Postprint
Chemosphere (2020) Vol. 261, art. no. 127721

Deciphering lead tolerance mechanisms in a population of the plant species *Biscutella auriculata* L. from a mining area: accumulation strategies and antioxidant defenses

Peco, J.D.[1,2]; Higueras, P.[2]; Campos, J.A.[1], Olmedilla, A.[3]; Romero-Puertas, M.C.[3] and Sandalio, L.M.[3]

1- Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Castilla-La Mancha (UCLM), Ronda de Calatrava, 7, 13071, Ciudad Real, Spain.

2- Instituto de Geología Aplicada, Universidad de Castilla-La Mancha (UCLM), Plaza de Manuel Meca, 1, 13400, Almadén, Ciudad Real, Spain.

3- Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Apartado 419, E-18080, Granada, Spain.

HIGHLIGHTS

- *Biscutella auriculata* tolerates high concentration of Pb without toxic symptoms being observed.
- Pb is mainly sequestered by PC2 and accumulated in the root cell wall and the vacuoles.
- Differential activation of antioxidant defenses was induced by Pb in leaves and roots.

Deciphering lead tolerance mechanisms in a population of the plant species *Biscutella auriculata* L. from a mining area: accumulation strategies and antioxidant defenses

Peco, J.D.[1,2]; Higueras, P.[2]; Campos, J.A.[1], Olmedilla, A.[3]; Romero-Puertas, M.C.[3] and Sandalio, L.M.[3]

1- Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Castilla-La Mancha (UCLM), Ronda de Calatrava, 7, 13071, Ciudad Real, Spain.

2- Instituto de Geología Aplicada, Universidad de Castilla-La Mancha (UCLM), Plaza de Manuel Meca, 1, 13400, Almadén, Ciudad Real, Spain.

3- Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Apartado 419, E-18080, Granada, Spain.

ABBREVIATIONS

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

ABSTRACT

The uptake and distribution of Pb and the mechanisms involved in the metal tolerance have been investigated in a mine population of *Biscutella auriculata*. Seedlings were exposed to 125 μM $Pb(NO_3)_2$ for 15 days under semihydroponic conditions. The results showed an increase in the size of Pb-treated seedlings and symptoms of toxicity were not observed. ICP-OES analyses showed that Pb accumulation was restricted to root tissue. Imaging of Pb accumulation by dithizone histochemistry revealed the presence of the metal in vacuoles and cell wall in root cells. The accumulation of Pb in vacuoles could be stimulated by an increase in phytochelatin PC2 content. Pb did not promote oxidative damage and this is probably due the increase of antioxidative defenses. In the leaves, Pb produced a significant increase in superoxide dismutase activity, while in roots an increase in catalase and components of the Foyer– Halliwell–Asada cycle were observed. The results indicated that *Biscutella auriculata* has a high capacity to tolerate Pb and this is mainly due to a very efficient mechanism to sequester the metal in roots and a capacity to avoid oxidative stress. This species could therefore be very useful for phytostabilization and repopulation of areas contaminated with Pb.

Keywords: Phytoremediation, Oxidative Stress, ROS, Phytochelatin, *Biscutella auriculata*

1. INTRODUCTION

Lead (Pb) is a potent heavy metal pollutant that is toxic for living organisms and has prolonged persistence in the environment due to its non-biodegradable nature (Wuana and Okieimen, 2014). The occurrence of Pb in the environment mainly results from mining, metallurgy, manufacturing and recycling activities and, in some countries, from the persistent use of Pb-based paints and leaded gasolines (Benavides et al., 2005; Kumar and Kumari, 2015). Pb persists in the soil and causes multiple direct and indirect toxic effects on plant growth and metabolism (Whitacre, 2015; Kumar and Prasad, 2018). High concentrations of Pb in the soil inhibit the germination of seeds and reduce plant growth due to the severe alteration of different metabolic pathways, including photosynthesis and transpiration, hormone balance, membrane permeability, mineral nutrition, ATP production and the promotion of oxidative damage due to the increased production of reactive oxygen species (ROS) (Kumar and Prasad, 2018).

Phytoremediation is a clean-up process that is effective, inexpensive and environmentally friendly for the remediation of metal-contaminated soils by using plants (Kidd et al., 2009; Dickinson, 2016). There are several different decontamination techniques within phytoremediation, namely phytofiltration, phytovolatilization, phytodegradation, phytoextraction and phytostabilization (Ali et al., 2013). Phytoextraction involves extraction of the metal from the soil by absorbing it in metal-accumulating plants and this approach is considered to be the main and most useful technique for the removal heavy metals and metalloids (Kumar and Prasad, 2018). The efficiency of phytoextraction depends on numerous factors such as the heavy metal characteristics and bioavailability, soil properties and plant species (Ali et al., 2013). Plants usually show little tolerance to the presence of heavy metals and they do not usually accumulate such metals within their tissues in appreciable amounts, but some

plants develop resilience mechanisms to overcome the restraints caused by the presence of high concentrations of pollutants. A thorough study of plant tolerance strategies is crucial to address the rehabilitation of degraded land (Cutright et al., 2012; Dickinson, 2016).

Different mechanisms for Pb tolerance and accumulation have been reported for plants (Kumar and Prasad, 2018). The most tolerant species accumulate around 95% of the absorbed Pb in root and only a small proportion is translocated to aerial parts of the plant (Ruiz et al., 2009; Gupta et al., 2009; Whitacre, 2015). Phytochelatin (PC) synthesis is a mechanism induced by Pb to neutralize and accumulate the metals in the vacuole (Andra et al., 2009; Wojas et al., 2010; Fischer et al., 2014; García et al., 2017). The metal accumulation capacity of plants is also affected by their ability to survive the oxidative stress caused by the production of reactive oxygen species (ROS) during Pb exposure (Sytar et al., 2013). Plants have complex enzymatic and non-enzymatic antioxidative defenses to maintain ROS levels that are compatible with the regular functioning of the cells (Romero-Puertas et al., 2018). The main enzymatic antioxidative defenses are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPOX), NADP-dependent dehydrogenases, glutathione S-transferase (GST) and the enzymes of the Foyer–Halliwell–Asada Cycle (ascorbate peroxidase [APX], monodehydroascorbate reductase [MDHAR], dehydroascorbate reductase [DHAR] and glutathione reductase [GR]) (Sandalio et al., 2012). Non-enzymatic components of the antioxidative defense system include ascorbate (AsA), glutathione (GSH), tocopherol, carotenoids and phenolic compounds, amongst others (Sharma et al., 2012). It has been reported that Pb exposure induces changes in antioxidant systems either by overexpressing or downregulating antioxidants to avoid damage caused by ROS production (Kumar and Prasad, 2018). Nitric oxide (NO) is a simple molecule

which acts as regulator of many physiological processes in plants, including the defence against heavy metal (Gill et al., 2013; Terrón-Camero et al., 2019; Terrón-Camero et al., 2020). It has been reported that NO can prevent oxidative damages by improving antioxidant defences and therefore it could be a key factor in the tolerance against heavy metals (Romero-Puertas et al., 2018; Souri et al., 2020).

Metal hypertolerance and hyperaccumulation are found in a large number of plant families, with the highest occurrence in the *Brassicaceae* family (Mohtadi et al., 2012). *Biscutella* is a genus herbal member of the *Brassicaceae* family that grows in areas bordering agricultural fields, roadsides and polluted areas (Peco et al., 2020). The species *Biscutella laevigata* has already been described as an accumulator with a tolerance to the metals thallium (LaCoste et al., 1999; 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). A population of *Biscutella auriculata* has been recently identified as one of the few species growing in a multimetal (Cu, Zn, Pb and Cd) contaminated area close to the San Quintin mine area located in Ciudad Real (Spain). This species has been reported as a Cd-tolerant plant which efficiently accumulates this metal in roots and trichomes and differentially regulate antioxidant damage prevention in roots and leaves (Peco et al., 2020). Given that Pb is one of the metals present in the soil from which the seeds were taken, we investigated Pb tolerance in *B. auriculata* and the mechanisms involved. The study therefore focuses on the effects of Pb on growth parameters, ROS and NO metabolism, Pb accumulation patterns, as well as leaf and root mineral status. Our results demonstrate that *B. auriculata* is a new Pb-tolerant growth-enhancing plant in the presence of high concentrations of Pb, which could be useful in restoring areas contaminated by this heavy metal.

2. MATERIAL AND METHODS

2.1 Plant materials and growth conditions

B. auriculata seeds were obtained from a natural population located in the San Quintin mining area (38°48'52.6"N 4°17'15.5"W) in Villamayor de Calatrava (Ciudad Real province, South Central Spain) (Supplementary Fig. 1A and B). This area has been altered by mining activity (Pb-Zn-Ag mine) and is contaminated Pb, Zn, Cu and Cd (Rodríguez et al., 2009). The seeds were hydrated for 24 h and then germinated on wet filter paper in Petri dishes at 25 ºC. Healthy homogenous seedlings were transferred to a semi-hydroponic system containing perlite (Flores-Cáceres et al., 2015) and a Hoagland nutrient solution (Hoagland and Arnon, 1950) for 15 days. After this period, seedlings were then irrigated with Hoagland nutrient solution supplemented with 0 or 125 µM of $Pb(NO_3)_2$ for 15 days (Pereira et al., 2016). Cultures were placed in a growth chamber under the following conditions: 24 ºC, 60% relativity humidity and 16/8 h light/dark photoperiod. The experiment was repeated three times. Finally, leaves and roots were processed separately, frozen in liquid nitrogen and stored at –80 ºC. Sterilized seeds were also germinated and were grown vertically in square Petri dishes (10 × 10 cm) containing MS medium supplemented with 0 or 125 µM of $Pb(NO_3)_2$ for 10 days (Sanz-Fernández et al., 2017). These plants were grown under the same growth chamber conditions as previously mentioned.

2.2 Growth parameters, photosynthesis data, photosynthetic pigments, phenolics and flavonoids content

Morphological and growth parameters were analyzed: leaf and root fresh weight, number of leaves and root length. Leaf area, trichomes and stomata density were analyzed from leaf images using ImageJ software. Gas exchange parameters (net photosynthesis rate, stomatal conductance, transpiration ratio and intercellular CO_2

concentration) were determined using a portable photosynthesis system (Ciras-3, PP Systems). Contents of chlorophylls, carotenoids and anthocyanins were analyzed by spectrophotometric methods according to Lichtenthaler and Buschmann (2001) and Sims and Gamon (2002), respectively. Total phenolics content was determined by a spectrophotometric technique according to Folin–Ciocalteau's method proposed by 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.

2.3 Mineral analysis and histochemical localization of Pb

In order to assess the mineral contents, plant samples were oven-dried at 60 ºC for 72 hours. Roots and leaves were weighed to determine the dry mass and were digested with an HNO_3/H_2O_2 mixture using a microwave digestion system (ETHOS 1, Milestone). Mineral composition was measured by inductively coupled plasma-optical emission spectrometry (ICP-OES, Varian 720-ES). The phytoextraction ability was determined using the translocation factor (TF) and the bioaccumulation factor (BF) equations described by Melo et al. (2009).

Pb storage was detected histochemically using the dithizone method described by Seregin and Kozhevnikova (2011). Leaves and roots were incubated in a solution of dithizone in acetone, glacial acetic acid and Milli-Q water (3:5:1) and incubated for one hour. Leaves were bleached by immersion in boiling ethanol. Stained roots were embedded in 5% low-melting agarose D1 EEO (Conda Pronadise) and cross-sections were obtained using a vibratome (VT1200/VT1200S Leica) and examined by optical microscopy (Leica DMI600B).

2.4 Quantification of glutathione, ascorbate and phytochelatins

Reduced and oxidized glutathione (GSH and GSSG) and total ascorbate (AsA) were determined by liquid chromatography-electrospray/mass spectrometry (LC-ES/MS) using a method described by El-Zohri et al. (2005). Plant extracts were prepared in HCl and analyzed on an HPLC system (H-Class, Waters, Mildford) coupled to a triple quadrupole mass spectrometer (Quattro-Micro, Waters, Mildford). PC2 and PC3 contents were analyzed in the same extracts by following the procedure described by El-Zohri et al. (2005). The supernatant was injected onto an Xselect CSH column (100 mm × 2.1 mm, Waters, Mildford) with a Vanguard Xselect CSH C18 cartridge (5 mm × 2.1 mm, Waters, Mildford). The results were calculated using pattern on PC2, PC3 and PC4 from Pepmic Co., Ltd (Suzhou, China). Biothiols and AsA were determined by multiple reaction monitoring (MRM) using positive electrospray and negative electrospray.

2.5 Lipid peroxidation, H_2O_2 content, NO content and total antioxidant capacity

Lipid peroxidation was determined in terms of malondialdehyde (MDA) concentration according to the method described by Buege and Aust (1978). The results were calculated using a calibration curve for MDA. H_2O_2 accumulation was analyzed by a spectrofluorometric method as described by Romero-Puertas et al. (2004). The results were calculated using a calibration curve for H_2O_2. NO content was determined by a spectrofluorometric method as described by Nakatsubo et al. (1988), using 4,5-diamino-fluorescein (DAF-2) and results are expressed in arbitrary fluorescence units. Total antioxidant capacity was determined according to the ABTS assay (Jiménez-Escrig et al., 2003) and the results were calculated using a Trolox calibration curve.

205 **2.6 Enzymatic assays**

206 Leaves and roots were ground in liquid nitrogen and the powder obtained was
207 homogenized in 0.1 M Tris-HCl pH 7.5, containing 0.1 mM EDTA, 0.2 % Triton X100,
208 2 mM DTT, 0.2 % PVP and 1X protease inhibitor cocktail (Sigma-Aldrich). The
209 homogenates were centrifuged at 14000 g for 20 min. Enzymatic activity was assayed
210 spectrophotometrically according to the following methods: CAT activity (EC
211 1.11.1.6); POD activity; GOX activity (EC 1.1.3.1); APX activity (EC 1.11.1.11), GR
212 activity (EC 1.6.4.2); MDHAR activity (EC 1.6.5.4) and DHAR activity (EC 1.8.5.1) as
213 reported by Hafsi et al. (2010); NADP-G6PDH (EC 1.1.1.49), NADP-IDH (EC
214 1.1.1.42) and NADP-MS (EC 4.1.3.2) as reported by León et al. (2002); GST activity
215 (EC 2.5.1.1) as described by Habig et al. (1974) and GSNOR (EC 1.2.1.46) as described
216 by Ortega-Galisteo et al. (2012). The enzymatic assays were carried out according to the
217 method described by Peco et al. (2020).

218 **2.7 Characterization of SOD isoenzyme and activity**

219 SOD activity (EC 1.15.1) was assayed by native polyacrylamide gel
220 electrophoresis (native-PAGE, 10% acrylamide/Bis) and activity was imaged in the gels
221 according to the photochemical method described by Beauchamp and Fridovich (1971).
222 The effects of 5 mM H_2O_2 and 2 mM KCN on SOD activity were evaluated to identify
223 the different SOD isoenzymes (Srivalli and Khanna-Chopra, 2001). The activity was
224 expressed as % of total SOD activity by determining the area under the peaks using
225 ImageJ. The different SOD bands obtained (Supplementary Fig. 2A) were analyzed by
226 MALDI-TOF mass spectrometry (UltrafleXtrem, Bruker). The sequences obtained were
227 compared with those found in UniProt for Fe SOD, Mn SOD and CuZn SOD of
228 *Arabidopsis thaliana*. Theoretical digestions of proteins, belonging to the three SOD
229 bands, were carried out while keeping the peptides corresponding to the conserved areas

obtained from the isoenzyme's alignment. Finally, peptides belonging to the different SOD isoenzymes were located in the mass spectrum using a MASCOT software.

2.8 Histochemical localization of H_2O_2 and $O_2^{\cdot -}$

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

2.9 Other assays

The protein contents in plant extracts were determined according to the method of Bradford (1976) using a bovine serum albumin (BSA) calibration curve.

2.10 Data analysis

Statistical analyses were carried out by a Student's t-test in IBM SPSS Statistics 24. Asterisks ($P<0.05$: *; $P<0.01$: **; $P<0.001$: ***) represent the level of significance in the figures. Images were analyzed using ImageJ software.

3. RESULTS

3.1 Effect of lead on plant growth and photosynthetic parameters

The effects of Pb on the growth and phenotype of *B. auriculata* plants growing in a semi-hydroponic medium are represented in Fig. 1A and Fig. 1C. Pb exposure for 15 days produced a significant increase in the FW of leaves and roots (1.4 fold), as well as in the leaf area (1.17 fold). However, changes were not observed in the number of leaves. The number of trichomes per area decreased in Pb-treated plants (1.7 fold), while the number of stomata per area increased in response to Pb-treatment (1.7 fold) (Fig 1C). Seedlings grown in square Petri dishes are shown in Fig. 1B and it can be observed that Pb induced the growth of the main root, while the secondary roots and hair roots were considerably reduced in number and size with respect to the control plants (Fig. 1B and Fig. 1D). Interestingly, Pb did not statistically affect the photosynthesis parameters, net photosynthesis rate, stomatal conductance, transpiration ratio, intercellular CO_2 concentration and leaf temperature (Supplementary Table 1). Pb exposure did not affect the chlorophylls and carotenoids contents, which remained unchanged, while the phenols content decreased slightly and the opposite trend was observed for the flavonoids content (Supplementary Table 1).

3.2 Lead uptake and accumulation

The mineral contents of roots and leaves in *B. auriculata* treated and untreated with Pb are shown in Supplementary Table 2. Pb was mainly accumulated in roots (3.686 mg g^{-1}) with a bioaccumulation factor of 1.081 and only a small content was translocated to the aerial part (0.028 mg g^{-1}), with the translocation factor being 0.007 (Supplementary Table 2). A statistically significantly decrease in zinc content in Pb-treated leaves and roots was observed but the other metals and macronutrients did not

276 change significantly in either roots or leaves. Dithizone histochemistry allows observing
277 Pb accumulation mainly in the cell wall and vacuole of root cortex cells (Fig. 2) but Pb
278 accumulation was not observed in root vascular bundles.

279 The results from the analysis of biothiols such as GSH and PCs, which could be
280 involved in metal sequestering, are shown in Fig. 3. GSH accounted for over 80 % of
281 biothiol content in leaves and for ~70 % in roots, with GSSG
282 accounting for ~15 % in leaves and ~10 % in roots; no statistically significant
283 differences between treatments were observed in either roots or leaves. PCs, which
284 were not found in leaves, made up approximately 20 % of biothiol content in roots.
285 While significant amounts of PC2-type were induced in the roots of Pb-treated plants,
286 no PC3 or PC4 were found.

