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*Expression patterns of antioxidant network in
pioneer plants from mine tailings polluted with
high levels of heavy metals*

*Técnicas Avanzadas en Investigación y Desarrollo Agrario y
Alimentario*

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**CONFORMIDAD DE SOLICITUD DE AUTORIZACIÓN DE DEPÓSITO DE
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D/D^a. Antonio A. Calderón García Director/a de la Tesis doctoral “Expression patterns of antioxidant network in pioneer plants from mine tailings polluted with high levels of heavy metals”.

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por D/D^a. Antonio López Orenes, dentro del Programa de Doctorado Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario, dando mi conformidad para que sea presentada ante el Comité de Dirección de la Escuela Internacional de Doctorado para ser autorizado su depósito.

La rama de conocimiento en la que esta tesis ha sido desarrollada es:

- ☒ Ciencias
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En Cartagena, a 11 de Diciembre de 2017

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El presente trabajo de investigación ha sido realizado en el Departamento de Ciencia y Tecnología Agraria de la Escuela Técnica Superior de Ingeniería Agronómica de la Universidad Politécnica de Cartagena con la ayuda del Ministerio de Educación Cultura y Deporte (AP2012-2559). Esta Tesis Doctoral se ha realizado bajo la financiación de los siguientes proyectos:

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Artículos originales de investigación derivados de la Tesis

1. **Antonio Lopez-Orenes**, Ascension Martínez-Perez, Antonio A. Calderon, María A. Ferrer (2014).

Pb-induced responses in *Zygophyllum fabago* plants are organ-dependent and modulated by salicylic acid. *Plant Physiology and Biochemistry*, 84, 57-66.

2. Isabel Párraga-Aguado, Maria-Nazaret González-Alcaraz, **Antonio López-Orenes**, María A. Ferrer-Ayala, Héctor Miguel Concesa (2016).

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3. **Antonio López-Orenes**, María C. Bueso, Héctor M. Conesa, Antonio A. Calderón, María A. Ferrer (2017).

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9. María M. Lajara, **Antonio Lopez-Orenes**, María A. Ferrer, Antonio A. Calderon (2015).

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- 1. Antonio López-Orenes, Isabel Párraga-Aguado, Héctor M. Conesa, Antonio A. Calderón, María A. Ferrer (2013).**
Analysis of components of the antioxidant systems in a pioneer plant species growing in heavy metal-polluted mine tailings.
XIII Congreso Luso-Español de Fisiología Vegetal.
- 2. López-Orenes A, Ferrer MA, Calderón AA. (2013)**
Patrones de expresión de sistemas antioxidantes en plantas pioneras presentes en balsas de estériles contaminadas con altas concentraciones de metales pesados.
II Workshop en Investigación Agroalimentaria.
- 3. Héctor M. Conesa, Jose-Ignacio Querejeta, Antonio López-Orenes, María Nazaret González-Alcaraz, María A. Ferrer, Antonio A. Calderón, Isabel Párraga-Aguado (2014).**
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- 4. López-Orenes A, Ferrer MA, Calderón AA. (2014)**
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III Workshop en Investigación Agroalimentaria - WiA14. Cartagena.
- 5. Antonio López-Orenes, Isabel Párraga-Aguado, Héctor M. Conesa, Antonio A. Calderón and María A. Ferrer (2015).**
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Resumen

La intensa actividad minera llevada a cabo en el distrito minero de Cartagena-La Unión durante más de dos mil quinientos años ha generado un gran volumen de residuos, muchos de los cuales se acumulan en balsas de estériles. Aunque la mayoría de estas balsas carecen de vegetación, en determinadas zonas proliferan ciertas especies pioneras que están sometidas a una exposición crónica a altas concentraciones de diversos metales pesados y metaloides. Por su carácter pionero y su tolerancia a metales, estas especies presentan un gran potencial en programas de fitomanejo encaminados a evitar la dispersión de contaminantes mediante el establecimiento de una cubierta vegetal estable y autosostenible a largo plazo. La colonización por especies vegetales de las balsas mineras de la Sierra de Cartagena no sólo está condicionada por la presencia de concentraciones fitotóxicas de metales, bajos niveles de nutrientes esenciales y alta salinidad, sino también por las condiciones de sequía y los altos niveles de radiación solar que son característicos de climas semiáridos. El estudio de las plantas pioneras que colonizan estas estructuras ofrece, por lo tanto, la oportunidad de encontrar mecanismos adaptativos a condiciones de estrés múltiple que permitan dar un salto cualitativo en la aplicación de las técnicas de fitomanejo. La presente Tesis Doctoral se centró, por un lado, en el estudio de sistemas antioxidantes, así como de diversos marcadores de estrés oxidativo y de otros parámetros relacionados con el estrés en tres especies de plantas pioneras crecidas de forma espontánea en balsas de estériles; dos de ellas herbáceas (*Zygophyllum fabago* y *Dittrichia viscosa*) y una arbórea (*Pinus halepensis*). Teniendo en cuenta el marco climático semiárido de este distrito minero, los análisis se realizaron tanto en primavera como en verano en dos años consecutivos (2012 y 2013). Por otro lado, considerando que esta zona minera contiene altos niveles de Pb, se estudió en condiciones controladas de laboratorio el efecto de la exposición, tanto aguda como crónica, a este metal en plantas de *Z. fabago*, con el fin de determinar los mecanismos de tolerancia que esta especie pone en marcha para contrarrestar la toxicidad del Pb.

En el primero de los estudios, los análisis multivariantes realizados mostraron claras diferencias en el patrón de expresión de los sistemas antioxidantes en las tres especies crecidas en las balsas en relación con plantas control (localizadas fuera de la zona degradada por la actividad minera, pero a escasa distancia de las poblaciones estudiadas). Entre especies, se registraron los mayores cambios en pino y en *Z. fabago*. En *D. viscosa*,

que mostró una gran capacidad de co-acumulación de Zn y Pb en hojas fotosintéticamente activas, no se observaron cambios significativos ni en la capacidad antioxidante ni en los niveles de ascorbato y glutatión. Una característica común en las tres especies estudiadas fue el mantenimiento del contenido hídrico relativo (RWC), incluso en el verano más seco (2013), si bien pudieron observarse diferencias entre especies en lo que respecta al tipo de osmorreguladores acumulados (compuestos orgánicos en *D. viscosa* y *Z. fabago* e iones en *P. halepensis*). En relación con el metabolismo fotosintético, éste no se vio afectado en plantas de *D. viscosa*, pero sí se observó una disminución en el contenido de clorofilas en pino y en *Z. fabago* en verano, hecho que puede considerarse como una estrategia de adaptación para reducir la absorción de luz y la formación de oxígeno singlete. Así mismo, en las tres especies estudiadas se observaron diferencias en el patrón de acumulación de compuestos fenólicos, así como cambios relevantes en las actividades de las enzimas fenilalanina amonio liasa (PAL) y peroxidasas (PRXs), tanto solubles como ligadas a las paredes celulares, lo que indica que el metabolismo fenólico desempeña un importante papel en la adaptación de estas especies a condiciones de estrés múltiple. Un análisis más detallado del tipo de compuestos fenólicos implicados en los mecanismos de tolerancia de cada una de las especies estudiadas permitió observar, como resultados más destacados, un incremento en los niveles de flavonoides en *Z. fabago*, de proantocianidinas (PAs) en pino y de ácido cafeico en *D. viscosa* en las poblaciones de las balsas mineras con respecto a sus correspondientes controles. Es importante señalar que, además de sus notables propiedades antioxidantes, los compuestos mencionados presentan una gran capacidad quelante de metales y el hecho de que, al menos en las dos últimas especies señaladas, estos fenoles aparecieran en fracciones obtenidas de paredes celulares, permiten asignar a este compartimento subcelular un papel relevante en la atenuación de la toxicidad asociada a altas concentraciones de metales pesados.

La segunda serie de estudios comprendidos en esta Tesis se llevó a cabo en condiciones controladas de laboratorio y utilizando plantas de *Z. fabago*. Uno de estos estudios se realizó con plántulas de 3 semanas, procedentes de semillas recolectadas en el pantano minero Mercader, tratadas con 0,5 mM de Pb durante una semana. En estas condiciones se observó una clara reducción en el crecimiento, así como en la actividad enzimática rubisco, fotosíntesis neta (A), eficiencia intrínseca en el uso del agua (A/gs), niveles de clorofila *a* y *b*, azúcares solubles y niveles de P, K y Zn. Además, los vástagos mostraron una menor capacidad antioxidante, mayores niveles de radical superóxido ($O_2^{\bullet-}$) y mayor

pérdida de electrolitos como consecuencia del daño oxidativo a las membranas celulares. Por el contrario, en las raíces se observaron menores niveles de Mn y Zn, una mayor capacidad antioxidante y menor pérdida de electrolitos, a pesar del aumento en los niveles de H_2O_2 y en el grado de oxidación de macromoléculas. Estos resultados evidencian la puesta en marcha de una respuesta antioxidante diferente en raíces y vástagos. Asimismo, la disminución de la actividad fotosintética y del crecimiento observados en el vástago pueden interpretarse como parte de una respuesta coordinada de la planta que resulta en una solución de compromiso entre crecimiento y defensa frente a la situación de estrés. El conjunto de cambios metabólicos y fisiológicos que experimenta la planta permitiría destinar mayores recursos para potenciar las respuestas defensivas, especialmente allí donde la intensidad del estrés es mayor (raíces) y contrarrestar los efectos tóxicos del Pb en detrimento del crecimiento.

Paralelamente, se evaluó el efecto del pretratamiento con un elicitor, el ácido salicílico (SA), sobre la respuesta antioxidante en plantas de *Z. fabago* tratadas con plomo. Para ello, utilizamos plántulas de 4 semanas, procedentes de semillas recolectadas en el pantano minero El Lirio, tratadas con 0,75 mM Pb durante una semana, en condiciones de hidroponía. Los resultados mostraron que, en general, todos los órganos ensayados, raíz, tallo y hojas, responden al tratamiento con Pb aumentando los niveles de ascorbato (AA) y GSH y manteniendo altos niveles de las formas reducidas de GSH y AA gracias a la actividad de la enzima deshidroascorbato reductasa (DHAR). Además, se observaron diferencias en el patrón de acumulación de prolina y de alcaloides carbolínicos en hojas y raíces que, podrían desempeñar un papel importante en los mecanismos de adaptación al Pb. Los pretratamientos con SA incrementaron de forma significativa la actividad superóxido dismutasa (SOD) en todos los órganos y estimularon la acumulación inducida por el Pb de prolina en las raíces y de alcaloides carbolínicos en los vástagos. Asimismo, el SA redujo la toma y la acumulación de Pb, sobre todo en los vástagos, lo que, desde un punto de vista aplicado, podría ser explotado en los programas de restauración de suelos degradados, con el fin de reducir el riesgo de entrada de este metal en la cadena trófica.

Finalmente, con el fin de evaluar si existen diferencias en los mecanismos adaptativos implicados en contrarrestar los efectos tóxicos del Pb entre poblaciones metalíferas (Agustín y Mercader) y no-metalíferas (NM) de *Z. fabago*, se realizó un experimento de exposición crónica, en el cual, semillas procedentes de las tres poblaciones fueron

expuestas a dosis sub-letales de $\text{Pb}(\text{NO}_3)_2$ (0, 25 y 50 μM) durante 30 días. El perfil ionómico de las semillas estuvo en consonancia con el de las condiciones edáficas de cada población, aunque también se evidenció la puesta en marcha de estrategias específicas para garantizar un suministro adecuado de macro- y micronutrientes en los tejidos de almacenamiento de las semillas, dado el bajo contenido en nutrientes presentes en las balsas de estériles mineros. Las plántulas procedentes de semillas metalíferas tuvieron, constitutivamente, niveles más altos de metabolitos implicados en rutas antioxidativas, en concreto, AA, carotenoides y fenoles solubles totales. Además, estas plántulas respondieron de forma más rápida y eficiente a la exposición al Pb, presentando, en consecuencia, un mejor desarrollo que fue más evidente en las plántulas de Mercader. El análisis de la expresión relativa de los genes que codifican para catalasa y ascorbato peroxidasa corroboraron la mayor efectividad en la eliminación de especies reactivas del oxígeno (ROS) de las poblaciones metalíferas. Curiosamente en estas poblaciones, el fortalecimiento de las rutas antioxidativas mostró un patrón diferente que, probablemente, podría estar determinado por las condiciones del suelo. De hecho, los niveles relativamente altos de Pb encontrados en las semillas de las poblaciones metalíferas podrían tener un efecto estimulante (“priming”) que daría lugar a una rápida activación de las respuestas defensivas, lo que, a su vez, permitiría contrarrestar los efectos tóxicos de la exposición al Pb. Aunque para confirmar esta hipótesis se requieren estudios más específicos, este trabajo proporciona un nuevo enfoque para comprender cómo las plantas se enfrentan al estrés mediado por metales pesados.

Abstract

The intense mining activity carried out in the mining district of Cartagena-La Unión for more than two thousand five hundred years has generated a large volume of mine tailings. Although most of these mine tailings remain unvegetated, in certain areas some pioneer species proliferate even though they are chronically exposed to high concentrations of various heavy metals and metalloids. Owing to their pioneer characteristic and their heavy metal tolerance, these species have a great potential in phytoremediation programs aimed at avoiding the dispersion of contaminants through the establishment of a stable and self-sustainable plant cover. The colonization by plant species into mine tailings in semi-arid climates is not only conditioned by the presence of phytotoxic concentrations of metals, low levels of essential nutrients and high salinity, but also by drought conditions and the high sunlight irradiance that are characteristic of these climatic areas. The study of the pioneer plants able to colonize these degraded zones offers the opportunity to find adaptive mechanisms under multi-stress scenarios that allow a qualitative leap in the application of phytomanagement techniques. The present PhD Thesis was focused, on the one hand, on the study of antioxidative response mechanisms, as well as on various oxidative stress markers in different pioneer plants species both herbaceous (*Zygophyllum fabago* and *Dittrichia viscosa*) and woody (*Pinus halepensis*) ones. Taking into account the semiarid climate framework of this mining district, the analyses were conducted both in spring and summer in two consecutive years (2012 and 2013). On the other hand, considering that this mining area contains high levels of Pb, the effect of both acute and chronic exposure to this metal in *Z. fabago* plants was studied under controlled laboratory conditions in order to determine the tolerance mechanisms that this species used to counteract Pb toxicity.

In plants grown in mine tailings, in relation to non-mining ones (control, located outside the degraded mining area, but close to the mining populations studied), the multivariate analysis showed clear differences in the expression pattern of the antioxidative systems among the three plant species analyzed, the greatest changes in the evaluated parameters being observed in pine and *Z. fabago* plants. In *D. viscosa*, which exhibited a high Zn and Pb co-accumulation capacity in photosynthetically active leaf tissues, no significant changes were observed in the antioxidant capacity nor in the levels of ascorbate and glutathione. A common feature in these pionner species was the maintenance of leaf

RWC even in the driest summer (2013), although changes were observed in the type of accumulated osmoprotectants (organic compounds in *D. viscosa* and *Z. fabago* and ions in *P. halepensis*). Photosynthetic metabolism was not affected in *D. viscosa* plants, but in pine and *Z. fabago* plants a decrease in the chlorophyll content was observed in summer, which can be considered as an adaptation strategy to reduce light absorption and $^1\text{O}_2$ formation. There were differences in the pattern of accumulation of phenolic compounds as well as relevant changes in the activities of the enzymes PAL and soluble and ionically-bound cell wall PRXs, suggesting that in these species phenylpropanoid metabolism plays a key role in the adaptive mechanisms to stress combinations. More specifically, an increase in the levels of flavonoids in *Z. fabago*, proanthocyanidins (PAs) in pine and caffeic acid in *D. viscosa* were observed in mining populations in comparison to non-mining ones. It is important to note that, in addition to their remarkable antioxidant properties, the aforementioned compounds have a great metal-chelating capacity and the fact that, at least in the last two species mentioned, these phenols appeared in fractions obtained from cell walls, suggests that this subcellular compartment plays an important role in attenuating the toxicity associated with high concentrations of heavy metals.

Under controlled laboratory conditions and using 3-week-old *Z. fabago* seedlings, from seeds collected in Mercader mine tailings, and treated with 0.5 mM Pb for one week, a clear reduction in growth was observed, as well as a drop in the enzymatic activity of rubisco, net photosynthesis rate (A), intrinsic WUE (A/g_s), chlorophyll contents, soluble sugars, and Zn, K and P levels. Shoots showed less antioxidant capacity, higher levels of $\text{O}_2^{\bullet-}$ and higher loss of cell membrane integrity. In its turn, roots had less Mn and Zn levels, and despite increases of H_2O_2 levels and biomolecules oxidation, they presented a robust antioxidant capacity which may support the observed cell membrane integrity. These results show that the antioxidant response in roots and shoots is different and the decrease in the photosynthetic activity and plant growth could be interpreted as a growth-defense trade-off towards a stress situation. The metabolic and physiological changes associated with this strategy would facilitate resource reallocation to enhance defensive responses, especially in roots where stress intensity is greater, in order to counteract the toxic effects of Pb in detriment of plant growth.

Concurrently, the effect of pretreatment with an elicitor, salicylic acid (SA), on the antioxidant response in *Z. fabago* plants was also evaluated. In this case, 4-week-old *Z. fabago* seedlings from seeds collected in El Lirio mine tailings were used. The seedlings were treated with 0.75 mM Pb for one week under hydroponics conditions. The results showed that in general, all organs (root, stem and leaf) reacted to Pb stress by increasing AsA and GSH concentrations and by maintaining a highly reduced GSH and AsA pools supported by DHAR. Moreover, the different organ accumulations of Pro and β -carboline alkaloids seem to play an important role in the adaptation to Pb stress. Pretreatment with SA had a significant effect on SOD activities in all organs and enhanced the Pb-induced accumulation of Pro in roots and alkaloids in shoots. In addition, SA inhibited Pb uptake and accumulation, especially in shoots. From a practical point of view, this finding could be exploited in land reclamation programs to reduce the risk of Pb entrance to food chains.

Finally, in order to evaluate whether there are differences in the adaptive mechanisms involved in counteracting Pb toxicity between metalliferous (Agustín and Mercader) and non-metalliferous (NM) *Z. fabago* populations, a chronic exposure experiment was conducted. To this end, seeds were collected from the three populations and germinated in controlled conditions in the presence of sub-lethal doses of $\text{Pb}(\text{NO}_3)_2$ (0, 25 and 50 μM) for 30 d. Ionic profiles of the seeds from NM and metallicolous populations developed in their natural environments were in line with the different edaphic conditions. However ionic profiles also indicated that the three populations carried out specific strategies in order to guarantee the appropriate supply of macro- and micronutrients in seed-storing tissues, in spite of the nutrient deficiencies present in mine soils. Metallicolous seedlings had constitutively higher levels of metabolites involved in antioxidative pathways, in particular ascorbate, carotenoids and total soluble phenols, and also responded more promptly and efficiently to chronic-Pb stress than NM seedlings and, hence M plants showed better performance and fitness, in particular those developed from seeds collected in Mercader mine tailings. The analyses of catalase and ascorbate peroxidase gene expression also supported a more efficient ROS-scavenging capability in M than in NM populations. Interestingly, in metallicolous populations, the strengthening of the antioxidative pathways followed different patterns that seem to be determined by soil conditions. In fact, the relatively high levels of Pb found in M seeds could act as a

priming stimulus, which facilitated a rapid response to counteract the toxic effects of chronic Pb exposure. Although more studies are needed to confirm this finding, this work provides a new approach to understanding how plants cope with heavy metal stress.

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CHAPTER 9: REFERENCES

Abbreviations

A , net CO ₂ assimilation rate	MDA , malondialdehyde
ABTS , 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate	MDHA , monodehydroascorbate
AG , Agustin (mine tailing)	MDHAR , monodehydroascorbate reductase
APX , ascorbate peroxidase	ME , Mercader (mine tailing)
AsA , ascorbic acid	NM , non mining (control sites)
C=O , carbonyl groups	NPT , soluble non-protein thiols
CAT , catalase	O₂^{•-} , superoxide radical
Chl , chlorophylls	PAL , phenylalanine amonio liase
Chla , Chlorophyll <i>a</i>	PAs , proanthocyanidins
Chlb , chlorophyll <i>b</i>	PC , phytochelatins
CO , control site, non mining	PCA , principal component analysis
CWP , cell wall-bound phenols	PLS-DA , partial least squares-discriminant analysis
DHA , dehydroascorbate	Pro , proline
DHAR , dehydroascorbate reductase	PSII , photosystem II
DPPH , 1,1-Diphenyl-2-picrylhydrazyl	RF , random forest
DW , dry weight	RI , Ripolles (mine tailing)
FA , total flavanols	ROS , reactive oxygen species
FO , total flavonoids	RWC , relative leaf water content
FRAP , Ferric Reducing Antioxidant Power	SA , salicylic acid
FW , fresh weight	SAG , 2-O-b-D-glucosylsalicylic acid
GR , glutathione reductase	SE , S , September (month)
GSH , glutathione	SOD , superoxide dismutase
GSSG , oxidized glutathione	sPRX , soluble peroxidase
H₂O₂ , hydrogen peroxide	TAA , total antioxidant activity
HCAs , hydroxycinnamic acid	TBA , thiobarbituricacid
HM , heavy metal	TBARS , thiobarbituric acid-reacting substances
HMMs , heavy metals/metalloids	TPC , total phenol content.
iPRX , ionically-bound cell wall peroxidase	VIP , variable importance in the projection
MA , M , May (month)	WI , Wikon (mine tailing)

1. INTRODUCTION

1.1. Heavy metals and their impact in the ecosystems

Heavy metals (HM) represent an ill-defined group of inorganic elements, with density higher than 5.0 g cm^{-3} , and they can be classified as either essential or non-essential (Wuana and Okieimen, 2011). Essential HMs are those needed by organisms for biological functions (*i.e.*, Fe, Mn, Cu, Zn, and Ni), whereas non-essential heavy metals are those not needed for any biological function (*i.e.*, cadmium (Cd), chromium (Cr), mercury (Hg), lead (Pb), aluminium (Al), silver (Ag), tin (Sn), among others) (Ali *et al.*, 2013).

In general, non-essential HMs are important environmental pollutants, particularly in areas where there is a high anthropogenic pressure (Wuana and Okieimen, 2011). Their presence in the atmosphere, soil and water, even in trace concentrations, can cause serious problems to both aquatic and terrestrial ecosystems, and HM bioaccumulation in the food chain can be highly dangerous (Sanità Di Toppi and Gabbrielli, 1999; Järup, 2003; Peralta-Videa *et al.*, 2009). This is because unlike organic contaminants, which can be oxidized to carbon (IV) oxide by microbial action, most metals do not undergo microbial or chemical degradation, and their total concentration in soils persists for a long time after their introduction (Wuana and Okieimen, 2011). Nevertheless, changes in their chemical forms (speciation) and bioavailability are, however, possible (Wuana and Okieimen, 2011). Moreover, this contamination poses a risk to human health because some HMs are carcinogenic, mutagenic, teratogenic and endocrine disruptors while others cause neurological and behavioral changes especially in children (Järup, 2003; Ali *et al.*, 2013).

The problem of HM-pollution is growing worldwide at an alarming rate due to the increase of industrialization and the disturbance of natural biogeochemical cycles (Ali *et al.*, 2013). For example, in the European Union (EU) it has been estimated that each year around 3 billion tonnes of solid wastes are thrown away, of which 90 million tonnes are potential hazardous (Panagos *et al.*, 2013). This amounts to about 6 tonnes of solid waste for every citizen in the EU (Panagos *et al.*, 2013). The main anthropogenic sources of HMs are present and former mining activities, foundries, smelters, sludge dumping,

industrial discharge, use of pesticides and (phosphate) fertilizers, etc. (Ali *et al.*, 2013; Panagos *et al.*, 2013). It is worth to notice that 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 promote their distribution to the surrounding areas (Mendez and Maier, 2008; Ali *et al.*, 2013). In arid and semiarid regions, the environmental impact of mine tailings disposal sites is tremendous, as unreclaimed mining sites generally remain without vegetation for tens to hundreds of years, and consequently, the exposed tailings can spread over tens of hectares by wind and water erosion (Mendez and Maier, 2008). In the Región de Murcia, the Cartagena-La Unión Mining District (37°37'20"N, 0°50'55"W–37°40'03"N, 0°48'12"W) contains one of the largest Pb and Zn content in the SW of Europe (Pérez-Sirvent *et al.*, 2012). The extension of this mining district is about 50 km², and there are more than 45 tailings dump sites that cover around 160 ha (Conesa and Schulin, 2010). These tailings have been clasified according to their geochemical characteristics into acid tailings, which showed high solubility of metals due to the very low pH (~3), and neutral tailings, which showed low metal water solubility but, they can be easily released in the presence of chelating agents such as EDTA and a pH ~7 (Conesa and Schulin, 2010). The major components of the tailings are Fe (11–26%), Si (8–13%), Al (2–4%) and S (2–3%), and among the hazardous HMs and metal(loid)s (thereafter, HMMs), Zn (9100 mg kg⁻¹), As (1900 mg kg⁻¹), Cd (10–34 mg kg⁻¹) and Pb (5000–7000 mg kg⁻¹) are of particular concern (Conesa and Schulin, 2010). Table 1.1 summarizes the concentrations of HMMs found in these tailings. Moreover, the presence of urban and agricultural areas close to the mining area, increase the risk of human exposure to HMMs via wind-borne dust or consumption of contaminated garden and agricultural crops (Conesa and Schulin, 2010).

Table 1.1. Concentration of heavy metals found in the tailings in the Cartagena-La Unión Mining District and comparison with threshold and guideline values for metals in soils. Adapted from Conesa and Schulin (2010) and Tóth *et al.* (2016). The guideline values have been defined on the basis of either ecological risks (e) or health risks (t).

	As	Cd	Cu	Pb	Zn	Reference
Mine tailings Cartagena-La Unión	n.a.	n.a.	150	4,000	12,000	Conesa <i>et al.</i> (2007)
	n.a.	n.a.	180	2,000	2,000	Conesa and Schulin (2010)
	1,900	8.8	380	7,000	5,400	Conesa <i>et al.</i> (2008)
	350	34	84	5,200	9,100	Conesa <i>et al.</i> (2008)
Threshold value (mg kg⁻¹)	5	1	100	60	200	Tóth <i>et al.</i> (2016)
Lower guideline value (mg kg⁻¹)	50 (e)	10 (e)	150 (e)	200 (t)	250 (e)	
Higher guideline value (mg kg⁻¹)	100 (e)	20 (e)	200 (e)	750 (e)	400 (e)	

1.1.1. General overview of restoration techniques for mine tailings

Taking into account that HMMs pose severe risks to the health of soil–plant–animal–human continuum, cleanup of HMM-contaminated soils is utmost necessary in order to minimize their impact. However, the cleanup of soils contaminated by hazardous HMM is a cost-intensive, technically complex procedure (Barceló and Poschenrieder, 2003). In the EU it has been estimated that soil remediation would cost €17.3 billion annually (CEC, 2006). According to the European Environmental Agency (EEA; <https://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites-3/assessment>), in 2011 local soil contamination was estimated at 2.5 million potentially contaminated sites in the EU-39, of which about 45% have been identified to date. About 15% of the identified contaminated sites (342,000) have been remediated. On average, 42% of the total expenditure on the management of contaminated sites comes from public budgets. Annual national expenditures for the management of contaminated sites are on average about €10.7 per capita. This corresponds to an average of 0.041 % of the national GDP (EEA, <https://www.eea.europa.eu/es>). In the EU the management of contaminated soils is based on “traditional” techniques and, particularly on excavation and off-site disposal, which accounts for about one third of management practices, whereas in-situ and ex-situ

remediation techniques for contaminated soils are applied more or less equally (EEA, <https://www.eea.europa.eu/es>).

The physical and chemical methods suffer from limitations like high cost, intensive labor, irreversible changes in soil properties and disturbance of native soil microflora. Chemical methods can also create secondary pollution problems (Tordoff *et al.*, 2000; Conesa *et al.*, 2012). Therefore, research is needed to develop cost effective, efficient and environmentally friendly remediation methods for decontamination of HMM-polluted soils. One such novel approach is phytoremediation, which is considered as a green alternative to solve the problem of HMM pollution and to meet the strictest current regulations regarding wastes with HMMs (Barceló and Poschenrieder, 2003; Pilon-Smits, 2005; Conesa *et al.*, 2012; Ali *et al.*, 2013). The term “phytoremediation” is a combination of two words: Greek phyto (meaning plant) and Latin remedium (meaning to correct or remove an evil). Since plants can be considered as “*miners*” of Earth's crust/soils they have an enormous ability to uptake pollutants from the environment and accomplish their detoxification by various mechanisms (Figure 1.1), which are not mutually exclusive, such as:

1. Phytoextraction (also known as phytoaccumulation, phytoabsorption or phytosequestration) refers to the uptake of contaminants from soil or water by plant roots and their translocation to and accumulation in aboveground biomass i.e., shoots.
2. Phytofiltration is the removal of pollutants from contaminated surface waters or waste waters by aquatic plants.
3. Phytovolatilization is the uptake of pollutants from soil by plants, their conversion to volatile form and subsequent release into the atmosphere. This technique can be used for organic pollutants and some heavy metals like Hg and Se.
4. Phytostabilization or phytoimmobilization refers to the immobilization of contaminants in soils by root uptake, adsorption onto roots or precipitation in the rhizosphere.

Figure 1.1. Possible fates of pollutants (heavy metals) during phytoremediation. The pollutant, which is represented by red circles, can be stabilized in the rhizosphere, sequestered inside the plant tissue, or volatilized. From Pilon-Smits (2005)

These phytoremediation techniques require low installation and maintenance costs compared to physical and chemical remediation options (Ali *et al.*, 2013) and their cost is regarded to be less than 5% of alternative clean-up methods (Prasad, 2003). Among the above-mentioned techniques, phytoextraction has been the focus of an intense research effort in order to find hyperaccumulator plant species (Barceló and Poschenrieder, 2003; Maestri *et al.*, 2010) or the use of genetic engineering to manipulate plant's capacity to tolerate, accumulate, and/or metabolize pollutants, and thus to create the ideal plant for environmental cleanup (Pilon-Smits and Pilon, 2002; Krämer, 2005). As a consequence, more than 450 hyperaccumulator herbaceous plants with a strong HM removal potential and high tolerance have been identified so far (Maestri *et al.*, 2010; Sheoran *et al.*, 2011; Ali *et al.*, 2013). In addition, despite the fact that woody plants are non-hyperaccumulators, some woody plants, such as *Populus* and *Salix* species, have been reported to be able to accumulate considerable levels of HMs in their aerial parts (Luo *et al.*, 2016).

However, from the practical point of view, the use of native plants is generally preferred for soil remediation processes because these plants are already adapted to local conditions and, consequently will have better chances of success than non-adapted ones. In fact, plant establishment on metalliferous wastes is impeded by a whole suite of physical, chemical and biological constraints – some of which may act synergistically – particularly residual high levels of heavy metals, macronutrient deficiencies and poor substrate structure (Tordoff *et al.*, 2000; Párraga-Aguado *et al.*, 2014a). Additionally in semi-arid regions, as it is the case for the current study, plant establishment is further constrained by drought and high temperatures (Párraga-Aguado *et al.*, 2014a). Párraga-Aguado *et al.* (2013) have carried out an exhaustive screening of the “pioneer vegetation” (Wong, 2003) in the Cartagena-La Unión Mining District where they have described the presence of several grass species (Poaceae), such as *Stipa tenacissima* and *Pipthaterum miliaceum*, in the slope of the tailings whereas at tailings’ plateau they identified several species belonging to the Asteraceae (Compositae) such as *Helichrysum decumbens*, and *Dittrichia viscosa*, as well as *Zygophyllum fabago* (Zygophyllaceae), which is classified as an invasive weed in the Database of Spanish National List of Weed Species (Sanz Elorza *et al.*, 2004), and woody species such as *Tetraclinis articulata* (Cupressaceae) and *Pinus halepensis* (Pinaceae).

In the last years, several authors spotlight the importance of the use of phytomanagement in terms of phytostabilisation as the most suitable alternative to stabilise soils affected by mining wastes particularly in arid and semi-arid conditions (Mendez and Maier, 2008; Conesa *et al.*, 2012; Párraga-Aguado *et al.*, 2014a). This technique involves the creation of a self-sustainable plant cover for the long-term stabilisation of metals within the rhizosphere (Párraga-Aguado *et al.*, 2014a). Implementation of phytostabilisation requires the selection of the plant species, the addition of soil amendments and inorganic fertilizers and evaluation of the grade of success (Mendez and Maier, 2008; Conesa *et al.*, 2012).

Since plant selection is considered a critical issue to remediate degraded soils, we present now an overview of the of physiological and biochemical mechanisms of HMM-induced plant stress response and tolerance. Special emphasis will be given to reactive oxygen species (ROS) signaling and the antioxidant defense system in plants.

1.2. HMM toxicity and plant responses

The phytotoxicity of HMMs is long established and it has been ascribed to three main reasons: (i) direct interaction with proteins due to their affinities for thioyl-, histidyl- and carboxyl-groups, causing the metals to target structural, catalytic and transport sites of the cell; (ii) stimulated generation of reactive oxygen species (ROS) that modify the antioxidant defence and elicit oxidative stress; and (iii) displacement of essential cations from specific binding sites, causing functions to collapse (Sharma and Dietz, 2009) (Figure 1.2).

Figure 1.2. Principal mechanisms of HM-induced damage development in sensitive plants and points of interaction and counteraction during acclimation in tolerant plants. Redox-active HMs directly elicit ROS generation. In general, HMs bind to and interfere with targets or compete for binding sites (-SH represents thiols, -COO⁻ represents carboxylic acids and imidazole represents histidyl residues), thereby altering target protein functions, which in turn causes changes in cell metabolism or triggers signaling events that can lead to acclimation. Activation of acclimation responses causes feedback loops with various sites of HM action, leading to, for example, the repair of damaged macromolecules, strengthening of the antioxidant defence system and lowering of HM concentrations in metabolically active cellular compartments. From Sharma and Dietz (2009).

Although most plant species are sensitive to HMM exposure, there is a group of plant species known as metallophytes that are specifically adapted to and thrive in heavy metal-rich soils (Krämer, 2010; Sheoran *et al.*, 2011; Ali *et al.*, 2013). In general, metallophytes have developed two major strategies to resist high HMM exposure. In the first strategy, “the *excluder strategy*”, plants are trying to keep the metal concentrations in roots low, despite high HMM concentration in the soil. Nevertheless, in high-HMM polluted areas, it is often too demanding to exclude toxic metals from plant roots due to the high metal concentrations in the soil (Lin and Aarts, 2012; Ali *et al.*, 2013). To withstand high metal exposure, plants have developed a different strategy, “the *tolerance strategy*”, which involves taking up metals and reducing the damage (Lin and Aarts, 2012). Again a division can be made, this time between plants that tolerate high uptake of metals, but restrict their accumulation to roots, and plants that accumulate metals and preferentially transport metals to the above-ground parts, which are known as hyperaccumulators, a term coined by Jaffré *et al.* (1976) (Figure 1.3). The hyperaccumulator concentration criterion is higher than $100 \mu\text{g g}^{-1}$ DW for Cd, or higher than $1000 \mu\text{g g}^{-1}$ DW for As, Cu, Pb, Ni, Se, or higher than $10000 \mu\text{g g}^{-1}$ DW for Mn and Zn in shoots (Krämer, 2010).

Figure 1.3. Molecular mechanism in response to HMM stress in plants depending on their sensitivity to heavy metal exposure (a–d). **a** HMM-sensitive plants, which cannot keep metals out of their roots or prevent transport to the shoot. **b** HMM-resistant excluder plants, which are able to keep metals outside the roots or take care of rapid efflux in case toxic metals have entered root cells. **c** Heavy metal-tolerant non-hyperaccumulator plants, in which metals can enter root cells where they are sequestered into root vacuoles, preventing translocation to shoots. **d** HMM-hypertolerant hyperaccumulator plants, in which metals are actively taken up through the root, and largely loaded into xylem for root to shoot transport. From Lin and Aarts (2012).

Whichever strategy plants use to limit the negative effects of HMM toxicity, the metal response needs to be tightly regulated to reduce damage by toxic metals but ensure proper homeostasis of essential minerals. Plant responses to HM stress are the combined results of cellular transport mechanisms and activation of signal transduction pathways. These processes are HMM-dependent and plant species-dependent (Lin and Aarts, 2012). In general, the accumulation of a given HMM is a function of uptake capacity and intracellular binding sites. In a multicellular organism, the situation is complicated by tissue- and cell-specific differences and also by intercellular transport (Clemens *et al.*, 2002). Figure 1.4 summarizes the proposed mechanisms involved in HM uptake and accumulation in plants, and Figure 1.5 gives a close-look of all of these processes in a single plant cell.

Figure 1.4. Molecular mechanisms proposed to be involved in HM accumulation by plants. (a) HM ions are mobilized by secretion of chelators and by acidification of the rhizosphere. (b) Uptake of hydrated HM ions or HM-chelate complexes is mediated by various uptake systems residing in the plasma membrane. Inside the cell, HMs are chelated and excess metal is sequestered by transport into the vacuole. (c) From the roots, HMs are transported to the shoot via the xylem. Inside the xylem, HMs are present as hydrated ions or as HM-chelate complexes. (d) After reaching the apoplast of the leaf, metals are differentially captured by different leaf cell types and move cell-to-cell through plasmodesmata. Storage appears to occur preferentially in trichomes. (e) Uptake into the leaf cells again is catalyzed by various transporters. Intracellular distribution of essential metals is mediated by specific metallochaperones and transporters localized in endomembranes. Abbreviations and symbols: CW, cell wall; M, metal/heavy metal; filled circles, chelators; filled ovals, transporters; bean-shaped structures, metallochaperones. From Clemens *et al.* (2002).

One of the mechanisms that plant cells possess to tolerate toxic HMMs involves the cell wall (see Fig. 1.5). Cell walls constitute a heterogeneous matrix that contains carboxylic groups of acidic polysaccharides (*i.e.*, homogalacturonan) and phenolic polymers (for example, lignin and suberin), which are able to bind divalent and trivalent metal cations (Krzesłowska, 2011). Moreover, HMMs could also be bound to extensins, a group of cell wall proteins rich in Cys (Hernández *et al.*, 2015). Moreover, cell wall composition is modified under HMM stress, and several genes involved in root lignin biosynthesis, suberin biosynthesis, and wax synthesis are overexpressed in *Thlaspi caerulescens* compared to *Arabidopsis thaliana* when exposed to Zn (Maestri *et al.*, 2010). In fact, the accumulation of lignin and suberin restricts the movement of metallic ions through the root apoplast, limiting their transfer to the xylem (Maestri *et al.*, 2010). In addition, exudates from roots and symbiotic mycorrhizal fungi can also affect the incorporation of HMMs into the rhizosphere (Figure 1.4a and Figure 1.5).

The next barrier of permeability is the plasma membrane, where numerous transport mechanisms have been characterized (Figure 1.5). Transporters or ion channels of essential nutrients facilitate the accumulation of hazardous HMMs in the cytosol, which are neutralized by active efflux to the apoplast and vacuole by transporters like P-type HEAVY METAL ATPASE (HMA2/4 and HMA3), ZINC ARABIDOPSIS TRANSPORTER (ZAT)-Zn/Cd, and/ or CATION EXCHANGER (CAX)-H⁺/Ca²⁺ antiporters (Hernández *et al.*, 2015). The membrane potential, which is negative on the inside of the plasma membrane and might exceed −200 mV in root epidermal cells, provides a strong driving force for the uptake of cations through secondary transporters (Clemens *et al.*, 2002).

Plant cells also synthesize different metabolites that bind the metal(loid)s ions to decrease their free cytosolic concentration (Figure 1.4 and Figure 1.5). An important group of ligands is formed by Cys-rich peptides like metallothioneins (MTs) and phytochelatins (PCs) (Cobbett and Goldsbrough, 2002). MTs are low molecular weight peptides that are transcriptionally regulated in a tissue-specific manner, whereas PCs are synthesized nontranslationally from reduced glutathione (GSH) in a transpeptidation reaction catalyzed by the enzyme PC synthase in the cytosol (Cobbett and Goldsbrough, 2002). Other metal-chelating molecules are, for example, histidine, nicotianamine and citrate (Clemens *et al.*, 2002). It has been established that HMM-chelation with MTs and PCs

might route metals mainly to root sequestration and HMM chelation with amino acids and/or organic acids appears to route metals primarily to the xylem (Clemens *et al.*, 2002). Phytochelatin-metal complexes can be transported to vacuoles through ABCC transporters (Mendoza-Cózatl *et al.*, 2010). Malfunctioning ABCC transporters can enhance the transfer of HMM to shoots, which compromises their detoxification (Hernández *et al.*, 2015).

Several studies have highlighted that the tolerance of plant cells to toxic elements, including HMMs, is highly dependent on GSH metabolism because it plays a central role as chelating agent (due to the high affinity of metals to its thiol (-SH) group and as a precursor of PCs as previously mentioned) but GSH is also an important antioxidant and signaling component (Jozefczak *et al.*, 2012; Hernández *et al.*, 2015). In fact, genes involved in the uptake of SO_4^{2-} , its reduction, or the synthesis of Cys are upregulated in plants exposed to toxic HMMs (Nocito *et al.*, 2006), pointed out the role of sulfur nutrition on the stress resistance in plants (Rausch and Wachter, 2005).

Figure 1.5. Mechanisms of metal (M^{2+}) detoxification and tolerance in plant cells. The metals bind to cell exudates or walls (1). Once metal enters the protoplast, the cytosolic concentration can be reduced by transport to the apoplast or vacuole by ZIP, CAX, and/or HMA2/4- and HMA3-ATPase transporters (2). The concentration of cytosolic free M^{2+} is also reduced by chelation with various ligands, such as MTs or PCs; complexes (M-PCs) are ultimately transported to the vacuole via ABCC-transporters (3). From Hernández *et al.* (2015).

1.2.1. ROS during abiotic stress

It is well known that organelles such as chloroplasts, mitochondria or peroxisomes with a highly oxidizing metabolic activity or with intense rate of electron flow are the major source of ROS in plant cells. Moreover, it has been estimated that 1–2% of O₂ consumed by plants is sidetracked to produce ROS in various subcellular compartments (Gill and Tuteja, 2010). Abiotic stress conditions, including drought, salinity, HM exposure and stress combination, induce the production of reactive oxygen species (ROS) (Table 1.2), such as superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radicals (•OH), and singlet oxygen (¹O₂) and, consequently break down the equilibrium between ROS generation and detoxification (Schützendübel and Polle, 2002; Gill and Tuteja, 2010). Abiotic stresses that limit CO₂ availability due to stomatal closure enhance the production of ROS such as O₂^{•-} and ¹O₂ in chloroplasts that, in turn, can initiate retrograde and anterograde signaling (Choudhury *et al.*, 2016). Chloroplastic ROS are mitigated by an array of ROS-scavenging enzymes and pathways such as Fe- and CuZn-Superoxide dismutases (SODs) and the Asada–Foyer–Halliwell pathway, as well as high concentrations of antioxidants such as ascorbic acid and GSH (Mittler *et al.*, 2004; Mignolet-Spruyt *et al.*, 2016). During abiotic stress, mitochondrial ROS accumulation is typically mediated via electron leakage from complex I and III to produce O₂^{•-}, which can be converted to H₂O₂ by Mn-SOD. This ROS accumulation can be mitigated by alternative oxidase (AOX), type II NAD(P)H dehydrogenase and uncoupling proteins in the inner mitochondrial membrane (Noctor *et al.*, 2007). Production of ROS in peroxisomes during abiotic stress is mainly the outcome of enhanced photorespiration resulting in the production of H₂O₂ by glycolate oxidase, which is primarily mitigated by catalase (CAT) (Del Río and López-Huertas, 2016). Production of ROS at the apoplast during abiotic stress is mediated via at least four different mechanisms. The most studied are the plasma membrane-bound NADPH oxidases (known in plants as RESPIRATORY BURST OXIDASE HOMOLOGUES [RBOH]) that link calcium and ROS signaling during stress and produce superoxide in the apoplast (Gilroy *et al.*, 2016). Apoplastic ROS production can also be mediated by class III peroxidases (Prxs) (O’Brien *et al.*, 2012). Another important ROS-producing enzyme in the apoplast during abiotic stress is oxalate oxidase, which is shown to be important for drought stress acclimation (Voothuluru and Sharp, 2013). Recently, xanthine dehydrogenase was proposed to play a role in stress ROS signaling (Ma *et al.*, 2016). To counteract apoplastic ROS levels, there

are CuZn-SODs, ascorbate peroxidases (APXs), cell wall-bound peroxidases, and low levels of ascorbate and GSH. Nevertheless, the apoplastic ROS-scavenging capacity is not as efficient as the intracellular ROS-scavenging systems, and allows the accumulation of ROS to high levels at the apoplast (Choudhury *et al.*, 2016).

