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Characterization of mechanisms involved in tolerance and accumulation of Cd in *Biscutella auriculata* L.

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ABSTRACT

Biscutella auriculata L. is one of the rare species that is able to grow in a very contaminated mining area in Villamayor de Calatrava (Ciudad Real, Spain). In an effort to understand the mechanisms involved in the tolerance of this plant to high metal concentrations, we grew *B. auriculata* in the presence of 125 μM $Cd(NO_3)_2$ for 15 days and analysed different parameters associated with plant growth, nitric oxide and reactive oxygen species metabolism, metal uptake and translocation, photosynthesis rate and biothiol (glutathione and phytochelatins) content. Treatment with Cd led to growth inhibition in both the leaves and the roots, as well as a reduction of photosynthetic parameters, transpiration and stomatal conductance. The metal was mainly accumulated in the roots and in the vascular tissue, although most Cd was detected in areas surrounding their epidermal cells, while in the leaves the metal accumulated mainly in spongy mesophyll, stomata and trichrome. Based on the Cd bioaccumulation (5.93) and translocation (0.15) factors, this species denoted enrichment of the metal in the roots and its low translocation to the upper tissues. Biothiol analysis showed a Cd-dependent increase of reduced glutathione (GSH) as well as the phytochelatins (PC2 and PC3) in both roots and leaves. Cd-promoted oxidative damage occurred mainly in the leaves due to disturbances in enzymatic and nonenzymatic antioxidants, while the roots did not show significant damage as a result of induction of antioxidant defences. It can be concluded that *B. auriculata* is a new Cd-tolerant plant with an ability to activate efficient metal-sequestering mechanisms in the root surface and leaves and to induce PCs, as well as antioxidative defences in roots.

Keywords: Cd; Biscutella; Phytorremediation; Oxidative stress; ROS.

ABBREVIATIONS

APX	Ascorbate peroxidase enzyme
AsA	Ascorbate
BF	Bioaccumulation factor
CAT	Catalase enzyme
DHAR	Dehydroascorbate reductase enzyme
GOX	Glycolate oxidase
GR	Glutathione reductase enzyme
GSH	Reduced glutathione
GSNO	S-Nitrosoglutathione
GSNOR	S-Nitrosoglutathione reductase
GSSG	Oxidised glutathione
GST	Glutathione S-Transferase enzyme
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase enzyme
NADP-G6PDH	$NADP^+$-dependent glucose-6-phosphate dehydrogenase enzyme
NADP-IDH	$NADP^+$-dependent isocitrate dehydrogenase enzyme
NADP-MS	$NADP^+$-dependent malate dehydrogenase enzyme
NO	Nitric oxide
PC	Phytochelatins
POD	Peroxidase enzyme
ROS	Reactive oxygen species
SOD	Superoxide dismutase enzyme
TF	Translocation factor

1. INTRODUCTION

Heavy metals constitute a very heterogeneous group of elements that vary markedly in their chemical properties and biological functions (Kalaivanan and Ganeshamurthy, 2016). Cadmium (Cd) is considered one of the most toxic heavy metals and it does not have any essential functions for plants. The accumulation of Cd in the soil is mainly due to anthropogenic causes, including power stations, heating systems, metal-working industries, waste incinerators and phosphate fertilizers (Hayat et al., 2019). Exposure to Cd, even in small doses, has a well-known adverse effect on plant growth and development (Sandalio et al., 2012). Particularly problematic are the mining areas where high concentrations of Cd are accumulated. The mining industry needs to take environmental concerns into account in order to prevent damage to the ecosystem and landscape. Revegetation of contaminated mining areas is a promising green and clean technique which would mitigate the dispersion of small contaminated particles and also help to maintain the ecosystem. In addition, there is a growing demand for cost-effective and environmentally friendly strategies, such as phytoremediation and phytostabilization, which can be implemented *in situ* (Haq et al., 2020). However, while most plants are quite sensitive to high metal concentrations, wild plants growing spontaneously in contaminated areas are good candidates for these strategies given their capacity to accumulate high metal concentrations and to survive under these conditions. Knowledge of the mechanisms involved in this natural tolerance of plants to metals is very important in order to design new phytoremediation strategies.

Cd-hyperaccumulating plants are able to grow at higher Cd concentrations (≥ 100 times) than non-hyperaccumulators and without showing important symptoms of toxicity (Suman et al., 2018). In the past, hyperaccumulator plants were defined as those able to accumulate more than 1 mg g^{-1} DW in the leaves. However, a new classification

according to the type of metal has been suggested and Mn and Zn hyperaccumulators will therefore be considered as plants capable of accumulating in their leaves concentrations greater than 10 mg g^{-1} of those metals, while for Co, Cu, Ni and Pb a the concentration should be higher than 1 mg g^{-1} and for Cd hyperaccumulators the value should be higher than 0.1 mg g^{-1} (Suman et al., 2018). The bioaccumulation factor (BF) and the translocation factor (TF) have been used to evaluate the phytoextraction potential of a given species. The efficiency of a plant in accumulating the heavy metal in comparison with its concentration in the soil is described by the BF, while TF indicates the plant's efficacy in the transport of heavy metals from roots to leaves (Melo et al., 2009; Liu et al., 2019). In metal excluder species the BF is less than 1, whereas in metal hyperaccumulator species the BF is greater than 1 (McGrath et al., 2002).

Plant species differ widely in the mechanisms of Cd accumulation and tolerance. Clearly, Cd tolerant species must be able either to prevent the absorption of excess Cd or to detoxify the metal after its absorption (Iqbal et al., 2018). Cd can be stored in a nontoxic way by chelation and compartmentalization in the apoplast or vacuoles. Within the vacuoles, Cd is sequestrated by phytochelatins, which are small peptides that are derived from glutathione and strongly induced by Cd toxicity (Song et al., 2014**)**. High levels of Cd inside the plants can produce negative effects, such as inhibition of plant growth, nutrient imbalance, inhibition of photosynthesis and respiration, and oxidative stress damage due to the accumulation of reactive oxygen species (Sandalio et al., 2001, Rodríguez-Serrano et al., 2006). The balance between the production and the scavenging of reactive oxygen species (ROS) is perturbed by Cd accumulation in plants. One of the main consequences of Cd toxicity is the increase of intracellular ROS levels, which can cause oxidative stress (Sandalio et al., 2001, Ali et al., 2019). Reactive oxygen species include radical and non-radical oxygen-derived molecules: $O_2^{\cdot -}$, H_2O_2,

$^1O_2$, $HO_2^{\cdot -}$, $OH^{\cdot}$, ROOH, $ROO^{\cdot}$, $RO^{\cdot}$, RCO and O_3, all of which are responsible for inducing oxidative damage to lipids, proteins, carbohydrates and DNA (Sies et al., 2017). In order to survive, plants can activate a variety of antioxidant mechanisms (enzymatic and non-enzymatic antioxidants) in order to reach ROS levels that are compatible with the normal functioning of the cells (You and Chan, 2015). The Foyer–Halliwell–Asada cycle consists of four enzymes, namely ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), and it has great importance in H_2O_2 elimination (Sandalio et al., 2012). Other relevant enzymes are catalase (CAT), peroxidases (POD) and superoxide dismutases (SODs), which are responsible for the detoxification of H_2O_2 and $O_2^{\cdot -}$, respectively (You and Chan, 2015). Additional enzymes that also act in the maintenance of the redox state in the cells are glutathione S-transferase (GST) and NADP-dependent dehydrogenases (Sandalio et al., 2012). The main non-enzymatic antioxidants are ascorbate (AsA) and glutathione (GSH), which participate as enzyme cofactors, which are precursors of other molecules and ROS sequesters (You and Chan, 2015). Flavonoids and carotenoids are also important non-enzymatic antioxidants (Ali et al., 2019).

Nitric oxide (NO) is attracting increasing attention as a regulator of many physiological processes in plants, including the defence against heavy metal (Gill et al., 2013, Terrón-Camero et al., 2019). It has been reported that NO can improve antioxidant defences by inducing the expression of genes that encode antioxidant enzymes and it is a key factor in the tolerance of cells to oxidative stress caused by Cd and other metals (Romero-Puertas and Sandalio, 2016, Romero-Puertas, et al., 2018, Souri et al., 2020). S-Nitrosoglutathione reductase (GSNOR) is an important enzyme that regulates intracellular levels of S-nitrosoglutathione (GSNO) and also indirectly

regulates NO levels and protein S-nitrosylation, a post-translational modification that affects the activity, location, and stability of proteins (Frungillo et al., 2014, Hu et al., 2019). GSNOR therefore plays an important role in the maintenance of reactive nitrogen species balance and in the control of the cellular redox state (Wen et al., 2019).