287 **3.3 Oxidative stress markers and antioxidant defenses**

288 Oxidative stress is a common effect caused by Pb and other metals in plants;
289 however, the H_2O_2 and MDA contents, used as oxidative markers in leaves (Fig. 4A),
290 suggest that Pb does not induce oxidative damage in *B. auriculata*. Imaging of H_2O_2
291 and $O_2^{\cdot -}$ accumulation in *B. auriculata* leaves did not show any significant changes in
292 Pb-treated plants (Supplementary Fig. 3), although microscopic images revealed a slight
293 accumulation of H_2O_2 in the central nerve (brown spots) of leaves, while $O_2^{\cdot -}$
294 accumulated in small spots (blue spots) on the base of trichomes. Glycolate oxidase
295 activity is a source of H_2O_2 from leaf photorespiration and changes in this activity were
296 not observed. Changes were also not detected in H_2O_2 and $O_2^{\cdot -}$ accumulation in roots
297 although slight staining was observed in vascular tissue and the zone of cell division
298 (Supplementary Fig. 4).

The ratio GSH/GSSG is used as a redox marker and this ratio did not change in Pb-treated plants, although the ascorbic acid content was statistically higher in Pb-treated leaves with respect to the control (Fig. 4A). The total antioxidant capacity did not change either in leaves or roots in response to Pb (Fig. 4A).The activity of enzymatic antioxidants CAT, GST, POD, MDHAR, DHAR, APX and GR were also analyzed, although APX and GR activity could not be detected (Fig. 4B) even on testing different buffers. The activity of GST and POD enzymes in leaves and roots remained unchanged between treatments, although Pb-treated roots showed an increase in CAT, MDHAR and DHAR with respect to untreated roots. Changes were also not observed in the activity of the NADP-dependent dehydrogenases analyzed (NADP-G6PDH, NADP-IDH, NADP-MS) in leaves due to Pb treatment (Fig. 4B). The activity of SOD was analyzed in leaves by native-PAGE and specific staining (Fig. 5). The results showed four different bands for SOD activity in *B. auriculata* and these increased markedly in Pb-treated leaves. In an effort to identify the different isoforms, the effects of two inhibitors specific for Fe-SOD and CuZn-SOD were analyzed (Fig. 5). The results showed a slight inhibition of the activity with 2 mM KCN and total inhibition with 5 mM H_2O_2, which ruled out the presence of Mn-SOD as this is resistant to both inhibitors, suggesting that all isoforms observed could be Fe-SODs. We proceeded to analyze three bands of SOD activity by MALDI/TOF mass spectrometry and identified some peptides that corresponded to the conserved sequences of Mn-SOD and Fe-SOD of *Arabidopsis thaliana*, but CuZn-SOD was not detected (Supplementary Fig. 2B and C).

Nitric oxide is also an important factor in the cell response to heavy metals and, for this reason, we analyzed the effect of Pb on the NO content in roots and leaves. A reduction in NO was observed in Pb-treated roots with respect to the control (Fig. 4C).

However, nitrosoglutathione reductase (GSNOR) activity, which regulates the level of nitrosoglutathione, a NO donor, decreased in leaves but did not differ significantly in roots in response to Pb treatment (Fig. 4C).

4. DISCUSSION

4.1 Lead stimulates the growth of *B. auriculata*

Lead is a toxic metal which causes inhibition of plant growth in different species such as *Brassica oleracea* (Sinha et al., 2006), *Lolium perenne (*Bai et al., 2014) and *Vigna unguiculate* (Bezerril et al., 2017), amongst others. However, in the present study, using a concentration of Pb in the range described as effective to cause significant inhibition of plants growth under hydroponic conditions (Kumar and Prassad, 2018), a significant increase in both leaf and root growth in *B. auriculata* was observed. Several studies have also shown the beneficial effect of Pb on plant growth, although in most cases plants were exposed to low concentrations of Pb (Seth et al., 2011; Batista et al., 2017; Sidhu et al., 2018). For example, in *Helianthus annuus* the exposure to concentrations of Pb up to 100 mg L^{-1} stimulated biomass production, while higher concentrations led to the opposite effect (Batista et al., 2017). *Lupinus albus* did not show any toxicity symptoms when grown with 180 µM Pb (García et al., 2017) and *Coronopus didymus* also tolerated increasing Pb concentrations up to 2.5 mg g^{-1}, with a concentration-dependent enhancement of root and shoot length being observed (Sidhu et al., 2018). This positive effect was explained by the phenomenon of hormesis, the stimulatory or beneficial effect induced by low doses of metals or toxic compounds (Calabrese and Blain, 2009; Seth et al., 2011; Batista et al., 2017). The mechanisms of hormesis have been widely discussed and the activation of specific and

general stress defenses is considered to be the main mechanism to explain this phenomenon (Poschenrieder et al., 2013). However, in this study *B. auriculata* was exposed to high Pb levels and therefore the induction of growth in this species cannot be explained as being due to hormesis. Taking into account that the seeds were isolated from a mine area that was heavily contaminated with Pb (San Quintin mine; Rodríguez et al., 2009), and other metals (Cu, Zn and Cd), a natural adaptation could occur to allow *B. auriculata* to grow under adverse conditions. A study carried out on two populations of *Biscutella laevigata*, one from waste heaps contaminated with Pb and Zn and the other from the mountain (non-contaminated area), showed a metal-dependent growth stimulation of *B. laevigata* from the waste-heap area when grown under hydroponic conditions, while the other population under the same conditions was inhibited by almost to 50 % (Wierzbicka and Pielichowska, 2004). Interestingly, in addition to the larger leaf size, the most remarkable issue observed in *B. auriculata* exposed to Pb was the increase in the principal root length and thickening, and the reduction in the number and the length of lateral and hair roots. The opposite effect was described by Koppitke et al. (2007) in a sensitive species *Vigna unguiculate*, which showed growth inhibition with a loss of apical dominance and a large number of lateral roots in response to Pb. Inhibition of root elongation was also reported in *Triticum aestivum* (Pena et al., 2015), *Vicia faba* (Mroczek-Zdyrska and Wójcik, 2012), *Silene vulgaris* and *Noccaea caerulescens* (Mohtadi et al., 2012), amongst others. Root architecture is controlled by auxin and exogenous auxin has been reported to inhibit primary root elongation and promote the formation of lateral roots (Alarcón et al., 2019). More recently, a mechanism linking cytokinin signaling and the auxin pool to tune root system architecture has been reported (Michniewicz et al., 2019). Therefore, the Pb-dependent changes observed in the root architecture in *B. auriculata* could be

due to changes in auxin efflux and influx and the balance of IAA and cytokinins. In *Zea mays* an improvement has been observed in Pb phytoextraction by IAA and gibberellins (Hadi et al., 2010). *B. auriculata* managed to grow in the presence of Pb without any symptoms of toxicity, complete its life cycle and to produce seeds in Pb-contaminated soils, thus demonstrating its high tolerance to Pb.

In line with the results observed for the growth parameters, Pb did not affect photosynthetic parameters, chlorophylls and carotenoids content in *B. auriculata*, while in Pb-sensitive species gas exchange inhibition and a reduction in photosynthetic pigments have been reported (Farooq et al., 2013; Bai et al., 2014; Bezerril et al., 2017). Leaf temperature, which has been reported as a good index of heavy metal stress (Thakur and Singh, 2012) did not change either, according with the absence of changes in leaf transpiration and stomatal conductance. The increase of stomata density per leaf area observed in *B. auriculata* could contribute to maintaining the gas exchange and photosynthesis rate in a similar way to that reported by Peco et al. (2020) in this plant species in response to Cd . These results demonstrate that *B. auriculata* leaves are not affected by Pb – probably because the metal is restricted to the root. Phenols and flavonoids are compounds that are strongly induced by heavy metals due to their role as antioxidants and metal chelators (Hernández et al., 2009; Sytar et al., 2013). In *B. auriculata* Pb produced a slight but statistically significant decrease in the phenol content and this was accompanied by a significant increase in the content of flavonoids in leaves. These results suggest that flavonoids can play a role in the tolerance to Pb in this species, as reported in *Lupinus luteus* (Pawlak-Sprada et al., 2011) and *Medicago sativa* (Sima et al., 2012). However, the contribution of flavonoids to Pb tolerance has not been established to date, although they are excellent antioxidants and also could regulate IAA transport (Kuhn et al., 2011). Interestingly, one of the major phenotypic

characteristics of *B. auriculata* in response to Cd was the high level of red/blue pigment anthocyanins in leaves (Peco et al., 2020), while anthocyanin accumulation was unaffected by Pb (Fig 1).

4.2 *B. auriculata* efficiently sequestered Pb in roots

One of the mechanisms involved in Pb tolerance of *B. auriculata* could be its high efficiency in uptaking and accumulating Pb in a non-toxic way to prevent toxicity and without disturbing the nutrient balance. Comparing with other non-hyperaccumulator plant species, shown in Supplemental Table 3, *B, auriculata* has one of the highest capacity to accumulate Pb in roots with a BF value of 1.08 and a TF of 0.01, showing even an increase of growth instead of reduction, which is a considerable advantage in phytoremediation processes. Similar values of Pb accumulation in roots have been reported in *Jatropha curcas*, *Pisum sativum* and *Coronopus didymus*, (see review by Kumar and Prassad, 2018), although these species were more sensitive than *B. auriculata* to Pb. Pb could be taken up by the root through Ca^{2+}-permeable channels (Pourrut et al., 2011) and, in fact, Pb caused a reduction of Ca^{2+} in *Oryza sativa*, probably due to competition between the two cations for the same Ca^{2+} channels (Kim et al., 2002). The contents of Zn, Mn, Ca and Fe were markedly affected by Pb exposure in *Zea mays* (Seregin et al., 2004), *Oryza sativa* (Chatterjee et al., 2004) and *Brassica oleracea* (Sinha et al., 2006). However, in *B. auriculata* only the content of Zn in the leaves and roots was statistically significantly reduced by Pb, thus suggesting competition between Pb/Zn for the same transporters. Another important factor that contributes to Pb tolerance in this species is the low translocation of the metal to the leaves by sequestration of the metal in cell wall and vacuoles, as observed in optical microscopy images of root cross sections. Most plants accumulate 90% of the total Pb in roots (Fahr, 2013). The cell wall is a mechanical barrier against Pb given its high

affinity for pectins and callose, which also restricts cell-to-cell Pb movement (Fahr, 2013). Other mechanisms that avoid Pb translocation include Pb precipitation by binding to the ion-exchangeable location in the cell walls (Kopittke et al., 2007; Islam et al., 2008; Zheng et al., 2012), precipitation as insoluble salts in intercellular spaces (Kopittke et al., 2007; Małecka et al., 2008) and vacuolar sequestration in the cortical and rhizodermal cells (Małecka et al., 2008; Meyers et al., 2008; Zheng et al., 2012). Interestingly, this species behaves differently in reaction to Cd, which accumulates in vascular bundles, the epidermis and in the amorphous structure surrounding epidermal root cells (Peco et al., 2020), thus demonstrating the different strategies adopted by the plant depending on the contaminating metal.

Previous research has suggested that trichomes can function as a site of accumulation and exclusion of heavy metals (Pielichowska and Wierzbicka, 2004; Peco et al., 2020) and an increase in the number of trichomes was observed in *Glycine max* treated with Pb (Weryszko-Chmielewska and Chwil, 2005). However, in this research Pb was not accumulated in trichomes and a reduction in the number of trichomes was observed in line with the low Pb translocation to the leaves. Intriguingly, trichome density and Cd accumulation in Cd-treated *B. auriculata* have been reported to increase in specific trichome rings (Peco et al., 2020), thus confirming that both Cd and Pb induce significantly different responses in *B. auriculata* that affect both plant phenotype and physiology.

Phytochelatins are oligomers of glutathione that are induced in response to heavy metal stress and these oligomers bind the metal and transport it to the vacuole to avoid metal toxicity (Whitacre, 2015; Turull et al., 2017). The high tolerance of *B. auriculata* to this metal could be due in part to its capacity to induce PC synthesis, as reported in other plant species (Supplementary Table 3), without, however, reducing the

GSH content required for antioxidant defenses. *B. auriculata* contains PC2 under control conditions and its biosynthesis is induced in response to Pb, while PC3 were not present neither in roots and leaves. *Lupinus albus* also showed a high tolerance to Pb accompanied by an increase in PCs in the roots (García et al. 2017) as well as it has been observed in other species (Supplemental Table 3). The essential role of PCs for Pb detoxification was demonstrated in *Arabidopsis thaliana* phytochelatin synthase mutants, which showed a higher sensitivity to Pb than wild type (Fischer et al. 2014). Interestingly *B. auriculata* responded to Cd by inducing PC2 and PC3, mainly in leaves (Peco et al., 2020), demonstrating that this species can discriminate between different metals triggering a differential response.

4.3 Lead does not induce oxidative stress in *B. auriculata*

One of the mechanisms of Pb toxicity that has been widely established in different plants species is the indirect ROS production and oxidative damage to lipids and proteins (Farooq et al., 2013; Bai et al., 2014; Saleem et al., 2018; Kumar and Prasad, 2018). However, Pb does not promote oxidative stress in *B. auriculata*, as suggested by the absence of changes in the lipid peroxidation marker MDA, H_2O_2 content, the balance between GSH/GSSG, which is considered as a good index of plant oxidative stress, as well as the activities of antioxidants analyzed in leaves, except for the SOD and the ASC contents, which increase in leaves. The activity of NADP-recycling enzymes was not affected by Pb in leaves and therefore NADPH availability is not a limitation in the response to Pb. The absence of oxidative stress damage could be due to the efficient mechanism for the accumulation of the metal in roots to avoid its toxicity, as mentioned previously, although an increase in the MDHAR and DHAR activities, components of the Foyer–Halliwell–Asada cycle, in roots could help to maintain the redox balance in the tissue. Interestingly, other activities in this cycle, APX

and GR, were not detected in the tissue of *B. auriculata* in spite of the use of different extraction buffers, which suggests that these activities could be very sensitive to proteolytic degradation or inactivation by unknown compounds present in the extracts. The Pb tolerance of plant species, *Coronopus didymus* and *Eclipta prostata* (Sidhu et al., 2016; Chandrasekhar et al., 2019), is also associated with factors such as increased enzymatic activity in the AsA-GSH cycle, suggesting that this cycle plays an important role in the prevention of Pb-related oxidative stress.

SOD is one of the primary antioxidative defenses against ROS accumulation in cells and it is induced by its substrate, $O_2^{\cdot -}$ (Del Río et al., 1991; Alscher et al., 2002). In our study, SOD activity considerably increased in Pb-treated leaves, probably in order to remove $O_2^{\cdot -}$ generated by Pb. However, opposite results were found in *B. auriculata* against Cu (data not shown) and Cd (Peco et al., 2020) with a strong SOD activity inhibition being observed, which could indicate that SOD makes a significant contribution to Pb tolerance in *B. auriculata*. Increased SOD activity in response to Pb has also been reported in other Pb-tolerant plants such as *Eclipta prostrate* (Chandrasekhar et al., 2019), *Coronopus didymus* (Sidhu et al., 2016), and *Peganum harmala* L. (Mahdavian et al., 2016). However, some discrepancies have been reported in the literature, with increases or decreases of SOD observed depending on the intensity and duration of Pb exposure and the plant species (Venkatachalam et al., 2017). Based on the inhibitory effect of H_2O_2 and CN^- on the SOD activity analyzed in native gels, *B. auriculata* only contains Fe-SOD. MALDI-TOF analysis demonstrated the absence of CuZn-SOD, while peptides with Fe-SOD and Mn-SOD homology were observed. Taking into account the high homology between both Mn- and Fe-SOD (del Río et al., 1991) and the results obtained with the inhibitors, one can conclude that *B.*

497 *auriculata* contains Fe-SODs while neither Mn- nor CuZn-SOD are present in this
498 species.

499 NO plays an important role in counteracting metal toxicity in different plant
500 species (Sandalio et al., 2012; Terrón-Camero et al,., 2019). In *B. auriculata*, Pb
501 treatment produces an increase in the NO content in roots. NO has been associated with
502 Pb uptake in *Pogonatherum crinitum* root cells (Yu et al., 2012) and inhibits the
503 translocation of Pb from roots to shoots in ryegrass (Bai et al., 2014), although the
504 mechanisms have not been establisehd as yet. NO has recently been reported to control
505 metal root uptake by regulating metal transporters such as proton pumps and antiporters
506 (CAX), NIP, NRAMP and ABC (reviewed in Terrón-Camero et al, 2019). Other
507 protective mechanisms regulated by NO could be the increases in pectin and
508 hemicellulose content (Xiong et al., 2009) and lignin (Zafari et al., 2017) in the root cell
509 wall to prevent accumulation of metals in the soluble fraction of the cells. NO could
510 also increase the tolerance to Pb by increasing the content of auxin, cytokinins and
511 gibberellins and by decreasing abscisic acid (Sadeghipour, 2017). In *B. auriculata*
512 exposed to Pb, the GSNOR activity which can regulate NO levels in the cell, did not
513 change in roots but it was significantly reduced in leaves – a finding that supports an
514 important role of both NO and GSNOR in Pb uptake and translocation. Recently, Li et
515 al. (2019), have reported that the differences of GSNOR expression might be
516 responsible for the natural variation of root tolerance to high Fe in different *Arabidopsis*
517 accessions. The antioxidant activity of NO reported in different plant species and in
518 response to different metals (Bai et al., 2014; Terrón-Camero et al., 2019) could also
519 contribute to the tolerance of *B. auriculata* to Pb.

520

521

5. CONCLUSIONS

The data obtained from the present study demonstrate that *B. auriculata* is a new Pb tolerant plant able to accumulate high concentrations of Pb in the roots while increasing its growth in presence of high Pb concentrations. However, *B. auriculata* can not be considered a hyper-accumulator because does not accumulate the metal in leaves, which demonstrate that Pb tolerance is not necessarily associated with Pb hyper-accumulation, such it has been reported in *N. caerulescens* (Mohtadi et al., 2012). Pb tolerance in *B. auriculata* could be due to its ability to accumulate Pb in the cell walls and vacuoles of root cells and the induction of PC2, as well as to the restriction of metal translocation to the shoot. Treatment with Pb produced a decrease in the Zn content in the plant, which could indicate that both elements may use the same channel/transporters. In line with the absence of symptoms of toxicity, Pb does not induce oxidative stress in this species – probably as a consequence of the low availability of the metal to participate in ROS production. However, the differential induction of some enzymatic antioxidant defense system such as SOD activity in leaf and CAT, and the Foyer-Halliwell-Asada cycle-activities in roots could also contribute to prevent ROS accumulation and oxidative damage. Differential changes in NO production and GSNOR activity could contribute to the tolerance to Pb in *B. auriculata*.. Therefore, *B. auriculata* may be useful for the phytostabilization and repopulation of areas contaminated with Pb and other metals.

ACKNOWLEDGMENTS

This work was supported by the Spanish Ministry of Science, Innovation and Universities (CGL2015-67644-R and PGC2018-098372-B-100), Castilla-La Mancha

Regional Government (SBPLY/17/180501/000273) and Internal Research Plan I+D+i of University of Castilla-La Mancha (Predoc contract [2016/14100]). The proteomic analysis was performed in the Proteomic Unit of IPBLN-CSIC, that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001.