Table 1.2. ROS in plant tissues and their basic properties. From Møller *et al.* (2007)

Property	Singlet oxygen ($^1\text{O}_2$)	Superoxide ($\text{O}_2^{\cdot-}$)	Hydrogen peroxide (H_2O_2)	Hydroxyl radical ($\text{HO}^{\cdot}$)
Half-life	1 μs	1 μs	1 ms	1 ns
Distance traveled	30 nm	30 nm	1 μm	1 nm
Cellular concentration	?	?	μM -mM	?
Reacts with:				
Lipids	Polyunsaturated fatty acids (PUFA)	Hardly	Hardly	Rapidly
DNA	Mainly guanine	No	No	Rapidly
Carbohydrates	No	No	No	Rapidly
Proteins	Trp, His, Tyr, Met, Cys	Fe-S centers	Cys	Rapidly

In this regard, it is important to notice, that beyond the traditional belief of ROS as mere toxic by-products of metabolic processes, the above-mentioned works strongly support that ROS primarily function in cells as signal transduction molecules involved in sensing and responding to stress, including HMM stress (Wrzaczek *et al.*, 2013; Choudhury *et al.*, 2016; Mittler, 2017). Moreover, ROS are connected to signaling events linked to oxidative changes which regulate plant-specific programmed cell death (Wrzaczek *et al.*, 2013). To prevent oxidative damage, cellular compartments are equipped with a complex network of antioxidative enzymes and low molecular weight antioxidants (Mittler *et al.*, 2004). However, the lifetime of ROS varies between different cellular compartments, because of differences in redox buffering, *i.e.*, the capacity of antioxidative systems to remove ROS (Foyer and Noctor, 2016). A set of discrete locations defines specificity in redox signaling in different compartments of the plant cell. Independent control and buffering permit redox-sensitive signal transduction to occur in locations such as the thylakoid lumen and the apoplast/cell wall, and across the membranes that separate these compartments from the highly redox-buffered spaces such as the stroma and cytosol

(Noctor and Foyer, 2016). Nevertheless, it is important to remember that H_2O_2 can be transported across the membrane system of plant cells via aquaporins (Møller and Sweetlove, 2010; Foyer and Noctor, 2016), is more stable, with a half-life of 1 ms, than the other ROS, and is regarded as a primary messenger (Møller and Sweetlove, 2010; Foyer and Noctor, 2016).

Because each cellular compartment establishes and controls its own ROS homeostasis, altogether the different ROS levels in the different compartments can be viewed as generating a particular ROS signature. This signature can change depending on the type of cell, its developmental stage, or stress level. Different abiotic stresses and/or different combinations of abiotic stresses (stress combination) are likely to cause the formation of different ROS signatures in plant cells (Choudhury *et al.*, 2016). Decoding these “global signatures” via different ROS sensors can create a stress-specific signal that will either tailor the acclimation response to the type of stress/combination affecting the plant, or induce apoptosis (De Gara *et al.*, 2010; Choudhury *et al.*, 2016).

It is thought that decoding ROS signals/signatures by the cell occur via different redox reactions of ROS and several proteins, causing in turn alterations in the control and regulation of plant metabolism, as well as the activation of transcriptional regulatory networks (Noctor and Foyer, 2016). Among the most ROS-induced protein post-translational modifications are sulfonylation, carbonylation, glutathionylation and S-nitrosylation (Choudhury *et al.*, 2016). Thus, for example H_2O_2 will oxidize sulfur-containing residues of proteins (e.g. the -SH group of cysteine) and, consequently alter protein structure and function (e.g. by the formation of disulfide bonds). Such alterations in protein structure/function can, for example, regulate the binding of transcription factors (TFs) to DNA and affect transcription (Dietz *et al.*, 2016; Choudhury *et al.*, 2016). Also, superoxide radicals ($\text{O}_2^{\bullet-}$) can affect the protein structure/function by interacting with the iron-sulfur (Fe-S) clusters of certain proteins (Noctor and Foyer, 2016).

One of best characterized case study for redox-regulated signaling proteins in plants (Wu *et al.*, 2012) is the interaction of TGA transcription factors with the co-activator NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), which also serves as a salicylic acid (SA) receptor (Wu *et al.*, 2012) (Figure 1.6). Under normal growth conditions, NPR1 is present as an oxidized, multimeric form in the cytoplasm but

upon changes in cellular redox state, NPR1 is reduced by thioredoxin and the freed monomeric forms translocate to the nucleus (Wrzaczek *et al.*, 2013). In fact, both NPR1 and its interactor TGA1 are also S-nitrosylated at cysteine residues, and S-nitrosylation of NPR1 stimulates its nuclear import (Wrzaczek *et al.*, 2013).

Figure 1.6. Proposed ROS signaling network in plants. Current evidence suggests that ROS induces Ca^{2+} influx into the cell while at the same time ROS production by RBOH proteins is regulated by Ca^{2+} and phosphorylation through both Ca^{2+} -dependent and other protein kinases. H_2O_2 , produced from dismutation of superoxide by the enzyme superoxide dismutase (SOD), can enter cells through aquaporins. Superoxide and H_2O_2 react with cell wall components, extracellular peptides or extracellular domains of receptors, such as receptor-like protein kinases (RLK). H_2O_2 also acts to regulate the K^+ channel SKOR in the plasma membrane through modification of a Cys residue on the apoplastic side. Oxidized extracellular peptides could bind to RLKs or oxidized RLK ectodomains could function in signaling. Oxidation and peptide binding might lead to differential RLK complex formation, phosphorylation events and subsequent signal transduction. These peptides and receptors are as yet hypothetical and have not been identified. Inside the cell ROS perception is integrated by one or more unknown integration nodes (X, Y) and intracellular organelles (mitochondria, peroxisomes, chloroplasts). Integrator Y might act on transcription factors (TFs), some of which are also redox regulated for the control of ROS-regulated gene expression. ROS signaling and redox balance is integrated with salicylic acid (SA) signaling. The TF TGA1 and the co-activator NPR1 are reduced and translocate to the nucleus where they participate in the regulation of SA-induced gene expression. TGA1 is S-nitrosylated on two cysteine residues. In addition, NPR1 is S-nitrosylated; however, the role and effect of this modification are still unclear. Apoplastic and symplastic signaling pathways connect adjacent cells where signals are integrated by unknown signaling nodes (Z) and result in (extracellular and intracellular) ROS production, signal amplification, signal spread and ultimately an acclimation response. From Wrzaczek *et al.* (2013).

The interaction between ROS signals and SA as well as with other phytohormones is well documented (Fujita *et al.*, 2006; Bartoli *et al.*, 2013). ROS signals are integrated with the action of plant hormones such as SA, ethylene, abscisic acid and jasmonates, among others in order to control growth and defence processes in response to environmental stresses (Fujita *et al.*, 2006; Bartoli *et al.*, 2013). Although all the phytohormones can interact with the cellular redox signaling hub, in this work, we focussed our attention on the role of SA, which is well known to be required in the signal transduction cascades that regulate plant defence mechanisms against both biotic, principally against biotrophic and hemibiotrophic pathogens (Vlot *et al.*, 2009), and abiotic stresses (Metwally *et al.*, 2003; Horváth *et al.*, 2007; Hayat *et al.*, 2010; Khan *et al.*, 2015)

1.2.2. Salicylic acid and plant defense

SA is a phenolic hormone (monohydroxybenzoic acid) that has been shown to regulate various aspects of plant growth and development (Rivas-San Vicente and Plasencia, 2011) as well as plays key signaling roles in plant stress responses (Vlot *et al.*, 2009;

Khan *et al.*, 2015). SA endogenous levels in plants not only differ widely among species, but also they can vary within an individual plant depending on the tissue type, subcellular compartment, and developmental stage; and after exposure to stress (Rivas-San Vicente and Plasencia, 2011; Klessig *et al.*, 2016).

SA is synthesized through two distinct and compartmentalized pathways that require the primary metabolite chorismate: the phenylpropanoid route in the cytoplasm initiates from phenylalanine, and the isochorismate pathway takes place in the chloroplast (Figure 1.7). Most of the SA synthesized in plants is glucosylated and/or methylated (Figure 1.7). Glucose conjugation at the hydroxyl group of SA results in formation of the SA glucoside [SA 2-*O*- β -D-glucoside] as a major conjugate by a SA glucosyltransferase (SAGT) localized in the cytosol. Glucose conjugation at the SA carboxyl group produces the SA glucose ester in minor amounts (Figure 1.7). The carboxyl group of SA can also be methylated to form methyl salicylate (MeSA) by a carboxyl methyltransferase that uses SAM as methyl donor. MeSA is converted back to SA by MeSA esterase, SABP2, or can be glucosylated to produce MeSA 2-*O*- β -D-glucose.

Figure 1.7. SA biosynthesis and metabolism. Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SA, salicylic acid; SAGT, SA glucosyltransferase; aa, amino acid; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase; SGE, salicyloyl glucose ester; SAG, SA O- β -glucoside; MeSA, methyl salicylate; MeSAG, methyl salicylate O- β -glucoside. From Vlot *et al.* (2009).

SA acts as a central regulator of cell fate by the reprogramming of gene expression (Vlot *et al.*, 2009; Khan *et al.*, 2015). This process involves the activation of NADPH oxidases, and in conjunction with cell wall Prxs, a rapid production and accumulation of active oxygen species - called oxidative burst - accompanying cytosolic Ca^{2+} release occurs in the apoplast in response to the perception of biotic and abiotic stresses (Kawano and Muto, 2000; Bartoli *et al.*, 2013). In fact, several studies pointed out that some of the SA-dependent ROS production in plant cells might depend on the interaction between SA and Prxs as well as the activation of NADPH oxidases (Almagro *et al.*, 2009). Moreover, it has been reported that SA can act as an inhibitor of the respiratory alternative oxidase (Hayat *et al.*, 2010), and among the more than 30 SA-binding proteins (SABPs), there are several enzymes belonging to the antioxidative systems such as CAT, APX, glutathione peroxidase, and thioredoxins (Klessig *et al.*, 2016). Therefore, this SA-mediated control of H_2O_2 accumulation has been attributed to be one of the beneficial effects of SA exogenous treatment in plants challenged with HMs (Pál *et al.*, 2002; Horváth *et al.*, 2007; Hayat *et al.*, 2010; Khan *et al.*, 2015). In addition to decrease oxidative injuries caused by HM exposure through the modulation of antioxidant system-components, SA treatment was also reported to improve growth rate, photosynthesis and carboxylation reactions (Pál *et al.*, 2002; Horváth *et al.*, 2007; Hayat *et al.*, 2010; Khan *et al.*, 2015). Several key enzymes involve in photosynthesis such as Rubisco, carbonic anhydrase and glyceraldehyde 3-phosphate dehydrogenase are also SABPs, and thus their enzymatic activities can be altered after SA-binding (Klessig *et al.*, 2016).

On the other hand, elevated SA levels have been reported to be a strong predictor of Ni hyperaccumulation in different *Thlaspi* species (Freeman *et al.*, 2005). Further, the high SA levels enhanced the activity of sulfur assimilation pathway (mainly the activity of serine acetyltransferase; SAT), leading to elevated GSH content and eventually increased Ni-resistance (Freeman *et al.*, 2005).

1.2.3. Antioxidant defense system in plants

Plants have evolved a particularly complex and redundant antioxidant defense system, in which enzymes and metabolites are linked in a network of reactions (De Gara *et al.*, 2010). The main components of the antioxidant machinery of plants are low molecular-weight antioxidant metabolites and enzymes (Mittler *et al.*, 2004). Enzymatic antioxidants include SOD, CAT, and the Asada–Foyer–Halliwell pathway, which involves successive oxidations and reductions of ascorbate, glutathione and NADPH (Figure 1.8 and Table 1.3). APX is the first enzyme and catalyzes the reduction of hydrogen peroxide to water by using AA as a specific electron donor. In this reaction, AA is oxidized to monodehydroascorbate (MDHA), which is an atypical radical intermediate that reacts only very slowly with oxygen and, thus unable to trigger oxidative cascade reactions that make radicals very harmful for metabolism (De Gara *et al.*, 2010). Spontaneously, two molecules of MDHA can undergo dismutation, producing AA and dehydroascorbate (DHA). MDHA is also reduced back to AA by an NAD(P)H-dependent oxido-reductase (MDHAR). To rescue DHA, which can undergo irreversible hydrolysis to 2,3-diketogulonic acid, DHA can be reconverted to AA by another oxido-reductase (DHAR) that uses GSH as an electron donor. The cycle is complete when the glutathione disulphide (GSSG) produced by DHAR activity is reduced to GSH by an NADPH-dependent oxido-reductase (GR) (De Gara *et al.*, 2010) (Figure 1.8).

Figure 1.8. Scheme of the AA–GSH cycle and connected ROS scavenging systems. From De Gara *et al.* (2010).

Table 1.3. Reactions and subcellular localization of antioxidant enzymes. Adapted from Gill and Tuteja (2010) and Noctor *et al.* (2016).

Antioxidant enzymes	Reactions catalyzed	Subcellular location	Number of genes
Ascorbate peroxidase (APX; EC 1.11.1.11)	$\text{H}_2\text{O}_2 + \text{AA} \rightarrow 2\text{H}_2\text{O} + \text{DHA}$	Cytosol, mitochondrion, plastid, peroxisome, root nodules	9 (Arabidopsis)
Catalase (CAT; EC 1.11.1.6)	$\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \frac{1}{2}\text{O}_2$	Peroxisome	3 (Arabidopsis, rice, maize, tobacco)
Dehydroascorbate reductase (DHAR; EC 1.8.5.1)	$\text{DHA} + 2\text{GSH} \rightarrow \text{AA} + \text{GSSG}$	Cytosol, root nodules	3 (Arabidopsis)
Glutathione peroxidase (GPX; EC 1.11.1.9)	$\text{H}_2\text{O}_2 + \text{GSH} \rightarrow \text{H}_2\text{O} + \text{GSSG}$	Cytosol, mitochondrion, plastid	7 (Arabidopsis)
Glutathione reductase (GR; EC 1.6.4.2)	$\text{GSSG} + \text{NAD(P)H} \rightarrow 2\text{GSH} + \text{NAD(P)}^+$	Cytosol, mitochondrion, plastid, root nodules	2 (Arabidopsis)
Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4)	$2\text{MDHA} + \text{NADH} + \text{H}^+ \rightarrow 2\text{AA} + \text{NAD}^+$	Cytosol, mitochondrion, plastid, root nodules	5 (Arabidopsis)
Peroxiredoxin (PrxR; EC 1.11.1.15)	$2 \text{R}'\text{-SH} + \text{ROOH} \rightarrow \text{R}'\text{-S-S-R}' + \text{H}_2\text{O} + \text{ROH}$	Chloroplast, mitochondrion	7 (Arabidopsis)
Superoxide dismutase (SOD; EC 1.15.1.1)	$\text{O}_2^{\bullet -} + \text{O}_2^{\bullet -} + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O}_2 + \text{O}_2$	* Grouped by metal cofactor	7 (Arabidopsis)
	Cu/Zn-SOD	Cytosol, plastid, peroxisome, root nodules	3 Cu/Zn-SODs
	Mn-SOD	Mitochondrion	1 Mn-SOD
	Fe-SOD	Plastid	3 Fe-SODs

Non-enzymatic antioxidants are GSH, AA (both water soluble and the main cellular redox buffers) and NAD y NADP, which are the major soluble non-protein redox couples in plant cells (Foyer and Noctor, 2011).

1.2.3.1. The primary antioxidant system

1.2.3.1.1. Ascorbate

Ascorbate is the most abundant, powerful and water soluble antioxidant in plants (Foyer and Noctor, 2011). It occurs in all plant tissues, particularly in photosynthetic tissues and nearly in all cell compartments (Table 1.4) (Noctor and Foyer, 2016). In plants, AA is synthesized in the mitochondria by L-galactono- γ -lactone dehydrogenase (Gill and Tuteja, 2010). It is a linchpin of cellular redox buffering, it can directly scavenge ROS, regenerates other metabolites, among which tocopherols, from oxidative damages and protects the catalytic site of a number of enzymes (e.g., hydroxylases) from irreversible oxidation (De Gara *et al.*, 2010). It can be used as substrate or enzyme cofactor in various biological reactions for example for violaxanthin de-epoxidase, a key enzyme in the regulation of photosystem II excitation capture (Demmig-Adams and Adams, 1996).

Table 1.4. Estimated or measured levels of H₂O₂, and the major soluble redox couples (ascorbate, GSH and NADP(H) and NAD(H)) in different subcellular compartments. From Noctor and Foyer (2016).

	H ₂ O ₂ mM	Ascorbate	Glutathione	NADP(H)	NAD(H)
Cytosol	≤ 0.01	21 (≥ 95)	3.5 (≥ 99)	0.3 (50)	0.7 (10)
Chloroplast	≤ 0.01	10 (≥ 95)	0.9 (≥ 99)	0.9 (40)	0.2 (70)
Mitochondrion	≤ 0.01	10 (≥ 95)	6.5 (≥ 99)	0.3 (80)	2 (70)
Peroxisome	≤ 0.01	23 (≥ 95)	3.6 (≥ 99)	n.m.	n.m.
Nucleus	≤ 0.01	16 (≥ 95)	4.9 (≥ 99)	n.m.	n.m.
Vacuole	≤ 0.01	2 (20)	0.03 (≥ 10)	n.m.	n.m.
Apoplast	1?	n.m. (20)	n.m. (≥ 10)	n.m.	n.m.
Typical leaf content* (nmol g ⁻¹ FW)	200	3,000 (90)	300 (95)	20 (50)	30 (20)

- Typical leaf content are given for Arabidopsis growing in moderate light and otherwise standard conditions.
- Figures in parentheses indicate approximate redox states (percentage reduced form).
- n m., Not measured.

1.2.3.1.2. *Glutathione*

Many of the functions of GSH are linked to defense, and thus, as previously mentioned, the tripeptide GSH (γ glu-cys-gly) plays a central role as chelating agent because of the high affinity of HMMs to its thiol (-SH) group and as a precursor of PCs (Cobbett and Goldsbrough, 2002). Apart from HMMs, GSH is used to detoxify xenobiotics or endogenous metabolites through glutathione *S*-transferases (GSTs). Moreover, GSH is necessary to maintain cellular redox homeostasis, which is also known to play a role in stress perception and signaling, defense reactions, and plant development (Hernández *et al.*, 2015). It is a potential scavenger of ROS including the highly toxic hydroxyl radical ($\bullet$ OH). As previously stated, GSH plays a paramount role in the antioxidative defense system by regenerating AA in the AA-GSH cycle (De Gara *et al.*, 2010). This role is reflected in the quasi-ubiquity of GSH in all living cells and all cell compartments (Table 1.4) (Noctor and Foyer, 2016).

Glutathione may be required as a relatively stable carrier of NO, in the form of *S*-nitrosoglutathione (GSNO), and several proteins in plant cells undergo *S*-glutathionylation, that is, formation of a stable mixed disulphide between GSH and a protein thiol (Choudhury *et al.*, 2016).

1.2.3.1.3. *NAD(H) and NADP(H)*

Under stress conditions NADPH is exceptionally important as a branchpoint between ROS-production and ROS-processing metabolism in plants (Noctor, 2006). Thus, cytosolic NAD(P)H provides the reductant for production of ROS through plasma membrane-bound NADPH oxidases, and in the stroma of chloroplasts, the concentration of NADP^+ available for reduction by ferredoxin-NADP⁺ reductase is considered important in determining electron flow to alternative acceptors such as oxygen. As mentioned in Figure 1.8, processing of ROS is also heavily dependent on NAD(P)H, and both NADPH and NADH are important as reductants for MDHAR. Reductive detoxification of ROS and organic peroxides occurs through the AA and GSH pools (Noctor, 2006). Oxo-derivatives of lipid peroxides can be detoxified by NAD(P)^+ -dependent oxidation or NADPH-dependent reduction catalysed by aldehyde dehydrogenases, aldo-keto reductases or alkenal reductases (Noctor, 2006).

1.2.3.2. *The secondary antioxidant system*

Apart from above-mentioned antioxidants, which constitute the first line of defence against oxidative stress, plant cells contain a large array of “extra” antioxidant enzymes including peroxiredoxins, glutathione peroxidases, and glutathione S-transferases, that reduce H₂O₂ and hydroperoxides by ascorbate-independent thiol-mediated pathways (Foyer and Noctor, 2009), as well as a suite of low molecular compounds that constitute a ‘secondary’ antioxidant system such as polyamines, the proteinogenic amino acid proline (Szabados and Saviouré, 2010) and diverse secondary metabolites, including the isoprenoids tocopherols and carotenoids located in plastids (Gill and Tuteja, 2010; Munné-Bosch, 2005), and phenolics (Brunetti *et al.*, 2015; Di Ferdinando *et al.*, 2014; Pourcel *et al.*, 2007).

In fact, there are extensive reports describing the enhancement of the biosynthesis of phenolic compounds in plants subjected to different stress conditions including HMM stress and stress combinations (Dixon and Paiva, 1995; Michalak, 2006; Vogt, 2010; Martinez *et al.*, 2016). Next, in the following sections a brief description of the role of proline and phenolics during stress tolerance will be carried out.

1.2.3.2.1. *Carotenoids*

Carotenoids have an important photoprotective role in plants based on their property to deactivate triplet chlorophyll (³Chl*) and singlet oxygen (¹O₂) (Demmig-Adams and Adams, 1996). Carotenoids have also a structural role and they are important for the photosystem I assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (Havaux, 1998). Current knowledge revealed that the xanthophyll lutein functions mainly in the deactivation of ³Chl*, while zeaxanthin is the major player in the deactivation of excited singlet Chl (¹Chl*) and thus, thermal dissipation of excess light energy (*i.e.*, the non-photochemical quenching). Besides, zeaxanthin also has important functions as an antioxidant in the lipid phase of the membrane (Jahns and Holzwarth, 2012).

1.2.3.2.2. *Proline accumulation and stress tolerance*

Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and the only one where the α -amino group is present as a secondary amine (Szabados and Savouré, 2010; Verslues and Sharma, 2010). Another distinctive feature of proline is that several types of plant stress, including HMM exposure, cause proline to accumulate to high levels in many plant species (Szabados and Savouré, 2010; Verslues and Sharma, 2010). For a long time, proline was considered as an inert compatible osmolyte that protects macromolecules under osmotic stress (Szabados and Savouré, 2010). Currently, extensive evidence suggests that proline itself as well as its metabolism have a prominent role in plant stress tolerance mechanisms. Proline has been reported (Szabados and Savouré, 2010; Verslues and Sharma, 2010; Ben Rejeb *et al.*, 2014) to be involved in the following functions, that are not mutually exclusive:

1. *Osmotic adjustment*: Accumulation of proline (and other solutes) decreases cellular osmotic potential to balance decreases in soil water potential while allowing turgor and water content to be maintained.
2. *Protection of cellular structure during dehydration*: Proline has properties (highly soluble, zwitterionic) observed in compatible solutes, thus can act as “water substitutes” to stabilize cellular structure through hydrophilic interactions and hydrogen bonding as well as a molecular chaperone, able to protect protein integrity.
3. *Redox buffering*: Synthesis of proline uses NADPH in the chloroplast or cytosol, whereas catabolism of proline releases reductant in the mitochondria. Adjusting the balance of proline synthesis and degradation can alleviate imbalances in cellular redox.
4. *Storage and transfer of reductant (and nitrogen)*: This function may be part of redox buffering at the whole plant level. Synthesis and catabolism of proline can be separated in either time (proline synthesis during stress; proline catabolism after stress release) or space (for example synthesis of proline in photosynthetic tissue, catabolism in other tissues such as root), suggest the importance of proline movement within the plant and separation of synthesis and catabolism between different tissues.
5. *A signaling molecule*: Transport of proline between different parts of the plant

may serve as a metabolic signal and influence programmed cell death.

6. *Reactive oxygen scavenging*: Proline itself can react with and detoxifies ROS, such as H_2O_2 and singlet oxygen, however the importance of proline relative to other ROS scavenging systems are still unclear.

1.2.3.2.3. Phenolic compounds and stress tolerance

Plants contain a remarkably diverse array of phenolic compounds, but all generally possess an aromatic arene (phenyl) ring with at least one hydroxyl group attached (Kutchan *et al.*, 2015). The compounds can be divided into four classes based on their chemical structures: simple phenols (e.g. phenol, catechol and pyrogallol), hydroxybenzoic acids (e.g. p -hydroxybenzoic, 3,4-dihydroxybenzoic, gallic and salicylic acids), phenylpropanoids (e.g. p-coumaric, caffeic, ferulic and sinapic acids) and flavonoids. Flavonoids are subdivided into at least 12 classes depending on the redox states of the C-ring, and are normally found as flavones (e.g. apigenin and luteolin), flavonols (e.g. kaempferol and quercetin), flavan-3-ols (e.g. catechin), anthocyanins (e.g. cyanidin, pelargonidin and delphinidin), and so forth (Pietta, 2000; Takahama, 2004; Perron and Brumaghim, 2009) (Figure 1.9).

Figure 1.9. Flavan general structure, showing the ring labeling and numbering system. Structures of catechol, gallol, and general structures of catechins, flavonols, flavones, and anthocyanins. R groups are typically H, OH, OCH_3 , galloyl esters, or carbohydrate groups, depending on the compound. From Perron and Brumaghim (2009)

Phenolics account for ~40% of organic carbon in plants and are derived primarily from phenylpropanoid and phenylpropanoid-acetate backbones (Kutchan *et al.*, 2015). Both pathways share the first three biochemical steps, the core phenylpropanoid pathway that begins with L-phenylalanine precursor and ends with the formation of 4-coumaroyl CoA (Figure 1.10). From there, branches dispatch precursors to the biosynthesis of the diverse phenylpropanoid classes (Boudet, 2007). L-phenylalanine (L-Phe) is synthesized through the shikimate pathway, in the plastids (Maeda and Dudareva, 2012). Phenylalanine ammonia lyase (PAL) catalyzes the first step in phenylpropanoid metabolism in which L-Phe undergoes deamination to yield *trans*-cinnamate and ammonia, which is toxic at high concentrations (Kutchan *et al.*, 2015). To prevent accumulation of ammonia and eliminate the requirement for N during phenolic synthesis the conversion of L-Phe into *trans*-cinnamate is engaged to a nitrogen cycle that utilizes glutamine synthetase and glutamate synthetase (GS and GOGAT) to produce glutamate, which is the amino donor in the reaction that forms arogenate from prephenate (Maeda and Dudareva, 2012; Kutchan *et al.*, 2015). Most plants have several PAL genes, ranging from four in *Arabidopsis thaliana* to close to two dozen in cultivated tomato (Kutchan *et al.*, 2015).

Figure 1.10. Schematic representation of the major branch pathways of flavonoid biosynthesis, starting with general phenylpropanoid metabolism. Enzyme abbreviation: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid 3' hydroxylase (F3'H) or flavonoid 3'5' hydroxylase (F3'5'H), isoflavone *O*-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), *O*-methyltransferase (OMT), Phe ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UGFT), and vestitone reductase (VR). From Winkel-Shirley (2001).

Literature is full of reports that describe the relationship between the increase in PAL gene/protein expression/activity and the increase in the levels of phenolics in response to different stimuli (Boudet, 2007). Phenolics have been reported to play key roles in plant structural integrity, UV photoprotection, reproduction and internal regulation of plant cell physiology and signaling (Ferrer *et al.*, 2008). Moreover, many phenolic compounds are potential antioxidants. For example, polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate (Blokhina *et al.*, 2003). The antioxidative properties of phenolics depend on their structure (Rice-Evans *et al.*, 1997), and are determined by: i) its reactivity as a hydrogen or electron-donating agent (which relates to its reduction potential), ii) the fate of the resulting antioxidant-derived radical, which is governed by its ability to stabilize and delocalize the unpaired electron, iii) its reactivity with other antioxidants, and iv) metal-chelating potential.

Evaluation of the antioxidant activities of different phenolic compounds (Rice-Evans *et al.*, 1997; Pietta, 2000) has established that the structural arrangements imparting greatest antioxidant activity are determined by the presence of: i) the ortho 3',4'-dihydroxy moiety in the B ring, ii) the meta 5,7-dihydroxy arrangements in the A ring, iii) the 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, for electron delocalization, as long as the *o*-dihydroxy structure in the B ring (however, alterations in the arrangement of the hydroxyl groups and substitution by glycosylation decreases the antioxidant activity), iv) for metal chelation, the *o*-diphenolic groups in the 3',4'-dihydroxy positions in the B ring of the flavonoid molecule, and the ketol structures 4-keto, 3-hydroxy or 4-keto and 5-hydroxy in the C ring of the flavonols (Fig. 1.11).

Figure 1.11. Proposed binding sites for trace metals to flavonoids. These sites are the catechol moiety in ring B, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring, and the 4-oxo, 5-hydroxyl groups between the heterocyclic and the A rings. From Pietta (2000).

On the other hand, it has been shown that phenolic compounds can be involved in the H_2O_2 -scavenging cascade in plant cells in the presence of AA and Prxs (EC 1.11.1.7) (Takahama and Oniki, 2000; Takahama, 2004). Prxs are typical enzymes of plants, form large multigenic families (Welinder *et al.*, 2002), and have been implicated in influencing cell wall properties and in a range of biotic and abiotic stresses (Passardi *et al.*, 2005). They are localized in the walls and vacuoles of plant cells, which are also the target compartments for the accumulation of most secondary metabolites, many of which may function as Prx substrates (Almagro *et al.*, 2009; Ferreres *et al.*, 2011). Thus, it has been suggested that Prx can act as an efficient H_2O_2 -scavenging system in the apoplast and in plant vacuoles in the presence of phenolics and AA (Takahama and Oniki, 2000; Takahama, 2004).

In this AA/phenolics/Prx system, phenolics (PhOH) are oxidized by Prxs to phenoxyl radicals ($\text{PhO}^\bullet$) which can be reduced back by AA. In fact, it has been established that phenoxyl radicals can accept electrons from AA more readily than from other radicals avoiding the formation of phenolic dimers (Pérez, 2002). The primary oxidation product of AA is the monodehydroascorbate radical ($\text{MDHA}^\bullet$) which disproportionates spontaneously to DHA. However, *in vivo* MDHA radical can also be reduced to AA and DHA in a reaction catalyzed by the MDHAR (reaction 3). DHA can be transported to cytoplasm and is reduced there by cytosolic DHAR (Noctor and Foyer, 2016). The transport of both ASC and DHA across tonoplast and between symplast and apoplast has

been observed (Noctor and Foyer, 2016). Thus, the functionality of the system will depend on the capacity of MDHA[•] and DHA to regenerate AA (Fig. 1.12), and on the cellular transport of DHA and AA between tonoplast and cytosol.

A scheme of the four reactions previously described in the presence of AA is:

where reaction 4 is the sum of the other reactions, see also Figure 1.12.

Figure 1.12. Possible reactions of AA/phenolics/Prx systems in a vacuole. This figure shows H₂O₂-scavenging when H₂O₂, which is generated by H₂O₂-generating systems (for example, chloroplasts, mitochondria and peroxisomes), crosses the tonoplast membrane and enters into the vacuole using tonoplast aquaporins (gray transporter in the figure). 1, Prx; 2, chemical reactions; 3, dehydroascorbate reductase; 4, glutathione reductase. AA, ascorbate; DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized form of glutathione; MDHA, monodehydroascorbate; PhOH, phenolics; PhO[•], phenoxyl radicals. Adapted from Takahama (2004).

In the absence of AA, the phenoxyl radicals can react between them giving rise to the corresponding polymers (Pérez, 2002). The scheme of the two reactions is the following:

Apart from Prxs, polyphenol oxidases (catechol oxidases and laccases) can also catalyze the oxidation of flavonoids, in the presence of oxygen, *in planta* (Pourcel *et al.*, 2007), and generate flavonoid radicals (e.g. phenoxyl radicals and flavonoid *o*-semiquinones) (Figure 1.13). Flavonoid radicals are highly reactive, with a lifetime of ~200 μs , and tend to form polymers in the absence of AA (Pourcel *et al.*, 2007). It has been reported that the formation of PAs might also function as a mechanism for H_2O_2 -scavenging, and therefore plant cell detoxification under different stress conditions (Hernández *et al.*, 2006; Pourcel *et al.*, 2007)

Figure 1.13. Enzymatic reactions of the three key enzymes involved in flavonoid oxidation in plants: polyphenol oxidases (catechol oxidase and laccase) and class III peroxidases. The three key enzymes for flavonoid oxidation in plants. From Pourcel *et al.* (2007).

1.3. Objectives

In view of the background described in the introduction, the main objectives of this work are focused on the examination of the oxidative stress signatures and the metabolic adjustments of plants in response to the adverse conditions prevailing in mine-tailings under semi-arid Mediterranean conditions. The study was conducted in different populations of pioneer plant species, including both herbaceous and woody plants, colonizing tailings of the Cartagena-La Unión Mining District. Since these mine tailings are highly polluted with Pb, the effects of acute and chronic lead exposure in one of these pioneer species, *Zygophyllum fabago*, under laboratory-controlled conditions, will also be analyzed in order to identify the possible tolerance mechanisms that this species used to counteract Pb toxicity.

The overall objectives described above were split into the following partial objectives:

- I.
 - i) To compare the antioxidative/oxidative profile along with key growth- and HMM stress-related parameters on field-grown pioneer plant species in both metalliferous mine tailings and in a non-mining site during Mediterranean spring and 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, Ni, Pb, Zn and Sb) associated with roots of these plants on the selected sites, and
 - iii) to identify inter-correlations among the different parameters evaluated using unsupervised and supervised multivariate statistical methods. The plant species selected in this study were the following:
 - a. *Zygophyllum fabago*
 - b. *Dittrichia viscosa*
 - c. *Pinus halepensis* Mill.
- II. Under laboratory-controlled conditions, and using *Z. fabago* plants, the partial objectives were:
 - a.
 - i) To examine the influence of SA pretreatment (24 h, 0.5 mM SA) on the accumulation and distribution of Pb in a metalicolous ecotype of *Z. fabago* during short-term (7 days) exposure to a high but still tolerable

concentration of Pb [0.75 mM Pb(NO₃)₂]. ii) To investigate the effects of Pb and SA pretreatment on antioxidative response, H₂O₂ production and SA content in leaves, stems and roots, and iii) to identify possible mechanisms of SA amelioration of Pb responses in this ecotype.

- b. To evaluate how acute Pb exposure modulates *Z. fabago* growth, photosynthetic reactions, carbohydrate and pigment contents, and rubisco activity and to identify the antioxidant strategy that this species used to counteract Pb toxicity in both shoots and roots.
- c. To analyze the gene expression and activity profiles of catalase and ascorbate-glutathione cycle enzymes in response to chronic Pb stress, with the main aim of establishing whether these genes/enzymes are relevant for adaptation to chronic lead exposure in both metalicolous and non-metallicolous *Z. fabago* seedlings.

Objectives: Graphical Abstract

Figure 1. Abstract of objectives.

PARTE I:
EXPRESSION PATTERNS OF ANTIOXIDANT
SYSTEMS IN PIONEER PLANTS GROWN IN
MINE TAILING

CHAPTER 2

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

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Graphical Abstract

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.

2.1. 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 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.

Keywords

Zygophyllum fabago; antioxidative/oxidative profiles; mine tailings piles; Mediterranean climate; biomarkers

2.2. 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 (Párraga-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; Párraga-Aguado *et al.*, 2014a).

For the last 30 years, more than 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; Párraga-Aguado *et al.*, 2014a; 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 amongst 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.

2.3. Materials and Methods

2.3.1. Plant sampling and soil analysis

Samples of *Z. fabago* L. leaves were obtained from plants growing spontaneously in the Cartagena-La Union 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 (Párraga-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 (ET_o) exceeded rainfall by a factor of 6 throughout the year (ET_o 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 greatest rainfall occurred and May 2013 followed a rainy month of April (80 mm rainfall) and was wetter than May 2012 (Supplemental Fig. S2).

Figure S1. Representative pictures of the three populations of *Z. fabago* plants growing in a non-mining area (A and B) and in Agustin (C and D) and Mercader (E and F) mine tailings piles in late spring (upper panel) and late summer (lower panel).

Figure S2. Seasonal variations in weather conditions (monthly precipitation, monthly average minimum and maximum temperatures (T), 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.

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 (Ca^{2+} , Mg^{2+} , K^{+} , Na^{+} , Cl^{-} , SO_4^{2-}), and HMM concentrations (As, Cd, Cu, Mn, Pb, Zn) were carried out according to standard methods (Párraga-Aguado *et al.*, 2014a).

2.3.2. 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 (%) [$\text{RWC} = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$]. 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.*, 2013a). 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 hours. Then, the tubes were placed at 100 °C for 5 min, cooled to room temperature and centrifuged (12,000 *g* for 10 min). The reducing sugar equivalents present in the supernatant were quantified using the anthrone assay.

2.3.3. 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 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was used for calibration. DPPH and ABTS radical scavenging activities were expressed as micromol of gallic acid equivalents (GAE) per gram fresh weight.

2.3.4. 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.*, 2013a). Total alkaloid content was determined spectrophotometrically, at 465 nm using the Dragendorff's reagent as previously described (López-Orenes *et al.*, 2014).

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

The 2,4-dinitrophenylhydrazone 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.*, 2013a). H₂O₂ contents were determined by the ferric-xylenol orange (FOX1) method (López-Orenes *et al.*, 2013a). The superoxide radical was detected by its ability to oxidize hydroxylamine to nitrite (Jiang and Zhang, 2001).

2.3.6. 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*-dimethylamino-cinnamaldehyde 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.*, 2013a).

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 Arnov'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 Arnou's reagent (10 % (w/v) NaNO_2 and 10% (w/v) Na_2MoO_4), 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 $\epsilon_{545} = 34.7 \text{ mM}^{-1} \text{ cm}^{-1}$ (Vermerris and Nicholson 2006).

2.3.7. 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.*, 2013a). PAL activity was determined spectrophotometrically by following the conversion of L-phenylalanine into *trans*-cinnamic acid ($\epsilon_{290} = 9.5 \text{ mM}^{-1} \text{ cm}^{-1}$). PRX activity was estimated at 30 °C using tetramethylbenzidine-HCl ($\epsilon_{652} = 39 \text{ mM}^{-1} \text{ cm}^{-1}$) 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.

2.3.8. 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.*, (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 (16,000 g 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 μ l of *Acinetobacter* sp. ADPWH-*lux* ($OD_{600}=0.4$) 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 μ M) and negative controls were read in parallel with the leaf samples. Four replicated measurements were run for each sample.

2.3.9. 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.

2.4. Results

2.4.1. 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 Agustín, 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, Hermann *et al.*, 2016). EC was higher in Agustín and Mercader tailings piles (2-fold) than in control soil samples.

Table 1. Main characteristics and metal(loid) concentrations in the rhizosphere soils of mining (Agustín 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	Agustín	Mercader
pH*	7.8 ± 0.1^a	7.2 ± 0.1^b	7.2 ± 0.1^b
EC* (dSm ⁻¹)	1.7 ± 0.3^b	$2.7 \pm 0.2^{a,b}$	3.5 ± 0.1^a
DOC (mg kg ⁻¹)	403.5 ± 48.3^a	35.1 ± 9.4^b	18.1 ± 2.8^b
TN (g kg ⁻¹)	4.3 ± 0.9^a	0.3 ± 0.1^b	0.2 ± 0.1^b
Available-P (mg kg ⁻¹)	194.8 ± 44.1^a	8.7 ± 1.7^b	7.4 ± 1.1^b
Water extractable ions ¹			
K ⁺ (mg kg ⁻¹)	965 ± 414^a	131 ± 23^b	36 ± 6^b
Na ⁺ (mg kg ⁻¹)	250 ± 55^a	288 ± 50^a	250 ± 75^a
Ca ²⁺ (mg kg ⁻¹)	1990 ± 225^b	3040 ± 145^a	3015 ± 5^a
Mg ²⁺ (mg kg ⁻¹)	90 ± 5^b	195 ± 20^b	640 ± 50^a
SO ₄ ²⁻ (mg kg ⁻¹)	995 ± 414^a	8035 ± 255^b	9736 ± 219^c
Cl ⁻ (mg kg ⁻¹)	775 ± 290^a	85 ± 7^c	300 ± 84^b
Total metal(loid)s ²			
As (mg kg ⁻¹)	15 ± 14^b	190 ± 26^a	414 ± 181^a
Cd (mg kg ⁻¹)	8 ± 2^b	45 ± 23^a	23 ± 10^a
Cu (mg kg ⁻¹)	47 ± 11^b	70 ± 4^a	90 ± 14^a
Mn (mg kg ⁻¹)	703 ± 147^b	7929 ± 59^a	12085 ± 2391^a
Pb (mg kg ⁻¹)	647 ± 56^b	3439 ± 363^a	2620 ± 290^a
Zn (mg kg ⁻¹)	547 ± 111^c	5368 ± 40^a	3517 ± 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)]

2.4.2. 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 greater than 0.95 and with more than 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).

Figure 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 nonmining (control, black color) and mining tailings piles Agustín (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.)

Figure 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.

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).

Figure 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.

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.

Figure 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.)

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 (log2 ratio between 0.2-0.3; Table 2).

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

Table 2. Normalized log 2 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 log 2 ratios higher than 1 are highlighted with red background and log 2 ratios lower than 1 are highlighted with blue background.

Population	Agustin								Mercader							
Month	May				September				May				September			
Year	2012		2013		2012		2013		2012		2013		2012		2013	
	Ratio	Pvalue	Ratio	Pvalue	Ratio	Pvalue	Ratio	Pvalue	Ratio	Pvalue	Ratio	Pvalue	Ratio	Pvalue	Ratio	Pvalue
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
HCAs	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

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, (López-Orenes *et al.*, 2014). 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 (log2 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 (log2 ratio between 1.4 and 2). A similar trend to that observed for lipid peroxidation was seen for H₂O₂ levels, and the decrease in the O₂^{•-} 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 (Figure 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 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).

Table S1. Mean and standard error values for all analyzed physiological and antioxidative/oxidative parameters measured in leaves of *Z. fabago* plants growing in non-mining (control) in both late spring and summer in 2012 and 2013.

Parameters	Control			
	Spring		Summer	
	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	1.84 ± 0.14	2.85 ± 0.10	3.16 ± 0.08	4.24 ± 0.16
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	0.63 ± 0.05	3.93 ± 0.10	7.39 ± 0.47	1.23 ± 0.06
Alkaloids (nmol harmaline eq. g^{-1} FW)	4.84 ± 0.28	9.79 ± 0.30	10.01 ± 0.56	13.82 ± 0.91
C=O (nmol C=O mg protein $^{-1}$)	31.36 ± 1.42	85.79 ± 1.84	43.18 ± 4.55	33.42 ± 1.15
Chl a ($\mu\text{g g}^{-1}$ FW)	892 ± 32	734 ± 17	469 ± 5	467 ± 8.93
Chl b ($\mu\text{g g}^{-1}$ FW)	701 ± 48	414 ± 6	326 ± 33	304 ± 4.21
DHA ($\mu\text{mol g}^{-1}$ FW)	1.77 ± 0.11	2.05 ± 0.07	1.31 ± 0.03	1.08 ± 0.07
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	2.00 ± 0.11	1.27 ± 0.09	2.73 ± 0.07	2.17 ± 0.11
Flavanols ($\mu\text{mol (+) catechin eq. g}^{-1}$ FW)	0.33 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
Flavonoids ($\mu\text{mol rutin eq. g}^{-1}$ FW)	1.84 ± 0.22	1.89 ± 0.03	0.61 ± 0.07	2.47 ± 0.05
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	9.66 ± 0.56	6.91 ± 0.34	12.47 ± 0.43	10.64 ± 0.34
GSH ($\mu\text{mol g}^{-1}$ FW)	1.42 ± 0.13	2.14 ± 0.07	2.30 ± 0.09	2.94 ± 0.11
GSSG ($\mu\text{mol g}^{-1}$ FW)	0.55 ± 0.04	0.35 ± 0.03	0.35 ± 0.02	0.27 ± 0.01
H ₂ O ₂ (nmol g^{-1} FW)	500 ± 37	249 ± 8	390 ± 16	307 ± 12
HCAAs ($\mu\text{mol caffeic acid eq. g}^{-1}$ FW)	1.31 ± 0.28	0.91 ± 0.17	0.48 ± 0.05	0.84 ± 0.12
iPRX (nkat mg $^{-1}$ protein)	1.32 ± 0.09	7.5 ± 0.76	1.69 ± 0.10	1.25 ± 0.04
Lignin (mg LTGA g $^{-1}$ DW)	8.00 ± 0.17	8.11 ± 0.07	7.70 ± 0.17	9.28 ± 0.28
MDA (nmol g^{-1} FW)	48.49 ± 3.70	22.27 ± 1.32	30.41 ± 2.18	51.86 ± 2.45
NPT ($\mu\text{mol g}^{-1}$ FW)	300 ± 7	593 ± 13	499 ± 4.10	391 ± 7.49
O ₂ $^{\bullet-}$ (nmol g^{-1} FW)	158 ± 11	127 ± 5	173 ± 17	101 ± 12
PAL (nmol <i>trans</i> -cinnamic acid h $^{-1}$ mg $^{-1}$ protein)	10.80 ± 1.20	15.00 ± 1.30	50.40 ± 2.40	21.60 ± 3.00
PAs ($\mu\text{mol cyanidin eq. g}^{-1}$ FW)	0.25 ± 0.01	0.24 ± 0.01	0.29 ± 0.01	0.22 ± 0.01
TPC ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	6.70 ± 0.27	7.99 ± 0.24	10.15 ± 0.33	12.87 ± 0.29
Proline ($\mu\text{mol g}^{-1}$ FW)	11.39 ± 0.32	10.46 ± 0.28	9.80 ± 0.41	9.46 ± 0.20
Protein (mg g^{-1} FW)	65.79 ± 1.91	83.61 ± 2.21	46.34 ± 1.36	49.55 ± 1.39
RWC (%)	79.80 ± 1.30	80.40 ± 0.80	70.80 ± 0.70	67.95 ± 0.50
SA (nmol g^{-1} FW)	15.62 ± 0.25	15.5 ± 0.11	17.94 ± 0.25	20.34 ± 0.25
SAG (nmol g^{-1} FW)	22.08 ± 0.37	21.07 ± 0.12	25.19 ± 0.44	17.81 ± 0.21
SOD (U mg $^{-1}$ protein)	42.17 ± 6.57	63.26 ± 4.07	20.07 ± 1.42	17.03 ± 2.29
sPRX (nkat mg $^{-1}$ protein)	0.46 ± 0.03	1.10 ± 0.02	4.88 ± 0.60	1.68 ± 0.07
Starch ($\mu\text{g g}^{-1}$ FW)	123 ± 5	142 ± 12	167 ± 6	159 ± 5
Sugars ($\mu\text{g g}^{-1}$ FW)	13.83 ± 0.39	12.44 ± 0.34	21.06 ± 0.86	18.77 ± 0.33

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).