Biscutella auriculata is a wild herbaceous plant that grows on pastureland, roadsides and degraded areas. Recently, we identified *B. auriculata* as one of the few species growing in a highly contaminated area close to the San Quintin mine located in Villamayor de Calatrava in the province of Ciudad Real in south-central Spain (Supplementary Figure 1). This area is contaminated with heavy metals, with the following ranges of metal concentrations found in mine tailings: 243.24–93900.87 mg kg^{-1} of Pb, 470.59–20911.78 mg kg^{-1} of Zn, 2.88–54.47 mg kg^{-1} of Cd and 44.10–716.5 mg kg^{-1} of Cu (Rodríguez et al., 2009). Although *B. auriculata* has not previously been identified as a metal-tolerant species, *Biscutella laevigata*, another species from the same genus, has been reported to be capable of hyperaccumulating and tolerating heavy metals, mainly thallium (Fellet et al., 2012, Pošćić et al., 2013, Wierzbicka et al., 2016, Pavoni et al., 2017), cadmium, lead and zinc (Pielichowska and Wierzbicka, 2004, Wierzbicka and Pielichowska, 2004, Escarré et al., 2011, Babst-Kostecka et al., 2020). However, the mechanisms involved in the metal tolerance of this genus have not been fully elucidated, while *B. auriculata,* in particular, has not previously reported to be a metal-tolerant plant species. In this study, we therefore aim to investigate the mechanisms involved in the tolerance of *B. auriculata,* which was isolated from this area, to Cd, one of the metals presents in the soil. To decipher the mechanisms involved in tolerance to high concentrations of Cd, we evaluated it effects on growth parameters, oxidative stress markers, enzymatic and non-enzymatic antioxidants, Cd accumulation patterns and mineral status in leaves and roots. This information is essential when

considering the possible use of *B. auriculata* in soil phytostabilization and for the revegetation of degraded areas polluted with Cd. The results obtained demonstrate for the first time that *B. auriculata* is a new Cd accumulator plant species which could be useful in the restoration of Cd-polluted areas, and the mechanisms involved are also discussed.

2. MATERIALS AND METHODS

2.1 Plant management and growth conditions

Biscutella auriculata seeds were collected in an area contaminated with heavy metals due to mining activity located in Villamayor de Calatrava, Ciudad Real province (South-Central Spain; 38°48'52.6"N 4°17'15.5"W). Seeds were soaked for 24 hours and then germinated on wet filter paper at 25 ºC. To be able to study specifically the effect of Cd on this species, germinated seeds were cultivated in a hydroponic system containing perlite and Hoagland nutrient solution (Hoagland and Arnon, 1950) for 15 days. After this period, seedlings were grown with nutrient solution supplemented with 0 or 125 µM of $Cd(NO_3)_2$ (Pereira et al., 2016) and maintained in a growth chamber under controlled conditions of temperature (24 ºC), relativity humidity (60%) and photoperiod (16 h) for 15 more days. The concentrations of Cd used were taken from data in the literature and following a preliminary analysis of growth with respect to Cd concentrations of 50, 100 and 125 µM. After the period of treatment, the aerial parts and roots were processed separately, frozen in liquid nitrogen and stored at –80 ºC. Additionally, to better analyse root architecture plants were grown vertically in square Petri dishes (10 × 10 cm) containing Murashige and Skoog (MS) medium supplemented with 0 or 125 µM of $Cd(NO_3)_2$ during 10 days (Sanz-Fernández et al., 2017).

2.2 Morphological parameters, photosynthesis data and pigment content

The following morphological and growth parameters were evaluated on images obtained by stereomicroscopy and Image J analyses: number of leaves, aerial part area and trichomes, stomata numbers and root fresh weight. Measurement of net photosynthesis rate, stomatal conductance, transpiration ratio and intercellular CO_2 concentration were determined using a portable photosynthesis system (Ciras-3, PP Systems). Chlorophylls, carotenoids and anthocyanin content were determined by a spectrophotometric method according to Lichtenthaler and Buschmann (2001) and Sims and Gamon (2002), respectively.

2.3 Trace element analysis

Leaves and roots were dried at 60 ºC for 72 hours and digested with an HNO_3/H_2O_2 mixture using a microwave digestion system (ETHOS 1, Milestone). The mineralized material was diluted with Milli-Q water and filtered through a 0.45 µm PVDF membrane. Trace element content was measured by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian 720-ES).

2.4 Evaluation of phytoextraction ability

The phytoextraction ability was determined using the translocation factor (TF) and the bioaccumulation factor (BF) described by Melo et al. (2009) according to the equations:

$$TF = \frac{[Cd]shoot}{[Cd]\,root} \qquad BF = \frac{[Cd]shoot}{[Cd]\,solution}$$

where [Cd] shoot and [Cd] root are the Cd concentrations (mg kg^{-1}) in the shoot and root, respectively, and [Cd] solution is the Cd concentration (mg L^{-1}) in the nutrient solution.

2.5 Histochemical analysis

Cadmium accumulation

Cadmium accumulation in roots and leaves was histochemically detected using the dithizone method (Seregin et al. 2004). Whole leaves and roots were immersed in a dithizone solution (0.5 mg ml^{-1}) prepared in acetone/glacial acetic acid/Milli-Q water (3:5:1) and incubated for one hour. After this period, the leaves were bleached by immersing them in boiling ethanol. After staining, pieces of roots and leaves were embedded in 5% low-melting agarose D1 EEO (Conda Pronadise) and transversal sections were obtained using a vibratome (VT1200 / VT1200S Leica) and examined by light microscopy (Leica DMI600B).

H_2O_2 and $O_2^{\cdot-}$ detection

The accumulation of H_2O_2 and $O_2^{\cdot-}$ was localized in leaves and roots according to Romero-Puertas et al. (2004). For H_2O_2 localization, whole leaves and roots were immersed in a 0.1% solution of DAB (3,3'-diaminobenzidine), vacuum-infiltrated for five minutes and then incubated at room temperature overnight in the dark. For $O_2^{\cdot-}$ localization, whole leaves and roots were immersed in a 0.1% solution of NBT (Nitro blue tetrazolium) and 10 mM Na-azide and samples were vacuum-infiltrated for 5–10 minutes and illuminated until dark spots appeared. In both cases, leaves were bleached by immersing them in boiling ethanol.

2.6 Quantification of GSH, AsA and PC

The oxidised (GSSG) and reduced (GSH) glutathione, and the total ascorbic acid (AsA) content were analysed by liquid chromatography-electrospray mass spectrometry (LC-ES/MS) according to El-Zohri et al. (2005). Plant extracts were prepared in 0.1 N HCl and, after centrifugation, the supernatant was injected onto an Atlantis T3 column (150 mm × 3 mm, Waters, Milford) with an Atlantis T3 pre-column (10 mm × 2.1 mm, Waters) using an HPLC (H-Class, Waters, Milford) coupled to a triple quadrupole mass spectrometer (Quattro-Micro, Waters, Milford). PC2 and PC3 contents were analysed following the procedure described by El-Zohri et al. (2005) and using 0.1 N HCl extracts. The supernatant was injected onto an Xselect CSH column (100 mm × 2.1 mm, Waters, Milford) with a Vanguard Xselect CSH C18 (5 mm × 2.1 mm, Waters, Milford). The PCs were calculated by multiple reaction monitoring (MRM) using positive and negative electrospray.

2.7 Analyses of lipid peroxidation, H_2O_2 and nitric oxide (NO) content

Lipid peroxidation was determined according to Buege and Aust (1978) using a standard curve for malondialdehyde (MDA). H_2O_2 accumulation was analysed by a spectrofluorometric method as described by Romero-Puertas et al. (2004) using a commercial H_2O_2 solution for the calibration curve. The accumulation of NO was determined by a spectrofluorometric method using 4,5-diaminofluorescein (DAF-2) according to Nakatsubo et al. (1988). The results are expressed in arbitrary units of fluorescence intensity.

