View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Repositori Institucional de la Universitat Jaume I

Spanish Journal of Agricultural Research
14(4), e0810, 12 pages (2016)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2016144-10117
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

NaCl protects against Cd and Cu-induced toxicity in the halophyte *Atriplex halimus*

Insaf Bankaji[1]*, Noomene Sleimi[1]*, Aurelio Gómez-Cadenas[2] and Rosa M. Pérez-Clemente[2]

[1] *UR: Matériaux, Nanomatériaux et Ecosystèmes, Faculté des Sciences de Bizerte, 7021 Jarzouna, Bizerte. Tunisie.* [2] *Universitat Jaume I, Departamento de Ciencias Agrarias y del Medio Natural. Castellón, Spain.*

** Both authors contributed equally to this work*

Abstract

The objective of the present work was to evaluate the extent of Cd- and Cu-induced oxidative stress and the antioxidant response triggered in the halophyte species *Atriplex halimus* after metallic trace elements exposure. Plants were treated for one month with Cd^{2+} or Cu^{2+} (400 µM) in the absence or presence of 200 mM NaCl in the irrigation solution. The interaction between salinity and heavy metal stress was analyzed in relation to plant growth, tissue ion contents (Na^+, K^+ and Mg^{2+}), oxidative damage and antioxidative metabolism. Data indicate that shoot and root weight significantly decreased as a consequence of Cd^{2+}- or Cu^{2+}-induced stress. Metallic stress leads to unbalanced nutrient uptake by reducing the translocation of K^+ and Mg^{2+} from the root to the shoot. The levels of malondialdehyde increased in root tissue when Cd, and especially Cu, were added to the irrigation solution, indicating that oxidative damage occurred. Results showed that NaCl gave a partial protection against Cd and Cu induced toxicity, although these contaminants had distinct influence on plant physiology. It can be concluded that salinity drastically modified heavy metal absorption and improved plant growth. Salinity also decreased oxidative damage, but differently in plants exposed to Cd or Cu stress.

Additional key words: *Atriplex halimus*; oxidative damage; trace element toxicity; salinity

Abbreviations used: APX (ascorbate peroxidase); CAT (catalase); DTNB (dinitrobenzoic acid); FW (fresh weight); GPX (guaiacol peroxidase); GSH (reduced glutathione); GSSG (oxidised glutathione); MDA (malondialdehyde); PC (phytochelatin); ROS (reactive oxygen species); SOD (superoxide dismutase); tGSH (total GSH)

Authors' contributions: Cation determination assays: IB. Coordinated the obtention and culture of the plants and was responsible for conducting different experiments and the evaluation of plants: NS. Responsible of the study of the antioxidative system, evaluating the antioxidant enzymatic activities as well as MDA; he also was in charge of glutathione and phytochelatin content determination: AGC. Coordination of the works and preparing the manuscript: RMPC.

Citation: Bankaji, I.; Sleimi, N.; Gómez-Cadenas, A.; Pérez-Clemente, R. M. (2016). NaCl protects against Cd and Cu-induced toxicity in the halophyte *Atriplex halimus*. Spanish Journal of Agricultural Research, Volume 14, Issue 4, e0810. http://dx.doi.org/10.5424/sjar/2016144-10117.

Received: 17 June 2016. **Accepted:** 17 Nov 2016.

Copyright © 2016 INIA. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial (by-nc) Spain 3.0 Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Funding: Ministerio de Economía y Competitividad, Spain (GL2013-42038-R; AGL2016-76574-R); Universitat Jaume I (P1-1B2012-06).

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Rosa M. Perez-Clemente: rosa.perez@uji.es

Introduction

Toxic trace elements are bioavailable to plants through their uptake from soil and water. Among pollutants, heavy metals such as cadmium (Cd), copper (Cu), lead and mercury are of particular concern as they are non-biodegradable and strongly phytotoxic. Furthermore, at high concentration, they not only limit plant productivity but also threaten human health (López-Climent *et al.*, 2011).

Based on their ability to cope with heavy metals in the medium where they grow, plants can be divided into three groups: i) indicator plants, sensitive to heavy metals; ii) excluder plants, being able to tolerate high heavy metal concentrations in the soil by preventing its accumulation the cytosol, and iii) hyperaccumulator plants, that tolerate high concentrations of specific trace elements in the medium, with an active uptake and specific accumulation of those elements (reviewed in Leitenmaier & Küpper, 2013).

Many arid and semi-arid regions of the world, that combine high soil salinity with aridity, have the additional problem that lands are even more degraded, consequence of the human activities such as mining,

agricultural activities, etc. In these particular soils it is important to consider all these stressful conditions (drought, salinity, heavy metal contamination) together, since isolating one adverse condition from the others is not possible. Under these particular circumstances, halophytes have been proposed as candidates for the removal of trace elements from the soil (Manousaki & Kalogerakis, 2009).

There are two main reasons to study Cd^{2+} and/or Cu^{2+} hyperaccumulator plants. First, there is an interest in acquiring basic knowledge on the mechanisms underlying environmental adaptation and cell detoxification of those trace metals. Second, the study of Cd^{2+}/Cu^{2+} hyperaccumulators can provide new tools to design genetically-modified plants useful for phytoremediation or phytoextraction (Clemens *et al*., 2013).

Cu is an essential micronutrient for a range of plant physiological processes via the action of Cu^{2+} -dependent enzymes. On the contrary, Cd is a non-essential heavy metal ion that causes disturbances in plant growth, photosynthesis, ion and water transport, as well as decreases in plant enzyme activities through reactions between the Cd^{2+} and thiol groups. When reaching a certain concentration in plant tissue, both metallic trace elements are involved in the generation of reactive oxygen species (ROS), which react with lipids, proteins, photosynthetic pigments, and nucleic acids, causing lipid peroxidation, membrane damage, metabolite degradation, inactivation of enzymes, and even cell death (Nagarani *et al*., 2012). The increase of lipid peroxidation products such as malondialdehyde (MDA) as a consequence of the oxidative stress has been considered as an indicator of free radical production and tissue damage. Actually, lipid peroxidation alters membrane properties such as fluidity and permeability and thereby its functions (da Silva *et al*., 2010).

Plants have developed different mechanisms to control the uptake of both essential and nonessential heavy metals. These mechanisms include the chelation and sequestration of ions by specific ligands to maintain them at low levels in the cytosol (Sbartai *et al.,* 2012). Chelation of trace elements is a ubiquitous detoxification strategy described in a wide range of plants (Yadav, 2010). The main group of substances acting as metal chelators in plants are phytochelatins (PCs), which are metal-binding peptides that also play similar detoxification roles in other eukaryotes, including fungi and microalgae (Hirata *et al.,* 2005). PCs, with a general structure of $(\gamma\text{-Glu-Cys})_n$–Gly (n=2–11), are rapidly synthesized by the cytoplasmic enzyme PC synthase in response to intoxication with heavy metals. Genetic studies have confirmed that glutathione (GSH) is pivotal for PC synthesis as GSH-deficient mutants of *Schizosaccharomyces pombe* and *Arabidopsis* sp are PC-deficient and hypersensitive to Cd (Lee *et al*., 2003). PCs production in plants is stimulated by the presence of heavy metals. PCs are metal-binding peptides and work by mobilizing heavy metal compounds in the cytosol and then sequestering PC-metal complexes in the vacuoles of plant cells. The absorbed heavy metal compounds are biochemically bonded by PCs in inert forms where they are eventually compartmentalized (Watanabe, 1997), so PCs could play a crucial role in phytodegradation and phytotransformation.

