Coordinated role of soluble and cell wall bound phenols is a key feature of the metabolic adjustment in a mining woody fleabane (*Dittrichia viscosa* L.) population under semi-arid conditions

Antonio López-Orenes[a], María C. Bueso[b], Isabel M. Párraga-Aguado, Antonio A. Calderón[a], María A. Ferrer[a*]

[a]Department of Agricultural Science and Technology, Universidad Politécnica de Cartagena, Paseo Alfonso XIII 48, 30203 Cartagena, Murcia (Spain)

[b]Department of Applied Mathematics and Statistics, Universidad Politécnica de Cartagena, Doctor Fleming s/n, 30202 Cartagena, Murcia (Spain)

Abstract

Environmental contamination by hazardous heavy metals/metalloids (metal(loid)s) is growing worldwide. To restrict the migration of toxic contaminants, the establishment of a self-sustainable plant cover is required. Plant growth in multi-polluted soils is a challenging issue not only by metal(loid) toxicities, but also by the co-occurrence of other stressors. *Dittrichia viscosa* is a pioneer Mediterranean species able to thrive in metal(loid)-enriched tailings in semi-arid areas. The aim of the present work was to examine the metabolic adjustments involved in the acclimation responses of this plant to conditions prevailing in mine-tailings during Mediterranean spring and summer. For this purpose, fully-expanded leaves, and rhizosphere soil of both mining and non-mining populations of *D. viscosa* grown spontaneously in south-eastern Spain were sampled in two consecutive years. Quantitative analysis of more than 50 biochemical, physiological and edaphic parameters were performed, including nutrient status, metal(loid) contents, leaf redox components, primary and secondary metabolites, salicylic acid levels, and soil physicochemical properties. Results showed that mining plants exhibited high foliar Zn/Pb co-accumulation capacity, without substantially affecting their photosynthetic metabolism or nutritional status even in the driest summer period. The comparison of the antioxidative/oxidative profile between mining and non-mining *D. viscosa* populations revealed no major seasonal changes in the content of primary antioxidants (ascorbate and GSH), or in the levels of ROS. Multivariate analysis showed that phenylalanine ammonia-lyase (PAL) and peroxidase (PRX) activities and soluble and cell wall-bound phenols were potential biomarkers for discriminating between both populations. During the dry season, a marked enhancement in the activity of both PAL and soluble PRX resulted in both a drop in the accumulation of soluble phenols and an increase of the strong metal chelator caffeic acid in the cell-

wall fraction, supporting the view that the plasticity of phenylpropanoid metabolism provide an effective way to counteract the effects of stress combinations.

Keywords

Mine tailings piles; Metal accumulator; Stress combinations; Mediterranean climate**;** Phenylpropanoid metabolism; Antioxidative/oxidative profiles

1. Introduction

The contamination of the environment with hazardous heavy metals/metalloids (hereafter termed metal(loid)s) is still growing worldwide at an alarming rate, which poses a serious ecological and human health threat of global dimension (Nsanganwimana et al., 2014; Panagos et al., 2013). In the European Union (EU), contamination by metal(loid)s is considered as one of the major threats to EU soil quality (Panagos et al., 2013). Worldwide there are estimated to be close to 22 million ha of land polluted by hazardous metal(loid)s, which has jeopardized people and environmental health as well as food and feed production (Nsanganwimana et al., 2014; Teng et al., 2010). Metalliferous mine tailings represent an important source of hazardous metal(loid) pollutants, which may spread to the surrounding areas leading to the deterioration of nearby agricultural fields and forests (Panagos et al., 2013; Tordoff et al., 2000). Although the reclamation of metalliferous mine wastes is a technically complex procedure (Barceló and Poschenrieder, 2003), in the last decades phytomanagement, which implies the establishment of a self-sustainable plant cover, has emerged as a cost-effective method for reducing water and wind erosion and the migration of hazardous contaminants in metalliferous substrates (Parraga-Aguado et al., 2013; Tordoff et al., 2000). Successful phytomanagement requires suitable plant species able to thrive under the harsh conditions prevailing in the metalliferous mine tailings and well adapted to the climatic conditions of the zone.

Dittrichia viscosa (L.) W. Greuter (woody fleabane) is an evergreen herbaceous perennial Mediterranean plant species (Asteraceae) found in ruderal environments (Al Hassan et al., 2016; Parolin et al., 2013), including mine tailings highly polluted by a broad range of toxic metal(loid)s, where it accumulates As, Cd, Pb, and Zn (Conesa et

al., 2011; Fernández et al., 2013; Pérez-Sirvent et al., 2012). Thus, taken into account its pioneer character and its ability to accumulate high metal concentrations in shoots, this species may improve edaphic properties of tailing soils, and thus, by ameliorating stressful conditions, may pave the way for the recruitment of other less tolerant plant species (Parraga-Aguado et al., 2013).

A common hallmark of plant response to abiotic stresses, including metal(loid) exposure, is an over-production of reactive oxygen species (ROS) into cells (Gill and Tuteja, 2010; Schützendübel and Polle, 2002). ROS in conjunction with the antioxidant network determine the cellular redox environment, which results in redox signaling appropriate to environmental stimuli and developmental cues, leading to growth and acclimation responses (Noctor et al., 2014). Despite the fact that tolerance to metal(loid)s can vary significantly amongst plant species, there is ample evidence that the strengthening of the antioxidant network would be essential for restoring cellular redox-homeostasis and metabolism functions under stress (for review, see Schützendübel and Polle 2002; Sharma and Dietz 2009; Gill and Tuteja 2010; Hossain et al. 2012; Singh et al. 2015). However, most studies dealing with the effect of metal(loid)s exposure on antioxidative/oxidative stress-related markers in plants have been performed under laboratory-controlled conditions. Scarce information can be found about stress biomarkers in plants growing in their natural environment, where they are concurrently exposed to diverse environmental stress factors. Therefore, the acclimation responses of plants to combined stress might require conflicting or antagonistic responses (Shaar-Moshe et al., 2017; Suzuki et al., 2014). Recent studies have shown that plant acclimation to stress combination elicits specific physiological and molecular responses that cannot be inferred from individual stress treatments, and such responses are characterized by changes in ROS levels, lipid peroxidation,

alterations in the expression/activity of ROS-scavenging enzymes, and higher content of antioxidants such as ascorbate (AA), glutathione (GSH), carotenoids and phenolic compounds (Choudhury et al., 2016; Martinez et al., 2016). Phenolic compounds comprise a wide and diverse group of secondary metabolites, and have been shown to play significant roles in plant defense, structural support, modulation of plant cell growth and differentiation, and survival (Ferrer et al., 2008). Among phenolics, flavonoids are considered powerful antioxidants due to their ability to prevent ROS production, as well as to quench ROS and to chelate metal ions, thus acting as modulators of ROS-signaling processes (Agati et al., 2012; Brunetti et al., 2015). Recent evidence has shown that environmental stresses like drought and heat could regulate the levels of nuclear flavonoids (Mouradov and Spangenberg, 2014), which, in turn, could act as regulators of the activity of various protein kinases involved in the ROS-signal transduction pathways that control cell growth and differentiation (Brunetti et al., 2015).

Since in Mediterranean areas high sunlight irradiance, high temperatures and severe drought are key factors limiting plant growth and development, and since, furthermore the cellular antioxidative/oxidative status plays a pivotal role in the capability of plants to cope with oxidative stress induced by environmental factors, the purpose of the current work was (i) to compare the antioxidative/oxidative profile along with key growth parameters of two populations of *D. viscosa*, one grown on a multi-metal(loid) polluted mine tailing (Agustin) and the other in a non-mining site (control), during late spring (May) and late summer (September) in two consecutive years (2012 and 2013); (ii) to identify any possible nutrient imbalance and to assess their bioaccumulation capacity of potentially harmful metal(loid)s under stress combination; (iii) to carry out an edaphic characterization of the rhizosphere soil (fertility parameters and total

concentrations of As, Cd, Cu, Mn, Ni, Pb, Zn and Sb) associated with *D. viscosa* roots on the selected sites, and (iv) to identify inter-correlations among the different biochemical parameters evaluated in both seasons as well as associations between plant markers and environmental factors using different multivariate statistical methods.

This study forms part of a wider investigation that has been undertaken to examine the oxidative stress signatures and the metabolic adjustments in response to the adverse conditions of mine-tailings in semi-arid regions in different pioneer plant species (López-Orenes et al., 2017). In this current work, and considering the metal accumulator properties of *D. viscosa* plants, we hypothesized that (1) the low soil fertility conditions in the tailings together with their high content of hazardous metal(loid)s would provoke a metabolic reprogramming to meet the demand for antioxidants and metal-chelating compounds, and (2) during summer the combination of nutrient deficiencies, metal(loid)-toxicities, high temperature and drought would require an effective photoprotective strategy to maintain the photosynthetic metabolism needed to perform the energy-requiring processes associated with metal uptake, transport and sequestration. The results obtained could contribute to improve our understanding of the acclimative responses of metal(loid)-accumulator plants to stress combinations under natural (field) conditions. Taking into account the potential of this species for thriving under harsh environmental conditions, it seems to be plausible its use in land reclamation programs, especially under the foreseeable future climate change scenario of increasing temperatures and decreasing precipitation.

