Seasonal changes in antioxidative/oxidative profile of mining and non-mining populations of Syrian beancaper as determined by soil conditions

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HIGHLIGHTS

- Seasonal redox changes in *Z. fabago* populations are determined by soil conditions.
- Higher C- and S- and lower N-based antioxidant levels were found in mining plants.
- The highest antioxidant capacity was found in mining populations at the end of summer.
- Proline or sugars, depending on soil N content, help maintain water balance in plants.
- Sugars and phenols in spring and alkaloid, PAL and PRX in summer typify populations.

GRAPHICAL ABSTRACT

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ABSTRACT

Soil pollution by heavy metals/metalloids (HMMs) is a problem worldwide. To prevent dispersion of contaminated particles by erosion, the maintenance of a vegetative cover is needed. Successful plant establishment in multi-polluted soils can be hampered not only by HMM toxicities, but also by soil nutrient deficiencies and the co-occurrence of abiotic stresses. Some plant species are able to thrive under these multi-stress scenarios often linked to marked fluctuations in environmental factors. This study aimed to investigate the metabolic adjustments involved in *Zygophyllum fabago* acclimative responses to conditions prevailing in HMM-enriched mine-tailings piles, during Mediterranean spring and summer. To this end, fully expanded leaves, and rhizosphere soil, of three contrasting mining and non-mining populations of *Z. fabago* grown spontaneously in south-eastern Spain were sampled in two consecutive years. Approximately 50 biochemical, physiological and edaphic parameters were examined, including leaf redox components, primary and secondary metabolites, endogenous levels of salicylic acid, and physicochemical properties of soil (fertility parameters and total concentration of HMMs). Multivariate data analysis showed a clear distinction in antioxidative/oxidative profiles between and within the populations studied. Levels of chlorophylls, proteins and proline characterized control plants whereas antioxidant capacity and C- and S-based antioxidant compounds were biomarkers of mining plants. Seasonal variations were characterized by higher levels of alkaloids and PAL and soluble peroxidase activities in summer, and by soluble sugars and hydroxycinnamic acids in spring irrespective of the population considered. Although the antioxidant systems are subjected to seasonal variations, the way and the intensity with which every population changes its antioxidative/oxidative profile seem to be determined by soil conditions. In short, *Z. fabago* displays

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a high physiological plasticity that allow it to successfully shift its metabolism to withstand the multiple stresses that plants must cope with in mine tailings piles under Mediterranean climatic conditions.

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1. Introduction

Soil pollution by heavy metals/metalloids (HMMs) is a problem worldwide, affecting not only terrestrial and aquatic ecosystems, but also wildlife and human health (Ali et al., 2013). In the European Union, ~340,000 polluted sites, most commonly contaminated by HMMs, have been identified (Panagos et al., 2013). Worldwide, it is estimated that about 22 million ha of land are contaminated by HMMs, and some of them are unsuitable and/or prohibited for food production (Nsanganwimana et al., 2014). Metalliferous mine tailings are considered the major source of pollution from mining activity due to their high metal content, and the high incidence of wind- and water-driven erosion events that promotes distribution to the surrounding areas (Ali et al., 2013; Mendez and Maier, 2008). A cost-effective tool for minimizing the erosion and dissemination of the contaminants in HMM-polluted areas is phytomanagement, which implies the establishment of a self-sustainable vegetal cover (Parraga-Aguado et al., 2013; Tordoff et al., 2000). However, in mine tailings the limited supply of essential nutrients, associated with high HMM content, and low water holding capacity may become a serious constraint for plant growth and survival. In semi-arid regions plant establishment on mine tailings is further hampered by heat and drought (Tordoff et al., 2000; Parraga-Aguado et al., 2014).

For the last 30 years, >450 hyperaccumulator plants with a strong HMM removal potential and high tolerance have been identified (Barceló and Poschenrieder, 2003; Maestri et al., 2010). However, in practice, there is a continuous interest in searching for native tolerant plants that are adapted to the local ecological environment, as a useful strategy to reach acceptable levels of plant cover in land reclamation processes (Mendez and Maier, 2008; Parraga-Aguado et al., 2014; Tordoff et al., 2000). In this regard, the Syrian beancaper *Zygophyllum fabago* (L.) is a succulent perennial shrub native from the desert of Syria, although nowadays it can be found in all Mediterranean countries and in the Southwest United States (Lefèvre et al., 2016). *Z. fabago* is considered a promising species for restoring HMM polluted soils in semi-arid regions, due to its outstanding pioneer characteristic that makes this species capable to colonize and persist in disturbed areas, including metalliferous mine tailings highly polluted by a broad range of HMMs like As, Pb, and Zn (Boojar and Tavakkoli, 2011; Conesa et al., 2006; Martínez-Sánchez et al., 2012).

In higher plants, a common consequence to environmental stresses, including HMM exposure, is an increased production of reactive oxygen species (ROS) in cells (Schützendübel and Polle, 2002). ROS in conjunction with redox active molecules set the redox environment of cells and tissues, which in turn results in signals that control the plant growth and development as well as initiate the appropriate acclimation responses to stress stimuli (Potters et al., 2010; Suzuki et al., 2012). Although the degree and mechanism of tolerance to HMMs can vary significantly among plant species (Clemens, 2006; Maestri et al., 2010), an overwhelming body of evidence shows that the reinforcement of antioxidant defense system would be essential for restoring redox-homeostasis and normal metabolism under stress (Gill and Tuteja, 2010). In this sense, higher transcript and enzymatic activity levels of several ROS-scavenging enzymes such as superoxide dismutases, peroxidases and catalases as well as greater ability to synthesize ascorbate (AA), glutathione (GSH) and phenolic compounds have been widely reported in different HMM-tolerant plants when compared with HMM-sensitive counterparts (Gill and Tuteja, 2010; Schützendübel and Polle, 2002; Sharma and Dietz, 2009). Apart from being essential compounds in the defense against ROS, GSH and phenolics have been

shown to detoxify HMM by chelation, followed by subsequent sequestration into vacuoles and cell walls (for review, see Singh et al., 2015). Several authors have also described that aside from phenolics (Díaz et al., 2001; Kováčik et al., 2011; Martínez-Alcalá et al., 2013; Michalak, 2006), there are other secondary metabolites such as alkaloids and/or saponins and the proteinogenic amino acid proline (Pro) which accumulated in plant cells under HMM stress (López-Orenes et al., 2014; Sharma and Dietz, 2006; Zhao et al., 2016). Secondary metabolites contribute to all aspects of plant responses to biotic and abiotic stimuli, and guarantee flexible adaptations of plants to ever-changing environmental conditions (Akula and Ravishankar, 2011; Hartmann, 2007). However, most studies dealing with the effect of HMMs exposure on plants have been carried out under hydroponics, in a fully nutrient-rich medium, and under laboratory-controlled conditions. Currently, there exists little information regarding HMM-stress-related markers in plants growing in their natural environment, where they are exposed simultaneously to other environmental constraints. Stress combination can have deleterious effect on plant growth, and prolonged exposure to abiotic stresses can result in the weakening of plant defenses (Rizhsky et al., 2004; Suzuki et al., 2014).