GSH, a non-enzymatic antioxidant, is a low-molecular-weight thiol involved in various metabolic processes that acts as an important plant defense molecule against environmental stresses, including trace elements (Hossain *et al*., 2011). It is one of the major antioxidant and redox buffers in plants, abundantly found in all cell compartments. GSH takes part in the control of H_2O_2 levels through the ascorbate-GSH cycle and also directly as a free-radical scavenger (Foyer & Noctor, 2005). Additionally, enzymatic activities greatly contribute to cope with the environmentally-induced oxidative stress and their activities have been used to evaluate stress responses in plants (Arbona *et al*., 2008). Antioxidant enzymes interrupt the cascades of uncontrolled oxidation by removing, neutralizing or scavenging oxy-radicals and their intermediates (Foyer & Noctor, 2005). Superoxide dismutase (SOD) acts as the first line defense against ROS, dismuting O_2^- to H_2O_2 which is still toxic and should be eliminated in subsequent reactions, through the action of catalase (CAT), other peroxidases and by the ascorbate-glutathione cycle. The equilibrium of SOD, ascorbate peroxidase (APX) and CAT activities is essential in order to determine the steady-state level of O_2^- and H_2O_2. For instance, when CAT activity is reduced in plants, other ROS-scavenging enzymes such as APX and guaiacol peroxidase (GPX) are up-regulated (Vandenabeele *et al*., 2004).

Atriplex halimus L. is a perennial, halophytic and nitrophilous shrub that generally forms part of the halophytic communities on saline soils and it represents one of the best options for the reclamation of degraded agricultural land in arid and semi-arid zones (reviewed in Walker *et al*., 2014). This species has been planted in the Mediterranean arid zone for livestock feed. In fact, the planted area is estimated at around 100,000 ha in Algeria, the Arabian Peninsula, Egypt, Iraq, Israel, Jordan, Libya, Morocco, Spain and Tunisia (Walker *et al.,* 2014). It has been previously reported that the addition of NaCl to the irrigation solution did not affect plant growth or chlorophyll concentration in *A. halimus.* On the contrary, there was a significant stimulation of its growth, which confirms the halophytic character of this species. Furthermore, a high concentration of NaCl

reverted some of the negative effects that trace element intoxication exerted on growth parameters of these plants (Bankaji *et al*., 2014).

Taking all this in consideration, the present study was designed to investigate (i) the extent of Cd^{2+}- and Cu^{2+}-induced oxidative stress in *A. halimus* plants, (ii) differences in the plant antioxidant responses to each metal, and (iii) the effect of Cd^{2+} and Cu^{2+} under salt stress conditions. These objectives were accomplished by determining lipid peroxidation, and also examining changes in antioxidant enzyme activities such as CAT and APX together with glutathione and PCs contents in different tissues of *A. halimus* plants exposed to toxic levels of Cu^{2+} and Cd^{2+} alone or in combination with high NaCl concentration in the irrigation solution.

Material and methods

Plant material and culture

Plants of *Atriplex halimus* (Chenopodiaceae) used in the experiments were obtained by cutting propagation: 5-cm long fragments of leafy stems were taken from mother plants, rinsed abundantly with distilled water, and placed for rooting in plastic pots (3 dm^3) containing a mixture of perlite/gravel (2:1) as a substrate. In these conditions, rooting occurred one month after planting (Bankaji *et al*., 2014). Young rooted cuttings were irrigated three times a week with 200 mL modified Hewitt nutritive solution (Hewitt, 1966) as described in Bankaji *et al*. (2014).

Experimental design

Acclimated rooted cuttings, homogeneous in size and looking were randomly distributed in control and five stress treatments. Briefly, 20 control plants and 20 plants per stress treatment were used. All plants were regularly irrigated (three times a week) with the same nutritive solution described above but for stressed plants it was supplemented with a) 400 µM $CdSO_4$; b) 400 µM $CuSO_4$); c) 200 mM NaCl; d) 400 µM $CdSO_4$ and 200 mM NaCl or e) 400 µM $CuSO_4$ and 200 mM NaCl. During the experimental period, plants were maintained in a greenhouse under natural photoperiod, average temperatures (night/day) of 12/25°C and relative humidity between 60-90%. According to preliminary experiments, concentration of 400 µM for $CdSO_4$ and $CuSO_4$ proved to be higher enough to induce negative effects on plant performance. It was expected that the negative effects could be reverted by the addition of NaCl to the irrigation solution. Treatments were maintained for one month.

At harvest, four weeks after the onset of treatments, plants of each treatment were randomly divided into groups of five plants for further analysis. In one group, leaves were separated from roots, washed with distilled water, frozen in liquid nitrogen and kept at -80 °C until GSH, PC and MDA analyses. Five different plants were processed similarly and used for the enzymatic activities assays. The remaining plants, which were used for the cation assays, were divided into leaves and roots, rinsed three times in cold water and blotted with filter paper. Treated roots were dipped in a cold solution of $CaCl_2$ during 5 min (Stolt *et al.,* 2003) to eliminate trace elements adsorbed at the root surface and then rinsed three times with cold distilled water. The fresh weight was immediately determined in roots and shoots before separating the leaves. The same experimental design was repeated twice with identical results.

Cation assays

Fresh plant material was digested by mixture of HNO_3:$HClO_4$ acids (4:1) (Barthwal *et al*., 2008). Mineralization was conducted gradually from 100 to 350°C for 2 hours. After that, the samples were taken into 50 mL of nitric acid 0.5%. Finally, these extracts were filtered and concentrations of Cu^{2+}, Cd^{2+}, K^+, Na^+ and Mg^{2+} in plant tissues were determined by atomic absorption spectrometry (Perkin Elmer PinAAcle 900T, USA). The blanks, used to set the zero atomic absorption spectrometer, were similarly processed as described above.

Antioxidant enzyme activity

For CAT, APX and GPX assays, extraction was performed as follows: 0.4 g of fresh leaf tissue was powdered using liquid nitrogen and extracted, at 4°C, with 2,0 ml of phosphate buffer containing 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 5 mM Na-ascorbate and 0.2 mM EDTA. After filtration through four layers of musseline cloth, the homogenate was centrifuged at 6000 rpm for 15 min and the supernatant recovered for enzyme activities determination using a UV/Visible spectrophotometer (Thermo Spectronic Genesis UV10, USA).

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC1.11.1.6) activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm (extinction coefficient of 0.036/mM·cm) of a reaction mixture containing 25 mM K- phosphate

buffer (pH 7.0), 10 mM H_2O_2 and 2 mL of enzyme extract (Aebi, 1984).

For GPX (Guaiacol: H_2O_2 oxidoreductase, EC1.11.1.7), the reaction mixture consisted of 25 mM K-phosphate buffer (pH 7.0), 10 mM H_2O_2, 9 mM guaiacol and 2 mL enzyme extract. The enzyme activity was measured by monitoring the increase in absorbance at 470 nm (extinction coefficient of 26.6/mM·cm) during polymerization of guaiacol (Fielding & Hall, 1978).

APX (L-Ascorbate: H_2O_2 oxidoreductase, EC1.11.1.11) was determined according to Nakano & Asada (1981) measuring ascorbate consumption at 290 nm (absorbance coefficient of 2.8/mM·cm). The reaction mixture contained 25 mM K-phosphate buffer (pH 7.0), 0.5 mM ascorbate, 2 mM H_2O_2, 0.1 mM EDTA and 2 mL of enzyme extract.

