Seasonal ionomic and metabolic changes in Aleppo pines growing on mine tailings under Mediterranean semi-arid climate.

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Abstract

Aleppo pine is the most abundant conifer species in Mediterranean basin. Knowledge of adaptive mechanisms to cope with different environmental stresses simultaneously is necessary to improve its resilience to the predicted climatic changes and anthropogenic stressors, such as heavy metal/metal(loid)s (HMMs) pollution. Here, one year-old needles and rhizosphere soil samples from five mining and non-mining (NM) populations of Aleppo pines grown spontaneously in SE Spain were sampled in two consecutive years during spring and summer. Quantitative determination of a wide suite of edaphic, biochemical, and physiological parameters was performed, including soil physicochemical properties, ionome profile, foliar redox components, primary and secondary metabolites. Mining rhizosphere soils were characterized by elevated contents of HMMs, particularly lead and zinc, and low carbon, nitrogen and potassium levels. Multivariate data analysis based on needle ionome and antioxidative/oxidative parameters revealed a clear distinction between seasons irrespective of the population considered. Spring needles were characterized by higher levels of HMMs, sulfur, glutathione (GSH), proanthocyanidins (PAs), and soluble phenols (TPC), whereas reduced chlorophylls and increased levels of carotenoids, relative water content and K^+, Na^+ and Cl^- typified summer needles. In general mining populations had higher levels of ascorbate, and TPC, and exhibited higher antioxidant activities than the NM population. This could contribute to prevent oxidative injury induced by HMMs. Taken together, results suggest that seasonal factors have a more marked effect on the metabolism of the Aleppo pine populations studied than that exerted by soil conditions. This effect could be mediated by water availability in surface soil layers. If this conclusion is right, predicted rainfall reduction and temperature increase in the Mediterranean basin associated to global climate change would lead to pine needle

metabolism to express the summer pattern for more prolonged periods. This, in turn, could negatively affect the performance of Aleppo pine populations.

Keywords

Antioxidative/oxidative profiles; Ionomic profiles; Mine tailings piles; Stress combinations; Mediterranean climate

1. Introduction

Aleppo pine (*Pinus halepensis* Mill.) is the most widely distributed conifer species in the Mediterranean region (Maseyk et al., 2008; Querejeta et al., 2008). In this area the species exhibits a bimodal growth pattern, with two optimal growth periods one in spring and a second in fall associated with favorable growing conditions (*i.e*., mild temperatures and adequate soil-water availability) (López-Serrano et al., 2005; Pacheco et al., 2017). Although this species is noted for its high resilience to heat and drought stress (Maseyk et al., 2008; Wellburn et al., 1996), climate warming is expected to have negative effects on tree survival and development, according to the Intergovernmental Panel on Climate Change 2014 (IPCC 2014; http://ipcc.ch/). Moreover, growth rate reductions of forest trees can be aggravated as a consequence of increased concentrations of hazardous pollutants such as heavy metal and metal(loid)s (HMMs), which are particularly prevalent in areas subjected to intense mining activity (Panagos et al., 2013). The ecological consequences of the simultaneous occurrence of natural and anthropogenic stressors are difficult to predict because they cannot be inferred from individual stress studies, especially if the stress combinations result in antagonistic or conflicting responses (Choudhury et al., 2017; Suzuki et al., 2014).

Under natural conditions, plants have evolved a complex network of signal transduction pathways to cope with multiple environmental stresses occurring simultaneously. Whether a plant is able to acclimate to these challenging environmental conditions or not is going to be, ultimately, determined by the appropriate signaling and coordination of plant responses (Harfouche et al., 2014; Urano et al., 2010; You and Chan, 2015). Extensive evidence now strongly supports that reactive oxygen species (ROS) are key signal transduction molecules in plant stress signaling (Mittler, 2017), although elevated

ROS levels, above a physiological threshold, can cause oxidative damage to biomolecules and cellular structures (De Gara et al., 2010). Increased production of ROS in plant cells has been widely shown under abiotic stress conditions, including HMM exposure (Gill and Tuteja, 2010; Schützendübel and Polle, 2002; You and Chan, 2015), as well as in different stress combinations (Choudhury et al., 2017; Suzuki et al., 2014). To keep ROS steady-state concentrations low, plants possess a particularly complex and redundant ROS-scavenging system, in which enzymes and metabolites are linked in a network of reactions (De Gara et al., 2010). Recent "omics" studies have highlighted that antioxidant defense machinery can play an important role not only in plant HMM-tolerance mechanisms (Dalcorso et al., 2013; Hossain and Komatsu, 2012; Singh et al., 2015) but also in the response of plants to stress combinations (Suzuki et al., 2014; Zandalinas et al., 2017). Indeed, the induction of ROS-scavenging enzymes, as well as a high content of both primary antioxidants, *i.e.*, ascorbate (AA) and glutathione (GSH), and secondary antioxidants, such as carotenoids, proline (Choudhury et al., 2017), and different phenolic compounds (Martinez et al., 2016), were found to have a key role in plant acclimation to stress combinations. In fact, the diversity and plasticity of phenolic compounds are considered to play a key role in plant defense mechanisms towards biotic and abiotic stresses (Agati et al., 2012; Brunetti et al., 2015; Pourcel et al., 2007). Phenylalanine ammonia-lyase (PAL), the key enzyme in controlling phenolic biosynthesis, and the large family of secreted class III plant peroxidases (PRX), which catalyze the oxidation of a wide variety of phenolic compounds using hydrogen peroxide as the electron acceptor, have also been reported to be stimulated by infection and environmental stress (Almagro et al., 2009; Dixon and Paiva, 1995). In addition, ionomics approaches have revealed that the study of shoot ionome, which represents the mineral nutrient and trace element composition of a plant

(Salt et al., 2008), could potentially be used as a tool to detect specific physiological responses to environmental variation, or nutritional statuses (Baxter et al., 2008).