Since the cellular antioxidative/oxidative status plays a pivotal role in the capability of plants to cope with oxidative stress induced by environmental factors, the aim of the current work was (i) to compare the antioxidative/oxidative profile along with key growth- and HMM stress-related parameters on three contrasting populations of *Z. fabago* grown spontaneously on two metalliferous mine tailings (Agustin and Mercader) and on a non-mining site (control) during late spring and late summer seasons in two consecutive years; (ii) to carry out an edaphic characterization of the rhizosphere soil (fertility parameters and total concentrations of As, Cd, Cu, Mn, Pb, and Zn) associated with *Z. fabago* roots on the three selected sites, and (iii) to identify inter-correlations among the different physiological and antioxidative/oxidative parameters evaluated in both seasons as well as associations between plant markers and environmental factors using both unsupervised and supervised multivariate statistical methods.

We hypothesized that (1) the low soil fertility conditions in the mine tailings together with their high content of hazardous HMMs would provoke important changes in the accumulation of N-containing metabolites and would enhance the accumulation of metal-chelating compounds, and (2) during summer the combination of nutrient deficiencies, HMM toxicities and high temperature and drought would drastically impact on both photosynthetic performance and the strengthening of the antioxidant network. The results obtained could contribute to improve our understanding of the acclimative responses of plants to stress combinations under natural conditions. Taking into account the potential of this species for thriving under extreme environmental conditions, use seems plausible in land reclamation programs.

2. Materials and methods

2.1. Plant sampling and soil analysis

Samples of *Z. fabago* L. leaves were obtained from plants growing spontaneously in the Cartagena-La Unión Mining District (37°37'20" N, 0°50'55" W–37°40'03" N, 0°48'12" W), specifically in two mine tailings (Agustin and Mercader) and in a non-mining area located about 1.5 km away from the tailings piles (Supplemental Fig. S1). Therefore, it was assumed that all plants from the three populations were exposed to similar weather conditions. The two mine tailings are located in a natural park which includes forests of *Pinus halepensis* and endemic

xerophytic thickets (Parraga-Aguado et al., 2013). The average annual rainfall of the zone was around 210 mm and 220 mm during 2012 and 2013, respectively (Supplemental Fig. S2). Potential evapotranspiration (ETo) exceeded rainfall by a factor of 6 throughout the year (ETo was 1312 and 1258 mm year⁻¹ during 2012 and 2013, respectively [Supplemental Fig. S2]). In these years the sampling date corresponding to September 2012 was that one in which the greatest rainfall occurred and May 2013 followed a rainy month of April (80 mm rainfall) and was wetter than May 2012 (Supplemental Fig. S2).

The uppermost fully expanded leaves from plants belonging to the three *Z. fabago* populations were collected the 3rd week of May (late spring) and the 3rd week of September (late summer) in two consecutive years (2012 and 2013). In all sampling periods, at least sixty leaves of each population were brought back to the laboratory at 4 °C and washed thoroughly with distilled water, gently blotted on filter paper, and randomly divided into five groups. One group (twelve leaves) were used to determine the relative water content, and the others four groups were immediately frozen in liquid nitrogen, finely ground in a liquid nitrogen-cooled analytical mill (IKA Labortechnik, Germany) and stored at -80 °C until analyzed. Rhizosphere soil samples were also collected from each selected plant. Soil pH, electrical conductivity (EC), dissolved organic carbon (DOC), total N, available phosphorus, water soluble ions (Ca²⁺, Mg²⁺, K⁺, Na⁺, Cl⁻, SO₄²⁻), and HMM concentrations (As, Cd, Cu, Mn, Pb, Zn) were carried out according to standard methods (Parraga-Aguado et al., 2014).

2.2. Plant performance measurements

The physiological status of the three *Z. fabago* populations was evaluated by measuring plant water status, photosynthetic pigment concentrations, total soluble protein levels, soluble sugars and starch contents. Plant water status was estimated by measuring the relative leaf water content (RWC) as RWC (%) [RWC = (fresh weight - dry weight) / (turgid weight - dry weight) × 100]. To determine the turgid weight, fresh leaves were kept in distilled water in darkness at 4 °C for 24 h. Leaf dry weight was obtained after 48 h at 60 °C. Four to six replicates were obtained per treatment. Chlorophylls (Chl) were extracted with 100% methanol (v/v), and total chlorophyll (Chl), Chl *a*, and Chl *b* levels were estimated spectrophotometrically as previously described (López-Orenes et al., 2013). The determination of soluble sugars was carried out using the anthrone method. Ethanol-insoluble polysaccharides were incubated in 1 mL of 0.1 M sodium acetate, pH 5 at 80 °C for 30 min. After cooling on ice, 3 units of α-amylase and 17.8 units of pullulanase were added, and the samples were incubated at 37 °C for 16 h. Then, the tubes were placed at 100 °C for 5 min, cooled to room temperature and centrifuged (12,000g for 10 min). The reducing sugar equivalents present in the supernatant were quantified using the anthrone assay.

2.3. Measurement of the total antioxidant capacity

The total antioxidant capacity was estimated by the DPPH (2,2-diphenyl-1-picrylhydrazyl radical), ABTS^{•+} (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate), and FRAP (ferric reducing/antioxidant power) assays as previously described (Pérez-Tortosa et al., 2012). The reducing power was expressed as micromol Fe(II) per gram fresh weight. A standard curve in the range 5–150 μM of FeSO₄·7H₂O was used for calibration. DPPH and ABTS radical scavenging activities were expressed as micromol of gallic acid equivalents (GAE) per gram fresh weight.