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

2.5. 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 & 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 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, Hubberten, Saito, & Hoefgen, 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 (S Munné-Bosch & 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 contribute to the survival of severely stressed plants by allowing nutrient recycling and reallocation from dying to developing tissues (Guiboileau *et al.*, 2010; Sergi Munné-Bosch & 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 $\text{O}_2^{\bullet-}$ 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 & Galisteo, 2003; D. H. 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; Andejelkovic *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 & 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.

PART I:
EXPRESSION PATTERNS OF ANTIOXIDANT
SYSTEMS IN PIONEER PLANTS GROWN IN
MINE TAILING

CHAPTER 3

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

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Graphical Abstract

Highlights

- No major seasonal changes in AA, GSH and ROS were observed in *D. viscosa* populations
- Phenolics seem to play a key role in *D. viscosa* acclimation to metal-polluted soils
- PAL, PRX, soluble and cell-wall phenolics help to discriminate *D. viscosa* populations
- High accumulation of Pb and Zn in leaves did not affect physiology of *D. viscosa*
- High levels of caffeic acid are present in cell walls of mining *D. viscosa* population

3.1. Abstract

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

Keywords

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

3.2. Introduction

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

Dittrichia viscosa (L.) W. Greuter (woody fleabane) is an evergreen herbaceous perennial Mediterranean plant species (Asteraceae) found in ruderal environments (Al Hassan *et al.*, 2016; Parolin *et al.*, 2013), including mine tailings highly polluted by a broad range of toxic metal(loid)s, where it accumulates As, Cd, Pb, and Zn (Conesa *et al.*, 2011; Fernández *et al.*, 2013; Pérez-Sirvent *et al.*, 2012). Thus, taken into account its pioneer character and its ability to accumulate high metal concentrations in shoots, this species may improve edaphic properties of tailing soils, and thus, by ameliorating stressful conditions, may pave the way for the recruitment of other less tolerant plant species (Párraga-Aguado *et al.*, 2013).

A common hallmark of plant response to abiotic stresses, including metal(loid) exposure, is an over-production of reactive oxygen species (ROS) into cells (Gill and Tuteja, 2010;

Schützendübel and Polle, 2002). ROS in conjunction with the antioxidant network determine the cellular redox environment, which results in redox signaling appropriate to environmental stimuli and developmental cues, leading to growth and acclimation responses (Noctor *et al.*, 2014). Despite the fact that tolerance to metal(loid)s can vary significantly amongst plant species, there is ample evidence that the strengthening of the antioxidant network would be essential for restoring cellular redox-homeostasis and metabolism functions under stress (for review, see Schützendübel and Polle 2002; Sharma and Dietz 2009; Gill and Tuteja 2010; Hossain *et al.* 2012; Singh *et al.* 2015). However, most studies dealing with the effect of metal(loid)s exposure on antioxidative/oxidative stress-related markers in plants have been performed under laboratory-controlled conditions. Scarce information can be found about stress biomarkers in plants growing in their natural environment, where they are concurrently exposed to diverse environmental stress factors. Therefore, the acclimation responses of plants to combined stress might require conflicting or antagonistic responses (Shaar-Moshe *et al.*, 2017; Suzuki *et al.*, 2014). Recent studies have shown that plant acclimation to stress combination elicits specific physiological and molecular responses that cannot be inferred from individual stress treatments, and such responses are characterized by changes in ROS levels, lipid peroxidation, alterations in the expression/activity of ROS-scavenging enzymes, and higher content of antioxidants such as ascorbate (AA), glutathione (GSH), carotenoids and phenolic compounds (Choudhury *et al.*, 2016; Martinez *et al.*, 2016). Phenolic compounds comprise a wide and diverse group of secondary metabolites, and have been shown to play significant roles in plant defense, structural support, modulation of plant cell growth and differentiation, and survival (Ferrer *et al.*, 2008). Among phenolics, flavonoids are considered powerful antioxidants due to their ability to prevent ROS production, as well as to quench ROS and to chelate metal ions, thus acting as modulators of ROS-signaling processes (Agati *et al.*, 2012; Brunetti *et al.*, 2015). Recent evidence has shown that environmental stresses like drought and heat could regulate the levels of nuclear flavonoids (Mouradov and Spangenberg, 2014), which, in turn, could act as regulators of the activity of various protein kinases involved in the ROS-signal transduction pathways that control cell growth and differentiation (Brunetti *et al.*, 2015). Since in Mediterranean areas high sunlight irradiance, high temperatures and severe drought are key factors limiting plant growth and development, and since, furthermore the cellular antioxidative/oxidative status plays a pivotal role in the capability of plants to cope with oxidative stress induced by environmental factors, the purpose of the current

work was (i) to compare the antioxidative/oxidative profile along with key growth parameters of two populations of *D. viscosa*, one grown on a multi-metal(loid) polluted mine tailing (Agustin) and the other in a non-mining site (control), during late spring (May) and late summer (September) in two consecutive years (2012 and 2013); (ii) to identify any possible nutrient imbalance and to assess their bioaccumulation capacity of potentially harmful metal(loid)s under stress combination; (iii) to carry out an edaphic characterization of the rhizosphere soil (fertility parameters and total concentrations of As, Cd, Cu, Mn, Ni, Pb, Zn and Sb) associated with *D. viscosa* roots on the selected sites, and (iv) to identify inter-correlations among the different biochemical parameters evaluated in both seasons as well as associations between plant markers and environmental factors using different multivariate statistical methods.

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

3.3. Materials and Methods

3.3.1. Plant and soil sampling and soil analysis

D. viscosa leaves were obtained from plants growing spontaneously in the Cartagena-La Unión Mining District (SE of the Iberian Peninsula) in one tailings pile (Agustín; 37°36'20" N, 0°50'15" W) and in a non-mining area (37°35'47" N, 0°49'26" W) located about 1.5 km away from this mining site (Supplemental Fig. S1). This mining area contains one of the largest Pb and Zn content in the SE of Europe (Pérez-Sirvent *et al.*, 2012), and the two sampling zones are located in a natural park which includes Aleppo pine forests and endemic xerophytic thickets (Párraga-Aguado *et al.*, 2013). During 2012 and 2013, the average annual rainfall of the zone was ~ 215 mm, the potential evapotranspiration was ~ 1285 mm yr⁻¹ and ~ 18 °C the annual average air temperature (Supplemental Fig. S2). Taking into account the proximity of the two sampling areas, it was assumed that all plants from the two populations (Supplemental Fig. S1) were exposed to similar weather conditions. 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).

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

Figure S2. Seasonal variations in weather conditions (monthly precipitation, monthly average minimum and maximum temperatures, and monthly average reference evapotranspiration [ETo]) from December 2011 to September 2013. Data were collected by an automatic weather station located near the experimental site. Each sampling time are indicated by asterisks.

The uppermost fully expanded leaves from *D. viscosa* plants belonging to the two 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) (Supplemental Fig. S3). In all sampling periods, at least one hundred leaves of each population were washed thoroughly with tap and distilled water, gently blotted on filter paper, and randomly divided into five groups. One group (twenty leaves) was used to determine the relative water content, and each one of the remaining four biological replicates, were divided into two subsamples, one of them was immediately frozen in liquid nitrogen, and stored at -80°C until analyzed, and the second one was dried at 60°C for 72 h for elemental analysis. Rhizosphere soil, sampled from the top 20 cm, were also collected from four selected plants, and transferred under aseptic conditions to laboratory. Soil analyses were carried out as described in Párraga-Aguado *et al.* (2014a). In short, soil pH, electrical conductivity (EC), dissolved organic carbon (DOC), and water extractable ions (Ca^{2+} , K^{+} , Mg^{2+} , Na^{+} , Cl^{-} , SO_4^{2-}) were determined in a 1:5 soil to water suspension after shaking for 2 h. Equivalent calcium carbonate (% CaCO_3) was estimated using the Bernard calcimeter method. Particle size distribution was determined following the method of Bouyoucos densimeter (Gee and Bauder, 1986). Total nitrogen (TN) was determined

using the Kjeldahl method (USDA, 1996). Total metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, Zn, and Sb) were measured by X-Ray Fluorescence (Buker S4 Pioneer).

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

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

Leaf dried tissues (~0.5 g), finely ground, were incinerated at 550 °C for 3 h prior to adding 1 mL of concentrated nitric acid. The resulting extracts were diluted to 25 mL

with MilliQ water and filtered through CHM F2041-110 ashless filter papers. The concentration of Cl, P and S were analyzed using an ion chromatographer (Metrohm). The Ca, K, Mg, and Na contents were analyzed using a flame atomic absorption spectrometer (Unicam 969 AA). Nitrogen in leaf samples was determined using a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK). Metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, and Zn) were determined by inductively coupled plasma-mass spectrometry (Agilent 7500A, detection limit 0.001 mg L^{-1}). Plant analyses were referenced using a CTA-VTL-2 certified material (Virginia tobacco leaves), and the percentage of recoveries ranged between 89 and 110%.

3.3.3. Plant performance measurements

The evaluation of the physiological status of the two *D. viscosa* populations was carried out by measuring leaf 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 (1 mL per 0.1 g tissue) using sonication (37 kHz) until the extracts were colorless (x2). The supernatants obtained after centrifugation (15,000xg for 15 min at 4°C) were used to determine the levels of photosynthetic pigments using the equations reported by Lichtenthaler and Wellburn (1983).

3.3.4. Total antioxidant activity and non-enzymatic antioxidants determinations

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

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

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

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

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

Supernatants of methanolic extracts, obtained as explained in section 2.3, were used for the spectrophotometric determination of different types of phenolics basically as described in a previous report (López-Orenes *et al.*, 2017). Briefly, the content of soluble total phenol compounds (TPC) was measured by the Folin-Ciocalteu method (Everette *et al.*, 2010) and expressed as gallic acid equivalents (GAE). Flavanols were determined using *p*-dimethylaminocinnamaldehyde (DMACA) reagent and expressed as (+)-catechin equivalents (López-Arnaldos *et al.*, 2001). Total flavonoids were measured by the aluminum chloride assay using rutin as a standard (Kim *et al.*, 2003), and total hydroxycinnamic acids (HCAs) were quantified using the Arnow's reagent and caffeic acid as a reference compound. The pellets of the methanol extracts, after thoroughly washing with ethanol, were air-dried at 60 °C and used for lignin determination by the lignin-thioglycolic acid method (Hatzilazarou *et al.*, 2006).

For the determination of the content of cell wall-associated proanthocyanidins (PAs), the absorbances at 545 nm of the supernatants obtained after the acid attack on washed cell

wall pellets were determined, and the results were expressed as cyanidin equivalents by using an $\varepsilon_{545} = 34.7 \text{ mM}^{-1} \text{ cm}^{-1}$ after Vermerris and Nicholson (2006).

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

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

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

RP-HPLC (Reversed phase-high pressure liquid chromatography) assays were performed with a liquid chromatographic system equipped with a Waters Alliance 2695 separations module (Waters, Milford, MA, USA), a variable-wavelength diode array detector Waters 2996 and controlled by Empower Pro software. A Luna C18 column (250 mm $\times$ 4.6 mm, 5 μm particle size; Phenomenex) was employed for separations. Chromatographic analyses were carried out at 40 °C as previously described (Xu *et al.*, 2017). The mobile phase consisted of 0.1% formic acid–water solution (solvent A), and methanol (solvent B). The gradient used was: 95% A, 0 min; 80% A, 15 min; 70% A, 20 min; 63% A, 25 min; 60% A, 40 min; 50% A, 60 min; 95% A, 63 min. The flow rate was 0.8 mL min⁻¹, and the injection volume was 10 μL . Identification of the major phenolic compounds was done by comparison of the retention times and UV spectra with those of reference compounds (caffeic acid, catechin, chlorogenic acid, coumaric acid, epicatechin, ferulic acid, gallic acid, p-hydroxybenzoic acid, protocatechuic acid, quercetin, and rutin). Calibration curves for quantification of analytes were generated by injection of standard

mixtures (standard amounts ranged from 0.1 to 5 nmol) and showed a good linearity ($r^2 > 0.99$) between standard amount and peak area in the chromatograms obtained at the wavelength corresponding to the maximum absorbance of the standard considered. Analysis of samples and standard solutions were done in triplicate. A mid-point calibration standard mixture was injected at the beginning and end of every sample batch (eight samples) in order to assess instrumentation drift in retention time and response factor. Possible analyte carry-over during batch analyses was checked by injecting pure methanol at the end of every sample batch. All solutions and *HPLC* mobile phases were prepared with freshly MilliQ water and *filtered through 0.45 μm nylon filters* (Millipore, Bedford, MA, USA).

3.3.8. Enzymatic assays

The extraction and assay of PAL and soluble and ionically-bound cell wall PRXs in leaf extracts were carried out as previously described (López-Orenes *et al.*, 2013a). PAL activity was determined by following the conversion of L-phenylalanine into *trans*-cinnamic acid (*t*-CA) ($\epsilon_{290} = 9.5 \text{ mM}^{-1}\text{cm}^{-1}$) at 290 nm using a microplate reader (Multiskan GO; Thermo Scientific) and 96-well UV plates (Corning). The activity of the enzyme was expressed in nmol of *t*-CA formation per hour per mg protein. PRX activity was estimated by following the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) ($\epsilon_{652} = 39 \text{ mM}^{-1}\text{cm}^{-1}$) in the presence of 2 mM H_2O_2 , at 652 nm. PRX activity was expressed in nkatal (nkat) per mg protein, which corresponds to the amount of enzyme that oxidizes 1.0 nmol of TMB per second per mg protein. Protein determinations were made with the Bradford protein assay kit (Bio-Rad Laboratories), using bovine serum albumin as a standard.

3.3.9. Quantification of free and conjugated salicylic acid

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

3.3.10. Statistical analysis

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

3.4. Results

3.4.1. Rhizosphere soil parameters

Table 1 depicts the results of rhizosphere soil analysis of Agustin mine tailing and control site where Tukey’s HSD post-hoc test was used to detect significant differences between both populations. Soil fertility parameters in Agustin rhizosphere soil samples were very low, especially the dissolved organic carbon (DOC) concentration, which was up to 12-fold lower ($\sim 9 \text{ mg kg}^{-1}$) than in non-mining samples ($\sim 109 \text{ mg kg}^{-1}$). Contrarily, the EC values and the content of total As, Pb and Zn in tailing samples were more than 10-fold higher than in controls. Moreover, Agustin samples were also characterized by high levels of water extractable divalent ions (SO_4^{2-} , Ca^{2+} , and Mg^{2+}), whereas monovalent ions (Cl^- , K^+ , and Na^+) remained at low levels about one-half or less than those found in rhizosphere control soils. Water extractable SO_4^{2-} and Ca^{2+} levels exhibited a strong correlation which can be related to secondary formation of gypsum in the tailings (Párraga-Aguado *et al.*, 2014a). Agustin samples had a sandy texture ($>70\%$), which negatively correlated with soil fertility parameters (OC, DOC and TN) ($r > -0.7$, $P < 0.05$, see Table S1).

Table 1. Main characteristics and metal(loid) concentrations in the rhizosphere soils of mining and non-mining *D. viscosa* plants. Data represent means ($n=4$) $\pm$ SE. Values followed by different letters in a row are significantly different at $P < 0.05$ by Tukey's HSD post-hoc test.

	Rhizosphere soil sample	
	Control	Agustin
Soil parameter		
pH*	7.99 $\pm$ 0.05 ^a	7.16 $\pm$ 0.04 ^b
CaCO ₃ (%)	20.32 $\pm$ 0.64 ^a	16.72 $\pm$ 1.41 ^a
EC* (dS m ⁻¹)	0.27 $\pm$ 0.02 ^b	2.72 $\pm$ 0.12 ^a
OC (%)	0.78 $\pm$ 0.09 ^a	0.27 $\pm$ 0.03 ^b
DOC (mg kg ⁻¹)	108.96 $\pm$ 18.24 ^a	8.75 $\pm$ 0.99 ^b
TN (g kg ⁻¹)	0.91 $\pm$ 0.07 ^a	0.28 $\pm$ 0.07 ^b
Texture (%)		
Clay	27.51 $\pm$ 1.16 ^a	2.32 $\pm$ 0.87 ^b
Silt	37.10 $\pm$ 4.31 ^a	26.54 $\pm$ 1.60 ^a
Sand	35.39 $\pm$ 3.20 ^b	71.14 $\pm$ 2.38 ^a
Water extractable ions (mg L⁻¹)*		
Cl ⁻	19.82 $\pm$ 2.91 ^a	7.08 $\pm$ 0.14 ^b
SO ₄ ²⁻	20.83 $\pm$ 0.58 ^b	1561.16 $\pm$ 8.10 ^a
Na ⁺	17.20 $\pm$ 2.76 ^a	8.63 $\pm$ 0.59 ^b
K ⁺	12.70 $\pm$ 1.24 ^a	5.54 $\pm$ 0.76 ^b
Ca ²⁺	35.15 $\pm$ 0.25 ^b	628.90 $\pm$ 3.55 ^a
Mg ²⁺	2.67 $\pm$ 0.44 ^b	15.72 $\pm$ 3.32 ^a
Total metal(loid)s (mg kg⁻¹)**		
As	18.25 $\pm$ 11.38 ^b	207.50 $\pm$ 13.77 ^a
Cd	17.25 $\pm$ 11.25 ^a	21.00 $\pm$ 5.07 ^a
Cu	44.83 $\pm$ 2.83 ^b	75.75 $\pm$ 0.63 ^a
Mn	1635.00 $\pm$ 61.71 ^b	9686.75 $\pm$ 609.57 ^a
Ni	39.25 $\pm$ 1.18 ^a	27.75 $\pm$ 3.71 ^b
Pb	397.50 $\pm$ 23.39 ^b	4116.25 $\pm$ 430.01 ^a
Zn	264.25 $\pm$ 16.0 ^b	4791.00 $\pm$ 234.04 ^a
Sb	1.00 $\pm$ 0.00 ^b	55.75 $\pm$ 6.03 ^a

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

**European Union threshold (mg kg⁻¹) for agricultural soils: As 5, Cd 1, Cu 100, Pb 60,

Zn 200 [extract, Tóth *et al.* (2016)].

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

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

1995). In fact, P is known to be one of the critical limiting elements for plant growth in terrestrial ecosystems (Güsewell, 2004).

The analyses of metal(loid)s showed that Agustín plants accumulated significantly higher levels (>20-fold) of As, Cd and Pb in leaf tissues compared to non-mining plants (Table 2). Generally, summer leaves accumulated higher levels of these metal(loid)s than spring ones. The highest accumulated metal(loid)s were Zn (~680 mg kg⁻¹ DW), Pb (~380 mg kg⁻¹ DW), and As (~30 mg kg⁻¹ DW). The amount of Zn and Pb accumulated exceeded the critical toxicity levels for plants, whose upper limits are 300 and 28 µg g⁻¹ DW, respectively (Krämer, 2010). Nevertheless, the mean bioaccumulation factors (BCF), defined as the ratio of total metal concentration in leaf biomass with respect to total metal concentration in the soil, for As, Pb and Zn were clearly lower than 1 (0.11, 0.06, and 0.10, respectively).

Table 2. Analysis of mineral nutrient and metal(loid) contents in Agustin *D. viscosa* leaves. Data are expressed as fold-differences relative to their corresponding non-mining (control) samples. The statistical significance of each ratio was tested by Student's test ($P < 0.05$; $n = 4$). Statistically significant fold-differences (mean ratios) >1.0 are highlighted in red while those <1.0 are highlighted in blue. The brighter the color, the higher the statistical significance (P -value). The scale bar is shown below the table. The mean control values are shown in parentheses and both macronutrient and metal(loid) values are expressed in mg kg^{-1} DW.

	Spring				Summer			
	2012		2013		2012		2013	
	Fold difference	P -value	Fold difference	P -value	Fold difference	P -value	Fold difference	P -value
Macronutrients*								
N	0.84 (15,440)	0.1389	0.96 (19,300)	0.7461	0.86 (13,000)	0.1425	1.06 (14,470)	0.1187
K	1.04 (8,988)	0.7589	0.63 (11,105)	0.0115	0.54 (10,947)	0.0070	0.72 (11,233)	0.0027
Ca	0.68 (7,471)	0.0734	1.21 (4,627)	0.2326	1.00 (5,611)	0.9845	0.81 (5,859)	0.0912
Mg	2.86 (1,665)	0.0003	3.60 (1,180)	0.0016	2.87 (2,474)	0.0002	3.06 (2,218)	0.0019
P	0.83 (984)	0.0966	1.04 (922)	0.7817	0.53 (1,096)	0.0018	0.82 (1,052)	0.2837
S	2.66 (2,879)	0.0005	1.03 (2,835)	0.6930	2.17 (3,555)	0.0025	2.19 (3,571)	0.0004
Metal(loid)s**								
As	21.71 (1.38)	0.0084	12.59 (1.37)	0.0007	28.47 (1.11)	0.0012	23.83 (0.63)	0.0026
Cd	3.16 (0.60)	0.0908	20.53 (0.18)	0.0158	22.38 (0.13)	0.0254	25.70 (0.05)	0.0020
Cu	0.74 (15.30)	0.1254	0.64 (15.05)	0.0077	0.87 (13.82)	0.3170	0.92 (11.99)	0.4642
Mn	1.77 (174.74)	0.0933	3.27 (127.88)	0.0032	3.85 (176.10)	0.0103	2.04 (164.26)	0.0088
Ni	0.97 (1.39)	0.7003	5.06 (0.69)	0.0038	1.22 (1.22)	0.0885	3.95 (1.27)	0.0021
Pb	16.47 (11.41)	0.0089	31.00 (7.08)	0.0021	38.36 (9.68)	0.0011	23.82 (7.08)	0.0017
Zn	4.88 (82.04)	0.0079	5.89 (64.24)	0.0013	11.86 (57.74)	0.0001	4.94 (72.94)	0.0002

*Average normal macronutrient concentrations (g kg^{-1} DW) in plants: N, 15; K, 10; Ca, 5; Mg, 2; P, 2; S, 1 [extract, Marschner (1995)].

**Critical toxicity levels in plants (mg kg^{-1} DW): As <2 -80, Cd 6-10, Cu 20-30, Mn 200-3500, Ni 10-50, Pb 0.6-28, Zn 100-300. Threshold for hyperaccumulators (mg kg^{-1} DW): As > 1000 , Cd > 100 , Cu > 300 , Mn > 10000 , Ni > 1000 , Pb > 1000 , Zn > 3000 [extract, Krämer (2010)].

≤ 0.05	≤ 0.005	≤ 0.001	≤ 0.001	≤ 0.005	≤ 0.05
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3.4.3. Multivariate analysis of physiological and antioxidative/oxidative data in *Dittrichia viscosa* leaves

Since metal(loid) toxicity is frequently driven by ROS generation, a wide range of antioxidants, oxidative stress markers, and some physiological parameters such as RWC, photosynthetic pigments, sugars and protein contents were analyzed. All these data were subjected to different multivariate statistical analyses in order to facilitate detection of statistically significant changes between the two *D. viscosa* populations studied. First, a principal component analysis (PCA), based on the correlation matrix as the similarity metric, was carried out for unsupervised dimension reduction. The first three principal components, which explained ~62% of variance, were plotted against each other (Figure 1). As can be seen, PC1 (~34% of the variance) separated the samples collected in spring 2013, *i.e.* the rainiest period, from the others. The first PCA axis was defined, on its positive side, by protein content, AA and soluble phenolic compounds (TPC, HCAs, flavonoids and flavanols), and by protein oxidation (carbonyl group content) and PAL and sPRX activities on the negative side of X-axis. PC2 (~15% of the variance) discriminated the samples from the different seasons and was defined by NPT, PAs and $O_2^{\bullet-}$ on the positive side of Y-axis and cell wall-bound phenols on the negative side. PC3 (~14% of the variance) separated control from Agustin samples, and was defined by TBARS (on the positive side of Y-axis) and RWC (on the negative side). Next, the data were analyzed using a supervised PLS-DA method, and the score and loading plots obtained were quite similar to those found in the PCA (data not shown). Since PLS-DA allows the identification of the most influential biomarkers based on the variable importance in the projection (VIP), we plotted the correlation coefficients for the first three components of PLS-DA and VIP score to clearly identify the potential biomarkers and interpret their physiological significance. As seen in Figure 2, the biomarkers with a VIP score >1 and with absolute correlation value greater than 0.5 with the first PLS-DA component, accounting for ~34% of the total variance, were soluble phenolic compounds (TPC, and flavonoids), protein and protein carbonyl contents, PAL and PRX activities, AA, and the stress-related phytohormone SA (Fig. 2A). For the second PLS-DA component (~15% of the variance; Fig. 2B) the markers were cell wall-bound phenols, proanthocyanidins (PAs), DHA, $O_2^{\bullet-}$ and NPT contents, and for the third component

(~14% of the total variance; Fig. 2C) the variables found were RWC, TBARS, lignin and iPRX.

Figure 1. Score (left) and correlation (right) plots of the first three components of the PCA applied to physiological and biochemical variables measured in leaves of *D. viscosa* plants growing in non-mining [control (Co), black] and mining tailings pile [Agustin (Ag), white] in late spring and summer in 2012 and 2013 (squares, May 2012; circles, September 2012; triangles, May 2013; inverted triangles, September 2013). Circles represent $r^2 = 50\%$ and 100% variability explained by the components. Abbreviations: AA, Ascorbate; ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity; Car, total carotenoids; Chla, Chlorophyll *a*; Chlb, chlorophyll *b*; CWP, cell wall-bound phenols; C=O, protein carbonyl groups; DHA, dehydroascorbate; DPPH, 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity; FA, total flavanols; FO, total flavonoids; FRAP, Ferric Reducing Antioxidant Power; iPRX, ionically-bound cell wall class III plant peroxidase activity; H₂O₂, hydrogen peroxide; HCAs, hydroxycinnamic acids; NPT, total soluble non-protein thiols; O₂⁻, superoxide radical; PAL, phenylalanine ammonia-lyase activity; PAs, cell wall-associated proanthocyanidins; Pro, proline; RWC, relative leaf water content; SA, salicylic acid; SAG, 2-*O*- β -D-glucosylsalicylic acid; sPrx, soluble class III plant peroxidase activity; TBARS, thiobarbituric acid reacting substances; TPC, total phenol content.

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

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

Random forest machine learning algorithm (RF) has recently emerged as an effective method for classification and feature selection in “omics” studies (Touw *et al.*, 2013). In RF, feature importance is measured by randomly permuting the feature in the out-of-bag (OOB) samples and calculating the decrease of classification accuracy (Touw *et al.*, 2013). Figure 3 reflects the ranking of the individual variables analyzed using the mean decrease in accuracy criterion. Thus, based on the change in the curve shape of the mean decrease in accuracy plot, it can be seen that 7 out of the top 11 descriptors are associated with phenolic metabolism, and cell wall-bound phenols was ranked as the most important biomarker. Given the RF ranking of variables (Fig. 3) and both the correlation coefficient and VIP scores for PLS-DA (Fig. 2), the parameters that could be considered as potential markers to differentiate between non-mining (control) and mining (Agustin) plants were cell wall-bound phenols, soluble phenols (TPC and flavonoids), PAL and PRX activities, and the phenolic hormone salicylic acid (SA).

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

To help visualization of the seasonal differences in the antioxidative/oxidative profile in the two *D. viscosa* populations studied, the ratio values (Agustín/control) were log₂-transformed and a two-way complete-linkage hierarchical clustering was performed by using a distance defined in terms of Pearson correlation and represented in a heatmap format (Fig. 4A). Additionally, the differences between sample groups were analyzed by a non-parametric Wilcoxon's test, and the mean ratios of fold changes and their associated *P*-values obtained are given in Supplemental Table 2. The values obtained for representative markers were also presented as box-and-whisker plots (Fig. 4B). As expected, the 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) grouping together (Figure 4A). Interestingly, the values of all the parameters used to assess the physiological status of the leaf in Agustín *D. viscosa* plants (RWC and the content of photosynthetic pigments, proteins, starch and soluble sugars) were quite similar to those found in control plants in both seasons, although a statistically significant increase in the total carotenoid content was noticed in summer samples. The analysis of ROS revealed that Agustín leaves had lower H₂O₂ levels, especially in summer, whereas the O₂^{•−} levels only significantly differed from controls in summer 2013 (mean ratio 1.5, Supplemental Table S2). Analyses of TBARS and carbonyl group contents, used as oxidative damage markers, were somewhat higher in Agustín leaves compared with controls although, the levels of the major redox buffers of the plant cells, reduced AA and GSH, were comparable between the two *D. viscosa* populations in both seasons (Fig. 4). The analyses of total NPT, which included GSH and phytochelatin, showed a very slight increase in Agustín leaves, whereas a significant reduction in the foliar concentrations of Pro (mean ratio ~0.5, Supplemental Table S2) as well as a decline, which was generally more pronounced in summer, in the contents of TPC,

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

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

	Spring				Summer			
	2012		2013		2012		2013	
	Ratio	<i>P</i> -value	Ratio	<i>P</i> -value	Ratio	<i>P</i> -value	Ratio	<i>P</i> -value
Ascorbate	1.08	0.0025	1.13	0.0005	1.01	0.9576	1.05	0.8501
ABTS	0.76	0.0005	0.94	0.0640	0.77	0.0005	0.86	0.0093
Carotenoids	1.13	0.4560	1.42	0.6657	1.59	0.0031	1.73	0.0210
Chl <i>a</i>	1.03	0.8501	1.06	0.1514	1.03	0.8501	1.18	0.0210
Chl <i>b</i>	1.04	0.0210	1.11	0.0015	1.06	0.4238	1.18	0.0122
Carbonyl	1.63	0.0034	1.20	0.0093	1.15	0.1294	2.05	0.0005
CW-Phenol	0.90	0.0005	1.38	0.0005	1.11	0.0005	1.41	0.0005
DHA	1.06	0.6772	1.01	0.9697	1.14	0.0068	1.08	0.0024
DPPH	0.82	0.0049	0.84	0.0005	0.87	0.0122	0.79	0.0049
Flavanols	0.76	0.0034	1.12	0.1099	0.90	0.1954	1.06	0.0034
Flavonoids	0.81	0.0005	0.98	0.9697	0.79	0.0005	0.77	0.0005
FRAP	0.80	0.0010	0.89	0.0005	0.87	0.0005	0.75	0.0269
GSH	0.92	0.2661	0.81	0.0005	0.98	0.4238	0.90	0.1514
H ₂ O ₂	0.89	0.3394	0.96	0.9097	0.40	0.0005	0.68	0.0342
HCAs	0.89	0.1763	1.00	0.8501	0.79	0.0049	0.78	0.0010
iPRX	0.73	0.0024	0.70	0.0025	0.77	0.0210	0.81	0.0005
Lignin	0.77	0.0005	0.67	0.0005	0.91	0.3394	0.78	0.0005
NPT	1.12	0.0049	1.11	0.0049	1.07	0.0024	1.14	0.0024
O ₂ ^{•-}	1.02	0.8501	1.06	0.0425	1.07	0.0771	1.52	0.0034
PAL	1.76	0.0005	1.85	0.0005	3.54	0.0025	4.17	0.0005
PAs	0.96	0.0024	0.93	0.1954	0.97	0.2535	0.98	0.5548
Proline	0.25	0.0005	0.58	0.0005	0.65	0.0005	0.57	0.0025
Protein	1.01	0.7910	1.23	0.0771	1.01	0.5693	1.01	0.6221
RWC	0.95	0.0012	0.95	0.0012	0.88	0.0012	0.91	0.0012
SA	0.65	0.0024	0.68	0.0024	0.65	0.0024	0.70	0.0024
SAG	0.96	0.1680	0.96	0.0303	0.87	0.0040	0.94	0.0303
sPRX	1.28	0.0024	1.30	0.0005	1.39	0.0025	1.32	0.0025
Starch	1.10	0.0454	1.30	0.0068	1.13	0.0024	1.37	0.0068
Sugars	1.07	0.0122	1.08	0.0024	1.13	0.0651	1.18	0.0025
TBARS	1.48	0.0005	1.67	0.0005	1.37	0.0005	1.19	0.0210
TPC	0.81	0.0005	0.91	0.0005	0.80	0.0005	0.82	0.0005

≤0.05	≤0.005	≤0.001	≤0.001	≤0.005	≤0.05
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Table S3. Mean and standard error values for all analyzed physiological and biochemical parameters measured in mining (Agustin) and non-mining (control) *D. viscosa* leaf samples in both late spring and summer in 2012 and 2013.

Parameters	Control				Agustin			
	Spring		Summer		Spring		Summer	
	2012	2013	2012	2013	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	2.53 $\pm$ 0.06	1.67 $\pm$ 0.03	2.65 $\pm$ 0.12	2.96 $\pm$ 0.07	2.73 $\pm$ 0.05	1.88 $\pm$ 0.03	2.66 $\pm$ 0.14	3.11 $\pm$ 0.12
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	37.55 $\pm$ 0.99	29.54 $\pm$ 1.03	62.89 $\pm$ 1.36	31.34 $\pm$ 1.22	28.54 $\pm$ 1.54	27.67 $\pm$ 0.83	48.17 $\pm$ 1.43	26.83 $\pm$ 0.91
Carbonyl groups (nmol C=O mg^{-1} protein)	4.91 $\pm$ 0.79	26.45 $\pm$ 0.97	10.16 $\pm$ 0.52	7.98 $\pm$ 0.35	7.99 $\pm$ 0.82	31.85 $\pm$ 1.32	11.65 $\pm$ 0.72	16.32 $\pm$ 0.74
Carotenoids ($\mu\text{g g}^{-1}$ FW)	7.22 $\pm$ 1.06	2.55 $\pm$ 0.56	3.61 $\pm$ 0.69	8.78 $\pm$ 1.12	8.17 $\pm$ 1.12	3.83 $\pm$ 1.02	5.62 $\pm$ 0.31	13.59 $\pm$ 2.68
Chla ($\mu\text{g g}^{-1}$ FW)	501.02 $\pm$ 2.31	512.38 $\pm$ 13.70	428.67 $\pm$ 23.51	438.74 $\pm$ 2.10	514.35 $\pm$ 9.84	542.08 $\pm$ 12.72	442.53 $\pm$ 33.78	517.10 $\pm$ 19.32
Chlb ($\mu\text{g g}^{-1}$ FW)	336.27 $\pm$ 3.72	322.83 $\pm$ 9.02	287.64 $\pm$ 13.01	275.26 $\pm$ 2.00	349.48 $\pm$ 5.07	356.55 $\pm$ 9.39	303.76 $\pm$ 18.37	324.31 $\pm$ 12.65
Cell wall phenols ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	65.91 $\pm$ 0.69	74.21 $\pm$ 1.50	81.80 $\pm$ 1.45	91.47 $\pm$ 1.87	59.24 $\pm$ 1.03	102.42 $\pm$ 1.81	90.74 $\pm$ 0.70	129.28 $\pm$ 2.17
DHA ($\mu\text{mol g}^{-1}$ FW)	0.79 $\pm$ 0.04	1.35 $\pm$ 0.07	0.93 $\pm$ 0.10	1.12 $\pm$ 0.05	0.84 $\pm$ 0.12	1.37 $\pm$ 0.07	1.06 $\pm$ 0.07	1.21 $\pm$ 0.04
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	72.50 $\pm$ 3.75	49.09 $\pm$ 1.05	95.77 $\pm$ 2.56	96.15 $\pm$ 7.18	59.26 $\pm$ 3.39	41.10 $\pm$ 1.64	83.01 $\pm$ 2.72	75.55 $\pm$ 5.75
Flavanols ($\mu\text{mol (+) catechin eq. g}^{-1}$ FW)	0.20 $\pm$ 0.01	0.11 $\pm$ 0.01	0.18 $\pm$ 0.01	0.20 $\pm$ 0.01	0.15 $\pm$ 0.01	0.13 $\pm$ 0.01	0.16 $\pm$ 0.01	0.21 $\pm$ 0.01
Flavonoids ($\mu\text{mol rutin eq. g}^{-1}$ FW)	106.49 $\pm$ 4.34	69.73 $\pm$ 3.78	162.53 $\pm$ 4.24	177.43 $\pm$ 6.81	86.28 $\pm$ 3.43	68.62 $\pm$ 1.71	127.57 $\pm$ 0.90	136.84 $\pm$ 4.10
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	298.97 $\pm$ 6.81	248.75 $\pm$ 6.21	359.42 $\pm$ 10.94	276.39 $\pm$ 28.26	238.21 $\pm$ 10.15	220.55 $\pm$ 3.28	311.08 $\pm$ 4.76	207.30 $\pm$ 25.41
GSH ($\mu\text{mol g}^{-1}$ FW)	48.09 $\pm$ 4.75	50.20 $\pm$ 3.53	48.79 $\pm$ 3.02	51.50 $\pm$ 3.19	44.14 $\pm$ 2.56	40.76 $\pm$ 1.43	47.71 $\pm$ 5.04	45.89 $\pm$ 3.98
H ₂ O ₂ (nmol g^{-1} FW)	196.25 $\pm$ 25.43	341.58 $\pm$ 20.14	328.02 $\pm$ 29.54	314.83 $\pm$ 32.89	173.81 $\pm$ 19.69	326.39 $\pm$ 18.93	131.13 $\pm$ 17.18	216.30 $\pm$ 48.96
HCAs ($\mu\text{mol caffeic acid eq. g}^{-1}$ FW)	162.78 $\pm$ 6.04	107.67 $\pm$ 5.09	204.35 $\pm$ 9.93	204.94 $\pm$ 9.92	144.16 $\pm$ 9.15	110.11 $\pm$ 6.57	160.50 $\pm$ 9.67	160.80 $\pm$ 8.71
iPRX (nkat mg^{-1} protein)	72.85 $\pm$ 1.35	160.53 $\pm$ 5.45	94.02 $\pm$ 6.75	71.72 $\pm$ 1.47	53.30 $\pm$ 3.28	112.24 $\pm$ 1.74	72.80 $\pm$ 5.58	58.03 $\pm$ 1.70
Lignin (mg LTGA g^{-1} DW)	16.88 $\pm$ 0.53	21.20 $\pm$ 1.73	12.56 $\pm$ 0.36	15.19 $\pm$ 0.46	12.97 $\pm$ 0.29	14.24 $\pm$ 0.49	11.39 $\pm$ 0.70	11.89 $\pm$ 0.39
NPT (nmol GSH eq. g^{-1} FW)	252.22 $\pm$ 8.01	228.77 $\pm$ 8.19	254.39 $\pm$ 2.32	191.63 $\pm$ 10.30	283.30 $\pm$ 8.37	253.60 $\pm$ 6.59	272.33 $\pm$ 4.16	217.46 $\pm$ 5.14
O ₂ ^{•-} (nmol g^{-1} FW)	238.09 $\pm$ 6.71	171.89 $\pm$ 10.33	178.21 $\pm$ 8.90	105.93 $\pm$ 7.15	242.20 $\pm$ 11.86	181.46 $\pm$ 4.04	189.70 $\pm$ 5.26	158.57 $\pm$ 11.99
PAL (nmol <i>trans</i> -cinnamic acid h^{-1} mg^{-1} protein)	21.48 $\pm$ 0.47	47.44 $\pm$ 3.29	11.03 $\pm$ 0.77	8.00 $\pm$ 0.77	37.73 $\pm$ 2.46	87.89 $\pm$ 8.44	38.08 $\pm$ 2.60	31.62 $\pm$ 4.16
PAs ($\mu\text{mol cyanidin eq. g}^{-1}$ FW)	0.56 $\pm$ 0.01	0.46 $\pm$ 0.02	0.37 $\pm$ 0.01	0.31 $\pm$ 0.01	0.54 $\pm$ 0.01	0.43 $\pm$ 0.02	0.35 $\pm$ 0.01	0.30 $\pm$ 0.01
Phytochelatins (NPT-GSH) (nmol GSH eq. g^{-1} FW)	204.14 $\pm$ 10.68	178.57 $\pm$ 9.34	205.60 $\pm$ 2.68	140.13 $\pm$ 11.79	239.16 $\pm$ 10.08	212.84 $\pm$ 6.92	224.62 $\pm$ 6.85	171.57 $\pm$ 8.11
Proline ($\mu\text{mol g}^{-1}$ FW)	1.66 $\pm$ 0.34	0.34 $\pm$ 0.03	1.25 $\pm$ 0.05	0.48 $\pm$ 0.07	0.42 $\pm$ 0.06	0.20 $\pm$ 0.01	0.81 $\pm$ 0.02	0.26 $\pm$ 0.02
Protein (mg g^{-1} FW)	36.62 $\pm$ 1.11	10.21 $\pm$ 0.53	28.40 $\pm$ 0.71	27.96 $\pm$ 1.40	36.89 $\pm$ 0.68	12.52 $\pm$ 1.11	28.80 $\pm$ 1.42	28.21 $\pm$ 0.78
RWC (%)	81.58 $\pm$ 0.47	82.50 $\pm$ 0.44	83.10 $\pm$ 0.64	80.73 $\pm$ 0.28	76.54 $\pm$ 0.38	77.22 $\pm$ 0.41	73.37 $\pm$ 0.47	73.53 $\pm$ 0.53
SA (nmol g^{-1} FW)	4.73 $\pm$ 0.31	3.71 $\pm$ 0.08	5.44 $\pm$ 0.32	7.85 $\pm$ 0.45	2.96 $\pm$ 0.10	2.49 $\pm$ 0.07	3.45 $\pm$ 0.15	5.42 $\pm$ 0.23
SAG (nmol g^{-1} FW)	2.50 $\pm$ 0.09	2.10 $\pm$ 0.06	2.75 $\pm$ 0.10	4.62 $\pm$ 0.16	2.38 $\pm$ 0.05	2.00 $\pm$ 0.03	2.37 $\pm$ 0.05	4.31 $\pm$ 0.07
sPRX (nkat TMB oxidized mg^{-1} protein)	3.26 $\pm$ 0.09	8.70 $\pm$ 0.19	3.61 $\pm$ 0.06	2.98 $\pm$ 0.07	4.17 $\pm$ 0.09	11.28 $\pm$ 0.23	5.00 $\pm$ 0.27	3.95 $\pm$ 0.11
Starch ($\mu\text{g g}^{-1}$ FW)	269.62 $\pm$ 24.57	294.62 $\pm$ 5.57	297.94 $\pm$ 10.21	276.48 $\pm$ 15.67	296.63 $\pm$ 11.79	382.87 $\pm$ 17.11	333.95 $\pm$ 4.64	374.55 $\pm$ 25.11
Sugars ($\mu\text{g g}^{-1}$ FW)	9.53 $\pm$ 0.07	8.89 $\pm$ 0.29	10.71 $\pm$ 0.21	10.06 $\pm$ 0.12	10.25 $\pm$ 0.23	9.60 $\pm$ 0.17	12.06 $\pm$ 0.55	11.88 $\pm$ 0.20
TBARS (nmol g^{-1} FW)	51.58 $\pm$ 0.81	33.03 $\pm$ 1.48	46.05 $\pm$ 2.17	52.18 $\pm$ 0.78	76.12 $\pm$ 4.01	55.06 $\pm$ 1.39	63.13 $\pm$ 1.55	62.01 $\pm$ 3.49
TPC ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	202.37 $\pm$ 2.52	121.27 $\pm$ 1.45	235.80 $\pm$ 4.75	259.51 $\pm$ 5.60	163.58 $\pm$ 4.38	111.11 $\pm$ 1.84	188.77 $\pm$ 3.38	213.32 $\pm$ 4.14

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

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

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

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

3.5. Discussion

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

This study clearly shows that Agustin *D. viscosa* plants exhibit multiple metal tolerance and high Zn and Pb co-accumulation capacity in photosynthetically active leaf tissues. Moreover, these metallicolous plants were able to accumulate the metalloid As up to 30 mg kg⁻¹ DW, which are in line with the values reported by Conesa *et al.* (2011) and Pérez-Sirvent *et al.* (2012) in mining ecotypes of the same plant species grown within this mining area and/or in its vicinity, and with those found by Pistelli *et al.* (2017) in a iron mining area on Elba Island (Italy). An index commonly used to evaluate the metal(loid) accumulation efficiency in plants is the bioaccumulation factor (BCF) (McGrath and

Zhao, 2003). Plants that have BCF values greater than 1 are considered suitable for phytoextraction, whereas plants with BCF values lower than 1 are preferred for phytostabilization (McGrath and Zhao, 2003). Here, the BCFs obtained for As, Pb and Zn in Agustín *D. viscosa* plants were lower than 1.0, and thus this species could be classified *sensu stricto* as good candidate for preventing the spread of metal(loid) contaminants by erosion. Low BCF values for these elements in different mining *D. viscosa* populations, can be worked out from data previously reported by other authors when the total soil metal concentration and metal(loid) content in leaf tissues are considered (Buscaroli *et al.*, 2016; Conesa *et al.*, 2011; Martínez-Sánchez *et al.*, 2012; Pistelli *et al.*, 2017).

