bullet-}$ and H_2O_2 into highly reactive radicals (i.e. $^{\bullet}OH$) through Fenton reactions (Kohen and Nyska, 2002). However, it is still a

debate the mechanism triggered by Cd, a non-reactive redox metal, that provokes the accumulation of ROS in exposed plants (Cuypers et al., 2010). We have clearly shown that Cd and Cu evoked a differential stress signature, implying that several cellular components were affected in case.

The exposure of alfalfa seedlings to the highest Cd concentrations caused a clear diminution in the activities of CAT, SOD in roots and to lesser extend in leaves, while suffered higher level of lipid peroxidation. Similar effects were reported in *Pisum sativum* and *Arabidopsis*, where CAT and SOD activity decreased particularly in roots (Rodríguez-Serrano et al., 2006; Smeets et al., 2009). Copper caused a milder phytotoxic effect on SOD, with a slight increase in SOD activity at both levels of Cu in shoots and roots, following a similar pattern as found in *Arabidopsis* (Drążkiewicz et al., 2004; Smeets et al., 2009; Cuypers et al., 2011). CAT activity was more sensitive, as it decreased significantly in the highest dose of Cu, showing again a similar behavior as observed in *Arabidopsis* (Smeets et al., 2009; Cuypers et al., 2011). Some authors suggest that the inhibition of CAT and SOD, important scavengers of ROS under abiotic stress, could be the cause of redox cellular imbalance detected (Chen *et al*., 2003; Razinger et al., 2008). In comparison, it is known that Cu participates directly in Fenton reactions that produce OH, a highly reactive radical capable of oxidizing rapidly lipids and proteins (Drążkiewicz et al., 2007; Sandalio et al., 2001). The toxic effects of Cu only appeared in the highest dose given to the plants (that is, CU2), as also was found in *Arabopsis thaliana* treated with 5 µM Cu, where root CAT activity was inhibited by half (Cuypers et al., 2011), response that was also observed in 7 days treated *Arabidopsis* (Drążkiewicz et al., 2004). This was also observed in the changes in antioxidant enzymatic activity could well only reflect that alterations in the cellular pools of ROS are readily occurring, as the sources of ROS might be well spread in different compartments of the cell, an a more profound characterization at the cellular level must be undertaken (Cuypers et al., 2010).

Alfalfa plants exposed to Cd and Cu suffered a significant and remarkable diminution in GSH concentration, in particular in the roots of plants treated with Cd or with the highest dose of Cu. Glutathione is the major non-protein thiol present in plants, and plays a relevant dual role in metal tolerance: is important for redox cellular homeostasis and is the precursor of PCs (Noctor et al., 2011). Phytochelatins are synthesized from GSH by the enzyme phytochelatin synthase, which generates a complex family of peptides rich in cysteine (Cobbett and Goldsborugh, 2002). The accumulation of PCs is important for tolerance to a number of metals and metalloids, such as Cd (Schat et al., 2002), As (Hartley-Whitaker et al., 2001), Hg (Carrasco-Gil et al., 2011) and Cu (Lee and Kang, 2005). Therefore, the depletion of GSH observed in roots could be explained partially by the synthesis of PCs, as was observed in alfalfa plants treated with similar doses of Cd (Sobrino-Plata et al., 2009). Such synthesis of PCs would eventually lead to a subsequent diminution in GSH concentration, as was observed in roots of Cd-treated plants (Seth et al., 2008, Sun et al., 2005).

The oxidative status of GSH can also be altered by the exposure of plants to metals, resulting in a diminution in the concentration of reduced GSH, while the proportion of GSSG augmented (Xiang and Oliver, 1998). Such changes were only observed under acute stress conditions in alfalfa seedlings grown hydroponically in a microscale assay under Cd and Hg stress (Ortega-Villasante et al., 2007).