2. Materials and Methods

2.1. Plant and soil sampling and soil analysis

145

146 *D. viscosa* leaves were obtained from plants growing spontaneously in the Cartagena-La
147 Union Mining District (SE of the Iberian Peninsula) in one tailings pile (Agustin;
148 37°36’20” N, 0°50’15” W) and in a non-mining area (37°35’47” N, 0°49’26” W)
149 located about 1.5 km away from this mining site (Supplemental Fig. S1). This mining
150 area contains one of the largest Pb and Zn content in the SE of Europe (Pérez-Sirvent et
151 al., 2012), and the two sampling zones are located in a natural park which includes
152 Aleppo pine forests and endemic xerophytic thickets (Parraga-Aguado et al., 2013).
153 During 2012 and 2013, the average annual rainfall of the zone was ~ 215 mm, the
154 potential evapotranspiration was ~ 1285 mm yr^{-1} and ~18 °C the annual average air
155 temperature (Supplemental Fig. S2). Taking into account the proximity of the two
156 sampling areas, it was assumed that all plants from the two populations (Supplemental
157 Fig. S1) were exposed to similar weather conditions. In these years the sampling date
158 corresponding to September 2012 was that one in which the greatest rainfall occurred
159 and May 2013 followed a rainy month of April (80 mm rainfall) and was wetter than
160 May 2012 (Supplemental Fig. S2).
161 The uppermost fully expanded leaves from *D. viscosa* plants belonging to the two
162 populations were collected the 3rd week of May (late spring) and the 3rd week of
163 September (late summer) in two consecutive years (2012 and 2013) (Supplemental Fig.
164 S3). In all sampling periods, at least one hundred leaves of each population were
165 washed thoroughly with tap and distilled water, gently blotted on filter paper, and
166 randomly divided into five groups. One group (twenty leaves) was used to determine
167 the relative water content, and each one of the remaining four biological replicates, were
168 divided into two subsamples, one of them was immediately frozen in liquid nitrogen,
169 and stored at –80°C until analyzed, and the second one was dried at 60ºC for 72 h for

elemental analysis. Rhizosphere soil, sampled from the top 20 cm, were also collected from four selected plants, and transferred under aseptic conditions to laboratory. Soil analyses were carried out as described in Parraga-Aguado et al. (2014). In short, soil pH, electrical conductivity (EC), dissolved organic carbon (DOC), and water extractable ions (Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, Cl^{-}, SO_4^{2-}) were determined in a 1:5 soil to water suspension after shaking for 2 h. Equivalent calcium carbonate (% $CaCO_3$) was estimated using the Bernard calcimeter method. Particle size distribution was determined following the method of Bouyoucos densimeter (Gee and Bauder, 1986). Total nitrogen (TN) was determined using the Kjeldahl method (USDA, 1996). Total metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, Zn, and Sb) were measured by X-Ray Fluorescence (Buker S4 Pioneer).

2.2. Macronutrient and metal(loid) determinations in leaf tissues

Leaf dried tissues (~0.5 g), finely ground, were incinerated at 550 ºC for 3 h prior to adding 1 mL of concentrated nitric acid. The resulting extracts were diluted to 25 mL with MilliQ water and filtered through CHM F2041-110 ashless filter papers. The concentration of Cl, P and S were analyzed using an ion chromatographer (Metrohm). The Ca, K, Mg, and Na contents were analyzed using a flame atomic absorption spectrometer (Unicam 969 AA). Nitrogen in leaf samples was determined using a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Chshire, UK). Metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, and Zn) were determined by inductively coupled plasma-mass spectrometry (Agilent 7500A, detection limit 0.001 mg L^{-1}). Plant analyses were referenced using a CTA-VTL-2 certified material (Virginia tobacco leaves), and the percentage of recoveries ranged between 89 and 110%.

195

196 *2.3. Plant performance measurements*

197

198 The evaluation of the physiological status of the two *D. viscosa* populations was carried
199 out by measuring leaf relative water content (RWC), photosynthetic pigment
200 concentrations, total soluble protein levels, soluble sugars and starch contents as
201 previously described (López-Orenes et al., 2017). Chlorophyll *a* (Chla), chlorophyll *b*
202 (Chlb) and total carotenoids were extracted with 100% methanol (1 mL per 0.1 g tissue)
203 using sonication (37 kHz) until the extracts were colorless (x2). The supernatants
204 obtained after centrifugation (15,000xg for 15 min at 4ºC) were used to determine the
205 levels of photosynthetic pigments using the equations reported by Lichtenthaler and
206 Wellburn (1983).

207

208 *2.4. Total antioxidant activity and non-enzymatic antioxidants determinations*

209

210 The analysis of the full spectrum of non-enzymatic antioxidant compounds were
211 estimated by the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH
212 (1,1-diphenyl-2-picrylhydrazyl) and FRAP (ferric reducing antioxidant potential) tests,
213 using methanolic extracts, according to Pérez-Tortosa et al. (2012). Quantification of
214 ascorbate (AA) and dehydroascorbate (DHA) were carried out using the bipyridyl
215 method as described by Gillespie and Ainsworth (2007). Reduced glutathione (GSH)
216 levels were determined fluorimetrically using *o*-phthalaldehyde after Senft et al. (2000).
217 The amount of free proline (Pro) was assayed using the acid-ninhydrin method (Bates et
218 al., 1973). The concentration of total soluble non-protein thiols (NPT) was analyzed
219 using DTNB [5,5′ dithio-(2-nitrobenzoic acid)] as described in Metwally et al. (2003),

and the concentration of phytochelatins was estimated from the difference between total soluble non-protein thiols and GSH (López-Orenes et al., 2014).

2.5. Determination of hydrogen peroxide, superoxide radicals, lipid peroxidation and protein oxidation

Hydrogen peroxide determination was carried out by the ferrous oxidation-xylenol orange method (Cheeseman, 2006). Superoxide anion radical concentrations were determined by the conversion of hydroxylamine into nitrite (Jiang and Zhang, 2001). The extent of lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reacting substances (TBARS) as reported by Hodges et al. (1999), and the extent of protein oxidation was estimated by measuring the protein carbonyl content using the dinitrophenylhydrazine assay (Levine et al., 1994).

2.6. Quantification of total soluble phenolic compounds, flavanols, total flavonoids, hydroxycinnamic acids, lignin, cell wall-associated proanthocyanidins and cell wall-bound phenols

Supernatants of methanolic extracts, obtained as explained in section 2.3, were used for the spectrophotometric determination of different types of phenolics basically as described in a previous report (López-Orenes et al., 2017). Briefly, the content of soluble total phenol compounds (TPC) was measured by the Folin-Ciocalteu method (Everette et al., 2010) and expressed as gallic acid equivalents (GAE). Flavanols were determined using *p*-dimethylaminocinnamaldehyde (DMACA) reagent and expressed as

(+)-catechin equivalents (López-Arnaldos et al., 2001). Total flavonoids were measured by the aluminum chloride assay using rutin as a standard (Kim et al., 2003), and total hydroxycinnamic acids (HCAs) were quantified using the Arnow's reagent and caffeic acid as a reference compound. The pellets of the methanol extracts, after thoroughly washing with ethanol, were air-dried at 60 ºC and used for lignin determination by the lignin-thioglycolic acid method (Hatzilazarou et al., 2006).

For the determination of the content of cell wall-associated proanthocyanidins (PAs), the absorbances at 545 nm of the supernatants obtained after the acid attack on washed cell wall pellets were determined, and the results were expressed as cyanidin equivalents by using an ε_{545} = 34.7 mM^{-1} cm^{-1} after Vermerris and Nicholson (2006).

The analysis of cell wall-bound phenols was carried out according to López-Arnaldos et al. (2001) with minor modifications. Briefly, the pellets of the methanol extracts were washed several times (x3) with pure methanol, and dried under nitrogen stream at 60ºC in a heating block (Techne Dri-Block, DB-3D). Then, dry cell wall materials were weighed and hydrolyzed with 2 M NaOH (1:100, w/v) for 16 h under nitrogen. The hydrolysates were acidified with concentrated HCl and extracted (x2) with diethyl ether. The pooled organic phases were dried under nitrogen stream, and the residue was dissolved in methanol and stored at −80°C until analyzed. Total phenolic contents in these fractions were assessed as indicated above for the total soluble phenolic compounds assay.

All the spectrophotometric determinations were done in quadruplicate. Calibration curves were generated for each assay session using the corresponding standard solutions. A good linearity ($r^2 > 0.99$) between standard concentration and absorbance was always observed for all the methods assayed.

2.7. HPLC analysis of soluble and cell wall-bound phenolic compounds

RP-HPLC (Reversed phase-high pressure liquid chromatography) assays were performed with a liquid chromatographic system equipped with a Waters Alliance 2695 separations module (Waters, Milford, MA, USA), a variable-wavelength diode array detector Waters 2996 and controlled by Empower Pro software. A Luna C18 column (250 mm × 4.6 mm, 5 μm particle size; Phenomenex) was employed for separations. Chromatographic analyses were carried out at 40 ºC as previously described (Xu et al., 2017). The mobile phase consisted of 0.1% formic acid–water solution (solvent A), and methanol (solvent B). The gradient used was: 95% A, 0 min; 80% A, 15 min; 70% A, 20 min; 63% A, 25 min; 60% A, 40 min; 50% A, 60 min; 95% A, 63 min. The flow rate was 0.8 mL min^{-1}, and the injection volume was 10 μL. Identification of the major phenolic compounds was done by comparison of the retention times and UV spectra with those of reference compounds (caffeic acid, catechin, chlorogenic acid, coumaric acid, epicatechin, ferulic acid, gallic acid, p-hidroxybenzoic acid, protocatechuic acid, quercetin, and rutin). Calibration curves for quantification of analytes were generated by injection of standard mixtures (standard amounts ranged from 0.1 to 5 nmol) and showed a good linearity ($r^2 > 0.99$) between standard amount and peak area in the chromatograms obtained at the wavelength corresponding to the maximum absorbance of the standard considered. Analysis of samples and standard solutions were done in triplicate. A mid-point calibration standard mixture was injected at the beginning and end of every sample batch (eight samples) in order to assess instrumentation drift in retention time and response factor. Possible analyte carry-over during batch analyses was checked by injecting pure methanol at the end of every sample batch. All solutions

and HPLC mobile phases were prepared with freshly MilliQ water and filtered through 0.45 μm nylon filters (Millipore, Bedford, MA, USA).

