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Mecanismos de resistencia a metales pesados en *Erica andevalensis*

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presentada por:

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MECANISMOS DE RESISTENCIA A METALES PESADOS EN *ERICA ANDEVALENSIS*

Memoria presentada por Mª BELÉN MÁRQUEZ GARCÍA, licenciada en Ciencias Ambientales para optar al Grado de Doctor por la Universidad de Huelva.

Memoria presentada dentro del marco del Programa de doctorado de "Gestión Ambiental" del Departamento de Biología Ambiental y Salud Pública (Facultad de Ciencias Experimentales, Universidad de Huelva)

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ABREVIATURAS

AA Antioxidant activity (Actividad antioxidante)
AMD Acid mine drainaje (Drenaje ácido de minas)
APX Ascorbate peroxidase (Ascorbato peroxidasa)
AsA Ascorbic acid (Ácido ascórbico)
DHA Oxidized ascorbic acid or dehidroascorbate (Ácido ascórbico oxidado o dehidroascorbato)
DPPH· 2,2-diphenyl-1-picryhydrazyl radical (Radical 2,2-difenil-1-picrihidrazilo)
EDTA Etilendiaminotetraacetic acid (Ácido etilendiaminotetraacético)
EROs Especies reactivas del oxígeno
FRAP Ferric reducing antioxidant power (Poder antioxidante reductor de hierro)
GA_3 Gibberellic acid (Ácido giberélico)
GLM General lineal model (Modelo lineal general)
GR Glutation reductase (Glutatión reductasa)
GSH Reduced glutathione (Glutatión reducido)
GSSG Oxidized glutathione (Glutatión oxidado)
IAA Indolacetic acid (Ácido indolacético)
LM Lloyd and McCown salt composition media (Composición de sales del medio de Lloyd y McCown)
MDA Malondealdehide (Malondealdehído)
MDHA Monodehidroascorbate (Monodehidroascorbato)
MS Murashige and Skoog salt composition media (Composición de sales del medio de Murashige y Skoog)
NADPH Nicotinamide adenine dinucleotide phosphate reduced form (Forma reducida de nicotinamida adenina dinucleótido fosfato)
POD Guayacol peroxidase (Guaiacol peroxidasa)
PC Phytochelatins (Fitoquelatinas)
PCA Principal component analysis (Análisis de componentes principales)
ROOH Hydroperoxides (Hidroperoxidos)
ROS Reactive oxigen species (Especies reactivas del oxígeno)
SOD Superoxido dismutase (Superóxido dismutasa)
TAC Total antioxidant capacity (Capacidad antioxidante total)
TBA Tiobarbituric acid (Ácido tiobarbiturico)
TE Trolox equivalents (Equivalentes de trolox)
TP Total phenolic compounds (Compuestos fenólicos totales)
VB Viénmont and Bejaurd salt composition media (Composición de sales del medio de Viénmont y Bejaurd)

RESUMEN DE LA TESIS

Erica andevalensis Cabezudo & Rivera es un brezo endémico que se caracteriza por vivir en los suelos ácidos y ricos en metales pesados de la Faja Pirítica del suroeste de España y Portugal. En esta tesis, como parte de la metodología empleada, se han realizado muestreos de campo para analizar la distribución de la especie, y se ha analizado el contenido de metales en el suelo y en los tejidos aéreos de la planta, en los que se encontraron concentraciones elevadas de Fe, Zn y Mn. A partir de los análisis bioquímicos realizados en las hojas de *E. andevalensis* recolectada del campo, se ha visto que esta especie se caracteriza por poseer niveles inferiores de ácido ascórbico y compuestos fenólicos que otras especies de su mismo género (*E. australis* y *E. arborea*). Ante el aumento de metales en el medio, la planta se defiende activando enzimas con actividad peroxidasas, principalmente ascorbato peroxidasa. Los metales, que en el interior de los tejidos vegetales pueden producir especies reactivas de oxígeno y provocar estrés oxidativo, no parecen afectar seriamente a *E. andevalensis*, como indican la relación entre la clorofila *a* y la *b* y los niveles de peroxidación lipídica. Las fitoquelatinas no parecen estar implicadas en la defensa frente a metales en *E. andevalensis*, ya que a pesar de crecer en suelos con concentraciones altas de metales y tener metales acumulados en las hojas, la concentración de fitoquelatinas encontrada es muy baja. Se ha conseguido cultivar la especie bajo condiciones controladas de laboratorio, punto que tiene gran importancia dado que es una especie catalogada “en peligro de extinción” por la legislación autonómica. Frente a la exposición a distintas concentraciones de Cd no se registraron cambios en los niveles de glutatión en las hojas, dato que apoya la no implicación de las fitquelatinas en la defensa de *E. andevalensis* frente a metales. La acumulación de Cd en el interior de la planta aumentó al incrementar la concentración a la que se expusieron. A concentraciones elevadas, otros sistemas antioxidantes no vinculados con el ciclo del ascorbato/glutatión son activados, aumentando la capacidad antioxidante total.

Con el objetivo de cultivar *E. andevalensis* bajo condiciones controladas de laboratorio, se ha desarrollado un protocolo de micropropagación de la misma, en el que a partir de fragmentos de tallo se regeneran plantas maduras.

Por otro lado, se han realizado estudios de germinación de semillas, que han puesto de manifiesto que condiciones de pH ácido, 2, y concentraciones elevadas de Fe estimulan la germinación, así como el crecimiento inicial de las plantas. Sin embargo el crecimiento se ve afectado a concentraciones elevadas de todos los metales ensayados (Fe, Mn, Cu y Zn). Se realizó un estudio del efecto del tratamiento de frío en la germinación de las semillas de *E. andevalensis* siendo los principales resultados que la tasa de germinación de semillas se incrementa al aumentar el tiempo que éstas son mantenidas en frío. Ha sido la primera vez que se describe el cultivo de *E. andevalensis* bajo condiciones controladas de laboratorio en tierra partiendo de semillas. Hemos observado que es una especie que se desarrolla mejor en suelos ácidos (de brezos) frente a substratos normales en condiciones controladas de laboratorio. El ácido giberélico (GA_3) estimula la germinación de *E. andevalensis* y afecta al desarrollo inicial de las plantas cuando se aplica en elevadas concentraciones. Una visión global de los resultados obtenidos revelan que *E. andevalensis* es una especie que está adaptada al medio en el que vive y estos resultados pueden ser aplicados en la elaboración de futuros planes de conservación de dicha especie.

ABSTRACT

Erica andevalensis Cabezudo & Rivera is an endemic heather with the characteristic of growing always in acidic and heavy metals enriched soils in the Iberian Pyritic Belt (SW Spain and Portugal). In this thesis, as part of the metodology used, trips to the country were made for analysing the distribution of the species and the heavy metal content has been analysed in soils and plant leaves. High concentraitons of Fe, Zn and Mn were found. The biochemical analysis of the leaves showed that *E. andevalensis* possess lower levels of ascorbic acid and phenolic compounds than other species of the same genera (*E. australis* and *E.*

arborea). In the case of an increase in the metal content in the soils, the plant defensive system is based in the increase of peroxidases enzymes, mainly ascorbate peroxidase. The metals, that can produce rective oxigen species in the vegetal cells and cause oxidative stress, seem not to affect to *E. andevalensis*, as was indicated by the ratio between the chlorophyll *a* and *b* and the lipid peroxidation levels. The phytochelatins did not seem to be implicated in *E. andevalensis* defence mechanisms against metals, despite the plant is growing in soils with high concentration of metals and the plant contain metals in the leaves, the phytochelatins concentration is too low. The species has been cultivated under controlled conditions in the laboratory and exposed to different concentrations of Cd. Any of the tested concentrations induce changes in the glutathione levels in the leaves. This data supported the no implication of phytochelatins in heavy metals defence in *E. andevalensis.* The Cd accumulation in plant leaves increaded when higher concentrations were added to the soil. High concentrations of Cd produce the activation of different antioxidant systems no related with the ascorbic acid/glutathione cycle, due to the increase in the total antioxidant activity.

With the aim of cultivate *E. andevalensis* in the laboratory, under controlled conditions, a micropropagation protocol has been developed, in which the whole plant is regenerated starting from a piece of stem.

The germination has been studied and acid pH and high concentrations of Fe resulted in stimulation of the germination, together with a better plant develpoment. However the growth was affected by the highest concentration in the case of all the tested metals (Fe, Mn, Cu and Zn). The effect of the cold treatment on the seed germination was analysed. The main results were the increase of the germination rate when the cold treatment was longer. It is the first time that the culture of *E. andevalensis* in soils has been described, starting from seeds. This plant shows a better development when acid soil is used under controlled conditions. Together with cold treatments, the effects of gibberellic acid (GA_3) on the germination and plant development has been described. A global view of the results got show that *E. andevalensis* is a well adapted species and these data could be taken into account in future conservation development programs.

ÍNDICE

INTRODUCCIÓN

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1. Los metales en el medio ambiente

El término metal es definido por la Real Academia Española de la Lengua como “elemento con brillo metálico, con capacidad de perder electrones para formar iones positivos y la habilidad de conducir el calor y la electricidad”. Sin embargo, aunque ésta es adecuada, es precisa una definición más estricta desde el punto de vista de la química.

Según la IUPAC (Unión Internacional de Química Pura y Aplicada), autoridad mundial en nomenclatura, terminología y métodos estandarizados de química, los metales se definen como elementos con conductividad eléctrica, brillo metálico, maleable y dúctil, que forman cationes y que tienen óxidos básicos. Esta definición es, no obstante, demasiado amplia, pudiéndose incluir en ella a casi toda la tabla periódica. Debido a ello, se hace necesario hacer una subclasificación para poder utilizar los metales en estudios biológicos y medioambientales. Esta clasificación incluye: metales, metaloides o semimetales, metales ligeros, metales pesados, metales beneficiosos, metales tóxicos, metales abundantes, metales disponibles, metales traza y micronutrientes (Duffus 2002).