Determination of total PC and GSH concentrations

Total non-protein thiol (TNP-SH) compound were extracted and assayed according to de Vos *et al*. (1992). Briefly, 100 mg of fresh weight (FW) plant tissue were rapidly frozen in liquid nitrogen and exhaustively extracted with 2 mL of 5% 5-sulphosalicylic acid with 6.3 mm diethylenetriaminepentaacetic acid (DETAPAC) using a pre-chilled mortar, pestle and quartz sand. The homogenate was filtered and centrifuged at 8000 rpm for 5 mn at 4°C. The N-P thiols were measured in the supernatant by diluting it with reagent containing 0.5 M K-phosphate buffer (pH 7.5) and 6 mM dinitrobenzoic acid (DTNB). Absorbance was recorded at 412 nm.

The DTNB– oxidised glutathione (GSSG) reductase recycling procedure of Anderson (1985) was used for the determination of both total [GSH (reduced glutathione) + GSSG] and oxidized glutathione. Briefly, 100 mg of fresh tissue was homogenized in 2 mL of 5% (w/v) cold metaphosphoric acid. Homogenate was centrifuged at 4°C to pellet debris and supernatant neutralized with 0.5 M phosphate buffer (pH 7.5). Total GSH (tGSH) was measured in a reaction mixture containing 125 mM Na-phosphate buffer (pH 7.5) with 6.3 mM Na-EDTA, 6 mM DTNB, 0.3 mM NADPH and the sample. Reaction was initiated by adding pure glutathione reductase (Sigma-Aldrich, Madrid, Spain) to the reaction mixture and monitored by measuring absorbance changes at 412 nm. For measuring GSSG, plant extracts were incubated at 25°C with 2-vinylpyridine and triethanolamine (Fluka, Madrid, Spain) for 1 h prior the determinations. The assay for GSSG was conducted as described above for tGSH. GSSG and tGSH contents were estimated by interpolating slope values in a calibration curve performed with pure standards. GSH content was calculated by subtracting GSSG content from the tGSH content. PC production was evaluated as PC-SH levels by subtracting the amount of GSH from the amount of TPN-SH compounds (de Vos *et al*., 1992).

MDA concentration

MDA concentration was measured following the procedure described in Hodges *et al*. (1999). Plant material (0.4 g root or leaf tissue) was homogenized in 5 mL of 80% cold ethanol (Panreac, Barcelona, Spain) using a tissue homogenizer (Ultra-Turrax; IKA-Werke, Staufen, Germany). Homogenates were centrifuged at 4°C to pellet debris and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid (TCA, Panreac) or a mixture of 20% TCA and 0.5% thiobarbituric acid (TBA, Sigma-Aldrich). Both mixtures were allowed to react in a water bath at 90°C for 1 h. After this time, samples were cooled down in an ice bath and centrifuged. Absorbance at 440, 534 and 600 nm was read in the supernatant against a blank. The MDA concentration in the extracts was calculated as in Arbona & Gomez-Cadenas (2008).

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) and the significance of the Tukey test was determined at 5% level. Statistical analyses were performed with the Statistica 8 for Windows software.

Results

Plant growth

Biomass production significantly decreased in shoots of *A. halimus* plants cultivated in a medium supplemented with Cd^{2+} (Fig. 1). Reductions in FW were detected both in shoots (37.5%) and in roots (24.1%) when 400 µM Cd^{2+} was added to the irrigation solution. Similarly, the addition of 400 µM Cu^{2+} to the irrigation solution reduced biomass production by 54.6% and 36.4% in shoots and roots, respectively. When the solution was supplemented with NaCl (200 mM) the negative effect of both trace elements was reverted, and FW in shoots and roots reached values even higher to those found in control plants (Fig. 1).

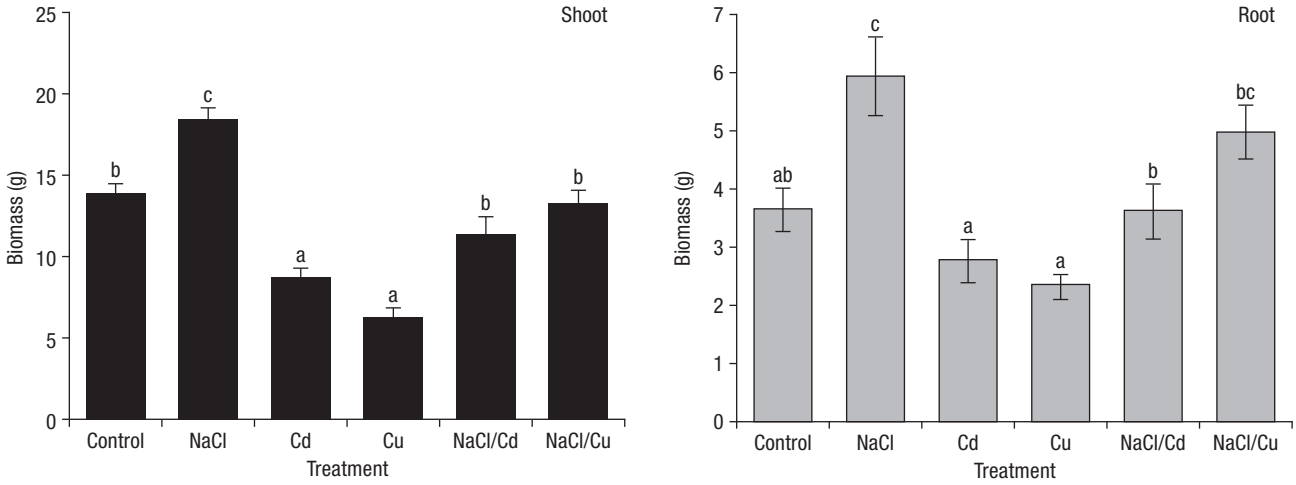

Figure 1. Biomass production in shoot and root tissue of *Atriplex halimus* exposed to 400 µM Cd^{2+} or Cu^{2+} in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values ± SE, n=10. Different letters denote significant differences at $p \leq 0.05$ on each treatment.