Metalliferous mining wastes represent very stringent conditions for plant growth because of nutrient deficiencies, high HMM content and salinity (Tordoff et al., 2000). Nevertheless, several studies have described the spontaneous colonization of HMM-enriched mine tailings by Aleppo pine in semi-arid areas (Parraga-Aguado et al. 2014, and refs. herein). Recently, woody and tree species have gained increasing interest in mine reclamation programs because of their massive and deep root systems (Luo et al., 2016). Although recent achievements in the study of the molecular responses to single stresses have been reported (Harfouche et al., 2014), the physiological and molecular mechanisms underlying the adaptation to HMMs under semi-arid Mediterranean conditions in woody plants are not clearly understood.

With this background, the overarching aim of the current work is to evaluate metabolic adjustments in response to the harsh conditions of mine tailings during both a favorable and a less-favorable growing season in Aleppo pines. To address this aim, a comparison of the antioxidative/oxidative profile, needle ionomics, physiological and edaphic parameters were carried out among five Aleppo pine populations growing either in a non-mining site (NM), or in multi-metal(loid) polluted mining tailings, located in the Cartagena-La Unión Mining District (SE Spain) during late spring (May) and late summer (September) in two consecutive years (2012 and 2013). Moreover, different dimensionality reduction and classification statistical methods were performed to identify inter-correlations among the different physiological and antioxidative/oxidative

parameters evaluated, as well as possible associations between plant markers, concentrations of nutrients/metal(loid)s and soil parameters.

This work is framed within a larger study devoted to examine the oxidative stress signatures and the metabolic adjustments in response to the adverse conditions of mine-tailings under semi-arid Mediterranean conditions in different pioneer plant species, including both herbaceous (López-Orenes et al., 2018, 2017) and woody plants.

2. Materials and Methods

2.1. Plant and soil sampling

Aleppo pine needles were obtained from mature trees growing spontaneously in the Cartagena-La Union Mining District (SE of the Iberian Peninsula) in four different tailings piles known as Agustin (37º36'20''N, 0º50'15''W), Mercader (37º36'15''N, 0º50'04''W), Ripolles (37° 36' 18" N – 0° 50' 10" W), and Wikon (37° 36' 15" N – 0° 50' 08" W), and in a non mining-impacted forest (37°35'47" N, 0°49'23" W) located about 1.5 km away from these mining sites (Supplemental Fig. S1). These tailings are located at a natural park which includes forests of Aleppo pine and endemic xerophytic thickets (Parraga-Aguado et al., 2014). This mining area contains one of the largest Pb and Zn content in the SW of Europe. Average annual rainfall of the area was around 210 mm and 220 mm during 2012 and 2013, respectively (Supplemental Fig. S2), and potential evapotranspiration (ETo) exceeded rainfall by sixfold (ETo was 1312 and 1258 mm yr^{-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

151 greatest rainfall occurred and May 2013 followed a rainy month of April (80 mm
152 rainfall) and was wetter than May 2012 (Supplemental Fig. S2).
153
154 Rhizosphere soil and plant samples were collected from four mature trees of similar size
155 per site (Agustin, Mercader, Ripolles, Wikon, and non mining-impacted forest) and
156 sampling date. Six to ten small branches, with current and one year-old needles, were
157 cut from different directions from the upper third of the crown of each tree
158 (Supplemental Fig. S3). In the laboratory, the current-year foliage was removed and
159 only the old (one-year) needles were selected (Supplemental Fig. S3). In all sampling
160 periods, at least 200 g of one year-old needles of each population were washed
161 thoroughly with tap and distilled water, gently blotted on filter paper. Then, the mixed
162 samples were randomly divided into five groups. One group was used to determine the
163 needle relative water content (RWC), and each one of the remaining four groups, were
164 divided into two subsamples, one of them was immediately frozen in liquid nitrogen,
165 and stored at –80°C for biochemical analysis, and, the second one was dried at 60 ºC for
166 72 h for elemental analysis. At the beginning of the sampling period (*i.e.*, May 2012),
167 four soil subsamples at each site were collected from the rhizosphere of Aleppo pine
168 trees, taking the top 20 cm of soil, and transferred under aseptic conditions to the
169 laboratory.
170
171 *2.2. Rhizosphere soil analysis*
172
173 The collected rhizosphere soil samples (n=4 per site) were air-dried, sieved through a 2
174 mm-sieve mesh, and subjected to several physicochemical analyses including pH,
175 electrical conductivity (EC), equivalent calcium carbonate (% $CaCO_3$), organic carbon

(OC), dissolved organic carbon (DOC), total nitrogen (TN), soil texture, water soluble ions (Cl^-, SO_4^{2-}, Na^+, K^+, Ca^{2+}, and Mg^{2+}), and metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, Zn, and Sb) (Parraga-Aguado et al. 2014). (See "Supplementary Material" for a full experimental procedure).

2.3. Elemental analysis in needle samples

About 500 mg of leaf-dried biomass (n=4 per site) were milled to powder in an analytical mill (IKA A11 basic). Samples were incinerated (550 ºC for 3 h) prior to adding 1 mL of concentrated HNO_3 (65%, Merck, Suprapur).. Digestion of the blank sample and reference materials (CTA-VTL-2 certified material, Virginia tobacco leaves) were carried out in the same way. Metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, Zn and Sb) were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500A) (Parraga-Aguado et al. 2014). Chloride, phosphate and sulfate were assessed by ion chromatography (Metrohm). Calcium, magnesium, potassium and sodium were determined by flame atomic absorption spectrometry (FAAS) using a Unicam 969 AA spectrometer. Nitrogen contents were measured on a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK).