En la actualidad, los términos metal o metal pesado están siendo utilizados de forma generalizada como sinónimo de elementos tóxicos y perjudiciales, en noticias tales como el accidente de la rotura de la Presa de Aznalcóllar en 1998. Sin embargo, el término metal pesado nunca ha sido definido por ninguna autoridad química tal como la IUPAC. Desde que el término se empezó a utilizar en química se le han dado un amplio rango de definiciones. No se ha encontrado ninguna relación entre la densidad de los metales, los conceptos fisicoquímicos que se han

utilizado para definirlos como “metales pesados” y la toxicidad o ecotoxicidad que se les atribuye. La biodisponibilidad es un punto clave en la toxicidad de los elementos metálicos y sus compuestos. La biodisponibilidad depende de parámetros biológicos y de propiedades fisicoquímicas de los elementos metálicos, sus iones y sus compuestos. Esto depende a su vez de la estructura atómica del elemento metálico, que se describe sistemáticamente en la tabla periódica. Por tanto, cualquier clasificación de los elementos metálicos para ser usada científicamente debe estar basada en la tabla periódica o en alguna subdivisión de ésta (Duffus 2002).

En esta tesis, el término “metales pesados” va a ser utilizado siguiendo la definición dada por Schützendübel y Polle (2002) como aquellos metales con densidad mayor que 5 g/cm^3. Entre estos metales se encuentran algunos de los elementos que, por su elevada toxicidad, son fuentes de contaminación como el cadmio, cromo, mercurio, plomo, etc. (Sanita di Toppi y cols. 1998; Clijsters y cols. 1999). Su acumulación en el suelo puede ser de origen natural, en función de los procesos de transformación físico-química de la roca madre en que se sustenta el suelo, o de origen antrópico, debido a actividades industriales y mineras (Macnair 1997; Shaw y cols. 2004).

2. Defensa de las plantas frente a los metales

El término metal, como anteriormente se ha expuesto, no es sinónimo de toxicidad, y las plantas requieren para su supervivencia una variedad de metales que, de forma genérica, se denominan “esenciales”, pues son requeridos por todas las plantas a concentraciones pequeñas, como elementos nutricionales. Hay otros elementos que se han comprobado que son esenciales sólo para algunas especies y otros que se ha comprobado que estimulan el crecimiento de las plantas, pero de los que no se conocen aún los procesos específicos en los que intervienen. Por

tanto, un elemento se define como **esencial** cuando no puede ser sustituido por otro por la especificidad en sus funciones bioquímicas y tiene una influencia directa en el organismo, el cual no puede crecer ni completar algún ciclo metabólico si este elemento no está presente (Kabata-Pendias y Pendias 2000). Estos metales son incorporados a través de las raíces, como micronutrientes u oligoelementos. Sin embargo, los metales esenciales pueden ser tóxicos si su concentración efectiva es muy elevada. Con alguna excepción, los metales pesados son **no esenciales** y, por tanto, son tóxicos en la naturaleza aún a concentraciones muy bajas (Clijsters y cols. 1999; Prasad 2004). Tanto la deficiencia como el exceso de elementos esenciales afectan al desarrollo óptimo de la planta, como se muestra en la Figura 1.

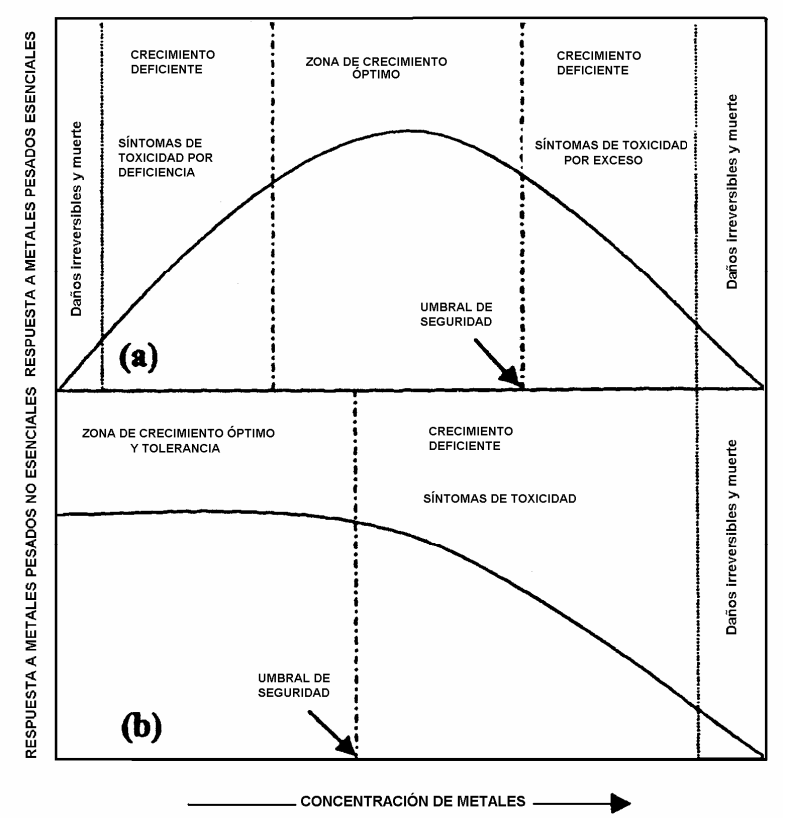


Figura 1. Curva dosis-respuesta del crecimiento de plantas. (a) Respuesta frente a metales pesados esenciales. (b) Respuesta frente a metales pesados no esenciales (adaptada de Shaw y cols. 2004).

Algunos metales desempeñan funciones de gran importancia en el metabolismo de las plantas, como puede observarse en la Tabla 1.

Tabla 1.
Principales funciones de elementos traza que son esenciales para las plantas (tomada de Kabata-Pendias y Pendias 2000).

Elemento	Forma parte de	Implicado en
Al		Controla propiedades coloidales en la célula, posible activación de algunas deshidrogenadas y oxidasas.
B	Fosfogluconatos	Metabolismo y transporte de carbohidratos, síntesis de flavonoides, síntesis de ácidos nucleicos, utilización del fosfato y producción de polifenoles.
Co	Coenzima	Fijación del N_2 en simbiosis, posiblemente también en plantas sin nódulos. Posible relación de los cambios de valencia con la síntesis de clorofilas y proteínas, aunque no está confirmado.
Cu	Varias oxidasas, plastocianinas y cenilsplasminas	Oxidación, fotosíntesis, metabolismo de las proteínas y los carbohidratos, metabolismo de la pared celular y posiblemente participa en la fijación del N_2 durante simbiosis
Fe	Hemoproteínas y non-hemo proteínas ferricas, deshidrogenasas y ferredoxinas	Fotosíntesis, fijación del N_2 y cambios de valencia.
Mn	Síntesis de varias enzimas	Fotoproducción de O_2 en el cloroplasto e indirectamente en la reducción del NO_3^-
Mo	Nitrato reductasa, nitrogenasas y oxidasas	Fijación del N_2, la reducción del NO_3^- y cambios de valencia
Si	Componentes estructurales	-
Zn	Anhidrasas, deshidrogenasas, proteinasas y peptidasas	Metabolismo de lípidos, carbohidratos y ácidos nucléicos.

Los metales, cuando se encuentran en exceso en las células vegetales pueden producir cambios de permeabilidad en las membranas celulares (Ag, Au, Br, Cd, Cu, Fe, Hg, I, Pb), reaccionar con los grupos tioles (Ag, Hg, Pb), competir por sitios especificos con metales esenciales (As, Sb, Se, Te, W, Fe) o sustituir iones esenciales (Cs, Li, Rb, Se, Sr) entre otros efectos (Kabata-Pendias y Pendias 2000, a partir de una recopilación de distintos autores).

Desde un punto de vista ambiental, no todos los metales producen la misma respuesta en las plantas, ni todas las plantas responden de la misma manera

frente al mismo metal. Existen metales que en general son más dañinos para las plantas que otros, como es el caso del Cu, Ni o Ag que son más tóxicos que el Pb, Mn o Al (Macnair 1997). Un factor importante a tener en cuenta, además del tipo y concentración del metal, es la especie o forma química en la que éste se encuentra, ya que la toxicidad depende de ésta. Algunos ejemplos de la relación entre toxicidad y especiación de metales son: el arsénico III es mucho más tóxico que el arsénico V (Kochian 1995); el cromo VI, que se suele encontrar formando cromato (CrO_4^{2-}), es más tóxico que el cromo III (Shanker y cols. 2005).

El hecho de que un metal esté presente en el suelo no significa que las plantas puedan asimilarlo. Se define la **biodisponibilidad** como la cantidad de un metal presente en suelo que puede ser incorporado por un ser vivo en las condiciones particulares que se establecen en la naturaleza. Este concepto indica que sólo una fracción del metal medido en suelo puede ser incorporado por una planta, lo cual depende de una serie de factores entre los que se incluyen no sólo la concentración del metal, sino una variedad de parámetros físico-químicos que determinan su especiación como el pH, el potencial redox, la hidrofobicidad, las interacciones con otros metales y no metales, etc. Un ejemplo es el Al, que a pesar de ser uno de los elementos más abundantes de la corteza terrestre, sólo es biodisponible en suelos ácidos y que, por tanto, sólo debe ser descrito como tóxico para las plantas en dicho tipo de suelos. En cualquier caso, existen mecanismos estrictamente biológicos que dependen, bien de la planta en cuestión, bien de organismos que generan un microambiente particular en el entorno de las raíces de la planta y que facilitan o dificultan el transporte y la incorporación del metal en las estructuras biológicas de la planta considerada (Greger 2004). Por consiguiente, el concepto de biodisponibilidad no es preciso en el sentido de que su valor depende tanto de factores ambientales diversos como de la biología particular de la planta de interés.