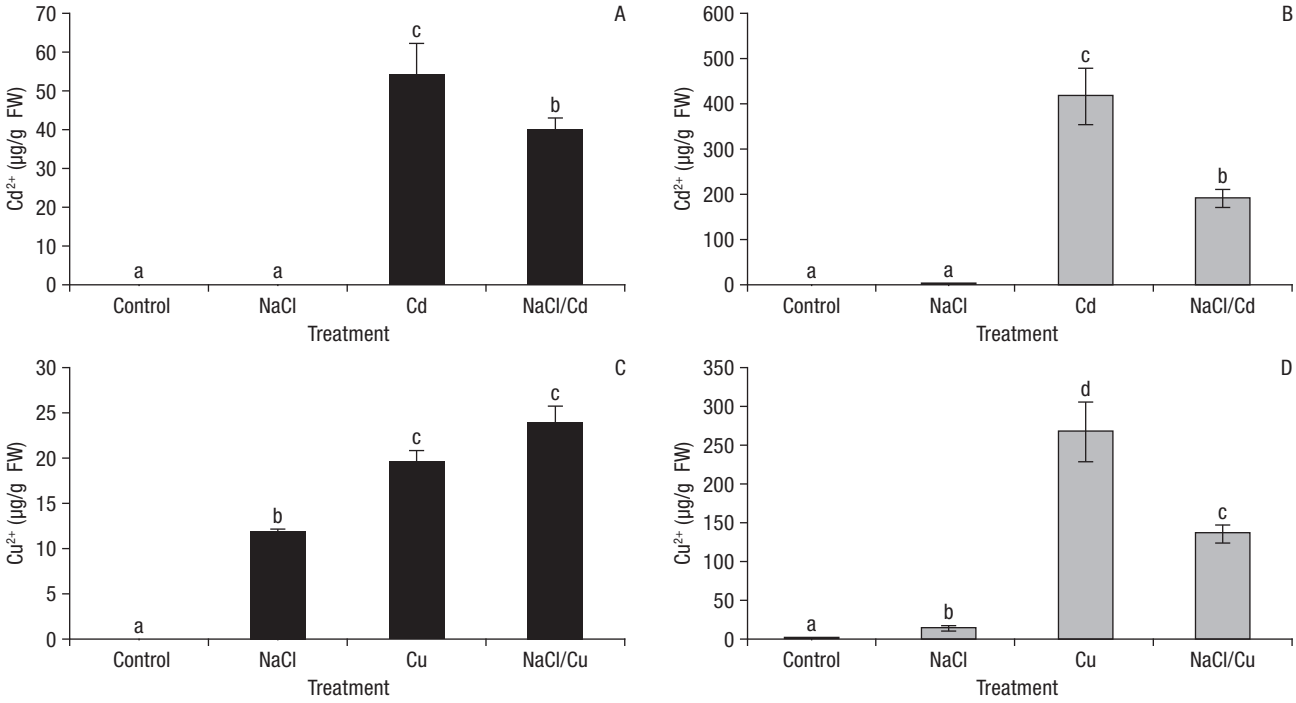

Figure 2. Cadmium (A,B) and copper (C,D) concentration in leaf (A,C) and root (B,D) tissue of *Atriplex halimus* exposed to 400 µM Cd^{2+} or Cu^{2+} in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values ± SE, n=10. Bars marked with the same letter are not significantly different at $p \leq 0.05$.

Shoot and root water contents did not show any significant alteration in response to NaCl or heavy metal treatment, showing values between 76.8 and 84.2% (data not shown).

Cu^{2+} and Cd^{2+} concentration

Cu^{2+} and Cd^{2+} concentration in leaves and roots of *A. halimus* plants were analyzed at the end of the experiment (after 4 weeks of treatment) and results are shown in Fig. 2. Cu^{2+} and Cd^{2+} concentrations in control plants were negligible or below the detection limit, both, in root and leaf tissues. When trace elements were added to the irrigation solution, Cd^{2+} and Cu^{2+} accumulation, was, as expected, higher in root than in leaf tissues. The concentration of Cd^{2+} was 417.1 and 54.0 µg/g FW in roots and leaves, respectively when plants were irrigated with a solution supplemented with 400 µM Cd^{2+}. On the other hand, Cu^{2+} content was

269.1 and 19.6 µg/g FW in roots and leaves, respectively when 400 µM Cu^{2+} was added to irrigation solution.

The addition of 200 mM NaCl to the irrigation solution significantly reduced the endogenous Cd^{2+} concentrations (Fig. 2A,B). In root tissue, the presence of NaCl induced a reduction of 53.4% whereas in leaf tissue, Cd^{2+} content was reduced by a 25.9%. When NaCl was simultaneously added with Cu^{2+}, a reduction of 48.7% in Cu^{2+} content was recorded in root tissue (Fig. 2D); on the contrary the endogenous Cu^{2+} concentration in leaf tissue was not significantly affected by the NaCl supply (Fig. 2C).

Nutrient concentration

Roots of plants treated with Cu^{2+} or Cd^{2} had Na^{+} contents similar to those measured in control ones. When Cu^{2+} was incorporated to the irrigation solution together with NaCl, the endogenous root Na^{+} concentration was significantly higher than that recorded in roots of plants irrigated with high concentrations of NaCl (without the trace element). In the case of Cd^{2+}, no differences in root Na^{+} concentration were recorded between plants irrigated with a solution supplemented with Cd alone or in combination with NaCl (Fig. 3A). The presence of Cd^{2+} and Cu^{2+} in the irrigation had different effect on leaf Na^{+} accumulation; while solution supplemented with Cd^{2+} did not affect accumulation of Na^{+} in the aerial part, the addition of Cu^{2+} caused a significant increase in Na+ concentration reaching values 1.8–fold higher than those measured in controls (Fig. 3A).

The presence of NaCl in the medium significantly decreased leaf K^{+} concentration from 9561.2 to 7679.0 µg/g FW. Similarly, a decrease in this parameter was observed in plants treated with Cd^{2+} either alone or in combination with NaCl (Fig. 3B). The addition of Cu^{2+}, in combination with NaCl, to the irrigation solution also reduced leaf K^{+} concentration, on the contrary, when Cu^{2+} was added alone, K^{+} concentration was similar to that measured in leaves of control plants (Fig. 3B).

The addition of NaCl or Cd^{2+} to the irrigation solution significantly decreased leaf Mg^{2+} concentration (33.4 and 20.4% reduction respectively). When both (NaCl and Cd^{2+}), were supplemented simultaneously, the reduction of this micronutrient content in leaves was more severe (54.6%). On the contrary, the addition of Cu^{2+} to the irrigation solution had no effect on this parameter although when this metal was provided simultaneously with NaCl, a significant reduction of leaf Mg^{2+} concentration (23.7%) was recorded. Root Mg^{2+} concentration significantly decreased in *A. halimus* under Cd^{2+} or Cu^{2+} stress, supplemented or not with NaCl (Fig. 3C).

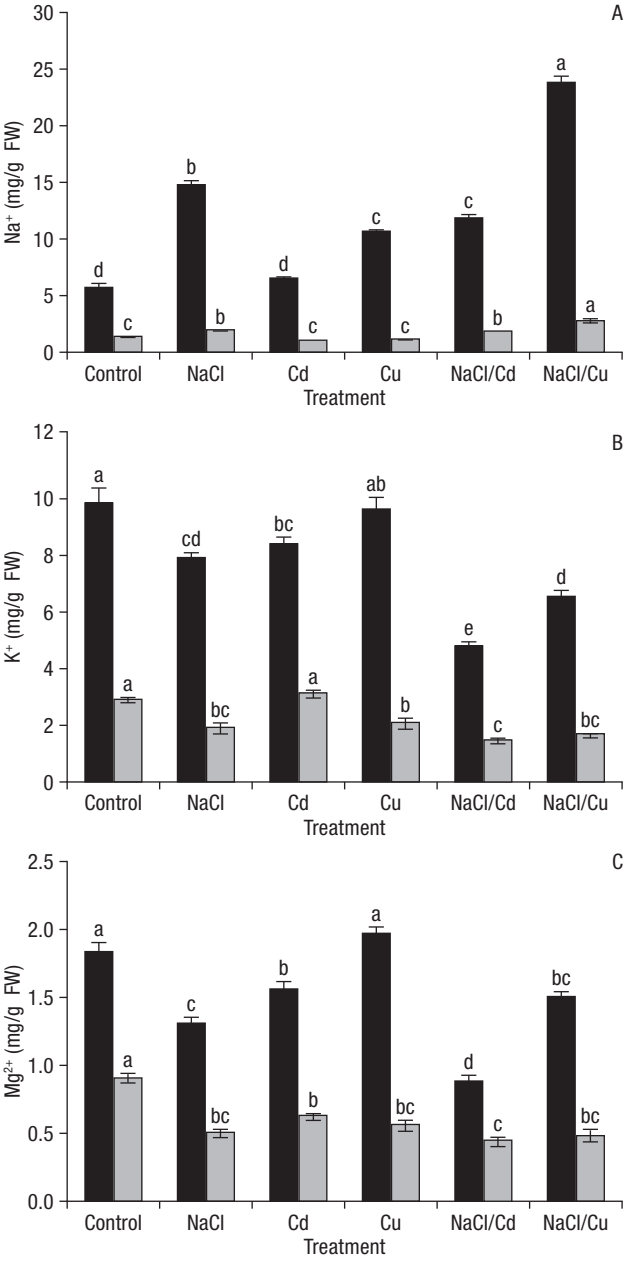

Figure 3. Sodium (A), potassium (B) and magnesium (C) concentration in leaves (bars in black) and roots (bars in grey) tissue of *Atriplex halimus* exposed to 400 µM Cd^{2+} or Cu^{2+} in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values ± SE, n=10. In each plant material, bars marked with the same letter are not significantly different at $p \leq 0.05$.