2.4. Physiological status measurements

All the spectrophotometric determinations were done in quadruplicate using a 96-well plate reader (Multiskan GO, Thermo Scientific). 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 observed for all the methods

assayed. Four replicate samples were taken per each site and sampling date all the biochemical/physiological determinations were repeated three times. (See "Supplementary Material" for a full experimental procedure).

The evaluation of the physiological status of the Aleppo pine populations was carried out by measuring needle relative water content (RWC), photosynthetic pigment concentrations, total soluble protein levels, soluble sugars and starch contents as previously described (López-Orenes et al. 2017). Chlorophyll *a* (Chla), chlorophyll *b* (Chlb), and total carotenoids were extracted with 100% methanol and quantified as described earlier (Lichtenthaler and Wellburn 1983).

2.5. Total antioxidant activity determinations

The total antioxidant activity was assessed by three different methods, namely DPPH (2,2-diphenyl-1-picrylhydrazyl radical), $ABTS^{\bullet+}$ (*2,2*'-azino-bis(3-ethylbenzothiazoline-6-sulphonate), and FRAP (ferric reducing/antioxidant power) assays as previously described (Pérez-Tortosa et al. 2012). DPPH and ABTS radical scavenging activities were expressed as μmol of gallic acid equivalents (GAE) per gram fresh weight. FRAP antioxidant activity was expressed as μmol Fe(II) per gram fresh weight.

2.6. Determination of ascorbate, dehydroascorbate, glutathione, proline and total soluble non-protein thiols

The contents of ascorbate (AA) and dehydroascorbate (DHA) were determined using the α-α′-bipyridyl-based spectrophotometric assay (Gillespie and Ainsworth, 2007).

Reduced glutathione (GSH) levels were determined fluorimetrically using an *o*-phthalaldehyde probe (Senft et al., 2000). The concentration of proline (Pro) was determined by the acid-ninhydrin method (Bates et al., 1973), and the levels of total soluble non-protein thiols (NPT) were estimated using the Ellman's reagent (Metwally et al., 2003).

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

The determination of hydrogen peroxide was carried out by the ferrous ion oxidation–xylenol orange (FOX) method (Cheeseman, 2006). Superoxide anion radical concentrations were measured by the conversion of hydroxylamine into nitrite, and quantified spectrophotometrically at 540 nm after azo coupling with sulfanilamide and naphthylamine (Jiang and Zhang, 2001). Lipid peroxidation products were determined by measuring the concentration of malondialdehyde (MDA), as the end product of the lipid peroxidation process, using the thiobarbituric acid reactive method (Hodges et al., 1999). Protein oxidation was quantified by measuring the protein carbonyl content using the dinitrophenylhydrazine assay (Levine et al., 1994).

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

The concentration of total soluble phenolic compounds (TPC) was determined in needle methanolic extracts by the Folin-Ciocalteu method (Everette et al., 2010) using gallic acid as standard. The determination of total soluble flavonoids (FO) was carried out

according to the aluminum chloride assay using rutin as standard (Kim et al., 2003). Total flavanol content (FA) was assessed using the *p*-dimethylaminocinnamaldehyde (DMACA) reagent and (+)-catechin as a reference (López-Arnaldos et al., 2001). Total hydroxycinnamic acids (HCAs) were measured using the Arnow's reagent and caffeic acid as standard. The pellets of the methanol extracts were used for lignin determination using thioglycolic acid (TGA) using a standard curve with alkali lignin (López-Orenes et al., 2013). The content of cell wall-associated proanthocyanidins (PAs) was determined as described by Vermerris and Nicholson (2006).

2.9. Enzymatic assays

The extraction and assay of PAL (EC 4.3.1.24) and soluble and ionically-bound cell wall Class III peroxidase activities (EC 1.11.1.7; hydrogen donor: H_2O_2 oxidoreductase, PRXs) in needle samples were performed as previously described (López-Orenes et al., 2013). Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

2.10. Quantification of free and conjugated salicylic acid

Quantification of free salicylic acid (SA) and conjugated SA (SAG, 2-*O*-*β*-D-glucosylsalicylic acid) were performed using the SA biosensor strain *Acinetobacter* sp. ADPWl*ux* (Huang et al. 2006 and 2005) with some modifications (see "Supplementary Material" for a full experimental procedure).

2.11. Statistical analysis

Exploratory analysis of experimental data have been carried out by box-and-whisker plots to compare populations and to detect outliers. Normal probability plots were also made to analyze the normality of data. Soil data were subjected to a one-way ANOVA with site (Agustin, Mercader, Ripolles, Wikon and non mining-impacted forest) as factor, and when the differences were significant at $P \leq 0.05$, a Tukey's HSD post-hoc test was conducted to detect differences between means. The resulting P-values were adjusted using the Benjamini and Hochberg method for multiple comparisons. For the multivariate analysis, log-transformed data were scaled and mean-centered to avoid the effect of the scale on the measurements of the data Dimensionality reduction and classification methods such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), or random forest (RF) were applied to all data sets. Finally, a heatmap analysis, combined with an agglomerative hierarchical clustering, using the complete-linkage and a distance based on Spearman rank correlation coefficient, were performed to detect differences of the parameters measured in pine needles between mining and NM populations. The clustering methods were visualized by using dendrogram-graphs where the grouped data in the same branch represent similar data. All statistical analysis and graphs were performed using the R Statistical Programming Environment (https://www.R-project.org/). (See "Supplementary Material").