2.8. Enzymatic assays

The extraction and assay of PAL and soluble and ionically-bound cell wall PRXs in leaf extracts were carried out as previously described (López-Orenes et al., 2013a). PAL activity was determined by following the conversion of L-phenylalanine into *trans*-cinnamic acid (*t*-CA) (ε_{290}= 9.5 $mM^{-1}cm^{-1}$) at 290 nm using a microplate reader (Multiskan GO; Thermo Scientific) and 96-well UV plates (Corning). The activity of the enzyme was expressed in nmol of *t*-CA formation per hour per mg protein. PRX activity was estimated by following the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) (ε_{652}= 39 $mM^{-1}cm^{-1}$) in the presence of 2 mM H_2O_2, at 652 nm. PRX activity was expressed in nkatal (nkat) per mg protein, which corresponds to the amount of enzyme that oxidizes 1.0 nmol of TMB per second per mg protein. Protein determinations were made with the Bradford protein assay kit (Bio-Rad Laboratories), using bovine serum albumin as a standard.

2.9. Quantification of free and conjugated salicylic acid

Quantification of free salicylic acid (SA) and conjugated SA (SAG, 2-*O*-*β*-D-glucosylsalicylic acid) were carried out using the SA biosensor strain *Acinetobacter* sp. ADPWl*ux* developed by Huang et al. (2006 and 2005) with some modifications (López-Orenes et al., 2017).

2.10. Statistical analysis

For each univariate variable, an exploratory analysis was carried out using box-and-whisker graphs to compare populations and to detect outliers. Rhizosphere soil data were subjected to the analysis of variance (ANOVA) with site (Agustin mine tailing and control) as factor, and when normality and homogeneity of variances assumptions were not met, the Box–Cox family of transformations was used to normalize residuals. Tukey's HSD test based on the range of the sample means was used as a post-hoc test. Biochemical and physiological parameters measured were mean-centered log-transformed and both unsupervised, principal component analysis (PCA), and supervised, partial least squares-discriminant analysis (PLS-DA), multivariate analysis were performed. A versatile classification algorithm, random forest (RF), as well as a heatmap analysis, combined with an agglomerative hierarchical clustering, were also carried out. All statistical analyses were conducted using the free software R (R Core Team, 2016).

3. Results

3.1. Rhizosphere soil parameters

Table 1 depicts the results of rhizosphere soil analysis of Agustin mine tailing and control site where Tukey's HSD post-hoc test was used to detect significant differences between both populations. Soil fertility parameters in Agustin rhizosphere soil samples were very low, especially the dissolved organic carbon (DOC) concentration, which was up to 12-fold lower (~9 mg kg^{-1}) than in non-mining samples (~109 mg kg^{-1}). Contrarily, the EC values and the content of total As, Pb and Zn in tailing samples were more than 10-fold higher than in controls. Moreover, Agustin samples were also

characterized by high levels of water extractable divalent ions (SO_4^{2-}, Ca^{2+}, and Mg^{2+}), whereas monovalent ions (Cl^{-}, K^{+}, and Na^{+}) remained at low levels about one-half or less than those found in rhizosphere control soils. Water extractable SO_4^{2-} and Ca^{2+} levels exhibited a strong correlation which can be related to secondary formation of gypsum in the tailings (Parraga-Aguado et al., 2014). Agustin samples had a sandy texture (>70%), which negatively correlated with soil fertility parameters (OC, DOC and TN) ($r > -0.7$, $P<0.05$, see Table S1).

3.2. Plant macronutrient and metal(loid) concentrations in Dittrichia viscosa leaves

In general, the macronutrient contents in Agustin leaves were closer to those observed in control leaves for Ca, N and P while K levels decreased in both seasons and Mg and S contents increased (up to 2-fold over controls) (Table 2). It is interesting to note that the foliar S concentration found in non-mining *D. viscosa* leaves was ~3-fold higher than the average normal S content found in plants, whereas the foliar content of P was about one-half lower than the average values previously reported for this macronutrient (Marschner, 1995). In fact, P is known to be one of the critical limiting elements for plant growth in terrestrial ecosystems (Güsewell, 2004).

The analyses of metal(loid)s showed that Agustin plants accumulated significantly higher levels (>20-fold) of As, Cd and Pb in leaf tissues compared to non-mining plants (Table 2). Generally, summer leaves accumulated higher levels of these metal(loid)s than spring ones. The highest accumulated metal(loid)s were Zn (~680 mg kg^{-1} DW), Pb (~380 mg kg^{-1} DW), and As (~30 mg kg^{-1} DW). The amount of Zn and Pb accumulated exceeded the critical toxicity levels for plants, whose upper limits are 300 and 28 μg g^{-1} DW, respectively (Krämer, 2010). Nevertheless, the mean

bioaccumulation factors (BCF), defined as the ratio of total metal concentration in leaf biomass with respect to total metal concentration in the soil, for As, Pb and Zn were clearly lower than 1 (0.11, 0.06, and 0.10, respectively).

3.3. Multivariate analysis of physiological and antioxidative/oxidative data in Dittrichia viscosa leaves

Since metal(loid) toxicity is frequently driven by ROS generation, a wide range of antioxidants, oxidative stress markers, and some physiological parameters such as RWC, photosynthetic pigments, sugars and protein contents were analyzed. All these data were subjected to different multivariate statistical analyses in order to facilitate detection of statistically significant changes between the two *D. viscosa* populations studied. First, a principal component analysis (PCA), based on the correlation matrix as the similarity metric, was carried out for unsupervised dimension reduction. The first three principal components, which explained ~62% of variance, were plotted against each other (Figure 1). As can be seen, PC1 (~34% of the variance) separated the samples collected in spring 2013, *i.e*. the rainiest period, from the others. The first PCA axis was defined, on its positive side, by protein content, AA and soluble phenolic compounds (TPC, HCAs, flavonoids and flavanols), and by protein oxidation (carbonyl group content) and PAL and sPRX activities on the negative side of X-axis. PC2 (~15% of the variance) discriminated the samples from the different seasons and was defined by NPT, PAs and $O_2^{\bullet -}$ on the positive side of Y-axis and cell wall-bound phenols on the negative side. PC3 (~14% of the variance) separated control from Agustin samples, and was defined by TBARS (on the positive side of Y-axis) and RWC (on the negative side). Next, the data were analyzed using a supervised PLS-DA method, and the score

394 and loading plots obtained were quite similar to those found in the PCA (data not
395 shown). Since PLS-DA allows the identification of the most influential biomarkers
396 based on the variable importance in the projection (VIP), we plotted the correlation
397 coefficients for the first three components of PLS-DA and VIP score to clearly identify
398 the potential biomarkers and interpret their physiological significance. As seen in Figure
399 2, the biomarkers with a VIP score >1 and with absolute correlation value greater than
400 0.5 with the first PLS-DA component, accounting for ~34% of the total variance, were
401 soluble phenolic compounds (TPC, and flavonoids), protein and protein carbonyl
402 contents, PAL and PRX activities, AA, and the stress-related phytohormone SA (Fig.
403 2A). For the second PLS-DA component (~15% of the variance; Fig. 2B) the markers
404 were cell wall-bound phenols, proanthocyanidins (PAs), DHA, $O_2^{\bullet -}$ and NPT contents,
405 and for the third component (~14% of the total variance; Fig. 2C) the variables found
406 were RWC, TBARS, lignin and iPRX.
407 Random forest machine learning algorithm (RF) has recently emerged as an effective
408 method for classification and feature selection in “omics” studies (Touw et al., 2013). In
409 RF, feature importance is measured by randomly permuting the feature in the out-of-bag
410 (OOB) samples and calculating the decrease of classification accuracy (Touw et al.,
411 2013). Figure 3 reflects the ranking of the individual variables analyzed using the mean
412 decrease in accuracy criterion. Thus, based on the change in the curve shape of the
413 mean decrease in accuracy plot, it can be seen that 7 out of the top 11 descriptors are
414 associated with phenolic metabolism, and cell wall-bound phenols was ranked as the
415 most important biomarker. Given the RF ranking of variables (Fig. 3) and both the
416 correlation coefficient and VIP scores for PLS-DA (Fig. 2), the parameters that could be
417 considered as potential markers to differentiate between non-mining (control) and
418 mining (Agustin) plants were cell wall-bound phenols, soluble phenols (TPC and

419 flavonoids), PAL and PRX activities, and the phenolic hormone salicylic acid (SA).
420 To help visualization of the seasonal differences in the antioxidative/oxidative profile in
421 the two *D. viscosa* populations studied, the ratio values (Agustin/control) were log2-
422 transformed and a two-way complete-linkage hierarchical clustering was performed by
423 using a distance defined in terms of Pearson correlation and represented in a heatmap
424 format (Fig. 4A). Additionally, the differences between sample groups were analyzed
425 by a non-parametric Wilcoxon's test, and the mean ratios of fold changes and their
426 associated *P*-values obtained are given in Supplemental Table 2. The values obtained
427 for representative markers were also presented as box-and-whisker plots (Fig. 4B). As
428 expected, the dendrogram showed a clear separation between spring and summer
429 samples, with the samples taken during the greatest rainfall period (*i.e.*, September 2012
430 and May 2013) grouping together (Figure 4A). Interestingly, the values of all the
431 parameters used to assess the physiological status of the leaf in Agustin *D. viscosa*
432 plants (RWC and the content of photosynthetic pigments, proteins, starch and soluble
433 sugars) were quite similar to those found in control plants in both seasons, although a
434 statistically significant increase in the total carotenoid content was noticed in summer
435 samples. The analysis of ROS revealed that Agustin leaves had lower H_2O_2 levels,
436 especially in summer, whereas the $O_2^{\bullet-}$ levels only significantly differed from controls
437 in summer 2013 (mean ratio 1.5, Supplemental Table S2). Analyses of TBARS and
438 carbonyl group contents, used as oxidative damage markers, were somewhat higher in
439 Agustin leaves compared with controls although, the levels of the major redox buffers
440 of the plant cells, reduced AA and GSH, were comparable between the two *D. viscosa*
441 populations in both seasons (Fig. 4). The analyses of total NPT, which included GSH
442 and phytochelatins, showed a very slight increase in Agustin leaves, whereas a
443 significant reduction in the foliar concentrations of Pro (mean ratio ~0.5, Supplemental