Plant uptake of As has been reported to be greater in sandy soils due to their low levels of Fe and Al oxides (Gulz *et al.*, 2005), thus the bioaccumulation of As found in Agustín leaves is not surprising considering the sandy texture of this mining pile. In addition, it is well known that arsenate, the most abundant environmental form of As in aerobic soils, is an antagonist of phosphate uptake, both competing for the same P_i transporters (Meharg and Hartley-Whitaker, 2002). This is also in line with our observation of a moderate negative correlation between leaf As content and foliar P concentration ($r=-0.42$, $P<0.05$; Supplemental Table S3), which may explain, at least in part, the lower levels of P found in summer 2012 Agustín leaves. Moreover, although it had been reported that Cd accumulation is a constitutive trait in this species (Fernández *et al.*, 2013), in our study the foliar accumulation of Cd did not surpass $4 \text{ mg kg}^{-1} \text{ DW}$. These results can be explained by taking into account the low levels of Cd found in the rhizosphere tailing soils ($21 \pm 5 \text{ mg kg}^{-1}$) in comparison to the several orders of magnitude higher concentration of Zn ($4,791 \pm 234 \text{ mg kg}^{-1}$) and Pb ($4,116 \pm 430 \text{ mg kg}^{-1}$) ions, with all three sharing similar chemical properties (Krämer, 2010).

On the other hand, the nutritional status of Agustín leaves was not affected either by the uptake and translocation of As, Pb and Zn to the aerial part of the plants or by the very low nitrogen ($0.28 \pm 0.07 \text{ g kg}^{-1}$) and dissolved organic carbon concentrations ($8.75 \pm 0.99 \text{ g kg}^{-1}$) found in the mining rhizosphere soils, since the content of the main macronutrients N, K, Ca, Mg, and P (the latter with the exception of the values noticed in summer 2012) in Agustín leaves were quite similar to those found in controls. One possible explanation for these results could be related to the fact that evergreen species, as

D. viscosa, exhibit an efficient internal remobilization of carbon and nutrients from dying leaves to developing tissues (Cherbuy *et al.*, 2001). In addition, the absence of competitors in mining soils (see Supplemental Figure S3D) can also contribute to maintain a scarce, but photosynthetic active leaf canopy under such hard stressful conditions.

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

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

Despite the higher Zn/Pb accumulation found in Agustín *D. viscosa* leaves, no significant differences were found in the levels of chlorophylls, proteins, starch and soluble sugars, suggesting that the photosynthetic metabolism of these leaves was not impaired even in the driest summer period studied (*i.e.*, September 2013). These results are in line with recent proteomic studies that emphasized the importance of maintaining net photosynthesis rate and energy production to perform the energy-demanding processes involved in metal uptake, transport and sequestration in both metal accumulator and hyperaccumulator plants (Bah *et al.*, 2010; Farinati *et al.*, 2009).

The basal levels of carotenoids increased in summer leaves in both *D. viscosa* populations, although more markedly in Agustín plants. Several studies have highlighted the efficiency of carotenoids in the photoprotection mechanisms in different native Mediterranean plant species during summer (Fenollosa *et al.*, 2017; Flexas *et al.*, 2014). It is well known that carotenoids play a dual role in photosynthesis, they can function as accessory light-harvesting pigments and as photoprotective molecules required not only to avoid the generation of single oxygen ($^1\text{O}_2$) from triplet excited chlorophylls but also to quench any $^1\text{O}_2$ produced (Niyogi, 2000). The production of $^1\text{O}_2$ has been reported to increase under high irradiance as well as under other environmental stress conditions which lead to closing of stomata, such as salinity and drought (Gill and Tuteja, 2010). Thus, by increasing the accumulation of these photoprotective pigments, Agustín leaves seemed to be more capable of avoiding the production of $^1\text{O}_2$ and, consequently, to maintain a high photosynthetic efficiency even in the dry season. This finding contrasts with the significant decline in Chl content found in Agustín *Zygophyllum fabago* populations under the same stressful conditions (López-Orenes *et al.*, 2017). Comparing with *D. viscosa*, this species exhibited lower foliar contents of Zn and Pb (~ 300 and ~ 7 mg kg⁻¹ DW, respectively, which fell within the critical range) (Párraga-Aguado *et al.*, 2016), indicating that both species presented a different adaptive strategy to withstand the adverse conditions of the mine tailing and the seasonal variations in this Mediterranean area.

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

The maintenance of the photosynthetic capacity under these drastic concurrent stressful conditions is only possible if ROS levels are kept at concentrations low enough for ensuring adequate metabolic functions in mesophyll cells. Our results revealed a tight control of $O_2^{\bullet-}$ and H_2O_2 levels in Agustín leaves in both seasons, although it was observed a certain degree of oxidative modifications of proteins and lipids (Fig. 4) that seemed to have no significant deteriorating consequences on photosynthetic metabolism, as evidenced by no changes in starch and soluble sugar concentrations, the end products of photosynthesis. In plants, AA and GSH are the major cellular antioxidants and redox buffers involved in redox homeostasis and ROS detoxification (Foyer and Noctor, 2005). In this study, no significant seasonal influence on the levels of AA and GSH were found between Agustín and control *D. viscosa* populations. Moreover, aside from these two major antioxidants, plants contain a wide range of secondary metabolites, most of which are redox-active compounds that may also be important in controlling ROS accumulation (Potters *et al.*, 2010). Even with some limitations, an integrated parameter to evaluate the full spectrum of antioxidant compounds present in a tissue is the total antioxidant capacity (Ghiselli *et al.*, 2000; López-Orenes *et al.*, 2013b). Here, we found that Agustín leaves exhibited only a slight reduction (mean ratio ~0.83, Supplemental Table S2) in the total antioxidant activity, measured as DPPH/ABTS scavenging ability, which was highly correlated with the reduction in the levels of soluble phenolic compounds. Nevertheless, it is important to highlight that the total antioxidant capacity in Agustín *D. viscosa* leaves was ca. 25-fold higher than that found in Agustín *Z. fabago* plants growing in the same conditions (López-Orenes *et al.*, 2017), and also that the levels of soluble TPC noticed in summer Agustín leaves were ca. 4-fold higher than the ones reported in leaves of Cd-exposed *D. viscosa* plants grown under hydroponics (Fernández *et al.*, 2013). Foliar accumulation of phenolic compounds has also been reported in different plant species exposed to heavy metal (Kováčik and Klejdus, 2008; Llugany *et al.*, 2013; Singh *et al.*, 2015, and refs. herein), and also in plants grown in multi-polluted soils (López-Orenes *et al.*, 2017; Martínez-Alcalá *et al.*, 2013).

A relevant aspect to consider is the metabolic cost associated with adaptation to chronic exposure to metal(loid)s, because these adaptive processes should be sustainable and effective, especially under nutrient-limited conditions (Maestri *et al.*, 2010). One of the mechanisms which fulfills both requirements is the metal(loid) sequestration/immobilization in plant cell walls rather than the induction of low molecular weight ligands such as phytochelatins due to the energy costs associated with sulfate reduction and phytochelatins synthesis (Maestri *et al.*, 2010, and refs. herein). Although GSH biosynthesis is regulated by sulfur assimilation and *D. viscosa* leaves showed a high foliar S content, the size of S-rich metal(loid)-binding peptides (*i.e.*, the non-protein thiol pool and GSH content) in Agustín leaves remained nearly unaffected, suggesting that metal(loid) detoxification via thiol-mediated complexation could have a minor role in these plants under the prevalent edaphoclimatic conditions.

Furthermore, it is well known that nutrient deficiencies can cause elevated levels of carbon-rich metabolites, such as phenolics (Fritz *et al.*, 2006). Here, we observed a significant enhancement of both PAL and sPRX activities in Agustín leaves as well as changes in the accumulation of both soluble and cell wall-bound phenolic compounds. Both hydroxycinnamic acid derivatives and flavonoids were the most abundant phenolics in the leaf soluble fraction, as has been previously reported (Mahmoudi *et al.*, 2016; Trimech *et al.*, 2014). The predominant phenolic monomer esterified and incorporated in the cell wall matrix in Agustín leaves was caffeic acid. HCAs, especially caffeic acid, and flavonoids have been reported to show high metal-chelation properties as well as strong antioxidant characteristics and ROS-scavenging activities (Agati *et al.*, 2012; Andejelkovic *et al.*, 2006; Michalak, 2006; Rice-Evans *et al.*, 1997).

In our study, high-to-moderate correlations between the foliar concentrations of metal(loid)s and the levels of both cell wall-bound phenols, soluble phenols, PAL and PRX activities were observed. What is more, all of these parameters were ranked among the top-10 significant biomarkers based on mean decrease in accuracy in RF, and had a VIP score > 1, suggesting that under natural (field) conditions phenylpropanoid metabolism could play an important role in the acclimation of mining *D. viscosa* plants not only to the high metal(loid) contents found in the mine tailing, but also to the adverse effects of other concurrent stressors (*i.e.*, nutrient deficiencies, high EC, and drought). Changes in phenolics in cell walls have also been reported in plants exposed to metals

(Kováčik and Klejdus, 2008), and drought (Hura *et al.*, 2017). In fact, several lines of evidence indicate that although the production of phenylpropanoid metabolites incurs metabolic costs, their biosynthesis can be offset by the multiplicity of functions that these compounds may play in plants exposed to a wide array of environmental constraints (Brunetti *et al.*, 2015, and refs. herein). Thus, the antioxidant properties ascribed to these phenolic compounds (*i.e.*, their ability to avoid ROS accumulation, to protect cells against ROS-induced damage, and to chelate metals) seem to be relevant in the acclimation response to the adverse conditions of the mine tailing, especially under the most stressful conditions (*i.e.*, summer 2013).

3.5.4. The endogenous levels of the phytohormone SA in leaves of metallicolous *D. viscosa* plants dropped irrespective of the sampling date

SA has long been recognized as a central signaling molecule in triggering defense responses against biotic and abiotic stress (Vlot *et al.*, 2009). Activation of the SA-mediated defense responses is associated with up-regulation of genes encoding defense-related proteins, and the accumulation of certain secondary metabolites. Here, we found good positive correlation between SA and soluble phenol contents ($r > 0.6$, $P < 0.01$, Supplemental Table S3). The amounts of SA, quantified using the SA biosensor strain *Acinetobacter* sp. ADP1_ux, were 2.70 ± 0.11 , and 3.87 ± 0.40 nmol g⁻¹ FW in spring and summer Agustín leaves, respectively. Moreover, the analyses of the phenolic phytohormone SA showed a significant drop ($P < 0.005$) in its endogenous level in Agustín leaves (mean ratio ~0.67) irrespective of the sampling date. In a previous work, we also found lower foliar content of free SA in leaves of two metallicolous populations of *Zygophyllum fabago* plants growing within this mining area relative to a non-mining population (López-Orenes *et al.*, 2017). At first glance, these results could be considered surprising because most of the studies in the literature reported that SA pretreatment contributed to the alleviation of metal(loid) toxicity in many plant species (for review, see Hayat *et al.*, 2010, and refs. herein). In a recent study using *Arabidopsis* SA-altering mutants lines, it was found that high endogenous levels of SA intensified the phytotoxicity induced by Pb and Cd ions (Tao *et al.*, 2013), what would support the observation that mining populations, which are chronically exposed to metal(loid)s, contained lower levels of SA than non-mining controls.

3.6. Conclusions

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

PART I:
EXPRESSION PATTERNS OF ANTIOXIDANT
SYSTEMS IN PIONEER PLANTS GROWN IN
MINE TAILING

CHAPTER 4

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

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Graphical Abstract

Highlights

- Marked seasonal changes in metabolism were observed in Aleppo pine populations
- Differences between mining and non-mining populations were more evident in spring
- S, metal(loid)s, GSH, PAs, and soluble phenols needle contents were higher in spring
- Low chlorophyll and high carotenoids, RWC, Cl; Na and K characterized summer samples
- Seasonal differences observed seem to be determined by topsoil water availability

4.1. Abstract

Aleppo pine is the most abundant conifer species in Mediterranean basin. Knowledge of adaptive mechanisms to cope with different environmental stresses simultaneously is considered essential to improve its resilience to the predicted climatic changes and anthropogenic stressors, such as heavy metals and metal(loid)s (HMMs). Here, one year-old needles and soil samples from mining and non-mining 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 biochemical, physiological and edaphic parameters was performed, including ionome profile, foliar redox components, primary and secondary metabolites, salicylic acid levels, and soil physicochemical properties. Marked seasonal differences in ionome and antioxidative /oxidative profiles were observed in all populations studied. Differences in these parameters between mining and non-mining samples were more apparent in spring than in summer samples. Enhanced foliar accumulation of S and HMMs in spring was accompanied by higher accumulation of GSH, proanthocyanidins (PAs), and other phenolics. The less-active growth period was characterized by a decrease in the content of chlorophylls and increased levels of monovalent ions (Cl, Na and K), needle relative water content and carotenoids. Overall, metabolic responses relative to control in mining populations are mainly grouped by season with the differences found in soil conditions playing a secondary role. Results obtained suggest that seasonal metabolic differences observed in Aleppo pine populations would probably be determined by water availability in surface soil layers. The present study gives some insights into how the predicted changes in climatic conditions in Mediterranean basin would affect the metabolic behavior of Aleppo pines growing on mine tailings.

Keywords

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

4.2. 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), where exhibits a bimodal growth pattern, with two optimal growth periods one in spring and a second in fall associated with favorable growing conditions (López-Serrano *et al.*, 2005; Pacheco *et al.*, 2017). This species is noted for its high resilience to heat and drought stress (Maseyk *et al.*, 2008; Wellburn *et al.*, 1996), as well as for its nitrogen-use efficiency (Michopoulos *et al.*, 2004), enabling it to grow in sparse soils with low water and nitrogen (N) contents (Wellburn *et al.*, 1996). Despite its high resilience to drought, 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.*, 2016; 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; Choudhury *et al.*, 2016). 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.*, 2016; 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; Foyer and Noctor, 2011). Recent “omics” studies have highlighted that antioxidant defense machinery can play an important role not only in plant HMM-tolerance mechanisms (Dalcorsio *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.*, 2016), 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; Passardi *et al.*, 2005). 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; Pii *et al.*, 2015).

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 (Párraga-Aguado *et al.* 2014b, 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.*, 2017, 2018) and woody plants.

4.3. Materials and Methods

4.3.1. Plant and soil sampling

Aleppo pine needles were obtained from mature trees growing spontaneously in the Cartagena-La Unión Mining District (SE of the Iberian Peninsula) in four different tailings piles known as Agustín (37°36'20''N, 0°50'15''W), Mercader (37°36'15''N, 0°50'04''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 area (37°35'47'' N, 0°49'23'' W) located about 1.5 km away from these mining sites (Supplemental Fig. S1). 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 (ET_o) exceeded rainfall by sixfold (ET_o 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

greatest rainfall occurred and May 2013 followed a rainy month of April (80 mm rainfall) and was wetter than May 2012 (Supplemental Fig. S2).

Figure S1. Geographical location of the study sites in the Cartagena-La Unión Mining District (Murcia, Spain). The different mine tailings piles (Agustín, Mercader, Ripolles and Wikon) and the control site are indicated in the map.

Figure S2. Seasonal variations in weather conditions (monthly precipitation, monthly average minimum and maximum temperatures, and monthly average reference evapotranspiration [ETo]) from December 2011 to September 2013. Data were collected by an automatic weather station located near the experimental site. Each sampling time are indicated by asterisks.

Four mature trees of similar size and age were sampled per site. Six to ten small branches, with current and one year-old needles, were cut from different directions from the upper third of the crown of each tree. In the laboratory, the current-year foliage was removed and only the old (one-year) needles were selected (Supplemental Fig. S3). In all sampling periods, at least 200 g of one year-old needles of each population were washed thoroughly with tap and distilled water, gently blotted on filter paper, and randomly divided into five groups. One group was used to determine the needle relative water content, and each one of the remaining four biological replicates, were divided into two subsamples, one of them was immediately frozen in liquid nitrogen, and stored at -80°C until analyzed, and the second one was dried at 60°C for 72 h for elemental analysis. At the beginning of the sampling period (*i.e.*, May 2012), four soil subsamples at each site were collected from the top 20 cm, and transferred under aseptic conditions to the laboratory.

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).

4.3.2. Soil analysis

The collected soil samples were air-dried, sieved through a 2 mm-sieve mesh, and subjected to several physicochemical analyses including pH, electrical conductivity (EC), equivalent calcium carbonate (% CaCO_3), organic carbon (OC), dissolved organic carbon (DOC), total N (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). These analyses were carried out as previously described (Párraga-Aguado *et al.* 2014b).

4.3.3. Elemental analysis in needle samples

About 500 mg of leaf-dried biomass, finely ground, were incinerated (550 °C for 3 h) prior to adding 1 mL of concentrated HNO_3 (65%, Merck, Suprapur). The resulting extracts were diluted to 25 mL with MilliQ water, and all sample solutions were filtered through CHM[®] ashless filter papers (F2041-110). 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) as previously described (Párraga-Aguado *et al.* 2014b). 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).

4.3.4. Physiological status measurements

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 (1 ml per 0.1 g tissue) in a water sonication bath, at 37 kHz for 40 min, until the extracts were colorless (x2). The supernatants obtained after centrifugation (15,000 $\times g$ for 15 min at 4 °C) were combined

and used for the quantification of photosynthetic pigments as described by Lichtenthaler and Wellburn (1983).

4.3.5. Total antioxidant activity determinations

The total antioxidant activity was assessed by three different methods, namely 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). 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.

4.3.6. 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).

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

The determination of hydrogen peroxide was carried out by the ferrous ion oxidation–xylene 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).

4.3.8. 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 was carried out according to the aluminum chloride assay using rutin as standard (Kim *et al.*, 2003). Total flavanol content 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, after thoroughly washing with ethanol and dried at 60 °C, were used for lignin determination using thioglycolic acid (TGA). The concentration of the lignin-TGA derivatives were measured at 280 nm by using a microplate reader (Multiskan GO; Thermo Scientific) and 96-well UV plates (Corning) from the standard curve prepared using commercial lignin alkali (471003, Sigma-Aldrich) (López-Orenes *et al.*, 2013a).

The content of cell wall-associated proanthocyanidins (PAs) was determined by measuring the absorbance at 545 nm of the supernatants obtained after the acid attack on washed cell wall pellets, and the results were expressed as cyanidin equivalents by using an $\varepsilon_{545} = 34.7 \text{ mM}^{-1} \text{ cm}^{-1}$ (Vermerris and Nicholson, 2006).

4.3.9. 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: H₂O₂ oxidoreductase, PRXs) in needle samples were performed as previously described (López-Orenes *et al.*, 2013a). Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

4.3.10 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.

ADPWlux (Huang *et al.* 2006 and 2005) with some modifications (López-Orenes *et al.* 2017).

4.3.11. Statistical analysis

Physicochemical characteristics and metal(loid) concentrations in soils from both mining and non-mining samples were subjected to a one-way ANOVA with site (Agustin, Mercader, Ripolles and Wikon mine tailings and control) 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. When the differences were significant at $P \leq 0.05$, a Tukey's HSD post-hoc test was conducted to detect differences between means. 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 the parameters measured in needles of Aleppo pine trees growing in non-mining and in mining tailings, which were previously mean-centered log-transformed to avoid the effect of the scale. Additionally, a heatmap analysis, combined with an agglomerative hierarchical clustering, was carried out to analyze the differences between mining and non-mining populations. All statistical analysis and graphs were performed using the R Statistical Programming Environment (<https://www.R-project.org/>).

4.4. Results

4.4.1. Multivariate analysis of soil parameters revealed distinct clustering differences among mining samples

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 soils were characterized by lower N and dissolved organic carbon (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 (SO₄²⁻, Ca²⁺, and Mg²⁺), whereas monovalent ions (Cl⁻, K⁺, and Na⁺) remained closer to the values found in control soils, except for Na in Agustin tailings. Significant negative moderate to high correlations were found

between divalent ion concentrations and the soil fertility parameters DOC and TN ($-0.95 > r > -0.6$, $P < 0.0001$; 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 formation of gypsum in the tailings (Párraga-Aguado *et al.* 2014b). 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, whereas Mercader one showed relatively low metal concentrations and the highest N and organic carbon contents (Table 1). All mining samples had a sandy texture (80%), with very low clay content ($<4\%$), and high negative correlations were noted between sand percentages and soil fertility parameters (DOC and TN; $r < -0.84$, $P < 0.0001$; Supplemental Table S1).

Multivariate analyses, using both unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA), revealed differences between non-mining (control) and mining soils and also showed clear clustering differences between, on the one hand, Mercader and Agustin samples and, on the other, Ripolles and Wikon samples, indicating close soil physicochemical similarities between samples sorted into the same cluster (Supplemental Fig. S4). The first PLS-DA component, which explained ~62 % of the total variance of the data set, was mainly influenced by soil fertility parameters (DOC and TN, all with positive loadings) and by metal(loid) soil concentrations (mainly Pb, Zn, As, and Cu). The second PLS-DA component (~10% of total variance) was influenced by K and Na levels and by organic carbon content (OC), all with positive loadings.

Table 1. Physicochemical characteristics and metal(loid) concentrations in soils from both mining (Agustin, Mercader, Ripolles and Wikon) and non-mining (NM) samples. 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 post-hoc test.

	Rhizosphere soil sample				
	NM	Agustin	Mercader	Ripolles	Wikon
Soil parameters					
pH (1:5)	7.99 $\pm$ 0.05 ^a	7.50 $\pm$ 0.06 ^b	7.53 $\pm$ 0.18 ^b	7.44 $\pm$ 0.06 ^{bc}	7.12 $\pm$ 0.03 ^c
EC (1:5) (dS m⁻¹)	0.27 $\pm$ 0.02 ^c	1.31 $\pm$ 0.45 ^{bc}	1.44 $\pm$ 0.53 ^b	0.80 $\pm$ 0.15 ^{bc}	2.81 $\pm$ 0.05 ^a
CaCO₃ (%)	20.32 $\pm$ 0.64 ^a	12.14 $\pm$ 3.58 ^b	9.23 $\pm$ 0.34 ^b	10.10 $\pm$ 1.76 ^b	6.27 $\pm$ 0.09 ^b
OC (%)	0.78 $\pm$ 0.09 ^{ab}	0.69 $\pm$ 0.02 ^{ab}	0.99 $\pm$ 0.13 ^a	0.59 $\pm$ 0.09 ^b	0.50 $\pm$ 0.05 ^b
DOC (mg kg⁻¹)	108.96 $\pm$ 18.24 ^a	52.61 $\pm$ 4.65 ^b	70.15 $\pm$ 7.51 ^{ab}	63.11 $\pm$ 12.61 ^{ab}	44.97 $\pm$ 12.01 ^b
TN (g kg⁻¹)	0.91 $\pm$ 0.07 ^a	0.32 $\pm$ 0.05 ^{bc}	0.59 $\pm$ 0.15 ^b	0.32 $\pm$ 0.06 ^{bc}	0.26 $\pm$ 0.04 ^c
Texture (%)					
Clay	27.51 $\pm$ 1.16 ^a	1.08 $\pm$ 0.00 ^c	2.10 $\pm$ 0.48 ^b	1.00 $\pm$ 0.00 ^c	3.72 $\pm$ 0.68 ^b
Silt	37.10 $\pm$ 4.31 ^a	13.97 $\pm$ 1.53 ^c	19.29 $\pm$ 2.53 ^b	10.77 $\pm$ 2.17 ^c	14.56 $\pm$ 1.51 ^{bc}
Sand	35.39 $\pm$ 3.20 ^c	84.95 $\pm$ 1.53 ^a	78.62 $\pm$ 2.64 ^b	88.23 $\pm$ 2.17 ^a	81.72 $\pm$ 1.43 ^b
Water extractable ions (1:5) (mg L⁻¹)					
Cl⁻	19.82 $\pm$ 2.91 ^a	11.94 $\pm$ 0.76 ^{ab}	8.72 $\pm$ 0.66 ^b	9.39 $\pm$ 0.76 ^b	14.80 $\pm$ 2.60 ^{ab}
SO₄²⁻	20.83 $\pm$ 0.58 ^c	1405.00 $\pm$ 134.46 ^a	676.08 $\pm$ 318.34 ^b	295.91 $\pm$ 75.97 ^{bc}	1610.99 $\pm$ 16.84 ^a
Na⁺	17.20 $\pm$ 2.76 ^b	94.72 $\pm$ 14.87 ^a	17.35 $\pm$ 0.90 ^b	11.42 $\pm$ 0.72 ^b	18.46 $\pm$ 6.63 ^b
K⁺	12.70 $\pm$ 1.24 ^{ab}	14.95 $\pm$ 2.99 ^a	13.09 $\pm$ 0.33 ^a	7.22 $\pm$ 0.65 ^{bc}	6.95 $\pm$ 0.49 ^c
Ca²⁺	35.15 $\pm$ 0.25 ^b	508.71 $\pm$ 53.44 ^a	240.74 $\pm$ 104.67 ^b	118.72 $\pm$ 37.95 ^b	630.18 $\pm$ 25.86 ^a
Mg²⁺	2.67 $\pm$ 0.44 ^b	27.70 $\pm$ 6.45 ^a	35.13 $\pm$ 9.77 ^a	17.14 $\pm$ 6.95 ^{ab}	17.08 $\pm$ 2.23 ^{ab}
Total metal(loid)s (mg kg⁻¹)					
As	18.25 $\pm$ 11.38 ^d	226.67 $\pm$ 23.63 ^c	697.67 $\pm$ 31.39 ^{ab}	559.75 $\pm$ 78.31 ^b	800.50 $\pm$ 46.19 ^a
Cd	17.25 $\pm$ 11.25 ^a	36.00 $\pm$ 10.21 ^a	22.67 $\pm$ 7.22 ^a	33.75 $\pm$ 4.85 ^a	45.50 $\pm$ 7.14 ^a
Cu	44.83 $\pm$ 2.83 ^b	86.47 $\pm$ 2.41 ^a	95.00 $\pm$ 0.87 ^a	114.38 $\pm$ 14.82 ^a	104.50 $\pm$ 3.66 ^a
Mn	1635.00 $\pm$ 61.71 ^c	7639.00 $\pm$ 442.22 ^b	10058.33 $\pm$ 96.25 ^a	9628.75 $\pm$ 570.83 ^a	10198.00 $\pm$ 275.29 ^a
Ni	39.25 $\pm$ 1.18 ^a	33.67 $\pm$ 2.26 ^{ab}	27.67 $\pm$ 1.89 ^{bc}	28.25 $\pm$ 2.29 ^{bc}	24.25 $\pm$ 2.66 ^c
Pb	397.50 $\pm$ 23.39 ^b	4665.33 $\pm$ 307.89 ^a	2873.33 $\pm$ 208.83 ^a	6288.25 $\pm$ 1779.73 ^a	5713.50 $\pm$ 329.77 ^a
Zn	264.25 $\pm$ 16.07 ^c	8685.33 $\pm$ 1095.77 ^a	4356.00 $\pm$ 245.83 ^b	9376.00 $\pm$ 381.55 ^a	9505.00 $\pm$ 440.54 ^a
Sb	1.00 $\pm$ 0.00 ^c	49.00 $\pm$ 12.26 ^b	60.67 $\pm$ 10.50 ^{ab}	66.50 $\pm$ 8.58 ^{ab}	98.50 $\pm$ 11.55 ^a

Figure S4. Score plots of PLS-DA of soil parameters from both mining (Agustin, Mercader, Ripolles and Wikon) and non-mining (NM) samples. Loadings of soil variables are indicated in the inset at the bottom-right corner of the figure. Sample codes: NM, filled black circle; Agustin, unfilled circle; Mercader, unfilled square; Ripolles, unfilled triangle; and Wikon, unfilled inverted triangle.

4.4.2. Needle ionome showed significant seasonal differences in nutrients and metal(loid)s concentrations among Aleppo pine populations

Given the differences between non-mining and mining soils and within mining groups the needle ionome was analyzed in order to establish whether these soil differences can be reflected in differences in the ionic profiles of the Aleppo pine samples. PCA on

needle ionome data revealed two principal components (PC) that together explained ~57% of the total variance (Fig. 1). 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 (Cl^- , Na^+ , and K^+). It is worth noting that the concentrations of As, Mn, Ni, Pb, and Zn in mining needle samples were between 3-10-fold higher than their corresponding non-mining (control) groups (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 $\mu\text{g g}^{-1}$, respectively, especially in spring needle samples (Supplemental Table S2). Regarding macronutrient levels, with the exception of N in Mercader, the concentrations of N, P and K were lower in the needles from mining populations than in those from NM ones. S and Mg^{2+} levels were higher in mining needles, and Ca^{2+} contents remained higher than (spring) or comparable to (summer) the values found in NM samples. It is important to highlight that, in general, the 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), especially for P, whose levels were ~0.6 g kg^{-1} and ~0.36 g kg^{-1} in NM and mining needle samples, respectively (Supplemental Table S2).

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

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 $\pm$ SE ($n=4$). Values above the normal range are shown in bold; values below the normal range are shown in bold and italics. nd, no data.

		NM			
		Spring		Summer	
	Average range	2012	2013	2012	2013
Macronutrients (g kg⁻¹ DW)					
N	9.22 - 14.28*	8.51 $\pm$ 0.38	10.08 $\pm$ 0.27	6.56 $\pm$ 0.15	7.58 $\pm$ 0.33
P	0.80 - 1.78*	0.65 $\pm$ 0.07	0.62 $\pm$ 0.07	0.67 $\pm$ 0.05	0.64 $\pm$ 0.03
K	3.20 - 8.67*	2.35 $\pm$ 0.11	2.40 $\pm$ 0.13	5.27 $\pm$ 0.15	7.44 $\pm$ 0.15
S	0.92 - 1.68*	1.45 $\pm$ 0.11	1.50 $\pm$ 0.03	1.47 $\pm$ 0.20	1.44 $\pm$ 0.06
Mg	1.84 - 2.89*	1.52 $\pm$ 0.19	1.39 $\pm$ 0.05	1.66 $\pm$ 0.12	1.49 $\pm$ 0.08
Ca	2.12 - 8.04*	2.63 $\pm$ 0.25	2.82 $\pm$ 0.17	1.43 $\pm$ 0.12	1.86 $\pm$ 0.04
Cl	0.34-0.64‡	0.67 $\pm$ 0.15	0.56 $\pm$ 0.04	0.96 $\pm$ 0.13	0.87 $\pm$ 0.12
Na	0.37-0.68‡	0.36 $\pm$ 0.05	0.34 $\pm$ 0.04	0.44 $\pm$ 0.12	0.40 $\pm$ 0.04
Metal(loid)s (mg kg⁻¹ DW)					
As	0.12 - 9.99§	0.45 $\pm$ 0.03	0.39 $\pm$ 0.04	0.25 $\pm$ 0.04	0.16 $\pm$ 0.02
Cd	0.05 - 0.5*	0.11 $\pm$ 0.01	nd	0.12 $\pm$ 0.05	0.01 $\pm$ 0.01
Cu	2.28 - 7.7*	2.66 $\pm$ 0.13	3.61 $\pm$ 0.14	2.64 $\pm$ 0.24	2.77 $\pm$ 0.04
Mn	32 - 912*	63.07 $\pm$ 10.17	50.33 $\pm$ 7.79	41.30 $\pm$ 2.86	42.59 $\pm$ 1.22
Ni	nd	0.33 $\pm$ 0.08	0.24 $\pm$ 0.17	0.14 $\pm$ 0.02	0.03 $\pm$ 0.02
Pb	0.14 - 5.59*	2.58 $\pm$ 0.28	1.16 $\pm$ 0.19	3.44 $\pm$ 1.33	1.14 $\pm$ 0.17
Zn	23 - 77.55*	33.75 $\pm$ 1.18	22.44 $\pm$ 1.21	34.78 $\pm$ 5.02	27.65 $\pm$ 1.97

*Data from Clarke *et al.*, (2008)

‡Data from Párraga-Aguado *et al.*, (2014b)

§Data from Patras *et al.*, (2005)

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 $\pm$ SE ($n=4$). Values above the normal range are shown in bold; values below the normal range are shown in bold and italics. nd, no data.

		Agustin				Mercader			
		Spring		Summer		Spring		Summer	
	Average range	2012	2013	2012	2013	2012	2013	2012	2013
Macronutrients (g kg⁻¹ DW)									
N	9.22 - 14.28*	5.43 $\pm$ 0.36	9.00 $\pm$ 1.86	5.85 $\pm$ 0.51	6.72 $\pm$ 0.61	15.83 $\pm$ 1.54	17.89 $\pm$ 0.81	7.99 $\pm$ 0.69	8.47 $\pm$ 0.58
P	0.80 - 1.78*	0.46 $\pm$ 0.07	0.42 $\pm$ 0.05	0.45 $\pm$ 0.03	0.46 $\pm$ 0.05	0.32 $\pm$ 0.01	0.33 $\pm$ 0.04	0.35 $\pm$ 0.01	0.41 $\pm$ 0.01
K	3.20 - 8.67*	1.73 $\pm$ 0.26	1.86 $\pm$ 0.12	5.66 $\pm$ 0.42	4.76 $\pm$ 0.85	1.56 $\pm$ 0.02	1.40 $\pm$ 0.18	5.11 $\pm$ 0.59	4.81 $\pm$ 0.22
S	0.92 - 1.68*	4.44 $\pm$ 0.34	4.40 $\pm$ 0.35	0.95 $\pm$ 0.49	1.39 $\pm$ 0.05	4.02 $\pm$ 0.29	4.32 $\pm$ 0.13	1.95 $\pm$ 0.12	1.80 $\pm$ 0.05
Mg	1.84 - 2.89*	3.14 $\pm$ 0.30	3.07 $\pm$ 0.06	1.92 $\pm$ 0.11	1.85 $\pm$ 0.13	2.89 $\pm$ 0.18	2.86 $\pm$ 0.09	1.90 $\pm$ 0.14	1.71 $\pm$ 0.17
Ca	2.12 - 8.04*	4.23 $\pm$ 0.90	3.13 $\pm$ 0.39	1.00 $\pm$ 0.11	1.74 $\pm$ 0.22	4.15 $\pm$ 0.11	3.61 $\pm$ 0.28	1.30 $\pm$ 0.15	1.99 $\pm$ 0.04
Cl	0.34-0.64‡	1.10 $\pm$ 0.04	1.11 $\pm$ 0.15	1.18 $\pm$ 0.12	1.34 $\pm$ 0.06	1.11 $\pm$ 0.13	0.96 $\pm$ 0.07	0.92 $\pm$ 0.01	0.93 $\pm$ 0.11
Na	0.37-0.68‡	0.98 $\pm$ 0.27	0.53 $\pm$ 0.13	1.38 $\pm$ 0.36	0.77 $\pm$ 0.27	0.58 $\pm$ 0.08	0.32 $\pm$ 0.01	0.72 $\pm$ 0.11	0.42 $\pm$ 0.03
Metal(loid)s (mg kg⁻¹ DW)									
As	0.12 - 9.99§	2.14 $\pm$ 0.18	1.97 $\pm$ 0.11	1.39 $\pm$ 0.20	1.01 $\pm$ 0.23	4.00 $\pm$ 0.21	3.08 $\pm$ 0.14	1.79 $\pm$ 0.34	1.25 $\pm$ 0.08
Cd	0.05 - 0.5*	0.22 $\pm$ 0.02	0.31 $\pm$ 0.06	0.16 $\pm$ 0.05	0.14 $\pm$ 0.02	0.50 $\pm$ 0.03	0.16 $\pm$ 0.03	0.15 $\pm$ 0.03	0.08 $\pm$ 0.01
Cu	2.28 - 7.7*	3.04 $\pm$ 0.23	3.21 $\pm$ 0.49	3.16 $\pm$ 0.50	2.89 $\pm$ 0.24	2.64 $\pm$ 0.43	4.42 $\pm$ 0.37	3.66 $\pm$ 0.31	3.09 $\pm$ 0.07
Mn	32 - 912*	256.07 $\pm$ 42.95	417.36 $\pm$ 40.98	213.02 $\pm$ 14.83	248.91 $\pm$ 23.17	169.01 $\pm$ 25.23	179.56 $\pm$ 19.37	123.98 $\pm$ 13.68	106.18 $\pm$ 5.89
Ni	nd	0.50 $\pm$ 0.04	0.18 $\pm$ 0.03	0.42 $\pm$ 0.07	0.26 $\pm$ 0.06	0.54 $\pm$ 0.04	0.46 $\pm$ 0.05	0.23 $\pm$ 0.08	0.02 $\pm$ 0.01
Pb	0.14 - 5.59*	11.44 $\pm$ 2.29	14.28 $\pm$ 2.80	12.25 $\pm$ 2.67	5.91 $\pm$ 1.47	24.85 $\pm$ 0.91	11.66 $\pm$ 1.46	7.56 $\pm$ 1.45	3.81 $\pm$ 0.30
Zn	23 - 77.55*	153.13 $\pm$ 7.93	130.54 $\pm$ 15.75	87.83 $\pm$ 11.38	86.81 $\pm$ 8.01	123.45 $\pm$ 10.89	97.65 $\pm$ 6.23	59.78 $\pm$ 2.51	59.53 $\pm$ 2.38

*Data from Clarke *et al.*, (2008)

‡Data from Párraga-Aguado *et al.*, (2014b)

§Data from Patras *et al.*, (2005)

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 $\pm$ SE ($n=4$). Values above the normal range are shown in bold; values below the normal range are shown in bold and italics. nd, no data.

		Ripolles				Wikon			
		Spring		Summer		Spring		Summer	
	Average range	2012	2013	2012	2013	2012	2013	2012	2013
Macronutrients (g kg⁻¹ DW)									
N	9.22 - 14.28*	7.83 $\pm$ 0.57	9.19 $\pm$ 0.48	6.61 $\pm$ 0.16	7.46 $\pm$ 0.22	7.89 $\pm$ 0.33	9.76 $\pm$ 0.74	6.10 $\pm$ 0.12	6.86 $\pm$ 0.05
P	0.80 - 1.78*	0.31 $\pm$ 0.02	0.31 $\pm$ 0.05	0.39 $\pm$ 0.03	0.37 $\pm$ 0.05	0.28 $\pm$ 0.01	0.28 $\pm$ 0.01	0.32 $\pm$ 0.02	0.32 $\pm$ 0.04
K	3.20 - 8.67*	1.90 $\pm$ 0.19	2.22 $\pm$ 0.50	3.36 $\pm$ 0.19	3.36 $\pm$ 0.04	1.87 $\pm$ 0.16	1.69 $\pm$ 0.11	5.31 $\pm$ 0.24	5.17 $\pm$ 0.78
S	0.92 - 1.68*	4.75 $\pm$ 0.12	4.87 $\pm$ 0.32	2.03 $\pm$ 0.12	2.02 $\pm$ 0.18	2.83 $\pm$ 0.17	2.88 $\pm$ 0.18	2.06 $\pm$ 0.05	2.22 $\pm$ 0.09
Mg	1.84 - 2.89*	3.10 $\pm$ 0.30	3.35 $\pm$ 0.42	2.10 $\pm$ 0.18	2.00 $\pm$ 0.14	2.35 $\pm$ 0.28	5.43 $\pm$ 0.38	1.70 $\pm$ 0.16	1.89 $\pm$ 0.19
Ca	2.12 - 8.04*	5.30 $\pm$ 0.67	3.80 $\pm$ 0.45	1.34 $\pm$ 0.28	1.97 $\pm$ 0.20	3.14 $\pm$ 0.25	3.14 $\pm$ 0.20	1.19 $\pm$ 0.07	2.52 $\pm$ 0.83
Cl	0.34-0.64‡	0.58 $\pm$ 0.04	0.67 $\pm$ 0.07	0.91 $\pm$ 0.10	0.87 $\pm$ 0.22	0.53 $\pm$ 0.04	0.51 $\pm$ 0.10	1.53 $\pm$ 0.13	1.38 $\pm$ 0.28
Na	0.37-0.68‡	0.45 $\pm$ 0.09	0.31 $\pm$ 0.07	0.76 $\pm$ 0.16	0.32 $\pm$ 0.09	0.51 $\pm$ 0.06	0.45 $\pm$ 0.08	0.82 $\pm$ 0.11	0.89 $\pm$ 0.34
Metal(loid)s (mg kg⁻¹ DW)									
As	0.12 - 9.99§	1.73 $\pm$ 0.10	1.60 $\pm$ 0.17	1.03 $\pm$ 0.06	0.52 $\pm$ 0.04	4.66 $\pm$ 0.38	5.09 $\pm$ 0.30	2.04 $\pm$ 0.22	1.33 $\pm$ 0.19
Cd	0.05 - 0.5*	0.29 $\pm$ 0.11	0.48 $\pm$ 0.17	0.07 $\pm$ 0.01	0.07 $\pm$ 0.03	0.14 $\pm$ 0.02	0.47 $\pm$ 0.05	0.22 $\pm$ 0.05	0.24 $\pm$ 0.12
Cu	2.28 - 7.7*	3.50 $\pm$ 0.61	3.10 $\pm$ 0.37	2.83 $\pm$ 0.15	2.61 $\pm$ 0.23	4.52 $\pm$ 0.61	3.82 $\pm$ 0.39	3.03 $\pm$ 0.24	2.80 $\pm$ 0.19
Mn	32 - 912*	421.75 $\pm$ 51.06	526.07 $\pm$ 84.23	344.73 $\pm$ 68.57	340.90 $\pm$ 91.81	172.36 $\pm$ 11.94	267.02 $\pm$ 10.80	179.62 $\pm$ 8.05	193.97 $\pm$ 11.48
Ni	nd	0.43 $\pm$ 0.02	0.21 $\pm$ 0.07	0.26 $\pm$ 0.11	0.15 $\pm$ 0.06	0.33 $\pm$ 0.01	0.13 $\pm$ 0.03	0.27 $\pm$ 0.16	0.07 $\pm$ 0.04
Pb	0.14 - 5.59*	9.77 $\pm$ 2.84	12.83 $\pm$ 1.98	3.69 $\pm$ 0.92	1.62 $\pm$ 0.16	13.29 $\pm$ 2.16	19.17 $\pm$ 2.43	9.35 $\pm$ 1.02	9.08 $\pm$ 2.58
Zn	23 - 77.55*	158.97 $\pm$ 21.76	116.36 $\pm$ 14.73	76.24 $\pm$ 8.70	85.56 $\pm$ 12.96	150.45 $\pm$ 5.73	117.36 $\pm$ 7.79	89.81 $\pm$ 3.04	89.41 $\pm$ 2.78

*Data from Clarke *et al.*, (2008)

‡Data from Párraga-Aguado *et al.*, (2014b)

§Data from Patras *et al.*, (2005)

Table S3. Mean ratio of fold changes and their associated P -values obtained by the t-student test ($P < 0.05$; $n = 12$) 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.

	Agustin				Mercader				Ripolles				Wikon			
	Spring		Summer		Spring		Summer		Spring		Summer		Spring		Summer	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
N	0.64	0.89	0.89	0.89	1.86	1.77	1.22	1.12	0.92	0.91	1.01	0.98	0.93	0.97	0.93	0.91
P	0.71	0.68	0.68	0.71	0.49	0.52	0.52	0.64	0.47	0.50	0.58	0.58	0.43	0.44	0.48	0.50
K	0.74	0.78	1.07	0.64	0.66	0.59	0.97	0.65	0.81	0.93	0.64	0.45	0.80	0.71	1.01	0.69
S	3.07	2.93	0.65	0.96	2.78	2.87	1.33	1.25	3.29	3.24	1.38	1.40	1.96	1.92	1.40	1.54
Mg	2.06	2.21	1.16	1.24	1.90	2.06	1.14	1.15	2.03	2.42	1.27	1.34	1.54	3.91	1.02	1.27
Ca	1.61	1.11	0.70	0.93	1.58	1.28	0.91	1.07	2.01	1.35	0.94	1.06	1.19	1.11	0.83	1.35
Cl	1.64	1.98	1.22	1.53	1.66	1.71	0.95	1.07	0.86	1.19	0.94	0.99	0.79	0.90	1.59	1.58
Na	2.75	1.56	3.13	1.92	1.61	0.94	1.62	1.05	1.25	0.92	1.72	0.81	1.41	1.32	1.87	2.22
As	4.80	4.99	5.56	6.49	8.96	7.81	7.14	8.06	3.87	4.05	4.13	3.32	10.44	12.90	8.14	8.58
Cu	1.14	0.89	1.20	1.04	0.99	1.23	1.39	1.12	1.32	0.86	1.07	0.94	1.70	1.06	1.15	1.01
Mn	4.06	8.29	5.16	5.84	2.68	3.57	3.00	2.49	6.69	10.45	8.35	8.00	2.73	5.31	4.35	4.55
Ni	1.52	0.74	3.02	9.00	1.65	1.87	1.69	0.84	1.31	0.84	1.89	5.05	1.01	0.55	1.95	2.47
Pb	4.43	12.35	3.56	5.17	9.64	10.09	2.20	3.33	3.79	11.10	1.07	1.42	5.15	16.59	2.72	7.94
Zn	4.54	5.82	2.53	3.14	3.66	4.35	1.72	2.15	4.71	5.19	2.19	3.09	4.46	5.23	2.58	3.23

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

To examine the effect of metal(loid) accumulation in the different Aleppo pine populations, a wide range of antioxidants, oxidative stress markers, and physiological parameters were analyzed (Supplemental Table S4) and subjected to multivariate statistical analysis, in an attempt to gain an unbiased view on the interactions among these markers. Firstly, a PCA was carried out for unsupervised dimension reduction. A plot of the first two PCs, that together explained ~50% of the total variance, showed a clear

separation between spring and summer samples, and also revealed evident clustering differences between non-mining 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.

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

Physiological and biochemical data were further processed by PLS-DA method in order to have a quantitative estimation of the most influential parameters based on the variable importance in the projection (VIP), defined as a weighted sum of squares of partial least squares loadings. 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 variable importance in the projection (VIP) score were plotted. As seen in Figure 3A, markers with the VIP threshold >1 and with high correlation with PLS-DA1, accounting for $\sim 32\%$ 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 $\sim 18\%$ 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 , $\text{O}_2^{\bullet-}$), 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 the growing season. Strong separation of the Mercader samples was caused by an increase in chlorophylls, and GSH, which showed a high correlation with foliar nitrogen content ($r >$

0.6, $P < 0.0001$, Supplemental Table S1), as well as by the absence of change in total antioxidant activities, soluble phenols (TPC and flavonoids), 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. S5).