Una vez en el interior de la raíz, y por tanto en el tejido vegetal, los metales causan daños a las plantas a través de distintos mecanismos (Ochiai 1987):

- bloqueando los grupos funcionales de moléculas de importancia biológica, como enzimas, polinucleótidos o sistemas de transporte de iones.
- desplazando o sustituyendo iones de metales esenciales de biomoléculas y unidades celulares funcionales.
- desnaturalizando e inactivando enzimas.
- rompiendo la integridad de membrana de células y orgánulos celulares.
- promoviendo la generación de especies reactivas que causan estrés oxidativo.

La tolerancia o resistencia de las plantas frente al exceso de metales en suelos es un concepto complejo que depende de una variedad de factores. En general, las plantas han desarrollado mecanismos genéricos de defensa ante la presencia de metales en el suelo, lo que en función de su eficacia determina su tolerancia o sensibilidad frente a la contaminación por metales. Los mecanismos de defensa pueden tener un **carácter primario o específico**, siendo determinados por una serie de genes que confieren resistencia a especies o variedades concretas frente a determinados metales. La expresión génica que determina la tolerancia puede tener **carácter constitutivo**, es decir, es independiente de la presencia de metales en el suelo, o tener **carácter inducido,** dependiendo la síntesis de las proteínas de la presencia y concentración de metales específicos en el suelo. La diferencia entre mecanismos primarios y secundarios es, normalmente, una cuestión de matiz ya que los procesos de defensa son cualitativamente similares aunque evidentemente difieren en su eficacia cuantitativa (Macnair 1997; Hall 2002).

Los mecanismos genéricos de defensa de las plantas frente a la contaminación por metales pueden tener carácter **extracelular** o **intracelular** (Tabla 2). Estos mecanismos suelen operar de forma conjunta y coordinada, e incluso estar apoyados por la presencia de organismos distintos a la propia planta que, como las micorrizas, le confieren resistencia en forma de barrera física

impidiendo el acceso de los metales a las células radicales de la planta afectada (Bradley y cols. 1981; 1982; Macnair 1997; Leyval y cols. 1997; Hall 2002; Cairney y Meharg 2003; Midgley y cols. 2004).

Tabla 2
Mecanismos generales de las plantas a nivel extracelular e intracelular (tabla adaptada de Macnair 1997).

Mecanismos de exclusión o barrera	Secreción de quelantes que disminuyen la solubilidad del metal o reducen la eficacia del transporte al interior de las células Secreción de compuestos que cambian el pH de la rizosfera y afectan a la especiación del metal. Producción de residuos aniónicos en la pared celular, donde quedan atrapados los iones metálicos.
Mecanismos defensivos intracelulares de control e inertización	Modificación de las propiedades de la membrana celular, o estructuras proteicas asociadas, reduciendo el transporte del metal hacia el citosol. Modulación de las vías de cotransporte (simporte o antiporte), disminuyendo la entrada neta del metal contaminante en la célula. Alteración (constitutiva o inducible) de enzimas sensibles para prevenir la inhibición por el metal. Producción de sustancias quelantes (fitoquelatinas y metalotioneinas) que se unen al metal en el interior de la célula, restándole toxicidad al impedir su interacción con diversas biomoléculas. Transporte del metal hacia la vacuola, donde se almacena y puede ser objeto de otros mecanismos de detoxificación como su unión a agentes quelantes

Por consiguiente, y en general, la estrategia de las plantas para prevenir los efectos tóxicos de los metales (Fig. 2) es impedir que éstos estén libres en el citosol y en los orgánulos celulares (Hall 2002). Por ello, hay plantas cuya tolerancia se basa en la **exclusión** de metales, de tal manera que la concentración intracelular de éstos es siempre inferior a la existente en el medio. En sentido contrario, hay grupos de plantas pueden **acumular** y aún **hiperacumular** metales en

compartimentos celulares aislados y/o ligados a moléculas quelantes que convierten al metal en inocuo (Baker 1981). Además se puede incluir en esta clasificación a un grupo de plantas denominadas **indicadoras** que incorporan y acumulan los metales en función de la concentración que se encuentra disponible en el sustrato (Ross and Kaye 1994). La relación entre la concentración de metal en el suelo y en la planta se muestra en el Fig. 2.

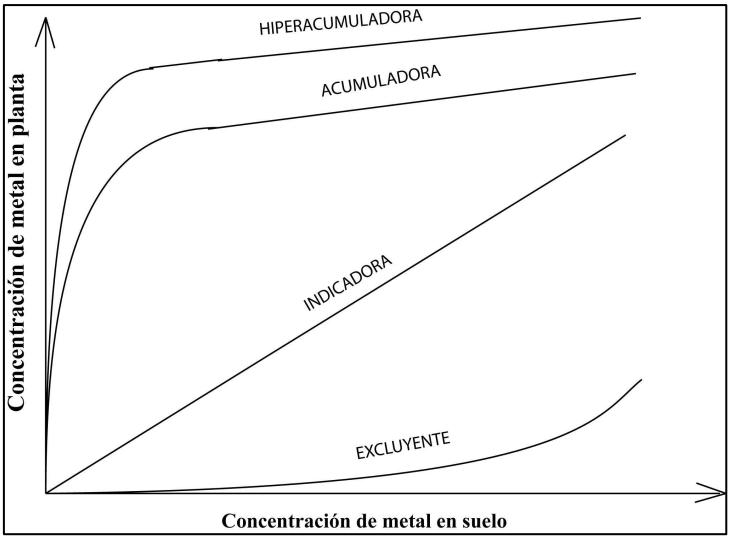


Figura 2. Respuesta de las plantas frente a la concentración de metales en el suelo, adaptada de Greger (2004).

Los mecanismos de defensa de las plantas frente a la contaminación por metales son altamente eficaces para aquellas variedades que se han adaptado a suelos específicos, siempre que la concentración de metales se mantenga relativamente estable (Hall 2002). No obstante, sea por causas naturales o por actividades humanas, si la concentración de metales aumenta excesivamente o si se incorporan al suelo metales no esenciales, las barreras defensivas de la planta pueden ser superadas. En estos casos, la planta se verá afectada por los metales, en mayor o menor grado, en función de la especiación del metal y su biodisponibilidad, pudiendo llegar a morir si la contaminación es excesiva. Pero cuando los niveles de metales tóxicos no superan valores letales y la exposición

tiene un carácter crónico, la planta desarrolla una sintomatología ligada al estrés oxidativo derivado de la generación de especies reactivas de oxígeno (EROs). Un estrés oxidativo persistente provocará una disminución de las capacidades fisiológicas de la planta con síntomas como clorosis, color amarillento, marchitez, atrofia, necrosis, etc. (Macnair 1997; Prasad 2004).

3. Estrés oxidativo

El oxígeno molecular (O_2) es uno de los elementos mas abundantes en la atmósfera terrestre, y es imprescindible para la vida aerobia, tanto terrestre como acuática. Los organismos fotosintéticos son los responsables de la existencia del O_2 en la atmósfera, ya que lo liberan cuando rompen la molécula de agua para obtener electrones durante la fotosíntesis. El O_2 permite a las células obtener energía mediante la combustión de compuestos orgánicos, pero al mismo tiempo se producen moléculas que pueden dañar a las células (Shaw y cols. 2004).

En su estado energético basal el O_2 no es reactivo, ya que, a pesar de tener dos electrones desapareados, lo que le convierte en un radical libre, éstos presentan un spin paralelo, teniendo la molécula la configuración denominada triplete $^3O_2$, que es la más estable posible (Fig. 3) (Halliwell y Gutteridge 1989; Ullah y Wilson 1995).

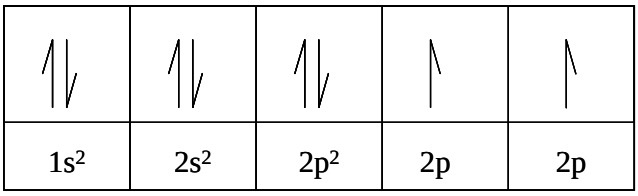


Figura 3. Configuración electrónica de la molécula de oxígeno ($^3O_2$).

Sin embargo, esta molécula de oxígeno ($^3O_2$) puede volverse reactiva a través de dos vías diferentes. Puede adquirir un electrón y convertirse en O_2^- ó uno de los electrones desapareados puede cambiar de spin y convertirse en oxígeno singlete ($^1O_2$) (Ullah y Wilson 1995). La activación física del oxígeno, esquematizada en la figura 4, con la consiguiente generación de las especies reactivas, se produce normalmente por la transferencia de energía de excitación desde un pigmento activado por irradiación solar o bien como resultado de la combustión de compuestos orgánicos durante la respiración celular (Perl-Treves y Aviahi 2002; Prasad 2004). Esta activación mediante reducción parcial está favorecida termodinámicamente, ya que la variación de energía libre de Gibbs tiene valor negativo para cada paso de incorporación de electrones, con la excepción de la incorporación de un primer electrón a la molécula de dioxígeno. Es así como se producen sucesivamente las diversas EROs: el radical superóxido ($O_2^{\bullet -}$), el peróxido de hidrógeno (H_2O_2), y el radical hidroxilo (HO•) (Halliwell y Gutteridge 1990; Ernst y cols. 1990; Prasad 2004).