Table S2) as well as a decline, which was generally more pronounced in summer, in the contents of TPC, flavonoids and HCAs were also noticed (Fig. 4). The drop in the levels of these soluble phenolic compounds (mean ratio ~0.8; Supplemental Table S2) was negatively correlated with sPRX activity (r> -0.4, P< 0.01; Supplemental Table S3) and with the content of carbonyl groups in proteins (r= -0.6, P< 0.01 with TPC, and r=-04, P< 0.01 with HCAs and flavonoids; Supplemental Table S3). Moreover, significant moderate correlations were noticed between soluble phenolic compounds and ABTS/DPPH radical-scavenging capacities (r> 0.4, P< 0.01; Supplemental Table S3). The most notorious change was the significant increase of PAL activity (Fig. 4), the first enzyme of the overall phenylpropanoid biosynthetic pathway, observed in both seasons in Agustin leaves, but especially in summer (mean ratio > 3.5, Supplemental Table S2). Furthermore, it is also worth stressing the opposite trend of change between total ionically-bound cell wall (iPRX) and soluble peroxidase (sPRX) activities in the two *D. viscosa* populations (Fig. 4). The substantial changes in the activity of PAL and PRX activities observed can be associated with the higher accumulation of cell wall-bound phenols found in Agustin leaves. The moderate-to-high correlations found between the foliar content of accumulated metal(loid)s and some biochemical markers such as the levels of cell wall-bound phenols, soluble phenolic compounds (TPC, HCAs, flavonoids and flavanols), PAL and PRX activities are summarized in Supplemental Table S1.

3.4. HPLC analysis of soluble and cell wall-bound phenolic compounds in Dittrichia viscosa leaves

Since phenolic compounds were identified as the most influential biomarkers and

exhibited moderate correlations with the accumulation of metal(loid)s, HPLC analyses were performed in order to verify possible changes in the accumulation of both soluble and cell wall phenolics in *D. viscosa* leaves. HPLC chromatograms of soluble methanolic extracts of *D. viscosa* leaves were characterized by the presence of both hydroxycinnamic derivatives and flavonoids, according to their UV–visible spectra and bibliographic sources (Mahmoudi et al., 2016; Trimech et al., 2014) (see Supplemental Fig. S4). Among the HCAs, the two large peaks with retention time of 33.8 min (peak 3) and 34.5 min (peak 4) were identified as dicaffeoyl quinic acid derivatives, and peak 1 (t_R=22.9 min) was identified as chlorogenic acid by comparison of its retention time and UV spectrum with those of authentic standard. Among the flavonoids, dihydroflavonols (taxifolin derivatives, peaks 2, 6, 8, 9, 10, and 11) were the most predominant compounds (Supplemental Fig. S4). However, no qualitative differences in the HPLC profiles were found either between the two populations or between seasons. In its turn, HPLC chromatograms of phenolics bound to the cell wall revealed the presence of a very large peak with a retention time of about 24 min (Peak 1), which represents >90% of the total peak area in the chromatogram, which was identified as caffeic acid (Figure 5). The HPLC profile was also characterized by the presence of one small peak (peak 2 at 29.7 min) corresponding to *p*-coumaric acid and other very small peaks, corresponding to trace amounts of protocatechuic acid, ferulic acid, and derivatives of the latter (Figure 5). The amount of these phenolic acids bound to the cell wall were higher in Agustin leaves, with the exception of summer 2012, and this trend was similar to that of the total phenolics content determined by the Folin-Ciocalteu method in the alcohol insoluble residues (AIR) of leaf cell wall material (Fig. 4).

4. Discussion

494

495 *4.1. Metallicolous D. viscosa plants exhibit multiple metal tolerance and high Zn and*
496 *Pb co-accumulation capacity in photosynthetic active tissues*

497

498 This study clearly shows that Agustin *D. viscosa* plants exhibit multiple metal tolerance
499 and high Zn and Pb co-accumulation capacity in photosynthetically active leaf tissues.
500 Moreover, these metallicolous plants were able to accumulate the metalloid As up to 30
501 mg kg^{-1} DW, which are in line with the values reported by Conesa et al. (2011) and
502 Pérez-Sirvent et al. (2012) in mining ecotypes of the same plant species grown within
503 this mining area and/or in its vicinity, and with those found by Pistelli et al. (2017) in a
504 iron mining area on Elba Island (Italy). An index commonly used to evaluate the
505 metal(loid) accumulation efficiency in plants is the bioaccumulation factor (BCF)
506 (McGrath and Zhao, 2003). Plants that have BCF values greater than 1 are considered
507 suitable for phytoextraction, whereas plants with BCF values lower than 1 are preferred
508 for phytostabilization (McGrath and Zhao, 2003). Here, the BCFs obtained for As, Pb
509 and Zn in Agustin *D. viscosa* plants were lower than 1.0, and thus this species could be
510 classified *sensu stricto* as good candidate for preventing the spread of metal(loid)
511 contaminats by erosion. Low BCF values for these elements in different mining *D.*
512 *viscosa* populations, can be worked out from data previously reported by other authors
513 when the total soil metal concentration and metal(loid) content in leaf tissues are
514 considered (Buscaroli et al., 2016; Conesa et al., 2011; Martínez-Sánchez et al., 2012;
515 Pistelli et al., 2017).
516 Plant uptake of As has been reported to be greater in sandy soils due to their low levels
517 of Fe and Al oxides (Gulz et al., 2005), thus the bioaccumulation of As found in
518 Agustin leaves is not surprising considering the sandy texture of this mining pile. In

addition, it is well known that arsenate, the most abundant environmental form of As in aerobic soils, is an antagonist of phosphate uptake, both competing for the same P_i transporters (Meharg and Hartley-Whitaker, 2002). This is also in line with our observation of a moderate negative correlation between leaf As content and foliar P concentration ($r=-0.42$, $P< 0.05$; Supplemental Table S3), which may explain, at least in part, the lower levels of P found in summer 2012 Agustin leaves. Moreover, although it had been reported that Cd accumulation is a constitutive trait in this species (Fernández et al., 2013), in our study the foliar accumulation of Cd did not surpass 4 mg kg^{-1} DW. These results can be explained by taking into account the low levels of Cd found in the rhizosphere tailing soils (21 ± 5 mg kg^{-1}) in comparison to the several orders of magnitude higher concentration of Zn ($4{,}791 \pm 234$ mg kg^{-1}) and Pb ($4{,}116 \pm 430$ mg kg^{-1}) ions, with all three sharing similar chemical properties (Krämer, 2010).

On the other hand, the nutritional status of Agustin leaves was not affected either by the uptake and translocation of As, Pb and Zn to the aerial part of the plants or by the very low nitrogen (0.28 ± 0.07 g kg^{-1}) and dissolved organic carbon concentrations (8.75 ± 0.99 g kg^{-1}) found in the mining rhizosphere soils, since the content of the main macronutrients N, K, Ca, Mg, and P (the latter with the exception of the values noticed in summer 2012) in Agustin leaves were quite similar to those found in controls. One possible explanation for these results could be related to the fact that evergreen species, as *D. viscosa*, exhibit an efficient internal remobilization of carbon and nutrients from dying leaves to developing tissues (Cherbuy et al., 2001). In addition, the absence of competitors in mining soils (see Supplemental Figure S3D) can also contribute to maintain a scarce, but photosynthetic active leaf canopy under such hard stressful conditions.

4.2. Leaves of metallicolous D. viscosa plants exhibited no seasonal changes in either leaf water status or photosynthetic metabolism even in the dry season

No significant seasonal influence on leaf RWC in Agustin plants, regardless of the difference in the rainfall patterns observed in the years analyzed was observed. Both, soluble sugars and free Pro are considered key osmolytes for osmotic adjustment in stressed plants (Suzuki et al., 2014). However, in our study Pro concentration in Agustin leaves dropped abruptly (mean ratio ~0.50, Supplemental Table S2), whereas the content of soluble sugars were closer to controls. These results contrasted with those observed on short-term (10 days) Cd-exposed *D. viscosa* plants grown under hydroponics in which the foliar Pro levels rose with increasing Cd concentration in the growth solution (Fernández et al., 2013). These authors found that the absolute content of free Pro in leaves of untreated plants was 1 μmol g^{-1} FW (Fernández et al., 2013), which is in agreement with the average Pro content that we found in non-mining controls (0.93 μmol g^{-1} FW). Nevertheless, the small accumulation of Pro found was too low to contribute to osmotic adjustment, which is in line with those reported by Al Hassan et al. (2016) in the same plant species exposed to salt and water stress. Extensive evidence now strongly supports that free Pro is a potent antioxidant, and several studies have demonstrated that Pro metabolism could have an important role in plant tolerance to environmental stress (Ben Rejeb et al., 2014). It had been observed high Pro levels in the phloem of stressed plants, pointing out the possible importance of Pro movement within photosynthetic and non-photosynthetic tissues to maintain plant metabolism during adverse environmental conditions (Verslues and Sharma, 2010, and refs. herein). Thus, it is plausible that the foliar reduction in free Pro levels in Agustin leaves could be related, at least in part, with its transport to other organs, although

569 further studies are needed to confirm and explain these observations.