3. Results

3.1. Multivariate analysis of rhizosphere soil data

The results of soil analysis for each Aleppo pine population are given in Table 1. Soil

pH remained around neutral to slightly alkaline in all collected samples. Mining rhizosphere soils were characterized by sandy texture, lower TN and DOC contents, higher EC values [equivalent to 5-20 dS m^{-1} in saturated paste (Walker and Bernal, 2008)], and elevated contents of water extractable divalent ions (SO_4^{2-}, Ca^{2+}, and Mg^{2+}) and metal(loid)s (particularly As, Pb and Zn). Significant negative correlations were found between soil fertility parameters (TN and DOC) and both divalent ion concentrations and sand percentages ($-0.95 > r > -0.6$, $P<0.0001$; Supplemental Table S1). . Moreover, water extractable SO_4^{2-} and Ca^{2+} levels exhibited a strong correlation ($r=0.97$, $P< 0.0001$; Supplemental Table S1), which can be linked to the secondary formation of gypsum in the tailings as previously outlined (Parraga-Aguado et al. 2014). In general, monovalent ions (Cl^-, K^+, and Na^+) remained closer to the values found in control soils, except for Na in Agustin tailings Rhizosphere mining samples showed 15- to 40-fold higher levels of total metal(loid) concentrations than non-mining (NM) samples. Within mining soils, Ripolles and Wikon exhibited the highest levels of Cu, Pb and Zn, and the lowest levels of K^+ and Mg^{2+} when compared with Agustin and Mercader rhizosphere soils. Mercader soil showed relatively low metal concentrations and the highest TN and organic carbon contents (Table 1).

Results of PCA and PLS-DA revealed differences between non-mining and mining soils due to the first component (Supplemental Fig. S4). The plot also showed a clear clustering between Mercader and Agustin samples and, Ripolles and Wikon samples. These differences indicated close soil physicochemical similarities between samples sorted into the same cluster (Supplemental Fig. S4). The first component of both PCA and, which explained ~62 % of the total variance of the data set, was mainly influenced by soil fertility parameters (DOC and TN) and by metal(loid) soil concentrations

(mainly Pb, Zn, As, and Cu). The second component of both PCA and PLS-DA (~10% of total variance) was influenced by K and Na levels and by organic carbon content (OC) (Supplemental Fig. S4)

3.2. Needle ionome of Aleppo pine populations

PCA on needle ionome data revealed two principal components (PC) that together explained ~57% of the total variance (Fig. 1). Similar results were obtained when the data were analyzed using PLS-DA (Supplemental Fig. S5). The first PC, which explained ~41 % of the total variance, clearly separated NM from mining samples, and was mainly influenced by foliar Mg and S and by metal(loid) concentrations (Pb, Zn, As, and Cd). The scatterplot of the first two principal components provided a clear separation between mining samples in the different seasons, and the highest positive influence on the PC2 was given by monovalent ions. The main differences between seasons were associated with N, S and Ca in spring and Cl^-, Na^+ and K^+ in summer.

Foliar concentration of macronutrient contents in both NM and mining samples were below the normal average values reported for *P. halepensis* grown on non-polluted soils (Clarke et al., 2008) (Supplemental Table S2), indicating a low nutritional status of pine trees. Foliar concentrations of As, Mn, Ni, Pb, and Zn in mining needle samples were between 3-10-fold higher than those found in NM samples (Supplemental Tables S2 and S3). However, the accumulated metal(loid) levels were within the reported normal range for *Pinus* species (Clarke et al., 2008; Pratas et al., 2005), with the notable exception of Pb and Zn, which exceeded the critical threshold value of 5.59 and 77.55 $\mu g\ g^{-1}$, respectively, especially in spring needle samples (Supplemental Table S2).

3.3. Multivariate analysis of physiological and antioxidative/oxidative data in Aleppo pine needles

Physiological and antioxidative/oxidative data were subjected to multivariate statistical analysis. Firstly, a PCA was carried out, where the first two components explained ~50% of the total variance (Fig. 2). This plot showed a clear separation between spring and summer samples. The PCA also revealed evident clustering differences between NM samples, that were situated in the lower left-quadrant, and mining samples that were positioned in both the upper left- and the lower right-quadrant (Fig. 2). The main differences between NM and mining populations were associated with soluble phenolic compounds [flavanols (FA), flavonoids (FO), HCAs and total phenol content (TPC)], total antioxidant activities (FRAP, ABTS and DPPH), ascorbate (AA) and protein content. The main differences between seasons were associated with GSH, chlorophyll and sugar levels, proanthocyanidins (PAs) and soluble peroxidase activity (sPRX) in spring and carotenoids and needle relative water content (RWC) in summer.

Physiological and biochemical data were further processed by PLS-DA method. PLS-DA results were similar to those obtained using PCA (see Supplemental Fig. S6). PLS-DA provides a a quantitative estimation of the most influential parameters based on the variable importance in the projection (VIP). Variables with VIP scores >1 were identified as the most important markers according to their ability to discriminate among groups. In order to visualize at a glance the most significant markers, the correlation coefficients for the first two components of PLS-DA and VIP score were plotted. As seen in Figure 3A, markers with the VIP threshold >1 and with high

correlation with PLS-DA1, accounting for ~32% of the variation, were those related with plant growth performance (RWC, chlorophyll and sugar contents), antioxidant compounds (AA, GSH, FA, and PAs), DPPH radical scavenging activities, and soluble peroxidase activity (sPRX). Parameters highly correlated with PLS-DA2, accounting for ~18% of the variation, and with the VIP threshold >1 were malondialdehyde (MDA), used as a lipid oxidation marker, and again chlorophylls, proteins, antioxidant compounds (AA, HCAs), DPPH radical scavenging activities, and PRX activities.

Then, random forest (RF) algorithm was also used to identify important variables based on mean decrease in accuracy criterion. As shown in Fig. 3B the variables with the greatest effect (mean decrease in accuracy ≥20) were PAs, GSH, photosynthetic pigments, proline and RWC, followed by a set of 11 variables (mean decrease in accuracy ≥15) related with phenol metabolism [PRX activities, lignin, HCAs, flavanols (FA)], AA and ROS levels (H_2O_2, $O_2^{\bullet -}$), and lipid oxidation (MDA).