Antioxidant enzyme activity

The increase in Cd^{2+} and Cu^{2+} content in plant tissue had significant effects on the activities of antioxidant enzymes (Fig. 4). In leaves, the addition of either Cd^{2+}

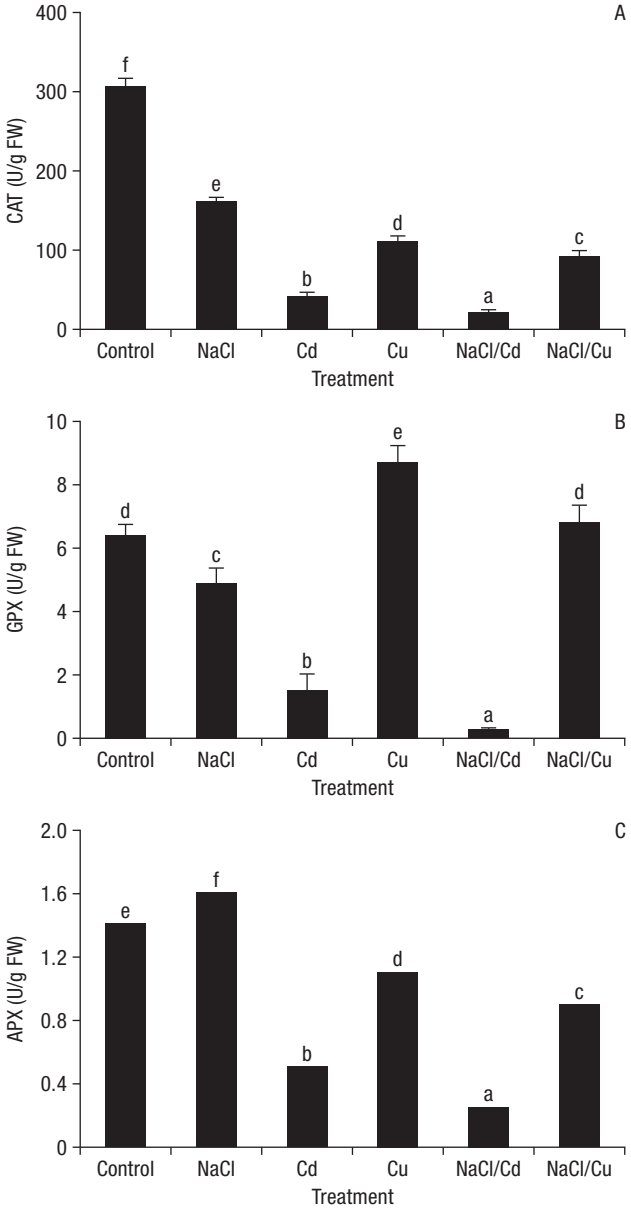

Figure 4. Enzymatic activity in leaves of *Atriplex halimus* exposed to 400 µM Cd^{2+} or Cu^{2+} in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values ± SE, n=10. Bars marked with the same letter are not significantly different at p=0.05. Catalase (A), guaiacol peroxidase (B) and ascorbate peroxidase (C).

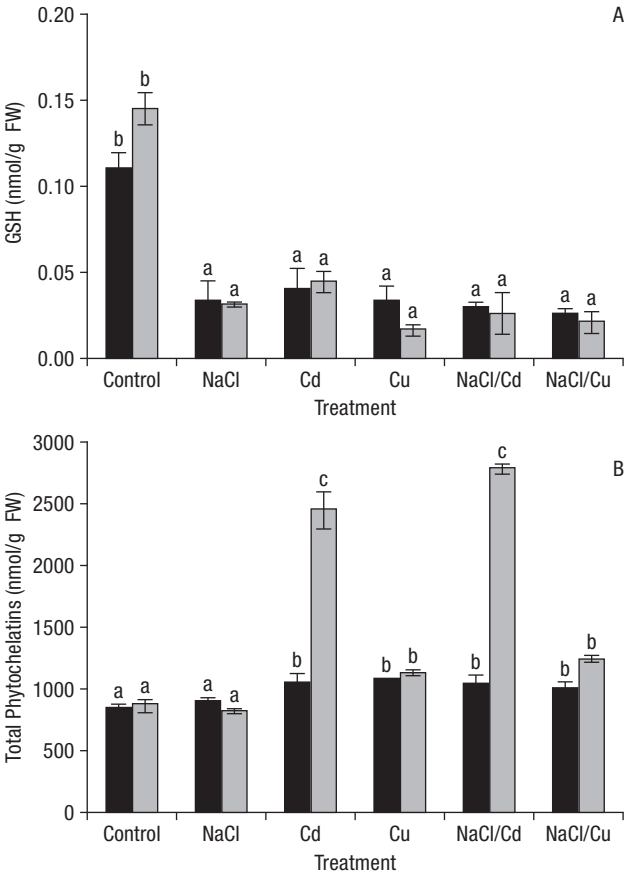

Figure 5. Reduced glutathione (A) and phytochelatins (B) content in leaves (bars in black) and roots (bars in grey) of *Atriplex halimus* under Cd or Cu stress. Data are mean values ± SE, n=10. In each plant material, bars marked with the same letter are not significantly different at $p \leq 0.05$.

or Cu^{2+} to the irrigation solution induced a significant decrease in CAT activity (86.3% and 64.2% after Cd^{2+} and Cu^{2+} treatments, respectively).

GPX activity decreased 75.8% under Cd^{2+} stress (Fig. 4B). In the case of APX activity it was recorded a reduction of 64.3% and 21.4% after Cd^{2+} and Cu^{2+} treatments respectively (Fig. 4C).

All studied enzymatic activities significantly decreased when Cd^{2+} and NaCl were added simultaneously to the irrigation solution, in comparison to those measured when Cd^{2+} or NaCl were added alone. Similar trend was observed for Cu^{2+} and NaCl treatment in the case of CAT and APX. Differently, GPX activity in NaCl plus Cu^{2+} or Cu^{2+} treated plants was significantly higher than in NaCl treated plants (Fig. 4B).

Glutathione and phytochelatin contents

As it is shown in Fig. 5, GSH levels experimented significant decrease in both roots and leaves of *A. halimus* when the irrigation solution was supplemented with Cd^{2+} (66.6% and 63.6% reduction, respectively). Similar results were observed in plants treated with Cu^{2+} (86.7% and 72.7% reduction in roots and leaves, respectively). When NaCl and Cd^{2+} or Cu^{2+} were simultaneously applied to the irrigation solution, there were no significant differences in GSH content both in leaf and root tissues in comparison with the values recorded when NaCl or the trace element were added to the solution in isolation.

The decrease of GSH levels was accompanied by increases in the synthesis of phytochelatins (Fig. 5B) mainly in roots exposed to high exogenous Cd^{2+} concentrations. In this condition PCs levels were 2.8- fold higher than to controls. Phytochelatin accumulation also increased in roots and leaves when the irrigation solution was supplemented with Cu^{2+} although to a lesser extent. The addition of NaCl to the irrigation solution containing either Cd^{2+} or Cu^{2+} did not alter the PCs levels determined when the solution was supplemented only with those metallic trace elements (Fig. 5B).