570

571 Despite the higher Zn/Pb accumulation found in Agustin *D. viscosa* leaves, no
572 significant differences were found in the levels of chlorophylls, proteins, starch and
573 soluble sugars, suggesting that the photosynthetic metabolism of these leaves was not
574 impaired even in the driest summer period studied (*i.e.*, September 2013). These results
575 are in line with recent proteomic studies that emphasized the importance of maintaining
576 net photosynthesis rate and energy production to perform the energy-demanding
577 processes involved in metal uptake, transport and sequestration in both metal
578 accumulator and hyperaccumulator plants (Bah et al., 2010; Farinati et al., 2009).
579 The basal levels of carotenoids increased in summer leaves in both *D. viscosa*
580 populations, although more markedly in Agustin plants. Several studies have
581 highlighted the efficiency of carotenoids in the photoprotection mechanisms in different
582 native Mediterranean plant species during summer (Fenollosa et al., 2017; Flexas et al.,
583 2014). It is well known that carotenoids play a dual role in photosynthesis, they can
584 function as accessory light-harvesting pigments and as photoprotective molecules
585 required not only to avoid the generation of single oxygen ($^1O_2$) from triplet excited
586 chlorophylls but also to quench any $^1O_2$ produced (Niyogi, 2000). The production of
587 $^1O_2$ has been reported to increase under high irradiance as well as under other
588 environmental stress conditions which lead to closing of stomata, such as salinity and
589 drought (Gill and Tuteja, 2010). Thus, by increasing the accumulation of these
590 photoprotective pigments, Agustin leaves seemed to be more capable of avoiding the
591 production of $^1O_2$ and, consequently, to maintain a high photosynthetic efficiency even
592 in the dry season. This finding contrasts with the significant decline in Chl content
593 found in Agustin *Zygophyllum fabago* populations under the same stressful conditions

(López-Orenes et al., 2017). Comparing with *D. viscosa*, this species exhibited lower foliar contents of Zn and Pb (~300 and ~7 mg kg^{-1} DW, respectively, which fell within the critical range) (Párraga-Aguado et al., 2016), indicating that both species presented a different adaptive strategy to withstand the adverse conditions of the mine tailing and the seasonal variations in this Mediterranean area.

4.3. Cellular antioxidant capability and ROS levels remained nearly unaffected whereas phenylpropanoid metabolism is enhanced in leaves of metallicolous D. viscosa plants especially in the driest period

The maintenance of the photosynthetic capacity under these drastic concurrent stressful conditions is only possible if ROS levels are kept at concentrations low enough for ensuring adequate metabolic functions in mesophyll cells. Our results revealed a tight control of $O_2^{\bullet -}$ and H_2O_2 levels in Agustin leaves in both seasons, although it was observed a certain degree of oxidative modifications of proteins and lipids (Fig. 4) that seemed to have no significant deteriorating consequences on photosynthetic metabolism, as evidenced by no changes in starch and soluble sugar concentrations, the end products of photosynthesis. In plants, AA and GSH are the major cellular antioxidants and redox buffers involved in redox homeostasis and ROS detoxification (Foyer and Noctor, 2005). In this study, no significant seasonal influence on the levels of AA and GSH were found between Agustin and control *D. viscosa* populations. Moreover, aside from these two major antioxidants, plants contain a wide range of secondary metabolites, most of which are redox-active compounds that may also be important in controlling ROS accumulation (Potters et al., 2010). Even with some limitations, an integrated parameter to evaluate the full spectrum of antioxidant

619 compounds present in a tissue is the total antioxidant capacity (Ghiselli et al., 2000;
620 López-Orenes et al., 2013b). Here, we found that Agustin leaves exhibited only a slight
621 reduction (mean ratio ~0.83, Supplemental Table S2) in the total antioxidant activity,
622 measured as DPPH/ABTS scavenging ability, which was highly correlated with the
623 reduction in the levels of soluble phenolic compounds. Nevertheless, it is important to
624 highlight that the total antioxidant capacity in Agustin *D. viscosa* leaves was ca. 25-fold
625 higher than that found in Agustin *Z. fabago* plants growing in the same conditions
626 (López-Orenes et al., 2017), and also that the levels of soluble TPC noticed in summer
627 Agustin leaves were ca. 4-fold higher than the ones reported in leaves of Cd-exposed *D.*
628 *viscosa* plants grown under hydroponics (Fernández et al., 2013). Foliar accumulation
629 of phenolic compounds has also been reported in different plant species exposed to
630 heavy metal (Kováčik and Klejdus, 2008; Llugany et al., 2013; Singh et al., 2015, and
631 refs. herein), and also in plants grown in multi-polluted soils (López-Orenes et al., 2017;
632 Martínez-Alcalá et al., 2013).
633 A relevant aspect to consider is the metabolic cost associated with adaptation to chronic
634 exposure to metal(loid)s, because these adaptive processes should be sustainable and
635 effective, especially under nutrient-limited conditions (Maestri et al., 2010). One of the
636 mechanisms which fulfills both requirements is the metal(loid)
637 sequestration/immobilization in plant cell walls rather than the induction of low
638 molecular weight ligands such as phytochelatins due to the energy costs associated with
639 sulfate reduction and phytochelatins synthesis (Maestri et al., 2010, and refs. herein).
640 Although GSH biosynthesis is regulated by sulfur assimilation and *D. viscosa* leaves
641 showed a high foliar S content, the size of S-rich metal(loid)-binding peptides (*i.e.*, the
642 non-protein thiol pool and GSH content) in Agustin leaves remained nearly unaffected,

643 suggesting that metal(loid) detoxification via thiol-mediated complexation could have a
644 minor role in these plants under the prevalent edaphoclimatic conditions.
645 Furthermore, it is well known that nutrient deficiencies can cause elevated levels of
646 carbon-rich metabolites, such as phenolics (Fritz et al., 2006). Here, we observed a
647 significant enhancement of both PAL and sPRX activities in Agustin leaves as well as
648 changes in the accumulation of both soluble and cell wall-bound phenolic compounds.
649 Both hydroxycinnamic acid derivatives and flavonoids were the most abundant
650 phenolics in the leaf soluble fraction, as has been previously reported (Mahmoudi et al.,
651 2016; Trimech et al., 2014). The predominant phenolic monomer esterified and
652 incorporated in the cell wall matrix in Agustin leaves was caffeic acid. HCAs,
653 especially caffeic acid, and flavonoids have been reported to show high metal-chelation
654 properties as well as strong antioxidant characteristics and ROS-scavenging activities
655 (Agati et al., 2012; Andejelkovic et al., 2006; Michalak, 2006; Rice-Evans et al., 1997).
656 In our study, high-to-moderate correlations between the foliar concentrations of
657 metal(loid)s and the levels of both cell wall-bound phenols, soluble phenols, PAL and
658 PRX activities were observed. What is more, all of these parameters were ranked among
659 the top-10 significant biomarkers based on mean decrease in accuracy in RF, and had a
660 VIP score > 1, suggesting that under natural (field) conditions phenylpropanoid
661 metabolism could play an important role in the acclimation of mining *D. viscosa* plants
662 not only to the high metal(loid) contents found in the mine tailing, but also to the
663 adverse effects of other concurrent stressors (*i.e.*, nutrient deficiencies, high EC, and
664 drought). Changes in phenolics in cell walls have also been reported in plants exposed
665 to metals (Kováčik and Klejdus, 2008), and drought (Hura et al., 2017). In fact, several
666 lines of evidence indicate that although the production of phenylpropanoid metabolites
667 incurs metabolic costs, their biosynthesis can be offset by the multiplicity of functions

668 that these compounds may play in plants exposed to a wide array of environmental
669 constraints (Brunetti et al., 2015, and refs. herein). Thus, the antioxidant properties
670 ascribed to these phenolic compounds (*i.e.*, their ability to avoid ROS accumulation, to
671 protect cells against ROS-induced damage, and to chelate metals) seem to be relevant in
672 the acclimation response to the adverse conditions of the mine tailing, especially under
673 the most stressful conditions (*i.e.*, summer 2013).
674
675 *4.4. The endogenous levels of the phytohormone SA in leaves of metallicolous D.*
676 *viscosa plants dropped irrespective of the sampling date*
677
678 SA has long been recognized as a central signaling molecule in triggering defense
679 responses against biotic and abiotic stress (Vlot et al., 2009). Activation of the SA-
680 mediated defense responses is associated with up-regulation of genes encoding defense-
681 related proteins, and the accumulation of certain secondary metabolites. Here, we found
682 good positive correlation between SA and soluble phenol contents ($r > 0.6$, $P < 0.01$,
683 Supplemental Table S3). The amounts of SA, quantified using the SA biosensor strain
684 *Acinetobacter* sp. ADP1_*lux*, were 2.70 ± 0.11, and 3.87 ± 0.40 nmol g^{-1} FW in spring
685 and summer Agustin leaves, respectively. Moreover, the analyses of the phenolic
686 phytohormone SA showed a significant drop ($P<0.005$) in its endogenous level in
687 Agustin leaves (mean ratio ~0.67) irrespective of the sampling date. In a previous work,
688 we also found lower foliar content of free SA in leaves of two metallicolous populations
689 of *Zygophyllum fabago* plants growing within this mining area relative to a non-mining
690 population (López-Orenes et al., 2017). At first glance, these results could be
691 considered surprising because most of the studies in the literature reported that SA
692 pretreatment contributed to the alleviation of metal(loid) toxicity in many plant species

(for review, see Hayat et al., 2010, and refs. herein). In a recent study using Arabidopsis SA-altering mutants lines, it was found that high endogenous levels of SA intensified the phytotoxicity induced by Pb and Cd ions (Tao et al., 2013), what would support the observation that mining populations, which are chronically exposed to metal(loid)s, contained lower levels of SA than non-mining controls.