El radical superóxido no es reactivo frente a la mayoría de las moléculas orgánicas y no es un oxidante a pH neutro. Es muy soluble en agua y por tanto no es transportado al interior de membranas ni penetra en las zonas hidrofóbicas de las células peroxidando lípidos. Sin embargo, un donador de electrones moderadamente eficiente lo puede reducir, como es el caso de los metales de transición, formándose peróxido de hidrógeno o agua oxigenada (H_2O_2). El H_2O_2 es tanto una EROs como una molécula de señalización celular. Es una molécula pequeña y estable, que no tiene carga, lo que le permite atravesar membranas y viajar libremente por el interior de las células, alcanzando distintos orgánulos y moléculas diana (Ullah y Wilson 1995). A su vez, el H_2O_2 puede seguir reduciéndose, dando lugar a la formación del radical hidroxilo (HO•). Este radical tiene un elevado poder oxidante que actúa quitando hidrógeno, hidroxilando o transfiriendo electrones, de cualquier molécula que se encuentre en su camino (Halliwell y Gutteridge 1990), dando lugar a otro radical, menos reactivo y con una vida media mayor. El HO• reacciona de forma no selectiva con cualquier molécula

que se encuentre cerca y por tanto no se desplaza. No hay ninguna molécula que lo neutralice y los mecanismos de defensa se basan en mantener bajas las concentraciones de sus precursores ($O_2^{\bullet -}$ y H_2O_2) así como de los metales que catalizan las reacciones de formación (Mano 2002).

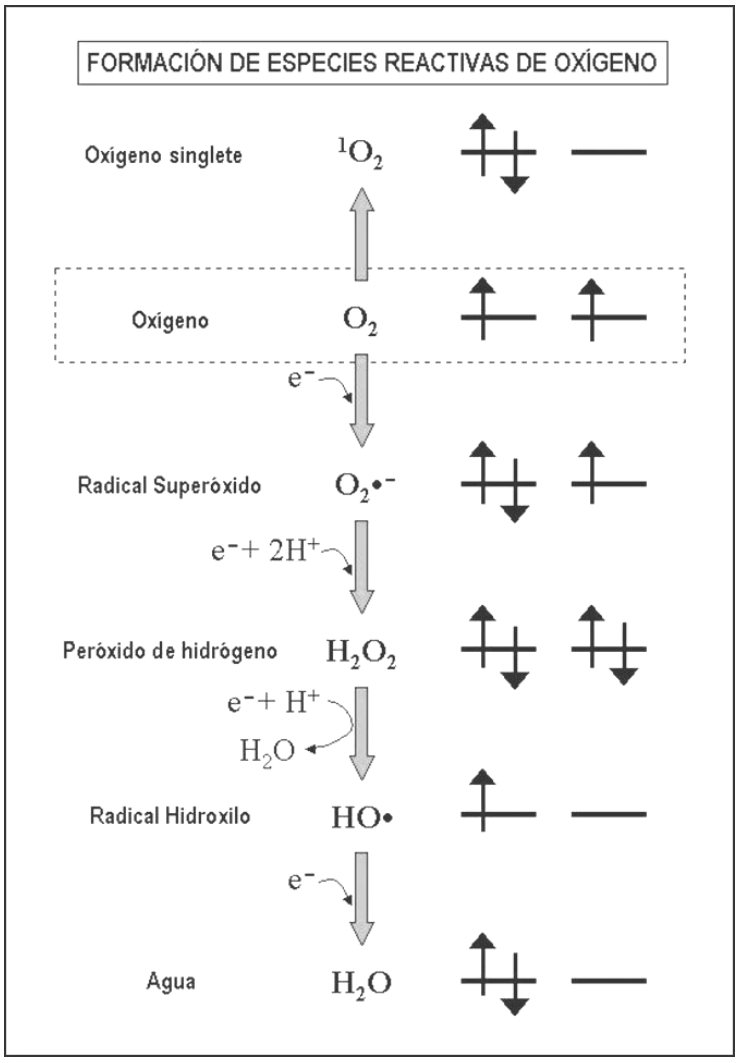


Figura 4. Esquema de los diferentes estados de oxidación del oxígeno. Se muestra en la columna de la derecha con una flecha, el spin que presentan los electrones de la última capa de cada uno de los radicales.

La reducción parcial del O_2 puede acelerarse mediante agentes catalíticos inorgánicos (sobre todo metales) y orgánicos, incluyendo una variedad de compuestos endógenos (enzimáticos y no enzimáticos). Por tanto, la contaminación por metales, así como otras muchas causas de estrés como la sequía, la congelación, la salinidad, el calor, la radiación ultravioleta, los contaminantes atmosféricos, el estrés mecánico, el déficit/exceso de nutrientes, el ataque de patógenos, pueden hacer que esta activación se eleve notablemente conduciendo a lo que se conoce con el nombre de **estrés oxidativo** (EO) (Mittler 2002).

Las EROs no son moléculas tóxicas por definición, ya que se generan siempre durante la fotosíntesis y las células han desarrollado mecanismos para metabolizarlas y mantenerlas a niveles no perjudiciales (Neill y cols. 2002), teniendo algunas de estas moléculas papel fisiológico o como segundos mensajeros (Inzé y Montagu 1995; Bohnert y Sheveleva 1998). Por ejemplo el H_2O_2 y el $^1O_2$ están implicados en la formación de lignina de las paredes celulares (Inzé y Montagu 1995), y además el H_2O_2 participa en la señalización celular en la defensa frente al ataque de patógenos (Ullah y Wilson 1995). Las EROs son tóxicas cuando su formación supera su tasa de eliminación por las células (Neill y cols. 2002). Este desequilibrio es el responsable del EO.

4. Metales y estrés oxidativo

Una vez definido el estrés oxidativo, y visto que los metales son uno de los agentes externos que pueden contribuir a aumentar la concentración de las EROs, se van a definir los principales mecanismos por los que los metales pueden inducir la formación de EROs como consecuencia de su actividad catalítica (Briat 2002).

Los metales de transición (poseen electrones desapareados) pueden actuar como catalizadores de la reducción del oxígeno, cediendo electrones y generando el radical superóxido, como se muestra en la reacción (1):

$$M^n + O_2 \rightarrow M^{n+1} + \mathbf{O_2}\bullet^- \quad (1)$$

En disolución acuosa a pH neutro, el radical superóxido puede generar peróxido de hidrógeno, el cual posteriormente se descompone produciendo el radical hidroxilo a través de la reacción de Haber-Weiss, la cual implica la presencia de un metal (M) como Cu o Fe, como se muestra en las reacciones (2 y 3):

$$M^{n+1} + O_2\bullet^- \rightarrow M^n + O_2 \quad (2)$$

$$M^n + H_2O_2 \rightarrow M^{n+1} + OH^- + OH\bullet \quad (3)$$

La suma de estas dos reacciones se suele resumir en (4), reacción denominada de Haber-Weiss y en el caso de que el metal implicado sea el Fe, la reacción pasa a denominarse reacción de Fenton (Briat 2002):

$$O_2\bullet^- + H_2O_2 \rightarrow \mathbf{HO}\bullet + HO^- + O_2 \quad (4)$$

En resumen, si las condiciones celulares son tales que existen concentraciones catalíticas de metales, además de oxígeno, se suceden una serie de reacciones que conducen a la formación del radical hidroxilo (OH•). Este radical actúa como un poderoso sumidero de protones, que obtendrá de cualquier molécula orgánica que tenga en su entorno, lo que explica sus efectos dañinos sobre la célula, ya que afecta a numerosas moléculas y estructuras celulares que resultan oxidadas en su presencia (Prasad 2004).

4.1 Daños en lípidos

Los daños en los lípidos causados por las EROs, conducen a la peroxidación lipídica y se producen como resultado de una cadena de reacciones que implican a los ácidos grasos poliinsaturados, y que da lugar a la desestabilización de las membranas celulares, pudiendo causar la muerte de la célula al afectar a una variedad de procesos de transporte, reconocimiento celular, señalización celular, etc.

La peroxidación lipídica se inicia cuando el radical OH• toma un protón de un ácido graso (RH), dando lugar a un radical alquilo (R•). Este radical tiende a estabilizarse mediante ajustes internos de la distribución de electrones de la molécula afectada, y mediante su combinación con oxígeno, lo que genera un radical hidroperóxido (ROO•), que provoca la propagación del ataque oxidativo, pues toma un protón de un ácido graso adyacente, produciendo un nuevo radical alquilo e hidroperóxido, y así sucesivamente. Estas reacciones en cadena hacen que la membrana afectada pierda estabilidad. En presencia de metales de transición, se producen reacciones tipo Fenton, generando nuevos radicales alquilo, con lo cual se agrava la situación. Las reacciones de peroxidación lipídica se pueden detener si la célula posee sistemas antioxidantes que impidan la reacción de iniciación o las de propagación (Figura 5) (Briat 2002; Blokhina y cols. 2003; Shaw y cols. 2004).

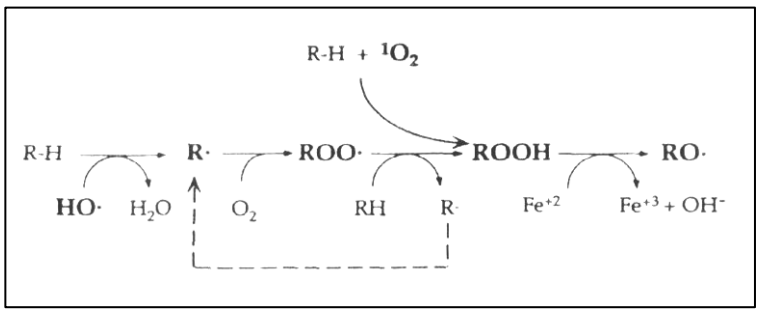


Figura 5. Esquema del proceso de peroxidación lipídica.