To determine in what extent seasonal changes affected the antioxidative/oxidative profile in the different Aleppo pine populations studied, the ratio values (mining/non-mining) were log2-transformed and a two-way complete-linkage hierarchical clustering was performed by using a distance defined in terms of Spearman rank correlation and represented in a heatmap (Figure 4). The statistical significance of fold changes was determined by a non-parametric Wilcoxon's test, and the mean values obtained are shown in Supplemental Table S5. The dendrogram showed a clear separation between spring and summer samples, which was already visible in the PCA analysis (see, Fig. 2), as well as a marked separation of Mercader samples from those of other populations, especially in spring. Strong separation of the Mercader samples was caused by an

increase in chlorophylls, and GSH and by the absence of change in total antioxidant activities, soluble phenols (TPC and FO), and iPRX activities (see Supplemental Table S4). Besides, there was a marked seasonal difference in total carotenoids/chlorophylls ratio among mining samples, particularly in Ripolles and Wikon samples (Supplemental Fig. S7). The dendrogram also revealed that Agustin summer samples were separated from those of Wikon and Ripolles. This separation can be caused by the reduction in the levels of photosynthetic pigments, although in spring these populations did not diverge and were clustered according to the year of sampling (Fig. 4).

Biochemical markers, illustrated in the rows of the heatmap in Fig. 4, were clustered into two large groups. The fist group was characterized by variables associated with the physiological status (photosynthetic pigments, RWC, sugars and starch contents; group 1, Fig. 4). The second group was characterized by markers related with phenol metabolism (HCAs, TPC, FA, FO, PRX and PAL activities), total antioxidant activities (FRAP, ABTS and DPPH), and AA content (group 2, Fig. 4). The heatmap also showed that the amplitude of changes in the biochemical markers clustered in the group 2 was larger than that of the group 1 during spring and summer seasons. These results indicated the important role of antioxidants and phenol metabolism in mining Aleppo pine populations (Fig. 4 and Supplemental Table S4). In fact, although a marked increase in the levels of DHA was noted particularly in spring (Fig. 4), all mining needles exhibited a high AA redox ratio [AA/(AA+DHA)] (Supplemental Fig. S6). This ratio is reported to be an important indicator of the redox status of the plant cell (Foyer and Noctor, 2016).

Finally, no general changes were observed in the endogenous levels of the stress-related phytohormone salicylic acid (SA) between NM and mining populations (Fig 4 and Supplemental Table S4), although a slight increase in SA levels was found in Mercader and Agustin summer samples. In general, conjugated SA (SAG) tended to decrease during the wetter spring period (*i.e.,* May 2013) and to increase during the wetter summer period (*i.e.,* September 2012).

4. Discussion

The present study aimed at evaluating the metabolic adjustments in response to the harsh conditions prevailing in mine-tailings under semi-arid Mediterranean conditions in four different mining Aleppo pine populations. Here, PCA and PLS-DA for rhizosphere soil data showed a clear separation between NM and mining soils. Within mining soils, the main differences were related with the levels of K^+, Na^+, Pb and Zn (Table 1 and Fig. S4). Moreover, PCA performed on needle ionome data discriminates between NM and mining populations, although cannot discriminate within mining pine populations (Fig. 1). Several studies have described that foliar ionome can be used as a rapid tool for biomonitoring HMMs contamination in highly polluted areas (Madejón et al., 2006, and refs. herein). Here, there were no good correlations between HMM concentrations in mining soils and their corresponding concentrations in pine needles (Fig. 1 and Table S1). These data are in agreement with those reported by Parraga-Aguado et al. (2014) for the same tree species within the same mining area. A plausible explanation for the lack of correlation between HMM concentrations in soils and needles could be related to the fact that Mediterranean Aleppo pine is well adapted to withstand drought because of its dimorphic root system (Voltas et al., 2015). In fact,

450 stable isotopes studies have supplied evidence of the ability of this species to acquire
451 mineral elements at different soil depth when surface soil layers become dry (Voltas et
452 al., 2015). Further investigation is needed to verify this assumption.
453
454 In our study, foliar concentrations of some nutrients (N, S, Mg, Mn and Ca) and
455 metal(loid)s were higher in spring than in summer (Fig. 1). The accumulation of these
456 nutrients can be related to the spring growth period of this species in the Mediterranean
457 area (Pacheco et al., 2017). An increase in the accumulation of K^+ was was observed in
458 summer needles. Potassium is the most abundant cation in plant cells, and plays a key
459 role in the water economy of plants, particularly by the maintenance of cell turgor
460 (Marschner, 1995; Shabala and Pottosin, 2014). Our results revealed a strong
461 correlation ($r > 0.8$, $P<0.0001$, Supplemental Table S1) between K levels and needle
462 RWC. Besides K^+, both foliar Na^+ and Cl^- levels also exhibited correlations with RWC
463 higher than 0.4 ($P< 0.0001$) (Supplemental Table S1). These results suggested that the
464 uptake of Na^+ together with the counterion Cl^- could also contribute to osmotic
465 adjustment during summer in mining samples. Aleppo pine is a moderately salt-tolerant
466 tree (Parraga-Aguado et al. 2014). There is extensive evidence that salt-tolerant species
467 could partially substitute K^+ for Na^+,, particularly under low K soils (Battie-Laclau et
468 al., 2014; Erel et al., 2014; Marschner, 1995),which are in line with our findings.
469 Despite the fact that soluble sugars and proline are considered key osmolytes
470 contributing to osmotic adjustment in stressed plants (Suzuki et al., 2014),, negative or
471 non-significant correlations between RWC and soluble sugars or proline were found
472 (Supplemental Table S1). These results suggest that these organic solutes appear to play
473 a minor role in the needle osmotic adjustment in Aleppo pine populations studied.
474 Osmotic adjustment via the accumulation of inorganic ions requires a lower energetic