5. Conclusions

The current study aims to get insight into oxidative stress signatures of metal(loid) tolerant plants grown under natural conditions and their acclimation responses to stress combinations. Our results showed that the mining (Agustin) *D. viscosa* plants, grown under semi-arid climate conditions, exhibit high Zn and Pb co-accumulation capacity in leaf tissues, without substantially affecting their photosynthetic metabolism or their nutritional status or RWC. Based on powerful multivariate statistics, both PAL and PRX activities, as well as soluble and cell wall bound phenol compounds were identified as potential markers for discriminating mining from non-mining plants, indicating that phenylpropanoid metabolism could play a coordinated role in plant acclimation to stress combinations. Moreover, the resilience to the harsh conditions prevailing in the mine tailings, especially during the dry seasons, together with its metal accumulation characteristics, makes *D. viscosa* plants potentially suitable candidates for being used in the phytoremediation, particularly in the phytostabilization, of contaminated soils under a climate change scenario.

Acknowledgements

This work was supported by the Ministerio de Ciencia e Innovación [grant number CTM2011-23958]; Ministerio de Ciencia y Tecnología [grant number CGL2006-11569]; and Fundación Séneca [grant number FB/23/FS/02]. AL-O holds a grant from the Ministerio de Educación Cultura y Deporte [grant number AP2012-2559]. Part of this work was carried out at the Instituto de Biotecnología Vegetal, UPCT.

References

Agati, G., Azzarello, E., Pollastri, S., Tattini, M., 2012. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci. 196, 67–76. doi:10.1016/j.plantsci.2012.07.014

Al Hassan, M., Chaura, J., López-Gresa, M.P., Borsai, O., Daniso, E., Donat-Torres, M.P., Mayoral, O., Vicente, O., Boscaiu, M., 2016. Native-invasive plants vs. halophytes in Mediterranean salt marshes: Stress tolerance mechanisms in two related species. Front. Plant Sci. 7, 473. doi:10.3389/fpls.2016.00473

Andejelkovic, M., Van Camp, J., De Meulenaer, B., Depaemelaere, G., Socaciu, C., Verloo, M., Verhe, R., 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. Food Chem. 98, 23–31. doi:10.1016/j.foodchem.2005.05.044

Bah, A.M., Sun, H., Chen, F., Zhou, J., Dai, H., Zhang, G., Wu, F., 2010. Comparative proteomic analysis of *Typha angustifolia* leaf under chromium, cadmium and lead stress. J. Hazard. Mater. 184, 191–203. doi:10.1016/j.jhazmat.2010.08.023

Barceló, J., Poschenrieder, C., 2003. Phytoremediation : principles and perspectives. Contrib. to Sci. Inst. d'Estudis Catalans, Barcelona 2, 333–344.

743 Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for
744 water-stress studies. Plant Soil 39, 205–207. doi:10.1007/BF00018060
745 Ben Rejeb, K., Abdelly, C., Savouré, A., 2014. How reactive oxygen species and
746 proline face stress together. Plant Physiol. Biochem. 80C, 278–284.
747 doi:10.1016/j.plaphy.2014.04.007
748 Brunetti, C., Guidi, L., Sebastiani, F., 2015. Isoprenoids and phenylpropanoids are key
749 components of the antioxidant defense system of plants facing severe excess light
750 stress. Environ. Exp. Bot. 119, 54–62. doi:10.1016/j.envexpbot.2015.04.007
751 Buscaroli, A., Zannoni, D., Menichetti, M., Dinelli, E., 2016. Assessment of metal
752 accumulation capacity of *Dittrichia viscosa* (L.) Greuter in two different Italian
753 mine areas for contaminated soils remediation. J. Geochemical Explor. 1–9.
754 doi:10.1016/j.gexplo.2016.10.001
755 Cheeseman, J.M., 2006. Hydrogen peroxide concentrations in leaves under natural
756 conditions. J. Exp. Bot. 57, 2435–2444. doi:10.1093/jxb/erl004
757 Cherbuy, B., Joffre, R., Gillon, D., Rambal, S., 2001. Internal remobilization of
758 carbohydrates, lipids, nitrogen and phosphorus in the Mediterranean evergreen oak
759 *Quercus ilex*. Tree Physiol. 21, 9–17. doi:10.1093/treephys/21.1.9
760 Choudhury, F.K., Rivero, R.M., Blumwald, E., Mittler, R., 2016. Reactive oxygen
761 species, abiotic stress and stress combination. Plant J. 1–12. doi:10.1111/tpj.13299
762 Conesa, H.M., María-Cervantes, A., Álvarez-Rogel, J., González-Alcaraz, M.N., 2011.
763 Influence of soil properties on trace element availability and plant accumulation in
764 a Mediterranean salt marsh polluted by mining wastes: Implications for
765 phytomanagement. Sci. Total Environ. 409, 4470–4479.
766 doi:10.1016/j.scitotenv.2011.07.049
767 Everette, J.D., Bryant, Q.M., Green, A.M., Abbey, Y. A., Wangila, G.W., Walker, R.B.,

2010. Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. J. Agric. Food Chem. 58, 8139–8144. doi:10.1021/jf1005935

Farinati, S., DalCorso, G., Bona, E., Corbella, M., Lampis, S., Cecconi, D., Polati, R., Berta, G., Vallini, G., Furini, A., 2009. Proteomic analysis of *Arabidopsis halleri* shoots in response to the heavy metals cadmium and zinc and rhizosphere microorganisms. Proteomics 9, 4837–4850. doi:10.1002/pmic.200900036

Fenollosa, E., Munné-Bosch, S., Pintó-Marijuan, M., 2017. Contrasting phenotypic plasticity in the photoprotective strategies of the invasive species *Carpobrotus edulis* and the coexisting native species *Crithmum maritimum*. Physiol. Plant. 160, 185–200. doi:10.1111/ppl.12542

Fernández, R., Bertrand, A., Reis, R., Mourato, M.P., Martins, L.L., González, A., 2013. Growth and physiological responses to cadmium stress of two populations of *Dittrichia viscosa* (L.) Greuter. J. Hazard. Mater. 244–245, 555–562. doi:10.1016/j.jhazmat.2012.10.044

Ferrer, J.L., Austin, M.B., Stewart, C., Noel, J.P., 2008. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol. Biochem. 46, 356–370. doi:10.1016/j.plaphy.2007.12.009

Flexas, J., Diaz-Espejo, A., Gago, J., Gallé, A., Galmés, J., Gulías, J., Medrano, H., 2014. Photosynthetic limitations in Mediterranean plants: A review. Environ. Exp. Bot. 103, 12–23. doi:10.1016/j.envexpbot.2013.09.002

Foyer, C.H., Noctor, G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17, 1866–75. doi:10.1105/tpc.105.033589

Fritz, C., Palacios-Rojas, N., Feil, R., Stitt, M., 2006. Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors

of phenylpropanoid metabolism. Plant J. 46, 533–548. doi:10.1111/j.1365-313X.2006.02715.x

Gee, G.W., Bauder, J.W., 1986. Particle-size analysis, in: Klute, A. (Ed.), Methods of Soil Analysis. Part 1. Physical and Mineralogical Methods. American Society of Agronomy and Soil Science Society of America, Madison, United States, pp. 383–410.

Ghiselli, A., Serafini, M., Natella, F., Scaccini, C., 2000. Total antioxidant capacity as a tool to assess redox status: Critical view and experimental data. Free Radic. Biol. Med. 29, 1106–1114. doi:10.1016/S0891-5849(00)00394-4

Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–30. doi:10.1016/j.plaphy.2010.08.016

Gillespie, K.M., Ainsworth, E. A, 2007. Measurement of reduced, oxidized and total ascorbate content in plants. Nat. Protoc. 2, 871–4. doi:10.1038/nprot.2007.101

Gulz, P.A., Gupta, S.K., Schulin, R., 2005. Arsenic accumulation of common plants from contaminated soils. Plant Soil 272, 337–347. doi:10.1007/s11104-004-5960-z

Güsewell, S., 2004. N : P ratios in terrestrial plants: variation and functional significance. New Phytol. 164, 243–266. doi:10.1111/j.1469-8137.2004.01192.x

Hatzilazarou, S.P., Syros, T.D., Yupsanis, T.A., Bosabalidis, A.M., Economou, A.S., 2006. Peroxidases, lignin and anatomy during *in vitro* and *ex vitro* rooting of gardenia (*Gardenia jasminoides* Ellis) microshoots. J. Plant Physiol. 163, 827–836. doi:10.1016/j.jplph.2005.06.018

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A., 2010. Effect of exogenous salicylic acid under changing environment: A review. Environ. Exp. Bot. 68, 14–25. doi:10.1016/j.envexpbot.2009.08.005