Uno de los productos finales de esta cadena de reacciones es el malondialdehido (MDA), compuesto que se forma por la oxidación de los ácidos grasos poliinsaturados en las células. Su concentración intracelular puede medirse fácilmente, siendo indicativa del grado de estrés oxidativo que padecen las células (Hodges y cols.1999).

4.2 Daños a proteínas

Las proteínas también pueden ser atacadas por las EROs, siendo el radical OH• la molécula que inicia las reacciones de oxidación. La oxidación de los aminoácidos (especialmente histidina, cisteína, tirosina y triptófano) afecta a las estructuras primaria, secundaria y terciaria de la proteína, provocando uniones internas entre cadenas peptídicas o roturas de las mismas, con la consiguiente pérdida de su actividad funcional, sea catalítica o estructural (Berlett y Stadman. 1997; Foyer y Noctor 2005).

La concentración intracelular de proteínas oxidadas, o productos generados de la oxidación, refleja el balance que existe entre la oxidación de proteínas y la degradación de éstas. Este balance depende de un gran número de factores, entre los que destacan la producción de EROs y la actividad de proteasas encargadas de la degradación. Algunas formas oxidadas de proteínas no sólo son resistentes a la actividad proteolítica de las proteasas, sino que también pueden inhibir a estas enzimas, impidiendo que desempeñen su función (Berlet y Stadman 1997) y agravando los daños producidos.

4.3 Daños al ADN

El radical hidroxilo puede alcanzar al ADN genómico o mitocondrial, con el que reacciona oxidándolo mediante la captación de protones de las bases nitrogenadas. Una vez que se oxidan estas bases, se pueden producir una variedad de alteraciones de carácter mutagénico por pérdida o deleción de fragmentos de ADN, formación de enlaces cruzados ADN/ADN, ADN/proteínas, etc. (Briat y Lebrun 1999; Briat 2002).

Además de estos daños que afectan directamente al ADN, la alteración por oxidación de una variedad de proteínas que catalizan las diversas fases de la expresión génica, dará lugar a daños epigenéticos que interferirán con numerosos procesos celulares. La consecuencia final de un estrés oxidativo prolongado será la muerte de la célula y, en el peor de los casos, la muerte del individuo afectado (Briat 2002).

5. Defensa de las plantas frente al estrés oxidativo

Las plantas, como todos los seres vivos, han desarrollado una variedad de mecanismos para defenderse del estrés oxidativo producido por las EROs. Estos mecanismos incluyen tanto sistemas enzimáticos como no enzimáticos que previenen la formación de EROs o impiden la propagación de las reacciones de oxidación, originadas por éstas, mediante la reducción de intermediarios oxidados (Clijsters y cols. 1999).

En general, los mecanismos de defensa de las plantas se basan en 3 tipos:

(1) La presencia de agentes reductores que interactúan directamente con las especies oxidadas (sean EROs o compuestos orgánicos), siendo los más

importantes el ácido ascórbico y el glutatión, aunque existen otras muchas moléculas reductoras que actúan como antioxidantes (vitaminas lipofílicas como tocoferol o coenzima Q; compuestos volátiles como terpenos, arenos y ésteres; polifenoles, antocianinas, flavonoides, carotenos; ácidos grasos poliinsaturados, etc.) (Blokhina y cols. 2003).

(2) La actividad de sistemas enzimáticos que catalizan la eliminación de EROs o la reducción de radicales de moléculas orgánicas, como las superóxido dismutasas, catalasas y peroxidasas (Clijsters y cols. 1999).

(3) El mantenimiento de un estado redox adecuado en la célula, vía la generación de piridín-nucleótidos reducidos originados por oxidación de compuestos orgánicos en reacciones catalizadas por la enzima málica, glucosa-6-fosfato deshidrogenasa, isocitrato deshidrogenasa o la glutamato deshidrogenasa. (Clijsters y cols. 1999).

Los mecanismos de protección frente al ataque oxidativo tienen una doble vertiente, pudiéndose clasificar como primarios y secundarios. Los mecanismos primarios interrumpen las reacciones en cadena de oxidación de compuestos orgánicos, generando formas menos activas; los mecanismos secundarios tienen carácter preventivo ya que eliminan las EROs directamente o impiden su formación (por ejemplo, un quelante de metales impediría que éstos catalizaran la formación de EROs).

6. Sistemas de defensa no enzimáticos en plantas

En las plantas, el papel del ácido ascórbico y del glutatión es especialmente relevante para la prevención de un posible estrés oxidativo derivado de la generación de EROs, aunque ambas moléculas tienen carácter multifuncional en

las células vegetales (regulando el ciclo celular, equilibrando el potencial redox, etc.). Desde el punto de vista de la defensa frente al estrés oxidativo, cabe subrayar que tanto el ácido ascórbico como el glutatión pueden presentarse en forma reducida o en forma oxidada. Las formas reducidas interaccionan directamente con las EROs o indirectamente, siendo sustratos de enzimas reductoras. Las formas reducidas predominan sobre las oxidadas en una planta que presenta un buen estado de salud, aunque esta proporción puede invertirse ante un ataque oxidativo. En situación de estrés oxidativo, donde el consumo de ácido ascórbico y glutatión reducidos es muy elevado, la planta puede adoptar dos sistemas para mantener unos niveles adecuados de ambos compuestos en estado reducido: su re-reducción a partir de sus formas oxidadas, o la síntesis *de novo* a partir de sus precursores.

6.1 Ácido ascórbico

El ácido L-ascórbico o vitamina C (AsA) es uno de los antioxidantes solubles clave en la defensa de las células frente al estrés oxidativo (Smirnoff 1996; 1998; 2000; Horemans y cols. 2000; Stadman 1991; Foyer 1993) y además juega un papel clave en la división y elongación celular (Córdoba y González-Reyes 1994; Horemans y cols. 2000).

El AsA tiene una complicada ruta de síntesis que implica a varios compartimentos celulares y a numerosas enzimas y metabolitos intermediarios, aunque la última fase de su síntesis se realiza en la membrana interna mitocondrial (Foyer 1993; Smirnoff 1996; 1998; 2000; Siendones y cols. 1999; Horemans y cols. 2000; Conklin 2001).

El AsA en los tejidos vegetales se encuentra en concentraciones de rango milimolar, aunque la concentración depende del tejido que se analice (Smirnoff 1996; Foyer y cols. 1991). Se encuentra en todos los orgánulos celulares,

incluyendo el apoplasto (Smirnoff 2000), pero son los tejidos fotosintéticos y los jóvenes los que presentan concentraciones superiores (Horemans y cols. 2000). Las reservas de AsA oxidado (DHA) aumentan cuando se da la combinación de aumento de la oxidación y una regeneración insuficiente (Conklin 2001). Esta molécula puede actuar como antioxidante reaccionando de forma no enzimática con el peróxido de hidrógeno, reduciéndolo a agua y oxidándose a monohidroascorbato (MDHA) o la reacción puede ser catalizada por la enzima ascorbato peroxidasa, tanto en los cloroplastos como en el citosol (Foyer 1993):

$$H_2O_2 + 2\ AaA \rightarrow 2H_2O + 2\ MDHA$$

El MDHA formado puede seguir oxidándose hasta DHA. Tanto el MDHA como el DHA en disolución acuosa son moléculas inestables, que pueden ser reducidos enzimáticamente de nuevo a AsA o si esto no sucede, el DHA, espontáneamente, se transforma en ácido dicetogulónico, a través de una reacción que es irreversible. En las células existe un ciclo de reducción de las formas oxidadas, que minimiza la síntesis *de novo* del AsA (Horemans y cols. 2000).

La concentración de AsA representa un reservorio del poder antioxidante y además esta molécula está implicada en los ciclos de otras moléculas antioxidantes, como el ciclo de las xantofilas, contribuyendo a mantener reducidos los antioxidantes de membrana (Horemans y cols. 2000) o como el α-tocoferol o la zeaxantina (Foyer 1993). La concentración de AsA en forma reducida es del 90 % bajo condiciones normales del metabolismo de las plantas (Smirnoff 1996; Foyer 1993).

6.2 Glutatión

El glutatión (GSH) es un tripéptido cuya síntesis requiere de la actividad catalítica de sólo dos enzimas: la γ-glutamilcisteina sintetasa y la glutatión sintetasa (May y cols. 1998; Noctor y cols. 2002; Foyer y Noctor 2005) y está formado por los aminoácidos glutamato, cisteína y glicina. (May y cols. 1998). Es la forma más abundante de azufre orgánico en las plantas, después del incorporado a las proteínas (Dixon y cols. 1998).