475 cost. Therefore, the accumulation of inorganic ions can be considered an efficient
476 carbon-saving strategy for maintaining cell turgor during the dry Mediterranean
477 summers under conditions of reduced photosynthetic potential. The advantages to using
478 inorganic ions for cell osmotic adjustment under different stress conditions have been
479 previously reported in different plant species (Orsini et al., 2011; Shabala and Lew,
480 2002).
481
482 In general, the foliar concentrations of K, N, and P found in pine populations were
483 below the normal range reported for this species (Clarke et al., 2008) and lower than
484 those described for several populations of Aleppo pine grown under similar semi-arid
485 Mediterranean conditions (López-Serrano et al., 2005; Querejeta et al., 2008). These
486 results suggest that K, N, and P are likely to be growth-limiting factors in this species
487 and can be related to the nutrient-limited conditions of the tailings. In contrast, the foliar
488 amount of S accumulated in mining pine populations exceeded by more than twice the
489 normal range (Clarke et al., 2008) (Tables S2 and S3). S content was correlated with the
490 levels of S-rich molecules (*i.e.*, the non-protein thiol pool, $r>0.4$, $P<0.0001$, and GSH
491 content, $r>0.6$, $P<0.0001$, Supplemental Table S1) found in needles.
492
493 Actually, the examination of overall antioxidative/oxidative profile evidenced that
494 spring needles, which showed higher accumulation of metal(loid)s, were characterized
495 by high GSH levels. Interestingly, GSH was ranked as one of the most important
496 variables based on VIP score and mean decrease in accuracy criterion in RF analysis
497 (Fig. 3). Moreover, foliar HMM concentrations exhibited correlations with GSH higher
498 than 0.4 ($P< 0.0001$) (Supplemental Table S1). High GSH content is considered
499 essential for detoxification of HMMs (Hernández et al., 2015; Jozefczak et al., 2012).

Thus, taken together, these results suggest that thiol-mediated complexation could be an important mechanism of metal(loid) detoxification in Aleppo pines under these edaphoclimatic conditions .

Proline was also placed among the top-5 significant biomarkers in RF analysis (Fig. 3B). Extensive evidence suggests that proline is a potent non-enzymatic antioxidant and contributes to the stabilization of redox systems such as the GSH pool (Liang et al., 2013; Szabados and Savouré, 2010). Our results showed a good correlation between proline and GSH levels ($r > 0.58$, $P < 0.0001$, Supplemental Table S1), which is in line with previous results demonstrating that the up-regulation of proline increased the levels of reduced GSH (for review see, Liang et al. 2013), thus reinforcing the above proposed essential role of GSH in HMM detoxification mechanisms in Aleppo pine.

Spring mining needles were also typified by increased concentrations of total soluble phenols (TPC), HCAs, flavonoids, flavanols, and particularly flavan-3-ol polymers (PAs) (Fig. 1 and Fig. 4). It is well established that nutrient deficiency and HMM stress lead to the accumulation of phenolic compounds by altering phenol metabolizing enzymes such as PAL and PRX (Boudet, 2007; Dixon and Paiva, 1995; Gill and Tuteja, 2010; Jouili et al., 2011). The data presented confirm the induction of PAL activity in all mining samples (Fig. 4 and Table S4). In its turn, soluble PRX (sPRX) and cell wall-bound PRX (iPRX) activities showed a different pattern in Mercader as compared with the other mining samples (Fig. 4 and Table S4). In particular, sPRX activity increased in Mercader but decreased or unchanged in the other mining samples, whereas iPRX activity remained unaffected in Mercader but decreased in the other populations. Taken together, these results suggest that the lower levels of soluble phenolics (HCAs,

525 flavonoids, and TPC) accumulated in Mercader needles could be related with the
526 different behavior of PRX and PAL activities found in this populations. Moreover, it is
527 worth noting that the contents of secondary metabolites are affected by foliar N levels
528 (Fritz et al., 2006). Therefore, the lower levels of soluble foliar phenolics found in
529 Mercader samples can also be related with their higher foliar N concentrations.
530
531 Phenolics have been described to be efficient antioxidants (Rice-Evans et al., 1997).
532 Phenol compounds are able to chelate metals, quench lipid peroxidation, scavenge ROS
533 and to protect or recycle endogenous antioxidants, such α-tocopherol and ascorbate
534 (Agati et al., 2012; Michalak, 2006). These antioxidant properties of phenolics depend
535 on their structure, mostly on the number and position of hydroxyl groups bound to the
536 aromatic ring (Andejelkovic et al., 2006; Rice-Evans et al., 1997). In this way, the
537 presence of *o*-diphenol groups enhances the radical scavenging and metal-chelating
538 capacities of phenolic compounds. Increased accumulation of phenolics has previously
539 been described in *Pinus* species challenged with heavy metals (Kareolewki and
540 Giertych, 1995; Roitto et al., 2005). In our study, high correlations between phenolic
541 compounds and foliar accumulation of metal(loid)s were noticed. These results suggest
542 that phenolics could have a role in detoxification and accumulation of metal(loid)s by
543 functioning as chelating ligands. Noteworthy, the oligomeric phenolics PAs were
544 ranked as the most important biomarker in RF analysis (Fig. 3B). This fact, together
545 with the trend observed in seasonal accumulation in needles, with higher accumulation
546 in spring (Fig. 4), which coincided with higher foliar accumulation of HMMs (Table
547 S3), seems to assign a relevant role to PAs in Aleppo pine tolerance to harsh mine
548 tailings conditions.
549