2.8 Enzymatic assays

SOD activity (EC 1.15.1) was assayed by native polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels and by a photochemical method using

nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The activity was expressed as % total SOD activity by measuring the area under the peaks using ImageJ. The rest of the enzymatic activities were analysed spectrophotometrically: CAT activity (EC 1.11.1.6); POD activity; GOX activity (EC 1.1.3.1); APX activity (EC 1.11.1.11), GR activity (EC 1.6.4.2); MDHAR activity (EC 1.6.5.4) and DHAR activity (EC 1.8.5.1) as reported by Hafsi et al. (2010); NADP-G6PDH (EC 1.1.1.49), NADP-IDH (EC 1.1.1.42) and NADP-MS (EC 4.1.3.2) as reported by León et al. (2002); GST activity (EC 2.5.1.1) as described by Habig et al. (1974) and GSNOR (EC 1.2.1.46) as described by Ortega-Galisteo et al. (2012). Details of the enzymatic assays are given in Supplementary Material and Methods.

2.9 Western blotting

Proteins were separated by denaturing electrophoresis (SDS-PAGE) on a Mini-Protean II slab cell (Bio-Rad) using 12% (w/v) separating gels and samples were transferred onto PVDF membranes in a Semi-dry Transfer Cell (Bio-Rad). Proteins were visualised using specific antibodies and the enhanced chemiluminescence method (Clarity™ Western ECL Substrate, Bio-Rad) (Romero-Puertas et al., 2007).

2.10 Other assays

Protein contents in plant extracts were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Total phenolics content was determined by Folin–Ciocalteau's method (Singleton and Rossi, 1965) using a calibration curve for gallic acid. Total flavonoids content was determined by the aluminium chloride spectrophotometric method (Zhishen et al., 1999) using a calibration curve for quercetin. Total antioxidant capacity was analysed according to the

ABTS assay (Jiménez-Escrig et al., 2003). The results were calculated using a calibration curve for Trolox.

2.11 Data analysis

Statistical analyses were performed by a Student's t-test in IBM SPSS Statistics 24. The significance level is represented in the figures by asterisks (P<0.05:*; P<0.01:**; P<0.001:***). Image analyses were carried out using Image Tool 3.0 software.

3. RESULTS

3.1 Effect of cadmium on the growth, phenotype and photosynthesis rate of *Biscutella auriculata*

Long term growth of *B. auriculata* with Cd led to a significant reduction of the fresh weight of leaves (45%) and roots (34%) and also the leaf area (48%), whereas the number of leaves was not affected (Table 1, Figure 1). In the leaves Cd induced a strong red/purple colour (Figure 1) due to anthocyanin accumulation (3561% of control) (Table 1). A significant increase (about 55%) in trichomes and stomata number per leaf area was also observed (Table 1). The negative effects of Cd on root growth mainly concerned the number of secondary roots and hair roots, as can be shown in plants grown in vertical plates (Figure 1B).

Analysis of the photosynthetic pigments in leaves showed a significant decrease in chlorophyll (chlorophyll a, chlorophyll b and total chlorophyll with 72%, 75% and 73%, respectively) and carotenoid contents in response to Cd treatment, as well as a significant reduction in the phenols content (Table 1), while flavonoids content remained unchanged (Table 1). Along with changes in photosynthetic pigments, Cd

affected the photosynthesis process and a considerably lower net photosynthetic rate was observed, along with lower stomatal conductance and transpiration (Figure 2). At the same time, a slight but statistically significant increase in leaf temperature and subcellular CO_2 concentration was observed in Cd-treated plants (Figure 2).

3.2 Cadmium accumulation and nutrient balance

Cadmium and nutrient contents in the roots and leaves of Cd-treated and untreated plants are shown in Supplementary Table 1. Cd accumulated mainly in the roots (1.184 mg g^{-1}) and to a lesser extent in leaves (0.174 mg g^{-1}). Manganese accumulation was statistically significantly lower in leaves of Cd-treated plants when compared to control plants; meanwhile, the rest of elements did not show any statistically significant change (Supplementary Table 1). The BF and TF values for Cd were 5.93 and 0.15, respectively (Supplementary Table 2), and this suggests a high efficiency for *B. auriculata* in the extraction of Cd from the nutrient solution and a medium efficiency in Cd translocation from the roots to the leaves. The accumulation of Cd was imaged in leaves and roots using histochemical staining with dithizone (Figure 3A and B). In the leaves (Figure 3A), Cd was accumulated in the spongy mesophyll, substomatal chamber, trichomes and secondary/central nerves. Cd was accumulated in the base of the trichome and inside in two well-differentiated vacuoles (Figures 3A). The accumulation of Cd in roots was evident inside the vascular bundles and on the epidermis of the root (Figure 3B). Interestingly, the highest Cd accumulation took place in an amorphous structure surrounding epidermal cells, which could be mucilage or the remains of border cells (Figure 3B).

Concerning the structures of leaves and roots, Cd promoted an increase in the cell size, mainly in palisade mesophyll, along with an increase in the number of cell

lines in the root cortex from two lines of cells found in control plants to three lines of cells observed in Cd-treated plants (Figure 3B).

3.3 Cadmium affects the content and redox state of glutathione and phytochelatin accumulation

The GSH and GSSG contents varied greatly in leaves of control and Cd-treated plants (Figure 4A). Cd treatment led to a statistically significantly increase in GSH content (50%) in leaves and a decrease in the oxidised form GSSG (332%), while in the roots statistically significantly differences were not observed (Figure 4A). The accumulation of PCs was not observed in the leaves of untreated plants, while in Cd-treated plants a statistically significantly accumulation of both PC2 and PC3 was observed (Figures 4A and 4B). However, roots from control plants contained PC2 but not PC3 and treatment with Cd promoted a statistically significantly accumulation of both PC2 and PC3 (Figures 4A and 4B). The highest PC accumulation was observed in leaves (Figure 4A). The relative concentrations (%) of both biothiols (GSH and PCs) in *B. auriculata* for both Cd-treated and untreated plants are shown in Figure 4B. It is worth highlighting the higher contribution of PCs to total thiols in both leaves and roots in Cd-treated plants, with PC2 having the highest proportion in both organs (Figure 4B).

3.4 Cadmium promotes oxidative stress

Cadmium-induced oxidative stress in leaves was evidenced by a statistically significantly increase in the H_2O_2 concentration (75%) and content of MDA (74%), which is used as a lipid peroxidation marker, in Cd-treated plants, while in roots significant differences were not observed (Figure 5). The histochemical localizations of $O_2^{\cdot -}$ (blue spots) and H_2O_2 (brown spots) in leaves are shown in Supplementary Figure 2. Cd-treated leaves showed a greater accumulation and intensity of blue spots, due to

the accumulation of $O_2^{\cdot-}$, than the leaves of untreated plants (Supplementary Figure 2A). Similar results were observed in the accumulation of H_2O_2, with a more intense brown colour observed due to the accumulation of H_2O_2 in Cd-treated leaves (Supplementary Figure 2B).

Interestingly the content of ascorbate in roots of Cd-treated plants was statistically significantly higher than in control plants; this trend was not observed in leaves (Figure 5). The GSH/GSSG ratio was markedly statistically significantly higher (587% of control) in leaves from Cd-treated plants but statistically significant differences were not found in roots (Figure 5). The analysis of total antioxidant capacity also showed different trends in roots and leaves, with a decrease observed in leaves and a significant increase in roots (Figure 5). The activity of GOX, an enzyme involved in photorespiration which contributes to H_2O_2 production, showed a statistically significantly increase to 72% of control, in leaves treated with Cd (Figure 5).

Concerning the enzymatic antioxidants, the activities of CAT, GST and POD, which are involved in removing H_2O_2 and peroxides, statistically significantly increased in roots with Cd by 68.4%, 108% and 61%, respectively (Figure 6A), while in leaves the CAT and GST activities were not significantly different and POD activity increased by 61% (Figure 6A). The analyses in gel of SOD activity in leaves showed the presence of at least four bands for control plants and considerable decreases in the number of bands (to one band: SOD-3) and total SOD activity upon treatment with Cd (Figure 6A). Analysis of the enzymatic activities involved in the Halliwell–Asada–Foyer cycle showed a statistically significantly increase in DHAR in leaves treated with Cd (181%), while MDHAR activity showed a statistically significantly induction in both leaves (143%) and roots (278%) of Cd-treated seedlings (Figure 6B). However, neither APX nor GR activities were detected in the extracts. Both activities were evaluated in

different crude extracts by changing the buffer compositions, protease inhibitor cocktails, and different AsA and DTT concentrations without any success. The analysis of GR activity in gel was also carried out but the results were negative (Figure 6B). However, an increase in the amount of GR protein was observed in leaves of Cd-treated plants by Western Blot using a specific antibody against GR (Figure 6B). Analysis of the activity of NADP-dependent dehydrogenases in leaves showed statistically significantly increases in the activity of NADP-G6PDH, NADP-IDH, NADP-MS in the Cd-treated plants by 257%, 77% and 245%, respectively, with the most marked differences observed in NADP-G6PDH associated with the pentose-phosphate cycle (Figure 6C).