Similarly, Szollosi et al. (2009) found an early increase of the GSH content in Indian mustard (*Brassica juncea* L.) seedlings, which was followed by a marked depletion at prolonged treatments. Copper caused also a severe depletion of GSH in *Arabidopsis*, supporting the idea that oxidative stress caused by a redox active and non-active metals (that is, Cu and Cd, respectively) may alter the GSH/GSSG redox pair (Cuypers et al., 2011). This notion is supported by the increase in GR activity found in alfalfa plants exposed to Cd and Cu, in agreement with the higher GR activity observed also in alfalfa plants but grown in a semi-hydroponic system treated with Cd (Sobrino-Plata et al., 2009). GR is responsible for the regeneration of GSH in a NADPH-dependent reaction, maintaining the reduced components of GSH-ascorbate cycle (Asada, 1999).

The activities of APX and GR, together with an adequate cellular level of GSH, are crucial in the operation of this H_2O_2-scanveging cycle, which operates in various subcellular compartments including chloroplasts, mitochondria and peroxisomes (Noctor and Foyer, 1998; Foyer and Noctor, 2005). In addition, novel investigations are revealing that GSH may play a relevant role in the plant responses to abiotic and biotic stress, modulating antioxidant enzymatic activity and the transcription of stress-related genes; metabolic changes important to prevent oxidative stress damages and to improve stress tolerance (Noctor et al., 2011).

Conclusion

In conclusion, our results suggest that alfalfa plants display a negative response of root and shoot growth and biomass production to Cd and high concentrations of Cu. The toxicity of these metals might be exerted through the depletion of key antioxidant enzymes activities such as CAT and SOD. The specific responses of alfalfa challenged with Cd and Cu, each showing a characteristic stress signature, might be considered as potential biomarkers to be applied in ecotoxicological research, particularly in soils devoted to organic agriculture in polluted environments in Tunisia. Finally,

our data also provides clues about the importance of GSH in the responses of alfalfa plants to Cd and Cu, which should prompt further research to understand the cellular mechanisms involved.

ACKNOWLEDGMENTS

The work was partially supported by funds from the Tunisian Ministry of Higher Education, Scientific Research and Technology, and by the Research Unit “Biochemistry and Ecotoxicology“. Financial support for this work was also provided by the cooperation grant from “Agencia Española para la Cooperación Internacional” (AECID), Spanish Ministry of Foreign Affairs and Cooperation (Grants A/023088/09 and A/030019/10).

REFERENCES

Asada K (1999). The water–eater cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Ann. Rev. Plant Physiol. Plant. Mol. Biol. 50:601-639.

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, Van der Lelie D (2004). Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nat. Biotech. 22:583-588.

Beladi M, Kashani A, Habibi D, Paknejad F, Golshan M (2011). Uptake and effect of lead and copper on three plant species in contaminated soils: Role of phytochelatin. Afr. J. Agric. Res. 6(15):3483-3492.

Bergmeyer HU, Gawenn K, Grassl M (1974). Enzymes as biochemical reagents. In: Methods in Enzymatic Analysis. Academic Press, New York.

Bhattacharjee S (2005). Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plants. Curr. Sci. 89:1113-21.

Carrasco JA, Armario P, Pajuelo E, Burgos A, Caviedes MA, Lopez R, Chamber MA, Palomares AJ (2005). Isolation and characterisation of symbiotically effective *Rhizobium* resistant to arsenic and heavy metals after the toxic spill at the Aznalcollar pyrite mine. Soil Biol. Biochem. 37:1131-1140.

Carrasco-Gil S, Álvarez-Fernández A, Sobrino-Plata J, Millán R, Carpena-Ruiz RO, Leduc DL, Andrews JC, Abadía J, Hernández LE (2011). Complexation of Hg with phytochelatins is important for plant Hg tolerance. Plant Cell. Environ. 34:778-791.

Chakravarty B, Srivastava S (1992). Toxicity of some heavy metals in vivo and in vitro in Helianthus annuus. Mutat. Res. 283:287-294.

Cho UH, Seo NH (2005). Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant. Sci. 168:113-120.

Clemens S (2006). Evolution and function of phytochelatin synthases. - J. Plant. Physiol. 163:319-332.

Cobbett C, Goldsbrough P (2002). Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. - Ann. Rev. Plant. Biol. 53:159-182.

Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Ravindran-Nair A, Munters E, Artois TJ, Nawrot T, Vangronsveld J, Smeets K (2010). Cadmium stress: an oxidative challenge. Biometals 23:927-940.