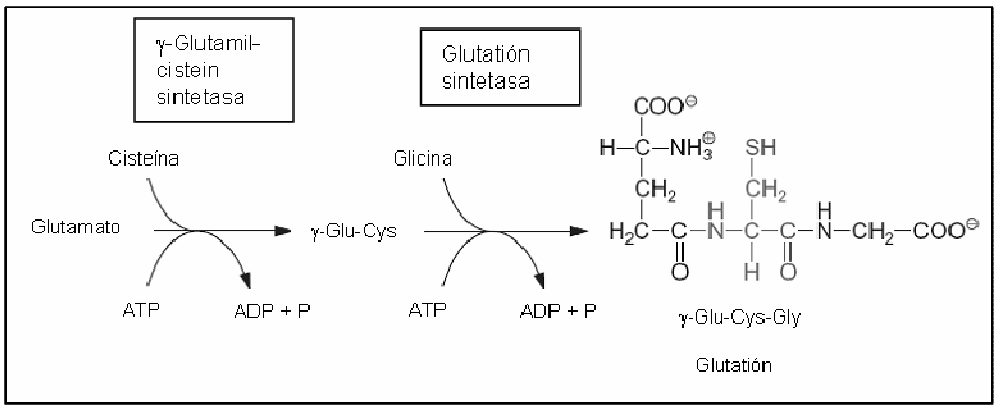


Figura 6. Esquema de la síntesis de glutatión (adaptada de Heldt 2004).

El GSH es un metabolito que desempeña múltiples funciones en las células, entre ellas defensa y protección. Es capaz de reaccionar con las EROs, y su función principal en este sentido es intervenir en el ciclo del ascorbato/glutatión para eliminar H_2O_2 y proteger a las proteínas frente a la desnaturalización provocada por la oxidación de los grupos tioles bajo condiciones de estrés (Noctor y cols. 1998). Todas estas reacciones conllevan la oxidación del grupo tiol, formando glutatión disulfuro o glutatión oxidado (GSSG). La proporción GSH/GSSG se mantiene por medio de la actividad glutatión reductasa (GR), que reduce al GSSG a GSH utilizando NADPH para llevar a cabo la reducción. La relación entre GSH/GSSG es un indicador del estado redox de la célula (Foyer y

Noctor 2005). Estos dos compuestos, bajo condiciones normales, se suelen encontrar en la proporción 100:1 (GSH/GSSG) (Maughan y Foyer 2006). La síntesis de GSH se activa cuando en el citosol existen condiciones de oxidación (Foyer y Noctor 2005).

6.3 Fitoquelatinas

Una de las defensas que las plantas tienen frente a la presencia de metales es la unión de éstos con moléculas orgánicas que ellas sintetizan y así evitar que queden libres en el citoplasma. Uno de los principales grupos de moléculas quelantes de metales en las plantas se denominan fitoquelatinas.

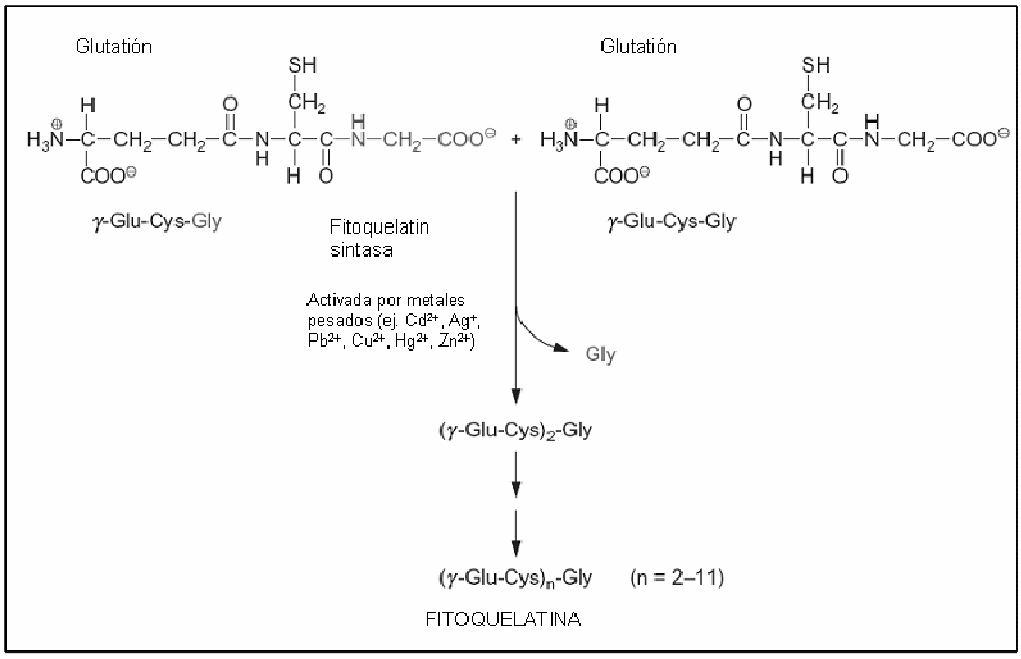


Figura 7. Esquema de la síntesis de fitoquelatinas (adaptada de Heldt 2004).

Las fitoquelatinas son pequeños péptidos ricos en cisteína, con la estructura general $(\gamma\text{-Glu-Cys})_n(\text{Gly})$, siendo n 2 a 11 (Leopold y cols. 1999; Cobbett 2000a; Briat 2002). Además de esta estructura general, se han descrito moléculas con estructura similar, cambiando la glicina por ß-alanina, serina o glutamina (Rauser 1995; Cobbett 2000b). Como puede observarse, son compuestos derivados del glutatión (Kneer y Zenk 1997) (Figura 7).

Las fitoquelatinas son metabolitos secundarios que no se sintetizan a partir de ARN mensajero, sino a través de la reacción catalizada por la enzima γ-glutamilcistein dipeptidil transpeptidasa (Turner 1994). Esta enzima se activa en presencia de metales como Cd, Ag, Pb, Cu, Hg, Zn, Sn, As (Schat y cols. 2002; Scarano y Morelli 2003; Landberg y Greger 2004). La actividad de esta enzima se ha detectado en casi todas las raíces de plantas, pero no siempre en tallos y hojas. Este hecho apoya la importancia que las fitoquelatinas tienen en la defensa frente a los metales pesados, pues la mayoría de ellos son absorbidos desde el suelo, siendo la raíz la primera barrera de defensa (Prasad 2004). Sin embargo, hay que tener en cuenta que los estudios realizados muestran que a pesar de que casi todos los metales inducen la síntesis de fitoquelatinas, sólo algunos forman complejos con éstas (Prasad 2004). Estos complejos se forman por unión con la cisteína, reduciendo de esta forma la concentración libre de metales (Scarano y Morelli 2003).

6.4 Compuestos fenólicos

Los fenoles son un conjunto de metabolitos secundarios sintetizados por las plantas tanto durante el desarrollo normal de éstas, como en respuesta a situaciones de estrés (Naczk y Shahidi 2006). Son un amplio conjunto de compuestos que se caracterizan por tener al menos un anillo aromático (C6) con uno o más grupos hidroxilos (Michalak 2006; Jung 2003). Se sintetizan principalmente a partir del

ácido cinámico, por acción de la enzima L-feniloalanina amonia-liasa (PAL), una enzima clave para el metabolismo primario y secundario (Dixon y Pavia 1995). Los fenoles se dividen en distintos grupos según el número de átomos de carbono que lo forman (Michalak 2006) y modificaciones en su estructura básica por oxidaciones, hidroxilaciones, glicosilaciones y metilaciones han dado lugar a un amplio grupo de compuestos con distintas funciones biológicas (Santiago y cols. 2000). La distribución de los fenoles en los tejidos vegetales, a nivel celular y subcelular no es uniforme (Naczk y Shahidi 2006).

Se ha observado que los fenoles tienen un papel en la defensa frente a los metales pesados, ya que su síntesis aumenta en plantas expuestas a concentraciones tóxicas de metales (Michalak 2006). La acción antioxidante de los compuestos fenólicos se deriva de sus propiedades redox, pudiendo actuar como donadores de electrones, como agentes reductores eliminando radicales libres (Rice-Evans y cols. 1997; Jung y cols. 2003; Grubešic y cols. 2005; Pourmorad y cols. 2006); además tienen una gran tendencia a quelar metales a través de los grupos hidroxilo y carboxilo, formando complejos estables con algunos de los metales tóxicos para las plantas, como Ni, Cu, Co, Fe, etc. (Jung y cols. 2003; Michalak 2006), evitando así la formación de radicales libres por acción de los metales. Sin embargo también se ha demostrado la actividad prooxidante de los fenoles en presencia de metales de transición como Fe o Cu (Sakihama y cols. 2002).

7. Sistemas de defensa enzimáticos en plantas

Una posible clasificación de los sistemas enzimáticos que presentan las células vegetales se muestra a continuación (Clijsters y cols. 1999):

Enzimas que metabolizan EROs	Peroxidasas (POD) Superóxido dismutasas (SOD) Catalasa (CAT)
Enzimas del ciclo ascorbato-glutatión	Ascorbato peroxidasa (APX) Monodehidroascorbato reductasa (MDHR) Dehidroascorbato reductasa (DHR) Glutatión reductasa (GR) Enzima málica (ME)
Enzimas reductoras de NAD(P)+	Glucosa-6-fosfato deshidrogenasa (G6PDH) Isocitrato deshidrogenasa (ICDH) Glutamato deshidrogenasa (GDH)

7.1 Superóxido dismutasa (SOD)

La enzima superóxido dismutasa (EC 1.15.1.1) está presente en casi todos los organismos aerobios. Es una de las enzimas clave en los mecanismos de defensa frente al estrés oxidativo (Van Breusegem y cols. 2002) ya que actúa sobre los radicales superóxido ($O_2^{\bullet -}$), como se muestra en la siguiente reacción (Rio y cols. 2002; Scandalios 1993):

$$2\ O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Esta enzima hace que la reacción sea 10.000 veces más rápida que la dismutación espontánea (Bowler y cols. 1992) y requiere de la presencia de un cofactor metálico (Inzé y Montagu, 1995). Se clasifica según el cofactor sea cobre o zinc, hierro y manganeso (Cu/ZnSOD, FeSOD y MnSOD, respectivamente). Normalmente, la MnSOD se localiza en las mitocondrias de todas las células eucariotas, mientras que las Cu/ZnSOD se han encontrado en el citosol, peroxisomas y cloroplastos de las plantas superiores (Van Breusegem y cols. 2002).