2.4. Quantification of ascorbate, glutathione, soluble non-protein thiols, proline and alkaloids

Dehydroascorbate (DHA) and AA levels were determined by the bipyridyl method and the concentration of soluble non-protein thiols

(NPT) was estimated by the ability of the sulfhydryl group to reduce 5,5'-dithiobis(2-nitro-benzoic acid) as previously described (López-Orenes et al., 2014). Oxidized and reduced GSH were evaluated fluorometrically using the fluorescent probe *o*-phthalaldehyde (Zawoznik et al., 2007). The proline content was determined spectrophotometrically in a sulfosalicylic acid extract using acid-ninhydrin reagent (López-Orenes et al., 2013). Total alkaloid content was determined spectrophotometrically, at 465 nm using the Dragendorff's reagent as previously described (López-Orenes et al., 2014).

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

The 2,4-dinitrophenylhydrazine assay was used for the determination of carbonyl groups in oxidatively modified proteins (Hernández et al., 2001) and the extension of lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reacting substances (TBARS) (López-Orenes et al., 2013). H₂O₂ contents were determined by the ferric-xylenol orange (FOX1) method (López-Orenes et al., 2013). The superoxide radical was detected by its ability to oxidize hydroxylamine to nitrite (Jiang and Zhang, 2001).

2.6. Quantification of total phenolics, flavanols, lignin, flavonoids, hydroxycinnamic acids, and cell wall-associated-proanthocyanidins

Methanolic extracts were used for the determination of total phenolics and flavanols using the Folin-Ciocalteu and *p*-dimethylaminocinnamaldehyde reagents, respectively, with calibration curves for gallic acid (phenolics) and (+)-catechin (flavanols). The pellets, after washing twice with 1% (w/v) Triton X-100, three times with MilliQ-water and five times with methanol and air-dried at 60 °C, were used for lignin determination using the lignin-thioglycolic acid (LTGA) reaction as previously described (López-Orenes et al., 2013).

Total flavonoid content was determined by the aluminium chloride method using rutin as a standard (Kim, 2003). The determination of total hydroxycinnamic acids (HCAs) content was carried out using the Arnow's reagent. In brief, 25 μL of a series of diluted leaf extracts were mixed with 50 μL of HCl 1.5 M, 100 μL of Arnow's reagent (10% (w/v) NaNO₂ and 10% (w/v) Na₂MoO₄), 50 μL of NaOH 2.0 M, and 25 μL of MilliQ-water. The absorbance was read at 490 nm using a 96-well plate reader (Multiskan GO, Thermo Scientific). The results were expressed as caffeic acid equivalents (mg CAE g⁻¹ FW), calculated from a standard curve prepared with caffeic acid.

The content of cell wall-associated proanthocyanidins (PAs) was estimated by measuring the absorbance at 545 nm of the supernatants obtained after the acid attack on washed cell wall pellets. Results were expressed as cyanidin equivalents by using an ε₅₄₅ = 34.7 mM⁻¹ cm⁻¹ (Vermerris and Nicholson 2006).

2.7. 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., 2013). PAL activity was determined spectrophotometrically by following the conversion of L-phenylalanine into trans-cinnamic acid (ε₂₉₀ = 9.5 mM⁻¹ cm⁻¹). PRX activity was estimated at 30 °C using tetramethylbenzidine-HCl (ε₆₅₂ = 39 mM⁻¹ cm⁻¹) as electron donor. Total SOD activity was assayed by the ferricytochrome c method (López-Orenes et al., 2014). Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as a standard.

2.8. Extraction and quantification of free and conjugated SA

Free SA and conjugated SA (SAG, 2-O-β-D-glucosylsalicylic acid) were quantified by using the SA biosensor strain *Acinetobacter* sp.

ADPWH_lux developed by Huang et al. (2006, 2005) with some modifications (Defraia et al., 2008). Briefly, SA was extracted by grinding 100 mg liquid nitrogen powdered leaf tissues with a micropestle in 250 μL of 0.1 M acetate buffer (pH 5.6). Then, the homogenates were centrifuged (16,000g for 15 min) and half of the supernatant (100 μL) was incubated with 4 U of β -glucosidase at 37 °C for 90 min for SAG determination, and the other half (100 μL) was kept on ice for free SA measurement. *Acinetobacter* sp. ADPWH_lux was grown overnight in LB media with shaking at 37 °C and then, 60 μL of LB, 20 μL of leaf extract (treated or not with β -glucosidase), and 50 μL of *Acinetobacter* sp. ADPWH-lux ($\text{OD}_{600} = 0.4$) were added to each well of an opaque 96-well plate. The plate was incubated at 37 °C for 60 min and luminescence signals were captured using a Fusion FX7 detection system and quantified with Bio1D software (Vilber Lourmat, France). A SA standard curve (SA final concentrations from 0 to 100 μM) and negative controls were read in parallel with the leaf samples. Four replicated measurements were run for each sample.

2.9. Statistical analysis

All statistical analysis and graphics were carried out using the language and environmental software R (<http://www.r-project.org>). All data were checked for normality and the homogeneity of variances. Where these assumptions were not met, the Box–Cox family of transformations was used to normalize residuals from the analysis of variance (ANOVA). Where appropriate transformations did not result in normal distributions, the non-parametric Wilcoxon test was used.

Both unsupervised, principal components analysis (PCA), and supervised, partial least squares-discriminant analysis (PLS-DA), multivariate analysis of the mean-centered log-transformed data were performed. Besides a versatile classification algorithm, random forest (RF), as well as a heat map analysis, combined with an agglomerative hierarchical clustering, were also performed to analyze similarities and differences among mining *Z. fabago* populations.

Table 1

Main characteristics and metal(loid) concentrations in the rhizosphere soils of mining (Agustin and Mercader) and non-mining *Zygophyllum fabago* plants. Data represent means ($n = 4$) $\pm$ SE. Values followed by different letters in a row are significantly different at $P < 0.05$, Tukey's HSD test.