REFERENCES

Alamri, S.A., Siddiqui, M.H., Al-Khaishany, M.Y.Y., Khan, M.N., Ali, H.M., Alaraidh, I.A., Alsahli, A.A., Al-Rabiah, H., Mateen, M., 2018. Kanuascorbic acid improves the tolerance of wheat plants to lead toxicity. J. Plant Interact. 13, 409–419. https://doi.org/10.1080/17429145.2018.1491067

Alarcón, M.V., Salguero, J., Lloret, P.G., 2019. Auxin modulated initiation of lateral roots is linked to pericycle cell length in maize. Front. Plant Sci. 10, 1–10. https://doi.org/10.3389/fpls.2019.00011

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals-Concepts and applications. Chemosphere. 91, 869-881. https://doi.org/10.1016/j.chemosphere.2013.01.075

Alscher, R. G., Erturk, N., Heath, L. S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331-1341. https://doi.org/10.1093/jexbot/53.372.1331

Andra, S.S., Datta, R., Sarkar, D., Makris, K.C., Mullens, C.P., Sahi, S. V., Bach, S.B.H., 2009. Induction of lead-binding phytochelatins in vetiver grass [*Vetiveria zizanioides* (L.)]. J. Environ. Qual. 38, 868–877. https://doi.org/10.2134/jeq2008.0316

569 Bai, X.Y., Dong, Y.J., Wang, Q.H., Xu, L.L., Kong, J., Liu, S., 2014. Effects of lead
570 and nitric oxide on photosynthesis, antioxidative ability, and mineral element
571 content of perennial ryegrass. Biol. Plant. 59, 163–170.
572 https://doi.org/10.1007/s10535-014-0476-8

573 Batista, A.A., Santos, J.A.G., Bomfim, M.R., Moreira, F.M., Leal, E.F., Conceição, J.N.
574 da, 2017. Induced changes in the growth of four plant species due to lead toxicity.
575 Rev. Bras. Eng. Agrícola e Ambient. 21, 327–332. https://doi.org/10.1590/1807-
576 1929/agriambi.v21n5p327-332

577 Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: Improved assays and an
578 assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.
579 https://doi.org/10.1016/0003-2697(71)90370-8

580 Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in Plants.
581 Toxic Metals in Plants. Brazilian J. Plant Physiol. https://doi.org/10.1590/S1677-
582 04202005000100004

583 Bezerril, N.M., Otoch, M. de L.O., Gomes-Rochette, N.F., Sobreira, A.C. de M.,
584 Barreto, A.A.G.C., de Oliveira, F.D.B., Costa, J.H., Borges, S. da S.S., do
585 Nascimento, R.F., Fernandes de Melo, D., 2017. Effect of lead on physiological
586 and antioxidant responses in two *Vigna unguiculata* cultivars differing in Pb-
587 accumulation. Chemosphere. 176, 397–404.
588 https://doi.org/10.1016/j.chemosphere.2017.02.072

589 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram
590 quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.
591 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

592 Buege, J.A., Aust, S.D., 1978. [30] Microsomal lipid peroxidation. Methods Enzymol.

52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

Calabrese, E.J., Blain, R.B., 2009. Hormesis and plant biology. Environ. Pollut. 157, 42–48. https://doi.org/10.1016/j.envpol.2008.07.028

Chandrasekhar, C., Ray, J.G., 2019. Lead accumulation, growth responses and biochemical changes of three plant species exposed to soil amended with different concentrations of lead nitrate. Ecotoxicol. Environ. Saf. 171, 26–36. https://doi.org/10.1016/j.ecoenv.2018.12.058

Chatterjee, C., Dube, B.K., Sinha, P., Srivastava, P., 2004. Detrimental effects of lead phytotoxicity on growth, Yield, and Metabolism of Rice. Commun. Soil Sci. Plant Anal. 35, 255–265. https://doi.org/10.1081/CSS-120027648

Cutright, T.J., Senko, J., Sivaram, S., York, M., 2012. Evaluation of the Phytoextraction Potential at an Acid-Mine-Drainage-Impacted Site. Soil Sediment Contam. 21, 970–984. https://doi.org/10.1080/15320383.2012.712070

del Río, L.A., Sevilla, F., Sandalio, L.M.,Palma, J.M., 1991. Nutritional effect and expression of SODs: Induction and gene expression; diagnostics; prospective protection against oxygen toxicity. Free Radic. Res. Commun. 12-13, 819-827. https://doi.org/10.3109/10715769109145863

Dickinson, N., 2016. Phytoremediation. Encycl. Appl. Plant Sci. 3, 327–331. https://doi.org/10.1016/B978-0-12-394807-6.00016-2

El-Zohri, M.H.A., Cabala, R., Frank, H., 2005. Quantification of phytochelatins in plants by reversed-phase HPLC–ESI–MS–MS. Anal. Bioanal. Chem. 382, 1871–1876. https://doi.org/10.1007/s00216-005-3331-0

Escarré, J., Lefèbvre, C., Raboyeau, S., Dossantos, A., Gruber, W., Cleyet Marel, J.C., Frérot, H., Noret, N., Mahieu, S., Collin, C., van Oort, F., 2011. Heavy Metal Concentration Survey in Soils and Plants of the Les Malines Mining District (Southern France): Implications for Soil Restoration. Water, Air, Soil Pollut. 216, 485–504. https://doi.org/10.1007/s11270-010-0547-1

Fahr, M., Laplaze, L., Bendaou, N., Hocher, V., El Mzibri, M., Bogusz, D., Smouni, A., 2013. Effect of lead on root growth. Front. Plant Sci. 4, 175. https://doi.org/10.3389/fpls.2013.00175

Farooq, M.A., Ali, S., Hameed, A., Ishaque, W., Mahmood, K., Iqbal, Z., 2013. Alleviation of cadmium toxicity by silicon is related to elevated photosynthesis, antioxidant enzymes; suppressed cadmium uptake and oxidative stress in cotton. Ecotoxicol. Environ. Saf. 96, 242–249. https://doi.org/10.1016/j.ecoenv.2013.07.006

Fellet, G., Pošćić, F., Casolo, V., Marchiol, L., 2012. Metallophytes and thallium hyperaccumulation at the former Raibl lead/zinc mining site (Julian Alps, Italy). Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 146, 1023–1036. https://doi.org/10.1080/11263504.2012.703250

Fischer, S., Kühnlenz, T., Thieme, M., Schmidt, H., Clemens, S., 2014. Analysis of plant Pb tolerance at realistic submicromolar concentrations demonstrates the role of phytochelatin synthesis for Pb detoxification. Environ. Sci. Technol. 48, 7552–7559. https://doi.org/10.1021/es405234p

Flores-Cáceres, M.L., Hattab, Sabrine, Hattab, Sarra, Boussetta, H., Banni, M., Hernández, L.E., 2015. Specific mechanisms of tolerance to copper and cadmium are compromised by a limited concentration of glutathione in alfalfa plants. Plant

Sci. 233, 165–173. https://doi.org/10.1016/j.plantsci.2015.01.013

García, S., Zornoza, P., Hernández, L.E., Esteban, E., Carpena, R.O., 2017. Response of *Lupinus albus* to Pb–EDTA indicates relatively high tolerance. Toxicol. Environ. Chem. 99, 1378–1388. https://doi.org/10.1080/02772248.2017.1387263

Gill, S.S., Hasanuzzaman, M., Nahar, K., Macovei, A., Tuteja, N., 2013. Importance of nitric oxide in cadmium stress tolerance in crop plants. Plant Physiol. Biochem. https://doi.org/10.1016/j.plaphy.2012.12.001

Gupta, D.K., Nicoloso, F.T., Schetinger, M.R.C., Rossato, L. V., Pereira, L.B., Castro, G.Y., Srivastava, S., Tripathi, R.D., 2009. Antioxidant defense mechanism in hydroponically grown *Zea mays* seedlings under moderate lead stress. J. Hazard. Mater. 172, 479–484. https://doi.org/10.1016/j.jhazmat.2009.06.141

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem, 249(22), 7130-7139.

Hadi, F., Bano, A., Fuller, M.P., 2010. The improved phytoextraction of lead (Pb) and the growth of maize (*Zea mays* L.): the role of plant growth regulators (GA3 and IAA) and EDTA alone and in combinations. Chemosphere. 80(4), 457-462.

Hafsi, C., Romero-Puertas, M. C., Gupta, D. K., del Río, L. A., Sandalio L. M., Abdelly, C., 2010. Moderate salinity enhances the antioxidative response in the halophyte Hordeum maritimum L. under potassium deficiency. Environ Exp Bot 69, 129–136. https://doi.org/10.1016/j.envexpbot.2010.04.008

Hernández, I., Alegre, L., Van Breusegem, F., Munné-Bosch, S., 2009. How relevant are flavonoids as antioxidants in plants? Trends Plant Sci. 14, 125–132.

https://doi.org/10.1016/j.tplants.2008.12.003

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. 347, 32 pp.

Islam, E., Liu, D., Li, T., Yang, X., Jin, X., Mahmood, Q., Tian, S., Li, J., 2008. Effect of Pb toxicity on leaf growth, physiology and ultrastructure in the two ecotypes of *Elsholtzia argyi*. J. Hazard. Mater. 154, 914–926. https://doi.org/10.1016/j.jhazmat.2007.10.121

Jiménez-Escrig, A., Dragsted, L.O., Daneshvar, B., Pulido, R., Saura-Calixto, F., 2003. In vitro antioxidant activities of edible artichoke (*Cynara scolymus* L.) and effect on biomarkers of antioxidants in rats. J. Agric. Food Chem. 51, 5540–5545. https://doi.org/10.1021/jf030047e

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: implications in phytoremediation. Environ. Exp. Bot. 67(1), 243-259. https://doi.org/10.1016/j.envexpbot.2009.06.013

Kim, Y.Y., Yang, Y.Y., Lee, Y., 2002. Pb and Cd uptake in rice roots. Physiol. Plant. 116, 368–372. https://doi.org/10.1034/j.1399-3054.2002.1160312.x

Kopittke, P.M., Asher, C.J., Menzies, N.W., 2007. Toxic effects of Ni^{2+} on growth of cowpea (*Vigna unguiculata*). Plant Soil 292, 283–289. https://doi.org/10.1007/s11104-007-9226-4

Kuhn, B.M., Geisle, M., Bigler, L., Ringli, C., 2011. Flavonols accumulate asymmetricallyand affect auxin transport in *Arabidopsis*. Plant Physiol. 156, 585–595. https://doi.org/10.1104/pp.111.175976

686 Kumar, G.H., Kumari, J.P., 2015. Heavy metal lead influative toxicity and its
687 assessment in phytoremediating plants - A Review. Water. Air. Soil Pollut. 226,
688 324. https://doi.org/10.1007/s11270-015-2547-7

689 Kumar, A., Prasad, M.N.V., 2018. Plant-lead interactions: Transport, toxicity, tolerance
690 and detoxification mechanisms. Ecotoxicol. Environ. Saf.
691 https://doi.org/10.1016/j.ecoenv.2018.09.113

692 LaCoste, C., Robinson, B., Brooks, R., Anderson, C., Chiarucci, A., Leblanc, M., 1999.
693 The phytoremediation potential of thallium-contaminated soils using *Iberis* and
694 *Biscutella* species. Int. J. Phytoremediation 1, 327–338.
695 https://doi.org/10.1080/15226519908500023

696 León, A.M., Palma, J.M., Corpas, F.J., Gómez, M., Romero-Puertas, M.C., Chatterjee,
697 D., Mateos, R.M., del Río, L.A., Sandalio, L.M., 2002. Antioxidative enzymes in
698 cultivars of pepper plants with different sensitivity to cadmium. Plant Physiol.
699 Biochem. 40, 813–820. https://doi.org/10.1016/S0981-9428(02)01444-4

700 Li, B., Sun, L., Huang, J., Göschl, C., Shi, W., Chory, J., Busch, W., 2019. GSNOR
701 provides plant tolerance to iron toxicity via preventing iron-dependent nitrosative
702 and oxidative cytotoxicity. Nat. Commun. 10(1), 1-13.
703 https://doi.org/10.1038/s41467-019-11892-5

704 Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and carotenoids: measurement
705 and characterization by UV-VIS spectroscopy. Curr. Protoc. Food Anal. Chem. 1,
706 F4.3.1-F4.3.8. https://doi.org/10.1002/0471142913.faf0403s01

707 Mahdavian, K., Ghaderian, S.M., Schat, H., 2016. Pb accumulation, Pb tolerance,
708 antioxidants, thiols, and organic acids in metallicolous and non-metallicolous
709 *Peganum harmala* L. under Pb exposure. Environ. Exp. Bot. 126, 21–31.

https://doi.org/10.1016/j.envexpbot.2016.01.010

Małecka, A., Piechalak, A., Morkunas, I., Tomaszewska, B., 2008. Accumulation of lead in root cells of *Pisum sativum*. Acta Physiol. Plant. 30, 629–637. https://doi.org/10.1007/s11738-008-0159-1

Melo, E.E.C., Costa, E.T.S., Guilherme, L.R.G., Faquin, V., Nascimento, C.W.A., 2009. Accumulation of arsenic and nutrients by castor bean plants grown on an As-enriched nutrient solution. J. Hazard. Mater. 168, 479–483. https://doi.org/10.1016/j.jhazmat.2009.02.048

Meyers, D.E.R., Auchterlonie, G.J., Webb, R.I., Wood, B., 2008. Uptake and localisation of lead in the root system of *Brassica juncea*. Environ. Pollut. 153, 323–332. https://doi.org/10.1016/J.ENVPOL.2007.08.029

Michniewicz, M., Ho, C. H., Enders, T. A., Floro, E., Damodaran, S., Gunther, L. K., Powers, K., Frick, E.M., Topp, C.N., Frommer, W.B., Strader, L. C., 2019. Transporter of IBA1 links auxin and cytokinin to influence root architecture. Developmental cell, 50(5), 599-609. https://doi.org/10.1016/j.devcel.2019.06.010

Mohtadi, A., Ghaderian, S.M., Schat, H., 2012. A comparison of lead accumulation and tolerance among heavy metal hyperaccumulating and non-hyperaccumulating metallophytes. Plant Soil. 352, 267–276. https://doi.org/10.1007/s11104-011-0994-5

Moslehi, A., Feizian, M., Higueras, P., Eisvand, H.R., 2019. Assessment of EDDS and vermicompost for the phytoextraction of Cd and Pb by sunflower (*Helianthus annuus* L.). Int. J. Phytoremediat. 21, 191–199. https://doi.org/10.1080/15226514.2018.1501336

733 Mroczek-Zdyrska, M., Wójcik, M., 2012. The influence of selenium on root growth and
734 oxidative stress induced by lead in *Vicia faba* L. minor plants. Biol. Trace Elem.
735 Res. 147, 320–328. https://doi.org/10.1007/s12011-011-9292-6

736 Nakatsubo, N., Kojima, H., Kikuchi, K., Nagoshi, H., Hirata, Y., Maeda, D., Imai, Y.,
737 Irimura, T., Nagano, T., 1998. Direct evidence of nitric oxide production from
738 bovine aortic endothelial cells using new fluorescence indicators:
739 Diaminofluoresceins. FEBS Lett. 427, 263–266. https://doi.org/10.1016/S0014-
740 5793(98)00440-2

741 Ortega-Galisteo, A.P., Rodríguez-Serrano, M., Pazmiño, D.M., Gupta, D.K., Sandalio,
742 L.M., Romero-Puertas, M.C., 2012. S-Nitrosylated proteins in pea (*Pisum sativum*
743 L.) leaf peroxisomes: Changes under abiotic stress. J. Exp. Bot. 63, 2089–2103.
744 https://doi.org/10.1093/jxb/err414

745 Pavoni, E., Petranich, E., Adami, G., Baracchini, E., Crosera, M., Emili, A., Lenaz, D.,
746 Higueras, P., Covelli, S., 2017. Bioaccumulation of thallium and other trace metals
747 in *Biscutella laevigata* nearby a decommissioned zinc-lead mine (Northeastern
748 Italian Alps). J. Environ. Manage. 186, 214–224.
749 https://doi.org/10.1016/J.JENVMAN.2016.07.022

750 Pawlak-Sprada, S., Arasimowicz-Jelonek, M., Podgórska, M., Deckert, J., 2011.
751 Activation of phenylpropanoid pathway in legume plants exposed to heavy metals .
752 Part I . Effects of cadmium and lead on phenylalanine ammonia-lyase gene
753 expression , enzyme activity and lignin content. Acta Biochim. Pol. 58, 211–216.
754 https://doi.org/10.18388/abp.2011

755 Peco, J.D., Campos, J.A., Romero-Puertas, M.C., Olmedilla, A., Higueras, P., Sandalio,
756 L.M., 2020. Characterization of mechanisms involved in tolerance and

accumulation of Cd in *Biscutella auriculata* L. Ecotoxicol. Environ. Saf. 201, 110784. https://doi.org/10.1016/j.ecoenv.2020.110784

Pena, L.B., Méndez, A.A., Matayoshi, C.L., Zawoznik, M.S., Gallego, S.M., 2015. Early response of wheat seminal roots growing under copper excess. Plant Physiol. Biochem. 87, 115–123. https://doi.org/10.1016/j.plaphy.2014.12.021

Pereira, M.P., Rodrigues, L.C. de A., Corrêa, F.F., de Castro, E.M., Ribeiro, V.E., Pereira, F.J., 2016. Cadmium tolerance in *Schinus molle* trees is modulated by enhanced leaf anatomy and photosynthesis. Trees - Struct. Funct. 30, 807–814. https://doi.org/10.1007/s00468-015-1322-0

Pielichowska, M. and Wierzbicka, M., 2004. Uptake and localization of cadmium by *Biscutella laevigata*, a cadmium hyperaccumulator. Acta Biol. Cracoviensia 46, 57–63.