Figure 3. Identification of the most influential physiological and biochemical biomarkers based on the variable importance in the projection (VIP) and the correlation coefficients for the first three components of PLS-DA (**A**) and on mean decrease in accuracy estimated by random forest machine learning algorithm (**B**). For abbreviations, see legend to Figure 2.

Figure 4. Heatmap and complete-linkage hierarchical clustering (by using a distance based on Spearman rank correlation coefficient) showing the seasonal fold change (mining vs. non-mining) of the physiological and biochemical parameters measured in needles of Aleppo pine trees growing in non-mining and in mining tailings piles in late spring and summer in 2012 and 2013. Log₂ ratios of fold changes relative to each respective control group are given by shades of red or blue colors according to the scale bar. For abbreviations, see legend to Figure 2.

The dendrogram also revealed that Agustin summer samples were separated from those of Wikon and Ripolles, this being caused by the reduction in the levels of photosynthetic pigments, although in the growing season these populations did not diverge and were clustered according to the year of sampling.

Biochemical markers, illustrated in the rows of the heatmap in Fig. 4, were clustered into two large groups which were characterized, on the one hand, by a number of variables associated with the physiological status (photosynthetic pigments, RWC, sugars and starch contents; group 1, Fig. 4) and, on the other hand, by markers related with phenol metabolism (HCAs, total phenol content, flavanols, flavonoids, 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, indicating 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), which 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).

Figure S5. Seasonal variations in the ratio of total carotenoids/chlorophylls 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.

Figure S6. 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.

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 $\pm$ SE (n=4).

	NM			
	Spring		Summer	
	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	27.62 $\pm$ 0.24	31.65 $\pm$ 2.27	28.75 $\pm$ 0.91	21.95 $\pm$ 0.59
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	65.34 $\pm$ 3.33	82.49 $\pm$ 3.91	68.15 $\pm$ 2.70	58.35 $\pm$ 2.36
Carotenoids ($\mu\text{g g}^{-1}$ FW)	1.99 $\pm$ 0.45	5.40 $\pm$ 0.65	26.40 $\pm$ 0.52	25.13 $\pm$ 0.99
Chla ($\mu\text{g g}^{-1}$ FW)	449.01 $\pm$ 6.39	462.23 $\pm$ 10.29	112.91 $\pm$ 7.34	94.80 $\pm$ 5.38
Chlb ($\mu\text{g g}^{-1}$ FW)	304.66 $\pm$ 2.25	293.75 $\pm$ 6.89	60.31 $\pm$ 3.52	59.11 $\pm$ 2.21
Carbonyl groups (nmol C=O mg ⁻¹ protein)	17.16 $\pm$ 0.85	4.63 $\pm$ 1.23	8.25 $\pm$ 0.93	11.19 $\pm$ 0.71
DHA ($\mu\text{mol g}^{-1}$ FW)	0.66 $\pm$ 0.12	1.13 $\pm$ 0.24	3.24 $\pm$ 0.43	1.19 $\pm$ 0.13
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	64.96 $\pm$ 2.10	64.94 $\pm$ 5.15	62.90 $\pm$ 3.13	42.06 $\pm$ 2.01
Flavanols (mmol (+) catechin eq. g ⁻¹ FW)	16.51 $\pm$ 0.46	15.83 $\pm$ 1.39	8.75 $\pm$ 0.51	9.05 $\pm$ 0.37
Flavonoids (mmol rutin eq. g ⁻¹ FW)	34.20 $\pm$ 1.04	32.15 $\pm$ 2.88	29.05 $\pm$ 1.20	21.97 $\pm$ 1.19
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	288.69 $\pm$ 7.66	272.94 $\pm$ 21.02	229.07 $\pm$ 7.59	189.86 $\pm$ 7.87
GSH ($\mu\text{mol g}^{-1}$ FW)	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01
H₂O₂ ($\mu\text{mol g}^{-1}$ FW)	1.83 $\pm$ 0.14	0.85 $\pm$ 0.04	2.05 $\pm$ 0.032	0.96 $\pm$ 0.06
HCAs (mmol caffeic acid eq. g ⁻¹ FW)	53.85 $\pm$ 1.22	98.20 $\pm$ 7.31	63.28 $\pm$ 2.66	58.32 $\pm$ 2.59
iPRX (nkat TMB oxidized mg ⁻¹ protein)	0.39 $\pm$ 0.03	0.36 $\pm$ 0.02	0.35 $\pm$ 0.05	0.21 $\pm$ 0.02
Lignin (mg LTGA g ⁻¹ DW)	66.34 $\pm$ 1.41	66.13 $\pm$ 0.32	70.12 $\pm$ 2.25	77.12 $\pm$ 0.97
MDA (nmol g ⁻¹ FW)	237.03 $\pm$ 2.62	365.48 $\pm$ 32.22	291.99 $\pm$ 15.59	340.49 $\pm$ 9.39
NPT (nmol GSH eq. g ⁻¹ FW)	420.44 $\pm$ 14.81	435.13 $\pm$ 11.26	422.36 $\pm$ 13.09	336.59 $\pm$ 10.86
O₂⁻ (nmol g ⁻¹ FW)	89.13 $\pm$ 8.36	69.67 $\pm$ 3.57	126.87 $\pm$ 5.46	75.62 $\pm$ 4.05
PAL (nmol <i>trans</i> -cinnamic acid h ⁻¹ mg ⁻¹ protein)	3.04 $\pm$ 0.36	1.89 $\pm$ 0.33	2.02 $\pm$ 0.30	1.95 $\pm$ 0.20
Proanthocyanidins (mmol cyanidin eq. g ⁻¹ FW)	3.01 $\pm$ 0.06	1.37 $\pm$ 0.05	1.95 $\pm$ 0.06	1.44 $\pm$ 0.02
Total phenol content (mmol gallic acid eq. g ⁻¹ FW)	160.67 $\pm$ 1.95	150.93 $\pm$ 7.20	127.60 $\pm$ 2.33	124.69 $\pm$ 3.01
Proline ($\mu\text{mol g}^{-1}$ FW)	0.90 $\pm$ 0.01	0.95 $\pm$ 0.03	0.83 $\pm$ 0.05	0.84 $\pm$ 0.06
Protein (mg g ⁻¹ FW)	29.02 $\pm$ 0.54	33.15 $\pm$ 3.63	37.40 $\pm$ 1.54	34.98 $\pm$ 1.27
RWC (%)	48.06 $\pm$ 0.13	47.19 $\pm$ 0.21	54.90 $\pm$ 0.08	53.63 $\pm$ 0.05
SA (nmol g ⁻¹ FW)	8.93 $\pm$ 0.26	8.65 $\pm$ 0.19	7.26 $\pm$ 0.30	7.37 $\pm$ 0.30
SAG (nmol g ⁻¹ FW)	8.57 $\pm$ 0.42	13.40 $\pm$ 2.04	6.79 $\pm$ 0.30	8.17 $\pm$ 0.25
sPRX (nkat TMB oxidized mg ⁻¹ protein)	1.34 $\pm$ 0.09	1.65 $\pm$ 0.03	0.49 $\pm$ 0.03	0.40 $\pm$ 0.01
Starch ($\mu\text{g g}^{-1}$ FW)	243.50 $\pm$ 7.78	220.17 $\pm$ 3.26	348.10 $\pm$ 25.97	217.27 $\pm$ 5.15
Sugars ($\mu\text{g g}^{-1}$ FW)	35.18 $\pm$ 1.04	36.81 $\pm$ 0.68	21.45 $\pm$ 0.51	20.21 $\pm$ 0.21

	Agustin			
	Spring		Summer	
	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	35.33 $\pm$ 2.07	33.61 $\pm$ 0.31	33.51 $\pm$ 1.40	22.65 $\pm$ 0.46
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	108.08 $\pm$ 3.82	97.33 $\pm$ 3.76	76.52 $\pm$ 3.95	76.47 $\pm$ 1.10
Carotenoids ($\mu\text{g g}^{-1}$ FW)	1.79 $\pm$ 0.14	8.88 $\pm$ 1.21	21.04 $\pm$ 1.40	20.57 $\pm$ 0.38
Chla ($\mu\text{g g}^{-1}$ FW)	341.01 $\pm$ 10.98	346.41 $\pm$ 12.97	70.88 $\pm$ 1.65	79.21 $\pm$ 0.83
Chlb ($\mu\text{g g}^{-1}$ FW)	261.71 $\pm$ 6.40	235.33 $\pm$ 9.77	42.16 $\pm$ 0.76	49.24 $\pm$ 0.33
Carbonyl groups (nmol C=O mg ⁻¹ protein)	9.41 $\pm$ 0.24	2.79 $\pm$ 0.85	4.16 $\pm$ 0.30	9.22 $\pm$ 0.84
DHA ($\mu\text{mol g}^{-1}$ FW)	1.38 $\pm$ 0.22	3.54 $\pm$ 0.39	5.15 $\pm$ 0.39	1.46 $\pm$ 0.25
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	91.59 $\pm$ 4.45	85.68 $\pm$ 4.45	72.65 $\pm$ 4.31	60.36 $\pm$ 1.51
Flavanols (mmol (+) catechin eq. g ⁻¹ FW)	24.02 $\pm$ 1.44	20.25 $\pm$ 1.15	11.28 $\pm$ 0.82	12.92 $\pm$ 0.17
Flavonoids (mmol rutin eq. g ⁻¹ FW)	53.17 $\pm$ 3.31	45.11 $\pm$ 3.86	30.96 $\pm$ 2.07	28.87 $\pm$ 0.51
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	390.46 $\pm$ 18.07	337.80 $\pm$ 11.93	264.04 $\pm$ 18.12	245.44 $\pm$ 7.07
GSH ($\mu\text{mol g}^{-1}$ FW)	0.21 $\pm$ 0.01	0.26 $\pm$ 0.01	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01
H₂O₂ ($\mu\text{mol g}^{-1}$ FW)	0.89 $\pm$ 0.09	0.75 $\pm$ 0.13	1.84 $\pm$ 0.06	0.59 $\pm$ 0.05
HCAs (mmol caffeic acid eq. g ⁻¹ FW)	86.62 $\pm$ 2.86	133.59 $\pm$ 3.54	80.77 $\pm$ 6.58	79.13 $\pm$ 1.13
iPRX (nkat TMB oxidized mg ⁻¹ protein)	0.16 $\pm$ 0.01	0.14 $\pm$ 0.01	0.16 $\pm$ 0.02	0.10 $\pm$ 0.01
Lignin (mg LTGA g ⁻¹ DW)	69.72 $\pm$ 1.91	69.55 $\pm$ 0.60	70.81 $\pm$ 2.44	82.11 $\pm$ 0.27
MDA (nmol g ⁻¹ FW)	357.41 $\pm$ 25.48	437.02 $\pm$ 9.95	393.06 $\pm$ 23.45	386.19 $\pm$ 18.92
NPT (nmol GSH eq. g ⁻¹ FW)	431.19 $\pm$ 9.36	440.95 $\pm$ 14.52	426.79 $\pm$ 6.69	341.77 $\pm$ 7.49
O₂⁻ (nmol g ⁻¹ FW)	130.20 $\pm$ 10.57	100.11 $\pm$ 4.02	122.73 $\pm$ 8.25	68.37 $\pm$ 7.38
PAL (nmol <i>trans</i> -cinnamic acid h ⁻¹ mg ⁻¹ protein)	4.83 $\pm$ 0.70	2.46 $\pm$ 0.25	2.56 $\pm$ 0.20	3.60 $\pm$ 0.23
Proanthocyanidins (mmol cyanidin eq. g ⁻¹ FW)	4.23 $\pm$ 0.06	2.10 $\pm$ 0.01	2.76 $\pm$ 0.17	1.59 $\pm$ 0.03
Total phenol content (mmol gallic acid eq. g ⁻¹ FW)	223.24 $\pm$ 8.82	184.53 $\pm$ 7.51	143.40 $\pm$ 5.93	164.71 $\pm$ 3.02
Proline ($\mu\text{mol g}^{-1}$ FW)	1.02 $\pm$ 0.06	1.00 $\pm$ 0.02	1.31 $\pm$ 0.12	1.03 $\pm$ 0.07
Protein (mg g ⁻¹ FW)	44.42 $\pm$ 2.19	37.83 $\pm$ 0.67	49.67 $\pm$ 1.84	40.45 $\pm$ 0.90
RWC (%)	47.97 $\pm$ 0.06	46.30 $\pm$ 0.21	54.25 $\pm$ 0.19	52.74 $\pm$ 0.15
SA (nmol g ⁻¹ FW)	10.11 $\pm$ 0.18	9.57 $\pm$ 0.27	8.67 $\pm$ 0.13	9.76 $\pm$ 0.31
SAG (nmol g ⁻¹ FW)	7.85 $\pm$ 0.13	9.48 $\pm$ 0.24	9.77 $\pm$ 0.17	9.34 $\pm$ 0.12
sPRX (nkat TMB oxidized mg ⁻¹ protein)	0.82 $\pm$ 0.04	1.13 $\pm$ 0.07	0.31 $\pm$ 0.04	0.39 $\pm$ 0.02
Starch ($\mu\text{g g}^{-1}$ FW)	360.53 $\pm$ 5.82	269.88 $\pm$ 3.89	358.13 $\pm$ 23.02	221.38 $\pm$ 3.72
Sugars ($\mu\text{g g}^{-1}$ FW)	28.61 $\pm$ 0.38	29.28 $\pm$ 1.24	20.86 $\pm$ 0.36	19.57 $\pm$ 0.26

	Mercader			
	Spring		Summer	
	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	36.10 $\pm$ 1.48	34.97 $\pm$ 1.84	40.83 $\pm$ 1.65	28.00 $\pm$ 0.64
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	70.55 $\pm$ 7.82	86.68 $\pm$ 1.93	91.58 $\pm$ 3.58	71.93 $\pm$ 2.35
Carotenoids ($\mu\text{g g}^{-1}$ FW)	15.51 $\pm$ 1.10	22.73 $\pm$ 1.43	29.10 $\pm$ 0.22	29.96 $\pm$ 0.87
Chla ($\mu\text{g g}^{-1}$ FW)	525.82 $\pm$ 20.13	572.94 $\pm$ 14.74	198.55 $\pm$ 12.21	135.06 $\pm$ 9.53
Chlb ($\mu\text{g g}^{-1}$ FW)	358.89 $\pm$ 11.16	358.32 $\pm$ 6.55	107.07 $\pm$ 5.40	82.40 $\pm$ 3.97
Carbonyl groups (nmol C=O mg ⁻¹ protein)	11.86 $\pm$ 0.40	4.37 $\pm$ 1.09	3.90 $\pm$ 0.26	9.43 $\pm$ 0.69
DHA ($\mu\text{mol g}^{-1}$ FW)	0.94 $\pm$ 0.20	4.35 $\pm$ 0.52	4.40 $\pm$ 1.01	1.21 $\pm$ 0.19
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	68.36 $\pm$ 4.91	69.62 $\pm$ 2.16	84.53 $\pm$ 3.49	57.44 $\pm$ 1.94
Flavanols (mmol (+) catechin eq. g ⁻¹ FW)	20.94 $\pm$ 1.23	17.36 $\pm$ 0.44	13.93 $\pm$ 0.70	13.16 $\pm$ 0.50
Flavonoids (mmol rutin eq. g ⁻¹ FW)	37.76 $\pm$ 3.00	33.96 $\pm$ 1.61	36.28 $\pm$ 1.82	29.82 $\pm$ 1.79
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	297.29 $\pm$ 12.93	280.80 $\pm$ 12.49	315.42 $\pm$ 12.41	250.19 $\pm$ 7.64
GSH ($\mu\text{mol g}^{-1}$ FW)	0.82 $\pm$ 0.04	0.40 $\pm$ 0.02	0.32 $\pm$ 0.02	0.24 $\pm$ 0.01
H₂O₂ ($\mu\text{mol g}^{-1}$ FW)	1.15 $\pm$ 0.05	0.68 $\pm$ 0.11	2.03 $\pm$ 0.16	0.72 $\pm$ 0.09
HCAs (mmol caffeic acid eq. g ⁻¹ FW)	55.82 $\pm$ 3.95	84.58 $\pm$ 5.04	84.04 $\pm$ 2.52	74.36 $\pm$ 2.33
iPRX (nkat TMB oxidized mg ⁻¹ protein)	0.39 $\pm$ 0.02	0.34 $\pm$ 0.02	0.22 $\pm$ 0.01	0.15 $\pm$ 0.01
Lignin (mg LTGA g ⁻¹ DW)	74.52 $\pm$ 0.61	78.06 $\pm$ 0.59	76.88 $\pm$ 0.36	83.02 $\pm$ 0.62
MDA (nmol g ⁻¹ FW)	374.84 $\pm$ 14.26	474.74 $\pm$ 28.52	459.55 $\pm$ 17.34	592.64 $\pm$ 21.70
NPT (nmol GSH eq. g ⁻¹ FW)	456.65 $\pm$ 9.49	463.66 $\pm$ 9.73	451.83 $\pm$ 22.74	363.22 $\pm$ 21.42
O₂⁻ (nmol g ⁻¹ FW)	115.43 $\pm$ 3.90	95.97 $\pm$ 2.37	109.57 $\pm$ 3.03	72.92 $\pm$ 5.76
PAL (nmol <i>trans</i> -cinnamic acid h ⁻¹ mg ⁻¹ protein)	4.12 $\pm$ 0.29	5.14 $\pm$ 0.44	4.24 $\pm$ 0.21	2.70 $\pm$ 0.33
Proanthocyanidins (mmol cyanidin eq. g ⁻¹ FW)	3.12 $\pm$ 0.05	1.89 $\pm$ 0.03	2.19 $\pm$ 0.06	1.45 $\pm$ 0.04
Total phenol content (mmol gallic acid eq. g ⁻¹ FW)	169.07 $\pm$ 5.75	155.51 $\pm$ 7.14	150.09 $\pm$ 8.04	140.99 $\pm$ 4.59
Proline ($\mu\text{mol g}^{-1}$ FW)	3.03 $\pm$ 0.03	2.38 $\pm$ 0.07	1.72 $\pm$ 0.05	2.07 $\pm$ 0.06
Protein (mg g ⁻¹ FW)	34.08 $\pm$ 2.62	35.27 $\pm$ 3.85	48.71 $\pm$ 1.49	41.55 $\pm$ 1.45
RWC (%)	46.19 $\pm$ 0.15	46.85 $\pm$ 0.31	52.79 $\pm$ 0.08	52.92 $\pm$ 0.06
SA (nmol g ⁻¹ FW)	9.71 $\pm$ 0.22	8.90 $\pm$ 0.16	8.42 $\pm$ 0.11	8.66 $\pm$ 0.29
SAG (nmol g ⁻¹ FW)	7.56 $\pm$ 0.52	8.11 $\pm$ 0.09	9.92 $\pm$ 0.15	8.91 $\pm$ 0.09
sPRX (nkat TMB oxidized mg ⁻¹ protein)	2.20 $\pm$ 0.25	2.85 $\pm$ 0.32	0.37 $\pm$ 0.02	0.27 $\pm$ 0.02
Starch ($\mu\text{g g}^{-1}$ FW)	279.22 $\pm$ 20.47	259.02 $\pm$ 7.70	356.53 $\pm$ 4.42	235.75 $\pm$ 5.58
Sugars ($\mu\text{g g}^{-1}$ FW)	30.32 $\pm$ 1.58	28.46 $\pm$ 1.17	21.36 $\pm$ 0.33	20.16 $\pm$ 0.15

	Ripolles			
	Spring		Summer	
	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	38.79 ± 1.72	39.02 ± 1.12	41.34 ± 0.77	30.98 ± 0.45
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	109.84 ± 4.35	98.19 ± 2.90	92.64 ± 1.61	77.62 ± 1.53
Carotenoids ($\mu\text{g g}^{-1}$ FW)	13.00 ± 2.28	23.04 ± 3.46	28.66 ± 0.34	27.63 ± 0.44
Chla ($\mu\text{g g}^{-1}$ FW)	447.84 ± 7.56	424.66 ± 15.76	92.24 ± 2.58	69.07 ± 1.11
Chlb ($\mu\text{g g}^{-1}$ FW)	301.14 ± 3.22	282.05 ± 6.21	53.95 ± 1.28	52.49 ± 1.24
Carbonyl groups (nmol C=O mg ⁻¹ protein)	9.93 ± 0.32	3.71 ± 0.88	2.75 ± 0.24	9.22 ± 0.44
DHA ($\mu\text{mol g}^{-1}$ FW)	2.42 ± 0.87	4.46 ± 0.44	3.92 ± 0.54	1.61 ± 0.33
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	99.18 ± 4.95	86.26 ± 3.24	89.58 ± 1.11	64.32 ± 1.06
Flavanols (mmol (+) catechin eq. g ⁻¹ FW)	27.21 ± 1.61	19.51 ± 0.47	13.39 ± 0.16	15.47 ± 0.47
Flavonoids (mmol rutin eq. g ⁻¹ FW)	58.68 ± 2.95	40.46 ± 2.33	40.89 ± 1.20	35.45 ± 1.38
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	435.09 ± 19.02	316.15 ± 9.45	338.80 ± 4.08	264.12 ± 3.45
GSH ($\mu\text{mol g}^{-1}$ FW)	0.28 ± 0.02	0.26 ± 0.01	0.17 ± 0.01	0.14 ± 0.01
H₂O₂ ($\mu\text{mol g}^{-1}$ FW)	0.91 ± 0.05	0.65 ± 0.04	1.72 ± 0.16	0.51 ± 0.06
HCAs (mmol caffeic acid eq. g ⁻¹ FW)	81.94 ± 3.68	107.81 ± 4.49	96.93 ± 1.40	84.53 ± 1.04
iPRX (nkat TMB oxidized mg ⁻¹ protein)	0.18 ± 0.01	0.25 ± 0.01	0.14 ± 0.02	0.09 ± 0.01
Lignin (mg LTGA g ⁻¹ DW)	76.58 ± 1.61	72.94 ± 1.25	70.38 ± 0.80	80.18 ± 1.63
MDA (nmol g ⁻¹ FW)	416.47 ± 14.09	478.06 ± 24.27	374.30 ± 12.14	494.46 ± 17.05
NPT (nmol GSH eq. g ⁻¹ FW)	475.69 ± 16.15	489.93 ± 8.95	433.32 ± 8.78	359.34 ± 26.20
O₂⁻ (nmol g ⁻¹ FW)	138.89 ± 8.74	141.58 ± 4.56	88.50 ± 1.80	49.27 ± 4.31
PAL (nmol <i>trans</i> -cinnamic acid h ⁻¹ mg ⁻¹ protein)	3.34 ± 0.13	5.01 ± 0.69	3.72 ± 0.46	3.76 ± 0.65
Proanthocyanidins (mmol cyanidin eq. g ⁻¹ FW)	4.99 ± 0.16	3.27 ± 0.42	2.24 ± 0.07	1.60 ± 0.05
Total phenol content (mmol gallic acid eq. g ⁻¹ FW)	239.41 ± 9.93	175.58 ± 4.96	169.00 ± 3.34	160.80 ± 3.03
Proline ($\mu\text{mol g}^{-1}$ FW)	1.23 ± 0.04	1.01 ± 0.02	1.32 ± 0.01	1.17 ± 0.06
Protein (mg g ⁻¹ FW)	49.64 ± 1.86	40.67 ± 1.80	53.19 ± 1.02	50.33 ± 1.49
RWC (%)	47.81 ± 0.23	46.79 ± 0.67	54.86 ± 0.15	53.22 ± 0.36
SA (nmol g ⁻¹ FW)	9.24 ± 0.15	9.05 ± 0.28	7.41 ± 0.33	7.56 ± 0.21
SAG (nmol g ⁻¹ FW)	8.35 ± 0.29	8.46 ± 0.09	8.38 ± 0.47	9.02 ± 0.15
sPRX (nkat TMB oxidized mg ⁻¹ protein)	0.64 ± 0.05	1.47 ± 0.04	0.20 ± 0.02	0.21 ± 0.02
Starch ($\mu\text{g g}^{-1}$ FW)	383.35 ± 14.88	280.18 ± 11.16	350.10 ± 7.00	301.80 ± 24.58
Sugars ($\mu\text{g g}^{-1}$ FW)	30.39 ± 1.15	31.50 ± 0.75	21.18 ± 0.42	20.10 ± 0.14

	Wikon			
	Spring		Summer	
	2012	2013	2012	2013
AA ($\mu\text{mol g}^{-1}$ FW)	39.27 $\pm$ 0.31	35.31 $\pm$ 0.61	43.56 $\pm$ 1.29	28.67 $\pm$ 1.13
ABTS ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	112.58 $\pm$ 2.37	88.81 $\pm$ 0.76	92.92 $\pm$ 3.61	82.98 $\pm$ 2.39
Carotenoids ($\mu\text{g g}^{-1}$ FW)	8.74 $\pm$ 0.91	22.00 $\pm$ 0.51	30.63 $\pm$ 1.21	26.81 $\pm$ 0.59
Chla ($\mu\text{g g}^{-1}$ FW)	435.45 $\pm$ 14.82	446.54 $\pm$ 9.44	102.99 $\pm$ 1.90	90.46 $\pm$ 3.34
Chlb ($\mu\text{g g}^{-1}$ FW)	301.42 $\pm$ 5.75	280.97 $\pm$ 4.65	54.40 $\pm$ 0.91	53.85 $\pm$ 1.05
Carbonyl groups (nmol C=O mg ⁻¹ protein)	11.08 $\pm$ 0.28	3.67 $\pm$ 0.33	5.97 $\pm$ 0.63	9.06 $\pm$ 0.56
DHA ($\mu\text{mol g}^{-1}$ FW)	2.34 $\pm$ 0.28	5.51 $\pm$ 0.51	3.34 $\pm$ 0.36	1.30 $\pm$ 0.18
DPPH ($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	95.68 $\pm$ 0.76	76.69 $\pm$ 1.77	89.39 $\pm$ 3.40	66.41 $\pm$ 2.83
Flavanols (mmol (+) catechin eq. g ⁻¹ FW)	30.04 $\pm$ 0.43	20.24 $\pm$ 0.60	14.68 $\pm$ 0.44	13.53 $\pm$ 0.44
Flavonoids (mmol rutin eq. g ⁻¹ FW)	64.46 $\pm$ 0.97	36.49 $\pm$ 0.93	41.82 $\pm$ 1.30	32.57 $\pm$ 1.00
FRAP ($\mu\text{mol Fe(II) g}^{-1}$ FW)	442.17 $\pm$ 4.41	326.25 $\pm$ 13.02	325.19 $\pm$ 13.63	265.90 $\pm$ 10.78
GSH ($\mu\text{mol g}^{-1}$ FW)	0.55 $\pm$ 0.02	0.32 $\pm$ 0.02	0.17 $\pm$ 0.01	0.15 $\pm$ 0.01
H₂O₂ ($\mu\text{mol g}^{-1}$ FW)	1.05 $\pm$ 0.08	0.54 $\pm$ 0.07	1.55 $\pm$ 0.03	0.53 $\pm$ 0.02
HCAs (mmol caffeic acid eq. g ⁻¹ FW)	85.43 $\pm$ 3.59	110.05 $\pm$ 2.62	99.98 $\pm$ 2.65	79.50 $\pm$ 1.90
iPRX (nkat TMB oxidized mg ⁻¹ protein)	0.13 $\pm$ 0.02	0.17 $\pm$ 0.03	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01
Lignin (mg LTGA g ⁻¹ DW)	81.96 $\pm$ 0.57	75.39 $\pm$ 0.64	79.44 $\pm$ 0.86	79.74 $\pm$ 0.40
MDA (nmol g ⁻¹ FW)	436.44 $\pm$ 3.42	490.72 $\pm$ 13.19	444.42 $\pm$ 22.67	509.32 $\pm$ 22.95
NPT (nmol GSH eq. g ⁻¹ FW)	460.08 $\pm$ 11.93	467.32 $\pm$ 8.71	426.93 $\pm$ 9.29	345.98 $\pm$ 3.09
O₂⁻ (nmol g ⁻¹ FW)	103.45 $\pm$ 3.67	118.34 $\pm$ 4.45	124.52 $\pm$ 6.70	46.65 $\pm$ 4.96
PAL (nmol <i>trans</i> -cinnamic acid h ⁻¹ mg ⁻¹ protein)	3.23 $\pm$ 0.25	4.29 $\pm$ 0.23	2.73 $\pm$ 0.48	2.89 $\pm$ 0.69
Proanthocyanidins (mmol cyanidin eq. g ⁻¹ FW)	4.02 $\pm$ 0.03	2.04 $\pm$ 0.05	2.06 $\pm$ 0.06	1.52 $\pm$ 0.03
Total phenol content (mmol gallic acid eq. g ⁻¹ FW)	233.65 $\pm$ 1.73	170.36 $\pm$ 3.69	175.96 $\pm$ 4.31	169.76 $\pm$ 3.45
Proline ($\mu\text{mol g}^{-1}$ FW)	1.33 $\pm$ 0.01	1.07 $\pm$ 0.06	1.49 $\pm$ 0.11	1.13 $\pm$ 0.13
Protein (mg g ⁻¹ FW)	47.64 $\pm$ 0.96	44.11 $\pm$ 0.63	57.39 $\pm$ 1.31	42.12 $\pm$ 1.32
RWC (%)	47.10 $\pm$ 0.04	46.95 $\pm$ 0.22	52.90 $\pm$ 0.09	53.18 $\pm$ 0.04
SA (nmol g ⁻¹ FW)	9.07 $\pm$ 0.14	8.74 $\pm$ 0.14	7.94 $\pm$ 0.21	7.55 $\pm$ 0.50
SAG (nmol g ⁻¹ FW)	8.31 $\pm$ 0.24	8.33 $\pm$ 0.11	8.92 $\pm$ 0.41	8.22 $\pm$ 0.18
sPRX (nkat TMB oxidized mg ⁻¹ protein)	0.81 $\pm$ 0.03	1.59 $\pm$ 0.08	0.33 $\pm$ 0.03	0.39 $\pm$ 0.01
Starch ($\mu\text{g g}^{-1}$ FW)	276.49 $\pm$ 7.15	298.50 $\pm$ 10.08	357.78 $\pm$ 27.42	271.53 $\pm$ 26.06
Sugars ($\mu\text{g g}^{-1}$ FW)	33.94 $\pm$ 1.08	33.18 $\pm$ 0.85	21.38 $\pm$ 0.35	20.12 $\pm$ 0.21

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.

	Agustin				Mercader			
	Spring		Summer		Spring		Summer	
	2012	2013	2012	2013	2012	2013	2012	2013
AA	1.28	1.06	1.17	1.03	1.31	1.10	1.42	1.28
ABTS	1.65	1.18	1.14	1.32	1.08	1.05	1.34	1.25
Car	0.90	1.64	0.79	0.82	7.81	4.21	1.10	1.20
Chla	0.76	0.75	0.63	0.85	1.17	1.24	1.76	1.46
Chlb	0.86	0.80	0.70	0.84	1.18	1.22	1.78	1.41
C=O	0.55	0.60	0.56	0.82	0.69	0.94	0.53	0.84
DHA	2.09	3.14	1.61	1.35	1.43	3.86	1.39	1.07
DPPH	1.41	1.32	1.18	1.43	1.05	1.07	1.35	1.37
Flavanols	1.45	1.28	1.32	1.43	1.27	1.10	1.59	1.45
Flavonoids	1.55	1.40	1.08	1.32	1.10	1.06	1.25	1.36
FRAP	1.35	1.24	1.16	1.30	1.03	1.03	1.38	1.32
GSH	1.14	1.01	1.02	1.01	4.44	1.57	2.34	1.85
H ₂ O ₂	0.49	0.89	0.90	0.63	0.63	0.80	0.99	0.75
HCAs	1.61	1.36	1.30	1.36	1.04	0.86	1.33	1.28
iPRX	0.40	0.39	0.50	0.48	1.00	0.96	0.69	0.70
Lignin	1.05	1.05	1.01	1.07	1.12	1.18	1.10	1.08
MDA	1.51	1.20	1.35	1.13	1.58	1.30	1.57	1.74
NPT	1.03	1.01	1.01	1.02	1.09	1.07	1.07	1.08
O ₂ ⁻	1.46	1.44	0.96	0.92	1.30	1.38	0.87	0.97
PAL	1.59	1.30	1.27	1.88	1.36	2.72	2.10	1.41
Pas	1.40	1.53	1.42	1.10	1.03	1.38	1.12	1.00
Phenols (TPC)	1.39	1.22	1.13	1.33	1.05	1.03	1.17	1.14
Proline	1.12	1.05	1.62	1.23	3.35	2.51	2.08	2.46
Protein	1.53	1.14	1.33	1.16	1.17	1.06	1.30	1.19
RWC	0.99	0.98	0.99	0.98	0.96	0.99	0.96	0.99
SA	1.13	1.11	1.20	1.33	1.09	1.03	1.17	1.17
SAG	0.92	0.71	1.44	1.14	0.88	0.60	1.47	1.09
sPRX	0.61	0.68	0.64	0.96	1.64	1.73	0.77	0.67
Starch	1.48	1.23	1.03	1.02	1.15	1.18	1.04	1.09
Sugars	0.81	0.80	0.97	0.97	0.86	0.77	1.00	1.00

	Ripolles				Wikon			
	Spring		Summer		Spring		Summer	
	2012	2013	2012	2013	2012	2013	2012	2013
AA	1.40	1.23	1.44	1.41	1.42	1.12	1.52	1.31
ABTS	1.68	1.19	1.36	1.34	1.72	1.08	1.37	1.44
Car	6.54	4.27	1.09	1.11	4.40	4.07	1.16	1.08
Chla	1.00	0.92	0.82	0.74	0.97	0.97	0.92	0.96
Chlb	0.99	0.96	0.90	0.89	0.99	0.96	0.91	0.91
C=O	0.58	0.80	0.38	0.82	0.65	0.79	0.74	0.81
DHA	3.65	3.95	1.23	1.38	3.54	4.88	1.05	1.21
DPPH	1.53	1.33	1.43	1.53	1.47	1.18	1.43	1.58
Flavanols	1.65	1.23	1.55	1.71	1.82	1.28	1.69	1.50
Flavonoids	1.72	1.26	1.42	1.63	1.88	1.13	1.44	1.50
FRAP	1.51	1.16	1.48	1.39	1.53	1.20	1.42	1.41
GSH	1.52	1.01	1.24	1.12	2.98	1.24	1.23	1.18
H ₂ O ₂	0.50	0.77	0.84	0.54	0.58	0.63	0.76	0.55
HCAs	1.52	1.10	1.54	1.45	1.59	1.12	1.58	1.37
iPRX	0.47	0.70	0.52	0.41	0.33	0.48	0.26	0.30
Lignin	1.15	1.10	1.01	1.04	1.24	1.14	1.14	1.03
MDA	1.76	1.31	1.28	1.45	1.84	1.34	1.52	1.50
NPT	1.13	1.13	1.03	1.07	1.09	1.07	1.01	1.03
O ₂ ⁻	1.56	2.03	0.70	0.65	1.16	1.70	0.99	0.63
PAL	1.10	2.65	1.84	1.92	1.06	2.27	1.35	1.57
Pas	1.66	2.39	1.15	1.11	1.33	1.49	1.05	1.05
Phenols (TPC)	1.49	1.16	1.33	1.30	1.45	1.13	1.38	1.37
Proline	1.36	1.06	1.61	1.39	1.47	1.13	1.80	1.35
Protein	1.71	1.23	1.42	1.44	1.64	1.33	1.54	1.21
RWC	0.99	0.99	0.99	0.99	0.98	0.99	0.96	0.99
SA	1.03	1.05	1.03	1.03	1.02	1.01	1.11	1.04
SAG	0.97	0.63	1.25	1.10	0.97	0.62	1.33	1.01
sPRX	0.48	0.89	0.42	0.52	0.60	0.96	0.67	0.96
Starch	1.57	1.27	1.02	1.39	1.14	1.36	1.03	1.25
Sugars	0.86	0.86	0.99	0.99	0.96	0.90	1.00	1.00

4.5. 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, although clear soil physicochemical differences were found among mining samples, the PCA performed on needle ionome data discriminates between NM and mining populations, although cannot discriminate within mining pine populations. It is well established that the shoot ionome reflects the summation of several biochemical and physiological processes, including rhizosphere chemistry, mineral uptake, translocation, transpiration and phloem recycling (Baxter *et al.*, 2008; Salt *et al.*, 2008). Therefore, it can be inferred that soil chemistry, in 0–20 cm soil depth, appeared not to be relevant to explain the needle ionome pattern observed. These results contrast with those reported by Sun *et al.* (2009), who found a strong correlation ($r > 0.9$) between heavy metal concentrations in soils and needles from Masson pines, but agree with those reported by Párraga-Aguado *et al.* (2014b). In general, in this upper soil layer the availability of nutrients is higher as well as the size and activity of soil microbial communities (Sainju and Good, 1993). Thus, the uppermost soil layer tends to have a high concentration of fine-roots that enable trees to acquire growth-limiting soil resources (McCormack *et al.*, 2015). However, other possible sources for water and nutrients cannot be discarded. In this way it has been described that these tailings contain impermeable clay layers at depth where water can be retained (Párraga-Aguado *et al.*, 2014b). Moreover, it has been suggested that Aleppo pine possesses a dimorphic root system that combines shallow lateral and deep roots, the latter being crucial for plant survival when surface soil layers become dry (Voltas *et al.*, 2015). In fact, stable isotopes studies conducted on several Mediterranean populations of Aleppo pine show that this species is able to change the depth at which water uptake preferentially occurs depending on water availability in upper soil layers (Voltas *et al.*, 2015). Therefore, it is possible that absorptive roots of pines growing on mine tailings can reach deeper soil layers, with different physicochemical properties from those found in the top 20 cm, in a process that can be favored by the low resistance to root-penetration offered by sandy materials (Párraga-Aguado *et al.*, 2014b).

In our study, foliar concentrations of some nutrients (N, S, Mg, Mn and Ca) and metal(loid)s were higher in spring, which can be related to the spring growth activity

period of this species in the Mediterranean area (López-Serrano *et al.*, 2005; Pacheco *et al.*, 2017), whereas in summer, an increase in the accumulation of K was noticed. It is well established that K, the most abundant cation in plant cells, plays a key role in the water economy of plants, particularly by the maintenance of cell turgor which is critical for cell activity and growth (Marschner, 1995; Shabala and Pottosin, 2014). Our results revealed a strong correlation ($r > 0.8$, $P < 0.0001$, Supplemental Table S1) between K levels and needle RWC. Besides K, both foliar Na and Cl levels also exhibited moderate correlations with RWC ($r > 0.4$, $P < 0.0001$, Supplemental Table S1), suggesting that the uptake of both ions could also contribute to osmotic adjustment during summers in mining samples. Aleppo pine is a moderately salt-tolerant tree (Párraga-Aguado *et al.* 2014b), and it is long recognized that salt-tolerant species could partially replace Na for K, particularly under low K conditions (Marschner, 1995). More recently, it has been reported the beneficial role of Na in the physiological adjustments in different tree species exposed to drought in K-deficient soils (Battie-Laclau *et al.*, 2014; Erel *et al.*, 2014), which are in line with our findings. Moreover, negative or non-significant correlations between RWC and soluble sugars or proline, which are considered key osmolytes contributing to osmotic adjustment in stressed plants (Suzuki *et al.*, 2014), were found (Supplemental Table S1), indicating that these organic solutes appear to play a minor role in the needle osmotic adjustment in Aleppo pine populations studied. Since the maintenance of turgor is critical for normal cell function, its regulation via the uptake of inorganic ions, which requires a lower energetic cost, can be considered an efficient carbon-saving strategy for maintaining osmotic adjustment during the dry Mediterranean summers under conditions of reduced photosynthetic potential. The advantages to using inorganic ions for cell osmotic adjustment have been previously reported in different plant species under different stress conditions such as hyperosmotic stress (Shabala and Lew, 2002) and salinity (Orsini *et al.*, 2011).

In general, the foliar concentrations of K, N, and P in both NM and mining pine populations were lower than those described for Aleppo pine in Mediterranean non-polluted sites (López-Serrano *et al.*, 2005; Querejeta *et al.*, 2008) but in accordance with those reported by Párraga-Aguado *et al.* (2014b) for *P. halepensis* trees grown on tailings within the same mining area. In contrast, the foliar amount of S accumulated exceeded by more than twice the normal range reported for this species (Clarke *et al.*, 2008), and was

correlated with the levels of S-rich molecules (*i.e.*, the non-protein thiol pool, $r > 0.4$, $P < 0.0001$, and GSH content, $r > 0.6$, $P < 0.0001$, Supplemental Table S1) found in needles.

Actually, the examination of overall antioxidative/oxidative profile evidenced that spring needles, which showed higher accumulation of metal(loid)s, were characterized by high GSH levels. Interestingly, GSH was ranked as one of the most important variables based on mean decrease in accuracy criterion in RF analysis, showed a high correlation with PLS-DA1 and had a VIP score > 1 . High GSH content is considered essential for detoxification of heavy metals, not merely because it plays a crucial role in controlling the antioxidative defense system and redox homeostasis in plant cells, but also because it serves as a metal chelator agent, due to the high affinity of metals for its thiol group, and is the direct precursor of metal-binding phytochelatins, which detoxify, and sequester HMMs in the vacuole (Freeman *et al.*, 2004; Gill and Tuteja, 2010; Hernández *et al.*, 2015; Jozefczak *et al.*, 2012; Luo *et al.*, 2016). In the present study, a high-to-moderate correlation between foliar HMM concentrations and GSH was found, suggesting that thiol-mediated complexation could be an important mechanism of metal(loid) detoxification in Aleppo pines under these edaphoclimatic conditions (*i.e.*, N-deficient soils with high levels of water extractable divalent ions and metal(loid)s).

Proline was also placed among the top-5 significant biomarkers in RF analysis. 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, HCAs, flavonoids, flavanols, and particularly flavan-3-ol polymers (PAs). It has long been acknowledged that both biotic and abiotic stresses lead to the accumulation of carbon-rich secondary metabolites, including phenolic compounds, by increasing PAL gene/protein expression/activity (Boudet, 2007; Dixon and Paiva, 1995). Moreover, several studies have also described the increase of PRX activity under HMM stress

conditions in different plant species (Cuypers *et al.*, 2002; Díaz *et al.*, 2001; Jouili *et al.*, 2011; Passardi *et al.*, 2005). The data presented confirm the induction of PAL activity, although PRX was slightly increased, unaffected or decreased in leaves from mining populations. In our study PAL, soluble and cell wall-bound PRX activities showed a different pattern in Mercader as compared with the other mining samples that could explain the different levels of phenolics (HCAs, flavonoids, and TPC) accumulated in Mercader needles. It is worth noting that the contents of secondary metabolites are affected by foliar C/N ratios (Fritz *et al.*, 2006), and Mercader samples showed the highest foliar N concentrations, whereas Agustin ones showed the lowest N levels.

Phenolics have been described to be efficient antioxidants due to their ability to chelate metals, quench lipid peroxidation and scavenge ROS as well as to their capacity to protect or recycle endogenous antioxidants, such α -tocopherol and ascorbate (Agati *et al.*, 2012; Andejelkovic *et al.*, 2006; Dai *et al.*, 2008; Michalak, 2006; Rice-Evans *et al.*, 1997). These antioxidant properties of phenolics depend on their structure, mostly on the number and position of hydroxyl groups bound to the aromatic ring (Andejelkovic *et al.*, 2006; Rice-Evans *et al.*, 1997). In this way, the presence of *o*-diphenol groups enhances the radical scavenging and metal-chelating capacities of phenolic compounds. Increased accumulation of phenolics has previously been described in *Pinus* species challenged with heavy metals (Kareolewski and Giertych, 1995; Roitto *et al.*, 2005) as well as under atmospheric pollution (Pasqualini *et al.*, 2003). In our study, high correlations between phenolic compounds and foliar accumulation of metal(loid)s were noticed, suggesting that phenolics could have a role in detoxification and accumulation of metal(loid)s by functioning as chelating ligands. In addition, the needle biomarker in the first position of the RF analysis showed in Fig. 3B is PAs content, and these oligomeric phenolics have been recognized as efficient metal chelators. This fact, together with the trend observed in seasonal accumulation in needles, seems to assign a relevant role to PAs in Aleppo pine tolerance to harsh mine tailings conditions.

Moreover, apart from GSH and phenolics, mining needles also showed higher antioxidant capacity, estimated by ABTS, DPPH and FRAP tests, higher AA levels and exhibited a high AA redox ratio. The increase in the levels of AA and GSH, the major cellular antioxidants and redox buffers in plant cells (Foyer and Noctor, 2011), proline, and other

redox-active non-enzymatic antioxidants (*i.e.*, phenolics) seemed to be effective in controlling $O_2^{\bullet-}$ and H_2O_2 levels in both seasons and, consequently, in reducing protein oxidation, measured as protein carbonyl content. Nevertheless our data also revealed a certain degree of lipid peroxidation in foliar mining samples in both seasons, as compared with NM ones, that appeared not to have a significant detrimental effect on photosynthetic metabolism, as evidenced by the higher starch concentrations found in mining samples ($\sim 300 \mu g g^{-1}$ FW) compared to NM ones ($\sim 250 \mu g g^{-1}$ 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). Since 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), 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 in order to reduce light absorption and 1O_2 formation and 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 in this season (average value of $6.29 g kg^{-1}$ DW). 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 particularly during the active-growth season. 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). The presence of a dimorphic root system in this species could account for a continuous supply of water, even during the driest periods. Reestablishment of wet conditions in surface soil layers would activate water and nutrient absorption by shallow roots, and, at this point, responses to topsoil conditions would affect plant performance.