Soil parameter	Rhizosphere soil sample		
	Control	Agustin	Mercader
pH ¹	7.8 $\pm$ 0.1 ^a	7.2 $\pm$ 0.1 ^b	7.2 $\pm$ 0.1 ^b
EC ¹ (dSm ⁻¹)	1.7 $\pm$ 0.3 ^b	2.7 $\pm$ 0.2 ^{a,b}	3.5 $\pm$ 0.1 ^a
DOC (g kg ⁻¹)	403.5 $\pm$ 48.3 ^a	35.1 $\pm$ 9.4 ^b	18.1 $\pm$ 2.8 ^b
TN (g kg ⁻¹)	4.3 $\pm$ 0.9 ^a	0.3 $\pm$ 0.1 ^b	0.2 $\pm$ 0.1 ^b
Available-P (g kg ⁻¹)	194.8 $\pm$ 44.1 ^a	8.7 $\pm$ 1.7 ^b	7.4 $\pm$ 1.1 ^b
Water extractable ions ¹			
K ⁺ (g kg ⁻¹)	965 $\pm$ 414 ^a	131 $\pm$ 23 ^b	36 $\pm$ 6 ^b
Na ⁺ (g kg ⁻¹)	201 $\pm$ 55 ^a	288 $\pm$ 505 ^a	249 $\pm$ 75 ^a
Ca ²⁺ (g kg ⁻¹)	1990 $\pm$ 225 ^b	3040 $\pm$ 145 ^a	3015 $\pm$ 5 ^a
Mg ²⁺ (g kg ⁻¹)	88 $\pm$ 5 ^b	195 $\pm$ 20 ^b	640 $\pm$ 50 ^a
SO ₄ ²⁻ (g kg ⁻¹)	995 $\pm$ 414 ^c	8035 $\pm$ 255 ^b	9736 $\pm$ 219 ^a
Cl ⁻ (g kg ⁻¹)	775 $\pm$ 290 ^a	85 $\pm$ 7 ^c	301 $\pm$ 84 ^b
Total metal(loid)s ²			
As (g kg ⁻¹)	15 $\pm$ 14 ^b	190 $\pm$ 26 ^a	414 $\pm$ 181 ^a
Cd (g kg ⁻¹)	8 $\pm$ 2 ^b	45 $\pm$ 23 ^a	23 $\pm$ 10 ^a
Cu (g kg ⁻¹)	47 $\pm$ 11 ^b	70 $\pm$ 4 ^a	90 $\pm$ 14 ^a
Mn (mg kg ⁻¹)	703 $\pm$ 147 ^b	7929 $\pm$ 59 ^a	12,085 $\pm$ 2391 ^a
Pb (mg kg ⁻¹)	647 $\pm$ 56 ^b	3439 $\pm$ 363 ^a	2620 $\pm$ 290 ^a
Zn (mg kg ⁻¹)	547 $\pm$ 111 ^c	5368 $\pm$ 40 ^a	3517 $\pm$ 192 ^b

¹ Data measured in a 1:5 soil to water ratio mixture.

² European Union threshold (mg kg⁻¹) for agricultural soils: As: 5, Cd: 1, Cu: 100, Pb: 60, Zn: 200 [extract, Tóth et al. (2016)].

3. Results

3.1. Rhizosphere soil characteristics

Rhizosphere soil characteristics of Agustin and Mercader mine tailings and control sites are depicted in Table 1. Plant essential nutrients (Total N, available-P and K⁺) were extremely low in rhizosphere soil samples from *Z. fabago* grown in both mine tailings piles. In fact, concentrations of these nutrients in Agustin rhizosphere samples were only about 4–13% of those found in control samples, and even lower (about 3–6%) in Mercader. Contrarily, total As, Cd, Cu, Mn, Pb, and Zn concentrations in both rhizosphere mine tailings were significantly higher (by about 13-fold, 6-fold, 1.5-fold, 11-fold, 5-fold, and 10-fold, respectively, in Agustin, and by about 28-fold, 3-fold, 2-fold, 17-fold, 4-fold, and 6.5-fold, respectively, in Mercader) compared to rhizosphere control samples. The concentration of all of these metal(loid)s was clearly above the EU threshold values for agricultural soils (Tóth et al., 2016). EC was higher in Agustin and Mercader tailings piles (2-fold) than in control soil samples.

3.2. Multivariate analysis of physiological and antioxidative/oxidative data

In order to facilitate detection of any seasonal pattern among the three different *Z. fabago* populations, and the inter-correlations among the different physiological and antioxidative/oxidative parameters evaluated, all the data obtained in this study were subjected to both unsupervised and supervised statistical methods. Firstly, a principal component analysis (PCA) was performed (Fig. 1). The first three principal components (PCs) captured 56.7% of variance between the samples. The first principal component (PC1), accounting for 33.8% of the total variance, separated the control samples, which were grouped on the negative side, from those of the mine tailings (MTs), which were clustered on the positive side. The first PCA axis was associated positively with phenols, redox compounds (AA and GSH), sPRX, total antioxidant capacity (DPPH and FRAP), soluble sugars, alkaloids and HCAs on the negative side of X-axis and Pro, protein content and chlorophylls on the positive side. The second principal component (PC2), accounting for 12.2% of the total variance, separated the MTs samples in the different seasons and was defined by PAL and ABTS (on the positive side of Y-axis) and sugar content and HCAs (on the negative side). The third principal component (PC3), accounting for 10.7% of the total variance, separated all the samples collected in spring 2013, i.e. the rainiest period, from the other samples (Fig. 1C), and was associated positively with protein oxidation (carbonyl group content), iPRX, and SA. Therefore, the PCA results suggested that there were gross seasonal changes between controls and MTs plants indicating that *Z. fabago* plants showed a high physiological plasticity and were able to shift their metabolism in response to HM stress in a season-dependent manner. Similar results were obtained when the data were analyzed using PLS-DA (data not shown). PLS-DA uses a regression technique that rotates the PCA components to sharpen the separation between the groups of samples, and provides a criterion to rank the overall contribution of each variable to the model, based on the variable importance in the projection (VIP). Consequently, in order to precisely identify biomarker candidates and interpret their physiological significance, the correlation coefficients for the first three components of PLS-DA and variable importance in the projection (VIP) score were plotted. In the Fig. 2, it can be seen that the biomarkers with a VIP score >0.95 and with >50% of the explained variance for the first component, accounting for 33.8% of the total variance, were phenols, protein content, Pro, total antioxidant capacity, NPT, HCAs, chlorophylls, sPRX and flavanols (Fig. 2A). PAL, Pro, flavanols, sugars and ABTS were the main markers for the second component, accounting for 12.2% of the total variance (Fig. 2B), and protein carbonyl groups, iPRX, SA, NPT, and MDA for the third component, accounting for 10.7% of the total variance (Fig. 2C).