Poschenrieder, C., Cabot, C., Martos, S., Gallego, B., Barceló, J., 2013. Do toxic ions induce hormesis in plants? Plant Sci. 212, 15–25. https://doi.org/10.1016/j.plantsci.2013.07.012

Pošćić, F., Marchiol, L., Schat, H., 2013. Hyperaccumulation of thallium is population-specific and uncorrelated with caesium accumulation in the thallium hyperaccumulator, *Biscutella laevigata*. Plant Soil 365, 81–91. https://doi.org/10.1007/s11104-012-1384-3

Pourrut, B., Shahid, M., Dumat, C., Winterton, P., Pinelli, E., 2011. Lead uptake, toxicity, and detoxification in plants. Rev. Environ. Contam. Toxicol. 213, 113–136. https://doi.org/10.1007/978-1-4419-9860-6_4

Rodríguez, L., Ruiz, E., Alonso-Azcárate, J., Rincón, J., 2009. Heavy metal distribution

and chemical speciation in tailings and soils around a Pb-Zn mine in Spain. J. Environ. Manage. 90, 1106–1116. https://doi.org/10.1016/j.jenvman.2008.04.007

Romero-Puertas, M.C., Rodríguez-Serrano, M., Corpas, F.J., Gomez, M.D., Del Rio, L.A., Sandalio, L.M., 2004. Cadmium-induced subcellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves. Plant. Cell Environ. 27, 1122–1134. https://doi.org/10.1111/j.1365-3040.2004.01217.x

Romero-Puertas, M.C., Terrón-Camero, L.C., Peláez-Vico, M.Á., Olmedilla, A., Sandalio, L.M., 2018. Reactive oxygen and nitrogen species as key indicators of plant responses to Cd stress. Environ. Exp. Bot. 161, 107–119. https://doi.org/10.1016/J.ENVEXPBOT.2018.10.012

Ruiz, E., Rodríguez, L., Alonso-Azcárate, J., Rincón, J., 2009. Phytoextraction of metal polluted soils around a Pb-Zn mine by crop plants. Int. J. Phytoremediat. 11, 360–384. https://doi.org/10.1080/15226510802565568

Sadeghipour, O., 2017. Nitric oxide increases Pb tolerance by lowering Pb uptake and translocation as well as phytohormonal changes in cowpea (*Vigna unguiculata* (L.) Walp.). Sains Malaysiana 46, 189–195. https://doi.org/10.17576/jsm-2017-4602-02

Saleem, M., Asghar, H.N., Zahir, Z.A., Shahid, M., 2018. Impact of lead tolerant plant growth promoting rhizobacteria on growth, physiology, antioxidant activities, yield and lead content in sunflower in lead contaminated soil. Chemosphere 195, 606–614. https://doi.org/10.1016/j.chemosphere.2017.12.117

Sandalio, L.M., Rodríguez-Serrano, M., Gupta, D.K., Archilla, A., Romero-Puertas, M.C., Del Río, L.A., 2012. Reactive Oxygen species and Nitric Oxide in plants under cadmium stress: From toxicity to signaling, in: Environmental Adaptations and Stress Tolerance of Plants in the Era of Climate Change. Springer New York,

804 New York, NY, pp. 199–215. https://doi.org/10.1007/978-1-4614-0815-4_9

805 Sanz-Fernández, M., Rodríguez-Serrano, M., Sevilla-Perea, A., Pena, L., Mingorance,
806 M.D., Sandalio, L.M., Romero-Puertas, M.C., 2017. Screening *Arabidopsis*
807 mutants in genes useful for phytoremediation. J. Hazard. Mater. 335, 143–151.
808 https://doi.org/https://doi.org/10.1016/j.jhazmat.2017.04.021

809 Seregin, I. V., Kozhevnikova, A.D., 2011. Histochemical methods for detection of
810 heavy metals and strontium in the tissues of higher plants. Russ. J. Plant Physiol.
811 58, 721–727. https://doi.org/10.1134/s1021443711040133

812 Seregin, I. V., Shpigun, L.K., Ivanov, V.B., 2004. Distribution and toxic effects of
813 cadmium and lead on maize roots. Russ. J. Plant Physiol. 51, 525–533.
814 https://doi.org/10.1023/B:RUPP.0000035747.42399.84

815 Seth, C.S., Misra, V., Singh, R.R., Zolla, L., 2011. EDTA-enhanced lead
816 phytoremediation in sunflower (*Helianthus annuus* L.) hydroponic culture. Plant
817 Soil 347, 231–242. https://doi.org/10.1007/s11104-011-0841-8

818 Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive Oxygen Species,
819 oxidative damage, and antioxidative defense mechanism in plants under stressful
820 conditions. J. Bot. 2012, 1–26. https://doi.org/10.1155/2012/217037

821 Sidhu, G.P.S., Bali, A.S., Singh, H.P., Batish, D.R., Kohli, R.K., 2018.
822 Phytoremediation of lead by a wild, non-edible Pb accumulator *Coronopus*
823 *didymus* (L.) Brassicaceae. Int. J. Phytoremediat. 20, 483–489.
824 https://doi.org/10.1080/15226514.2017.1374331

825 Sidhu, G.P.S., Singh, H.P., Batish, D.R., Kohli, R.K., 2016. Effect of lead on oxidative
826 status, antioxidative response and metal accumulation in *Coronopus didymus*. Plant

827 Physiol. Biochem. 105, 290–296. https://doi.org/10.1016/j.plaphy.2016.05.019

828 Sima, G., Fatemeh, Z., Niknam, V., 2012. Determination of peroxidase activity, total
829 phenolic and flavonoid compounds due to lead toxicity in *Medicago sativa* L. Adv.
830 Environ. Biol. 6, 2357–2364.

831 Sims, D.A., Gamon, J.A., 2002. Relationships between leaf pigment content and
832 spectral reflectance across a wide range of species, leaf structures and
833 developmental stages. Remote Sens. Environ. 81, 337–354.
834 https://doi.org/10.1016/S0034-4257(02)00010-X

835 Singleton, V.L., Rossi, J.A., 1965. Colorimetry of Total Phenolics with
836 Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 16, 144 LP
837 – 158.

838 Sinha, P., Dube, B.K., Srivastava, P., Chatterjee, C., 2006. Alteration in uptake and
839 translocation of essential nutrients in cabbage by excess lead. Chemosphere 65,
840 651–656. https://doi.org/10.1016/j.chemosphere.2006.01.068

841 Souri, Z., Karimi, N., Farooq, M.A., Sandalio, L.M., 2020. Nitric oxide improves
842 tolerance to arsenic stress in *Isatis cappadocica* desv. Shoots by enhancing
843 antioxidant defenses. Chemosphere 239, 124523.
844 https://doi.org/10.1016/j.chemosphere.2019.124523

845 Srivalli, B., Khanna-Chopra, R., 2001. Induction of new isoforms of superoxide
846 dismutase and catalase enzymes in the flag leaf of wheat during monocarpic
847 senescence. Biochem. Biophys. Res. Commun. 288, 1037–1042.
848 https://doi.org/10.1006/bbrc.2001.5843

849 Wojas, S., Ruszczyńska, A., Bulska, E., Clemens, S., Antosiewicz, D.M., 2010. The

role of subcellular distribution of cadmium and phytochelatins in the generation of distinct phenotypes of AtPCS1- and CePCS3-expressing tobacco. J. Plant Physiol. 167, 981–988. https://doi.org/10.1016/j.jplph.2010.02.010

Sytar, O., Kumar, A., Latowski, D., Kuczynska, P., Strzałka, K., Prasad, M.N. V., 2013. Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. Acta Physiol. Plant. 35, 985–999. https://doi.org/10.1007/s11738-012-1169-6

Thakur, A. K., Singh, K. J., 2012. Leaf temperature as thermal bio-indicator of heavy metal pollutants. J. Agr. Sci. Tech. 2(1A), 131.

Terrón-Camero, L.C., Peláez-Vico, M.Á., Del-Val, C., Sandalio, L.M., Romero-Puertas, M.C., Brouquisse, R., 2019. Role of nitric oxide in plant responses to heavy metal stress: Exogenous application versus endogenous production. J. Exp. Bot. https://doi.org/10.1093/jxb/erz184

Terrón-Camero, L.C., del Val, C., Sandalio, L.M., Romero-Puertas, M.C., 2020. Low endogenous NO levels in roots and antioxidant systems are determinants for the resistance of Arabidopsis seedlings grown in Cd. Environ. Pollut. 256, 113411. https://doi.org/10.1016/j.envpol.2019.113411

Turull, M., Grmanova, G., Dago, À., Ariño, C., Díez, S., Díaz-Cruz, J.M., Esteban, M., 2017. Phytochelatin synthesis in response to Hg uptake in aquatic plants near a chlor-alkali factory. Chemosphere 176, 74–80. https://doi.org/10.1016/j.chemosphere.2017.02.092

Venkatachalam, P., Jayalakshmi, N., Geetha, N., Sahi, S. V., Sharma, N.C., Rene, E.R., Sarkar, S.K., Favas, P.J.C., 2017. Accumulation efficiency, genotoxicity and antioxidant defense mechanisms in medicinal plant *Acalypha indica* L. under lead

874 stress. Chemosphere 171, 544–553.
875 https://doi.org/10.1016/j.chemosphere.2016.12.092

876 Weryszko-Chmielewska, E., Chwil, M., 2005. Lead-Induced histological and
877 ultrastructural changes in the leaves of Soybean (*Glycine max* (L.) Merr.). Soil Sci.
878 Plant Nutr. 51, 203–212. https://doi.org/10.1111/j.1747-0765.2005.tb00024.x

879 Whitacre, D.M., 2015. Lead Uptake, Toxicity, and detoxification in plants. Rev.
880 Environ. Contam. Toxicol. 233. https://doi.org/10.1007/978-3-319-10479-9

881 Wierzbicka, M., Pielichowska, M., 2004. Adaptation of *Biscutella laevigata* L, a metal
882 hyperaccumulator, to growth on a zinc–lead waste heap in southern Poland: I:
883 Differences between waste-heap and mountain populations. Chemosphere 54,
884 1663–1674. https://doi.org/10.1016/J.CHEMOSPHERE.2003.08.031

885 Wierzbicka, M., Pielichowska, M., Abratowska, A., Wiłkomirski, B., Wysocka, I.,
886 Panufnik-Mędrzycka, D., Bulska, E., 2016. Thallium hyperaccumulation in Polish
887 populations of *Biscutella laevigata* (Brassicaceae). Acta Biol. Cracoviensia s. Bot.
888 58.

889 Wuana, R.A., Okieimen, F.E., 2014. Heavy metals in contaminated soils: A review of
890 sources, chemistry, risks, and best available strategies for remediation. Heavy Met.
891 Contam. Water Soil Anal. Assessment, Remediat. Strateg. 2011, 1–50.
892 https://doi.org/10.1201/b16566

893 Xiong, J., An, L., Lu, H., Zhu, C., 2009. Exogenous nitric oxide enhances cadmium
894 tolerance of rice by increasing pectin and hemicellulose contents in root cell wall.
895 Planta 230, 755–765. https://doi.org/10.1007/s00425-009-0984-5

896 Yu, Q., Sun, L., Jin, H., Chen, Q., Chen, Z., Xu, M., 2012. Lead-induced nitric oxide

generation plays a critical role in lead uptake by *Pogonatherum crinitum* root cells. Plant Cell Physiol. 53(10), 1728-1736. https://doi.org/10.1093/pcp/pcs116

Zafari, S., Sharifi, M., Chashmi, N. A., 2017. Nitric oxide production shifts metabolic pathways toward lignification to alleviate Pb stress in *Prosopis farcta*. Environ. Exp. Bot. 141, 41-49. https://doi.org/10.1016/j.envexpbot.2017.06.011

Zheng, L., Peer, T., Seybold, V., Lütz-Meindl, U., 2012. Pb-induced ultrastructural alterations and subcellular localization of Pb in two species of *Lespedeza* by TEM-coupled electron energy loss spectroscopy. Environ. Exp. Bot. 77, 196–206. https://doi.org/10.1016/j.envexpbot.2011.11.018

Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

Deciphering lead tolerance mechanisms in a population of the plant species *Biscutella auriculata* L. from a mining area: accumulation strategies and antioxidant defenses

Peco, J.D.[1,2]; Higueras, P.[2]; Campos, J.A.[1], Olmedilla, A.[3]; Romero-Puertas, M.C.[3] and Sandalio, L.M.[3]

1- Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Castilla-La Mancha (UCLM), Ronda de Calatrava, 7, 13071, Ciudad Real, Spain.

2- Instituto de Geología Aplicada, Universidad de Castilla-La Mancha (UCLM), Plaza de Manuel Meca, 1, 13400, Almadén, Ciudad Real, Spain.

3- Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Apartado 419, E-18080, Granada, Spain.

ABBREVIATIONS

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

ABSTRACT

The uptake and distribution of Pb and the mechanisms involved in the metal tolerance have been investigated in a mine population of *Biscutella auriculata*. Seedlings were exposed to 125 μM $Pb(NO_3)_2$ for 15 days under semihydroponic conditions. The results showed an increase in the size of Pb-treated seedlings and symptoms of toxicity were not observed. ICP-OES analyses showed that Pb accumulation was restricted to root tissue. Imaging of Pb accumulation by dithizone histochemistry revealed the presence of the metal in vacuoles and cell wall in root cells. The accumulation of Pb in vacuoles could be stimulated by an increase in phytochelatin PC2 content. Pb did not promote oxidative damage and this is probably due the increase of antioxidative defenses. In the leaves, Pb produced a significant increase in superoxide dismutase activity, while in roots an increase in catalase and components of the Foyer– Halliwell–Asada cycle were observed. The results indicated that *Biscutella auriculata* has a high capacity to tolerate Pb and this is mainly due to a very efficient mechanism to sequester the metal in roots and a capacity to avoid oxidative stress. This species could therefore be very useful for phytostabilization and repopulation of areas contaminated with Pb.

Keywords: Phytoremediation, Oxidative Stress, ROS, Phytochelatin, *Biscutella auriculata*

1. INTRODUCTION

Lead (Pb) is a potent heavy metal pollutant that is toxic for living organisms and has prolonged persistence in the environment due to its non-biodegradable nature (Wuana and Okieimen, 2014). The occurrence of Pb in the environment mainly results from mining, metallurgy, manufacturing and recycling activities and, in some countries, from the persistent use of Pb-based paints and leaded gasolines (Benavides et al., 2005; Kumar and Kumari, 2015). Pb persists in the soil and causes multiple direct and indirect toxic effects on plant growth and metabolism (Whitacre, 2015; Kumar and Prasad, 2018). High concentrations of Pb in the soil inhibit the germination of seeds and reduce plant growth due to the severe alteration of different metabolic pathways, including photosynthesis and transpiration, hormone balance, membrane permeability, mineral nutrition, ATP production and the promotion of oxidative damage due to the increased production of reactive oxygen species (ROS) (Kumar and Prasad, 2018).

Phytoremediation is a clean-up process that is effective, inexpensive and environmentally friendly for the remediation of metal-contaminated soils by using plants (Kidd et al., 2009; Dickinson, 2016). There are several different decontamination techniques within phytoremediation, namely phytofiltration, phytovolatilization, phytodegradation, phytoextraction and phytostabilization (Ali et al., 2013). Phytoextraction involves extraction of the metal from the soil by absorbing it in metal-accumulating plants and this approach is considered to be the main and most useful technique for the removal heavy metals and metalloids (Kumar and Prasad, 2018). The efficiency of phytoextraction depends on numerous factors such as the heavy metal characteristics and bioavailability, soil properties and plant species (Ali et al., 2013). Plants usually show little tolerance to the presence of heavy metals and they do not usually accumulate such metals within their tissues in appreciable amounts, but some

plants develop resilience mechanisms to overcome the restraints caused by the presence of high concentrations of pollutants. A thorough study of plant tolerance strategies is crucial to address the rehabilitation of degraded land (Cutright et al., 2012; Dickinson, 2016).

Different mechanisms for Pb tolerance and accumulation have been reported for plants (Kumar and Prasad, 2018). The most tolerant species accumulate around 95% of the absorbed Pb in root and only a small proportion is translocated to aerial parts of the plant (Ruiz et al., 2009; Gupta et al., 2009; Whitacre, 2015). Phytochelatin (PC) synthesis is a mechanism induced by Pb to neutralize and accumulate the metals in the vacuole (Andra et al., 2009; Wojas et al., 2010; Fischer et al., 2014; García et al., 2017). The metal accumulation capacity of plants is also affected by their ability to survive the oxidative stress caused by the production of reactive oxygen species (ROS) during Pb exposure (Sytar et al., 2013). Plants have complex enzymatic and non-enzymatic antioxidative defenses to maintain ROS levels that are compatible with the regular functioning of the cells (Romero-Puertas et al., 2018). The main enzymatic antioxidative defenses are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPOX), NADP-dependent dehydrogenases, glutathione S-transferase (GST) and the enzymes of the Foyer–Halliwell–Asada Cycle (ascorbate peroxidase [APX], monodehydroascorbate reductase [MDHAR], dehydroascorbate reductase [DHAR] and glutathione reductase [GR]) (Sandalio et al., 2012). Non-enzymatic components of the antioxidative defense system include ascorbate (AsA), glutathione (GSH), tocopherol, carotenoids and phenolic compounds, amongst others (Sharma et al., 2012). It has been reported that Pb exposure induces changes in antioxidant systems either by overexpressing or downregulating antioxidants to avoid damage caused by ROS production (Kumar and Prasad, 2018). Nitric oxide (NO) is a simple molecule

which acts as regulator of many physiological processes in plants, including the defence against heavy metal (Gill et al., 2013; Terrón-Camero et al., 2019; Terrón-Camero et al., 2020). It has been reported that NO can prevent oxidative damages by improving antioxidant defences and therefore it could be a key factor in the tolerance against heavy metals (Romero-Puertas et al., 2018; Souri et al., 2020).

Metal hypertolerance and hyperaccumulation are found in a large number of plant families, with the highest occurrence in the *Brassicaceae* family (Mohtadi et al., 2012). *Biscutella* is a genus herbal member of the *Brassicaceae* family that grows in areas bordering agricultural fields, roadsides and polluted areas (Peco et al., 2020). The species *Biscutella laevigata* has already been described as an accumulator with a tolerance to the metals thallium (LaCoste et al., 1999; 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). A population of *Biscutella auriculata* has been recently identified as one of the few species growing in a multimetal (Cu, Zn, Pb and Cd) contaminated area close to the San Quintin mine area located in Ciudad Real (Spain). This species has been reported as a Cd-tolerant plant which efficiently accumulates this metal in roots and trichomes and differentially regulate antioxidant damage prevention in roots and leaves (Peco et al., 2020). Given that Pb is one of the metals present in the soil from which the seeds were taken, we investigated Pb tolerance in *B. auriculata* and the mechanisms involved. The study therefore focuses on the effects of Pb on growth parameters, ROS and NO metabolism, Pb accumulation patterns, as well as leaf and root mineral status. Our results demonstrate that *B. auriculata* is a new Pb-tolerant growth-enhancing plant in the presence of high concentrations of Pb, which could be useful in restoring areas contaminated by this heavy metal.

2. MATERIAL AND METHODS

2.1 Plant materials and growth conditions

B. auriculata seeds were obtained from a natural population located in the San Quintin mining area (38°48'52.6"N 4°17'15.5"W) in Villamayor de Calatrava (Ciudad Real province, South Central Spain) (Supplementary Fig. 1A and B). This area has been altered by mining activity (Pb-Zn-Ag mine) and is contaminated Pb, Zn, Cu and Cd (Rodríguez et al., 2009). The seeds were hydrated for 24 h and then germinated on wet filter paper in Petri dishes at 25 ºC. Healthy homogenous seedlings were transferred to a semi-hydroponic system containing perlite (Flores-Cáceres et al., 2015) and a Hoagland nutrient solution (Hoagland and Arnon, 1950) for 15 days. After this period, seedlings were then irrigated with Hoagland nutrient solution supplemented with 0 or 125 µM of $Pb(NO_3)_2$ for 15 days (Pereira et al., 2016). Cultures were placed in a growth chamber under the following conditions: 24 ºC, 60% relativity humidity and 16/8 h light/dark photoperiod. The experiment was repeated three times. Finally, leaves and roots were processed separately, frozen in liquid nitrogen and stored at –80 ºC. Sterilized seeds were also germinated and were grown vertically in square Petri dishes (10 × 10 cm) containing MS medium supplemented with 0 or 125 µM of $Pb(NO_3)_2$ for 10 days (Sanz-Fernández et al., 2017). These plants were grown under the same growth chamber conditions as previously mentioned.