4.6. 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 than non-mining ones in the two seasons studied, but differences in these parameters were more pronounced in spring samples. The latter could be due to greater water availability in surface soil layers, which would expose shallow absorptive roots to topsoil components. The results obtained suggest that the effects induced by water supply during the growing season could have significantly more impact on the physiological and biochemical statuses of mining needle samples than inter-population differences. Taking into account the predicted precipitation scenarios in the Mediterranean basin in the context of climate change, with significant rainfall reductions, the present study gives some clues about the future metabolic behavior of Aleppo pines growing on mine tailings.

PART II:
PIONEER SPECIES GROWN UNDER
CONTROLLED ENVIRONMENTAL
CONDITIONS

CHAPTER 5

Pb-induced responses in *Zygophyllum fabago* plants are organ-dependent and modulated by salicylic acid

5.1. Abstract

Zygophyllum fabago is a promising species for restoring heavy metal (HM) polluted soils, although the mechanisms involved in HM tolerance in this non-model plant remain largely unknown. This paper analyses the extent to which redox-active compounds and enzymatic antioxidants in roots, stems and leaves are responsible for Pb tolerance in a metallicolous ecotype of *Z. fabago* and the possible influence of salicylic acid (SA) pretreatment (24 h, 0.5 mM SA) in the response to Pb stress. SA pretreatment reduced both the accumulation of Pb in roots and even more so the concentration of Pb in aerial parts of the plants, although a similar drop in the content of chlorophylls and in the maximum quantum yield of photosystem II was observed in both Pb- and SA-Pb-treated plants. Pb increased the endogenous free SA levels in all organs and this response was enhanced in root tissues upon SA pretreatment. Generally, Pb induced a reduction in catalase, ascorbate peroxidase and glutathione reductase specific activities, whereas dehydroascorbate reductase was increased in all organs of control plants. SA pretreatment enhanced the Pb-induced H₂O₂ accumulation in roots by up-regulating Fe-superoxide dismutase isoenzymes. Under Pb stress, the GSH redox ratio remained highly reduced in all organs while the ascorbic acid redox ratio dropped in leaf tissues where a rise in lipid peroxidation products and electrolyte leakage was observed. Finally, an organ-dependent accumulation of proline and β -carboline alkaloids was found, suggesting these nitrogen-redox-active compounds could play a role in the adaptation strategies of this species to Pb stress.

Keywords

Alkaloids, Cellular redox state, Lead stress, Proline, Salicylic acid, *Zygophyllum fabago*

5.2. Introduction

Lead (Pb) is a highly toxic and extremely persistent environmental pollutant, which poses serious threats to human health in industrialized countries (Gupta *et al.*, 2013). In fact, it is ranked second of all hazardous substances by the U.S. Agency for Toxic Substances and Disease Registry (ATSDR, 2011). Its concentration is increasing rapidly in soil largely through automotive emissions, mining, and industrial processes, which has been shown to cause a range of adverse effects on plant growth (Gupta *et al.*, 2013). Certain species, however, have evolved a complex network of homeostatic mechanisms that allow them to thrive in the presence of high soil Pb concentrations that are lethal to most species (Gupta *et al.*, 2013; Maestri *et al.*, 2010). Although over the last 10-20 years our basic understanding of the mechanisms underlying plant tolerance to non-essential toxic metals has greatly improved (Clemens, 2006; Gupta *et al.*, 2013; Maestri *et al.*, 2010), the biochemical mechanisms that mediate Pb tolerance and adaptation still remain largely unknown.

The toxicity of Pb has been associated with an increase in lipid peroxidation generated by reactive oxygen species (ROS) and/or by both the lipoxygenase enzyme and alterations in the antioxidative defense system (Gupta *et al.*, 2013, and references herein). In fact, multiple studies have demonstrated the heavy metal (HM) induction of H₂O₂ and other ROS. Plants possess a complex antioxidant network composed of metabolites, including ascorbate (AsA), glutathione (GSH) and carotenoids, and enzymes, including superoxide dismutases (SOD), catalase (CAT), peroxidases and the enzymes of the AsA-GSH cycle that maintains ROS levels and cellular redox homeostasis within certain limits (Noctor and Foyer, 1998). Changes in H₂O₂ production and in the cellular redox environment, mainly through the AsA/dehydroascorbate and GSH/oxidized glutathione redox couples, result in signals that influence the biological status of cells and tissues in response to stress (Noctor and Foyer, 1998).

Salicylic acid (SA) is a phenolic hormone that plays a crucial role in the induction of defense mechanisms in plants to combat both biotic (Vlot *et al.*, 2009) and abiotic stress factors, including HMs (Horváth *et al.*, 2007; Metwally *et al.*, 2003). SA also influences plant growth and development (Rivas-San Vicente and Plasencia, 2011) and contributes to maintaining cellular redox homeostasis (Mateo *et al.*, 2006). Furthermore, SA

signaling depends on SA-induced changes to the redox environment that result in the deoligomerization of the key transcriptional coactivator non-expressor of pathogenesis-related genes 1 (NPR1) leading to nuclear import of NPR1 monomers (Vlot *et al.*, 2009). Interestingly, NPR1 and its paralogues NPR3 and NPR4 have been proposed as SA receptors, and the binding of SA to NPR1 has been determined to occur via the transition metal copper through two cysteine residues (Janda and Ruelland, 2014; Pajerowska-Mukhtar *et al.*, 2013).

Moreover, a constitutive elevated level of SA has been correlated with increased Ni tolerance across different *Thlaspi* hyperaccumulator species (Freeman *et al.*, 2005). Such SA-mediated Ni tolerance has been associated with an enhancement of serine acetyltransferase activity, leading to the accumulation of GSH, which in turn allows *Thlaspi* to defend against Ni-induced lipid peroxidation (Freeman *et al.*, 2005). Besides being an essential co-substrate and a reductant in the control of ROS concentration, GSH is the precursor for the biosynthesis of phytochelatins (PC), which detoxify HM by chelation (Clemens, 2006).

It has recently been reported that exogenous application of SA also increases the proline (Pro) content in *Cistus* shoots (López-Orenes *et al.*, 2013a). Pro accumulation is recognized as being another mechanism through which plants may prevent oxidative damage caused by HMs (Ben Rejeb *et al.*, 2014; Sharma and Dietz, 2006). Moreover, transcriptomics studies have also highlighted the up-regulation of genes encoding enzymes involved in antioxidative defense, thiol and proline metabolism, and biosynthesis of various secondary metabolites during Pb stress (Liu *et al.*, 2009).

The Syrian beancaper (*Zygophyllum fabago* L.) is a succulent perennial shrub of the Zygophyllaceae family. *Z. fabago* emerges as an early colonizer of disturbed areas, including soils affected by mining activities and therefore highly polluted by a broad range of HMs such as Pb, Zn and Cu (Lefèvre *et al.*, 2014; Párraga-Aguado *et al.*, 2013). This pioneering character highlights the importance of the species for establishing “fertility islands” in polluted areas, improving soil conditions and allowing the subsequent colonization by late successional plant species (Párraga-Aguado *et al.*, 2013). No information, however, is available about the influence of SA on Pb uptake and Pb tolerance in this species. Therefore, clarifying the possible mechanisms that could

improve *Z. fabago* performance under Pb stress, would contribute to increase the effectiveness of reclamation technology in the future. For these reason, the objectives of the present work were: *i*) to examine the influence of SA pretreatment (24 h, 0.5 mM SA) on the accumulation and distribution of Pb in a metallicolous ecotype of *Z. fabago* during short-term (7-days) exposure to a high but still tolerable concentration of Pb [0.75 mM $\text{Pb}(\text{NO}_3)_2$], which is commonly found in mining soils, *ii*) to investigate the effects of Pb and SA pretreatment on antioxidative response, H_2O_2 production and SA content in leaves, stems and roots, and *iii*) to identify possible mechanisms of SA amelioration of Pb responses in this metal-tolerant ecotype.

5.3. Materials and methods

5.3.1. Plant material and culture conditions

Seeds of *Z. fabago* were collected from the mine tailing “El Lirio” in Cartagena-La Unión Mining District (SE Spain) and germinated on filter paper moistened with distilled water. The seedlings were grown for 15 days in 1:1 (v/v) mixture of sand and vermiculite, irrigated with half-strength Hoagland solution, and grown hydroponically for 2 weeks in aerated one-quarter strength Hoagland solution. The nutrient solution was renewed every 2 days. Plants were grown in a culture chamber (Sanyo MLR-351, Japan) under a 16 h photoperiod with 25/20°C day/night temperature, with a photon flux density of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. After 30 d of growth, the nutrient solution was replaced and one half of the plants were grown for 24 h in 0.5 mM SA solution buffered to pH 5.0 with 3 mM MES/KOH, and the remainder in 3 mM MES/KOH, pH 5.0. Then, the plants at six-node stage were cultured for 7 d with either 0.75 mM $\text{Pb}(\text{NO}_3)_2$ or 1.5 mM KNO_3 solutions, which were buffered to pH 5.0 with 3 mM MES/KOH. The solutions were renewed every 2 days. Four individual plants were considered as one replicate. After 7 days of treatments, roots were washed for 10 min in 5 mM CaCl_2 , washed thoroughly with distilled water, blotted with filter paper and then, separated into roots, stems and leaves (young and mature leaves). Samples were immediately frozen in liquid nitrogen, pulverized in a liquid nitrogen-cooled A-11 analytical mill (IKA-Labortechnik, Germany) and stored at -80°C for further analysis. Whole-shoot transpiration was estimated by

measuring the volume of hydroponic solutions at the beginning and at the end of the trial period.

5.3.2. Pb determination

Dried samples were reduced to ashes in a muffle furnace (Select-Horn furnace P-Selecta) at 450 °C for 8 h and digested in a heat block with HNO₃ (70 %). Pb concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500A).

5.3.3. Fluorescence measurements

The measurements of chlorophyll fluorescence parameters were made on the upper (adaxial) surface of leaves belonging to the 4th node using a PAM-210 chlorophyll fluorometer system (Heinz Waltz, Germany). Plants were kept for 25 min in darkness to determine F_o (minimum fluorescence yield) and F_m (maximum fluorescence yield) and, then exposed for 15 min to actinic light (140 $\mu\text{mol m}^{-2} \text{s}^{-1}$) to calculate F'_o and F'_m . Calculations for maximum quantum efficiency of photosystem II (PSII) photochemistry $[(F_m - F_o)/F_m]$ and quantum efficiency of PSII $[\Phi_{\text{PSII}} = (F'_m - F_s)/F'_m]$; where F_s corresponds to steady state fluorescence yield] were carried out following the recommendations described by Maxwell and Johnson (2000)

5.3.4. Chlorophylls and carotenoids contents

Chlorophylls (Chl) and carotenoids were extracted in 100% methanol and their concentrations were calculated as previously described (López-Orenes *et al.*, 2013a).

5.3.5. Determination of electrolyte leakage percentage

Three leaf discs, 6 mm in diameter, were cut with a cork borer, washed with MilliQ water and incubated with the adaxial side up on 10 mL of 0.4 M mannitol for 5 h. Following incubation, the conductivity of the solution was measured, and then the samples were autoclaved at 121°C for 20 min. When the solution was cooled to room temperature, the conductivity was measured again and the results were expressed as relative conductivity

$[(\text{conductivity after incubation}/\text{conductivity after autoclaving}) \times 100]$.

5.3.6. Determination of lipid peroxidation, H_2O_2 , ascorbate, glutathione and soluble non-protein thiols

Lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) as previously described (López-Orenes *et al.*, 2013a).

The H_2O_2 contents were determined by the ferric-xylenol orange (FOX1) method as previously described (López-Orenes *et al.*, 2013a). Oxidized and reduced forms of ascorbate were measured using the α - α' -bipyridyl method (Gillespie and Ainsworth, 2007). Oxidized and reduced forms of glutathione and soluble non-protein thiols (NPT) were measured according to Queval and Noctor (2007). The assays were adapted for the use of 96-well plates and a microplate reader (Multiskan GO, Thermo Scientific). Total PCs concentration expressed as GSH equivalents was estimated from the difference between total non-protein thiols and GSH.

5.3.7. Enzymatic assays

Liquid nitrogen powdered samples (100 mg fresh weight) were homogenized in 1.2 mL of ice-cold 100 mM Na phosphate buffer, pH 7.8, containing 0.5 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 % (w/v) polyvinylpolypyrrolidone using a micropestle. For APX activity, 5.0 mM AsA was included instead of DTT in the extraction medium. Homogenates were centrifuged (20000 g for 30 min at 4 °C) and the supernatants were desalted by chromatography on Sephadex G-25 columns (NAP5, Pharmacia Biotech.) equilibrated with the appropriate buffer. Protein was measured by the Bradford protein assay kit (Bio-rad Laboratories) using bovine serum albumin as a standard.

SOD (EC 1.15.1.1), APX (EC 1.11.1.11), DHAR (EC 1.8.5.1), MDHAR (EC 1.6.5.4), and GR (EC 1.8.1.7) activities were assayed as described previously (Hernández *et al.*, 2001). CAT (EC 1.11.1.6) assay was performed according to Gillespie *et al.* (2011). A unit of SOD activity was defined as the amount of SOD required to inhibit cytochrome c

reduction by 50 % in a coupled system with xanthine oxidase at pH 7.8 at 25 °C. The assays were adapted for the use of 96-well plates and a microplate reader.

SOD isozymes were separated by native PAGE on 10 % (w/v) acrylamide gels and visualized in gels by a photochemical method according to Hernández *et al.* (2001). Isoenzyme identification was performed by selective inhibition with 5 mM KCN or 5 mM H₂O₂. The percentage of activity for the different SODs was quantified by image analysis using ImageJ software (<http://imagej.nih.gov/ij/>).

5.3.8. Extraction and quantification of Pro and alkaloids

The Pro content was determined spectrophotometrically in a sulphosalicylic acid extract, using acid ninhydrin reagent (López-Orenes *et al.*, 2013a).

Total alkaloid content was determined spectrophotometrically, at 465 nm using the Dragendorff's reagent (Sigma-Aldrich), according to the method of Sreevidya and Mehrotra (2003). GC-MS analysis was carried out using a 6890N gas chromatograph (Agilent) equipped with a mass spectrometric detector (Agilent 5975i) and an Agilent HP-5MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) capillary column. Methanolic extracts (1 µl) were injected on column into a flow of helium gas held constant at 1.0 ml min⁻¹. The oven temperature was programmed from 50 to 320 °C. Alkaloid metabolites were identified by comparing the retention times and mass spectra with spectral data of commercial standards (harmaline and harmine, Sigma-Aldrich) and from the NIST 2011 mass spectral library.

5.3.9. Extraction and quantification of free and conjugated SA

Extraction, HPLC analyses, and quantification of free and conjugated SA (SAG, 2-O-β-D-glucosylsalicylic acid) were performed as previously described (López-Orenes *et al.*, 2013a).

5.3.10. Statistical analysis

Data are presented as mean $\pm$ standard errors (SE) from three independent experiments. The statistical significance of the differences ($P < 0.05$) between groups was tested using one-way analysis of variance (ANOVA) combined with Tukey's honestly significant difference (HSD) post hoc test. All the statistical analyses were performed using the SPSS software (version 19.0; SPSS Inc., USA).

5.4. Results

5.4.1. Effect of SA treatment on Pb accumulation

SA pretreatment (24 h, 0.5 mM SA) before exposure to Pb resulted in a dramatic reduction in the concentration of Pb in aboveground tissues (~4-fold lower than those observed in Pb-controls), while in roots the reduction was less pronounced (~1.7-fold lower than those of Pb-controls) (Table 1).

Table 1. Effect of SA pretreatment (0.5 mM for 24 h) on lead accumulation (ng g DW^{-1}) in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM $\text{Pb}(\text{NO}_3)_2$ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of five individual plants per replicate. Values followed by different letters in a column are significantly different at $P < 0.05$, Tukey's HSD test.

Treatment	Pb accumulation (ng g DW^{-1})		
	Root	Stem	Leaf
Control + Pb	30579.7 ± 476.0^a	1781.2 ± 75.3^a	39.4 ± 13.5^a
SA + Pb	18072.9 ± 987.9^b	431.4 ± 183.8^b	9 ± 4.9^b

5.4.2. Effects of Pb on chlorophyll a fluorescence and photosynthetic pigment content in leaves of *Z. fabago*

As shown in Table 2, there was a small drop (less than 6 %) in the F_v/F_m ratio in Pb-, SA-, and SA-Pb-treated plants with respect to control values, although all the F_v/F_m values obtained fell into the 0.74–0.85 range, suggesting that neither Pb nor SA treatments affected the photochemical efficiency of PSII. The quantum yield of PSII (Φ_{PSII})

measures the proportion of light absorbed by Chl associated with PSII that is used in photochemistry (Maxwell and Johnson, 2000). After Pb exposure, there was a slight decrease in Φ_{PSII} but not statistically significant (Table 2), indicating that the efficiency of PSII photochemistry was not impaired by Pb.

SA alone provoked a marked increase in total carotenoid content, while the levels of Chl remained unaffected. Carotenoid levels were also increased by Pb treatment, whereas the levels of Chl decreased although the Chl a/b ratio remained unchanged (Table 2).

5.4.3. Effects of SA on H_2O_2 level and lipid peroxidation in *Z. fabago* under Pb stress

No statistically significant changes were observed in the H_2O_2 content in leaves and stems of Pb-treated plants or those pretreated with SA before Pb exposure, although there was some elevation of H_2O_2 levels in roots, especially in those pretreated with SA (Fig. 1). As expected, lipid peroxidation products increased by about 21-49 % in Pb-treated plants compared to the control groups. SA alone induced a significant increase in leaf MDA content (Fig. 1) and electrolyte leakage (Table 2).

Table 2. Effect of SA pretreatment (0.5mM for 24 h) on chlorophyll fluorescence parameters, photosynthetic pigment content, and electrolyte leakage in leaves of *Z. fabago* plants exposed to 0.75 mM $\text{Pb}(\text{NO}_3)_2$ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of six individual plants per replicate. Values followed by different letters in a row are significantly different at $P < 0.05$, Tukey's HSD test.

Parameters	Control	Control + Pb	SA	SA + Pb
Chlorophyll fluorescence parameters				
F_v/F_m	0.819 ± 0.003^a	0.771 ± 0.010^b	0.769 ± 0.005^b	0.773 ± 0.011^b
Φ_{PSII}	0.664 ± 0.015^a	0.642 ± 0.009^b	0.655 ± 0.016^b	0.636 ± 0.017^b
Photosynthetic pigment content ($\mu\text{g g}^{-1}$ FW)				
Chl a	933 ± 62^a	661 ± 48^c	$899 \pm 54^{a,b}$	$769 \pm 73^{b,c}$
Chl b	583 ± 36^a	434 ± 25^b	587 ± 39^a	486 ± 35^b
Carotenoids	19.0 ± 1.9^c	28.7 ± 1.7^b	39.1 ± 1.9^a	39.6 ± 2.9^a
Electrolyte leakage (%)				
5 h	23.7 ± 1.9^b	44.3 ± 2.7^a	36.1 ± 1.7^a	38.9 ± 2.5^a

5.4.4. Effect of Pb on the free and bound SA levels

In control plants Pb treatment led to both a decrease in the content of SAG as well as an increase in the level of free SA, especially in stem tissues where a 9.4-fold increase was found (Table 3). A significant increase in SAG levels was noticed with all SA treatments, but this increase was less pronounced in SA-Pb plants. In these plants, the levels of free SA were closer to those observed in Pb-treated controls, with the exception of the values found in roots in which free SA content showed a 4.8-fold increase. In addition, an increase of free SA in root and stem tissues of SA-pretreated plants was also found.

Figure 1. Effects of Pb and SA pretreatment (0.5 mM for 24 h) on (A) H₂O₂ concentration and (B) lipid peroxidation in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM Pb(NO₃)₂ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of four individual plants per replicate. Values followed by different letters are significantly different at $P < 0.05$, Tukey's HSD test.

Table 3. Effects of Pb and SA pretreatment (0.5 mM for 24 h) on the accumulation of free and bound salicylic acid (SAG) in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM Pb(NO₃)₂ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of four individual plants per replicate. Values followed by different letters in a row are significantly different at $P < 0.05$, Tukey's HSD test.

SA content ($\mu\text{mol g}^{-1}$ FW)	Treatment			
	Control	Control + Pb	SA	SA + Pb
Leaf				
Free SA	0.412 ± 0.096^b	0.731 ± 0.075^a	0.398 ± 0.069^b	0.543 ± 0.076^b
SAG	1.882 ± 0.084^b	0.847 ± 0.076^c	3.946 ± 0.318^a	2.165 ± 0.293^b
Stem				
Free SA	0.087 ± 0.013^c	0.821 ± 0.117^a	0.466 ± 0.038^b	0.710 ± 0.097^a
SAG	1.900 ± 0.042^c	1.434 ± 0.088^d	11.273 ± 0.631^a	5.003 ± 0.933^b
Root				
Free SA	0.094 ± 0.017^d	0.174 ± 0.0125^c	1.16 ± 0.096^a	0.833 ± 0.088^b
SAG	1.854 ± 0.079^c	1.038 ± 0.028^d	25.050 ± 0.594^a	15.240 ± 0.727^b

5.4.5. Effects of SA on antioxidant enzymes in *Z. fabago* under Pb stress

SA alone induced SOD (by about 3.4- to 4-fold) and, to a lesser extent, CAT (1.7- to 1.9-fold) specific activities, whereas APX and GR specific activities showed either increases or decreases depending on the organ analyzed (Fig. 2). Generally, Pb treatment provoked a reduction in the activities of CAT, APX, and GR while SOD and MDHAR were unaffected, and DHAR specific activity increased (Fig. 2). The values of enzymatic activities found in SA-Pb treated plants were closer to those of Pb-control treatments with the notable exception of SOD, in which 1.6-fold, 2.5-fold and 3.1-fold increases were observed in leaves, stems and roots of SA-Pb plants, respectively, with regard to the corresponding Pb-controls (Fig. 2A).

Figure 2. Effects of Pb and SA pretreatment (0.5 mM for 24 h) on (A) SOD, (B) CAT, (C) APX, (D) MDHAR, (E) DHAR, and (F) GR specific activities in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM $\text{Pb}(\text{NO}_3)_2$ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of four individual plants per replicate. For each organ, different letters indicate significantly different values following Tukey's HSD test ($P < 0.05$).

SOD isoenzyme patterns showed the presence of up to ten bands on gels regarding the treatment applied (Fig. 3). Based on inhibition studies, the three main types of SODs could be identified on gels: Isoenzymes 1-4 in Fig. 3 would correspond to Mn-SODs, isoenzymes 5-8 could be identified as Fe-SODs, and, finally, isoenzymes 9-10 would be CuZn-SODs. Relative abundance of the isoenzymes varied in an organ-dependent fashion with respect to control upon Pb treatment (Fig. S1). Thus, Fe-SODs 5 and 6 decreased

their relative levels in leaves and SODs 5 and 7 in stems of Pb-treated plants, whereas CuZn-SOD 9 levels increased in these organs; however, the opposite trend was observed in SODs 6, 7 and 9 from roots (Fig. S1). The most noticeable effect of SA pretreatments was an increase in the number of bands on gels (Fig. 3). The newly displayed bands mainly corresponded to Mn-SODs. SA also provoked a marked increase in CuZn-SOD 9 in stems and roots, but not in leaves where this isoenzyme was practically unaffected with respect to control plants. Finally, SA-Pb treatments resulted in similar (SOD 6 from leaves, SOD 7 from stems, SOD 9 from the three organs), reverted (SOD 5 from both leaves and stems, SOD 7 from roots), enhanced (SOD 6 from both stems and roots), and depressed (SOD 7 from leaves, SOD 8 from both stems and roots) relative levels of SOD isoenzymes with respect to non-SA-pretreated Pb-treated plants.

Figure 3. Effects of Pb (0.75 mM for 7 days) and SA pretreatment (0.5 mM for 24 h) on SOD isoenzyme patterns corresponding to leaves, stems, and roots of *Z. fabago* after separation by native PAGE. For quantification of band intensities, see supplemental Fig. 1. L, leaves; S, stems; R, roots.

Fig. S1. Relative abundance of numbered bands obtained from SOD isozyme electrophoreograms corresponding to extracts from leaves, stems, and roots of *Z. fabago* incubated for 7 days in the presence of 0.75 mM Pb with or without SA pretreatment (0.5 mM for 24 h).

The levels of total soluble proteins were significantly ($P < 0.05$) increased by Pb exposure and SA-pretreatment, especially in stems and roots (see Table S1).

Table S1. Effects of Pb and SA pretreatment (0.5 mM for 24 h) on the protein content in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM Pb(NO₃)₂ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of four individual plants per replicate. Values followed by different letters in a row are significantly different at $P < 0.05$, Tukey's HSD test. Data in parentheses are the percentage of control treatments.

	Treatment			
	Control	Control + Pb	SA	SA + Pb
Protein content				
Leaf	8.16 $\pm$ 0.46 ^b	10.55 $\pm$ 0.39 ^a (129,3 %)	7.38 $\pm$ 0.61 ^b (90,4 %)	9.97 $\pm$ 0.44 ^a (122,2 %)
Stem	5.13 $\pm$ 0.42 ^b	6.74 $\pm$ 0.41 ^a (131,4 %)	7.60 $\pm$ 0.50 ^a (148,1 %)	7.67 $\pm$ 0.47 ^a (149,5 %)
Root	4.92 $\pm$ 0.59 ^b	6.68 $\pm$ 1.19 ^a (135,8 %)	7.02 $\pm$ 0.67 ^a (142,7 %)	6.98 $\pm$ 0.66 ^a (141,9 %)

5.4.6. Effects of SA on AsA, GSH, NPT and PC contents in *Z. fabago* under Pb stress

As shown in Figure 4A, the ascorbate pools were affected by Pb exposure. Compared to untreated controls, the total contents of AsA and DHA were higher in leaves, stems and roots of both Pb- (by about 50 %, 41 % and 43 %, respectively) and SA-Pb plants (by about 56 %, 22 % and 36 %, respectively). Under Pb stress, the AsA redox ratio [AsA/(AsA+DHA)] dropped in aboveground organs, especially in leaves of both Pb- and SA-Pb plants due to a relatively large 3-fold increase in DHA. In SA alone pretreatments, AsA and DHA levels increased in aboveground organs, but the levels in roots were similar to those found in untreated controls.

The levels of GSH were slightly increased upon Pb exposure in Pb-control plants and decreased in both SA- and SA-Pb-treated plants. SA alone caused some elevation of total GSSG levels especially in aboveground organs, and these increments resembled those observed in both Pb- and SA-Pb treated plants. Despite these changes, the GSH redox ratio [GSH/(GSH+GSSG)] remained above 0.79 in all organs (Fig. 4B).

The analyses of total NPT showed that the addition of Pb produced a slight increase in the NPT content in roots (by about 17 %), while no major changes occurred in aboveground organs. SA alone had no effect on NPT content, but an enhancement by 39 % was observed in roots of SA-Pb plants compared to untreated controls (Fig. 4C). A slight increase in the levels of PCs, estimated from the difference of NPT and GSH, was observed in roots of SA-Pb-treated plants (Fig. 4C).

Figure 4. Effects of Pb and SA pretreatment (0.5 mM for 24 h) on the content of (A) ascorbic (AsA) and dehydroascorbic acids, (B) reduced and oxidized glutathione (GSH), and (C) total non-protein thiol compounds (NP) and phytochelatins (PC) in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM Pb(NO₃)₂ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of four individual plants per replicate. Values followed by different letters are significantly different at $P < 0.05$, Tukey's HSD test. DHA, dehydroascorbic acid; GSSG, oxidized glutathione.

5.4.7. Effects of SA on Pro and total alkaloid contents in *Z. fabago* under Pb stress

SA alone caused a 2-fold increase in root Pro contents (Fig. 5A). Upon Pb exposure, the level of proline increased in all organs. The most prominent increase was observed in roots of both Pb-treated (by about 2.2-fold) and SA-Pb-treated (by about 2.9-fold) plants as compared to controls.

Total alkaloid contents noticeably increased upon Pb exposure in leaves of Pb- (10.5-fold) and SA-Pb (13-fold) plants, whereas a less marked increase was detected in stems (24 % and 43 %, respectively) and was unaffected in root tissues. SA pretreatment also provoked a 4-fold increase in the levels of total alkaloids in leaf tissues (Fig. 5B). GC-MS analysis showed a higher accumulation in shoots of a compound tentatively identified as a β -carboline derivative upon SA-, Pb- and SA-Pb treatments (Fig. 6). This increase was particularly outstanding in the case of leaves from SA-Pb treatments and less evident in roots.

Figure 5. Effects of Pb and SA pretreatment (0.5 mM for 24 h) on the content of (A) proline and (B) alkaloids in leaves, stems and roots of *Z. fabago* plants exposed to 0.75 mM Pb(NO₃)₂ for 7 days. Data represent means $\pm$ SE of three independent replicates. Each data point represents the measurements of four individual plants per replicate. Values followed by different letters are significantly different at $P < 0.05$, Tukey's HSD test.

Figure 6. Chromatograms extracted at m/z 211 of leaf methanolic extracts analyzed by GC-MS (A). Inset shows the mass spectrum of the peak at 15.02 min and the structure of the putative β -carboline derivative according to NIST database. (B) Relative quantification of peak areas corresponding to the compound with retention time 15.02 min in extracts from leaves, stems and roots of *Z. fabago* exposed to Pb for 7 days. Values followed by different letter are significantly different at $P < 0.05$, Tukey's HSD test.

5.5. Discussion

5.5.1. SA reduces Pb accumulation and attenuates the loss of chlorophylls

This study was carried out to analyze the possible effects of SA pretreatment on Pb tolerance in *Z. fabago* plants exposed for seven days to Pb under hydroponic conditions. Using this system, it was observed that most of the Pb taken up by *Z. fabago* plants was found in root tissues and only a small amount was translocated to the aboveground organs. The preferential accumulation of non-essential HMs like Pb^{2+} within the root is well documented. Pb retention in the roots is considered an important mechanism for protecting against the spread of Pb ions *in planta* via apoplast (Clemens, 2006). SA pretreatment reduced both the accumulation of Pb in roots and even more so the concentration of Pb in aerial parts of the plants. Although there was a decline in transpiration rate in Pb-, SA-, and SA-Pb-treated plants (by about 17 %, 11 % and 21 %, respectively; data not shown), the differences were not statistically significant, ruling out the effect of transpiration rate on the reduction of Pb organ contents in SA-Pb plants. SA reduction of Pb uptake has also been previously observed in *Vallisneria natans* plants (Wang, Zhang, Wang, & Lu, 2012). It has been suggested that SA can induce H^+ -ATPase activity, which in turn could influence metals absorption into roots via divalent cation transporters (Clemens, 2006). In addition, several other mechanisms such as the release of HM-binding ligands and the pumping of HMs out of cells through P-type ATPases (Clemens, 2006) are thought to also be involved in the reduced uptake of non-essential metals, and it is plausible that SA can also modify these processes associated with HM uptake. Nevertheless, further studies are needed to confirm and explain this finding.

The most widespread visual symptoms of Pb toxicity in plants are a reduction in growth and the development of foliar chlorosis (Gupta *et al.*, 2013). However, in the present study, although there was a reduction in the levels of Chl under Pb treatment, the Chl a/Chl b ratio remained unaltered, suggesting that neither the photosystem I/photosystem II ratio nor the antenna size were affected by Pb exposure. Moreover, the increase in the carotenoid levels observed may protect chloroplast against free radical formation induced by Pb (Gupta *et al.*, 2013, and references herein). Taken together, these data could explain why the efficiency of PSII photochemistry was only slightly affected by Pb exposure in both control and SA-pretreated plants.

5.5.2. Pb increases the endogenous free SA levels in all organs and this response is enhanced in root tissues upon SA pretreatment

SA was proposed to play an essential role in the regulation of cellular redox homeostasis that could be essential to orchestrate appropriate defense responses in different stress conditions (Mateo *et al.*, 2006). To this respect, the enhanced free SA content detected in all organs of Pb-treated plants may be involved in cell response to Pb toxicity. This free SA increase might result from a release from the conjugates, given that SAG levels declined concomitantly. Moreover, a subsequent metabolism of part of the SA produced in other compounds such as dihydroxybenzoates and/or methyl salicylate cannot be ruled out (Vlot *et al.*, 2009). Thus, in chamomile (Kováčik, Grúz, Hedbavny, Klejdus, & Strnad, 2009) and pea (Rodriguez-Serrano *et al.*, 2006) plants, a Cd-dependent accumulation of 2,5-dihydroxybenzoate and methyl salicylate, respectively, has been observed.

5.5.3. SA enhances the Pb-induced H₂O₂ accumulation in roots by up-regulating Fe- SOD isoenzymes

The analysis of H₂O₂ content revealed that Pb induced the accumulation of H₂O₂ in roots, whereas no major changes were observed in the aboveground organs of Pb-treated plants. The accumulation of H₂O₂ in roots has been described during the early stages of heavy-metal exposure in roots of *Vicia faba* treated with Pb (Pourrut *et al.*, 2008) and in roots of *Arabidopsis* treated with Cd (Cuypers *et al.*, 2011). In addition, H₂O₂ increases in root tissues have also been found after long-term exposure (14 days) to Cd in pea plants (Rodriguez-Serrano *et al.*, 2006).

In the regulation of H₂O₂ levels, the balance between SODs and H₂O₂-scavenging enzymes is crucial (Noctor & Foyer, 1998). CAT and APX, as well as those enzymes involved in the AsA-GSH cycle (i.e., MDHAR, DHAR and GR), are key enzymes responsible for H₂O₂ decomposition (Noctor & Foyer, 1998). In our experiments, the reduction in CAT, APX and GR specific activities might favor the accumulation of H₂O₂ in roots by Pb²⁺ treatment (Fig. 2). Moreover, the higher H₂O₂ levels noticed in SA-Pb-treated roots could be explained, at least partially, by both the strong reduction of CAT activity (< 10-fold) induced by Pb (Fig. 2B) and the increased activity of SOD (> 3-fold)

induced by SA pretreatment (Fig. 2A). Rao *et al.* (1997) reported that SA-enhanced H₂O₂ levels were related to increased activities of CuZn-SODs. Here a strong enhancement in the activity of CuZn-SOD isoforms in stem and root tissues of SA-treated plants was also observed. Under Pb stress, a marked increase in Fe-SOD activities was found in roots, suggesting that these isoenzymes could be involved in the H₂O₂ accumulation found in this organ in both Pb- and SA-Pb treated plants. Based on the minor alteration in the levels of lipid peroxidation found in roots of Pb-treated plants, it is plausible that the accumulation of H₂O₂ found in this organ might play a regulatory role, as has been proposed earlier (Gill & Tuteja, 2010; Rodriguez-Serrano *et al.*, 2006).

Interestingly, the only enzyme induced by Pb exposure in all organs was DHAR (Fig. 2), which catalyzes the conversion of DHA to AsA using GSH as an electron donor. The data presented here suggest that GSH makes an important contribution to maintaining a highly reduced AsA pool of Pb-treated plants supported by DHAR, as has also been shown previously for Pb-accumulating and non-accumulating ecotypes of *Sedum alfredii* under Pb stress (D. Liu *et al.*, 2008). Although Pb induced an enhancement of DHAR activity in shoots, the levels of DHA remained too high, especially in leaf tissues. Thus, the reduction observed in the AsA redox ratio (Fig. 4) together with the decrease in the APX and CAT activities (Fig. 2) might partially explain the higher extent of lipid peroxidation, evaluated by electrolyte leakage and MDA, noticed in leaf tissues of *Z. fabago* plants treated with Pb. Nevertheless, the participation of lipoxygenase in lipid peroxidation under Pb stress cannot be ruled out (Huang, *et al.*, 2012). Despite the fact that Pb-mediated reduction in GR activity can affect the GSH/(GSH+GSSG) ratio, the GSH redox state remained highly reduced. Accumulating evidence suggests that long-term responses or acclimation conditions are correlated with the maintenance of a highly reduced GSH pool (Locato, Gadaleta, De Gara, & De Pinto, 2008).

5.5.4. SA pretreatment slightly increases the NPT in roots but enhances the Pb-induced accumulation of Pro in roots and alkaloids in shoots

Although Pb can interact with thiol groups (Gupta, *et al.*, 2013), only a slight increase in the levels of NPT in roots was observed, suggesting that sequestration of Pb²⁺ ions in *Z. fabago* plants can be carried out by others mechanisms such as cell wall immobilization. PCs synthesis after Pb stress has been reported in the Pb-hyperaccumulator aquatic fern

Salvinia minima (Estrella-Gómez *et al.*, 2009) and in selected clones of *Melilotus alba* and *Melilotus officinalis* (Fernández, *et al.*, 2012). However, several authors have questioned the role of PC in the development of HM tolerance due to the energy costs associated with sulfate reduction and PC synthesis (Gupta, Huang, Yang, Razafindrabe, & Inouhe, 2010).

Proline is suggested to act as a potent nonenzymatic antioxidant and to relieve the oxidative burden from the glutathione system (Ben Rejeb, *et al.*, 2014; Sharma and Dietz, 2006). In this study, Pb induced Pro accumulation in all organs, but the increment was more pronounced in roots. Thus, considering the concentration of Pb, the absence of membrane damage (evaluated as MDA content), and the GSH redox ratio found in root tissues, it is plausible that Pro should act as an antioxidant as has been proposed earlier (Sharma and Dietz, 2009, and references herein). Moreover, the data presented here also suggest the upregulation of Pro levels by SA pretreatment, especially under Pb stress. A stimulation of proline accumulation due to up- and down-regulation of Pro biosynthesis and catabolism, respectively, by SA pretreatment was also observed in lentil seedlings under both non-saline and saline conditions (Misra & Saxena, 2009).

The Zygophyllaceae family is characterized by the accumulation of indolic (β -carboline) alkaloids, which have been reported to act as scavengers of ROS in yeasts (Moura, *et al.*, 2007), and in different animal models (Kim, *et al.*, 2001 and references herein). In the present study, exposure to Pb increased alkaloid levels in aboveground organs, but not in roots, and the Pb response was enhanced in leaf tissues of SA-pretreated plants. It has recently been shown that the accumulation of brachycerine, a monoterpene indole alkaloid, may serve as a ROS scavenger in leaves of *Psychotria brachyceras* upon metal stress (do Nascimento, *et al.*, 2013). Thus, taken together, the distinct accumulation pattern of Pro and alkaloids found in both root and shoot tissues in Pb-stressed *Z. fabago* plants may have an important role in Pb stress adaptation, possibly contributing to the maintenance of the cellular redox state and preventing oxidative injury.

5.6. Conclusion

The data presented here demonstrate that the *Z. fabago* plant is a Pb-tolerant species even under acute Pb exposure. Generally, all organs reacted to Pb stress by increasing AsA and GSH concentrations and by maintaining a highly reduced GSH and AsA pools supported by DHAR. Moreover, the different organ accumulations of Pro and β -carboline alkaloids seem to play an important role in the adaptation to Pb stress. Pretreatment with SA had a significant effect on SOD activities in all organs and enhanced the Pb-induced accumulation of Pro in roots and alkaloids in shoots. In addition, SA-inhibited Pb uptake and accumulation, especially in shoots. From a practical point of view, this finding could be exploited in land reclamation programs to reduce the risk of Pb entrance to food chains.

PART II:
PIONEER SPECIES GROWN UNDER
CONTROLLED ENVIRONMENTAL
CONDITIONS

CHAPTER 6

Different mechanisms of the metalliferous *Zygophyllum fabago* shoots and roots to cope with Pb toxicity

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6.1. Abstract

Lead (Pb) remains classified as a priority pollutant, urging the need to identify Pb-tolerant plants to implement successful phytoremediation programmes. *Zygophyllum fabago* is considered an early colonizer of heavy metal-polluted soils under semiarid conditions, though mechanisms underlying that tolerance remain unclear. In order to characterize *Z. fabago* plants' performance on Pb-contaminated soils, we evaluated how Pb influenced roots and shoots growth, carbon metabolism and oxidative status. For that, 30 days-old seedlings from one population colonizing a mine tailing ("Mercader" at Murcia (southeast Spain) were exposed to 500 μM $\text{Pb}(\text{NO}_3)_2$ for 1 week. Results showed that this high dose of Pb induced no plant mortality nor senescence, though promoting plant nanism. Around 20% of the absorbed Pb was translocated to shoot tissues, and a decline in Zn, K and P was evident in shoots, together with a decrease of the net photosynthetic rate (A), RuBisCO activity, chlorophyll contents and soluble sugars. Moreover, shoots showed high levels of the highly aggressive $\text{O}_2^{\cdot-}$, and less antioxidant capacity, culminating with higher loss of cell membrane integrity. Exposed roots had less Mn and Zn levels, and despite increases of H_2O_2 levels and biomolecules oxidation, they presented a robust antioxidant capacity which may support the observed cell membrane integrity. Also, roots modulated total ascorbate and non-protein thiols levels in response to Pb. These findings, supported by a PCA analysis, contribute to elucidate this species' mechanisms to cope Pb toxicity, and show that roots evolved an efficient antioxidant capacity, demonstrating the importance of organ specificity in *Z. fabago* plants. Considering the tolerance of this species to Pb (and to other metals) we suggest that this species could be an ideal tool in phytoremediation programmes of sites contaminated with multiple metals.

Key words: Pb stress, photosynthesis, oxidative stress, *Zygophyllum fabago*

6.2. Introduction

Heavy metal contamination of soils is a worldwide problem with ecological and human health impacts, including the contamination transfer to higher strata of the food chain (Peralta-Videa *et al.*, 2009). For example, according to European Environmental Agency, in Europe, there are more than 2.5 million polluted sites, most commonly contaminated by heavy metals and mineral oil (Panagos *et al.*, 2013). Therefore, the development of efficient strategies for soil phytoremediation and/or colonization is urgent. To this regard, several plant characteristics such as heavy metal tolerance and bioaccumulation, size, growth rate, root depth, and climatic adaptation are considered important (Pilon-Smits 2005).

Zygophyllum fabago is a succulent perennial shrub of the *Zygophyllaceae* family, which emerges as an early colonizer in soils affected by mining activities, highly polluted by heavy metal and metalloids under Mediterranean conditions (Lefèvre *et al.*, 2014; Párraga-Aguado *et al.*, 2013). Despite not being a hyperaccumulating species, it was able to rapidly accumulate in shoots up to 280 mg kg^{-1} dry weight (DW) of Cd and 600 mg kg^{-1} DW of Zn, in response to the addition of $10\text{-}\mu\text{M}$ Cd or $100\text{-}\mu\text{M}$ Zn, respectively, in nutrient solution (Lefèvre *et al.*, 2010), although a low Pb bioaccumulation in above-ground tissues has been reported in 0.75-mM Pb-treated *Z. fabago* plants under hydroponic conditions (López-Orenes *et al.*, 2014).

To this regard, it was well-documented that the activity of heavy metal-sequestering pathways in root cells as well as the regulation of heavy metal root-to-shoot translocation are important mechanisms leading to exclusion or accumulation of heavy metal in the aerial parts (Clemens 2006). The phytotoxicity symptoms of heavy metal on plants directly depend on their localization in the plant tissues and their coordination with ligands. Visible effects of Pb exposure include chlorotic spots, necrotic lesions in leaf surface, senescence of the leaf, and stunted growth. Seed germination is drastically affected at higher metal concentrations and the seedling root and shoot development is also affected, with roots being more sensitive (Li *et al.*, 2005). Lead also reduces the uptake and transport of nutrients in plants, such as Ca, Fe, Mg, Mn, P, and Zn, by blocking the entry or binding of the ions to ion-carriers making them unavailable for uptake and transport from roots to shoots (Rodríguez *et al.*, 2015). This in turn interferes

with several physiological and biochemical processes, among which photosynthesis is one of the most affected (Rodriguez *et al.*, 2015). Photosynthesis sensitivity to Pb includes the substitution of the central atom of chlorophyll (Chl) (Mg) by Pb, which decreases photosynthetic light-harvesting in the affected Chl molecules (Küpper *et al.*, 1996), as well as the reduction in the activity of Calvin cycle enzymes, mainly RuBisCO (Rodriguez *et al.*, 2015). Moreover, a proteomic study was conducted in the metal tolerant *Typha angustifolia*, showing that Pb up-regulated photosynthesis-related proteins (RuBisCO activase, Mg-protoporphyrin IX chelatase, fructokinase, and plastocyanin), carbohydrate metabolic pathway enzymes (fructokinase), and defense-related proteins (Bah *et al.*, 2010). The authors suggested that the protection of photosynthesis by re-establishment of carbohydrate metabolism homeostasis could be an important mechanism that this species use to cope with Pb toxicity.

Pb is known to trigger lipid peroxidation by increasing reactive oxygen species (ROS), and several authors have shown that the activation of antioxidative defenses is beneficial for plant performance against Pb toxicity (Gill and Tuteja 2010; Gupta *et al.*, 2013).

With this background and taking into account the high ability of *Z. fabago* to survive to metalliferous mine wastes (often contaminated with multiple metals), it is of vital importance to clarify the different mechanisms that this species uses, in shoots and roots, to cope with high Pb doses. The main objectives of this work were as follows: (a) to evaluate how acute Pb exposure modulates *Z. fabago* growth, photosynthetic reactions, carbohydrate and pigment contents, and RuBisCO activity and (b) to identify the antioxidant strategy that *Z. fabago* used to counteract Pb toxicity in both shoots and roots.