Analysis of the NO content in leaves showed that statistically significant differences did not occur due to the Cd-treatment, while in roots a statistically significantly decrease (55%) was observed compared to control plants (Supplementary Figure 3). In turn, GSNOR activity decreased sharply (55%) in leaves in Cd-treated plants, while significant differences were not observed in roots (Supplementary Figure 3).

4. DISCUSSION

4.1 Cadmium induces changes in plant phenotype

Biscutella auriculata is able to growth in very contaminated mine soils that contain Pb, Zn, Cd and Cu (Figure 1) and, as shown in this work, this species can survive high Cd concentrations, although this situation has a detrimental effect on growth that affects both leaves and roots. The inhibitory effect of Cd on growth has been reported for different plant species (Sandalio et al., 2001, Rodríguez-Serrano et al.,

2009, Nahar et al., 2016, Deng et al., 2017, Huybrechts et al., 2019, Liu et al., 2019, Huihui et al., 2020).

In *Biscutella auriculata* the presence of Cd mainly reduced the number of secondary roots and hair roots. Inhibiton of root growth may be attributed to a reduction in the mitotic activities of meristematic cells induced by Cd (Sobkowiak and Deckert 2004, Seth et al., 2008). Another characteristic effect of Cd on *B. auriculata*, which is common to other plants (Rodríguez-Serrano et al., 2006, Maksimović et al., 2007), is an increase in cortex size, which probably affects the resistance to the radial flow of water and mineral nutrients, thus affecting root and shoot growth (Maksimović et al., 2007). However, changes were not observed in xylem number and vessel size, as reported previously for pea roots exposed to Cd (Rodríguez-Serrano et al., 2006, Belimov et al., 2015). In leaves the presence of Cd considerably reduced leaf area, although it promoted the enlargement of mesophyll cells, in a similar way to that reported for pea (Sandalio et al., 2001) and maize plants (Maksimović et al., 2007). Growth inhibition could be due to the negative effects of Cd on cell division and hormonal balance (Huybrechts et al., 2019). The reduction of almost 30% in the net rate of photosynthesis observed in *B. auriculata* is in line with results reported in different plant species (Sandalio et al., 2001, Bloomfield et al., 2014, An et al., 2018, Huihui et al., 2020) and could contribute to growth inhibition induced by Cd. The inhibition of photosynthesis could be a consequence of the significant reduction (73%) in chlorophyll, probably caused by photooxidative damage or the direct effect of Cd by inhibiting chlorophyll biosynthesis (Stobart et al., 1985). However, the inhibition of photosynthesis could also be due to stomatal closure, as indicated by the 40% and 25% reductions in stomatal conductance and transpiration, respectively. Similar results were reported in pea plants (Sandalio et al., 2001) and *Vicia faba* (Neelu et al., 2000). Leaf temperature, which

itself is indicative of changes in leaf transpiration and stomatal conductance (Chaerle et al., 2007), which showed a slight but significant increase in Cd-treated plants, has been reported to be a good indicator of plant metal stress (Thakur and Singh, 2012). Thus, changes ranging from 1ºC to 3ºC in leaf temperature, depending on the period of Cd treatment, has been reported in *Glycine max* under Cd stress (Thakur and Singh, 2012), with reductions in leaf temperature under long term exposure indicating an attempt to adapt to stress conditions probably through the induction of defense mechanisms such as phytochelatin synthesis (Thakur and Singh, 2012). The ability of *B. auriculata* to maintain leaf temperatures under Cd stress conditions could be an adaptive mechanism.

Interestingly, the density of stomata per leaf area increased in *B. auriculata* as a result of Cd treatment. Similar results were observed in *Helianthus annuus* treated with Pb, Cd, Cu and Zn (Kastori et al., 1992) and in *Brachiaria decumbens* (Gomes et al., 2011). The increase in stomata density observed in this study could be an example of anatomic plasticity compensating for the reduced area of transpiration by maintaining CO_2 flow, as reported with respect to *Brachiaria decumbens* (Gomes et al., 2011) and other plant species (Rucinska-Sobkowiak, 2016). However, we have to keep in mind that, rather than enhancing stomatal formation, the Cd-dependent water deficit in leaves could give rise to a reduction in the size of guard cells and therefore increase stomatal density per leaf area, as described by Neelu et al. (2000) and Tram et al. (2013). The intercellular CO_2 value, which can be used to determine whether the effect of Cd on photosynthesis is stomata- or nonstomata-dependent, increased due to the Cd treatment of *B. auriculata*, suggesting that stomatal limitation is not the principal factor affecting photosynthesis. The induction of photorespiration, as suggested by the increase in GOX activity, which could supply endogenous CO_2 for photosynthesis, may contribute to the increase in intercellular CO_2.

In adittion to a reduction in leaf area, the most characteristic phenotype of *B. auriculata* in response to Cd was the red/purple colour of the leaf due to the accumulation of anthocyanins. This is a very specific response because other metals, such as Cu, do not give rise to this phenotype in *B. auriculata* (data not shown). It has been reported that anthocyanins participate in the defence against photoinhibition under environmental conditions (light, temperature and metals) giving rise to an imbalance between light energy and photosynthesis rate and acting as free radical scavengers (Gould et al., 2018). However, anthocyanins can also chelate metals and they could therefore regulate metal homeostasis and thus contribute to the attenuation of metal toxicity (Landi, 2015). The accumulation of anthocyanins in response to contamination with heavy metals has already been described in other species, such as *Biscutella laevigata* (Babst-Kostecka et al., 2016), *Zea mays* (Javed et al., 2017) and *Brassica sp*. (Hale et al., 2002). A close relationship between anthocyanin accumulation and tolerance against heavy metals has been demonstrated in petunia plants overexpressing the transcription factor RsMYB1, from *Raphanus sativus*, which regulates anthocyanin synthesis (Al et al., 2018). The tolerance of the Cd-accumulator genotype CB671 of *Brassica napus* has also been correlated to its ability to preferentially channel flavonoid materials towards anthocyanin production. Despite the increase in anthocyanin content in *B. auriculata*, a reduction in total phenols and carotenoids, with total flavonoids remaining unchanged, was observed in plants exposed to Cd.

4.2 *Biscutella* efficiently accumulate Cd without promoting micronutrient imbalance

Biscutella auriculata accumulated Cd mainly in the roots, although the metal can be efficiently translocated to the leaves. TF and BF are commonly used to determine the effectiveness of a plant in the translocation and bioaccumulation of a

metal, respectively (Melo et al., 2009, Liu et al., 2019). *B. auriculata* had a BF value of 5.9, which is considered characteristic of hyperaccumulating plants and is similar to that of other Cd-hyperaccumulating plants (Supplementary Table 3). A significant proportion of the metal, around 174 mg Kg^{-1} DW, was translocated to the leaves, with a TF of 0.15, in a similar way to other Cd-hyperaccumulators such as *Lonicera japonica*, *Atriplex halimus* and *Iris lactea* (Supplementary Table 3). Based on these data we can consider *B. auriculata* as a Cd accumulator plant.

Cadmium is a divalent cation that can compete with other cations for transporters. Interestingly, the analysis of elements by ICP showed only a significant decrease in Mn concentration in leaves from plants treated with Cd, in contrast to the situation for other species, where Cd treatment led to decreases in Ca, Fe, Mg and even Zn contents (Sandalio et al., 2001, Gomes et al., 2012). NRAMP and ZIP families are transporters involved in Mn^{2+} uptake but they can also transport Cd^{2+} (Peng et al., 2008, Pittman, 2005). Consequently, it is likely that both Cd^{2+} and Mn^{2+} could compete for the same transporter and this may explain the reduction of Mn content in Cd-treated plants.