Cuypers A, Smeets K, Ruytinx J, Opdenakker K, Keunen E, Remans T, Horemans N, Vanhoudt N, Van Sanden S, Van Belleghem F, Guisez Y, Colpaert J, Vangronsveld J (2011). The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. J. Plant. Physiol. 168:309-316.

Das P Samantaray S, Rout GR (1997) Studies on cadmium toxicity in plants: a review. Environ Pollut 98:29–36.

Davletova S, Rizhsky L, Liang H, Shengqiang Z, Oliver D J, Coutu J, Shulaev V, Schlauch K, Mittler R (2005). Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. Plant Cell 17:268-281.

De Vos CHR, Bookum WMT, Vooijs R, Schat H, De Kok LJ (1993). Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*. Plant Physiol. Biochem. 31:151-158.

Drążkiewicz M, Skórzyńska-Polit E, Krupa Z (2004). Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. Biometals 17:379-387.

Drążkiewicz M, Skórzyńska-Polit E, Krupa Z (2007). The redox state and activity of superoxide dismutase classes in *Arabidopsis thaliana* under cadmium or copper stress. Chemosphere 67:188-193.

Foyer CH, Noctor G (2005). Oxidant and antioxidant signalling in plants: are-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28:1056-1071.

Fry SC, Miller JG, Dumville JC (2002). A proposed role for copper ions in cell wall loosening. Plant Soil 247:57-67.

Gichner T, Patková Z, Száková J, Demnerová K (2004). Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves. Mutat. Res. 559:49-57.

Gratão 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.

Hagemeyer J (2004). Ecophysiology of plant growth under heavy metal stress. In: Prasad NMV(eds) Heavy metal stress in plants: from biomolecules to ecosystems. Springer, Berlin Heidelberg. pp. 201–222.

Hartley-Whitaker J, Ainsworth G, Vooijs R, Ten Bookum W, Schat H, Meharg AA (2001). Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. Plant Physiol. 126:299-306.

Hattab S, Chouba L, Ben Khedher M, Mehouachi T, Boussetta H (2009a). Cadmium and Copper induced DNA damage in *Pisum sativum* roots and leaves as determined by the Comet assay. Plant Biosys. 143:S6-S11.

Hattab S, Dridi B, Ben-Khedher M, Boussetta H (2009b). Photosynthesis and growth responses of pea *Pisum sativum* L. grown under heavy metals stress. J. Environ. Sci. 21:1552-1556.

Heath RL, Packer K (1968). Leaf senescense: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32:93-101.

Hissin PJ, Hilf RA (1976). Fluorimetric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74:214-226.

Hou W, Chen X, Song G, Wang Q, Chang CC (2007). Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). Plant Physiol. Biochem. 45:62-69.

Kohen R, Nyska A (2002). Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. 30:620-650.

Lee S, Kang BS (2005). Phytochelatin is not a primary factor in determining copper tolerance. J. Plant. Biol. 48:32-38.

Martí MC, Camejo D Fernández-García N, Rellán-Alvarez R, Marques S, Sevilla F, Jiménez A (2009). Effect of oil refinery sludges on the growth and antioxidant system of alfalfa plants. J Hazard Mater 171:879-85.

McCarthy I, Romero-Puertas MC, Palma JM, Sandalio LM, Corpas FJ, Gomez M, Del Rio LA (2001). Cadmium induces senescence symptoms in leaf peroxisomes of pea plants. Plant Cell Environ. 24:1065-1073.

McCord J, Fridovich I (1969). Superoxide dismutase: an enzymatic function for erythrocuperein (hemocuprein). J. Biol. Chem. 244:6049-6055.

Noctor G, Foyer CH (1998). Ascorbate and glutathione: keeping active oxygen under control. Ann. Rev. Plant. Physiol. Plant. Mol. Biol. 49:249-279.

Noctor G (2006). Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ. 29:409-425.

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011). Glutathione. The Arabidopsis Book. 9:1-32.

Quartacci MF, Cosi E, Navari-Izzp F (2001). Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. J. Exp. Bot. 354:77-84.

Ortega-Villasante C, Rellán-Álvarez R, del Campo FF, Carpena-Ruiz RO, Hernández LE (2005). Cellular damage induced by cadmium and mercury in *Medicago sativa*. J. Exp. Bot. 56:2239-2251.