Lipid peroxidation

Lipid peroxidation was measured and expressed in terms of MDA. The addition of 200 mM NaCl to the irrigation solution did not have any effect on MDA content, neither in root or leaf tissues (Fig. 6). Plants irrigated with this high saline solution exhibited MDA levels similar to those recorded in control plants. The concentration of this metabolite increased in root tissue when plants were irrigated with a solution containing Cd^{2+} or Cu^{2+} reaching 1.6- and 5.9-fold increase, respectively, compared to controls. Although the addition of NaCl alone to the irrigation solution did not alter basal levels of MDA in root tissue, when Cu^{2+} was simultaneously added, significantly higher values of MDA were recorded. In leaf tissue, MDA concentration was similar among treatments (Fig. 6).

Discussion

Atriplex halimus L. is a perennial halophytic shrub which grows throughout the Mediterranean basin in areas of low annual rainfall and high potential evapotranspiration, many of which can be classified as arid or semi-arid.

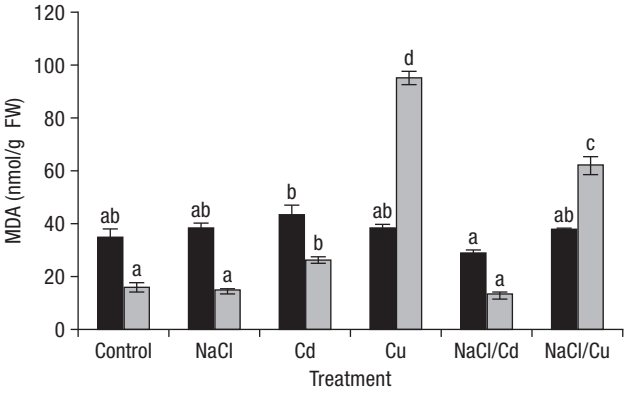

Figure 6. Malondialdehyde content in leaves (bars in black) and roots (bars in grey) of *Atriplex halimus* under Cd or Cu stress. Data are mean values ± SE, n=10. In each plant material, bars marked with the same letter are not significantly different at $p \leq 0.05$.

Although there are some works that, through the study of different physiological parameters, attempted to address the responses of different halophyte species exposed to abiotic stress caused by the presence of heavy metals in the soil, to our knowledge, there is no information on how, in these conditions, elevated salinity in the substrate may affect plant performance.

This could have practical implications in phytoremediation, since in many arid and semi-arid regions of the world relatively high soil salinity is present in heavy metal contaminated soils. Taking into consideration the results described in this work, it could be also considered the potential use of this species as an alternative method for soil desalination, where salt could be removed from the soil instead of being washed downwards by water.

It has been described that high concentrations of metallic trace elements in the soil may cause plant growth impairment. The inhibition of shoot growth could be due to a decrease in photosynthesis, a disturbance in the mineral nutrition and water balance, and/or to changes in the hormonal status. Results described in this work indicate that shoot and root- biomass of *A. halimus* plants decreased significantly as a consequence of Cd^{2+} or Cu^{2+}-induced stress. However, the negative effect of these metallic trace elements was reverted when NaCl was added to the irrigation solution. This finding is in concordance with those described in *Solanum nigrum,* which has been proposed as a Cd-accumulator species (Xu *et al.*, 2010). An important mechanism of plant adaptation to environmental adverse conditions is the structural alteration of the root system. Supplementation of the irrigation solution with NaCl would increase the area and thickness of the leaves and the number of lateral roots, causing increases in the efficiency of photosynthesis and the uptake of water and nutrition in the root system, thereby increasing the biomass of the plants under Cd^{2+} or Cu^{2+} stress conditions.

Indeed, our results are in agreement with those reported by Lefévre *et al*. (2009) and Han *et al.* (2012) in the halophyte species *A. halimus* and *Kosteletzkya virginica*. According to these authors, a beneficial impact of exogenous NaCl on plant response to Cd^{2+} may be, at least partly, linked to NaCl-induced growth stimulation classically reported for halophyte at moderate salinities, which therefore leads to a dilution effect.

According to Zu *et al*. (2005), hyperaccumulators are plants that can accumulate more than 10 to 500 times concentration of element trace in their aerial parts than a normal plant does. Taking this into consideration, the results described in this and previous works

(Bankaji *et al*., 2014) suggest that *A. halimus* is a Cu^{2+} and Cd^{2+} hyperaccumulator species.

A. halimus is used for soil stabilization against erosive forces since its deep root system reduces soil compaction and improves aeration (Clemente *et al*., 2012). In this species, the addition of NaCl to the irrigation solution significantly reduced the endogenous Cd^{2+} concentration in different tissues (Fig. 2). On the contrary, salinity induced an increase of leaf Cu^{2+} concentration and a significant reduction in Cu^{2+} content in root tissues. These results are in clear contrast to those reported by Lopez-Chuken & Young (2005) showing that salinity induces an increase in Cd^{2+} levels rather than a decrease in halophyte species. According to these studies, this increase is the consequence of an impact of salinity on the soil and not on the plant. Addition of chloride salts to soil induces an increase in Cd^{2+} free ion activity following cation exchange with the salt cation (Lefévre *et al*., 2009). This effect, however, could be ruled out in the present work since the experiments here described were performed using a chemically inert substrate, where the formation of such complexes do not occur, and then, trace element absorption is not facilitated. It could be interesting to confirm this idea by experiments using soil as substrate, which take into consideration the bioavailability of some other ions presents in the substrate.

Meanwhile, it might be possible that in the conditions described in this work, salinity has an effect reducing the capacity of roots for ion uptake or even that Na^+ competitively inhibits Cd^{2+} uptake (Bankaji *et al*., 2014).

Halophytes are characterized by their ability to replace K^+ by Na^+ for specific functions, as for maintaining vacuolar osmotic equilibrium (Gattward *et al*., 2012). As it has been reported for other halophytes (Pan *et al*., 2011), the addition of NaCl to the irrigation solution significantly decreased shoot K^+ content as the absorption of this ion was reduced. The effect of the addition of Cd^{2+} or Cu^{2+} on K^+ content differs since Cd^{2+} significantly reduced shoot K^+ concentration while Cu^{2+} did not affect this parameter, suggesting that Cd^{2+} impaired K^+ uptake in the roots or translocation to the aerial tissues.

The addition of Cu^{2+} or Cd^{2+} to the medium was accompanied by an alteration of ionic equilibrium within the shoot and root tissues, and again Cd y Cu differed in this respect. Cd^{2+} inhibited Mg^{2+} (an essential mineral nutrient for photosynthetic metabolism and for phloem loading) uptake and translocation. On the contrary, after Cu^{2+} treatment, shoots exhibited similar Mg^{2+} values than those recorded in controls. Results pointed out in this work demonstrate that heavy metals present in solution have an impact on Na^+ absorption, and once more, Cd and Cu had different effect. Cd^{2+} did not cause Na^+ accumulation in shoots, but an increase in shoot Na^+ concentration was observed after Cu^{2+} exposure. In terms of Na^+ shoot concentration, a synergistic effect of both, Cu^{2+} and NaCl was observed. The specific increase in Na^+ concentration observed in root and leaf tissues of plants simultaneously exposed to NaCl and Cu^{2+} suggests that Na^+ could play a specific role in response to abiotic stress. Similarly, in other C4 species an increase in Na^+ endogenous content has been found after plant exposure to different abiotic stress conditions, this could be because Na is needed for an efficient phosphoenolpyruvate regeneration (Lutts *et al*., 2004).