Fig. 1. Score (left) and correlation (right) plots of the first three components of the PCA applied to physiological and biochemical variables in leaves of *Z. fabago* plants growing in non-mining (control, black color) and mining tailings piles Agustin (blue color) and Mercader (green color) 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Recently, machine learning algorithms, such as RF, have been demonstrated to be an effective tool in analyzing large data sets in life sciences (Touw et al., 2013). RF algorithm has become very popular mainly because it provides high prediction accuracy and information on variable importance for classification. One of the measures of variable importance in RF is the mean decrease in accuracy, which corresponds to the increase of the out-of-bag (OOB) error upon permutations (Touw et al., 2013). The top 15 significant biomarkers based on mean decrease in accuracy (Fig. 3) coincided in a great extent with those showed in Fig. 2. Thus, taken together, these results suggested that these parameters could be considered as potential markers to differentiate between control (Chl, protein content, and Pro) and MTs populations (phenols, total antioxidant capacity, AA, and GSH) and between seasons: summer (sPRX, PAL, and alkaloid content) and spring (HCAs and sugars).

In order to have an overall visual representation of the seasonal differences between the two MT populations, the ratio values (MTs/control) were log-transformed (base 2) and a hierarchical clustering analysis was performed and presented in a heatmap (Fig. 4). The differences between MT groups were analyzed by a non-parametric

Wilcoxon's test, and the mean ratios of fold changes and their associated *P*-values obtained were summarized in Table 2. As can be seen in Fig. 4, the clustering tree diagram (dendrogram) showed a clear separation between spring and summer samples, with the samples taken during the greatest rainfall period (i.e. September 2012 and May 2013) clustering together. The upper cluster in the biomarker hierarchy contains nine markers that clearly differed between both season in MT populations, whereas the lower cluster contains markers that both increased (e.g., HCAs, starch, phenols and NPT) and decreased (e.g., Pro and Chl) in plants growing in MTs.

Among the parameters used to assess the physiological status of *Z. fabago* MT plants (RWC and the content of Chl, proteins and soluble sugars), only the RWC remained unaffected (with log 2 ratio between 0.8 and 1; Table 2), whereas both soluble sugar and starch concentrations increased, and the foliar concentrations of chlorophylls, soluble proteins and Pro decreased. In this respect, it is noteworthy to highlight the striking low Pro level found in Mercader leaves in both seasons (log 2 ratio between 0.2 and 0.3; Table 2).

The Zygophyllaceae family is chemically characterized by the presence of indole alkaloids, particularly β -carboline derivatives, and their

Fig. 2. Correlation coefficient scores of PLS-DA analysis vs variable importance in the projection (VIP) of the three PLS-DA components. Variables were represented by inverted triangles.

levels have been reported to markedly increase in leaves of Pb-treated *Z. fabago* plants (López-Orenes et al., 2014). In this study, the foliar concentration of total alkaloids in control plants was slightly lower in spring (about 5–10 nmol harmaline eq. g⁻¹ FW; Supplemental Table S1) than in summer (about 10–14 nmol nmol harmaline eq. g⁻¹ FW), and a rise in the alkaloid content was found in MT leaves in both seasons, especially in Agustin *Z. fabago* plants.

Fig. 3. Importance measure (permutation-based mean decrease accuracy) provided by the random forest analysis of the physiological and biochemical parameters measured in *Z. fabago* leaves.

Leaf extracts from MT plants showed superior Fe-reducing power and ABTS/DPPH radical-scavenging ability, higher content of phenolics (total soluble phenols, HCAs, flavonoids and PAs), AA, GSH, and total non-protein thiol (NPT) compounds. As expected, high correlations between these antioxidant tests and specific antioxidant compounds, such as DPPH and FRAP tests with phenols ($r = 0.7$, $P < 0.0001$), GSH ($r = 0.6$, $P < 0.0001$) and AA ($r = 0.5$, $P < 0.0001$), and ABTS with total thiol content ($r = 0.6$, $P < 0.0001$) were observed (Supplemental Table S2). The increase in the level of these antioxidant compounds could explain the reduction in the content of malondialdehyde (MDA), a marker of lipid peroxidation, found in MT leaves (log 2 ratio between 0.5 and 0.89; Table 2), although a significant increase in protein oxidation levels was detected in MT plants in all sampling periods (log 2 ratio between 1.4 and 2). A similar trend to that observed for lipid peroxidation was seen for H_2O_2 levels, and the decrease in the O_2^- levels in MT leaves could be associated with the increase in the total SOD specific activity, which is the unique enzyme responsible for the dismutation of superoxide radicals (Fig. 4 and Table 2).

On the other hand, it should be noted that the marked accumulation of phenolic compounds in summer leaves could be related to the

Fig. 4. Heatmap of Pearson's rank correlation coefficients and hierarchical clustering showing the fold change of physiological and biochemical parameters in leaves of *Z. fabago* plants growing in Agustín and Mercader mining tailings piles in late spring and summer in 2012 and 2013. Log 2 ratios of fold changes relative to each respective control group are given by shades of red or blue colors according to the scale bar. Data represent the mean log 2 values of four individual biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increase of PAL activity, the first enzyme of the overall phenylpropanoid biosynthetic pathway, observed in this season. Other important enzymes involved in phenol metabolism are class III peroxidases (PRX). Interestingly, the response pattern of both ionically-bound cell wall peroxidases (iPRX) and soluble peroxidases (sPRX) was opposite: iPRX activities dropped whereas sPRX activities increased in MT leaf tissues. As far as the levels of free and conjugated SA were concerned, the highest levels of free and conjugated SA were found in summer in all the populations (Supplemental Table S1). In MT leaves, the content of both SA and SAG were slightly inferior to those from controls in both seasons (Fig. 4 and Table 2).

Additionally, to identify associations between antioxidative/oxidative biomarkers and rhizosphere soil parameters a Pearson's r correlation coefficient analysis was performed. In this respect, some clear-cut correlations were found between both Pro and protein levels and N in soil ($r > 0.7$, $P < 0.0001$), foliar GSH content and S and Cd concentrations in soil ($r \geq 0.6$, $P < 0.0001$), and HcAs, phenols, and total antioxidant activity (FRAP and DPPH) and the concentration of As, Cd, Cu, Mn, Pb and Zn in soils ($r > 0.5$, $P < 0.0001$) (Table 3).