Concerning the histological distribution and accumulation of Cd, it is interesting to note that a significant proportion of the metal in roots was accumulated outside epidermal cells, which represents an extracellular-trap mechanism in which mucilage, exudates (Javed et al., 2017) or border-like cells can be involved. It has been reported that border cells formation can be induced against heavy metals and could represent a tolerance mechanism for metals and contaminants (Hawes et al., 2016). Cross sections of roots also showed Cd accumulation mainly in vascular tissue. The deposition of Cd in the cell walls of the root is another possibility to accumulate Cd (Huybrechts et al. 2019), as described previously in several species such as *Zea mays* (Javed et al., 2017) and *Oryza sativa* (Xiong et al., 2009) amongst others. From the xylem, Cd can be

translocated to leaves and it is accumulated in the central nerve, trichomes, spongy parenchyma and stomata. Inside trichomes, Cd accumulates in two well-differentiated ring structures that could be associated with vacuoles, although Cd was also accumulated in the base of the trichome. In the metal hyper-accumulator *Arabidopsis halleri*, Zn and Cd are sequestered mainly in a ring of cells around the trichome base (Küpper et al., 2000), while Mn is accumulated in cells at the base of trichomes in *Alysum murale* (McNear and Hupper, 2014) and *Odontarrhena muralis* (do Nascimento et al., 2020).The accumulation of Cd inside the trichrome has already been documented in previous studies on *Biscutella laevigata* suggesting that it could be a mechanism to remove Cd from the interior of the plant to avoid toxicity (Pielichowska and Wierzbicka, 2004). In *Arabidopsis thaliana* Cd-GSH complexes have been observed in trichomes, where the GSH biosynthetic pathway is highly active (Domínguez-Solis et al., 2004) and PC synthase expression has been detected in the same area of the Arabidopsis trichomes, thus supporting the idea that these structures could be the main location for the sequestration of PC–Cd complexes (Lee et al., 2002). In tobacco plants the exclusion of toxic Cd was observed by forming and excreting Cd/Ca-containing crystals through the head cells of trichomes (Choi et al., 2001). Differences observed in metal distribution in trichome could be attributed to the physiological differences between different species (Karabourniotis et al., 2020). The presence of phenolic compounds in trichome cells could contribute to enhancing metal chelating activity in these structures (Karabourniotis et al., 2020). The increase in the number of trichomes per leaf area observed in response to Cd in *B. auriculata* supports the role of these specialized epidermal cells in Cd detoxification and accumulation. The pattern and density of trichomes are highly variable in natural plant populations, suggesting that trichomes play an important role in plant defenses against different biotic and abiotic stress factors (Hauser, 2014). Therefore, increasing trichome density could have great

potential to improve plant metal tolerance, although the molecular basis of trichome patterning in response to heavy metals requires further research. Other benefits of increased trichome density are the prevention of water loss through transpiration and indirectly the regulation of the energy balance and thus of leaf lamina temperatures (Karabourniotis et al., 2020), which could explain the absence of any significant changes in the temperature of *Biscutela auriculata* leaves exposed to Cd.

In cross sections of *Biscutellla auriculata* leaves the highest Cd accumulation was found in the spongy parenchyma and stomata/substomatal chamber, probably due to the transpiration stream. Zn and Cd in mesophyll cells have also been reported to accumulate in *Arabidopsis halleri* with high concentrations of Cd (Küpper et al., 2000), while Cd has been reported to accumulate in the abaxial leaf epidermis and stomata of *Thlaspi caerulescens* (Wójcik et al., 2005).

4.3 Biothiols content in response to Cd treatment

The content of biothiols increased considerably upon Cd treatment in *B. auriculata* leaves and roots, with phytochelatins PC2 and PC3 being the most abundant biothiols. Neither PC1 nor PC4 were observed. PC production is one of the most important mechanisms of Cd detoxification in plants and their synthesis is induced by an increase in the intracellular Cd concentration (Wojcik and Tukiendorf, 2004), as reported for several species such as *Vignia radiata* (Nahar et al., 2016), *Medicago sativa* (Flores-Cáceres et al., 2015) and *Arabidopsis thaliana* (Wojcik and Tukiendorf, 2004). The sensitivity of some *Arabidopsis thaliana* mutants to Cd and other metals has been associated with PC deficiency (Howden et al., 1995) and the tolerance and metal hyperaccumulation characteristics of different plant species have been associated with their ability to produce PCs (Supplementary Table 3). The antioxidant GSH plays an important role in several physiological processes, including signal transduction,

conjugation of metabolites, detoxification, as a substrate for enzymatic antioxidants and in the regulation of stress-responsive gene expression (Ali et al., 2019), but it is also the precursor of phytochelatins (Yadav, 2010). A significant increase in the concentration of GSH was observed in *B. auriculata* leaves in response to Cd treatment, which in addition to contributing to phytochelatin production could participate directly in chelating Cd, as reported for other plant species in response to different metals such Cd (Domínguez-Solis et al., 2004) and As (Souri et al., 2020) and improving antioxidant defenses. *Arabidopsis* mutants overexpressing the enzyme adenosine 5′-phosphosulfate reductase 2 (APR2), which plays an important role in the reductive sulfate assimilation pathway, positively regulated Cd tolerance via the glutathione-dependent pathway, thus improving GSH-dependent antioxidant capability and Cd-chelation by PCs (Xu et al., 2020). The conjugation of metals and xenobiotics to GSH is governed by GST (Edwards and Dixon, 2005), which explains the increase in GST activity observed in *B. auriculata* exposed to Cd. Both PCs and GSH-metal complexes could therefore contribute to the storage of metals in the cell vacuole and trichomes to prevent Cd toxicity.

4.4 Cadmium promotes differential effects on oxidative stress and antioxidative defences in leaves and roots

One of the primary events taking place in response to Cd and other metals is the production of ROS. It has recently been reported that after 15 minutes of Cd treatment *Arabidopsis thaliana* cells experienced changes in peroxisome dynamics, which were driven by ROS, and one hour after Cd treatment the induction of ROS-dependent genes such as GST and RRTF1 was observed (Rodríguez-Serrano et al., 2016). However, long-term Cd treatment triggers an increase in ROS accumulation and this causes oxidative damage to proteins, lipids and DNA (Ali et al., 2019). In *B. auriculata* plants

Cd induced oxidative stress in leaves but not in roots, as suggested by the accumulation of ROS and MDA, a marker of lipid peroxidation. Oxidative stress is a common response to Cd in plants (Sandalio et al., 2001, Kaur et al., 2019, Liu et al., 2019, Huihui et al., 2020, Çatav et al., 2020). NADPH oxidase associated with the plasma membrane is one of the most important sources of ROS under Cd stress, as reported in *Arabidopsis* (Remans et al., 2010) and *Pisum sativum* (Romero-Puertas et al., 2004), but Cd can also boost ROS generation by altering the electron transport chain in mitochondria and chloroplast (Heyno et al., 2008, Keunen et al., 2011). However, glycolate oxidase, a key enzyme of photorespiration, could also contribute, as observed in this work and previous work carried out on *Arabidospsis thaliana* (Gupta et al., 2017), *Glycine max* (Pérez-Chaca et al., 2014) and *Pisum sativum* (Romero-Puertas et al., 2004), amongst others (Sandalio et al., 2012).

In an effort to understand the oxidative stress observed in leaves we analysed the activity of antioxidative enzymes such as SOD, which constitute the first line of defence against $O_2^{\cdot -}$ and give rise to H_2O_2 and O_2 (You and Chan, 2015). Four SOD isoforms were detected in leaves, and three of them almost disappeared in response to Cd. Similar results were obtained in *Pisum sativum* where the inactivation of Mn-SOD by oxidative modification and the downregulation of predominant CuZn-SOD gene expression were observed (Romero-Puertas et al., 2007). However, a Cd-dependent increase of SOD activity in many plant species has been reported in response to Cd: *Brassica juncea* (Kaur et al., 2019), *Mentha arvensis* (Zaid et al., 2020, *Triticum aestivum* (Çatav et al., 2020), while changes in the activity of SOD were not observed in *Arabidopsis thaliana* plants exposed to low Cd concentration (Smeets et al., 2009). The differences observed could be due to the experimental conditions, Cd concentration, period of treatment and/or the specific strategies used for the different species against the metal (Sandalio et

al., 2012). Catalase activity did not change in leaves while it increased in roots, where peroxidase activity was also higher than in leaves, thus suggesting that roots, as opposed to leaves, are quite effective in removing H_2O_2. CAT activity has been observed to decrease under Cd-treatment, for example, in *Pisum sativum* (Sandalio et al., 2001), *Brassica napus* (Nouairi et al., 2009) and *Triticum aestivum* (Çatav et al., 2020), while an increase was observed in *Brassica juncea* (Nouairi et al., 2009), Mentha arvensis (Zaid et al., 2020) and *Lantana camara (*Liu et al., 2019*).* The total antioxidant capacity in roots also increases in *B. auriculata* plants treated with Cd, probably due to the increase of GSH and AsA, amongst other factors. In leaves the ascorbate-glutathione cycle could be activated in response to Cd as a result of the increase of DHAR and MDHAR activities, as well as the GSH content. However, the activity of two components of this cycle, APX and GR, could not be analysed in the extracts, although Western blot of GR demonstrated an increase of GR protein. Inactivation of GR in plants has been demonstrated under in vitro conditions in response to phenols (Zhang et al., 1997) and flavonoids (Elliott et al., 1992). Given its central role in maintaining the cellular redox state, the GSH/GSSG ratio can be considered to be an indicator of plant oxidative stress (Foyer and Noctor, 2005), in this study the higher rate observed in the leaves of *B. auriculata* indicate a functional GR. The functionality of the AsA-GSH cycle in leaves could be supported by the increase of different NADP-dehydrogenase activities, i.e., NADP-G6PDH, NADP-IDH and NADP-MS, which supply the reduction power needed for the AsA-GSH cycle. This set of enzymes would play an important role in plant response to Cd toxicity (*León et al., 2002*) and the increase in their activity has been reported to be associated with Cd tolerance in different pepper cultivars and soybean plants (León et al., 2002, Pérez-Chaca et al., 2014).