Cu^{2+} and Cd^{2+} induce the formation of harmful ROS, which causes irreversible damage to macromolecules such as lipid and proteins. Lipid peroxidation is widely used as marker of stress-induced damage. In this study, MDA content, a by-product of lipid peroxidation (da Silva *et al*., 2010) was used as an indirect measurement. Our results indicate that MDA levels increased in root tissue when Cd^{2+}, and especially Cu^{2+}, were added to the irrigation solution, indicating that oxidative damage occurred. When NaCl was added to the irrigation solution simultaneously with Cd or Cu, MDA levels were reduced, which suggests protective role of NaCl against the stress caused by heavy metals. According to Mourato *et al*. (2012), it can be indicated that the toxic effect of trace elements is probably exerted through free radical generation.

ROS have to be detoxified for the protection of plant cells against the toxic effect of these species. The differences in subcellular localization and biochemical properties of antioxidant enzymes and the distinct responses in gene expression, in addition to the presence of non-enzymatic mechanisms, result in a versatile and flexible antioxidant system able to control the optimum ROS levels (Vranova *et al*., 2002). However, the antioxidant response of plants under heavy metal stress depends greatly on the genotype, metal species and stress treatment duration. It has been reported that exposure of pea (*Pisum sativum* L.) plants to cadmium altered enzymatic and non-enzymatic antioxidant defenses (Romero *et al*., 2007). On the contrary, in coffee plants, the activity of APX increased at lower cadmium concentration but it was not detectable in cells subjected to a higher cadmium level (Gomes-Junior *et al*., 2006). In rice, the transcript levels of all APX encoding genes significantly increased after eight hours of exposure to 20 ppm aluminum (Rosa *et al*., 2010). In pea plants, gene expression of the cytosolic APX isoform increased or declined depending on the aluminum concentration; however, APX enzyme activity did not show any significant change in response to aluminum (Panda & Matsumoto, 2010). In cell suspensions of *Coffea arabica* L., treatment with nickel induced a rapid in-

crease in APX activity, with differences in the degree of induction associated to the heavy metal concentration (Gomes-Junior *et al.*, 2006). Our results show that the addition of Cd^{2+} to the irrigation solution induced a significant decrease in different enzymatic activities (CAT, GPX and APX) in *A. halimus*. This decrease could result from the damage caused by metal ion-induced oxygen species (Kachout *et al.,* 2009). When NaCl was simultaneously added, the reduction was more drastic. Similarly, Cu^{2+} induced a decreased in CAT and APX activities but increased GPX activity. It is known that while Cu acts as catalysts in the formation of reactive oxygen species (Fenton reaction) and causes GSH depletion, Cd does not appear to generate free radicals, but it increases lipid peroxidation and decrease GSH content, which results indirectly in active oxygen species production. A previous report has shown that these oxygen species cause oxidative damage to antioxidant enzymes diminishing their activities (Kachout *et al*., 2009). All these findings emphasize the importance and complexity of the interactions of the antioxidant enzymatic activities with other antioxidants in fine tuning of plant antioxidant metabolism.

GSH is a key metabolite in PC synthesis and therefore essential for toxic metal neutralization (López-Climent *et al*., 2014), its concentration after Cd or Cu exposure, alone or together with NaCl, decreased in *A. halimus* together with an increased synthesis of PCs, mainly in roots of Cd^{2+}- treated plants. This depletion may be attributed to the involvement of GSH in the synthesis of PCs induced by Cd^{2+} and the formation of Cd-GSH complexes (Mendoza & Moreno, 2006).

As a protection from heavy metal intoxication, plants have developed mechanisms to immediately inactivate metal ions entering the cytosol (López-Climent *et al*., 2014). PCs are rapidly induced *in vivo* by a wide range of heavy metal ions, including Cd^{2+} and Cu^{2+}. Data presented herein demonstrate that Cd^{2+} and Cu^{2+} stress caused PC accumulation in *A. halimus* that can be considered as hyperaccumulator species.

As conclusion, salinity drastically modified heavy metal accumulation and improved plant growth. High levels of NaCl also decreased oxidative damage, but differently in plants exposed to Cd or Cu stress. Under high concentrations of Cd^{2+}, oxidative damage was reduced by NaCl treatment in both, root and shoots tissue whereas under Cu^{2+} stress, the NaCl-induced reduction in oxidative damage only took place in root tissue.

References

Aebi H, 1984. Catalase *in vitro*. Method Enzymol 105: 121-126. https://doi.org/10.1016/S0076-6879(84)05016-3

Anderson ME, 1985. Determination of glutathione and glutathione disulfide in biological samples. Method Enzymol 113: 548-555. https://doi.org/10.1016/S0076-6879(85)13073-9

Arbona V, Gómez-Cadenas A, 2008. Hormonal modulation of citrus responses to flooding. J Plant Growth Regul 27: 241-250. https://doi.org/10.1007/s00344-008-9051-x

Arbona V, Hossain Z, López-Climent MF, Pérez-Clemente RM, Gómez-Cadenas A, 2008. Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. Physiol Plant 132: 452-466. https://doi.org/10.1111/j.1399-3054.2007.01029.x

Bankaji I, Sleimi N, López-Climent MF, Perez-Clemente RM, Gomez-Cadenas A, 2014. Effects of combined abiotic stresses on growth, trace element accumulation, and phytohormone regulation in two halophytic species. J Plant Growth Regul 33: 632-643. https://doi.org/10.1007/s00344-014-9413-5

Barthwal J, Nair S, Kakkar P, 2008. Heavy metal accumulation in medicinal plants collected from environmentally different sites. Biomed Environ Sci 4: 319-324. https://doi.org/10.1016/S0895-3988(08)60049-5

Clemens S, Aarts MGM, Thomine S, Verbruggen N, 2013. Plant science: The key to preventing slow cadmium poisoning. Trends Plant Sci 18: 92-99. https://doi.org/10.1016/j.tplants.2012.08.003

Clemente R, Walkerb DJ, Pardo T, Martínez-Fernández D, Bernala MP, 2012. The use of a halophytic plant species and organic amendments for the remediation of a trace elements-contaminated soil under semi-arid conditions. J Hazard Mater 223-224: 63-71. https://doi.org/10.1016/j.jhazmat.2012.04.048

da Silva CTB, Souza VK, Da Silva APF, Lyra Lemos RP, Conserva LM, 2010. Determination of the phenolic content and antioxidant potential of crude extracts and isolated compounds from leaves of Cordia *multispicata* and *Tournefortia bicolor*. Pharm Biol 48: 63-69. https://doi.org/10.3109/13880200903046146

de Vos CHR, Vonk MJ, Vooijs R, Schat H, 1992. Glutathione depletion due to copper induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol 98: 853-885. https://doi.org/10.1104/pp.98.3.853

Fielding JL, Hall JL, 1978. A biolchemical and cytochemical study of peroxidase activity in roots of *Pisum sativum*: I. A comparison of dab-peroxidase and guaiacol-peroxidase with particular emphasis on the properties of cell wall activity. J Exp Bot 4: 969-981. https://doi.org/10.1093/jxb/29.4.969

Foyer CH, Noctor G, 2005. Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. Plant Cell 7: 1866-1875. https://doi.org/10.1105/tpc.105.033589

Gattward JN, Almeida AAF, Souza JO, Gomes FP, Kronzucker HJ, 2012. Sodium-potassium synergism in *Theobroma cacao*: Stimulation of photosynthesis, water-use efficiency and mineral nutrition. Physiol Plant 146: 350-362. https://doi.org/10.1111/j.1399-3054.2012.01621.x

Gomes-Junior RA, Moldes CA, Delite FS, Pompeu GB, Gratão PL, Mazzafera P, Lea PJ, Azevedo RA, 2006. Antioxidant metabolism of coffee cell suspension cultures

in response to cadmium. Chemosphere 65: 1330-1337. https://doi.org/10.1016/j.chemosphere.2006.04.056

Han RM, Lefèvre I, Ruan CJ, Qin P, Lutts S, 2012. NaCl differently interferes with Cd and Zn toxicities in the wetland halophyte species *Kosteletzkya virginica* (L.) Presl. Plant Growth Regul 68: 97-109. https://doi.org/10.1007/s10725-012-9697-z

Hewitt EJ, 1966. Sand and water culture methods used in the study of plant nutrition. Technical Communication No. 22, 2nd ed. Com. Bur. of HortiCul. and Plant Crops East Malling. Maidstore, Kent.