4. Discussion

A remarkable feature of *Z. fabago* is its ability to thrive in mine tailings under semi-arid environment. Acclimation to the harsh conditions prevailing in the tailings piles is expected to provoke profound changes

in gene expression, which can result in strong molecular and physiological changes. Moreover under natural conditions, conflicting or antagonistic plant responses can be triggered when different stress conditions (e.g., drought and high temperatures) are combined (Suzuki et al., 2014).

Here, as expected in a water-limited Mediterranean area, rainfalls favored *Z. fabago* growth as reflected in PCA analysis (Fig. 1B), especially during spring rainfall (i.e., May 2013) although summer rainfall (i.e., September 2012) exerted less influence on plant growth, especially in MT plants.

Interestingly, our findings revealed no significant seasonal influence on leaf RWC in *Z. fabago* plants growing in tailings piles, regardless of the difference in the rainfall patterns observed in the years analyzed. This suggested that cell turgor in photosynthetic active leaves of MT plants was not seriously impaired, even in the driest summer period studied (September 2013). Free Pro and soluble sugars are key osmolytes contributing towards osmotic adjustment in stressed plants (Suzuki et al., 2014; Szabados and Saviouré, 2010). In fact, an increase in Pro (Lefèvre et al., 2014; López-Orenes et al., 2014) and soluble sugars concentrations (Lefèvre et al., 2014) has been previously reported in leaves of *Z. fabago* plants exposed to HM under hydroponics conditions in a fully nutrient-rich medium. In this study, however, a high accumulation of soluble sugars was found, while the foliar concentration of Pro in MT leaves was significantly lower than that from controls. Nevertheless, the results obtained in this study are not surprising given the very low

Table 2
Normalized log₂ ratio of fold change and their associated *P*-values obtained by the non-parametric Wilcoxon's test ($P < 0.05$; $n = 4$) of the physiological and biochemical parameters measured in *Z. fabago* leaves. The brighter the colors, the higher the probability values. The scale bar is shown below the table. Normalized log₂ ratios higher than 1 are highlighted with red background and log₂ ratios lower than 1 are highlighted with blue background.

Population	Agustin								Mercader							
Month	Spring				Summer				Spring				Summer			
Year	2012		2013		2012		2013		2012		2013		2012		2013	
	Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value	Ratio	P value
PAL	1.05	0.5047	1.04	0.7240	1.84	0.0093	2.20	0.0122	1.03	0.1335	0.94	0.0361	1.44	0.0020	1.46	0.0269
Sugars	1.87	0.0005	1.87	0.0005	0.80	0.0005	0.76	0.0005	1.74	0.0005	1.60	0.0005	1.07	0.0522	1.29	0.0005
Flavanols	1.01	0.3266	1.00	0.7240	0.21	0.0025	0.29	0.0025	0.99	0.9687	0.74	0.0053	0.40	0.0005	0.34	0.0025
DPPH	1.72	0.0005	2.00	0.0005	1.36	0.0005	1.11	0.0425	1.39	0.0010	1.56	0.0005	1.66	0.0005	1.87	0.0005
FRAP	1.72	0.0005	1.89	0.0005	1.38	0.0005	1.12	0.0522	1.47	0.0005	1.56	0.0005	1.52	0.0005	1.63	0.0005
AA	1.71	0.0210	1.65	0.0005	1.14	0.0024	0.86	0.0005	1.46	0.0005	1.28	0.0005	1.56	0.0005	1.63	0.0005
RWC	0.79	0.0015	0.82	0.0015	1.04	0.0015	1.25	0.0015	0.94	0.0020	1.03	0.0020	0.90	0.0015	0.93	0.0015
DHA	1.17	0.1763	1.15	0.0010	0.47	0.0005	0.33	0.0005	0.64	0.0005	0.72	0.0210	0.42	0.0005	0.57	0.0210
Protein	0.57	0.0005	0.59	0.0005	0.45	0.0024	0.39	0.0005	0.48	0.0025	0.43	0.0005	0.43	0.0005	0.42	0.0005
HCA	2.95	0.0005	1.92	0.0024	4.59	0.0005	2.51	0.0005	2.70	0.0005	2.09	0.0025	6.19	0.0005	4.57	0.0025
Flavonoids	1.49	0.0425	1.25	0.0005	2.48	0.0005	1.11	0.0122	1.57	0.0005	1.16	0.0053	3.47	0.0005	1.38	0.0093
Starch	1.32	0.0010	1.46	0.0025	1.06	0.1696	1.47	0.0210	1.39	0.0005	1.30	0.0108	0.94	0.2661	1.32	0.0005
SOD	1.83	0.0923	1.17	0.3668	2.18	0.0253	1.44	0.0049	1.80	0.0020	1.07	0.9097	2.85	0.0020	1.30	0.2334
Phenols	2.03	0.0005	1.84	0.0005	1.84	0.0005	1.28	0.0093	1.57	0.0005	1.36	0.0010	2.03	0.0005	2.18	0.0005
NPT	1.37	0.0005	1.30	0.0005	1.29	0.0005	1.24	0.0010	1.27	0.0005	0.97	0.0522	1.42	0.0005	1.55	0.0005
Alkaloids	2.05	0.0025	1.44	0.0041	3.54	0.0005	1.38	0.0025	2.08	0.0005	1.25	0.0022	1.43	0.0025	1.38	0.0024
O ₂ ^{•−}	0.79	0.0005	0.59	0.0024	0.77	0.0010	0.53	0.0022	0.60	0.0376	0.44	0.0025	0.42	0.0005	0.35	0.0023
sPRX	1.48	0.0005	1.27	0.0005	1.46	0.0005	2.29	0.0005	4.20	0.0005	2.75	0.0005	1.18	0.0210	3.93	0.0005
SAG	0.95	0.0020	0.87	0.0020	0.87	0.0236	0.87	0.0020	0.82	0.0015	0.71	0.0015	0.84	0.0015	0.75	0.0015
SA	0.97	0.0035	0.91	0.0020	0.85	0.0020	0.86	0.0020	0.83	0.0015	0.74	0.0015	0.82	0.0015	0.77	0.0015
Proline	0.25	0.0005	0.55	0.0005	0.82	0.0210	0.56	0.0005	0.18	0.0025	0.30	0.0005	0.19	0.0025	0.20	0.0005
ABTS	2.08	0.0005	1.32	0.0025	1.23	0.0005	1.38	0.0005	1.69	0.0005	1.09	0.0010	1.09	0.0015	1.51	0.0005
MDA	0.50	0.0005	0.85	0.0210	0.71	0.0010	0.50	0.0005	0.50	0.0005	0.89	0.0034	0.63	0.0005	0.55	0.0005
H ₂ O ₂	0.59	0.0342	0.70	0.0005	0.40	0.0005	0.56	0.0005	0.53	0.0005	0.69	0.0024	0.79	0.0015	0.37	0.0005
iPRX	0.44	0.0005	0.27	0.0005	0.69	0.0005	0.45	0.0005	0.59	0.0005	0.48	0.0005	0.64	0.0005	1.02	0.6772
Pas	0.95	0.0042	1.07	0.0050	1.15	0.0010	1.19	0.0024	1.41	0.0022	1.14	0.0024	1.18	0.0005	1.06	0.5548
GSSG	0.32	0.0005	0.60	0.0005	0.69	0.0049	0.65	0.0005	0.46	0.0005	0.58	0.0005	0.56	0.0005	0.92	0.3394
GSH	2.71	0.0005	1.45	0.0005	1.78	0.0025	1.07	0.3394	1.79	0.0005	1.31	0.0005	1.65	0.0005	0.98	0.5186
Lignin	0.83	0.0025	0.98	0.5186	1.06	0.0210	0.91	0.0253	0.96	0.0342	0.97	0.0210	1.06	0.0161	0.81	0.0005
C=O	1.54	0.0134	1.50	0.0005	1.44	0.0052	1.99	0.0010	1.36	0.0269	1.49	0.0005	1.43	0.0210	2.02	0.0005
Chl b	0.94	0.8501	0.64	0.0005	0.51	0.0005	0.17	0.0005	0.51	0.0005	0.57	0.0005	0.53	0.0005	0.15	0.0005
Chl a	0.75	0.0005	0.58	0.0005	0.33	0.0005	0.19	0.0005	0.52	0.0005	0.51	0.0005	0.39	0.0005	0.18	0.0005