818 Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the
819 thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in
820 plant tissues containing anthocyanin and other interfering compounds. Planta 207,
821 604–611. doi:10.1007/s004250050524
822 Hossain, M.A., Piyatida, P., da Silva, J. A. T., Fujita, M., 2012. Molecular mechanism
823 of heavy metal toxicity and tolerance in plants: Central role of glutathione in
824 detoxification of reactive oxygen species and methylglyoxal and in heavy metal
825 chelation. J. Bot. 2012, 1–37. doi:10.1155/2012/872875
826 Huang, W.E., Huang, L., Preston, G.M., Naylor, M., Carr, J.P., Li, Y., Singer, A.C.,
827 Whiteley, A.S., Wang, H., 2006. Quantitative in situ assay of salicylic acid in
828 tobacco leaves using a genetically modified biosensor strain of *Acinetobacter* sp.
829 ADP1. Plant J. 46, 1073–1083. doi:10.1111/j.1365-313X.2006.02758.x
830 Huang, W.E., Wang, H., Zheng, H., Huang, L., Singer, A C., Thompson, I., Whiteley,
831 a S., 2005. Chromosomally located gene fusions constructed in *Acinetobacter* sp
832 ADP1 for the detection of salicylate. Environ. Microbiol. 7, 1339–1348.
833 doi:10.1111/j.1462-2920.2005.00821.x
834 Hura, T., Tyrka, M., Hura, K., Ostrowska, A., Dziurka, K., 2017. QTLs for cell wall-
835 bound phenolics in relation to the photosynthetic apparatus activity and leaf water
836 status under drought stress at different growth stages of triticale. Mol. Genet.
837 Genomics 292, 415–433. doi:10.1007/s00438-016-1276-y
838 Jiang, M., Zhang, J., 2001. Effect of abscisic acid on active oxygen species,
839 antioxidative defence system and oxidative damage in leaves of maize seedlings.
840 Plant Cell Physiol. 42, 1265–1273. doi:10.1093/pcp/pce162
841 Kim, D., Jeong, S.W., Lee, C.Y., 2003. Antioxidant capacity of phenolic
842 phytochemicals from various cultivars of plums. Food Chem. 81, 321–326.

doi:10.1016/S0308-8146(02)00423-5

Kováčik, J., Klejdus, B., 2008. Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots. Plant Cell Rep. 27, 605–615. doi:10.1007/s00299-007-0490-9

Krämer, U., 2010. Metal hyperaccumulation in plants. Annu. Rev. Plant Biol. 61, 517–534. doi:10.1146/annurev-arplant-042809-112156

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233, 346–357. doi:10.1016/S0076-6879(94)33040-9

Lichtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591–592. doi:10.1042/bst0110591

Llugany, M., Tolrà, R., Martín, S.R., Poschenrieder, C., Barceló, J., 2013. Cadmium-induced changes in glutathione and phenolics of *Thlaspi* and *Noccaea* species differing in Cd accumulation. J. Plant Nutr. Soil Sci. 176, 851–858. doi:10.1002/jpln.201300096

López-Arnaldos, T., Muñoz, R., Ferrer, M.A., Calderón, A.A., 2001. Changes in phenol content during strawberry (*Fragaria* x *ananassa*, cv. Chandler) callus culture. Physiol. Plant. 113, 315–322. doi:10.1034/j.1399-3054.2001.1130303.x

López-Orenes, A., Bueso, M.C., Conesa, H.M., Calderón, A.A., Ferrer, M.A., 2017. Seasonal changes in antioxidative/oxidative profile of mining and non-mining populations of Syrian beancaper as determined by soil conditions. Sci. Total Environ. 575, 437–447. doi:10.1016/j.scitotenv.2016.10.030

López-Orenes, A., Martínez-Moreno, J.M., Calderón, A.A., Ferrer, M.A., 2013a. Changes in phenolic metabolism in salicylic acid-treated shoots of *Cistus*

heterophyllus. Plant Cell, Tissue Organ Cult. 113, 417–427. doi:10.1007/s11240-012-0281-z

López-Orenes, A., Martínez-Pérez, A., Calderón, A.A., Ferrer, M.A., 2014. Pb-induced responses in *Zygophyllum fabago* plants are organ-dependent and modulated by salicylic acid. Plant Physiol. Biochem. 84, 57–66. doi:10.1016/j.plaphy.2014.09.003

López-Orenes, A., Ros-Marín, A.F., Ferrer, M.A., Calderón, A.A., 2013b. Antioxidant capacity as a marker for assessing the in vitro performance of the endangered *Cistus heterophyllus*. Sci. World J. 2013, 1–10. doi:10.1155/2013/176295

Maestri, E., Marmiroli, M., Visioli, G., Marmiroli, N., 2010. Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. Environ. Exp. Bot. 68, 1–13. doi:10.1016/j.envexpbot.2009.10.011

Mahmoudi, H., Hosni, K., Zaouali, W., Amri, I., Zargouni, H., Hamida, N. Ben, Kaddour, R., Hamrouni, L., Nasri, M. Ben, Ouerghi, Z., 2016. Comprehensive phytochemical analysis, antioxidant and antifungal activities of *Inula viscosa* Aiton leaves. J. Food Saf. 36, 77–88. doi:10.1111/jfs.12215

Marschner, H., 1995. Mineral Nutrition of Higher Plants, Second Edi. ed. Academic Press, London; San Diego.

Martínez-Alcalá, I., Hernández, L.E., Esteban, E., Walker, D.J., Bernal, M.P., 2013. Responses of *Noccaea caerulescens* and *Lupinus albus* in trace elements-contaminated soils. Plant Physiol. Biochem. 66, 47–55. doi:10.1016/j.plaphy.2013.01.017

Martínez-Sánchez, M.J., García-Lorenzo, M.L., Pérez-Sirvent, C., 2012. Trace element accumulation in plants from an aridic area affected by mining activities. J. Geochemical Explor. 123, 8–12. doi:10.1016/j.gexplo.2012.01.007

893 Martinez, V., Mestre, T.C., Rubio, F., Girones-Vilaplana, A., Moreno, D.A., Mittler, R.,
894 Rivero, R.M., 2016. Accumulation of flavonols over hydroxycinnamic acids favors
895 oxidative damage protection under abiotic stress. Front. Plant Sci. 7, 1–17.
896 doi:10.3389/fpls.2016.00838
897 McGrath, S.P., Zhao, F.J., 2003. Phytoextraction of metals and metalloids from
898 contaminated soils. Curr. Opin. Biotechnol. 14, 277–282. doi:10.1016/S0958-
899 1669(03)00060-0
900 Meharg, A.A., Hartley-Whitaker, J., 2002. Arsenic uptake and metabolism in arsenic
901 resistant and nonresistant plant species. New Phytol. 154, 29–43.
902 doi:10.1046/j.1469-8137.2002.00363.x
903 Metwally, A., Finkemeier, I., Georgi, M., Dietz, K., 2003. Salicylic acid alleviates the
904 cadmium toxicity in barley seedlings. Plant Physiol. 132, 272–281.
905 doi:10.1104/pp.102.018457.duced
906 Michalak, A., 2006. Phenolic compounds and their antioxidant activity in plants
907 growing under heavy metal stress. Polish J. Environ. Stud. 15, 523–530.
908 Mouradov, A., Spangenberg, G., 2014. Flavonoids: a metabolic network mediating
909 plants adaptation to their real estate. Front. Plant Sci. 5, 1–16.
910 doi:10.3389/fpls.2014.00620
911 Niyogi, K.K., 2000. Safety valves for photosynthesis. Curr. Opin. Plant Biol. 3, 455–
912 460. doi:10.1016/S1369-5266(00)00113-8
913 Noctor, G., Lelarge-Trouverie, C., Mhamdi, A., 2014. The metabolomics of oxidative
914 stress. Phytochemistry 112, 33–53. doi:10.1016/j.phytochem.2014.09.002
915 Nsanganwimana, F., Pourrut, B., Mench, M., Douay, F., 2014. Suitability of *Miscanthus*
916 species for managing inorganic and organic contaminated land and restoring
917 ecosystem services. A review. J. Environ. Manage. 143, 123–134.

doi:10.1016/j.jenvman.2014.04.027

Panagos, P., Hiederer, R., Van Liedekerke, M., Bampa, F., 2013. Contaminated sites in Europe: Review of the current situation based on data collected through a European network. J. Environ. Public Health Artical ID, 1–11. doi:DOI 10.1016/j.ecolind.2012.07.020

Parolin, P., Scotta, M.I., Bresch, C., 2013. Biology of *Dittrichia viscosa*, a Mediterranean ruderal plant. Phyt. – Int. J. Exp. Bot. 83, 251–262.

Parraga-Aguado, I., Gonzalez-Alcaraz, M.N., Alvarez-Rogel, J., Jimenez-Carceles, F.J., Conesa, H.M., 2013. The importance of edaphic niches and pioneer plant species succession for the phytomanagement of mine tailings. Environ. Pollut. 176, 134–43. doi:10.1016/j.envpol.2013.01.023

Párraga-Aguado, I., González-Alcaraz, M.N., López-Orenes, A., Ferrer, M.A., Conesa, H.M., 2016. Evaluation of the environmental plasticity in the xerohalophyte *Zygophyllum fabago* L. for the phytomanagement of mine tailings in semiarid areas. Chemosphere 161, 259–265. doi:10.1016/j.chemosphere.2016.07.024

Parraga-Aguado, I., Querejeta, J.-I., González-Alcaraz, M.-N., Jiménez-Cárceles, F.J., Conesa, H.M., 2014. Usefulness of pioneer vegetation for the phytomanagement of metal(loid)s enriched tailings: grasses vs. shrubs vs. trees. J. Environ. Manage. 133, 51–8. doi:10.1016/j.jenvman.2013.12.001

Pérez-Sirvent, C., Martínez-Sánchez, M.J., Martínez-López, S., Bech, J., Bolan, N., 2012. Distribution and bioaccumulation of arsenic and antimony in *Dittrichia viscosa* growing in mining-affected semiarid soils in southeast Spain. J. Geochemical Explor. 123, 128–135. doi:10.1016/j.gexplo.2012.08.002

Pérez-Tortosa, V., López-Orenes, A., Martínez-Pérez, A., Ferrer, M.A., Calderón, A.A., 2012. Antioxidant activity and rosmarinic acid changes in salicylic acid-treated