Hirata K, Tsuji N, Miyamoto K, 2005. Biosynthetic regulation of phytochelatins, heavy metal-binding peptides. J Biosci Bioeng 100: 593-599. https://doi.org/10.1263/jbb.100.593

Hodges DM, DeLong JM, Forney CF, Prange RK, 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207: 604-611. https://doi.org/10.1007/s004250050524

Hossain MA, Hasanuzzaman M, Fujita M, 2011. Coordinate induction of antioxidant defense and glyoxalase system by exogenous proline and glycinebetaine is correlated with salt tolerance in mung bean. Front Agric in China 5: 1-14. https://doi.org/10.1007/s11703-010-1070-2

Kachout SS, Mansoura AB, Leclerc JC, Jaffel K, Rejeb MN, Ouerghi Z, 2009. Effects of heavy metals on antioxidant activities of: *Atriplex hortensis* and *Atriplex rosea*. J Appl Bot Food Qual 83: 37-43. http://pub.jki.bund.de/index.php/JABFQ/article/view/2123

Lee S, Moon JS, Ko TS, Petros D, Goldsbrough PB, Korban SS, 2003. Overexpression of Arabidopsis phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiol 131: 656-663. https://doi.org/10.1104/pp.014118

Lefévre I, Marchal G, Meerts, P, Corréal E, Lutts S, 2009. Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ Exp Bot 65: 142-152. https://doi.org/10.1016/j.envexpbot.2008.07.005

Leitenmaier B, Küpper H, 2013. Compartmentation and complexation of metals in hyperaccumulator plants. Front Plant Scie 4 (20): 374. https://doi.org/10.3389/fpls.2013.00374

Lopez-Chuken UJ, Young SD, 2005. Plant screening of halophyte species for cadmium phytoremediation. Z Naturforsch C 3: 236-243.

López-Climent MF, Arbona V, Pérez-Clemente RM, Gómez-Cadenas A, 2011. Effects of cadmium on gas exchange and phytohormone contents in citrus. Biol Plant 55: 187-190. https://doi.org/10.1007/s10535-011-0028-4

López-Climent MF, Arbona V, Pérez-Clemente RM, Zandalinas SI, Gómez-Cadenas A, 2014. Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants. Plant Biol 16: 79-87. https://doi.org/10.1111/plb.12006

Lutts S, Lefevre I, Delpérée, Kivits S, Dechamps C, Robledo A, Correal E, 2004. Heavy metal accumulation by the halophyte species Mediterranean saltbush. J Environ Qual 33: 1271-1279. https://doi.org/10.2134/jeq2004.1271

Manousaki E, Kalogerakis N, 2009. Phytoextraction of Pb and Cd by the Mediterranean saltbuss (*Atriplex halimus* L.): metal uptake in relation to salinity. Environ Sci Pollut Res 16: 844-854. https://doi.org/10.1007/s11356-009-0224-3

Mendoza-Cózatl DG, Moreno-Sánchez R, 2006. Control of glutathione and phytochelatin synthesis under cadmium stress. Pathway modeling for plants J Theor Biol 238: 919-936. https://doi.org/10.1016/j.jtbi.2005.07.003

Mourato M, Reis R, Martins LL, 2012. Characterization of plant antioxidative system in response to abiotic stresses: a focus on heavy metal toxicity. In: Advances in selected plant physiology aspects; Montanaro G (ed.). pp: 23-44. InTech, Croatia. https://doi.org/10.5772/34557

Nagarani N, JanakiDevi V, YokeshBabu M, Kumaraguru AK, 2012. Protective effect of *Kappaphycus alvarezii* (Rhodophyta) extract against DNA damage induced by mercury chloride in marine fish. Toxicol Environ Chem 94: 1401-1410. https://doi.org/10.1080/02772248.2012.707792

Nakano Y, Asada K, 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Phys 22: 867-880.

Pan X, Yang J, Zhang D, Chen X, Mu S, 2011. Cu(II) complexation of high molecular weight (HMW) fluorescent substances in root exudates from a wetland halophyte (*Salicornia europaea* L.). J Biosci Bioeng 111: 193-197. https://doi.org/10.1016/j.jbiosc.2010.09.017

Panda SK, Matsumoto H, 2010. Changes in antioxidant gene expression and induction of oxidative stress in pea (*Pisum sativum* L.) under Al stress. Biometals 23: 753-762. https://doi.org/10.1007/s10534-010-9342-0

Romero-Puertas MC, McCarthy I, Sandalio LM, Palma JM, Corpas FJ, Gomez M, Del Rio LA, 1999. Cadmium toxicity and oxidative metabolism of pea leaf peroxisomes. Free Radical Research 31: 25-31. https://doi.org/10.1080/10715769900301281

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira JAG, Ferreira-Silva SL Abreu-Neto J, Margis R, Margis-Pinheiro M, 2010. Cytosolic APx knockdown indicates an ambiguous redox responses in rice. Phytochemistry 71: 548-558. https://doi.org/10.1016/j.phytochem.2010.01.003

Sbartai H, Djebar MR, Sbartai I, Berrabbah H, 2012. Bioaccumulation of cadmium and zinc in tomato (*Lycopersicon esculentum* L.) C R Biol 335: 585-593. https://doi.org/10.1016/j.crvi.2012.08.001

Stolt JP, Sneller FEC, Brynelsson T, Lundborg T, Schat H, 2003. Phytochelatin and cadmium accumulation in wheat. Environ Exp Bot 49: 21-28. https://doi.org/10.1016/S0098-8472(02)00045-X

Vandenabeele S, Vanderauwera S, Vuylsteke M, Rombauts S, Langebartels C, Seidlitz HK, Zabeau M, Van Montagu M, Inzé D, Van Breusegem F, 2004. Catalase deficiency drastically affects gene expression induced by high light in *Arabidopsis thaliana*. Plant J 39: 45-58. https://doi.org/10.1111/j.1365-313X.2004.02105.x

Vranová E, Inzé D, Van Breusegem F, 2002. Signal transduction during oxidative stress. J Exp Bot 53: 1227-1236. https://doi.org/10.1093/jexbot/53.372.1227

Walker DJ, Lutts S, Sánchez-García M, Correal E, 2014. *Atriplex halimus* L.: Its biology and uses. J Arid Environ 100-101: 111-121. https://doi.org/10.1016/j.jaridenv.2013.09.004

Watanabe ME, 1997. Phytoremediation on the brink of commercialisation. Environ Sci Tech 31: 182-186. https://doi.org/10.1021/es972219s

Xu J, Yin H, Liu X, Li X, 2010. Salt affects plant Cd-stress responses by modulating growth and Cd accumulation. Planta 231: 449-459. https://doi.org/10.1007/s00425-009-1070-8

Yadav SK, 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S Afr J Bot 76: 167-179. https://doi.org/10.1016/j.sajb.2009.10.007

Zu Y, Li YT, Chen J, Chen H, Qin L, Schvartz C, 2005. Hyperaccumulation of Pb, Zn and Cd in herbaceous grown on lead-zinc mining area in Yunnan, China. Environ Int 31: 755-762. https://doi.org/10.1016/j.envint.2005.02.004