More than 1	Less than 1
≤ 0.001	≤ 0.001
≤ 0.005	≤ 0.005
≤ 0.05	≤ 0.05
> 0.05	> 0.05

levels of essential nutrients such as N, P and K found in the rhizosphere tailing soils. In fact, it is well known that during mineral nutrient deficiency a perturbation of amino acid metabolism occurs (Watanabe et al., 2010). Thus, the results presented here suggest that the low levels of essential nutrient found in MT soils, especially in Mercader tailings, would force *Z. fabago* to accumulate more C-containing compounds associated with photosynthesis (i.e., sugars) and less N-containing compounds (i.e., Pro) for osmotic adjustment. The replacement of Pro by sugars as major osmoprotectants has also been described in plants exposed to a combination of stress factors (for review, see Suzuki et al., 2014, and refs. herein).

Moreover, a significant decline in Chl content, which was particularly drastic in September 2013, was also found in MT leaves. In fact, based on multivariate data analysis, Chl content was identified as one of the

most significant parameters for discrimination between non-mining and mining *Z. fabago* populations. Actually, chlorophyll loss is one of the common visual symptoms of HMM exposure (for review, see Viehweger, 2014; Singh et al., 2015), and has been postulated as a simple and reliable indicator of HM toxicity in plants (Gratão et al., 2005; Martínez-Alcalá et al., 2013). However, it is important to highlight that Chl loss can also be considered as an adaptive feature in plants grown under stressful climatic conditions to protect the photosynthetic apparatus from photoinhibition and photooxidation (Munné-Bosch and Alegre, 2000). Besides, it should be noted that during summer *Z. fabago* mining plants are subjected not only to HMM-stress and nutrient deficiencies, but also to a combination of heat and drought. These adverse environmental conditions have been reported to induce premature leaf senescence, which is considered one of the strategies that

Table 3

Pearson's correlation coefficients for selected markers with significant correlation ($P < 0.0001$) between log-transformed plant markers and soil parameters. Asterisk indicates $P < 0.001$ (see Supplementary Table S2 for more details).

	N	S	As	Cd	Cu	Mn	Pb	Zn
AA			0.426		0.432	0.417	0.368	0.363
Alkaloids			0.414	0.475	0.370	0.433	0.473	0.474
Chl a	0.581		−0.575	−0.512	−0.554	−0.580	−0.565	−0.561
Chl b	0.487		−0.491	−0.402	−0.482	−0.490	−0.460	−0.456
DPPH			0.565	0.488	0.548	0.567	0.546	0.542
Flavonoids			0.474	0.376	0.470	0.472	0.437	0.433
FRAP			0.64	0.575	0.614	0.646	0.632	0.628
GSH		0.595	0.548	0.645	0.484	0.576	0.636	0.638
HCAs			0.698	0.598	0.679	0.700	0.671	0.666
NPT		0.401	0.389	0.375	0.367	0.397	0.400	0.399
PAs		0.375	0.402		0.420	0.388	0.322	0.316*
Phenols			0.630	0.580	0.601	0.638	0.632	0.629
Proline	0.775							
Protein	0.732							
sPRX			0.441		0.460	0.426	0.353	0.346

contribute to the survival of severely stressed plants by allowing nutrient recycling and reallocation from dying to developing tissues (Guiboileau et al., 2010; Munné-Bosch and Alegre, 2004).

Under these nutrient-limited conditions and high concentration of metal(loid)s in soil, the analysis of the full spectrum of non-enzymatic antioxidant compounds, estimated by the ABTS, DPPH and FRAP tests, revealed that MT leaves exhibited a higher antioxidant capacity and higher levels of antioxidant compounds (AA, GSH, NPT, and phenols). In addition, the activities of antioxidant enzymes, such as sPRX and SOD, in MT leaves showed a significant increase relative to controls. Taken together, these data indicate that the up-regulation of the antioxidant network could explain, at least partly, the decrease in both H_2O_2 and $O_2^{\bullet-}$ contents and the low oxidative damage to lipids (measured in terms of MDA formed) noticed in MT leaf tissues in all sampling periods. Consistent with previous laboratory studies (for review, see Gill and Tuteja, 2010; Singh et al., 2015), our results not only support the notion that the reinforcement of antioxidant defenses could lead to improved HMM stress-tolerance in *Z. fabago* mining plants, but also underpin the emerging role of the antioxidant network in the protection of plants to a combination of severe environmental conditions (Suzuki et al., 2014).