2.2 Growth parameters, photosynthesis data, photosynthetic pigments, phenolics and flavonoids content

Morphological and growth parameters were analyzed: leaf and root fresh weight, number of leaves and root length. Leaf area, trichomes and stomata density were analyzed from leaf images using ImageJ software. Gas exchange parameters (net photosynthesis rate, stomatal conductance, transpiration ratio and intercellular CO_2

concentration) were determined using a portable photosynthesis system (Ciras-3, PP Systems). Contents of chlorophylls, carotenoids and anthocyanins were analyzed by spectrophotometric methods according to Lichtenthaler and Buschmann (2001) and Sims and Gamon (2002), respectively. Total phenolics content was determined by a spectrophotometric technique according to Folin–Ciocalteau's method proposed by 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.

2.3 Mineral analysis and histochemical localization of Pb

In order to assess the mineral contents, plant samples were oven-dried at 60 ºC for 72 hours. Roots and leaves were weighed to determine the dry mass and were digested with an HNO_3/H_2O_2 mixture using a microwave digestion system (ETHOS 1, Milestone). Mineral composition was measured by inductively coupled plasma-optical emission spectrometry (ICP-OES, Varian 720-ES). The phytoextraction ability was determined using the translocation factor (TF) and the bioaccumulation factor (BF) equations described by Melo et al. (2009).

Pb storage was detected histochemically using the dithizone method described by Seregin and Kozhevnikova (2011). Leaves and roots were incubated in a solution of dithizone in acetone, glacial acetic acid and Milli-Q water (3:5:1) and incubated for one hour. Leaves were bleached by immersion in boiling ethanol. Stained roots were embedded in 5% low-melting agarose D1 EEO (Conda Pronadise) and cross-sections were obtained using a vibratome (VT1200/VT1200S Leica) and examined by optical microscopy (Leica DMI600B).

2.4 Quantification of glutathione, ascorbate and phytochelatins

Reduced and oxidized glutathione (GSH and GSSG) and total ascorbate (AsA) were determined by liquid chromatography-electrospray/mass spectrometry (LC-ES/MS) using a method described by El-Zohri et al. (2005). Plant extracts were prepared in HCl and analyzed on an HPLC system (H-Class, Waters, Mildford) coupled to a triple quadrupole mass spectrometer (Quattro-Micro, Waters, Mildford). PC2 and PC3 contents were analyzed in the same extracts by following the procedure described by El-Zohri et al. (2005). The supernatant was injected onto an Xselect CSH column (100 mm × 2.1 mm, Waters, Mildford) with a Vanguard Xselect CSH C18 cartridge (5 mm × 2.1 mm, Waters, Mildford). The results were calculated using pattern on PC2, PC3 and PC4 from Pepmic Co., Ltd (Suzhou, China). Biothiols and AsA were determined by multiple reaction monitoring (MRM) using positive electrospray and negative electrospray.

2.5 Lipid peroxidation, H_2O_2 content, NO content and total antioxidant capacity

Lipid peroxidation was determined in terms of malondialdehyde (MDA) concentration according to the method described by Buege and Aust (1978). The results were calculated using a calibration curve for MDA. H_2O_2 accumulation was analyzed by a spectrofluorometric method as described by Romero-Puertas et al. (2004). The results were calculated using a calibration curve for H_2O_2. NO content was determined by a spectrofluorometric method as described by Nakatsubo et al. (1988), using 4,5-diamino-fluorescein (DAF-2) and results are expressed in arbitrary fluorescence units. Total antioxidant capacity was determined according to the ABTS assay (Jiménez-Escrig et al., 2003) and the results were calculated using a Trolox calibration curve.

205 **2.6 Enzymatic assays**

206 Leaves and roots were ground in liquid nitrogen and the powder obtained was
207 homogenized in 0.1 M Tris-HCl pH 7.5, containing 0.1 mM EDTA, 0.2 % Triton X100,
208 2 mM DTT, 0.2 % PVP and 1X protease inhibitor cocktail (Sigma-Aldrich). The
209 homogenates were centrifuged at 14000 g for 20 min. Enzymatic activity was assayed
210 spectrophotometrically according to the following methods: CAT activity (EC
211 1.11.1.6); POD activity; GOX activity (EC 1.1.3.1); APX activity (EC 1.11.1.11), GR
212 activity (EC 1.6.4.2); MDHAR activity (EC 1.6.5.4) and DHAR activity (EC 1.8.5.1) as
213 reported by Hafsi et al. (2010); NADP-G6PDH (EC 1.1.1.49), NADP-IDH (EC
214 1.1.1.42) and NADP-MS (EC 4.1.3.2) as reported by León et al. (2002); GST activity
215 (EC 2.5.1.1) as described by Habig et al. (1974) and GSNOR (EC 1.2.1.46) as described
216 by Ortega-Galisteo et al. (2012). The enzymatic assays were carried out according to the
217 method described by Peco et al. (2020).

218 **2.7 Characterization of SOD isoenzyme and activity**

219 SOD activity (EC 1.15.1) was assayed by native polyacrylamide gel
220 electrophoresis (native-PAGE, 10% acrylamide/Bis) and activity was imaged in the gels
221 according to the photochemical method described by Beauchamp and Fridovich (1971).
222 The effects of 5 mM H_2O_2 and 2 mM KCN on SOD activity were evaluated to identify
223 the different SOD isoenzymes (Srivalli and Khanna-Chopra, 2001). The activity was
224 expressed as % of total SOD activity by determining the area under the peaks using
225 ImageJ. The different SOD bands obtained (Supplementary Fig. 2A) were analyzed by
226 MALDI-TOF mass spectrometry (UltrafleXtrem, Bruker). The sequences obtained were
227 compared with those found in UniProt for Fe SOD, Mn SOD and CuZn SOD of
228 *Arabidopsis thaliana*. Theoretical digestions of proteins, belonging to the three SOD
229 bands, were carried out while keeping the peptides corresponding to the conserved areas

obtained from the isoenzyme's alignment. Finally, peptides belonging to the different SOD isoenzymes were located in the mass spectrum using a MASCOT software.

2.8 Histochemical localization of H_2O_2 and $O_2^{\cdot -}$

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

2.9 Other assays

The protein contents in plant extracts were determined according to the method of Bradford (1976) using a bovine serum albumin (BSA) calibration curve.

2.10 Data analysis

Statistical analyses were carried out by a Student's t-test in IBM SPSS Statistics 24. Asterisks ($P<0.05$: *; $P<0.01$: **; $P<0.001$: ***) represent the level of significance in the figures. Images were analyzed using ImageJ software.

3. RESULTS

3.1 Effect of lead on plant growth and photosynthetic parameters

The effects of Pb on the growth and phenotype of *B. auriculata* plants growing in a semi-hydroponic medium are represented in Fig. 1A and Fig. 1C. Pb exposure for 15 days produced a significant increase in the FW of leaves and roots (1.4 fold), as well as in the leaf area (1.17 fold). However, changes were not observed in the number of leaves. The number of trichomes per area decreased in Pb-treated plants (1.7 fold), while the number of stomata per area increased in response to Pb-treatment (1.7 fold) (Fig 1C). Seedlings grown in square Petri dishes are shown in Fig. 1B and it can be observed that Pb induced the growth of the main root, while the secondary roots and hair roots were considerably reduced in number and size with respect to the control plants (Fig. 1B and Fig. 1D). Interestingly, Pb did not statistically affect the photosynthesis parameters, net photosynthesis rate, stomatal conductance, transpiration ratio, intercellular CO_2 concentration and leaf temperature (Supplementary Table 1). Pb exposure did not affect the chlorophylls and carotenoids contents, which remained unchanged, while the phenols content decreased slightly and the opposite trend was observed for the flavonoids content (Supplementary Table 1).

3.2 Lead uptake and accumulation

The mineral contents of roots and leaves in *B. auriculata* treated and untreated with Pb are shown in Supplementary Table 2. Pb was mainly accumulated in roots (3.686 mg g^{-1}) with a bioaccumulation factor of 1.081 and only a small content was translocated to the aerial part (0.028 mg g^{-1}), with the translocation factor being 0.007 (Supplementary Table 2). A statistically significantly decrease in zinc content in Pb-treated leaves and roots was observed but the other metals and macronutrients did not

change significantly in either roots or leaves. Dithizone histochemistry allows observing Pb accumulation mainly in the cell wall and vacuole of root cortex cells (Fig. 2) but Pb accumulation was not observed in root vascular bundles.

The results from the analysis of biothiols such as GSH and PCs, which could be involved in metal sequestering, are shown in Fig. 3. GSH accounted for over 80 % of biothiol content in leaves and for ~70 % in roots, with GSSG accounting for ~15 % in leaves and ~10 % in roots; no statistically significant differences between treatments were observed in either roots or leaves. PCs, which were not found in leaves, made up approximately 20 % of biothiol content in roots. While significant amounts of PC2-type were induced in the roots of Pb-treated plants, no PC3 or PC4 were found.

3.3 Oxidative stress markers and antioxidant defenses

Oxidative stress is a common effect caused by Pb and other metals in plants; however, the H_2O_2 and MDA contents, used as oxidative markers in leaves (Fig. 4A), suggest that Pb does not induce oxidative damage in *B. auriculata*. Imaging of H_2O_2 and $O_2^{\cdot -}$ accumulation in *B. auriculata* leaves did not show any significant changes in Pb-treated plants (Supplementary Fig. 3), although microscopic images revealed a slight accumulation of H_2O_2 in the central nerve (brown spots) of leaves, while $O_2^{\cdot -}$ accumulated in small spots (blue spots) on the base of trichomes. Glycolate oxidase activity is a source of H_2O_2 from leaf photorespiration and changes in this activity were not observed. Changes were also not detected in H_2O_2 and $O_2^{\cdot -}$ accumulation in roots although slight staining was observed in vascular tissue and the zone of cell division (Supplementary Fig. 4).

The ratio GSH/GSSG is used as a redox marker and this ratio did not change in Pb-treated plants, although the ascorbic acid content was statistically higher in Pb-treated leaves with respect to the control (Fig. 4A). The total antioxidant capacity did not change either in leaves or roots in response to Pb (Fig. 4A).The activity of enzymatic antioxidants CAT, GST, POD, MDHAR, DHAR, APX and GR were also analyzed, although APX and GR activity could not be detected (Fig. 4B) even on testing different buffers. The activity of GST and POD enzymes in leaves and roots remained unchanged between treatments, although Pb-treated roots showed an increase in CAT, MDHAR and DHAR with respect to untreated roots. Changes were also not observed in the activity of the NADP-dependent dehydrogenases analyzed (NADP-G6PDH, NADP-IDH, NADP-MS) in leaves due to Pb treatment (Fig. 4B). The activity of SOD was analyzed in leaves by native-PAGE and specific staining (Fig. 5). The results showed four different bands for SOD activity in *B. auriculata* and these increased markedly in Pb-treated leaves. In an effort to identify the different isoforms, the effects of two inhibitors specific for Fe-SOD and CuZn-SOD were analyzed (Fig. 5). The results showed a slight inhibition of the activity with 2 mM KCN and total inhibition with 5 mM H_2O_2, which ruled out the presence of Mn-SOD as this is resistant to both inhibitors, suggesting that all isoforms observed could be Fe-SODs. We proceeded to analyze three bands of SOD activity by MALDI/TOF mass spectrometry and identified some peptides that corresponded to the conserved sequences of Mn-SOD and Fe-SOD of *Arabidopsis thaliana*, but CuZn-SOD was not detected (Supplementary Fig. 2B and C).

Nitric oxide is also an important factor in the cell response to heavy metals and, for this reason, we analyzed the effect of Pb on the NO content in roots and leaves. A reduction in NO was observed in Pb-treated roots with respect to the control (Fig. 4C).

However, nitrosoglutathione reductase (GSNOR) activity, which regulates the level of nitrosoglutathione, a NO donor, decreased in leaves but did not differ significantly in roots in response to Pb treatment (Fig. 4C).

4. DISCUSSION

4.1 Lead stimulates the growth of *B. auriculata*

Lead is a toxic metal which causes inhibition of plant growth in different species such as *Brassica oleracea* (Sinha et al., 2006), *Lolium perenne (*Bai et al., 2014) and *Vigna unguiculate* (Bezerril et al., 2017), amongst others. However, in the present study, using a concentration of Pb in the range described as effective to cause significant inhibition of plants growth under hydroponic conditions (Kumar and Prassad, 2018), a significant increase in both leaf and root growth in *B. auriculata* was observed. Several studies have also shown the beneficial effect of Pb on plant growth, although in most cases plants were exposed to low concentrations of Pb (Seth et al., 2011; Batista et al., 2017; Sidhu et al., 2018). For example, in *Helianthus annuus* the exposure to concentrations of Pb up to 100 mg L^{-1} stimulated biomass production, while higher concentrations led to the opposite effect (Batista et al., 2017). *Lupinus albus* did not show any toxicity symptoms when grown with 180 µM Pb (García et al., 2017) and *Coronopus didymus* also tolerated increasing Pb concentrations up to 2.5 mg g^{-1}, with a concentration-dependent enhancement of root and shoot length being observed (Sidhu et al., 2018). This positive effect was explained by the phenomenon of hormesis, the stimulatory or beneficial effect induced by low doses of metals or toxic compounds (Calabrese and Blain, 2009; Seth et al., 2011; Batista et al., 2017). The mechanisms of hormesis have been widely discussed and the activation of specific and

general stress defenses is considered to be the main mechanism to explain this phenomenon (Poschenrieder et al., 2013). However, in this study *B. auriculata* was exposed to high Pb levels and therefore the induction of growth in this species cannot be explained as being due to hormesis. Taking into account that the seeds were isolated from a mine area that was heavily contaminated with Pb (San Quintin mine; Rodríguez et al., 2009), and other metals (Cu, Zn and Cd), a natural adaptation could occur to allow *B. auriculata* to grow under adverse conditions. A study carried out on two populations of *Biscutella laevigata*, one from waste heaps contaminated with Pb and Zn and the other from the mountain (non-contaminated area), showed a metal-dependent growth stimulation of *B. laevigata* from the waste-heap area when grown under hydroponic conditions, while the other population under the same conditions was inhibited by almost to 50 % (Wierzbicka and Pielichowska, 2004). Interestingly, in addition to the larger leaf size, the most remarkable issue observed in *B. auriculata* exposed to Pb was the increase in the principal root length and thickening, and the reduction in the number and the length of lateral and hair roots. The opposite effect was described by Koppitke et al. (2007) in a sensitive species *Vigna unguiculate*, which showed growth inhibition with a loss of apical dominance and a large number of lateral roots in response to Pb. Inhibition of root elongation was also reported in *Triticum aestivum* (Pena et al., 2015), *Vicia faba* (Mroczek-Zdyrska and Wójcik, 2012), *Silene vulgaris* and *Noccaea caerulescens* (Mohtadi et al., 2012), amongst others. Root architecture is controlled by auxin and exogenous auxin has been reported to inhibit primary root elongation and promote the formation of lateral roots (Alarcón et al., 2019). More recently, a mechanism linking cytokinin signaling and the auxin pool to tune root system architecture has been reported (Michniewicz et al., 2019). Therefore, the Pb-dependent changes observed in the root architecture in *B. auriculata* could be

due to changes in auxin efflux and influx and the balance of IAA and cytokinins. In *Zea mays* an improvement has been observed in Pb phytoextraction by IAA and gibberellins (Hadi et al., 2010). *B. auriculata* managed to grow in the presence of Pb without any symptoms of toxicity, complete its life cycle and to produce seeds in Pb-contaminated soils, thus demonstrating its high tolerance to Pb.

In line with the results observed for the growth parameters, Pb did not affect photosynthetic parameters, chlorophylls and carotenoids content in *B. auriculata*, while in Pb-sensitive species gas exchange inhibition and a reduction in photosynthetic pigments have been reported (Farooq et al., 2013; Bai et al., 2014; Bezerril et al., 2017). Leaf temperature, which has been reported as a good index of heavy metal stress (Thakur and Singh, 2012) did not change either, according with the absence of changes in leaf transpiration and stomatal conductance. The increase of stomata density per leaf area observed in *B. auriculata* could contribute to maintaining the gas exchange and photosynthesis rate in a similar way to that reported by Peco et al. (2020) in this plant species in response to Cd . These results demonstrate that *B. auriculata* leaves are not affected by Pb – probably because the metal is restricted to the root. Phenols and flavonoids are compounds that are strongly induced by heavy metals due to their role as antioxidants and metal chelators (Hernández et al., 2009; Sytar et al., 2013). In *B. auriculata* Pb produced a slight but statistically significant decrease in the phenol content and this was accompanied by a significant increase in the content of flavonoids in leaves. These results suggest that flavonoids can play a role in the tolerance to Pb in this species, as reported in *Lupinus luteus* (Pawlak-Sprada et al., 2011) and *Medicago sativa* (Sima et al., 2012). However, the contribution of flavonoids to Pb tolerance has not been established to date, although they are excellent antioxidants and also could regulate IAA transport (Kuhn et al., 2011). Interestingly, one of the major phenotypic

characteristics of *B. auriculata* in response to Cd was the high level of red/blue pigment anthocyanins in leaves (Peco et al., 2020), while anthocyanin accumulation was unaffected by Pb (Fig 1).

4.2 *B. auriculata* efficiently sequestered Pb in roots

One of the mechanisms involved in Pb tolerance of *B. auriculata* could be its high efficiency in uptaking and accumulating Pb in a non-toxic way to prevent toxicity and without disturbing the nutrient balance. Comparing with other non-hyperaccumulator plant species, shown in Supplemental Table 3, *B, auriculata* has one of the highest capacity to accumulate Pb in roots with a BF value of 1.08 and a TF of 0.01, showing even an increase of growth instead of reduction, which is a considerable advantage in phytoremediation processes. Similar values of Pb accumulation in roots have been reported in *Jatropha curcas*, *Pisum sativum* and *Coronopus didymus*, (see review by Kumar and Prassad, 2018), although these species were more sensitive than *B. auriculata* to Pb. Pb could be taken up by the root through Ca^{2+}-permeable channels (Pourrut et al., 2011) and, in fact, Pb caused a reduction of Ca^{2+} in *Oryza sativa*, probably due to competition between the two cations for the same Ca^{2+} channels (Kim et al., 2002). The contents of Zn, Mn, Ca and Fe were markedly affected by Pb exposure in *Zea mays* (Seregin et al., 2004), *Oryza sativa* (Chatterjee et al., 2004) and *Brassica oleracea* (Sinha et al., 2006). However, in *B. auriculata* only the content of Zn in the leaves and roots was statistically significantly reduced by Pb, thus suggesting competition between Pb/Zn for the same transporters. Another important factor that contributes to Pb tolerance in this species is the low translocation of the metal to the leaves by sequestration of the metal in cell wall and vacuoles, as observed in optical microscopy images of root cross sections. Most plants accumulate 90% of the total Pb in roots (Fahr, 2013). The cell wall is a mechanical barrier against Pb given its high

affinity for pectins and callose, which also restricts cell-to-cell Pb movement (Fahr, 2013). Other mechanisms that avoid Pb translocation include Pb precipitation by binding to the ion-exchangeable location in the cell walls (Kopittke et al., 2007; Islam et al., 2008; Zheng et al., 2012), precipitation as insoluble salts in intercellular spaces (Kopittke et al., 2007; Małecka et al., 2008) and vacuolar sequestration in the cortical and rhizodermal cells (Małecka et al., 2008; Meyers et al., 2008; Zheng et al., 2012). Interestingly, this species behaves differently in reaction to Cd, which accumulates in vascular bundles, the epidermis and in the amorphous structure surrounding epidermal root cells (Peco et al., 2020), thus demonstrating the different strategies adopted by the plant depending on the contaminating metal.