7.2 Catalasa (CAT)

La enzima catalasa (EC 1.11.1.6) tiene como sustrato el H_2O_2 (Inzé y Montagu 1995; Mittler 2002; Van Breusegem y cols. 2002). Tiene una alta capacidad de reacción, pero muy poca afinidad por el H_2O_2 (Willekens y cols. 1997; Smirnoff 1998). Esta baja afinidad por el sustrato hace pensar que su papel principal sea la eliminación de H_2O_2 cuando está a elevadas concentraciones bajo condiciones de estrés (Mittler 2002; Smirnoff 1998). A diferencia de otras peroxidasas, la catalasa no requiere de un sustrato reductor para poder actuar (Inzé y Montagu 1995). Se localiza en los peroxisomas, glioxisomas y mitocondrias (Mittler 2002; Inzé y Montagu 1995; Asada 1992; Van Breusegem y cols. 2002). Los peroxisomas son orgánulos con diversas funciones en las células de las plantas y hongos, como la fotorrespiracion, la oxidación de los lípidos, el ciclo del glicoxilato y el metabolismo de las especies reactivas del oxígeno entre otras. Por ello el importante papel que tiene la catalasa en la eliminación de EROs en estos orgánulos (Rio y cols. 2002). Se han descrito tres variedades de esta enzima, con diferentes funciones. La *cat1*, encargada de eliminar el H_2O_2 producido en la fotorrespiracion, la *cat2* tiene la función de eliminar el H_2O_2 que se forma durante el estrés oxidativo y la *cat3* que elimina el H_2O_2 generado durante la oxidación de los ácidos grasos (Inzé y Montagu 1995).

7.3 Guaiacol peroxidasa (POD)

Se denomina guaiacol peroxidasa (EC 1.11.1.7) a un conjunto de enzimas presentes en el citosol, la vacuola y la pared celular en las células vegetales que, al igual que la catalasa y la ascorbato peroxidasa, tienen como sustrato al H_2O_2, al cual reducen hasta agua (Asada 1992; Mehlhorn y cols. 1996). Tanto esta enzima como la ascorbato peroxidasa (que se describe a continuación) son inducidas por la presencia de metales pesados (Clijsters y cols. 1999).

7.4 Ascorbato peroxidasa (APX)

La ascorbato peroxidasa (EC 1.11.1.11) es una enzima que tiene como sustrato el H_2O_2, pero tiene una afinidad mayor que la catalasa, sugiriendo que el papel de esta enzima es regular el H_2O_2 cuando las concentraciones no son demasiado elevadas, como sucede en el metabolismo normal de las células, cuando no existe ningún tipo de estrés. (Mittler 2002; Smirnoff 1998). Se localiza en los cloroplastos, peroxisomas, citosol y mitocondrias (Conklin 2001). La ascorbato peroxidasa reduce el agua oxigenada a agua, usando como agente reductor el ascorbato (AsA), oxidándolo a monodehidroascorbato (MDHA) (Smirnoff 2000), catalizando la siguiente reacción:

$$H_2O_2 + AsA \rightarrow H_2O + MDHA$$

7.5 Glutatión reductasa (GR)

La glutatión reductasa (EC 1.6.4.2) es una enzima que actúa reduciendo el glutatión disulfuro (GSSG) a glutatión (GSH) y necesita de la presencia de NADPH como agente reductor. Tiene un importante papel en la regeneración del glutatión, y es también parte del ciclo de Halliwell-Asada. En este ciclo, el glutatión se oxida para regenerar el ascorbato, como muestra la reacción:

$$GSSG + NADPH \xrightarrow[GR]{} GSH + NADP^+$$

Es la última enzima implicada en el ciclo de Halliwell-Asada, además de jugar un importante papel en numerosos procesos celulares (Inzé y Montagu 1995). Esta enzima se localiza en las plantas superiores en los plástidos y en el citosol. (Creissen y Mullineaux 2002).

7.6 Ciclo de Halliwell-Asada

Como se ha ido exponiendo anteriormente, el reciclado de las formas reducidas del ácido ascórbico y del glutatión a partir de sus formas oxidadas les obliga a depender mutuamente el uno del otro. Esto se manifiesta claramente cuando se analiza el **ciclo de Halliwell-Asada**, donde el ácido ascórbico oxidado (DHA) es reducido con electrones que proceden del GSH mediante la actividad catalítica de la DHAR (Foyer 1993; Foyer y cols. 1994). El glutatión se regenera, tomando electrones cedidos por el NADPH, a través de la actividad glutatión reductasa. Junto a este ciclo, en la figura 8 se muestra la interacción de una variedad de sistemas defensivos ante la presencia de las EROs (Asada 1992; Scandalios 1993; Inzé y Montagu 1995; Smirnoff 1998; Clijsters y cols. 1999; Smirnoff 2000; Conklin 2001; Mittler 2002).

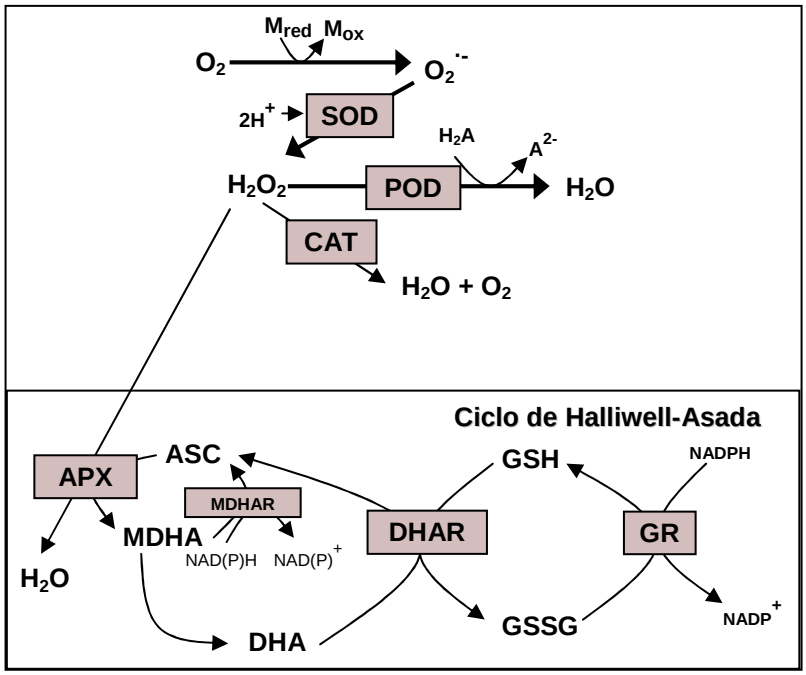


Figura 8. Esquema de algunas de las enzimas implicadas en la defensa frente a las EROs. SOD superóxido dismutasa; PER peroxidasas; CAT catalasa; APX ascorbato peroxidasa; MDHAR monodehidroascorbato reductasa; DHAR dehidroascorbato reductasa; GR glutatión reductasa; ASC ascorbato reducido; MDHA monodehidroascorbato; DHA dehidroascorbato; GSH glutatión reducido; GSSG glutatión oxidado.

8. La faja pirítica, un ambiente extremo en la provincia de Huelva

La provincia de Huelva está situada al sur de la Península Ibérica, junto a la frontera con Portugal. En esta provincia se encuentra la comarca del Andévalo entre la Sierra de Aracena al norte y la costa al sur. Esta comarca se sitúa sobre la faja pirítica (Figura 9), uno de los mayores depósitos de sulfuros y manganeso del oeste de Europa (Montes-Botella y Tenorio 2003). La faja pirítica tiene una extensión de unos 200 km, desde las montañas de Caveira (en el sur de Portugal) hasta Aznalcóllar (Sevilla) (Davis y cols. 2000; Montes-Botella y Tenorio 2003). De norte a sur tiene una anchura de unos 30-40 km (Davis y cols. 2000).

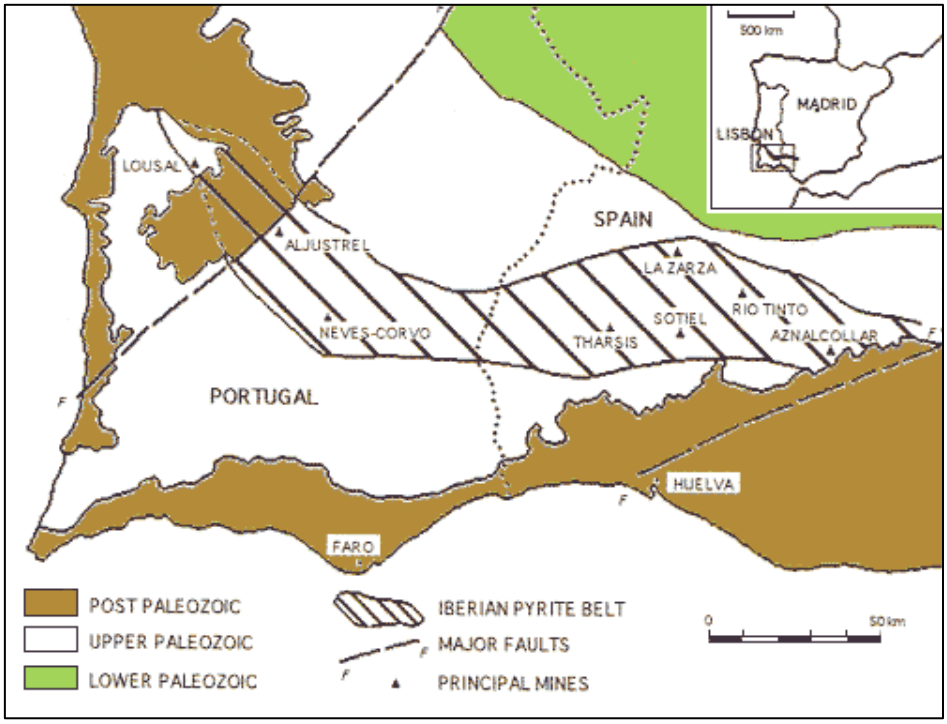


Figura 9. Esquema de la Faja Pirítica (tomada de Real 1999).