Ortega-Villasante C, Hernandez LE, Rellan-Alvarez R, del Campo FF, Carpena-Ruiz RO (2007). Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings. New Phytol 176:96-107.

Pastor J, Hernandez AJ, Prieto N, Fernandez-Pascual M (2003). Accumulating behaviour of *Lupinus albus* L. growing in a normal and a decalcified calcic luvisol polluted with Zn. J. Plant Physiol. 160: 1457-1465.

Peralta-Videa JR, De la Rosa G, Gonzalez JH, Gardea-Torresdey JL (2004). Effects of the growth stage on the heavy metal tolerance of alfalfa plants. Adv. Environ. Res. 8:679-685.

Razinger J, Dermastia M, Koce JD, Zrimec A (2008). Oxidative stress in duckweed (*Lemna minor* L.) caused by short-term cadmium exposure. Environ. Pollut. 153:687-694.

Rodríguez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gomez M, Del Rıo LA, Sandalio LM (2006). Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. - Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. Plant. Cell Environ. 29:1532-1544.

Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas M, del Rıo LA (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J. Exp. Bot. 52:2115-26.

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM (2002). The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non hyperaccumulator metallophytes. J. Exp. Bot. 53:2381-2392.

Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Goldbold D L, Polle A (2001). Cadmium-induced changes in antioxidant systems, hydrogen peroxide content and differentiation in Scot pine roots. Plant. Physiol. 127:887-898.

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

Seth C S, Chaturvedi P K, Misra V (2008). The role of phytochelatins and antioxidants in tolerance to Cd accumulation in *Brassica juncea* L. Ecotoxicol. Environ. Safety 71:76-85.

Sharma SS, Dietz KJ (2009). The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci. 14:43-50.

Smeets K, Opdenakker K, Remans T, Van Sanden S, Van Belleghem F, Semane B, Horemans N, Guisez Y, Van gronsveld J, Cuypers A (2009). Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multi-pollution context. J. Plant Physiol. 166:1982-1992.

Sobrino-Plata J, Ortega-Villasante C, Flores-Cáceres ML, Escobar C, Del Campo FF, Hernández LE (2009). Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa. Chemosphere 77:946-954.

Sriprang R, Hayashi M, Yamashita M, Ono H, Saeki K, Murooka YA (2002). Novel bioremediation system for heavy metals using the symbiosis between leguminous plant and genetically engineered rhizobia. J. Biotech. 99:279-293.

between cadmium and zinc on phytochelatin and glutathione production in wheat (*Triticuma estivum* L.). – Environ. Toxicol. 20:195-201.

Szollosi R, Varga IS, Erdei L, Mihalik E (2009). Cadmium-induced oxidative stress and antioxidative mechanisms in germinating Indian mustard (*Brassica juncea L.*) seeds. – Ecotoxicol. Environ. Safety. 72: 1337-1342.

Valverde M, Trejo C, Rojas E (2001). Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? – Mutagenesis. 16: 265-270.

Vatamaniuk OK, Mari S, Lang A, Chalasani S, Demkiv L O, Rea P (2004). Phytochelatin synthase, a dipeptidyl transferase that undergoes multisite acylation with gammaglutamylcysteine during catalysis. Stoichiometric and site-directed mutagenic analysis of AtPCS1-catalyzed phytochelatin synthesis. - J. Biol. Chem. 279:22449-22460.

Wagner GJ (1993). Accumulation of cadmium in crop plants and its consequences to human health. - Adv. Agron. 51:173-212.

Weckx JEJ, Clijsters HMM (1996). Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. – Physiol. Plant. 96: 506-512.

Xiang C, Oliver D J (1998). Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. – Plant. Cell. 10:1539-1550.

Zhang X, Li C, Nan Z (2010). Effects of cadmium stress on growth and anti-oxidative systems in *Achnatherum inebrians* symbiotic with *Neotyphodium gansuense*. - J. Haz. Mat. 175:703-709.

Zhou ZS, Guo K, Elbaz AA, Yang ZM (2009). Salicylic acid alleviates mercury toxicity by preventing oxidative stress in roots of Medicago sativa. – Environ. Exp. Bot. 65:27-34.