A further interesting feature is that despite the fact that the total N content found in rhizosphere MT soils was very low (by a factor of 10 compared to controls), MT leaves accumulated higher levels of alkaloids than did the controls. These nitrogen-compounds have been reported to act as scavengers of ROS in several organisms, including yeast (Moura et al., 2007), and animals (Herraiz and Galisteo, 2003; Kim et al., 2001). Thus, it is plausible that the foliar accumulation of alkaloids in MT plants could counteract ROS accumulation and, consequently could contribute to prevent oxidative injury in leaf tissues of *Z. fabago* mining plants. Despite possessing an apparently powerful antioxidant system, there was an increase in the levels of protein carbonyl groups in MT leaves compared to controls. To this respect, Pena et al. (2008) have reported the inhibition of the proteolytic systems by HMs and the progressive accumulation of oxidized proteins in sunflower leaves. Therefore, it is tempting to speculate that the higher intracellular levels of oxidized proteins found in MT *Z. fabago* leaves could be attributed, at least in part, to the HMs-induced inhibition of proteasome activity.

The examination of overall antioxidative/oxidative profile in spring and summer leaves revealed some differences between mining and non-mining *Z. fabago* plants. Thus, summer MT leaves were characterized by high PAL and sPRX activities and by higher concentration of compounds with antioxidant function and metal-chelation properties, such as HCAs and flavonoids (Agati et al., 2012; Andejkovic et al., 2006; Rice-Evans et al., 1997). Accumulation of phenolic compounds

in leaf tissues was also observed in different plant species exposed to heavy metal (Llugany et al., 2013; Singh et al., 2015, and refs. herein), and also in plants grown in multi-polluted soils (Márquez-García et al., 2009; Martínez-Alcalá et al., 2013). In our study, this observation was supported by correlation tests that indicated a high-to-moderate correlation between the concentrations of HMMs in soils and the levels of soluble phenols, HCAs and flavonoids as well as sPRX activity. Interestingly, sPRX was ranked as the most important biomarker in RF analysis (Fig. 3), showed a high correlation with PLS-DA components (Fig. 2A), and also had a VIP score > 1 , which is considered influential in discriminating between sample groups. All of these data are in line with previous results showing that PRXs are quite sensitive to the presence of heavy metal in soils (Passardi et al., 2005 and refs. herein).

In the plant cell, PRXs are mainly localized in the cell wall and in the vacuole, which are also the target compartments for the accumulation of phenolic compounds, many of which may function as PRX substrates (Ferrerres et al., 2011). In our study, soluble and cell wall-bound PRXs exhibited a distinct behavior in MT leaves in comparison with controls. The rise of sPRX activity and the reduction of iPRX may affect the phenol profile in both cell compartments. Thus, in regard to the family of flavanols, there was a decrease in the levels of soluble flavanols and an increase in the content of oligomers of flavanols (proanthocyanidins, PAs), which correlated with the rise of sPRX. Moreover, the different levels of flavonoids and HCAs observed in MT leaves in both seasons could be related, at least in part, to the different regulation of the expression and/or activity of these PRX isoenzyme groups. In this sense, differential regulation of the expression and/or activity of PRX isoenzymes by AA (Takahama and Oniki, 2000) and SA (Almagro et al., 2009; Lajara et al., 2015; Mika et al., 2010) have been widely reported in the literature.

Levels of SA in MT leaves were lower than those of control plants irrespective of the sampling date. At first sight, this could be considered surprising because most of the studies in the literature reported enhancements of tolerance to Pb and other HMs in many plant species upon SA treatment (for review, see Horváth et al., 2007, and refs. herein). What is more, treatment of *Z. fabago* plants with Pb under hydroponic conditions was described to result in both higher levels of endogenous free SA and decreased Pb uptake relative to control plants (López-Orenes et al., 2014). These facts seem to be a contradiction to the results of the present work. However, it must be taken into account that lab/greenhouse assay conditions are far from those prevailing in real environments. Moreover, in studies carried out under controlled conditions plants are usually challenged with HMMs for a limited period of time, in contrast to chronic exposure to HMMs taking place under field conditions. Interestingly, Tao et al. (2013), working with SA-altering mutants of *Arabidopsis*, found that high endogenous levels of SA

made the phytotoxicity induced by Pb and Cd worse, what would support the observation that MT plants contained lower levels of SA than control plants.

Finally, although the antioxidant systems of the three *Z. fabago* populations studied are subjected to seasonal variations, the way and the intensity with which every population changes its antioxidative/oxidative profile are determined by soil conditions. So, as mentioned above, low nitrogen availability in the rhizosphere of MT plants reduced the accumulation of proline and increased the levels of other osmotic active compounds. In this way, the enhanced increases in the levels of phenolics (and in the activities of phenol metabolizing enzymes), and AA and S-based antioxidants in the leaves of MT plants relative to non-mining plants could be a reflection of the higher concentrations of heavy metal, redox-active metal, and metalloids (As) present in mine tailings piles.

5. Conclusions

Z. fabago plants showed specific seasonal antioxidative/oxidative profiles when grown under different multi-polluted soils and nutrient-limited conditions, being those parameters related to the maintenance of the cellular redox homeostasis (AA, GSH, antioxidant capacity, phenols, alkaloids and sPRX activity) critical in modulating the acclimation response to the harsh conditions prevailing in the tailings piles. Meanwhile, a high foliar RWC was maintained through the replacement of Pro by sugars as major osmoprotectants in photosynthetically active MT leaves, allowing the continuation of the cell metabolic activity. Moreover, keeping in mind that climatic conditions are the same for the three emplacements considered in the present study, the decreases observed in chlorophylls, soluble protein and Pro contents can also be considered as sensitive biochemical markers of the acclimative responses of *Z. fabago* plants to the harsh conditions imposed by soil characteristics in the tailings piles, especially by the nutrient limitation and the high levels of HMMs.

To date, scarce information exists regarding the stress signatures of HMM tolerant plants grown under natural conditions. Such studies provide valuable information not only to design more realistic experimental approaches and interpret the evidences obtained with model plants under laboratory-controlled conditions but also to provide an insight into common stress tolerance strategies with potential applicability for *in situ* phytoremediation programs on semi-arid HMM-polluted sites.

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