6.3. Materials and methods

6.3.1. Plant material and culture conditions

Z. fabago seeds from plants growing at the mine tailing Mercader (Cartagena-La Union Mining District, SE Spain) were germinated on perlite with distilled water. After germination, the seedlings were transferred to perlite as the solid substrate of a hydroponic system with one fourth Hoagland solution (pH 5.0) (Sigma-Aldrich, St. Louis, MO, USA), which was renewed every 3 days. Plant growth took place at a 16-h

photoperiod, $470\text{-}\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation, and $25/20\text{-}^{\circ}\text{C}$ day/night temperature. Two treatments were assigned, control ($0\text{-}\mu\text{M Pb}$) and Pb ($500\text{-}\mu\text{M Pb}$). After 30 days of growth, in both treatments, the irrigation solution was renewed by a nutrient solution. However, in the Pb treatment, $\text{Pb}(\text{NO}_3)_2$ was added to the nutrient solution to achieved a final concentration of $500\text{-}\mu\text{M Pb}(\text{NO}_3)_2$. After 7 days of treatment, photosynthetic data was collected and samples (shoots/leaves and roots) were harvested and washed (10 min in 0.5-mM CaSO_4 followed by distilled water). For relative water content (RWC), RuBisCO activity, and cell membrane permeability (CMP) determination, fresh samples were used. The other assays used samples frozen with liquid N_2 and stored at $-80\text{ }^{\circ}\text{C}$.

6.3.2. Growth and relative water content analysis

Samples were used to measure growth and RWC. Leaf and root size and fresh weight were measured. Then, shoots and roots were dried to constant weight at $60\text{ }^{\circ}\text{C}$. RWC was assessed using the following formula: $\% \text{RWC} = [(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight})] \times 100$.

6.3.3. Pb determination and nutrient analysis

Plants were washed with a CaSO_4 solution to remove Pb^{2+} adsorbed to the root and dried to constant weight at $60\text{ }^{\circ}\text{C}$. Dried tissues from shoots and roots were incinerated at $450\text{ }^{\circ}\text{C}$ for 8 h (P-Selecta muffle furnace, Spain) and digested with hot HNO_3 (70%). Pb and elemental form of nutrients (K, Mg, Mn, P, Zn, and Fe) were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500A, USA).

6.3.4. Photosynthetic pigment content

Chls and carotenoids were extracted from frozen leaf discs with methanol (100%) and their concentrations quantified as reported by López-Orenes *et al.*, (2013a).

6.3.5. Gas exchange and chlorophyll fluorescence measurements

In situ, net photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E), and the intercellular CO_2 concentration/ambient CO_2 ratio (C_i/C_a) were measured with a portable IRGA (LCpro+, ADC, Hoddesdon, UK) under growth chamber conditions. All measurements were performed on the second or third older leaf.

Chlorophyll *a* fluorescence was analysed with a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). Maximum quantum efficiency of photosystem II (PSII) was calculated as $F_v / F_m = (F_m - F_0) / F_m$ by measuring the fluorescence signal from dark-adapted leaves when all reaction centers are open using a low-intensity pulsed measuring light source (F_0) and during a pulse saturating light (0.7-s pulse of 15000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light) when all reactions centers are closed (F_m) (Öquist *et al.*, 1992).

6.3.6. Determination of RuBisCO activity

Immediately after harvesting, fresh leaves (with 0.5 cm^2 on average) were homogenized with 1-mL extraction buffer according to Dias *et al.*, (2014a). The extraction buffer contained 50-mM Tris/HCl (pH 7.9), 1-mM EDTA, 5-mM Na-pyruvate, 8-mM MgCl_2 , 20-mM dithiothreitol, 2-mM K_2HPO_4 , and 0.3% (m/v) BSA. After extraction, the homogenate was incubated for 20 min with 20-mM MgCl_2 and 10-mM NaHCO_3 , centrifuged at $9000 \times g$ (~ 10 min), and the maximum RuBisCO (EC 4.1.1.39) activity was measured spectrophotometrically at 340 nm according to Lilley and Walker (1974).

Soluble sugar quantification: Frozen shoot and root samples were homogenized with 80% (v/v) ethanol at 80 °C, during 20 min. After centrifugation at $10,000 \times g$ (~ 10 min), the supernatant was used to determine the total soluble sugars according to Dias *et al.*, (2013).

6.3.7. Cell membrane permeability

Cell viability was assessed by measuring membrane permeability to electrolytes (Azevedo *et al.*, 2005). For that, intact and washed leaves and roots were incubated overnight (25 °C, 85 rpm) in deionized water (< 2-μS conductivity). Then, the leakage of electrolytes from cells was determined by measuring water conductivity (μS, Consort C830) before (L_0) and after (L_t) autoclaving (10 min, 121 °C). CMP was calculated as the ratio L_0/L_t (Dias *et al.*, 2014b).

6.3.8. Total antioxidant activities

The total antioxidant activity (TAA) of shoots and roots was determined using the following assays: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), the FRAP (ferric reducing antioxidant power) assay, and the ABTS^{•+} assay (using the radical cation 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)), as described by Pérez-Tortosa *et al.*, (2012).

6.3.9. Determination of phenolics, flavanols, flavonoids, hydroxycinnamic acids, and lignin

Shoot and root samples (~ 0.1-g powdered tissue) were homogenized with 100% methanol, incubated in the dark for 1 h with slow agitation, and centrifuged (15,000 ×g, 15 min, 4 °C). The supernatant was used to assess: total phenolics with the Folin-Ciocalteu assay and gallic acid was used as standard (López-Orenes *et al.*, 2013b); flavanols using *p*-dimethylamino- cinnamaldehyde (DMACA) reagent, with (+) catechin being used as standard (López-Arnaldos *et al.*, 2001); flavonoids with the aluminium chloride reagent using rutin as standard (Kim *et al.*, 2003); hydroxycinnamic acids (HCAs) with the Arnow's assay using caffeic acid as standard. Lignin levels were determined with the thioglycolic acid assay, using commercial lignin alkali (471003, Sigma-Aldrich) as standard (López-Orenes *et al.*, 2013a).

6.3.10. Determination of lipid peroxidation, protein oxidation, H_2O_2 , O_2^- , ascorbate, soluble non-protein thiols, and proline

Oxidative status was determined in shoots and roots. The lipid peroxidation product malondialdehyde (MDA) was determined according to López-Orenes *et al.*, (2013a). Protein carbonyl groups were determined by the 2,4-dinitrophenylhydrazine assay (Hernández *et al.*, 2001). The levels of H_2O_2 were measured using the ferric-xylenol orange (FOX1) assay (López-Orenes *et al.*, 2013a). The superoxide radical was measured using the hydroxylamine/nitrite method (Jiang and Zhang 2001). Ascorbate, dehydroascorbate, and soluble non-protein thiols (NPT) were quantified as described by López-Orenes *et al.*, (2014). Finally, proline levels were quantified using the acid-ninhydrin reagent (López-Orenes *et al.*, 2013a). All the assays were performed with a microplate UV-Vis spectrophotometer reader (Multiskan GO, Thermo Scientific).

6.3.11. Statistical analysis

The experiment was conducted with a total number of 132 plants (66 plants for the control and 66 plants for the Pb treatment). All data were presented as a mean $\pm$ standard error (SE, the number of replicates for each parameter is detailed in the “Results”). Differences ($p < 0.05$) between groups were tested using a one-way analysis of variance (ANOVA) followed by the Tukey’s post hoc test. These analyses were performed with a SPSS program (version 19.0; SPSS Inc., USA). Principal component analysis (PCA) was carried out to explore the physiological/biochemical profiles of *Z. fabago* of all treatments (control and Pb), by reducing the multivariate data matrix to an interpretable bidimensional biplot that explains the highest proportion of variation in the data (more than 68% in both shoots and roots). The PCA was performed with the program “CANOCO for Windows” v4.02 (Ter Braak and Smilauer 1999) using default settings, except for the centering and standardization window where the “center and standardize” option was selected. Eight data pairs ($n = 8$, as control and Pb) were used.

6.4. Results

6.4.1. Growth and water content analysis

All plants survived to the Pb treatment. However, *Z. fabago* plants grown at 0- and 500- μ M Pb had different growth rates, though maintaining morphological similarities. In both groups, leaves were well developed, with only occasional chlorosis symptoms, and no necrotic spots nor leaf senescence were observed. Values for biomass, size, and leaf area are presented in Table 1. Plants exposed to 500- μ M Pb suffered significant decreases in both leaf area and root length, while shoot length was not affected. The variation in dry mass of shoots and roots also decreased ($p < 0.05$) to around half the values of control. Regarding water status after Pb treatment, the RWC in roots and leaves remained similar ($p \geq 0.05$) to the control (Table 1).

Table 1. Variation in leaf area, shoot and root size, dry mass and relative water content in *Z. fabago* plants exposed to 0 and 500 μ M Pb during 1 week. Values are given as mean $\pm$ standard error for each condition ($n=6$). For each row, different letters represent significant differences at $p < 0.05$.

		Pb treatment	
		0 μ M	500 μ M
Area (mm^2)	Leaves	162.84 $\pm$ 8.89 a	91.28 $\pm$ 13.62 b
Size (cm)	Shoot	3.89 $\pm$ 0.58 a	3.34 $\pm$ 0.49 a
	Roots	7.74 $\pm$ 0.59 a	5.91 $\pm$ 0.21 b
Biomass (mg)	Shoot	125.24 $\pm$ 4.90 a	77.12 $\pm$ 7.35 b
	Roots	68.86 $\pm$ 7.35 a	38.24 $\pm$ 9.77 b
RWC (%)	Shoot	91.79 $\pm$ 3.98 a	88.62 $\pm$ 1.53 a
	Roots	83.58 $\pm$ 6.31 a	89.76 $\pm$ 5.04 a

6.4.2. Pb and mineral nutrient quantification

Table 2 depicts the effect of Pb exposure on mineral nutrient content and Pb distribution in both root and shoot tissues. The content of Pb in roots and shoots significantly increased with the Pb concentration present in the growth medium (Table 2). The information of Tables 1 and 2 was used to calculate the Pb uptake average for shoot and root [$\text{ng Pb g}^{-1} \text{DW}$]. Average uptake was respectively for

control 0.91 ng Pb in shoots and 3.35 ng Pb in roots and for exposed plants was respectively 16.40 ng Pb in shoots and 30.60 ng Pb in roots (Table 2).

Table 2. Pb content and nutrient accumulation in shoots and roots of control and Pb-treated *Z. fabago* plants. Values are given as mean $\pm$ standard error for each condition ($n=6$). For each row, Different letters represent significant differences at $p < 0.05$. (nd = no determined)

		Pb treatment	
		0 μ M	500 μ M
Pb (ng g ⁻¹ DW)	Shoots	7.24 $\pm$ 0.59 b	213.25 $\pm$ 40.05 a
	Roots	48.66 $\pm$ 8.92 b	799.73 $\pm$ 94.59 a
P (mg g ⁻¹ DW)	Shoots	1.30 $\pm$ 0.13 b	1.63 $\pm$ 0.06 a
	Roots	2.40 $\pm$ 0.16 a	2.25 $\pm$ 0.03 a
K (mg g ⁻¹ DW)	Shoots	8.47 $\pm$ 1.59 b	12.43 $\pm$ 0.43 a
	Roots	21.54 $\pm$ 1.85 a	16.37 $\pm$ 4.97 a
Mg (mg g ⁻¹ DW)	Shoots	3.84 $\pm$ 0.60 a	4.97 $\pm$ 0.38 a
	Roots	nd	nd
Mn (μ g g ⁻¹ DW)	Shoots	10.33 $\pm$ 1.46 a	14.02 $\pm$ 2.23 a
	Roots	13.35 $\pm$ 1.42 a	3.38 $\pm$ 2.53 b
Fe (μ g g ⁻¹ DW)	Shoots	68.25 $\pm$ 26.29 a	92.45 $\pm$ 0.97 a
	Roots	553.39 $\pm$ 133.35 a	290.24 $\pm$ 61.01 a
Zn (μ g g ⁻¹ DW)	Shoots	74.68 $\pm$ 10.49 a	40.34 $\pm$ 7.17 b
	Roots	185.16 $\pm$ 18.88 a	31.73 $\pm$ 10.57 b

Exposure to Pb affected the content of some mineral nutrients in roots and, mostly, in the aerial part (Table 2). In roots, Mn and Zn levels were significantly higher in control (~ 4-fold, and 6-fold, respectively) compared to Pb-treated plants. In the aerial part, Pb treatment significantly decreased Zn contents, whereas P and K contents significantly increased.

6.4.3. Photosynthetic pigment content, Chl fluorescence, gas exchange, RuBisCO activity and soluble sugars

Carotenoid contents remained unaffected by Pb exposure, whereas both Chl *a* and Chl *b* levels decreased significantly, not affecting the ratio Chl *a/b* (Table 3). The F_v/F_m mean values of both groups ranged between 0.74 and 0.85, being the average values of the control slightly higher than those of exposed plants (Table 3).

Table 3. Chlorophyll *a* (*Chl a*), chlorophyll *b* (*Chl b*) and carotenoids contents, *Chl a/b*, F_v/F_m , gas-exchange, RuBisCo activity, and soluble sugars content in leaves of *Z. fabago* plants exposed to 500 μM Pb after 1 week. Values are mean $\pm$ standard error ($n=6$). For each row, different letters represent significant differences at $p < 0.05$.

Pb treatment		
	0 μM	500 μM
<i>Chl a</i> ($\mu\text{g g}^{-1}$ FW)	184.70 $\pm$ 2.38 a	129.71 $\pm$ 2.47 b
<i>Chl b</i> ($\mu\text{g g}^{-1}$ FW)	105.14 $\pm$ 0.86 a	74.80 $\pm$ 1.53 b
<i>Chl a/b</i>	1.76 $\pm$ 0.04 a	1.73 $\pm$ 0.07 a
Carotenoids ($\mu\text{g g}^{-1}$ FW)	19.30 $\pm$ 0.76 a	19.00 $\pm$ 0.55 a
F_v/F_m	0.82 $\pm$ 0.00 a	0.79 $\pm$ 0.01 b
E ($\text{mmol m}^{-2} \text{s}^{-1}$)	0.82 $\pm$ 0.07 a	0.76 $\pm$ 0.08 a
g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	53.06 $\pm$ 5.11 a	46.23 $\pm$ 5.00 a
A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	3.07 $\pm$ 0.41 a	1.50 $\pm$ 0.20 b
A/g_s	58.18 $\pm$ 6.21 a	32.35 $\pm$ 3.19 b
C_i/C_a	0.74 $\pm$ 0.03 b	0.85 $\pm$ 0.01 a
RuBisCO ($\text{nmol s}^{-1} \text{g}^{-1}$ FW)	18.20 $\pm$ 1.21 a	7.89 $\pm$ 0.84 b
Soluble sugars ($\mu\text{g g}^{-1}$ FW)	Shoots	15.46 $\pm$ 0.38 a
	Roots	11.00 $\pm$ 0.39 b
		9.12 $\pm$ 0.22 b

Concerning the gas exchange parameters, plants exposed to Pb displayed significant changes in A , A/g_s , and C_i/C_a (Table 3). Compared to control plants, Pb treatment led to both a reduction (approximately 50%) in A and the A/g_s ratio and a slight increase in C_i/C_a ratio. The E and g_s remained unaltered by Pb supply. In addition, a significant reduction (in $\sim 57\%$) of RuBisCO activity was observed in Pb-treated plants as compared to controls (Table 3). The levels of soluble sugars in Pb-treated organs showed a general trend to decrease ($p < 0.05$, Table 3).

6.4.4. Oxidative stress markers: lipid and protein oxidation, cell membrane permeability, H_2O_2 , $\text{O}_2^{\cdot-}$ and proline content

In order to assess the oxidative damage, MDA content and the carbonyl group content of proteins were evaluated. A trend to increase lipid (MDA) and protein oxidation were observed in both shoots and, mostly, in roots (Table 4). Electrolyte

leakage (a parameter of cell membrane damage) increased significantly in exposed leaves, while exposed roots showed less electrolyte leakage ($p < 0.05$).

Table 4. Effect of Pb exposure on MDA, carbonyl concentration, electrolyte leakage, H_2O_2 , O_2^- and proline concentration in shoots and roots of *Z. fabago* plants. Results are expressed as the mean $\pm$ standard error ($n=4-5$). For each row and tissue type, different letters indicate significant differences between treatments at $p < 0.05$.

Measure	Shoots		Roots	
	0 μM	500 μM	0 μM	500 μM
MDA content (nmol g^{-1} FW)	5.4 ± 0.47 a	6.81 ± 0.80 a	1.90 ± 0.58 b	4.15 ± 1.01 a
Carbonyl concentration ($\text{nmol mg protein}^{-1}$)	19.11 ± 0.50 b	27.61 ± 1.09 a	37.44 ± 3.84 b	72.09 ± 8.81 a
Electrolyte leakage (%)	9.23 ± 1.39 b	20.11 ± 2.70 a	35.83 ± 2.78 a	24.09 ± 1.70 b
H_2O_2 (nmol g^{-1} FW)	410.98 ± 9.91 a	301.03 ± 3.52 b	297.20 ± 23.06 b	383.28 ± 4.19 a
O_2^- (nmol g^{-1} FW)	16.75 ± 1.28 b	26.98 ± 4.99 a	20.24 ± 3.60 a	10.36 ± 3.91 b
Proline ($\mu\text{mol g}^{-1}$ FW)	1.02 ± 0.02 a	0.64 ± 0.01 b	1.41 ± 0.03 a	1.01 ± 0.02 b

An accumulation of H_2O_2 was observed in exposed roots, while decreasing in exposed shoots (Table 4). Conversely, Pb exposure increased the levels of O_2^- in shoots but decreased the levels of this ROS in exposed roots (Table 4). Finally, the concentration of the stress metabolite proline decreased by around 28% in roots and 37% in shoots treated with Pb.

6.4.5. Total antioxidant capacity and antioxidant compounds

The TAA was measured by the ABTS, the DPPH, and the FRAP methods in order to assess the full spectrum of low molecular weight antioxidant compounds present in shoots and roots (Table 5). Only Pb-exposed shoots showed a significant decrease in the FRAP values, as well as in the ABTS radical-scavenging ability ($p < 0.05$). No significant changes in DPPH radical-scavenging ability were noted between control and Pb-exposed plants (Table 5).

Table 5. Total antioxidant activity, levels of total phenolics (TPC), hydroxycinnamic acid (HCA) derivatives, flavonoids, flavonols, lignin, AsA (ascorbate), DHA (dehydroascorbate), AsA/DHA and soluble total non-protein thiols (NPT) in shoots “S” and roots “R” of *Z. fabago* plants exposed to 500 μM Pb after 1 week. Data are presented as mean $\pm$ standard error ($n=6$). For each row, different letters represent significant differences at ($p < 0.05$).

			Pb treatment	
			0 μM	500 μM
Total antioxidant activity	ABTS	S	2.50 $\pm$ 0.05 a	2.07 $\pm$ 0.07 b
	($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	R	1.32 $\pm$ 0.11 a	1.17 $\pm$ 0.06 a
	DPPH	S	1.31 $\pm$ 0.18 a	1.14 $\pm$ 0.10 a
	($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	R	2.24 $\pm$ 0.14 a	2.44 $\pm$ 0.17 a
	FRAP	S	8.30 $\pm$ 0.23 a	5.86 $\pm$ 0.18 b
	($\mu\text{mol Fe(II) g}^{-1}$ FW)	R	6.17 $\pm$ 0.18 a	6.05 $\pm$ 0.26 a
Phenolic compounds	TPC	S	11.35 $\pm$ 0.13 a	8.97 $\pm$ 0.21 b
	($\mu\text{mol gallic acid eq. g}^{-1}$ FW)	R	4.14 $\pm$ 0.16 a	4.08 $\pm$ 0.15 a
	Hydroxycinnamic acids	S	3.05 $\pm$ 0.11 a	2.39 $\pm$ 0.26 b
	($\mu\text{mol caffeic acid eq. g}^{-1}$ FW)	R	0.87 $\pm$ 0.09 a	0.92 $\pm$ 0.08 a
	Flavonoids	S	1.10 $\pm$ 0.10 a	0.28 $\pm$ 0.04 b
	($\mu\text{mol rutin eq. g}^{-1}$ FW)	R	nd	nd
	Flavanols	S	41.75 $\pm$ 1.31 a	23.31 $\pm$ 0.72 b
	(nmol catechin eq. g $^{-1}$ FW)	R	nd	nd
Ascorbic acid and NPT	Lignin	S	9.74 $\pm$ 0.21 a	8.01 $\pm$ 0.36 a
	(mg LTGA g $^{-1}$ DW)	R	31.47 $\pm$ 0.27 a	25.23 $\pm$ 0.40 b
	AsA	S	0.57 $\pm$ 0.01 a	0.39 $\pm$ 0.01 b
	($\mu\text{mol g}^{-1}$ FW)	R	0.25 $\pm$ 0.01 a	0.22 $\pm$ 0.01 b
	DHA	S	0.20 $\pm$ 0.02 a	0.13 $\pm$ 0.01 b
	($\mu\text{mol g}^{-1}$ FW)	R	0.15 $\pm$ 0.01 b	0.25 $\pm$ 0.03 a
	Ratio AsA/ DHA	S	2.83 $\pm$ 0.34 a	3.04 $\pm$ 0.31 a
		R	1.60 $\pm$ 0.18 a	0.86 $\pm$ 0.15 b
	NPT	S	329.18 $\pm$ 11.71 a	216.09 $\pm$ 10.09 b
	(nmol GSH eq. g $^{-1}$ FW)	R	292.41 $\pm$ 21.64 b	363.29 $\pm$ 12.19 a

Under control conditions, shoots are richer in total phenolic content (TPC, Table 5) than roots. TPC decreased by 20% ($p < 0.05$) in shoots of Pb-exposed plants, remaining constant ($p \geq 0.05$) in roots. A similar profile for shoots and roots was found for hydroxycinnamic acids when exposed to Pb (Table 5). Flavonoids and flavanols were only detected in shoots (Table 5). Pb exposure reduced (by about 44%, $p < 0.05$) the flavanols in shoots compared to controls. A similar trend was found for flavonoids but with a higher decrease ($\sim 75\%$ lower than values of control) (Table 5). Finally, lignin concentration was higher in roots than in shoots, and only in the first ones Pb exposure induced a significant decrease ($\sim 20\%$ the control values) (Table 5).

As shown in Table 5, for control plants, the AsA content was approximately double (2.3-fold and 1.8-fold) in shoots than in roots. In both organs, these levels decreased significantly in Pb-exposed plants, being this decrease (32%) more severe in shoots. DHA decreased significantly in shoots of plants exposed to Pb, while in roots, it increased significantly (~ 1.7-fold, compared to control, Table 5). The ratio AsA/DHA decreased significantly only in contaminated roots (Table 5). Finally, the analyses of total NPT showed that the addition of Pb slightly increased (~ 24%, $p < 0.05$) the NPT levels in roots whereas decreased significantly (~ 34%) in the shoots.

6.4.6. Multivariate approach: global overview of the variables related to photosynthesis, growth, and antioxidant capacity/oxidative stress

PCA was carried out to identify parameters that can best describe the *Z. fabago* performance upon Pb treatment in both roots and shoots. Figure 1a shows that in shoots, the first principal component (PC1), which explained 68.1% of the total variance, was heavily loaded on parameters related to growth (area and biomass), photosynthesis (Chl, F_v/F_m , sugars, RuBisCO), antioxidant and protection compounds (proline, AsA, TPC, flavonoids, flavanols, and NPT), TAA (ABTS and FRAP) on the positive side of X-axes, and stress markers (MDA, carbonyl group contents, and ion leakage), O_2^- , AsA/DHA, RWC, and C_i/C_a on the negative side. The second principal component (PC2), which explained 8.9% of the total variance, was defined by carotenoids (on the positive side of Y-axes), g_s and E (on the negative side). Moreover, the projection of the samples on the PC axes showed a clear separation between control samples (which were located quite clustered on the right part of the plot) and Pb samples (which were located on the left part of the plot).

Figure 1b shows that in roots, the first principal component (PC1), which explained 49.4% of the total variance, was heavily loaded on parameters related to growth (size and biomass), antioxidant compounds (AsA, AsA/DHA), proline and lignin on the negative side of X-axes, and stress markers (MDA, carbonyl group contents, and DHA) and RWC on the positive side. The second principal component (PC2), which explained 18.9% of the total variance, was defined by antioxidant compounds (TPC and HCAs) and TAA (ABTS, DPPH, and FRAP). Moreover, the projection of the

samples on the PC axes showed a clear separation between control samples (which were located quite clustered on the left part of the plot) and Pb samples (which were located on the right part of the plot).

Figure 1. PCA analyses of functional responses of shoots (a) and roots (b) in *Z. fabago* plants exposed to 0- and 500- μ M Pb for 1 week. Loading plots for the first axis (explained variation is 68.1% in shoots and 49.4% in roots) and second axis (explained variation is 8.9% in shoots and 18.9% in roots). Eight data pairs ($n = 8$, as control and Pb) were used

6.5. Discussion

Lead toxicity has been reported for several plant species that are sensitive to doses lower than the one tested here for *Z. fabago* (for review see Pourrut *et al.*, 2011; Rodriguez *et al.*, 2015). The nanism (not paralleled with senescence/necrotic symptoms) observed in *Z. fabago* may be regarded as a defense strategy, rather than a negative impact of the metal. In their review on plant growth–defense trade-offs, Huot *et al.*, (2014) stressed that plant defense strategies require resources and ultimately lead to growth reduction. Those authors also stressed the role of diminished photosynthesis (and consequent decrease of the overall pool of energy reserves) on growth reduction, as was observed in *Z. fabago* plants.

Considering the RWC, the absence of significant disturbances in exposed *Z. fabago* indicates the species ability to control water balance in both roots and leaves. These results are in line with other findings where *Z. fabago* plants exposed to Zn or Cd showed maintenance of RWC (Lefèvre *et al.*, 2014, 2016). Contrarily, species sensitive to metals as Pb (Parys *et al.*, 1998) or Al (Silva *et al.*, 2012) showed high disturbance of RWC.

The content of Pb in plants exposed to 500 μM increased from 7.24 (control) to 213.20 ng/gDM in shoots (~ 30-fold increment) and from 48.60 (control) to 799.73 ng/gDM in roots (~ 16-fold increment). Even considering the loss of DM in both organs of exposed plants (around 38% loss in shoots and 44% in roots), the increase of total Pb content per organ mass remains higher in shoots than in roots (~ 18 and 9 -fold, respectively). These data show that, besides the efficiency of roots to accumulate Pb, shoots also demonstrate an increasing efficiency to translocate and accumulate Pb. Species like *Thlaspi rotundifolium* and *Brassica juncea* also showed high levels of Pb in both organs (e.g., Sharma and Dubey 2005).

It remains unclear how Pb enters the root (Peralta-Videa *et al.*, 2009; Pourrut *et al.*, 2013). It was proposed that it may involve non-selective processes together with an H^+ /ATPase pump activity to ensure the negative membrane potential in rhizoderm cells (Pourrut *et al.*, 2013). Our data show that even at the high level of Pb studied here, no major impairments (except for decreases of Zn and Mn) were found in root

nutrient balances, supporting that this organ can handle this Pb level. Pb exposure, however, increased the translocation of K and P to shoots, while the decreased Zn uptake in roots was also manifested in shoots. It was shown that Pb interferes with K^+ -ATPases of cell membranes and may induce an efflux of K^+ from roots (Pourrut *et al.*, 2013). Considering our data, for this tolerant species, it may be proposed that the K efflux may also occur in inner cells to the xylem cells, leading to its increased translocation to upper parts. Being a mobile element, K is also involved in osmotic regulation, which supports an ability of osmoregulation and control of the RWC in Pb-treated leaves. In pea, Rodriguez *et al.*, (2015) found a decrease in K as a consequence of Pb exposure, particularly in roots. Similar to the observed in our work, Breckle and Kahle (1992) showed that beech plants were highly sensitive to Pb, and that roots suffered a decrease in K, Mn, and Fe levels (among others).

In this study, Pb exposure strongly reduced Chl *a* and Chl *b* contents, though not affecting carotenoids contents. Chlorophyll loss, due to pigment or its precursors degradation, may be a consequence of oxidative stress, since negative correlations with the oxidative damage parameters (carbonyls, MDA, electrolyte leakage, and O_2^-) were found in the PCA (Fig. 1a). Also, as suggested for other species exposed to abiotic stress (Dias *et al.*, 2016, Vasques *et al.*, 2016), chlorophyll content reduction can be seen as a photoprotection mechanism (Bjorkman and Demmig-Adams 1994): upon Pb exposure, *Z. fabago* plants decreased chlorophyll content to reduce leaf light absorption, protecting plants from photoinhibition. This is also supported by the correlation between the chlorophylls decrease and F_v/F_m (Fig. 1A). Despite the slight (< 4%) decline of the F_v/F_m in plants exposed to Pb, the values found are within the range of values (0.75–0.85) referred for healthy plants (Dias *et al.*, 2013). Similarly to *Z. fabago*, Pb exposure did not induce photoinhibition in species as *Pelargonium* (KrishnaRaj *et al.*, 2000), rattlebush (Ruley *et al.*, 2006), cucumber (Burzynski and Klobus 2004), and pea (Rodriguez *et al.*, 2015). It was proposed for other species that Pb exposure did not affect the levels of chlorophyll, suggesting that for those species it did not impair the photosynthetic light harvesting complexes (Choudhury and Behera 2001; Rodriguez *et al.*, 2015). Despite the decrease of the contents of both chlorophylls, the balance between them was not disturbed in the photosynthetic core complexes. Some authors suggest that the maintenance of a high Chl *a/b* ratio may represent an adaptation to endure stress conditions (Li *et al.*, 2011). One should

highlight that the overall response of photosynthetic pigments to Pb is rather complex, as it depends on the species, exposure conditions, etc., and some heterogeneous responses have been described in other studies (John *et al.*, 2009; Cenkeci *et al.*, 2012; Rodriguez *et al.*, 2015).

Gas exchange-related parameters (A , E , g_s , and C_i/C_a) were reported in different species to be influenced by metal stress (Kaznina *et al.*, 2005; Dias *et al.*, 2013), but these studies were particularly focused on crops with higher sensitivity to Pb than *Z. fabago*. Pb exposure affected negatively A and A/g_s and increased the C_i/C_a but did not influence the stomatal aperture (E). Considering that Pb has little or no effect on stomatal aperture and increases CO_2 availability, A impairment can be mostly attributed to non-stomatal effects. Limitations of photosynthesis by reduced activity of the primary enzyme of the Calvin cycle, RuBisCO, were already reported for several plant species exposed to Pb (Parys *et al.*, 1998; Rodriguez *et al.*, 2015). Also, a putative influence of the decreased RuBisCO activity (56.6%) on the limitation of CO_2 uptake (decrease of A) cannot be discharged, as it is supported by the PCA data (Fig. 1a). Heavy metals, such as Cr and Cd, are described to induce abnormalities in the structure, expression, or activity of RuBisCO resulting in severe disruption of photosynthesis (Pietrini *et al.*, 2003; Dias *et al.*, 2016). Besides, it is known that oxidative stress (that increased in our Pb exposed plants) may also decrease RuBisCO activity (Pietrini *et al.*, 2003), and our data demonstrate also a negative correlation between MDA, carbonyls, electrolyte leakage, and RuBisCO (Fig. 1a). The final result of photosynthesis is the synthesis of carbohydrates, which may be evaluated by assessing the levels of the end-products soluble sugars and the carbohydrate of reserve, starch. Soluble sugar contents may increase under stress conditions, to maintain leaf cell turgor, and/or act as energy source and metabolite signaling molecules (Rosa *et al.*, 2009). In Pb-exposed *Z. fabago*, soluble sugars decreased, which by the PCA data could be related mostly to the reduction of A (Fig. 1a). Consequently, under these stress conditions, low sugar availability reduced also plant growth investment, as sustained by the positive correlation between biomass, sugars and A (Fig. 1a). Also, considering the photosynthesis-related parameters and the plant biomass data, the strong correlation (Fig. 1a) between the decrease of plant biomass and photosynthetic parameters, including

chlorophylls, A , F_v/F_m , sugars, and RuBisCO activity, supports that those changes in photosynthesis resulted ultimately in a general decline of plant growth.

Plant redox status depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (Sharma and Dubey 2007). In stressful conditions, including metal toxicity, this equilibrium is highly dependent on the antioxidant machinery activity. The present study was undertaken in order to understand how the antioxidant system responds to Pb exposure and the relationship between Pb toxicity, oxidative stress, and antioxidant systems in *Z. fabago*. In general, our results strengthen that the oxidative status of shoots and roots was differently affected by Pb treatment.

While O_2^- levels increased in shoots and decreased in roots upon Pb treatment, H_2O_2 contents decreased in shoots and increased in roots, which may be supported by differences in the expression of antioxidant systems in these organs, as already reported for other metal-stressed species (Silva *et al.*, 2012). For example, the increased levels of H_2O_2 in roots may be due to a better activity of enzymes (e.g., SOD) converting O_2^- into H_2O_2 . In shoots, besides SOD activity, other enzymes (catalase and peroxidases) that subsequently remove H_2O_2 are putatively being more stimulated decreasing H_2O_2 . A major consequence of an uncontrolled increase of ROS is the oxidation of biomolecules, namely lipids and proteins (e.g., Silva *et al.*, 2012). The increase of H_2O_2 in roots was positively correlated with MDA and carbonyl formation (Fig. 1b). On other hand, the MDA and carbonyl negative correlation with O_2^- (Fig. 1b) may be justified because this is a major ROS involved in lipid peroxidation initiation and in reversible oxidation of methionine sulfoxide and protein carbonyl formation (Dickinson and Chang 2011). In shoots, the increase of O_2^- seems to be more related to membrane damages, as sustained by the strong positive correlation found between this ROS and MDA, carbonyls, and electrolyte leakage. The fact that electrolyte leakage only occurred in leaves may be explained not only by the higher oxidative power of O_2^- when compared with H_2O_2 (supported by the PCA) but also by the assumption that organs display different protective mechanisms to Pb toxicity (Monteiro *et al.*, 2012) also demonstrated by the different PCA profiles.

Under control conditions, *Z. fabago* shoots are rich on secondary compounds as TPC, flavonoids, flavanols, and lignin, but the severe decrease in these compounds after exposure to Pb strongly suggests that this metal interferes, in a yet unclear way, with secondary metabolism pathways in shoots (and much less in roots). It has been established that the antioxidant activity of plant extracts is well correlated with their phenolic concentrations (Rice-Evans *et al.*, 1997; Zheng and Wang 2001). Our PCA data corroborate these findings, suggesting that most of these phenolic compounds may contribute to the TAA (mostly ABTS, FRAP) in both organs, and in shoots, its decrease after Pb exposure is correlated (Fig. 1a) to the increase of membrane damages and ROS (O_2^-).

Also, AsA is an important antioxidant, reacting with e.g., $OH^\cdot$ and peroxy ($ROO^\cdot$) radicals, and may be found in the symplast and in the apoplast (Yin *et al.*, 2010), particularly in peripheral root cells (Liu *et al.*, 2008). So, keeping a high AsA content is important for ROS detoxification, in both symplast and apoplast. Reduced levels of AsA and increased levels of the oxidized form are often a marker for oxidative stress conditions (Yin *et al.*, 2010). In the present work, shoots and roots showed slight differences regarding the use of this important antioxidant cycle. Despite the changes in AsA and DHA levels in roots and shoots, its ratio only decreased in Pb exposed roots. AsA/DHA decline in roots was associated to DHA increased, as sustained by the negative correlation showed in PCA (Fig. 1b). The specific differences in the AsA/DHA profiles in shoots and roots support that this cycle may not be a major strategy to the antioxidant capacity in roots. Other strategies, such as thiol, including NPT, can play more important protective roles (Fig. 1B). According to Zhao *et al.*, (2015), one of the most important mechanisms for heavy metal detoxification in plants is the complexation with strong ligands, such as NPT. So, high levels of NPT in roots exposed to Pb indicate high ability to tolerate cellular metal load, as suggested by Zhao *et al.*, (2015) for *Athyrium wardii*. Similar conclusions were reported by Kandziora-Ciupa *et al.*, (2013) in *Vaccinium myrtillus* leaves exposed to several heavy metals (Cd, Pb, and Zn). Moreover, NPT accumulation in *Z. fabago* roots possibly reflect a defense reaction to enhanced production of ROS, in particular H_2O_2 , as suggested by their positive correlation (Mishra *et al.*, 2006). In leaves of poplar, total NPT (mainly GSH and phytochelatins) were not stimulated by Cd suggesting that these molecules were not fundamental for tolerance to Cd and

other defence systems were activated (Baccio *et al.*, 2014), as demonstrated for *Z. fabago* leaves/shoots.

In summary, our data strongly demonstrate different mechanisms of shoots and roots in *Z. fabago* to cope with oxidative stress induced by Pb, which is also demonstrated by the PCA correlations seen in Fig. 1. The polar plots of data (Fig. 2) help to visualize the profiles of response to Pb in *Z. fabago* roots and shoots. These plots together with PCA (Fig. 1) support the view that shoots and roots respond in a different way to the metal challenge. Being O_2^- , membrane degradation (electrolyte leakage and MDA), and protein oxidation (carbonyl) more evident in shoots, while in roots, apart from some oxidative markers (mainly MDA, carbonyl, DHA, and H_2O_2), the defence biomolecule NPT increased. Since Pb content remains higher in roots than in shoots in *Z. fabago*, the different responses observed are probably reflecting different mechanisms of Pb interactions in each organ.

Figure 2. Polar analyses of functional responses of shoots and roots of *Z. fabago* plants exposed to 0 μM and 500 μM Pb A) Polar analyses of shoots and B) Polar analyses of roots. Data are expressed as percentage of the control.

6.6. Conclusions

In conclusion, the major responses of *Z. fabago* plants to short-term Pb exposure are summarized in Fig. 3. Overall, this work demonstrates that this species can cope with high doses of Pb, using defence mechanisms related with nanism and photosynthetic decrease. In particular, in response to the Pb-induced stress, several photoprotective mechanisms, such as reductions of the Chl *a* content, were activated preventing plant from suffering photoinhibition. However, Pb exposure impairs *A* and the increase of C_i/C_a pinpoints that biochemical effects (e.g., RuBisCO activity) can be the main cause of *A* reduction. The reduction of *A* affects also sugar production resulting in a

decrease of plant growth. This work also demonstrates that roots and shoots differ in the effects/responses at the oxidative level. For example, AsA/DHA ratio was maintained in shoots, while TAA (ABTS and FRAP), AsA, DHA, NPT, TPC, flavonoids, hydroxycinnamic acids, and flavonols decreased; roots suffer a decrease mostly in lignin, AsA, and AsA/DHA, but TPC and HCAs were unaltered and NPT levels increased. Taken together, these data demonstrate the adaptation of *Z. fabago* to highly contaminated soils, explaining the colonizing capacity of this species.

Figure 3. Functional responses of shoots and roots of *Z. fabago* plants exposed to 0 μ M and 500 μ M Pb. Arrows represent the increase or decrease in the respective endpoint measured in *Z. fabago* plants exposed to Pb comparatively to control plants.

PART II:
PIONEER SPECIES GROWN UNDER
CONTROLLED ENVIRONMENTAL
CONDITIONS

CHAPTER 7

Is Pb tolerance in *Zygophyllum fabago* due to the strengthening of the antioxidant pathways caused by *metal priming* of seeds?

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Highlights

- Specific differences in seed accumulation of Pb were shown in *Z. fabago* populations.
- Soil conditions modified the effectiveness of antioxidative pathways in *Z. fabago* progeny.
- Metallicolous (M) seedlings exhibited constitutively activated defense mechanisms.
- Lipid peroxidation increased under chronic Pb exposure in non-M seedlings.
- M seedlings respond to Pb stress by altering antioxidative metabolism more promptly and efficiently than non-M.

7.1. Abstract

Zygophyllum fabago is a pioneer species that thrives in mine soils characterized by different levels of heavy metals as well as nutrient deficiencies. In order to obtain information on the adaptive mechanisms that enable *Z. fabago* to counteract heavy metal toxicity, seeds were collected from three populations grown in natural environments characterized by different levels of lead (Pb) and essential nutrients, and germinated in controlled conditions in the presence of sub-lethal doses of $\text{Pb}(\text{NO}_3)_2$ (0, 25 and 50 μM) for 30 d. Ionic profiles of the seeds from non-metallicolous (NM) and metallicolous (M) populations developed in their natural environments were in line with the different edaphic conditions. However ionic profiles also indicated that the three populations carried out specific strategies in order to guarantee the appropriate supply of macro- and micronutrients in seed-storing tissues, in spite of the nutrient deficiencies present in mine soils. Their phenotyping characterization and germinability as well as seedling fitness clearly showed that M populations were well adapted to cope with chronic exposure to Pb. M seedlings had constitutively higher levels of metabolites involved in antioxidative pathways, in particular ascorbate, carotenoids and total soluble phenols. In addition, when NM and M seedlings were exposed to different Pb concentrations, the antioxidative metabolism was altered probably in order to better counteract Pb toxicity. Analyses of catalase and ascorbate peroxidase gene expression also supported a more efficient ROS-scavenging capability in M than in NM populations. Interestingly, in M populations, the strengthening of the antioxidative pathways followed different patterns that seem to be determined by soil conditions. In fact, the relatively high levels of Pb found in M seeds could act as a priming stimulus, which facilitated a rapid response to counteract the toxic effects of chronic Pb exposure.

Key words: ascorbate-gluthatione cycle, catalase, heavy metal, ionome, lead, redox homeostasis.

7.2. Introduction

Lead (Pb) is one of the most toxic and widespread hazardous metals in both terrestrial and aquatic ecosystems, and poses serious threats to environmental quality and human health (Gupta *et al.*, 2013; Singh *et al.*, 2015). The high degree of Pb contamination depends on its retention time of 150-5000 years, and on its extensive use in industry and agriculture. Sources of Pb pollution mainly arise from mining and smelting, traffic, as well as from the disposal of contaminated municipal sewage sludge and industrial waste (Yang *et al.*, 2000). Pb exposure, even at micromolar concentrations, causes toxicity in most plants, leading to a decrease in seed germination, plant growth, chlorophyll production as well as disturbances in antioxidant defenses (for review, see Pourrut *et al.*, 2011).

A few plant species have been identified in anthropogenically Pb-contaminated soils that are able to tolerate Pb toxicity, although the biochemical mechanisms that mediate Pb tolerance and adaptation remain largely unknown (for review, see Gupta *et al.*, 2013).

Abiotic stress conditions, including heavy metal exposure, are able to break the equilibrium between reactive oxygen species (ROS) generation and detoxification causing an impairment in redox homeostasis (Gill and Tuteja, 2010; Schützendübel and Polle, 2002). Extensive evidence now strongly supports that ROS act as signal molecules in cells (Choudhury *et al.*, 2016; De Pinto *et al.*, 2015; Mittler, 2017; Paradiso *et al.*, 2016).

However, they are also toxic byproducts of aerobic metabolism that can cause considerable damage to cellular structures and metabolic pathways (De Gara *et al.*, 2010). To prevent oxidative damage, cells possess a complex network of antioxidative enzymes and metabolites that remove and keep ROS at basal non-toxic levels (Mittler *et al.*, 2004). As signal molecules, ROS also play a key role in stress by promoting several signaling transduction pathways that result in the activation of acclimatory or defense responses against stresses or in the induction of programmed cell death (Choudhury *et al.*, 2016). The capacity of a plant cell to maintain ROS concentration at a basal level during stress conditions is essential to maintaining its own metabolism (de Pinto *et al.*, 2006; Mittler, 2017).

In fact, there is substantial evidence from genetic, transcriptomic, proteomic and biochemical studies linking heavy metal tolerance to cellular antioxidative defense systems (Sharma and Dietz, 2009; Singh *et al.*, 2015). Results from these studies performed in different heavy metal-tolerant plants have shown changes in the expression/activity of different ROS-scavenging enzymes including catalase and the enzymes of the ascorbate/glutathione (ASC-GSH) cycle, as well as changes in the content of non-enzymatic antioxidants such as ascorbate (ASC) and glutathione (GSH) (Dalcorsio *et al.*, 2013; Hossain and Komatsu, 2012; Lin and Aarts, 2012; Paradiso *et al.*, 2008; Sharma and Dietz, 2009; Singh *et al.*, 2015; van de Mortel *et al.*, 2006). Ascorbate peroxidase, the first enzyme of the ASC-GSH cycle, is considered to play a critical role in the fine regulation of H₂O₂ levels in plant cells and/or within specific cellular compartments (De Gara *et al.*, 2010). Although several ascorbate peroxidase isoenzymes have been characterized in different cellular compartments such as cytosol, chloroplasts, microbodies and mitochondria (Locato *et al.*, 2009; Shigeoka, 2002), cytosolic ascorbate peroxidase (APX) appears to be highly responsive to environmental stress, including drought, heat, salt, and high-intensity irradiation (Diaz-Vivancos *et al.*, 2013; Funatsuki *et al.*, 2003; Ishikawa *et al.*, 2005; Yabuta, 2004) and to stress combinations (Koussevitzky *et al.*, 2008).

The Syrian beancaper (*Zygophyllum fabago* L.), a succulent perennial shrub of the Zygophyllaceae family, is one of the few species capable of growing in poor soils that have been severely contaminated with Pb and Zn under semi-arid conditions (Boojar and Tavakkoli, 2011; López-Orenes *et al.*, 2017; Martínez-Sánchez *et al.*, 2012; Parraga-Aguado *et al.*, 2013). *Z. fabago* is a facultative metallophyte that tolerates high levels of Cd and Zn (Lefèvre *et al.*, 2014) as well as Pb (López-Orenes *et al.*, 2014) under hydroponic conditions.

Because of these properties, *Z. fabago* is considered a good candidate for the *in situ* reclamation of mine waste in the Mediterranean area (López-Orenes *et al.*, 2017). In fact, this process requires plant species that are tolerant to different combinations of climatic and edaphic constraints, involving severe drought, high temperature as well as nutrient deficiencies, salinity and high concentrations of heavy metals.