Ascorbate (AsA) is considered to be a potent antioxidant because of its capacity to donate electrons in a large number of enzymatic and non-enzymatic reactions. AsA acts as an antioxidant and participates in different physiological processes of great relevance in plants, such as growth and differentiation (Ali et al., 2019). The AsA content was enhanced in *B. auriculata* roots treated with Cd, while in leaves significant differences were not observed. The increase of AsA in Cd-treated plants was described in leaves of *Bechmeria nivea* (Liu et al., 2007) and *Hibiscus cannabinus* (Deng et al., 2017) and roots of *Pisum sativum* (Rodríguez-Serrano et al., 2006) and *Phragmites australis* (Iannelli et al., 2002). The decline in ascorbic acid content has been associated with cadmium toxicity in rice seedlings and pretreatment with AsA reduced the subsequent Cd-induced toxicity in this species (Chao et al., 2010). The sensitivity of the *Brassica napus* genotype ZD622 to Cd is associated, among other factors, with the decrease in ascorbate levels (Mwamba et al, 2020), demonstrating the important role played by this antioxidant in Cd tolerance.

The GST enzymes are a large and diverse group of enzymes that catalyse the conjugation of electrophilic xenobiotic substrates with GSH and they remove cytotoxic or genotoxic compounds that can damage the cell (Kumar and Trivedi, 2018). In *B. auriculata* the activity of GST showed an increase in response to Cd-treatment in root, while in leaves this activity remained unchanged. The increase in GST activity in response to Cd treatment has also been observed in *Brassica juncea* (Kaur et al., 2019) and *Vignia radiata* (Nahar et al., 2016). GST overexpression enhances tolerance to Cd in rice (Zhao et al., 2009). In maize plants it has been reported that there is a close relationship between anthocyanins and one type of GST (BZ-2), which catalyses the formation of anthocyanin-GSH conjugates and allows its transport to the vacuoles (Marrs et al., 1995, Alfenito et al., 1998). In *B. auriculata* we observed an increase in

both GST activity and anthocyanin content in response to Cd; consequently, it is likely that in addition to other functions, the GST has an important role in the accumulation of anthocyanins in Cd-treated plants. However, one must remember that GST can also prevent oxidative stress by acting on organic hydroperoxides formed during oxidative stress as a glutathione peroxidase (Kumar and Trivedi, 2018). The total peroxidase activity, analysed as guaiacol peroxidase, showed an increase in response to Cd in both roots and leaves and therefore this could contribute to the antioxidative defences; however, peroxidases also participate in lignin biosynthesis, which is an important physiological defence against toxic heavy metals (Hegedüs et al., 2001). The induction of POD activity has been observed as a general response of higher plants to Cd (Sandalio et al., 2001, Kaur et al., 2019, Liu et al., 2019, Zaid et al., 2020, Çatav et al., 2020).

The production of endogenous NO in response to heavy metals in plants has been extensively analysed, with NO increasing under short periods of treatment while long-term Cd treatment and high concentrations of metals give rise to a reduction of this molecule (Xiong et al., 2009, Sandalio et al., 2012, Pérez-Chaca et al., 2014, Romero-Puertas et al., 2018; Terrón-Camero et al., 2019). Several studies have shown that NO-dependent post-transcriptional modifications of proteins can regulate ROS metabolism, phytochelatins and proteolysis processes in plant response to Cd and other heavy metals (Terrón-Camero et al., 2019, Souri et al., 2020). In our study, long term Cd treatment produced a reduction in the NO content in *B. auriculata* roots but changes were not observed in leaves, which suggest a differential regulation of NO in both organs. This result is supported by the changes observed in the pattern of GSNOR activity, which regulates GSNO levels that is considered a natural reservoir of NO. Thus, GSNOR activity decreased in leaves but it did not change in roots. Several studies have produced

controversial results concerning GSNOR activity in response to Cd and therefore its role is not clear (Wang et al., 2015, Hu et al., 2019). However, the importance of GSNOR in metal response has been demonstrated using *gsnor1-3* Arabidopsis mutants, which are more susceptible to Cu toxicity (Petö et al., 2013).

5. CONCLUSIONS

Biscutella auriculata, despite suffering a growth reduction, can survive under high concentrations of Cd due to its ability to develop strategies to efficiently accumulate and tolerate Cd to withstand its toxicity. One of these strategies is related to the ability to trap the metal outside of root by mucilage or border-like cells, and inside the tissue by sequestering Cd in phytochelatins and probably by inducing GSH-complex to be stored in the vacuoles. The large accumulation of Cd observed in trichomes and stomata, as well as the changes observed in both their densities, suggest that these structures could also be involved in the metal tolerance strategy of *B. auriculata*. The differential regulation of antioxidant defences in roots and leaves to cope with oxidative damage induced by Cd, as well as the GSH and ASC components of the ascorbate-glutathione cycle, play a central role in this strategy. The induction of anthocyanins can be considered as a specific mechanism of *B. auriculata* which participates in both Cd accumulation and antioxidative responses. Our findings show that *B. auriculata* is a new Cd-tolerant and Cd-accumulating plant, which, given its capacity to grow in soils contaminated by multiple pollutants could be used effectively in the restoration of Cd-contaminated soils.

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Table 1. Effect of Cd on growth and phenotype.

	0 μM $Cd(NO_3)_2$	125 μM $Cd(NO_3)_2$
Leaves FW (g)	1.79 ± 0.15	0.98 ± 0.09***
Root FW (g)	0.36 ± 0.03	0.24 ± 0.02**
Leaves area (cm^2)	57.11 ± 4.98	29.75 ± 2.64***
Leaf number	9.45 ± 0.34	9.5 ± 0.34
Number of trichomes/mm^2	22.37 ± 3.03	34.64 ± 3.27**
Number of stomata/mm^2	189.86 ± 9.19	291 ± 16.71***
Chlorophyll a (μg g^{-1} FW)	1017.82 ± 68.28	284.25 ± 38.04***
Chlorophyll b (μg g^{-1} FW)	611.77 ± 78.65	150.33 ± 26.23***
Total chlorophyll (μg g^{-1} FW)	1629.59 ± 135.07	434.58 ± 56.43***
Carotenoids (μg g^{-1} FW)	28152.15 ± 4161.58	11547.01 ± 2029.12**
Phenols (μg galic acid g^{-1} FW)	2171.34 ± 183.25	1545.67 ±132.97*
Flavonoids (mg quercetin g^{-1} FW)	112.57 ± 8.02	112.01 ± 6.47
Anthocyanins (μg g^{-1} FW)	1.17 ± 0,17	42.91 ± 4.06***

Asterisk indicates that the mean value is significantly different between treatments and controls (±standard error) (*$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$).

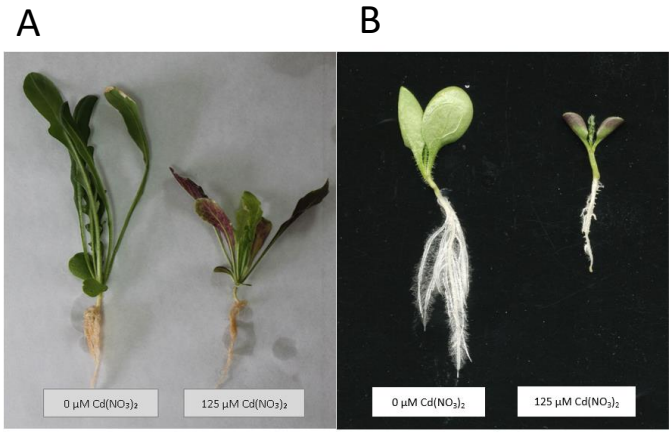

Figure 1. *B. auriculata* seedlings grown in hydroponic culture treated with 0 and 125 µM $Cd(NO_3)_2$, after 30 days (**A**) and grown in square Petri dishes, after 10 days (**B**).