943 *Thymus membranaceus* shoots. Food Chem. 130, 362–369.
944 doi:10.1016/j.foodchem.2011.07.051
945 Pistelli, L., D'Angiolillo, F., Morelli, E., Basso, B., Rosellini, I., Posarelli, M.,
946 Barbafieri, M., 2017. Response of spontaneous plants from an ex-mining site of
947 Elba island (Tuscany, Italy) to metal(loid) contamination. Environ. Sci. Pollut.
948 Res. 24, 7809–7820. doi:10.1007/s11356-017-8488-5
949 Potters, G., Horemans, N., Jansen, M.A.K., 2010. The cellular redox state in plant stress
950 biology - a charging concept. Plant Physiol. Biochem. PPB 48, 292–300.
951 doi:10.1016/j.plaphy.2009.12.007
952 R Core Team, 2016. R: A Language and Environment for Statistical Computing.
953 Vienna, Austria. URL https://www.R-project.org/.
954 Rice-Evans, C., Miller, N., Paganga, G., 1997. Antioxidant properties of phenolic
955 compounds. Trends Plant Sci. 2, 152–159. doi:10.1016/S1360-1385(97)01018-2
956 Schützendübel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-
957 induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351–
958 65.
959 Senft, A.P., Dalton, T.P., Shertzer, H.G., 2000. Determining glutathione and glutathione
960 disulfide using the fluorescence probe *o*-phthalaldehyde. Anal. Biochem. 280, 80–
961 86. doi:10.1006/abio.2000.4498
962 Shaar-Moshe, L., Blumwald, E., Peleg, Z., 2017. Unique physiological and
963 transcriptional shifts under combinations of salinity, drought, and heat. Plant
964 Physiol. 174, 421–434. doi:10.1104/pp.17.00030
965 Sharma, S.S., Dietz, K.-J., 2009. The relationship between metal toxicity and cellular
966 redox imbalance. Trends Plant Sci. 14, 43–50. doi:10.1016/j.tplants.2008.10.007
967 Singh, S., Parihar, P., Singh, R., Singh, V.P., Prasad, S.M., 2015. Heavy metal tolerance

968 in plants: Role of transcriptomics, proteomics, metabolomics, and ionomics. Front.
969 Plant Sci. 6, 1143. doi:10.3389/fpls.2015.01143
970 Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and
971 biotic stress combinations. New Phytol. 203, 32–43. doi:10.1111/nph.12797
972 Tao, S., Sun, L., Ma, C., Li, L., Li, G., Hao, L., 2013. Reducing basal salicylic acid
973 enhances Arabidopsis tolerance to lead or cadmium. Plant Soil 372, 309–318.
974 doi:10.1007/s11104-013-1749-2
975 Teng, Y., Ni, S., Wang, J., Zuo, R., Yang, J., 2010. A geochemical survey of trace
976 elements in agricultural and non-agricultural topsoil in Dexing area, China. J.
977 Geochemical Explor. 104, 118–127. doi:10.1016/j.gexplo.2010.01.006
978 Tordoff, G.M., Baker, A.J., Willis, A.J., 2000. Current approaches to the revegetation
979 and reclamation of metalliferous mine wastes. Chemosphere 41, 219–28.
980 Touw, W.G., Bayjanov, J.R., Overmars, L., Backus, L., Boekhorst, J., Wels, M., van
981 Hijum, S.A.F.T., 2013. Data mining in the Life Sciences with Random Forest: a
982 walk in the park or lost in the jungle? Brief. Bioinform. 14, 315–326.
983 doi:10.1093/bib/bbs034
984 Trimech, I., Weiss, E.K., Chedea, V.S., Marin, D., Detsi, A., Ioannou, E., Roussis, V.,
985 Kefalas, P., 2014. Evaluation of anti-oxidant and acetylcholinesterase activity and
986 identification of polyphenolics of the invasive weed *Dittrichia viscosa*.
987 Phytochem. Anal. 25, 421–428. doi:10.1002/pca.2510
988 USDA, 1996. Soil Survey Laboratory methods Manual. Soil Survey Investigations
989 Report No. 42.
990 Vermerris, W., Nicholson, R., 2006. Phenolic Compound Biochemistry. Springer
991 Netherlands, Dordrecht. doi:10.1007/978-1-4020-5164-7
992 Verslues, P.E., Sharma, S., 2010. Proline metabolism and its implications for plant-

993 environment interaction. Arabidopsis Book 8, e0140. doi:10.1199/tab.0140
994 Vlot, A.C., Dempsey, D.A., Klessig, D.F., 2009. Salicylic acid, a multifaceted hormone
995 to combat disease. Annu. Rev. Phytopathol. 47, 177–206.
996 doi:10.1146/annurev.phyto.050908.135202
997 Xu, D.P., Zheng, J., Zhou, Y., Li, Y., Li, S., Li, H. Bin, 2017. Ultrasound-assisted
998 extraction of natural antioxidants from the flower of *Limonium sinuatum*:
999 Optimization and comparison with conventional methods. Food Chem. 217, 552–
1000 559. doi:10.1016/j.foodchem.2016.09.013
1001

Figure legends

Figure 1. Score (left) and correlation (right) plots of the first three components of the PCA applied to physiological and biochemical variables measured in leaves of *D. viscosa* plants growing in non-mining [control (Co), black] and mining tailings pile [Agustin (Ag), white] in late spring and summer in 2012 and 2013 (squares, May 2012; circles, September 2012; triangles, May 2013; inverted triangles, September 2013). Circles represent r^2 = 50% and 100% variability explained by the components. Abbreviations: AA, Ascorbate; ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity; Car, total carotenoids; Chla, Chlorophyll *a*; Chlb, chlorophyll *b*; CWP, cell wall-bound phenols; C=O, protein carbonyl groups; DHA, dehydroascorbate; DPPH, 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity; FA, total flavanols; FO, total flavonoids; FRAP, Ferric Reducing Antioxidant Power; iPRX, ionically-bound cell wall class III plant peroxidase activity; H_2O_2, hydrogen peroxide; HCAs, hydroxycinnamic acids; NPT, total soluble non-protein thiols; $O_2^{\cdot-}$, superoxide radical; PAL, phenylalanine ammonia-lyase activity; PAs, cell wall-associated proanthocyanidins; Pro, proline; RWC, relative leaf water content; SA, salicylic acid; SAG, 2-*O*-*β*-D-glucosylsalicylic acid; sPrx, soluble class III plant peroxidase activity; TBARS, thiobarbituric acid reacting substances; TPC, total phenol content.

Figure 2. Identification of the most influential physiological and biochemical biomarkers based on the variable importance in the projection (VIP) and the correlation coefficients for the first three components of PLS-DA. For abbreviations, see legend to Figure 1.

Figure 3. Identification of the most influential physiological and biochemical biomarkers based on mean decrease in accuracy estimated by random forest machine learning algorithm. For abbreviations, see legend to Figure 1.

Figure 4. (A) Heatmap and complete-linkage hierarchical clustering (by using a distance in terms of based on Pearson's correlation coefficient) showing the seasonal fold change (mining vs. non-mining) of the physiological and biochemical parameters measured in leaves of *D. viscosa* plants growing in non-mining and in Agustin mining tailings pile in late spring and summer in 2012 and 2013. Log2 ratios of fold changes relative to each respective control group are given by shades of red or blue colors according to the scale bar. Asterisk denotes the rainiest sampling periods. For abbreviations, see legend to Figure 1. (B) Foliar levels of selected parameters. Values are expressed as box-and-whisker plots with the bottom and top of the box indicating the 25% and 75% percentiles, bold line in box the median, individual points the outliers and whiskers the lowest and highest values, excluding the outliers.

Figure 5. Comparison of HPLC chromatograms of cell wall-bound phenolics from alcohol insoluble residue (AIR) fraction of leaf material of non-mining (control) and mining (Agustin) *D. viscosa* plants in late spring and summer in 2012 and 2013 (M12, May 2012; S12, September 2012; M13, May 2013; S13, September 2013). The inset shows the UV-spectra of caffeic acid (Peak 1) and *p*-coumaric acid (Peak 2).

Supporting information

Table S1. Pearson's r correlation coefficients among rhizosphere soil parameters, foliar accumulated metal(loid)s and biochemical biomarkers measured in mining (Agustin) and non-mining *Dittrichia viscosa* plants. Asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$).

Table S2. Mean ratios of fold changes (mining vs. non-mining) and their associated *P*-values obtained by the non-parametric Wilcoxon's test ($P < 0.05$) of the physiological and biochemical parameters measured in *D. viscosa* leaves. The brighter the color, the higher the statistical significance (*P*-value). The scale bar is shown below the table. Mean ratios higher than 1 are highlighted with red background and mean ratios lower than 1 are highlighted with blue background.

Table S3. Mean and standard error values for all analyzed physiological and biochemical parameters measured in mining (Agustin) and non-mining (control) *D. viscosa* leaf samples in both late spring and summer in 2012 and 2013.

Figure S1. Geographical location of the study sites in the Cartagena-La Unión Mining District (Murcia, Spain). Agustin mine tailings pile and control site are indicated in the map.

Figure S2. Seasonal variations in weather conditions (monthly precipitation, monthly average minimum and maximum temperatures, and monthly average reference evapotranspiration [ETo]) from December 2011 to September 2013. Data were

collected by an automatic weather station located near the experimental site. Each sampling time are indicated by asterisks.

Figure S3. Representative pictures of the two populations of *D. viscosa* plants growing in a non-mining area (A, B and C) and in Agustin mine tailings pile (D, E and F) in late summer.

Figure S4. Representative HPLC chromatograms of soluble phenol compounds from methanolic extracts from leaves of non-mining (control) and mining (Agustin) *D. viscosa* plants. The samples corresponding to late spring in 2012. UV-VIS spectra (top panel) of the corresponding peaks. Peak 1, chlorogenic acid; peak 2, taxifolin derivatives; peak 3 and 4, dicaffeoyl quinic acid derivatives; peak 5; peak 7; peaks 6, 8, 9, 10, and 11, taxifolin derivatives.