Apart from GSH and phenolics, mining needles also showed higher antioxidant capacity, estimated by ABTS, DPPH and FRAP tests. Higher AA levels and a high AA redox ratio were also observed in these samples (Fig. 4 and Fig. S8). The increase in the levels of AA, GSH, proline, and phenolics seemed to be effective in controlling $O_2^{\bullet -}$ and H_2O_2 levels in both seasons. In fact, a reduction in protein oxidation, measured as protein carbonyl content, was observed. Nevertheless our data also revealed a certain degree of lipid peroxidation in foliar mining samples in both seasons, as compared with NM ones. This lipid peroxidation appeared not to have a significant detrimental effect on photosynthetic metabolism, as evidenced by the higher starch concentrations found in mining samples (~300 μg g^{-1} FW) compared to NM ones (~250 μg g^{-1} FW). In this regard, it is important to highlight both the marked increase in the carotenoid content, particularly in spring, and the decrease in the levels of chlorophylls in summer mining samples. It is well established that carotenoids have a dual role in plants as both accessory light-harvesting pigments and photoprotective molecules by quenching triplet chlorophyll, singlet oxygen ($^1O_2$) and other free radicals (Young, 1991). Chlorophylls in the triplet excited state can lead to the formation of $^1O_2$, especially under high irradiance as well as under stress conditions that induce closing of stomata (Gill and Tuteja, 2010). Thus, the increase observed in the ratio of total carotenoids to chlorophylls, especially in summer and in Ripolles and Wikon samples, can be considered an adaptation strategy to prevent irreversible damage to the photosynthetic apparatus. The increase in the ratio carotenoids/chlorophylls can contribute, on the one hand, to reduce light absorption and $^1O_2$ formation, and on the other hand, to increase the photoprotection capacity of needles under these drastic concurrent stressful conditions, as previously outlined (Haldimann, 1998). It is worthy to mention that the low levels of chlorophylls found in Agustin summer needles can be related to the particularly low levels of N found in these

samples. This view is reinforced by the trend observed in Mercader samples, whose N levels were the highest in all populations and also showed the highest levels of chlorophylls.

Finally, as water scarcity is characteristic in semi-arid Mediterranean regions, it is not surprising to found that rainfalls favored Aleppo pine growth. In fact, several studies have highlighted that long-lived tree species from Mediterranean climates are plastic enough to cope with erratic rainfall patterns (Camarero et al., 2010).

5. Conclusions

Our results revealed a strong seasonality in needle ionome and antioxidative/oxidative profiles that correlates with the seasonal variation of growth rate in Mediterranean Aleppo trees. Mining samples exhibited higher contents of antioxidative metabolites (AA and soluble phenols) than NM ones in the two seasons studied. The associated higher antioxidant activity could contribute to prevent oxidative injury induced by the higher concentrations of HMM found in these populations. Moreover, the magnitude of the changes in the physiological and antioxidative/oxidative parameters was more pronounced in spring samples, coinciding with the period of active growth of Mediterranean Aleppo pine. Despite the fact that spring needles accumulated higher levels of HMMs, particularly Pb and Zn, pine performance seem not to be affected. Data analysis assigned to PAs and GSH a key role in spring needle metabolism. This could be related to the involvement of these compounds in HMM detoxification mechanisms in Aleppo pines. During summer, needles maintained high RWC through

599 the accumulation of inorganic ions and increased photoprotection capacity what seem to
600 be critical for tree acclimation to the dry season.
601 Predicted precipitation scenarios in the Mediterranean basin within the context of
602 climate change, with significant rainfall reductions, would lead to a shift in the
603 metabolic behavior of Aleppo pines growing on mine tailings. The present study points
604 to future reductions in photosynthetic capacity of pine populations due to the more
605 prolonged dry periods.
606
607
608 **Acknowledgements**
609 This work was supported by the Ministerio de Ciencia e Innovación [grant number
610 CTM2011-23958]; Ministerio de Economía, Industria y Competitividad [grant number
611 CGL2014-54029-R]; Ministerio de Ciencia y Tecnología [grant number CGL2006-
612 11569]; and Fundación Séneca [grant number FB/23/FS/02]. AL-O holds a grant from
613 the Ministerio de Educación Cultura y Deporte [grant number AP2012-2559]. Part of
614 this work was carried out at the Instituto de Biotecnología Vegetal, UPCT.
615
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853 **Figure legends**

854

855 **Figure 1.** Principal component analysis based on correlation matrix applied to needle
856 ionome data of non-mining (NM) and mining Aleppo pine populations, in late spring
857 and summer in 2012 and 2013. Circles represent r^2 = 50% and 100% variability
858 explained by the components. Population codes: NM, filled black color; Agustin, filled
859 red color; Mercader, unfilled red color; Ripolles, filled blue color; and Wikon, unfilled
860 blue color. Season and year codes: May 2012, squares; September 2012, circles; May
861 2013, triangles; September 2013, inverted triangles.