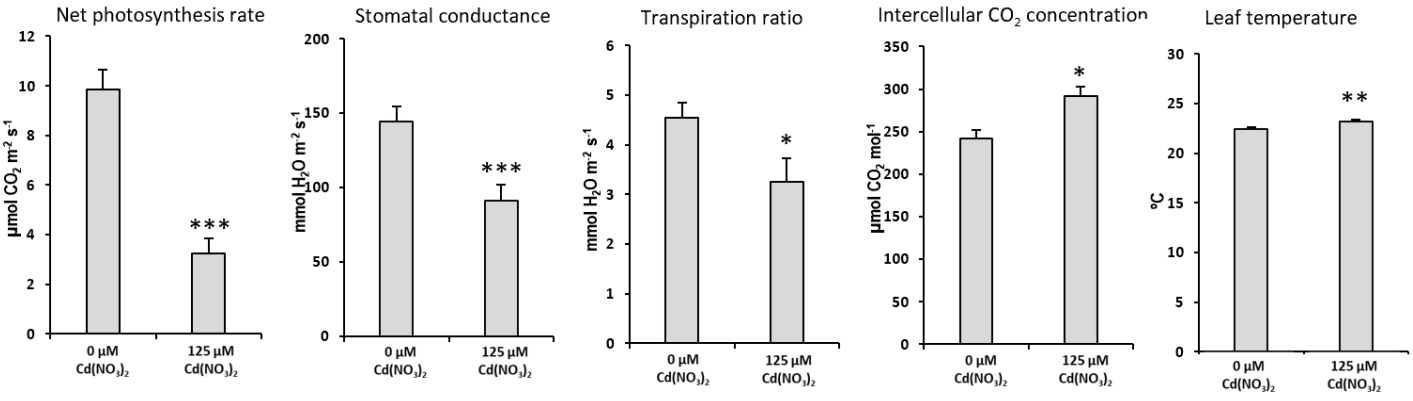

Figure 2. Net photosynthetic rate, stomatal conductance, transpiration ratio, intercellular CO_2 concentration and leaf temperature in leaves of *B. auriculata* treated with 0 and 125 µM $Cd(NO_3)_2$. Values represent the mean ± standard error and asterisks indicates significant differences between treatments and controls (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

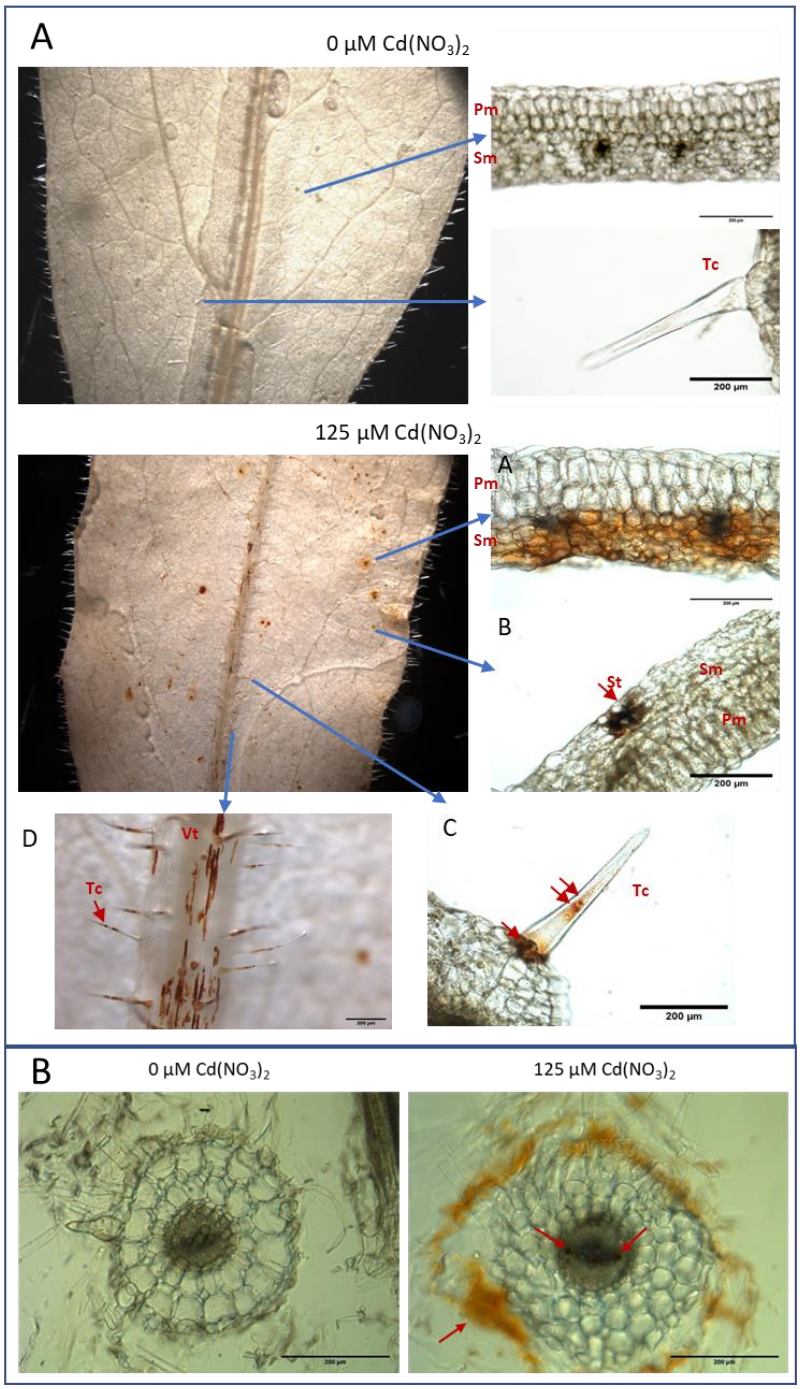

Figure 3. Histochemical localization of Cd in leaves **(A)** and roots **(B)** of plants treated with or without Cd $(NO_3)_2$. Cd is visualized using dithizone staining (brown colour). Palisade mesophyll (Pm), spongy mesophyll (Sm), vascular tissue (Vt), stomata (St) and trichomes (Tc). Red arrows show Cd localization in leaves (**A**) and roots (**B**). Blue arrows show higher magnification of cross sections.

A

	0 µM Cd $(NO_3)_2$		125 µM Cd $(NO_3)_2$	
	Leaves	Root	Leaves	Root
GSH (µg g^{-1} FW)	40.233 ± 1.028	38.755 ± 3.299	60.433 ± 4.711*	36.358 ± 4.409
GSSG (µg g^{-1} FW)	8.150 ± 1.333	4.633 ± 1.199	1.883 ± 0.522*	2.867 ± 0.846
PC2 (µg g^{-1} FW)	< LD	10.469 ± 0.249	196.4 ± 46.863***	147.175 ± 20.423***
PC3 (µg g^{-1} FW)	< LD	< LD	129.15 ± 27.521***	118.508 ± 29.96***

B

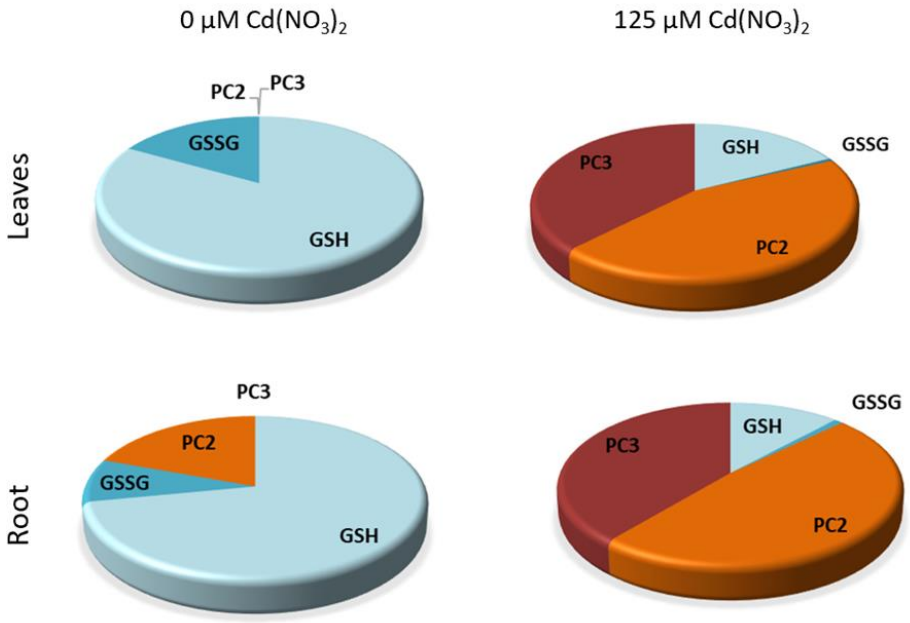

Figure 4. Biothiols content (**A**) and relative biothiols concentration (%) (**B**) in Cd-treated and Cd-untreated leaves and roots from *B. auriculata*. Values represent the mean ± standard error and asterisks indicates significant differences between treatment and control plants (*$p \leq 0.05$; *** $p \leq 0.001$).