Previous research has suggested that trichomes can function as a site of accumulation and exclusion of heavy metals (Pielichowska and Wierzbicka, 2004; Peco et al., 2020) and an increase in the number of trichomes was observed in *Glycine max* treated with Pb (Weryszko-Chmielewska and Chwil, 2005). However, in this research Pb was not accumulated in trichomes and a reduction in the number of trichomes was observed in line with the low Pb translocation to the leaves. Intriguingly, trichome density and Cd accumulation in Cd-treated *B. auriculata* have been reported to increase in specific trichome rings (Peco et al., 2020), thus confirming that both Cd and Pb induce significantly different responses in *B. auriculata* that affect both plant phenotype and physiology.

Phytochelatins are oligomers of glutathione that are induced in response to heavy metal stress and these oligomers bind the metal and transport it to the vacuole to avoid metal toxicity (Whitacre, 2015; Turull et al., 2017). The high tolerance of *B. auriculata* to this metal could be due in part to its capacity to induce PC synthesis, as reported in other plant species (Supplementary Table 3), without, however, reducing the

GSH content required for antioxidant defenses. *B. auriculata* contains PC2 under control conditions and its biosynthesis is induced in response to Pb, while PC3 were not present neither in roots and leaves. *Lupinus albus* also showed a high tolerance to Pb accompanied by an increase in PCs in the roots (García et al. 2017) as well as it has been observed in other species (Supplemental Table 3). The essential role of PCs for Pb detoxification was demonstrated in *Arabidopsis thaliana* phytochelatin synthase mutants, which showed a higher sensitivity to Pb than wild type (Fischer et al. 2014). Interestingly *B. auriculata* responded to Cd by inducing PC2 and PC3, mainly in leaves (Peco et al., 2020), demonstrating that this species can discriminate between different metals triggering a differential response.

4.3 Lead does not induce oxidative stress in *B. auriculata*

One of the mechanisms of Pb toxicity that has been widely established in different plants species is the indirect ROS production and oxidative damage to lipids and proteins (Farooq et al., 2013; Bai et al., 2014; Saleem et al., 2018; Kumar and Prasad, 2018). However, Pb does not promote oxidative stress in *B. auriculata*, as suggested by the absence of changes in the lipid peroxidation marker MDA, H_2O_2 content, the balance between GSH/GSSG, which is considered as a good index of plant oxidative stress, as well as the activities of antioxidants analyzed in leaves, except for the SOD and the ASC contents, which increase in leaves. The activity of NADP-recycling enzymes was not affected by Pb in leaves and therefore NADPH availability is not a limitation in the response to Pb. The absence of oxidative stress damage could be due to the efficient mechanism for the accumulation of the metal in roots to avoid its toxicity, as mentioned previously, although an increase in the MDHAR and DHAR activities, components of the Foyer–Halliwell–Asada cycle, in roots could help to maintain the redox balance in the tissue. Interestingly, other activities in this cycle, APX

and GR, were not detected in the tissue of *B. auriculata* in spite of the use of different extraction buffers, which suggests that these activities could be very sensitive to proteolytic degradation or inactivation by unknown compounds present in the extracts. The Pb tolerance of plant species, *Coronopus didymus* and *Eclipta prostata* (Sidhu et al., 2016; Chandrasekhar et al., 2019), is also associated with factors such as increased enzymatic activity in the AsA-GSH cycle, suggesting that this cycle plays an important role in the prevention of Pb-related oxidative stress.

SOD is one of the primary antioxidative defenses against ROS accumulation in cells and it is induced by its substrate, $O_2^{\cdot -}$ (Del Río et al., 1991; Alscher et al., 2002). In our study, SOD activity considerably increased in Pb-treated leaves, probably in order to remove $O_2^{\cdot -}$ generated by Pb. However, opposite results were found in *B. auriculata* against Cu (data not shown) and Cd (Peco et al., 2020) with a strong SOD activity inhibition being observed, which could indicate that SOD makes a significant contribution to Pb tolerance in *B. auriculata*. Increased SOD activity in response to Pb has also been reported in other Pb-tolerant plants such as *Eclipta prostrate* (Chandrasekhar et al., 2019), *Coronopus didymus* (Sidhu et al., 2016), and *Peganum harmala* L. (Mahdavian et al., 2016). However, some discrepancies have been reported in the literature, with increases or decreases of SOD observed depending on the intensity and duration of Pb exposure and the plant species (Venkatachalam et al., 2017). Based on the inhibitory effect of H_2O_2 and CN^- on the SOD activity analyzed in native gels, *B. auriculata* only contains Fe-SOD. MALDI-TOF analysis demonstrated the absence of CuZn-SOD, while peptides with Fe-SOD and Mn-SOD homology were observed. Taking into account the high homology between both Mn- and Fe-SOD (del Río et al., 1991) and the results obtained with the inhibitors, one can conclude that *B.*

497 *auriculata* contains Fe-SODs while neither Mn- nor CuZn-SOD are present in this
498 species.

499 NO plays an important role in counteracting metal toxicity in different plant
500 species (Sandalio et al., 2012; Terrón-Camero et al,., 2019). In *B. auriculata*, Pb
501 treatment produces an increase in the NO content in roots. NO has been associated with
502 Pb uptake in *Pogonatherum crinitum* root cells (Yu et al., 2012) and inhibits the
503 translocation of Pb from roots to shoots in ryegrass (Bai et al., 2014), although the
504 mechanisms have not been establisehd as yet. NO has recently been reported to control
505 metal root uptake by regulating metal transporters such as proton pumps and antiporters
506 (CAX), NIP, NRAMP and ABC (reviewed in Terrón-Camero et al, 2019). Other
507 protective mechanisms regulated by NO could be the increases in pectin and
508 hemicellulose content (Xiong et al., 2009) and lignin (Zafari et al., 2017) in the root cell
509 wall to prevent accumulation of metals in the soluble fraction of the cells. NO could
510 also increase the tolerance to Pb by increasing the content of auxin, cytokinins and
511 gibberellins and by decreasing abscisic acid (Sadeghipour, 2017). In *B. auriculata*
512 exposed to Pb, the GSNOR activity which can regulate NO levels in the cell, did not
513 change in roots but it was significantly reduced in leaves – a finding that supports an
514 important role of both NO and GSNOR in Pb uptake and translocation. Recently, Li et
515 al. (2019), have reported that the differences of GSNOR expression might be
516 responsible for the natural variation of root tolerance to high Fe in different *Arabidopsis*
517 accessions. The antioxidant activity of NO reported in different plant species and in
518 response to different metals (Bai et al., 2014; Terrón-Camero et al., 2019) could also
519 contribute to the tolerance of *B. auriculata* to Pb.

520

521

5. CONCLUSIONS

The data obtained from the present study demonstrate that *B. auriculata* is a new Pb tolerant plant able to accumulate high concentrations of Pb in the roots while increasing its growth in presence of high Pb concentrations. However, *B. auriculata* can not be considered a hyper-accumulator because does not accumulate the metal in leaves, which demonstrate that Pb tolerance is not necessarily associated with Pb hyper-accumulation, such it has been reported in *N. caerulescens* (Mohtadi et al., 2012). Pb tolerance in *B. auriculata* could be due to its ability to accumulate Pb in the cell walls and vacuoles of root cells and the induction of PC2, as well as to the restriction of metal translocation to the shoot. Treatment with Pb produced a decrease in the Zn content in the plant, which could indicate that both elements may use the same channel/transporters. In line with the absence of symptoms of toxicity, Pb does not induce oxidative stress in this species – probably as a consequence of the low availability of the metal to participate in ROS production. However, the differential induction of some enzymatic antioxidant defense system such as SOD activity in leaf and CAT, and the Foyer-Halliwell-Asada cycle-activities in roots could also contribute to prevent ROS accumulation and oxidative damage. Differential changes in NO production and GSNOR activity could contribute to the tolerance to Pb in *B. auriculata*.. Therefore, *B. auriculata* may be useful for the phytostabilization and repopulation of areas contaminated with Pb and other metals.

ACKNOWLEDGMENTS

This work was supported by the Spanish Ministry of Science, Innovation and Universities (CGL2015-67644-R and PGC2018-098372-B-100), Castilla-La Mancha

Regional Government (SBPLY/17/180501/000273) and Internal Research Plan I+D+i of University of Castilla-La Mancha (Predoc contract [2016/14100]). The proteomic analysis was performed in the Proteomic Unit of IPBLN-CSIC, that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001.

REFERENCES

Alamri, S.A., Siddiqui, M.H., Al-Khaishany, M.Y.Y., Khan, M.N., Ali, H.M., Alaraidh, I.A., Alsahli, A.A., Al-Rabiah, H., Mateen, M., 2018. Kanuascorbic acid improves the tolerance of wheat plants to lead toxicity. J. Plant Interact. 13, 409–419. https://doi.org/10.1080/17429145.2018.1491067

Alarcón, M.V., Salguero, J., Lloret, P.G., 2019. Auxin modulated initiation of lateral roots is linked to pericycle cell length in maize. Front. Plant Sci. 10, 1–10. https://doi.org/10.3389/fpls.2019.00011

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals-Concepts and applications. Chemosphere. 91, 869-881. https://doi.org/10.1016/j.chemosphere.2013.01.075

Alscher, R. G., Erturk, N., Heath, L. S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331-1341. https://doi.org/10.1093/jexbot/53.372.1331

Andra, S.S., Datta, R., Sarkar, D., Makris, K.C., Mullens, C.P., Sahi, S. V., Bach, S.B.H., 2009. Induction of lead-binding phytochelatins in vetiver grass [*Vetiveria zizanioides* (L.)]. J. Environ. Qual. 38, 868–877. https://doi.org/10.2134/jeq2008.0316

569 Bai, X.Y., Dong, Y.J., Wang, Q.H., Xu, L.L., Kong, J., Liu, S., 2014. Effects of lead
570 and nitric oxide on photosynthesis, antioxidative ability, and mineral element
571 content of perennial ryegrass. Biol. Plant. 59, 163–170.
572 https://doi.org/10.1007/s10535-014-0476-8

573 Batista, A.A., Santos, J.A.G., Bomfim, M.R., Moreira, F.M., Leal, E.F., Conceição, J.N.
574 da, 2017. Induced changes in the growth of four plant species due to lead toxicity.
575 Rev. Bras. Eng. Agrícola e Ambient. 21, 327–332. https://doi.org/10.1590/1807-
576 1929/agriambi.v21n5p327-332

577 Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: Improved assays and an
578 assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.
579 https://doi.org/10.1016/0003-2697(71)90370-8

580 Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in Plants.
581 Toxic Metals in Plants. Brazilian J. Plant Physiol. https://doi.org/10.1590/S1677-
582 04202005000100004

583 Bezerril, N.M., Otoch, M. de L.O., Gomes-Rochette, N.F., Sobreira, A.C. de M.,
584 Barreto, A.A.G.C., de Oliveira, F.D.B., Costa, J.H., Borges, S. da S.S., do
585 Nascimento, R.F., Fernandes de Melo, D., 2017. Effect of lead on physiological
586 and antioxidant responses in two *Vigna unguiculata* cultivars differing in Pb-
587 accumulation. Chemosphere. 176, 397–404.
588 https://doi.org/10.1016/j.chemosphere.2017.02.072

589 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram
590 quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.
591 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

592 Buege, J.A., Aust, S.D., 1978. [30] Microsomal lipid peroxidation. Methods Enzymol.

593 52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

594 Calabrese, E.J., Blain, R.B., 2009. Hormesis and plant biology. Environ. Pollut. 157,
595 42–48. https://doi.org/10.1016/j.envpol.2008.07.028

596 Chandrasekhar, C., Ray, J.G., 2019. Lead accumulation, growth responses and
597 biochemical changes of three plant species exposed to soil amended with different
598 concentrations of lead nitrate. Ecotoxicol. Environ. Saf. 171, 26–36.
599 https://doi.org/10.1016/j.ecoenv.2018.12.058

600 Chatterjee, C., Dube, B.K., Sinha, P., Srivastava, P., 2004. Detrimental effects of lead
601 phytotoxicity on growth, Yield, and Metabolism of Rice. Commun. Soil Sci. Plant
602 Anal. 35, 255–265. https://doi.org/10.1081/CSS-120027648

603 Cutright, T.J., Senko, J., Sivaram, S., York, M., 2012. Evaluation of the Phytoextraction
604 Potential at an Acid-Mine-Drainage-Impacted Site. Soil Sediment Contam. 21,
605 970–984. https://doi.org/10.1080/15320383.2012.712070

606 del Río, L.A., Sevilla, F., Sandalio, L.M.,Palma, J.M., 1991. Nutritional effect and
607 expression of SODs: Induction and gene expression; diagnostics; prospective
608 protection against oxygen toxicity. Free Radic. Res. Commun. 12-13, 819-827.
609 https://doi.org/10.3109/10715769109145863

610 Dickinson, N., 2016. Phytoremediation. Encycl. Appl. Plant Sci. 3, 327–331.
611 https://doi.org/10.1016/B978-0-12-394807-6.00016-2

612 El-Zohri, M.H.A., Cabala, R., Frank, H., 2005. Quantification of phytochelatins in
613 plants by reversed-phase HPLC–ESI–MS–MS. Anal. Bioanal. Chem. 382, 1871–
614 1876. https://doi.org/10.1007/s00216-005-3331-0

615

Escarré, J., Lefèbvre, C., Raboyeau, S., Dossantos, A., Gruber, W., Cleyet Marel, J.C., Frérot, H., Noret, N., Mahieu, S., Collin, C., van Oort, F., 2011. Heavy Metal Concentration Survey in Soils and Plants of the Les Malines Mining District (Southern France): Implications for Soil Restoration. Water, Air, Soil Pollut. 216, 485–504. https://doi.org/10.1007/s11270-010-0547-1

Fahr, M., Laplaze, L., Bendaou, N., Hocher, V., El Mzibri, M., Bogusz, D., Smouni, A., 2013. Effect of lead on root growth. Front. Plant Sci. 4, 175. https://doi.org/10.3389/fpls.2013.00175

Farooq, M.A., Ali, S., Hameed, A., Ishaque, W., Mahmood, K., Iqbal, Z., 2013. Alleviation of cadmium toxicity by silicon is related to elevated photosynthesis, antioxidant enzymes; suppressed cadmium uptake and oxidative stress in cotton. Ecotoxicol. Environ. Saf. 96, 242–249. https://doi.org/10.1016/j.ecoenv.2013.07.006

Fellet, G., Pošćić, F., Casolo, V., Marchiol, L., 2012. Metallophytes and thallium hyperaccumulation at the former Raibl lead/zinc mining site (Julian Alps, Italy). Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 146, 1023–1036. https://doi.org/10.1080/11263504.2012.703250

Fischer, S., Kühnlenz, T., Thieme, M., Schmidt, H., Clemens, S., 2014. Analysis of plant Pb tolerance at realistic submicromolar concentrations demonstrates the role of phytochelatin synthesis for Pb detoxification. Environ. Sci. Technol. 48, 7552–7559. https://doi.org/10.1021/es405234p

Flores-Cáceres, M.L., Hattab, Sabrine, Hattab, Sarra, Boussetta, H., Banni, M., Hernández, L.E., 2015. Specific mechanisms of tolerance to copper and cadmium are compromised by a limited concentration of glutathione in alfalfa plants. Plant

Sci. 233, 165–173. https://doi.org/10.1016/j.plantsci.2015.01.013

García, S., Zornoza, P., Hernández, L.E., Esteban, E., Carpena, R.O., 2017. Response of *Lupinus albus* to Pb–EDTA indicates relatively high tolerance. Toxicol. Environ. Chem. 99, 1378–1388. https://doi.org/10.1080/02772248.2017.1387263

Gill, S.S., Hasanuzzaman, M., Nahar, K., Macovei, A., Tuteja, N., 2013. Importance of nitric oxide in cadmium stress tolerance in crop plants. Plant Physiol. Biochem. https://doi.org/10.1016/j.plaphy.2012.12.001

Gupta, D.K., Nicoloso, F.T., Schetinger, M.R.C., Rossato, L. V., Pereira, L.B., Castro, G.Y., Srivastava, S., Tripathi, R.D., 2009. Antioxidant defense mechanism in hydroponically grown *Zea mays* seedlings under moderate lead stress. J. Hazard. Mater. 172, 479–484. https://doi.org/10.1016/j.jhazmat.2009.06.141

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem, 249(22), 7130-7139.

Hadi, F., Bano, A., Fuller, M.P., 2010. The improved phytoextraction of lead (Pb) and the growth of maize (*Zea mays* L.): the role of plant growth regulators (GA3 and IAA) and EDTA alone and in combinations. Chemosphere. 80(4), 457-462.

Hafsi, C., Romero-Puertas, M. C., Gupta, D. K., del Río, L. A., Sandalio L. M., Abdelly, C., 2010. Moderate salinity enhances the antioxidative response in the halophyte Hordeum maritimum L. under potassium deficiency. Environ Exp Bot 69, 129–136. https://doi.org/10.1016/j.envexpbot.2010.04.008

Hernández, I., Alegre, L., Van Breusegem, F., Munné-Bosch, S., 2009. How relevant are flavonoids as antioxidants in plants? Trends Plant Sci. 14, 125–132.

https://doi.org/10.1016/j.tplants.2008.12.003

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. 347, 32 pp.