862

863 **Figure 2.** Score (left) and correlation (right) plots of the first two components of the
864 PCA applied to physiological and biochemical variables measured in needles of Aleppo
865 pine trees growing in non-mining (NM) and mining tailings pile (Agustin, Mercader,
866 Ripolles and Wikon) in late spring and summer in 2012 and 2013. Circles represent r^2 =
867 50% and 100% variability explained by the components. For population, season and
868 year codes, see legend to Figure 1. Abbreviations: AA, ascorbate; ABTS, 2,2′ -azino-
869 bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity; Car, total
870 carotenoids; Chla, Chlorophyll *a*; Chlb, chlorophyll *b*; C=O, protein carbonyl group
871 content; DHA, dehydroascorbate; DPPH, 1,1-Diphenyl-2-picrylhydrazyl radical
872 scavenging activity; FA, total flavanols; FO, total flavonoids; FRAP, ferric
873 reducing/antioxidant power; iPRX, ionically-bound cell wall class III plant peroxidase
874 activity; H_2O_2, hydrogen peroxide; HCAs, hydroxycinnamic acids; MDA,
875 malondialdehyde; NPT, total soluble non-protein thiols; $O_2^{\cdot-}$, superoxide radical; PAL,
876 phenylalanine ammonia-lyase activity; PAs, cell wall-associated proanthocyanidins;
877 Pro, proline; RWC, relative water content; SA, salicylic acid; SAG, 2-*O*-*β*-D-

878 glucosylsalicylic acid; sPrx, soluble class III plant peroxidase activity; TPC, total
879 phenol content.
880
881 **Figure 3**. Identification of the most influential physiological and biochemical
882 biomarkers based on the variable importance in the projection (VIP) and the correlation
883 coefficients for the first three components of PLS-DA (**A**) and on mean decrease in
884 accuracy estimated by random forest machine learning algorithm (**B**). For abbreviations,
885 see legend to Figure 2.
886
887 **Figure 4.** Heatmap and complete-linkage hierarchical clustering (by using a distance
888 based on Spearman rank correlation coefficient) showing the seasonal fold change
889 (mining vs. non-mining) of the physiological and biochemical parameters measured in
890 needles of Aleppo pine trees growing in non-mining and in mining tailings piles in late
891 spring and summer in 2012 and 2013. Log2 ratios of fold changes relative to each
892 respective control group are given by shades of red or blue colors according to the scale
893 bar. For abbreviations, see legend to Figure 2.
894

Supporting information

Table S1. Pearson's r correlation coefficients among rhizosphere soil parameters, foliar accumulated metal(loid)s and biochemical biomarkers measured in minings (Mercader, Agustin, Ripolles and Wikon) and non-mining Aleppo pine trees. Asterisks indicate statistical significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

Table S2. Macronutrient and metal(loid) concentrations in needles from non-mining (NM) and mining (Agustin, Mercader, Ripolles and Wikon) samples collected in both late spring and summer in 2012 and 2013. Data represented mean ± SE (*n*=4). Values above the normal range are shown in bold; values below the normal range are shown in bold and italics

Table S3. Mean ratio of fold changes and their associated *P*-values obtained by the t-student test ($P < 0.05$; $n = 4$) of macronutrient and metal(loid) contents in Aleppo pine needles. 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 S4. Physiological and biochemical parameters measured in needles from non-mining (NM) and mining (Agustin, Mercader, Ripolles and Wikon) Aleppo pine trees collected in both late spring and summer in 2012 and 2013. Data represented mean ± SE (*n*=4).

Table S5. 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 Aleppo needles. 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.

Figure S1. Geographical location of the study sites in the Cartagena-La Unión Mining District (Murcia, Spain). The different mine tailings piles (Agustin, Mercader, Ripolles and Wikon) and the control (non-mining) 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 five populations of *P. halepensis* trees growing in a non-mining area (NM) and in mine tailings piles (Agustin, Mercader, Ripolles and Wikon) in late summer (upper panel). Images of the branches collected from each Aleppo pine populations, with current and one year-old flushes, (middle panel). Images of the one year-old needles used in this study from the five Aleppo pine populations (lower panel).

Figure S4. Score (A and C) and correlation (B and D) plots of the first two components of both Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) applied to soil parameters from both mining (Agustin, Mercader, Ripolles and Wikon) and non-mining (NM) samples. Sample codes: NM, filled black

946 circle; Agustin, unfilled circle; Mercader, unfilled square; Ripolles, unfilled triangle;
947 and Wikon, unfilled inverted triangle. Abbreviations: DOC, dissolved organic carbon;
948 EC, electrical conductivity; OC, organic carbon content; TN, total nitrogen.
949
950 **Figure S5.** Score (left) and correlation (right) plots of the first two components of the
951 Partial Least Squares-Discriminant Analysis (PLS-DA) based on correlation matrix
952 applied to needle ionome data of non-mining (NM) and mining Aleppo pine
953 populations, in late spring and summer in 2012 and 2013. Circles represent r^2 = 50%
954 and 100% variability explained by the components. For population, season and year
955 codes, see legend to Figure 1.
956
957 **Figure S6.** Score (left) and correlation (right) plots of the first two components of the
958 PLS-DA applied to physiological and biochemical variables measured in needles of
959 Aleppo pine trees growing in non-mining (NM) and mining tailings pile (Agustin,
960 Mercader, Ripolles and Wikon) in late spring and summer in 2012 and 2013. Circles
961 represent r^2 = 50% and 100% variability explained by the components. For population,
962 season and year codes, see legend to Figure 1. For abbreviations, see legend to Figure 2.
963
964 **Figure S7.** Seasonal variations in the ratio of total carotenoids/chlorophylls in needles
965 of Aleppo pine trees growing in non-mining (NM) and mining tailings piles (Agustin,
966 Mercader, Ripolles and Wikon) in late spring and summer in 2012 and 2013. Values are
967 expressed as box-and-whisker plots. The box represents the interquartile range (IQR),
968 the bold line in box the median, the whiskers represent 1.5 times the IQR, and the single
969 dots (•) represent outlier points.
970

Figure S8. Seasonal variations in the ascorbate redox state in needles of Aleppo pine trees growing in non-mining (NM) and mining tailings piles (Agustin, Mercader, Ripolles and Wikon) in late spring and summer in 2012 and 2013. Values are expressed as box-and-whisker plots. The box represents the interquartile range (IQR), the bold line in box the median, the whiskers represent 1.5 times the IQR, and the single dots (•) represent outlier points.