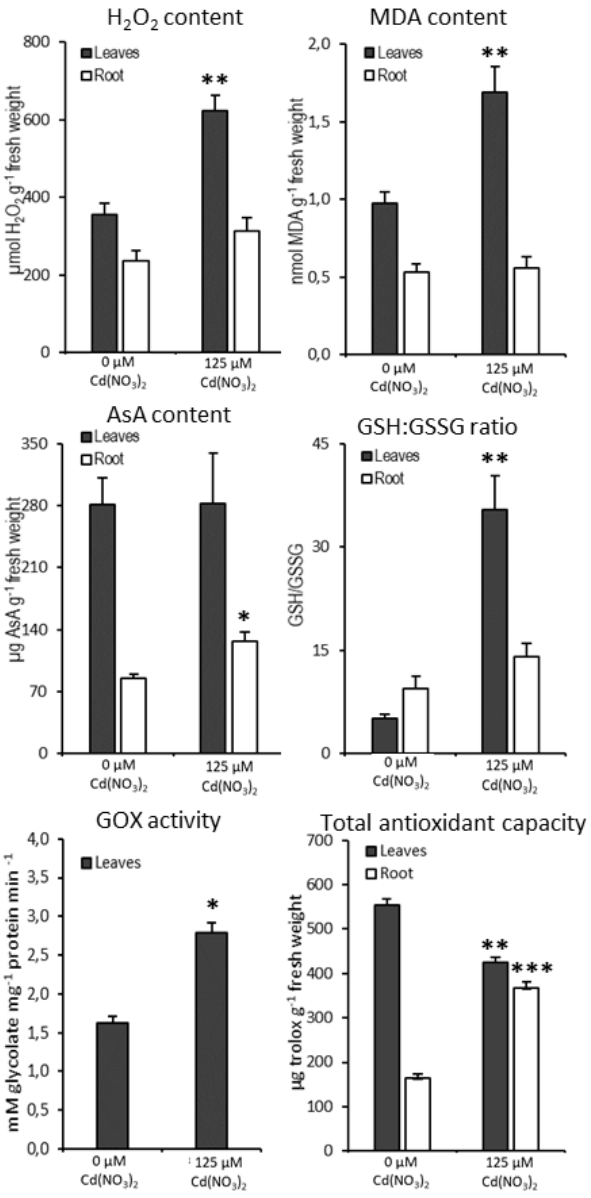

Figure 5. Effects of $Cd(NO_3)_2$ on H_2O_2 content , lipid peroxidation, AsA content, GSH/GSSG ratio, GOX activity and total antioxidant capacity of *B. auriculata* leaves and roots. Values represent the mean ± standard error and asterisks indicates significant differences between treatment and control plants (*$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$).

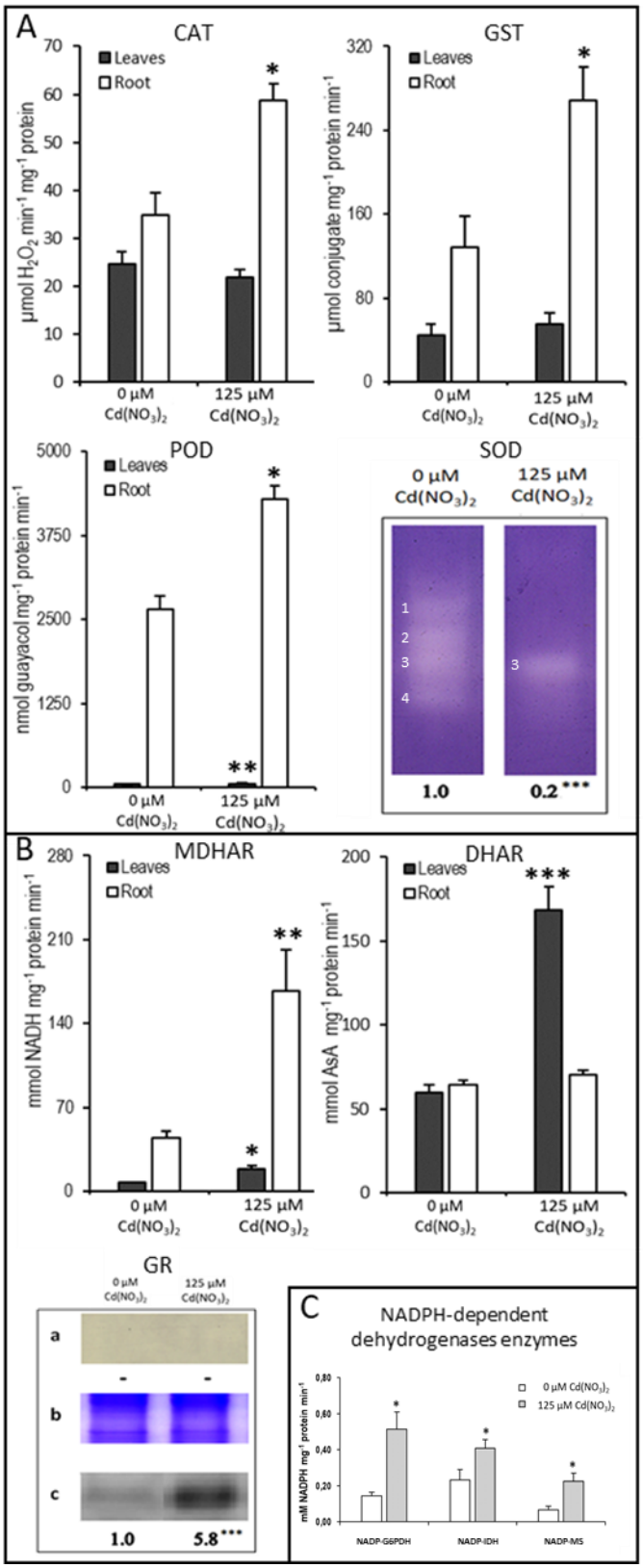
A
CAT
Leaves
Root
µmol H_2O_2 min^{-1} mg^{-1} protein
0 µM $Cd(NO_3)_2$
125 µM $Cd(NO_3)_2$
GST
µmol conjugate mg^{-1} protein min^{-1}
POD
nmol guayacol mg^{-1} protein min^{-1}
SOD
1.0
0.2 ***
B
MDHAR
mmol NADH mg^{-1} protein min^{-1}
DHAR
mmol AsA mg^{-1} protein min^{-1}
GR
a
b
c
1.0
5.8 ***
C
NADPH-dependent dehydrogenases enzymes
mM NADPH mg^{-1} protein min^{-1}
NADP-G6PDH
NADP-IDH
NADP-ME

Figure 6. Effects of $Cd(NO_3)_2$ on enzymatic antioxidants (A and B) and NADP-dependent dehydrogenases (C) of *Biscutella auriculata* leaves and roots. CAT, catalase activity; GST, glutathione S-transferase activity; POD, peroxidase activity; SOD, superoxide dismutase activity; MDHAR, monodehydroascorbate reductase enzyme; DHAR, dehydroascorbate reductase enzyme; GR, glutathione reductase; NADP-G6PDH, $NADP^+$-dependent glucose-6-phosphate dehydrogenase enzyme; NADP-IDH, $NADP^+$-dependent isocitrate dehydrogenase enzyme; NADP-MS, $NADP^+$-dependent malate dehydrogenase. Values represent the mean ± standard error and asterisks indicates significant differences between treatment and control plants (*$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$).