Islam, E., Liu, D., Li, T., Yang, X., Jin, X., Mahmood, Q., Tian, S., Li, J., 2008. Effect of Pb toxicity on leaf growth, physiology and ultrastructure in the two ecotypes of *Elsholtzia argyi*. J. Hazard. Mater. 154, 914–926. https://doi.org/10.1016/j.jhazmat.2007.10.121

Jiménez-Escrig, A., Dragsted, L.O., Daneshvar, B., Pulido, R., Saura-Calixto, F., 2003. In vitro antioxidant activities of edible artichoke (*Cynara scolymus* L.) and effect on biomarkers of antioxidants in rats. J. Agric. Food Chem. 51, 5540–5545. https://doi.org/10.1021/jf030047e

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: implications in phytoremediation. Environ. Exp. Bot. 67(1), 243-259. https://doi.org/10.1016/j.envexpbot.2009.06.013

Kim, Y.Y., Yang, Y.Y., Lee, Y., 2002. Pb and Cd uptake in rice roots. Physiol. Plant. 116, 368–372. https://doi.org/10.1034/j.1399-3054.2002.1160312.x

Kopittke, P.M., Asher, C.J., Menzies, N.W., 2007. Toxic effects of Ni^{2+} on growth of cowpea (*Vigna unguiculata*). Plant Soil 292, 283–289. https://doi.org/10.1007/s11104-007-9226-4

Kuhn, B.M., Geisle, M., Bigler, L., Ringli, C., 2011. Flavonols accumulate asymmetricallyand affect auxin transport in *Arabidopsis*. Plant Physiol. 156, 585–595. https://doi.org/10.1104/pp.111.175976

686 Kumar, G.H., Kumari, J.P., 2015. Heavy metal lead influative toxicity and its
687 assessment in phytoremediating plants - A Review. Water. Air. Soil Pollut. 226,
688 324. https://doi.org/10.1007/s11270-015-2547-7

689 Kumar, A., Prasad, M.N.V., 2018. Plant-lead interactions: Transport, toxicity, tolerance
690 and detoxification mechanisms. Ecotoxicol. Environ. Saf.
691 https://doi.org/10.1016/j.ecoenv.2018.09.113

692 LaCoste, C., Robinson, B., Brooks, R., Anderson, C., Chiarucci, A., Leblanc, M., 1999.
693 The phytoremediation potential of thallium-contaminated soils using *Iberis* and
694 *Biscutella* species. Int. J. Phytoremediation 1, 327–338.
695 https://doi.org/10.1080/15226519908500023

696 León, A.M., Palma, J.M., Corpas, F.J., Gómez, M., Romero-Puertas, M.C., Chatterjee,
697 D., Mateos, R.M., del Río, L.A., Sandalio, L.M., 2002. Antioxidative enzymes in
698 cultivars of pepper plants with different sensitivity to cadmium. Plant Physiol.
699 Biochem. 40, 813–820. https://doi.org/10.1016/S0981-9428(02)01444-4

700 Li, B., Sun, L., Huang, J., Göschl, C., Shi, W., Chory, J., Busch, W., 2019. GSNOR
701 provides plant tolerance to iron toxicity via preventing iron-dependent nitrosative
702 and oxidative cytotoxicity. Nat. Commun. 10(1), 1-13.
703 https://doi.org/10.1038/s41467-019-11892-5

704 Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and carotenoids: measurement
705 and characterization by UV-VIS spectroscopy. Curr. Protoc. Food Anal. Chem. 1,
706 F4.3.1-F4.3.8. https://doi.org/10.1002/0471142913.faf0403s01

707 Mahdavian, K., Ghaderian, S.M., Schat, H., 2016. Pb accumulation, Pb tolerance,
708 antioxidants, thiols, and organic acids in metallicolous and non-metallicolous
709 *Peganum harmala* L. under Pb exposure. Environ. Exp. Bot. 126, 21–31.

https://doi.org/10.1016/j.envexpbot.2016.01.010

Małecka, A., Piechalak, A., Morkunas, I., Tomaszewska, B., 2008. Accumulation of lead in root cells of *Pisum sativum*. Acta Physiol. Plant. 30, 629–637. https://doi.org/10.1007/s11738-008-0159-1

Melo, E.E.C., Costa, E.T.S., Guilherme, L.R.G., Faquin, V., Nascimento, C.W.A., 2009. Accumulation of arsenic and nutrients by castor bean plants grown on an As-enriched nutrient solution. J. Hazard. Mater. 168, 479–483. https://doi.org/10.1016/j.jhazmat.2009.02.048

Meyers, D.E.R., Auchterlonie, G.J., Webb, R.I., Wood, B., 2008. Uptake and localisation of lead in the root system of *Brassica juncea*. Environ. Pollut. 153, 323–332. https://doi.org/10.1016/J.ENVPOL.2007.08.029

Michniewicz, M., Ho, C. H., Enders, T. A., Floro, E., Damodaran, S., Gunther, L. K., Powers, K., Frick, E.M., Topp, C.N., Frommer, W.B., Strader, L. C., 2019. Transporter of IBA1 links auxin and cytokinin to influence root architecture. Developmental cell, 50(5), 599-609. https://doi.org/10.1016/j.devcel.2019.06.010

Mohtadi, A., Ghaderian, S.M., Schat, H., 2012. A comparison of lead accumulation and tolerance among heavy metal hyperaccumulating and non-hyperaccumulating metallophytes. Plant Soil. 352, 267–276. https://doi.org/10.1007/s11104-011-0994-5

Moslehi, A., Feizian, M., Higueras, P., Eisvand, H.R., 2019. Assessment of EDDS and vermicompost for the phytoextraction of Cd and Pb by sunflower (*Helianthus annuus* L.). Int. J. Phytoremediat. 21, 191–199. https://doi.org/10.1080/15226514.2018.1501336

Mroczek-Zdyrska, M., Wójcik, M., 2012. The influence of selenium on root growth and oxidative stress induced by lead in *Vicia faba* L. minor plants. Biol. Trace Elem. Res. 147, 320–328. https://doi.org/10.1007/s12011-011-9292-6

Nakatsubo, N., Kojima, H., Kikuchi, K., Nagoshi, H., Hirata, Y., Maeda, D., Imai, Y., Irimura, T., Nagano, T., 1998. Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: Diaminofluoresceins. FEBS Lett. 427, 263–266. https://doi.org/10.1016/S0014-5793(98)00440-2

Ortega-Galisteo, A.P., Rodríguez-Serrano, M., Pazmiño, D.M., Gupta, D.K., Sandalio, L.M., Romero-Puertas, M.C., 2012. S-Nitrosylated proteins in pea (*Pisum sativum* L.) leaf peroxisomes: Changes under abiotic stress. J. Exp. Bot. 63, 2089–2103. https://doi.org/10.1093/jxb/err414

Pavoni, E., Petranich, E., Adami, G., Baracchini, E., Crosera, M., Emili, A., Lenaz, D., Higueras, P., Covelli, S., 2017. Bioaccumulation of thallium and other trace metals in *Biscutella laevigata* nearby a decommissioned zinc-lead mine (Northeastern Italian Alps). J. Environ. Manage. 186, 214–224. https://doi.org/10.1016/J.JENVMAN.2016.07.022

Pawlak-Sprada, S., Arasimowicz-Jelonek, M., Podgórska, M., Deckert, J., 2011. Activation of phenylpropanoid pathway in legume plants exposed to heavy metals . Part I . Effects of cadmium and lead on phenylalanine ammonia-lyase gene expression , enzyme activity and lignin content. Acta Biochim. Pol. 58, 211–216. https://doi.org/10.18388/abp.2011

Peco, J.D., Campos, J.A., Romero-Puertas, M.C., Olmedilla, A., Higueras, P., Sandalio, L.M., 2020. Characterization of mechanisms involved in tolerance and

accumulation of Cd in *Biscutella auriculata* L. Ecotoxicol. Environ. Saf. 201, 110784. https://doi.org/10.1016/j.ecoenv.2020.110784

Pena, L.B., Méndez, A.A., Matayoshi, C.L., Zawoznik, M.S., Gallego, S.M., 2015. Early response of wheat seminal roots growing under copper excess. Plant Physiol. Biochem. 87, 115–123. https://doi.org/10.1016/j.plaphy.2014.12.021

Pereira, M.P., Rodrigues, L.C. de A., Corrêa, F.F., de Castro, E.M., Ribeiro, V.E., Pereira, F.J., 2016. Cadmium tolerance in *Schinus molle* trees is modulated by enhanced leaf anatomy and photosynthesis. Trees - Struct. Funct. 30, 807–814. https://doi.org/10.1007/s00468-015-1322-0

Pielichowska, M. and Wierzbicka, M., 2004. Uptake and localization of cadmium by *Biscutella laevigata*, a cadmium hyperaccumulator. Acta Biol. Cracoviensia 46, 57–63.

Poschenrieder, C., Cabot, C., Martos, S., Gallego, B., Barceló, J., 2013. Do toxic ions induce hormesis in plants? Plant Sci. 212, 15–25. https://doi.org/10.1016/j.plantsci.2013.07.012

Pošćić, F., Marchiol, L., Schat, H., 2013. Hyperaccumulation of thallium is population-specific and uncorrelated with caesium accumulation in the thallium hyperaccumulator, *Biscutella laevigata*. Plant Soil 365, 81–91. https://doi.org/10.1007/s11104-012-1384-3

Pourrut, B., Shahid, M., Dumat, C., Winterton, P., Pinelli, E., 2011. Lead uptake, toxicity, and detoxification in plants. Rev. Environ. Contam. Toxicol. 213, 113–136. https://doi.org/10.1007/978-1-4419-9860-6_4

Rodríguez, L., Ruiz, E., Alonso-Azcárate, J., Rincón, J., 2009. Heavy metal distribution

780 and chemical speciation in tailings and soils around a Pb-Zn mine in Spain. J.
781 Environ. Manage. 90, 1106–1116. https://doi.org/10.1016/j.jenvman.2008.04.007

782 Romero- Puertas, M.C., Rodríguez- Serrano, M., Corpas, F.J., Gomez, M.D., Del Rio,
783 L.A., Sandalio, L.M., 2004. Cadmium-induced subcellular accumulation of $O_2^{\cdot-}$
784 and H_2O_2 in pea leaves. Plant. Cell Environ. 27, 1122–1134.
785 https://doi.org/10.1111/j.1365-3040.2004.01217.x

786 Romero-Puertas, M.C., Terrón-Camero, L.C., Peláez-Vico, M.Á., Olmedilla, A.,
787 Sandalio, L.M., 2018. Reactive oxygen and nitrogen species as key indicators of
788 plant responses to Cd stress. Environ. Exp. Bot. 161, 107–119.
789 https://doi.org/10.1016/J.ENVEXPBOT.2018.10.012

790 Ruiz, E., Rodríguez, L., Alonso-Azcárate, J., Rincón, J., 2009. Phytoextraction of metal
791 polluted soils around a Pb-Zn mine by crop plants. Int. J. Phytoremediat. 11, 360–
792 384. https://doi.org/10.1080/15226510802565568

793 Sadeghipour, O., 2017. Nitric oxide increases Pb tolerance by lowering Pb uptake and
794 translocation as well as phytohormonal changes in cowpea (*Vigna unguiculata* (L.)
795 Walp.). Sains Malaysiana 46, 189–195. https://doi.org/10.17576/jsm-2017-4602-02

796 Saleem, M., Asghar, H.N., Zahir, Z.A., Shahid, M., 2018. Impact of lead tolerant plant
797 growth promoting rhizobacteria on growth, physiology, antioxidant activities, yield
798 and lead content in sunflower in lead contaminated soil. Chemosphere 195, 606–
799 614. https://doi.org/10.1016/j.chemosphere.2017.12.117

800 Sandalio, L.M., Rodríguez-Serrano, M., Gupta, D.K., Archilla, A., Romero-Puertas,
801 M.C., Del Río, L.A., 2012. Reactive Oxygen species and Nitric Oxide in plants
802 under cadmium stress: From toxicity to signaling, in: Environmental Adaptations
803 and Stress Tolerance of Plants in the Era of Climate Change. Springer New York,

New York, NY, pp. 199–215. https://doi.org/10.1007/978-1-4614-0815-4_9

Sanz-Fernández, M., Rodríguez-Serrano, M., Sevilla-Perea, A., Pena, L., Mingorance, M.D., Sandalio, L.M., Romero-Puertas, M.C., 2017. Screening *Arabidopsis* mutants in genes useful for phytoremediation. J. Hazard. Mater. 335, 143–151. https://doi.org/https://doi.org/10.1016/j.jhazmat.2017.04.021

Seregin, I. V., Kozhevnikova, A.D., 2011. Histochemical methods for detection of heavy metals and strontium in the tissues of higher plants. Russ. J. Plant Physiol. 58, 721–727. https://doi.org/10.1134/s1021443711040133

Seregin, I. V., Shpigun, L.K., Ivanov, V.B., 2004. Distribution and toxic effects of cadmium and lead on maize roots. Russ. J. Plant Physiol. 51, 525–533. https://doi.org/10.1023/B:RUPP.0000035747.42399.84

Seth, C.S., Misra, V., Singh, R.R., Zolla, L., 2011. EDTA-enhanced lead phytoremediation in sunflower (*Helianthus annuus* L.) hydroponic culture. Plant Soil 347, 231–242. https://doi.org/10.1007/s11104-011-0841-8

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive Oxygen Species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 1–26. https://doi.org/10.1155/2012/217037

Sidhu, G.P.S., Bali, A.S., Singh, H.P., Batish, D.R., Kohli, R.K., 2018. Phytoremediation of lead by a wild, non-edible Pb accumulator *Coronopus didymus* (L.) Brassicaceae. Int. J. Phytoremediat. 20, 483–489. https://doi.org/10.1080/15226514.2017.1374331

Sidhu, G.P.S., Singh, H.P., Batish, D.R., Kohli, R.K., 2016. Effect of lead on oxidative status, antioxidative response and metal accumulation in *Coronopus didymus*. Plant

827 Physiol. Biochem. 105, 290–296. https://doi.org/10.1016/j.plaphy.2016.05.019

828 Sima, G., Fatemeh, Z., Niknam, V., 2012. Determination of peroxidase activity, total
829 phenolic and flavonoid compounds due to lead toxicity in *Medicago sativa* L. Adv.
830 Environ. Biol. 6, 2357–2364.

831 Sims, D.A., Gamon, J.A., 2002. Relationships between leaf pigment content and
832 spectral reflectance across a wide range of species, leaf structures and
833 developmental stages. Remote Sens. Environ. 81, 337–354.
834 https://doi.org/10.1016/S0034-4257(02)00010-X

835 Singleton, V.L., Rossi, J.A., 1965. Colorimetry of Total Phenolics with
836 Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 16, 144 LP
837 – 158.

838 Sinha, P., Dube, B.K., Srivastava, P., Chatterjee, C., 2006. Alteration in uptake and
839 translocation of essential nutrients in cabbage by excess lead. Chemosphere 65,
840 651–656. https://doi.org/10.1016/j.chemosphere.2006.01.068

841 Souri, Z., Karimi, N., Farooq, M.A., Sandalio, L.M., 2020. Nitric oxide improves
842 tolerance to arsenic stress in *Isatis cappadocica* desv. Shoots by enhancing
843 antioxidant defenses. Chemosphere 239, 124523.
844 https://doi.org/10.1016/j.chemosphere.2019.124523

845 Srivalli, B., Khanna-Chopra, R., 2001. Induction of new isoforms of superoxide
846 dismutase and catalase enzymes in the flag leaf of wheat during monocarpic
847 senescence. Biochem. Biophys. Res. Commun. 288, 1037–1042.
848 https://doi.org/10.1006/bbrc.2001.5843

849 Wojas, S., Ruszczyńska, A., Bulska, E., Clemens, S., Antosiewicz, D.M., 2010. The

role of subcellular distribution of cadmium and phytochelatins in the generation of distinct phenotypes of AtPCS1- and CePCS3-expressing tobacco. J. Plant Physiol. 167, 981–988. https://doi.org/10.1016/j.jplph.2010.02.010

Sytar, O., Kumar, A., Latowski, D., Kuczynska, P., Strzałka, K., Prasad, M.N. V., 2013. Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. Acta Physiol. Plant. 35, 985–999. https://doi.org/10.1007/s11738-012-1169-6

Thakur, A. K., Singh, K. J., 2012. Leaf temperature as thermal bio-indicator of heavy metal pollutants. J. Agr. Sci. Tech. 2(1A), 131.

Terrón-Camero, L.C., Peláez-Vico, M.Á., Del-Val, C., Sandalio, L.M., Romero-Puertas, M.C., Brouquisse, R., 2019. Role of nitric oxide in plant responses to heavy metal stress: Exogenous application versus endogenous production. J. Exp. Bot. https://doi.org/10.1093/jxb/erz184

Terrón-Camero, L.C., del Val, C., Sandalio, L.M., Romero-Puertas, M.C., 2020. Low endogenous NO levels in roots and antioxidant systems are determinants for the resistance of Arabidopsis seedlings grown in Cd. Environ. Pollut. 256, 113411. https://doi.org/10.1016/j.envpol.2019.113411

Turull, M., Grmanova, G., Dago, À., Ariño, C., Díez, S., Díaz-Cruz, J.M., Esteban, M., 2017. Phytochelatin synthesis in response to Hg uptake in aquatic plants near a chlor-alkali factory. Chemosphere 176, 74–80. https://doi.org/10.1016/j.chemosphere.2017.02.092

Venkatachalam, P., Jayalakshmi, N., Geetha, N., Sahi, S. V., Sharma, N.C., Rene, E.R., Sarkar, S.K., Favas, P.J.C., 2017. Accumulation efficiency, genotoxicity and antioxidant defense mechanisms in medicinal plant *Acalypha indica* L. under lead

874 stress. Chemosphere 171, 544–553.
875 https://doi.org/10.1016/j.chemosphere.2016.12.092

876 Weryszko-Chmielewska, E., Chwil, M., 2005. Lead-Induced histological and
877 ultrastructural changes in the leaves of Soybean (*Glycine max* (L.) Merr.). Soil Sci.
878 Plant Nutr. 51, 203–212. https://doi.org/10.1111/j.1747-0765.2005.tb00024.x

879 Whitacre, D.M., 2015. Lead Uptake, Toxicity, and detoxification in plants. Rev.
880 Environ. Contam. Toxicol. 233. https://doi.org/10.1007/978-3-319-10479-9

881 Wierzbicka, M., Pielichowska, M., 2004. Adaptation of *Biscutella laevigata* L, a metal
882 hyperaccumulator, to growth on a zinc–lead waste heap in southern Poland: I:
883 Differences between waste-heap and mountain populations. Chemosphere 54,
884 1663–1674. https://doi.org/10.1016/J.CHEMOSPHERE.2003.08.031

885 Wierzbicka, M., Pielichowska, M., Abratowska, A., Wiłkomirski, B., Wysocka, I.,
886 Panufnik-Mędrzycka, D., Bulska, E., 2016. Thallium hyperaccumulation in Polish
887 populations of *Biscutella laevigata* (Brassicaceae). Acta Biol. Cracoviensia s. Bot.
888 58.