To our knowledge and despite its practical relevance, neither genomic nor transcriptomic data are currently available on this non-model plant species. The mechanisms allowing populations of *Z. fabago* to normally complete their life cycle in a very contaminated environment have also not been investigated in depth.

In order to better evaluate whether different populations of this facultative metallophyte species have acquired specific defense mechanisms, seeds produced in natural environments were analysed regarding their ionic profiles and phenotypic characterization. Seedlings of different populations grown under the same treatments with sub-lethal Pb doses (30 days, 25 and 50 μM) were also studied in terms of alterations in their ionome, gene expression and metabolite levels of antioxidative networks, as well as growth and fitness.

The results obtained highlight that the M populations, despite the different specificities, are more versatile in terms of enhancing the antioxidative network than NM populations, probably as an adaptation against this heavy metal.

7.3. Material and methods

7.3.1. Seedling growth and Pb treatment

Seeds of *Z. fabago* were collected from plants growing spontaneously in the Cartagena-La Unión Mining District (Murcia, Spain) in the mine tailings known as Agustín (37°36'19''N, 0°50'14''W) and Mercader (37°36'19''N, 0°50'03''W) and in a nearby non-mining site (37°36'19''N, 0°49'14''W). Seeds were surface-sterilized for 30 min in 20% (v/v) Domestos® (4.3% sodium hypochlorite, Unilever, UK), rinsed 3 times in sterile distilled water and left for imbibition overnight. Then, the seeds of each population were placed in nine trays (50 seeds per tray and three replicate trays per treatment for each population) containing a mixture of sand, vermiculite and peat (1:1:0.05, v/v/v). The trays were watered, at the beginning of the experiments, with equal amounts (800 mL) of N-solution, so that one-third of the trays were watered with 100 μM KNO_3 (control), one-third watered with 25 μM $\text{Pb}(\text{NO}_3)_2$ and 50 μM KNO_3 , and the remaining third with 50 μM $\text{Pb}(\text{NO}_3)_2$. All the solutions were buffered to pH 5.0 with MES/KOH (3

mM) to prevent Pb precipitation. The trays were arranged in a randomised complete block in a culture chamber under a 16 h photoperiod at 25/20 °C day/night temperature, with a photon flux density of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, and then were irrigated only with distilled water. After 30 days of growth, the seedlings were removed and their roots were washed for 5 min in 5 mM CaCl_2 to remove apoplastic Pb (Wang *et al.*, 2007), rinsed in distilled water and blotted with filter paper. Then, the seedlings were weighted and the aerial parts (shoots) were separated from the roots. Samples were immediately frozen in liquid nitrogen, pulverized and stored at -80°C for further analysis. Shoot seedling size was also measured as the length from the base to the upper end of the longest leaf. The low dose of Pb used was chosen taking into account the recommended maximum values for irrigation water (5 mg L^{-1}) reported by U.S. Environmental Protection Agency (US Environmental Protection Agency, 2012) and FAO (<http://www.fao.org/docrep/003/t0234e/t0234e06.htm>).

7.3.2. Nutrient and Pb concentrations

Total elemental analysis in seeds, shoots and roots were determined by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7900). Briefly, fine powder of dry samples (~200 mg) were incinerated at 450 °C for 8 h and digested in 1 mL concentrated HNO_3 (65% Merck, Suprapur). Then, the resulting extracts were diluted to 25 mL with Milli-Q water, filtered on ashless filter papers (Whatman 42) and stored at 4°C until analysis.

7.3.3. Analyses of photosynthetic pigment, total soluble phenols, ascorbate, glutathione and lipid peroxidation

For photosynthetic pigment analysis, liquid nitrogen-powdered shoots (~0.1 g) were extracted with 100% methanol (1.2 mL) and sonicated at 40 °C for 30 min in a water bath sonicator (FB-15049, Fisher Scientific). After centrifugation ($15,000 \times g$ for 15 min at 4°C), aliquots of the supernatants were used for the spectrophotometric determination of chlorophyll and carotenoid contents as previously described (López-Orenes *et al.*, 2013a). The resulting supernatants were also used for the determination of soluble total phenol content (TPC), using the Folin-Ciocalteu reagent and gallic acid (25-2000 μM) as a

standard (Everette *et al.*, 2010).

For ASC and GSH determinations, 0.3 g of liquid nitrogen-powdered samples were homogenized with six volumes of cold 5% (w/v) meta-phosphoric acid at 4°C with mortar and pestle. The homogenates were centrifuged at 20,000 x *g* for 15 min at 4 °C, and the supernatants were collected and used for the analysis of ASC and GSH levels and redox state according to de Pinto *et al.* (1999).

Lipid peroxidation was assessed by measuring the concentration of malondialdehyde (MDA) as the end product of the lipid peroxidation process according to Hodges *et al.* (1999).

7.3.4. Western blotting

Liquid nitrogen-powdered shoots (~0.1 g fresh weight) were homogenized in 1.2 mL of ice-cold 0.1 M phosphate buffer (pH 7.8) containing 0.5 mM EDTA, 5 mM MgCl₂, 5 mM ASC, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 % (w/v) polyvinylpolypyrrolidone (PVPP). The slurry was then incubated on ice for 15 min with shaking, after which it was centrifuged at 20,000 × *g* at 4°C for 15 min. The cleared supernatants were desalted using NAP-5 columns equilibrated with Tris-acetate buffer (25 mM, pH 6.4). For immunoblot analyses, proteins were denatured in Laemmli sample buffer containing 2.5% SDS, 10% glycerol, 0.5 % 1,4-dithiothreitol, bromophenol blue and 75 mM Tris-HCl (pH 6.8) at 100°C for 5 min. Denatured proteins were then separated on 10% SDS-PAGE, and blotted to nitrocellulose membranes (Whatman®, Protan) in transfer buffer (25 mM Tris, 190 mM Gly, and 20% (v/v) methanol), at 100 V for 60 min, using a Bio-Rad Mini Trans-Blot cell. The membrane was then blocked with a solution containing 5% (w/v) skim milk powder, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 0.05% (v/v) Tween 20 for 90 min and incubated with anti-cytosolic APX rabbit polyclonal antibody (1:5,000 dilution, 28 kDa, AS06180, Agrisera) and with goat anti-rabbit IgG-HRP conjugated (1:5,000 dilution, Santa Cruz Biotechnology). Antigenic polypeptides were visualized by chemiluminescence (Clarity Western ECL, Biorad, Hercules, CA) and quantified with the ChemiDoc MP system imager using the sotware ImageLab 4.1 (Bio-Rad).

7.3.5. Total RNA extraction and Real Time RT-PCR expression analysis

At the beginning of total RNA extraction, RNase Zap Decontamination Solution was used to remove RNase contaminant from work surfaces. RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality and quantity were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Residual DNA was removed from RNA samples by TURBO DNA-free™ Kit (Thermo Fisher Scientific) and first-strand cDNA was synthesized using High Capacity RNA-to-cDNA Kit (Applied Biosystems) starting from 1 µg of total RNA. The cDNA was used for real-time RT-PCR analysis.

Real-time RT-PCR was carried out using SYBR Green PCR Master Mix (Thermo Fisher Scientific). Three technical replicates for each biological replicate were used for each sample and template-free negative control. The melting curve, at the end of the qPCR amplification program, was used to evaluate the formation of non-specific PCR products. *ACT2*, *YLS8*, *SAND*, and *Fbox* were previously evaluated as candidate reference genes for their expression stability (data not shown). *Actin2* had the most stable expression during Pb exposure among the three *Z. fabago* populations and so it was used as housekeeping gene for Real Time RT-PCR. The list of primers used for the gene expression analysis of CAT, APX, dehydroascorbate reductase and glutathione reductase is reported in Supplementary information Table S1. The attempt to design primers for monodehydroascorbate reductase were not successful.

7.3.6. Statistical analysis

Data are presented as mean $\pm$ standard error (SE) from three independent experiments. The statistical significance of the differences ($P < 0.05$) between groups was tested using one-way analysis of variance (ANOVA) combined with Tukey's honestly significant difference (HSD) post-hoc test. All the statistical analyses were performed using the SPSS software (version 24.0; SPSS Inc., USA).

7.4. Results

7.4.1. Ionomic analysis and phenotyping of seeds from *Z. fabago* populations grown in their natural environments

Seeds of *Z. fabago* were collected from plants grown in two mining areas (Agustin and Mercader) and a nearby non-mining area (see Materials and methods). The mining areas contain one of the largest Pb and Zn content in the SW of Europe, and these mine soils are characterized by high sand proportions, reduced nutrients (N, P, K) and organic matter, and high concentration of heavy metals (Pb, Zn, Mn, Fe and Cd) (López-Orenes *et al.*, 2017). Table 1 shows the ionomic profile of the seeds of the three populations grown in their natural environments. In spite of the great differences in P contents in the rhizosphere soils, comparable P levels were shown in NM and M seeds. The seeds of the three populations showed different levels of storage of other macronutrients, which were not always in line with their concentration in the soils. In particular Ca and S levels were lower in NM and Agustin than in Mercader seeds, while Mg levels were 50% higher in the two M populations compared to NM seeds. The K level was about a half-fold lower in M seeds than in NM seeds. The analyses of micronutrients showed that M seeds, particularly those in Mercader, accumulated significantly higher levels of Fe, Mn and Zn compared to NM seeds, while Cu concentration was comparable in the three different populations.

As expected, the Pb levels were different in the seeds of the three populations: in NM seeds the Pb level was $\sim 3 \text{ ng g}^{-1} \text{ DW}$, whereas in Agustin and Mercader, the Pb levels in the seeds were 10-fold ($\sim 30 \text{ ng g}^{-1} \text{ DW}$, 0.03 ppm) and 36-fold higher ($\sim 100 \text{ ng g}^{-1} \text{ DW}$, 0.1 ppm), respectively (Table 1). In addition, phenotypic differences were investigated in the seeds grown in their natural environments. A significant difference in mean seed mass was observed among NM ($3.73 \pm 0.03 \text{ mg}$), Agustin ($4.85 \pm 0.04 \text{ mg}$) and Mercader ($4.49 \pm 0.03 \text{ mg}$) *Z. fabago* populations (see Supplementary information Fig. S1).

Table 1. Seed ionome profile of non-metallicolous (NM) and metallicolous (Agustin and Mercader) *Zygophyllum fabago* populations. The data represents the mean value of three experiments $\pm$ SE. Values in each row with different letters are significantly different (Tukey's HSD post-hoc test, $P < 0.05$). Numbers in parentheses represent percentage of control values.

	NM	Agustin	Mercader
Macronutrients (mg g ⁻¹ DW)			
Ca	13.32 $\pm$ 0.32 ^b (100%)	12.58 $\pm$ 0.26 ^b (94%)	16.39 $\pm$ 0.45 ^a (123%)
K	5.2 $\pm$ 0.31 ^a (100%)	2.67 $\pm$ 0.06 ^b (51%)	3.08 $\pm$ 0.16 ^b (59%)
Mg	2.60 $\pm$ 0.11 ^b (100%)	3.89 $\pm$ 0.04 ^a (150%)	4.32 $\pm$ 0.11 ^a (166%)
P	2.89 $\pm$ 0.16 ^a (100%)	2.93 $\pm$ 0.01 ^a (101%)	3.08 $\pm$ 0.10 ^a (107%)
S	1.22 $\pm$ 0.05 ^b (100%)	1.40 $\pm$ 0.01 ^b (115%)	2.00 $\pm$ 0.01 ^a (164%)
Micronutrients (μg g ⁻¹ DW)			
Cu	15.2 $\pm$ 3.4 ^a (100%)	18.8 $\pm$ 1.5 ^a (123%)	12.6 $\pm$ 0.2 ^a (83%)
Fe	43.5 $\pm$ 2.3 ^c (100%)	1230.1 $\pm$ 44.3 ^b (2830%)	4062.8 $\pm$ 201.7 ^a (9348%)
Mn	30.0 $\pm$ 1.1 ^c (100%)	96.6 $\pm$ 1.4 ^b (323%)	226.9 $\pm$ 4.5 ^a (758%)
Zn	37.3 $\pm$ 1.1 ^c (100%)	110.2 $\pm$ 3.5 ^b (296%)	135.6 $\pm$ 3.1 ^a (364%)
Lead (μg g ⁻¹ DW)			
Pb	2.87 $\pm$ 0.33 ^c (100%)	29.70 $\pm$ 0.69 ^b (1035%)	104.58 $\pm$ 0.54 ^a (3644%)

Figure S1. Phenotypic differences in seed size of non-metallicolous (NM) and metallicolous (Agustín and Mercader) *Z. fabago* populations grown in the presence of increasing Pb concentration. Seed biomass was expressed as the average mass per seed based on 50 seeds of each populations. Different letters indicate significantly different values following Tukey's HSD post-hoc test ($P < 0.05$).

7.4.2. Seed germination and seedling growth under chronic Pb exposure

When the seeds were grown in the same conditions and under chronic Pb exposure, a significant reduction in germination ($>35\%$) occurred in NM, while no significant difference was observed in the two M populations of *Z. fabago* (Table 2). After four weeks of Pb exposure, a significant dose-dependent decrease in biomass, was observed in NM seedlings (Table 2), although no visible symptoms of toxicity were noted (Supplementary Fig. S2). At a lower Pb concentration ($25 \mu\text{M}$, which is the maximum legally permissible concentration for Pb in irrigation water), a significant decrease in shoot size was already evident in NM plants. At the highest dose of Pb ($50 \mu\text{M}$), the growth of NM shoots was much more affected (Table 2). In contrast, no significant differences in germination nor in growth parameters were found in the two M (Agustín and Mercader) seedlings grown under Pb exposure, compared to their respective controls or to each other.

The growth reduction of NM seedlings by 50 μM Pb was accompanied by a significant decrease in both chlorophyll (-30%) and carotenoid (-63%) content, while the lower concentration of Pb had no effect on the content of photosynthetic pigments (Table 3). On the other hand in M plants, Pb exposure induced an increase in the chlorophyll content, but a decrease in the carotenoid concentration, although less evident than those observed in NM plants (Table 3).

In order to verify whether Pb treatment induced oxidative stress in the three populations, the levels of lipid peroxidation were analyzed. Interestingly, under control conditions (no Pb exposure), NM had significant higher levels of lipid peroxidation. Pb exposure induced a slight increase in lipid peroxidation in both NM and Agustín, although the levels of MDA were always higher in NM than in Agustín. No significant increase in lipid peroxidation was evident in Mercader (Table 3).

Table 2. Seed germination, biomass and shoot size of non-metallicolous (NM) and metallicolous (Agustín and Mercader) *Z. fabago* seedlings grown in the presence of increasing Pb (0, 25, and 50 μM) concentration for 4 weeks. Seed germination percentage was recorded after 10 d of imbibition. Values represent the means $\pm$ SE of three experiments. For each parameter, different letters denote significantly different values following Tukey's HSD post-hoc test ($P < 0.05$).

Parameters	Population	0 μM Pb	25 μM Pb	50 μM Pb
Germination (%)	NM	80.3 $\pm$ 2.0 ^a	52.0 $\pm$ 1.7 ^b	50.7 $\pm$ 1.8 ^b
	Agustín	85.1 $\pm$ 4.6 ^a	87.3 $\pm$ 5.3 ^a	83.7 $\pm$ 3.5 ^a
	Mercader	83.0 $\pm$ 3.6 ^a	86.3 $\pm$ 2.4 ^a	79.7 $\pm$ 2.2 ^a
Seedling biomass (mg)	NM	436 $\pm$ 16 ^a	319 $\pm$ 51 ^a	199 $\pm$ 28 ^b
	Agustín	416 $\pm$ 25 ^a	440 $\pm$ 24 ^a	425 $\pm$ 19 ^a
	Mercader	404 $\pm$ 23 ^a	428 $\pm$ 27 ^a	419 $\pm$ 21 ^a
Shoot size (cm)	NM	3.63 $\pm$ 0.15 ^a	2.90 $\pm$ 0.41 ^b	2.03 $\pm$ 0.09 ^b
	Agustín	3.48 $\pm$ 0.11 ^a	3.48 $\pm$ 0.35 ^a	3.65 $\pm$ 0.22 ^a
	Mercader	3.33 $\pm$ 0.35 ^a	3.58 $\pm$ 0.28 ^a	3.35 $\pm$ 0.35 ^a

Table 3. Content of photosynthetic pigments and lipid peroxidation in shoots from non-metallicolous (NM) and metallicolous (Agustin and Mercader) *Z. fabago* seedlings grown in the presence of increasing Pb (0, 25, and 50 μM) concentration for 4 weeks. Values represent the means $\pm$ SE of three experiments. For each parameter, different letters denote significantly different values following Tukey's HSD post hoc test ($P < 0.05$).

Parameters	Population	0 μM Pb	25 μM Pb	50 μM Pb
Chl <i>a</i> ($\mu\text{g g}^{-1}$ FW)	NM	230.6 $\pm$ 5.6 ^a	240.8 $\pm$ 7.2 ^a	157.8 $\pm$ 5.8 ^c
	Agustin	183.2 $\pm$ 7.6 ^b	237.3 $\pm$ 8.9 ^a	240.3 $\pm$ 9.8 ^a
	Mercader	150.6 $\pm$ 6.5 ^c	211.1 $\pm$ 4.8 ^b	200.1 $\pm$ 8.6 ^b
Chl <i>b</i> ($\mu\text{g g}^{-1}$ FW)	NM	122.48 $\pm$ 8.90 ^a	128.8 $\pm$ 7.8 ^a	93.6 $\pm$ 7.5 ^c
	Agustin	98.92 $\pm$ 7.72 ^c	124.1 $\pm$ 6.9 ^a	127.9 $\pm$ 8.2 ^a
	Mercader	81.05 $\pm$ 7.76 ^d	110.0 $\pm$ 5.4 ^b	103.3 $\pm$ 8.0 ^b
Total Chl ($\mu\text{g g}^{-1}$ FW)	NM	353.7 $\pm$ 9.1 ^a	370.8 $\pm$ 10.9 ^a	252.1 $\pm$ 9.3 ^d
	Agustin	282.9 $\pm$ 8.2 ^c	362.5 $\pm$ 9.8 ^a	369.3 $\pm$ 8.8 ^a
	Mercader	232.3 $\pm$ 9.2 ^d	322.0 $\pm$ 10.2 ^b	304.4 $\pm$ 10.6 ^b
Carotenoids ($\mu\text{g g}^{-1}$ FW)	NM	22.45 $\pm$ 1.21 ^b	22.57 $\pm$ 1.42 ^b	8.27 $\pm$ 0.22 ^c
	Agustin	32.97 $\pm$ 2.38 ^a	27.78 $\pm$ 1.57 ^{a,b}	21.79 $\pm$ 2.51 ^b
	Mercader	34.27 $\pm$ 2.59 ^a	27.60 $\pm$ 1.65 ^{a,b}	25.37 $\pm$ 1.43 ^b
Lipid peroxidation (nmol MDA g^{-1} FW)	NM	11.76 $\pm$ 0.49 ^b	12.95 $\pm$ 0.66 ^a	13.11 $\pm$ 0.68 ^a
	Agustin	10.31 $\pm$ 0.22 ^c	11.79 $\pm$ 0.47 ^{ab}	11.41 $\pm$ 0.39 ^b
	Mercader	9.20 $\pm$ 0.57 ^c	9.79 $\pm$ 0.71 ^c	11.20 $\pm$ 0.54 ^{bc}

Figure S2. Photographs show 30-day-old seedlings from the three *Z. fabago* populations after 4 weeks of continuous Pb (0, 25 or 50 μM) exposure.

7.4.3. Ionomic analysis of *Z. fabago* seedlings grown under chronic Pb exposure

The ionomic profile was similar in the shoots of the three different populations grown in the same conditions and in the absence of Pb, with the exception of Zn, the content of which was significantly higher in NM than in M shoots (Table 4). Moreover, Pb exposure did not lead to an imbalance in the uptake of macro- and micronutrients (Table 4).

In the roots grown in the absence of Pb, the significant differences were only observed in the levels of Mg (lower in Agustin than in the other populations) and Fe (higher in Mercader than in the other populations). The uptake of macro- and micronutrients did not significantly change in the roots as a consequence of the Pb exposure, with the exception of Fe which decreased in Mercader under 50 μM Pb, and Cu which increased in Agustin under both Pb concentrations (Table 4).

In control conditions, the Pb levels were higher in the shoots of the M than the NM populations (Table 4). Under Pb exposure, an increase in the uptake and translocation to shoots was only found in NM, while the Pb content remained almost constant in both Agustin and Mercader shoots. In roots, no significant differences were observed under control growth conditions among the three populations. However, under Pb exposure, an increase in Pb content was evident in all the three populations, although its uptake was significantly lower in Mercader (Table 4).

Table 4. Ionic profile in both shoots and roots from non-metallicolous (NM) and metallicolous (Agustin and Mercader) *Z. fabago* seedlings grown in the presence of increasing Pb (0, 25, and 50 μM) concentration for 4 weeks. Values represent the means $\pm$ SE of three experiments. Different letters indicate significantly different values between the treatments. *P*-values of the two-way ANOVA for the effects of populations (P), Pb treatment (T) and their interaction (PxT) are indicated in columns. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant.

	Pb (0 μM)			Pb (25 μM)			Pb (50 μM)			<i>P</i> -values		
	NM	Agustin	Mercader	NM	Agustin	Mercader	NM	Agustin	Mercader	P	T	PxT
Shoots												
Macronutrient (mg g⁻¹ DW)												
K	17.46 $\pm$ 0.75 ^a	14.38 $\pm$ 0.27 ^a	14.76 $\pm$ 0.62 ^a	19.65 $\pm$ 0.45 ^a	15.34 $\pm$ 0.35 ^a	15.49 $\pm$ 0.68 ^a	19.26 $\pm$ 0.38 ^a	16.25 $\pm$ 0.52 ^a	16.33 $\pm$ 0.88 ^a	**	NS	NS
Ca	14.59 $\pm$ 1.44 ^a	14.40 $\pm$ 0.42 ^a	10.68 $\pm$ 0.67 ^a	17.07 $\pm$ 1.15 ^a	16.12 $\pm$ 1.25 ^a	11.90 $\pm$ 0.72 ^a	20.46 $\pm$ 1.37 ^a	15.79 $\pm$ 2.08 ^a	13.65 $\pm$ 1.23 ^a	***	*	NS
Mg	11.39 $\pm$ 0.35 ^a	10.93 $\pm$ 0.72 ^a	8.13 $\pm$ 0.44 ^a	12.71 $\pm$ 0.69 ^a	11.67 $\pm$ 0.58 ^a	9.97 $\pm$ 0.37 ^a	14.27 $\pm$ 4.31 ^a	14.85 $\pm$ 1.15 ^a	10.37 $\pm$ 0.59 ^a	*	NS	NS
P	2.33 $\pm$ 0.33 ^a	2.22 $\pm$ 0.29 ^a	2.14 $\pm$ 0.30 ^a	2.78 $\pm$ 0.39 ^a	2.45 $\pm$ 0.33 ^a	1.18 $\pm$ 0.27 ^a	3.14 $\pm$ 0.35 ^a	2.03 $\pm$ 0.27 ^a	2.10 $\pm$ 0.31 ^a	NS	NS	NS
S	1.61 $\pm$ 0.41 ^a	1.50 $\pm$ 0.84 ^a	1.37 $\pm$ 0.56 ^a	1.72 $\pm$ 0.67 ^a	1.69 $\pm$ 0.55 ^a	1.44 $\pm$ 0.43 ^a	1.82 $\pm$ 0.56 ^a	1.83 $\pm$ 0.57 ^a	1.65 $\pm$ 0.65 ^a	NS	NS	NS
Micronutrient ($\mu\text{g g}^{-1}$ DW)												
Fe	46.04 $\pm$ 4.99 ^a	52.90 $\pm$ 9.32 ^a	58.76 $\pm$ 5.22 ^a	44.05 $\pm$ 9.70 ^a	50.37 $\pm$ 6.60 ^a	55.41 $\pm$ 23.78 ^a	41.80 $\pm$ 8.03 ^a	53.49 $\pm$ 12.67 ^a	55.38 $\pm$ 10.49 ^a	NS	NS	NS
B	52.31 $\pm$ 8.47 ^{a,b}	43.68 $\pm$ 1.75 ^{a,b}	39.56 $\pm$ 1.81 ^{a,b}	55.81 $\pm$ 5.38 ^a	33.99 $\pm$ 2.33 ^{a,b}	38.53 $\pm$ 1.68 ^{a,b}	45.99 $\pm$ 9.26 ^{a,b}	49.36 $\pm$ 4.32 ^{a,b}	32.26 $\pm$ 3.43 ^b	*	NS	NS
Mn	16.32 $\pm$ 1.54 ^a	18.27 $\pm$ 1.43 ^a	13.17 $\pm$ 1.07 ^a	17.84 $\pm$ 1.85 ^a	15.41 $\pm$ 1.39 ^a	19.34 $\pm$ 0.81 ^a	15.54 $\pm$ 2.02 ^a	20.65 $\pm$ 1.57 ^a	18.27 $\pm$ 2.40 ^a	NS	NS	NS
Zn	23.45 $\pm$ 4.80 ^{a,b}	18.91 $\pm$ 2.69 ^b	10.39 $\pm$ 1.85 ^c	28.19 $\pm$ 4.34 ^a	19.78 $\pm$ 1.62 ^b	10.92 $\pm$ 2.51 ^c	33.83 $\pm$ 6.73 ^a	15.01 $\pm$ 3.40 ^{b,c}	12.72 $\pm$ 3.69 ^{b,c}	**	NS	NS
Cu	54.52 $\pm$ 5.34 ^a	57.15 $\pm$ 13.25 ^a	39.19 $\pm$ 3.80 ^a	56.46 $\pm$ 6.47 ^a	42.95 $\pm$ 6.91 ^a	37.17 $\pm$ 3.78 ^a	45.70 $\pm$ 10.04 ^a	39.81 $\pm$ 11.98 ^a	33.09 $\pm$ 12.10 ^a	NS	NS	NS
Pb ($\mu\text{g g}^{-1}$ DW)												
	0.56 $\pm$ 0.36 ^b	2.11 $\pm$ 0.45 ^a	1.84 $\pm$ 0.79 ^a	0.63 $\pm$ 0.04 ^b	1.38 $\pm$ 0.17 ^a	2.19 $\pm$ 0.61 ^a	2.39 $\pm$ 0.78 ^a	1.52 $\pm$ 0.47 ^a	1.50 $\pm$ 0.40 ^a	*	NS	**
Roots												
Macronutrient (mg g⁻¹ DW)												
K	31.85 $\pm$ 2.54 ^{a,b}	36.20 $\pm$ 0.54 ^a	36.23 $\pm$ 0.72 ^a	35.69 $\pm$ 0.74 ^a	36.41 $\pm$ 0.37 ^a	35.79 $\pm$ 0.40 ^a	25.93 $\pm$ 0.84 ^b	32.46 $\pm$ 2.31 ^{a,b}	36.22 $\pm$ 1.36 ^a	**	NS	NS
Ca	7.62 $\pm$ 1.15 ^a	5.35 $\pm$ 0.62 ^{a,b}	6.08 $\pm$ 0.20 ^{a,b}	4.41 $\pm$ 0.45 ^b	4.21 $\pm$ 0.37 ^b	3.77 $\pm$ 0.38 ^b	6.32 $\pm$ 0.20 ^{a,b}	6.46 $\pm$ 0.13 ^{a,b}	6.43 $\pm$ 0.245 ^{a,b}	NS	*	NS
Mg	5.63 $\pm$ 0.82 ^a	3.77 $\pm$ 0.26 ^b	4.10 $\pm$ 0.25 ^{a,b}	3.37 $\pm$ 0.30 ^b	3.49 $\pm$ 0.17 ^b	3.81 $\pm$ 0.25 ^b	4.58 $\pm$ 0.27 ^{a,b}	4.64 $\pm$ 0.31 ^{a,b}	3.50 $\pm$ 0.18 ^b	NS	NS	NS
P	2.24 $\pm$ 0.27 ^a	2.25 $\pm$ 0.34 ^a	1.93 $\pm$ 0.86 ^a	2.53 $\pm$ 0.42 ^a	2.23 $\pm$ 0.32 ^a	1.12 $\pm$ 0.90 ^a	1.62 $\pm$ 0.21 ^a	1.88 $\pm$ 0.23 ^a	2.00 $\pm$ 0.88 ^a	NS	NS	NS
S	1.23 $\pm$ 0.66 ^a	1.22 $\pm$ 0.44 ^a	1.30 $\pm$ 0.42 ^a	1.20 $\pm$ 0.46 ^a	1.19 $\pm$ 0.063 ^a	1.37 $\pm$ 0.34 ^a	1.21 $\pm$ 0.53 ^a	1.34 $\pm$ 0.60 ^a	1.29 $\pm$ 0.42 ^a	NS	NS	NS
Micronutrient ($\mu\text{g g}^{-1}$ DW)												
Fe	241 $\pm$ 20 ^b	176 $\pm$ 16 ^b	343 $\pm$ 58 ^a	231 $\pm$ 68 ^b	165 $\pm$ 20 ^{b,c}	295 $\pm$ 49 ^{a,b}	203 $\pm$ 68 ^b	189 $\pm$ 30 ^b	119 $\pm$ 19 ^c	**	NS	***
B	28.26 $\pm$ 10.12 ^a	28.65 $\pm$ 8.52 ^a	21.11 $\pm$ 1.56 ^a	23.93 $\pm$ 2.59 ^a	28.52 $\pm$ 10.62 ^a	22.87 $\pm$ 2.93 ^a	34.60 $\pm$ 14.41 ^a	23.68 $\pm$ 6.65 ^a	18.62 $\pm$ 0.83 ^a	NS	NS	NS
Mn	13.42 $\pm$ 2.00 ^{a,b}	11.24 $\pm$ 0.98 ^{a,b}	14.60 $\pm$ 0.69 ^{a,b}	11.64 $\pm$ 0.77 ^{a,b}	9.74 $\pm$ 0.51 ^{a,b}	10.72 $\pm$ 0.80 ^b	10.19 $\pm$ 1.14 ^{a,b}	15.94 $\pm$ 4.64 ^a	11.55 $\pm$ 0.65 ^{a,b}	NS	NS	NS
Zn	59.11 $\pm$ 14.46 ^a	63.20 $\pm$ 17.86 ^a	51.31 $\pm$ 1.97 ^a	40.19 $\pm$ 3.94 ^a	61.50 $\pm$ 9.81 ^a	68.69 $\pm$ 10.00 ^a	54.95 $\pm$ 12.25 ^a	42.60 $\pm$ 0.30 ^a	49.32 $\pm$ 0.29 ^a	NS	NS	NS
Cu	31.40 $\pm$ 1.83 ^b	35.49 $\pm$ 8.6 ^b	44.61 $\pm$ 1.18 ^b	44.53 $\pm$ 13.16 ^{a,b}	57.00 $\pm$ 9.76 ^a	42.53 $\pm$ 2.17 ^b	43.31 $\pm$ 8.43 ^{a,b}	52.57 $\pm$ 6.18 ^a	47.43 $\pm$ 3.95 ^{a,b}	**	*	*
Pb ($\mu\text{g g}^{-1}$ DW)												
	0.33 $\pm$ 0.14 ^c	0.29 $\pm$ 0.14 ^c	0.29 $\pm$ 0.09 ^c	1.16 $\pm$ 0.17 ^b	1.94 $\pm$ 0.38 ^a	0.73 $\pm$ 0.06 ^b	2.01 $\pm$ 0.26 ^a	1.78 $\pm$ 0.27 ^a	0.89 $\pm$ 0.07 ^b	**	***	*

7.4.4. Effects of chronic Pb exposure on ROS scavenging networks

Because ASC and GSH are key players in cellular redox homeostasis, the effect of chronic Pb exposure on the ASC and GSH contents and redox states were determined in the shoots of the three *Z. fabago* populations. In the absence of Pb, the concentrations of total and reduced ASC were higher in both Agustín (~30 %) and Mercader (~40 %) than in NM-seedlings (Fig. 1A). However upon Pb exposure, their levels decreased in the two M-populations, especially in Agustín at 50 μ M Pb, but remained unchanged in NM-seedlings (Fig. 1A). The ASC redox state was not modified by Pb treatment in the three *Z. fabago* populations, maintaining values around 0.8-0.9 (Fig. 1A).

The levels of total and reduced GSH in control conditions were higher in NM (~20 %) than in M seedlings (Fig. 1B). Under Pb exposure, GSH levels were increased (~30%) in Agustín, while they remained unaffected in Mercader and NM-seedlings compared to their respective controls (Fig. 1B). Pb treatment also induced slight increases in GSSG levels in the three populations (Fig. 1B). Despite these changes, the GSH redox ratio [GSH/(GSH+GSSG)] remained above 0.9 in all the samples (Fig. 1B).

Since the role of phenols in protecting against oxidative stress is well known, as well as their capability to detoxify heavy metals by chelation (Michalak, 2006; Singh *et al.*, 2015), the levels and Pb-induced alteration of total soluble phenols were also studied. Under control conditions, M shoots contained higher levels of phenolic compounds than NM. Under Pb exposure, their levels also remained unaltered in NM shoots, while phenolic concentrations significantly decreased in M shoots (Fig. 1C).

The oxidative stress induced by heavy metals can be counteracted by ROS-scavenging enzymes. Therefore, the effects of chronic Pb exposure were studied on the gene expression of catalase and enzymes of the ASC-GSH cycle. As shown in Fig. 2A, in seedlings grown under control conditions, the level of CAT gene expression, normalized on the basis of the housekeeping gene Actin2, was higher in Agustín than in the other populations.

Pb exposure also led to an increase in CAT gene expression in all the populations. However in NM and Agustín this was only after exposure to the highest Pb concentration

(50 μ M); while in Mercader this increase was already evident after Pb 25 μ M exposure. On the other hand, APX gene expression strongly decreased in NM exposed to the highest Pb concentration, while it was not altered in Agustin and Mercader (Fig 2B). The behavior of this enzyme was confirmed by Western blot analysis, since a significant decrease in the amount of proteins reacting with an antibody against cytosolic APX was evident only in NM, but not in Agustin and Mercader (Fig 3). The gene expressions of GSSG reductase and DHA reductase, enzymes involved in the recycling of the oxidized form of GSH and ASC, were not altered by Pb exposure (data not shown).

Figure 1. Levels and redox state of ascorbate (A), glutathione (B) pools and contents of soluble total phenols (C) in shoots from non-metallicolous (NM) and metallicolous (Agustin and Mercader) *Z. fabago* seedlings grown in the presence of increasing Pb (0, 25, and 50 μM) concentrations for 4 weeks. Values represent the means $\pm$ SE of three experiments. Different letters indicate significantly different values following Tukey's HSD post-hoc test ($P < 0.05$).

Figure 2. Effect of Pb on the gene expression of catalase (A) and APX (B) in shoots from non-metallicolous (NM) and metallicolous (Agustin and Mercader) *Z. fabago* seedlings. The expression level of each gene was normalized to that of actin. The data represents the mean value of four experiments $\pm$ SE. Different letters indicate significantly different values following Tukey's HSD post-hoc test ($P < 0.05$).

Figure 3. Relative intensity of the western blotting of APX in non-metallicolous (NM) and metallicolous (Agustin and Mercader) *Z. fabago* shoots. The values were calculated as percentage of the intensity of each band relative to the intensity of the band of non-metallicolous, non Pb-treated seedlings taken as control. Error bars represent SE based on triplicate. Different letters indicate significantly different values following Tukey's HSD post-hoc test ($P < 0.05$).

Table S1. Degenerate PCR primers used in this study

Gene symbol	Polarity	Primer sequence
ACT2	Forward	5'-GATGATGCKCCYAGRGCWGT-3'
	Reverse	5'-TGATGCCARATCTTYTCCAT-3'
CAT	Forward	5'-GBATGAARTTYCCDGAYATGGT-3'
	Reverse	5'-CCATRTGBCTRATAATCTTGTGG-3'
APX	Forward	5'-CATGGCACTCTGCTGGWA-3'
	Reverse	5'-GGCTCCAAAAGYCTAACRGC-3'
GR	Forward	5'-TGGWTTTGATGGAVHTATGA-3'
	Reverse	5'-AYCTTYCCMCGVCCTTCDAT-3'
DHAR	Forward	5'-GAYGGAACWGARCARRYRTTRCT-3'
	Reverse	5'-TCWGGHAYDGWCCADTTCTT-3'
	Forward	5'- YCARAG GGTDTTGYT DAC-3'
	Reverse	5'-TCWGGHAYDGWCCADTTCTT-3'
	Forward	5'- GGHGAYTGYCCHTTYWSYC-3'
	Reverse	5'- AGYAAHRYYTGYTCWGTTC-3'

7.5. Discussion

The role of Pb-hypertolerance in the facultative metallophyte *Z. fabago* is still poorly understood. Our ionic and phenotyping analyses of the seeds collected from plants grown in their natural environments and exposed to different amounts of Pb, highlighted that this species adapts to the presence of very high levels of the heavy metal in soil. Indeed, the seeds of M populations have a larger size and biomass (Fig. S1). In spite of the great differences in the amounts of macro- and micro-nutrients present in the natural environments, the seed ionome of *Z. fabago* also revealed a selective increase in the stored macro- and micronutrients (Table 1). Thus, M seeds were able to uptake the same amount of P as did the seeds grown in the NM-environment, despite the low available P found on rhizosphere mining soils. The only common deficiency concerns K (about 50% less in M than NM), although this has no impact on germinability and seedling fitness (Table 2). In fact, variations in seed biomass are considered to be an adaptive feature for survival under unpredictable conditions in pioneer plant species (Luzuriaga *et al.*, 2006). A higher seed biomass has been correlated with higher food reserves and higher germination potential (Kołodziejek, 2017).

Excess Pb in soil often leads to an impairment in seed germination and a retarded seedling growth (Pourrut *et al.*, 2011), suggesting that growth reduction induced by Pb may be mainly due to a nutrient imbalance (Gopal and Rizvi, 2008) and/or to a depression in the photosynthetic rate (Islam *et al.*, 2008). In our study, the analyses of macro- and micro-nutrients in the roots or shoots of the seedlings showed that Pb exposure does not induce significant changes between NM and M, in spite of a clear growth decrease in NM seedlings. Thus, the growth reduction observed in NM seedlings after Pb exposure, could partly be due, to a decrease in both chlorophylls and carotenoid levels, with a consequent reduction in the photosynthetic rate. These data agree with previous results obtained in non-metallicolous plant species in which a degradation of chlorophylls and/or inhibition of their synthesis have been suggested as responsible for stunted growth in Pb-treated plants (Hattab *et al.*, 2016; Sharma and Dubey, 2005).

The analysis of Pb contents in roots and shoots of NM and M *Z. fabago* seedlings exposed to chronic Pb treatment also revealed important differences among populations. In NM seedlings, Pb accumulation in roots and its translocation to shoots depended on the

Pb concentration applied. On the other hand, no changes in the patterns of Pb accumulation were observed in the seedlings of M populations regardless of the dose employed. This thus suggests differences in the control of Pb uptake and translocation between M and NM populations.

Confirmation that specific mechanisms control the uptake and distribution of minerals in the different populations came from the analysis of the Pb content found in M population seeds grown in their natural environments. Indeed, despite the fact that the total Pb concentration was higher in Agustín than in Mercader tailing soils, the Pb concentration was more than 3-fold higher in the seeds collected from plants grown in Mercader than those collected in Agustín (Table 1). The presence of non-essential metals within the seeds of metal-tolerant ecotypes is well documented (Kranner and Colville, 2011; Vogel-Mikuš *et al.*, 2007) although their biological role is still unclear. Here, the Pb content found in shoots from M seedlings, under Pb-free conditions, was $\sim 2 \text{ ng g}^{-1} \text{ DW}$, which can be attributed to the high Pb levels found in M seeds, which were redistributed in the seedling tissue during germination.

Biochemical and molecular studies suggest that alterations in the plant oxidative metabolism are one of the first responses to Pb exposure (Auguy *et al.*, 2016; Liu *et al.*, 2009; Pourrut *et al.*, 2008; Wang *et al.*, 2016). In fact, Pb phytotoxicity has been associated with an increase in lipid peroxidation (Dazy *et al.*, 2009; Gupta *et al.*, 2013). In our study, a higher lipid peroxidation was found in NM compared with M seedlings, both in control conditions and after Pb exposure (Table 3). This thus implies that Pb ions are detoxified more successfully in M populations and/or that these populations exhibited constitutively activated defense mechanisms to cope with oxidative stress. In line with this, M populations possess constitutively higher levels of ASC, carotenoids and total soluble phenols that have well-known antioxidative properties, as we previously found in field studies (López-Orenes *et al.*, 2017). Surprisingly, the GSH content, which plays a pivotal role in defense reactions against toxic metals (Hernández *et al.*, 2015; Jozefczak *et al.*, 2012; Paradiso *et al.*, 2008), was lower in both M groups compared with NM. Several studies have shown that metal-hypertolerant populations originating in soil with high concentrations of sulfate have lower S-containing compounds than populations from sites rich in metal carbonates (Ernst *et al.*, 2008). Thus, the low GSH levels found in M populations can be explained in terms of an adaptive mechanism to the high concentration

of SO_4^{2-} found in Agustín and Mercader tailings soils (which were in the range of 8 to 10 g kg^{-1}).

Maintaining ROS homeostasis, through the modulation of the antioxidant network is critical for plant growth and survival under abiotic stress conditions (Choudhury *et al.*, 2016; De Gara *et al.*, 2010; Gill and Tuteja, 2010; Schützendübel and Polle, 2002). Interestingly in our study, no pronounced changes were observed for ASC and GSH contents upon exposure to Pb in NM seedlings. However a decrease in the total amount of ASC and increase in that of GSH were observed in M seedlings, although the two M populations showed different dose-dependent behaviors (Fig. 1), which could be caused by a different proneness to accumulate Pb in the two M populations (Table 1). Our results also suggest that ASC and GSH pools have a more prominent role in the metabolism of M than in the metabolism of NM seedlings. It is also possible that the perturbation of these redox pairs, which was boosted by relatively low Pb doses, could play a key role in the induction of Pb tolerance mechanisms in *Z. fabago* M populations. Exposure to heavy metals leads to alterations in ASC and GSH levels, however the direction of the response can differ depending on the metal concentration, period of treatment and species studied (Cuypers *et al.*, 2011; López-Orenes *et al.*, 2017, 2014; Paradiso *et al.*, 2008; Rodríguez-Serrano *et al.*, 2006).

The decrease in soluble phenolic compounds observed in M populations could also be related to the formation of polymeric phenolics, including cell wall-immobilized phenolics, which could contribute to the cell wall immobilization of Pb^{2+} ions. In fact, the cell wall is an important mechanical barrier to heavy metal accumulation within plant cells (Ovečka and Takáč, 2014), and the main cellular compartment where Pb particles are mostly deposited (Inoue *et al.*, 2013; Wang *et al.*, 2015). In fact, when exposed to Pb, the M populations showed a lower increase in Pb translocation to shoots than NM population (Table 4). Phenolic polymerization, which involves phenoxy radicals, might contribute to the control of cellular ROS homeostasis through the direct and indirect, peroxidase-mediated, interaction between active forms of oxygen, or oxygen-centered radicals, and phenolic compounds (Agati *et al.*, 2013). Therefore, the involvement of these reactions of oxidative phenolic coupling in ROS control would also result in the depletion of the soluble phenolic pool.

CAT and the enzymes of the ASC-GSH cycle also play a pivotal role in controlling H₂O₂ levels in plant cells and, consequently, in determining plant responses to stress (De Gara *et al.*, 2010; Mhamdi *et al.*, 2010; Noctor and Foyer, 1998). In our study, the analyses of gene expression indicate a different enzymatic ROS-scavenging capability in NM and M seedlings. Under control conditions, CAT appeared to be more expressed in Agustín than in Mercader and NM shoots. When the seedlings were exposed to Pb, CAT expression in NM reached the levels of Agustín only after exposure to 50 µM Pb, while this value was reached by Mercader more promptly. In addition, Pb exposure led to a decrease in the APX-dependent ROS removal only in NM shoots, while it remained unaltered in the M shoots (Fig. 3). This is thus further evidence that M populations have a higher efficiency in ROS-control/detoxification than NM ones, although the way in which each M population strengthens the antioxidative pathways followed different patterns upon Pb exposure. The difference in this response between Agustín and Mercader could be related to differences in their natural environments. In fact, when components of the antioxidative networks were determined in *Z. fabago* plants grown spontaneously on those mine tailings some differences were observed (López-Orenes *et al.*, 2017), supporting the influence of soil conditions on plant performance.

7.6. Conclusions

Our results revealed that M seedlings not only had constitutively higher levels of metabolites involved in antioxidative pathways, but also responded more promptly and efficiently to chronic-Pb stress than NM seedlings and, hence M plants showed better performance and fitness, in particular those developed from seeds collected in Mercader mine tailings. Genetic intra-species variations could be responsible for the differential responses to Pb observed here, although the involvement of other mechanisms cannot be ruled out. An epigenetic mechanism could also contribute to the performance of plants arising from seeds developed in different environments. Over the last few years, research on plant chemical priming (including seed priming) has been deciphering the molecular basis behind the improvement in plant performance under specific conditions. Since it has been reported that ROS and other redox-unbalancing molecules can be generated in response to Pb treatments (Corpas and Barroso, 2017), it is tempting to suggest that this metal could also act as a priming agent. The relatively high levels of Pb that we found in seeds from M populations, especially in Mercader, could act as a priming stimulus on

these seeds (Martinez-Medina *et al.*, 2016). Therefore, this "metal priming" could be involved in the induction of the higher Pb tolerance observed in M populations compared to NM plants in response to Pb exposure (Martinez-Medina *et al.*, 2016). Although more studies are needed to confirm this finding, we believe that this work provides a new approach to understanding how plants cope with heavy metal stress.

8. REFERENCES

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