889 Wuana, R.A., Okieimen, F.E., 2014. Heavy metals in contaminated soils: A review of
890 sources, chemistry, risks, and best available strategies for remediation. Heavy Met.
891 Contam. Water Soil Anal. Assessment, Remediat. Strateg. 2011, 1–50.
892 https://doi.org/10.1201/b16566

893 Xiong, J., An, L., Lu, H., Zhu, C., 2009. Exogenous nitric oxide enhances cadmium
894 tolerance of rice by increasing pectin and hemicellulose contents in root cell wall.
895 Planta 230, 755–765. https://doi.org/10.1007/s00425-009-0984-5

896 Yu, Q., Sun, L., Jin, H., Chen, Q., Chen, Z., Xu, M., 2012. Lead-induced nitric oxide

generation plays a critical role in lead uptake by *Pogonatherum crinitum* root cells. Plant Cell Physiol. 53(10), 1728-1736. https://doi.org/10.1093/pcp/pcs116

Zafari, S., Sharifi, M., Chashmi, N. A., 2017. Nitric oxide production shifts metabolic pathways toward lignification to alleviate Pb stress in *Prosopis farcta*. Environ. Exp. Bot. 141, 41-49. https://doi.org/10.1016/j.envexpbot.2017.06.011

Zheng, L., Peer, T., Seybold, V., Lütz-Meindl, U., 2012. Pb-induced ultrastructural alterations and subcellular localization of Pb in two species of *Lespedeza* by TEM-coupled electron energy loss spectroscopy. Environ. Exp. Bot. 77, 196–206. https://doi.org/10.1016/j.envexpbot.2011.11.018

Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

Response to reviewers/editor in question & answer format (word file)

Typo mistakes have been corrected according with the reviewers's suggestions

CRediT author statement:

Jesus Peco: Investigation, Writing - Original Draft. **Juan Antonio Campos**: Validation, Supervision, Conceptualization, Visualization. **María C. Romero-Puertas**: Supervision. **Adela Olmedilla:** Supervision. **Pablo L. Higueras**: Resources, Funding acquisition, Visualization. **Luisa M. Sandalio**: Conceptualization, Methodology, Resources, Writing - Review & Editing, Visualization, Funding acquisition

*Declaration of Interest Statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dear Dr Teresa Cutright

We have corrected the manuscript following the suggestions by the reviewers. I expect the new manuscript could be suitable for publication in Chemosphere

Looking forward to hearing from you soon

Luisa M. Sandalio

Graphical Abstract

High $Pb(NO_3)_2$ concentrations stimulate *Biscutella auriculata* growth

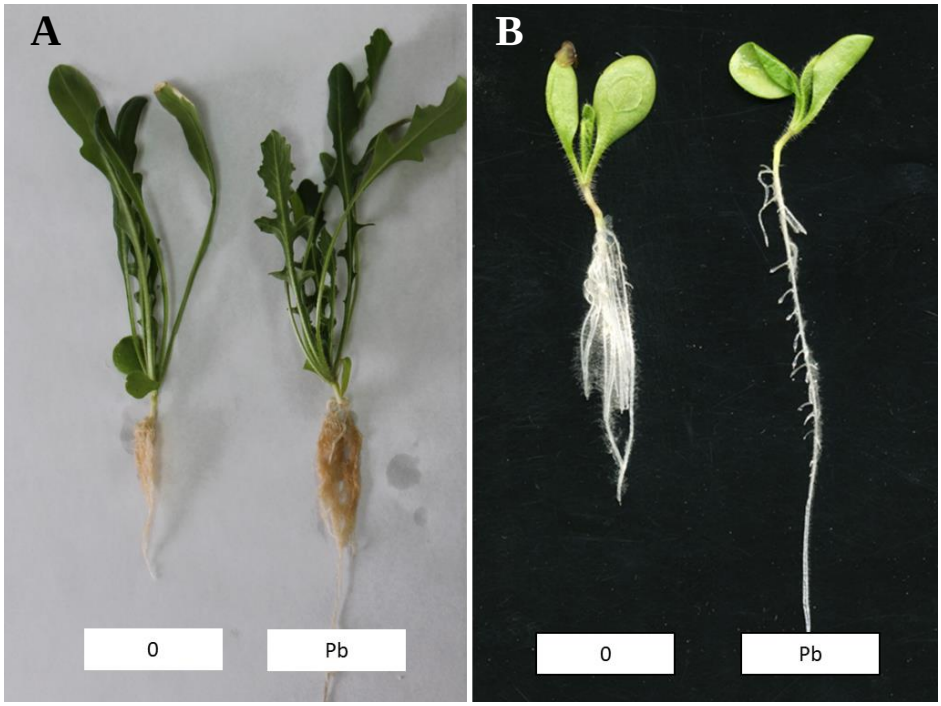
Pb tolerance mechanisms

- Accumulation of Pb in vacuoles and cell wall by induction of PC2.
- Prevents Pb translocation to the aerial part.
- Activation of antioxidative defenses:
  - SOD in leaves.
  - CAT, MDHAR and DHAR in roots.

The uptake and distribution of Pb and the mechanisms involved in the metal tolerance have been investigated in a mine population of *Biscutella auriculata*. Seedlings were exposed to 125 μM $Pb(NO_3)_2$ for 15 days under semihydroponic conditions. The results showed an increase in the size of Pb-treated seedlings and symptoms of toxicity were not observed. ICP-OES analyses showed that Pb accumulation was restricted to root tissue. Imaging of Pb accumulation by dithizone histochemistry revealed the presence of the metal in vacuoles and cell wall in root cells. The accumulation of Pb in vacuoles could be stimulated by an increase in phytochelatin PC2 content. Pb did not promote oxidative damage and this is probably due the increase of antioxidative defenses. In the leaves, Pb produced a significant increase in superoxide dismutase activity, while in roots an increase in catalase and components of the Foyer– Halliwell–Asada cycle were observed. The results indicated that *Biscutella auriculata* has a high capacity to tolerate Pb and this is mainly due to a very efficient mechanism to sequester the metal in roots and a capacity to avoid oxidative stress. This species could therefore be very useful for phytostabilization and repopulation of areas contaminated with Pb.

FIGURES

Figure 1. Phenotype of *Biscutella auriculata* seedlings treated with 0 and 125 µM Pb $(NO_3)_2$ grown in: hydroponic culture for 30 days (**A**, **C**) and square Petri dishes for 10 days (**B and D**). Asterisk indicates that the mean value is significantly different between treatments and controls (±standard error) (*$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$). (1.5 column)



C

	Leaves DW (g)	Root DW (g)	Leaves area (cm^2)	Leaf number	Number of trichomes / mm^2	Number of stomata / mm^2
0	1.69 ± 0.12	0.35 ± 0.02	54.80 ± 4.87	9.40 ± 0.37	44.73 ± 6.78	140.5 ± 10.62
Pb	2.38 ± 0.12***	0.50 ± 0.04**	64.64 ± 3.28	9.45 ± 0.34	26.47 ± 1.48*	239.14 ± 14.42***

D

	Primary roots size (cm)	Number of lateral roots	Lateral roots size (cm)
0	7.08 ± 0.29	15.60 ± 0.93	1.99 ± 0.14
Pb	9.40 ± 0.37*	10.40 ± 2.84*	0.53 ± 0.05***

Figure 2. Pb accumulations in roots of *B. auriculata* growth in hydroponic conditions with and without Pb for 15 days. Pb is visualized using the dithizone staining (brown colour). Red arrows show Pb localization. (1.5 column)

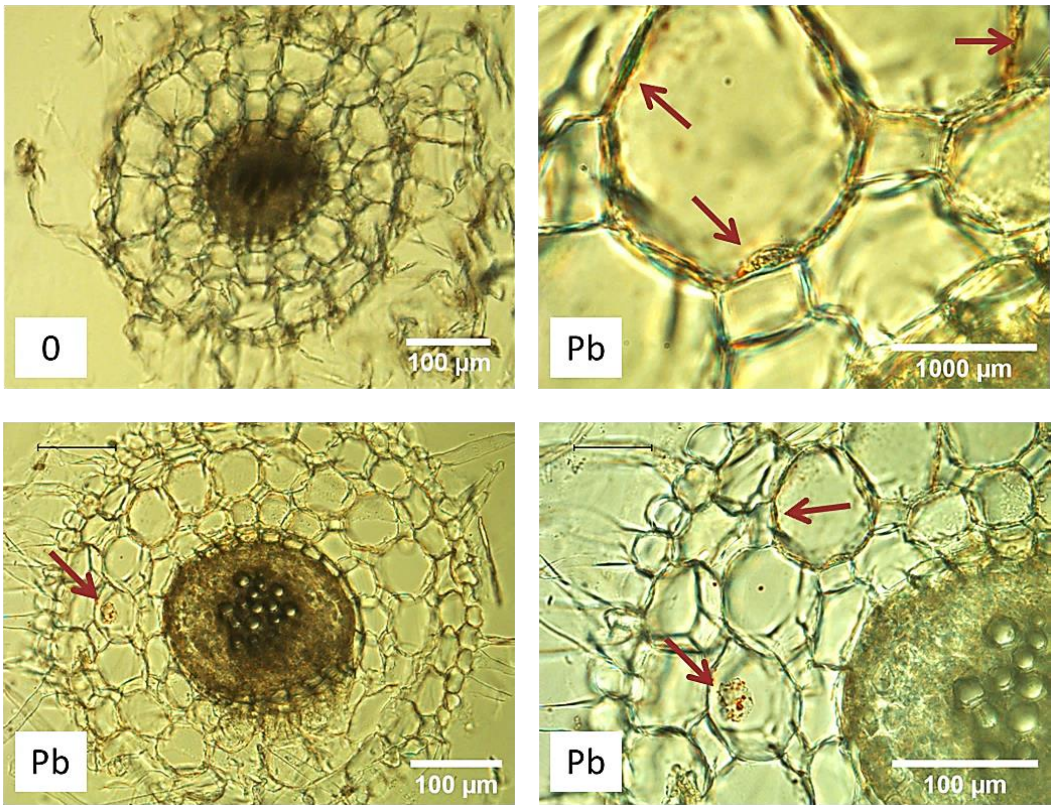


Figure 3. Effect of Pb (125 µM) on biothiols content in *B. auriculata* plants (**A**) and relative biothiols concentration (%) (**B**) in leaves and roots of *B. auriculata.* Values represent the mean ± standard error and asterisks indicates significant differences between treatment and control plants (*p ≤ 0.05). (1.5 column)

A

	0		Pb	
	Leaves	Root	Leaves	Root
GSH (µg g^{-1} FW)	40.23 ± 1.03	38.76 ± 3.3	56.18 ± 6.4	43.51 ± 3.23
GSSG (µg g^{-1} FW)	8.15 ± 1.33	4.63 ± 1.2	8.78 ± 2.03	6.57 ± 1.79
PC2 (µg g^{-1} FW)	< LD	10.47 ± 0.25	< LD	13.43 ± 0.85*
PC3 (µg g^{-1} FW)	< LD	< LD	<LD	<LD
PC4 (µg g^{-1} FW)	< LD	< LD	<LD	<LD
Total biothiols (µg g^{-1} FW)	48.38 ± 1.39	53.86 ± 4.75	64.96 ± 8.43	63.51 ± 5.87

B

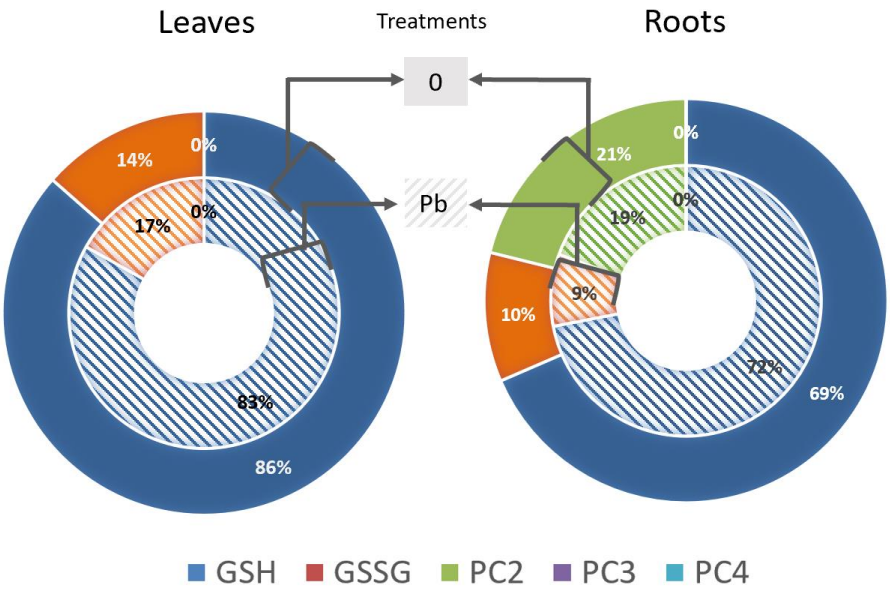


Figure 4. Effects of Pb (125 µM) on ROS and NO metabolism in *B. auriculata* plants grown in hydroponic conditions for 15 days. (**A**) oxidative stress parameters (H_2O_2 content, lipid peroxidation, GOX activity, ascorbate content (AA), GSH/GSSG ratio and total antioxidant capacity, (**B**) enzymatic antioxidant activities (CAT, GST, POD, MDHAR, DHAR and NADPH-dependent dehydrogenase enzymes. (**C**) NO content and GSNOR activity in *B. auriculata* leaves and roots. Asterisk indicates that the mean value is significantly different between treatments and controls (±standard error) (*$p \leq 0.05$). (1.5 column)

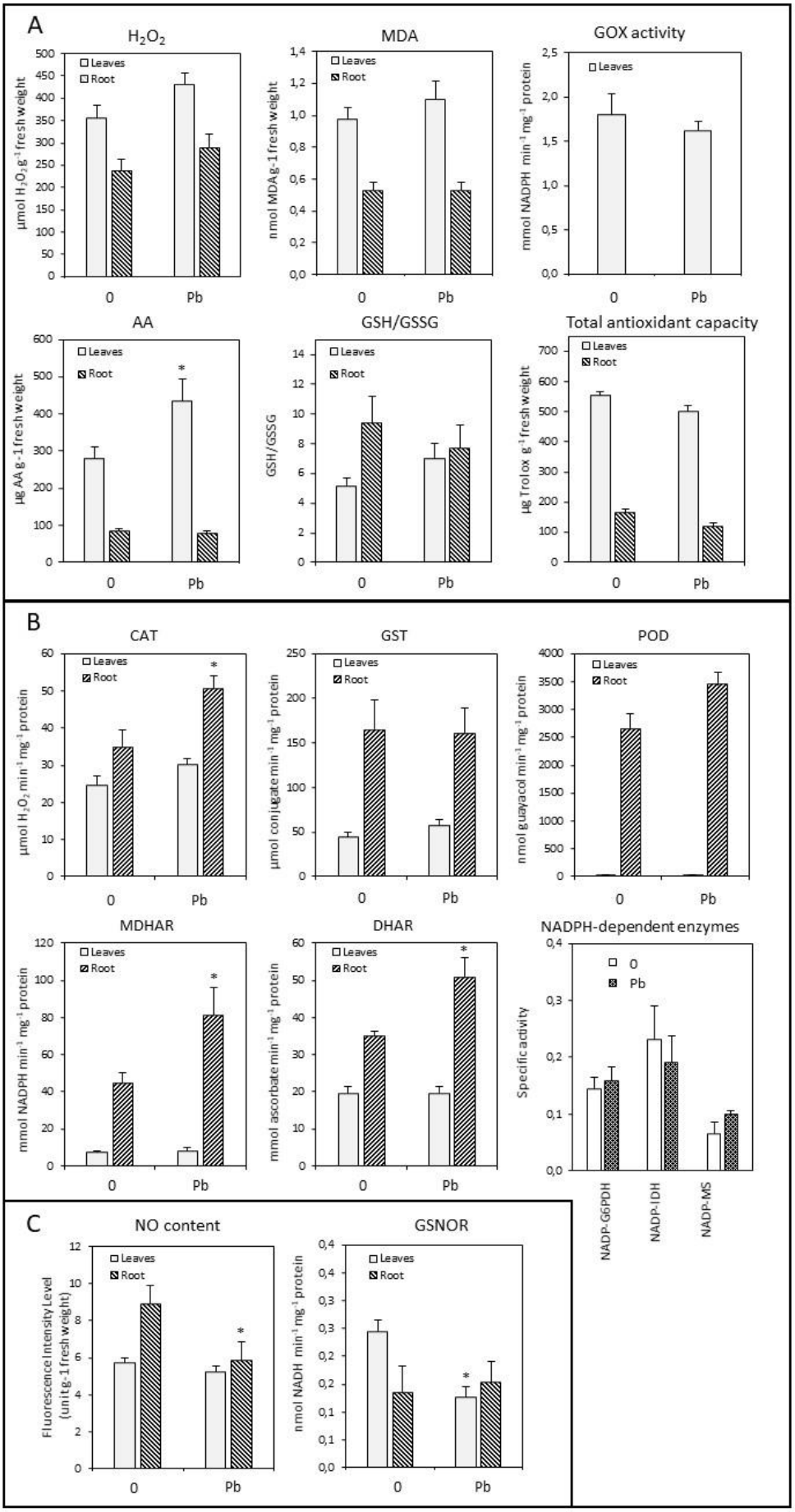
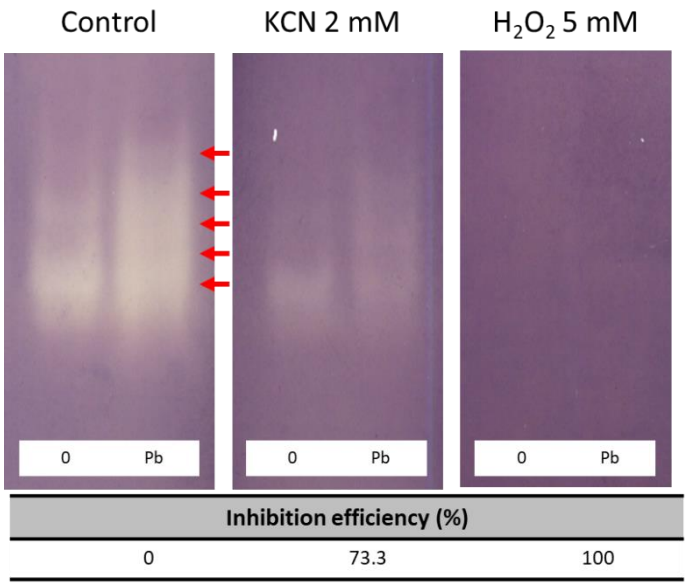

A
H2O2
Leaves
Root
µmol H2O2 g-1 fresh weight
0
Pb
MDA
nmol MDA g-1 fresh weight
GOX activity
mmol NADPH min-1 mg-1 protein
AA
µg AA g-1 fresh weight
GSH/GSSG
Total antioxidant capacity
µg Trolox g-1 fresh weight
B
CAT
µmol H2O2 min-1 mg-1 protein
GST
µmol conjugate min-1 mg-1 protein
POD
nmol guayacol min-1 mg-1 protein
MDHAR
mmol NADPH min-1 mg-1 protein
DHAR
mmol ascorbate min-1 mg-1 protein
NADPH-dependent enzymes
Specific activity
NADP-G6PDH
NADP-IDH
NADP-MS
C
NO content
Fluorescence Intensity Level (unit g-1 fresh weight)
GSNOR
nmol NADH min-1 mg-1 protein

Figure 5. Effects of Pb (125 µM) on SOD activity analysed by native-PAGE. Inhibitory efficiency of KCN 2 mM and H_2O_2 5 mM is shown. (Single column)



Inhibition efficiency (%)		
0	73.3	100