El origen de estos ricos yacimientos minerales data del Carbonífero (Moreno 1993), cuando se produjo una mineralización de tipo hidrotermal en los fondos marinos durante un periodo de intensa actividad volcánica, lo que provocó

la acumulación de depósitos masivos muy ricos en Fe, Cu, Zn, As, Pb, Ag y Au (Davis y cols. 2000).

Debido a la riqueza en metales en el suelo de la faja pirítica, la actividad minera se remonta a hace más de 5000 años, datándose sus inicios con las civilizaciones de Iberos y Tartesos que vivieron en el lugar (Davis y cols. 2000). La extracción de metales del suelo se hizo tanto en minas a cielo abierto como en galerías (Davis y cols. 2000), y los metales que desde el principio se buscaban y extraían eran cobre, plata y oro (Hudson-Edwards y cols. 1999). La primera mina está datada sobre el año 3000 a.C. y se situaba en lo que hoy es el pueblo de Nerva (Davis y cols. 2000). Civilizaciones posteriores también sacaron provecho de la riqueza del suelo y Fenicios y Romanos siguieron explotando las minas y haciendo nuevas explotaciones (Davis y cols. 2000). Esta intensa actividad minera ha seguido hasta nuestros días, clausurándose la última mina, en Riotinto, en el año 2002.

Las minas más importantes en los siglos XIX y XX han sido las de Riotinto, Tharsis y Sotiel-Coronada (Montes-Botella y Tenorio 2003).

9. El drenaje ácido de minas

El drenaje ácido de minas (AMD, son las siglas inglesas) es uno de los mayores problemas ambientales asociados a las minas de sulfuros. Los sulfuros, bajo condiciones reductoras son estables y muy insolubles. De forma natural, la pirita y otros minerales asociados están enterrados en condiciones de anoxia y solo una pequeña parte está expuesta al exterior. La oxidación de los sulfuros tiene lugar cuando los minerales son expuestos a las condiciones atmosféricas generando acidez, sulfatos y liberando hierro y otros metales y metaloides presentes en el mineral original. La reacción global que controla este proceso es la que se expone

en la reacción (1) (Nordstrom y Alpers 1999; Cánovas y cols. 2007; Nieto y cols. 2007):

$$FeS_{2(s)} + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad (1)$$

En presencia de oxígeno, los iones ferrosos reaccionan, dando lugar al ión férrico, reacción (2):

$$Fe^{2+} + ¼\ O_{2\,(aq)} + H^+ \rightarrow Fe^{3+} + ½\ H_2O \qquad (2)$$

El ión férrico puede reaccionar oxidando una nueva pirita (3) o precipitar en forma de hidróxido ($Fe(OH)_3$) (4):

$$FeS_{2(s)} + 14\ Fe^{3+} + 8H_2O \rightarrow 15\ Fe^{2+} + 2SO_4^{=} + 16\ H^+ \qquad (3)$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_{3(S)} + 3H^+ \qquad (4)$$

Este conjunto de reacciones generan acidez (H^+) y liberan sulfatos ($SO_4^{=}$), Fe^{2+} y metales y metaloides que se encuentran en este tipo de depósitos como As, Cd, Co, Cu, Zn, Ni, Pb. Además, el ácido generado reacciona con los silicatos cercanos liberando también numerosos iones (Al, Ca, Mg, Mn, Si) (Cánovas y cols. 2007). Este proceso de oxidación química también es catalizado por bacterias que oxidan sulfuros, acelerando la reacción (2) (Lopez-Archilla y Amils 1999) (se comenta posteriormente).

La explotación minera en la provincia de Huelva cesó en el año 2002, con el cierre de Riotinto, después de años y años de explotación. Sin embargo, las escombreras siguen estando en contacto con el aire y el agua de la lluvia, manteniéndose las reacciones de oxidación y el AMD (Cánovas y cols. 2007). Recientemente, en noviembre de 2007, la mina de Aguas Teñidas, en el término municipal de Almonaster la Real, ha sido reabierta (Prensa local). Esta nueva explotación tiene en proyecto una total limpieza de los efluentes generados, lo que en principio no debería suponer un gran impacto a las aguas de la zona (comunicación personal), y no cabe esperar aumento de depósitos mineros ni AMD como consecuencia de dicha actividad.

10. Tinto y Odiel, ríos que surcan la faja pirítica en la provincia de Huelva

La provincia de Huelva es surcada de norte a sur por dos ríos, el Tinto y el Odiel, atravesando la faja pirítica y desembocando en la Ría de Huelva al océano Atlántico (Fig. 10).

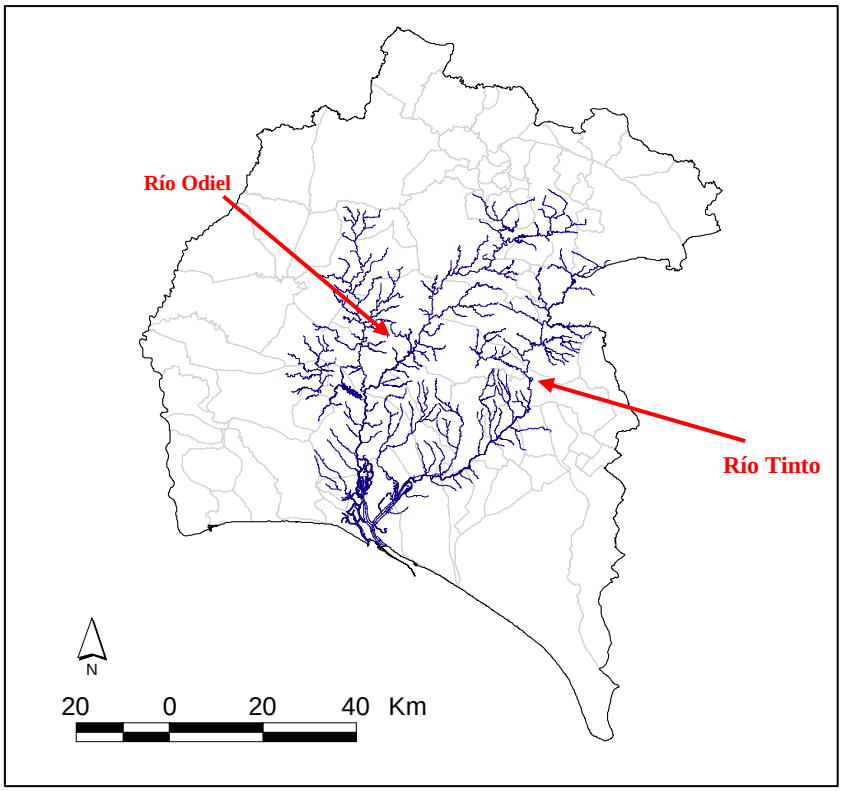


Figura 10. Provincia de Huelva con las cuencas de los ríos Odiel y Tinto.

El río Odiel nace en el Parque Natural de Sierra de Aracena y Picos de Aroche. Su longitud es de 128 km – 140 km (variación debida a que la desembocadura sea contabilizada o no) (López-Archilla y Amils 1999; Montes-Botella y Tenorio 2003; Sainz y cols. 2003), con un caudal medio de 460 hm^3/año, aunque con variaciones anuales (Sainz y cols. 2003), y una cuenca de unos 2300 km^2 (López-Archilla y Amils 1999; Sánchez-España y cols. 2005). Este río

discurre por la provincia de Huelva de norte a sur, y en su recorrido recibe afluentes ácidos y cargados en metales pesados procedentes de la faja pirítica (Sánchez-España y cols. 2005). Como consecuencia de esto, es un río muy ácido, con valores de pH en el rango de 2,76 a 3,71 en algunos de sus tramos (Montes-Botella y Tenorio 2003).

El río Tinto nace en Peña del Hierro (López-Archilla y cols. 2001; Cánovas y cols. 2008), en el término municipal de Nerva, ya dentro de la faja pirítica. Tiene una longitud de 92 km y una cuenca de 1676 km^2 (López-Archilla y Amils 1999). Las aguas de este río, rojas y ácidas desde su nacimiento (Fig. 11), generan un ambiente extremo caracterizado por su pH extremadamente bajo, con un valor de 2,2 de promedio, y una alta concentración de metales pesados en suspensión (Fe 2,3 g/L; Zn 0,22 g/L; Cu 0,11 g/L). Estas condiciones son el resultado de reacciones químicas y de la actividad metabólica de microorganismos quimiolitotróficos. La cadena alimenticia de este río es sólo microbiana (López-Archilla y cols. 2001).

Figura 11. Imagen del Río Tinto cerca de su nacimiento.

Tanto el Tinto como el Odiel son dos ríos contaminados, aunque dicha contaminación tiene un origen diferente. La contaminación del río Tinto es, en gran medida de origen natural, consecuencia de los procesos geológicos naturales que se

dan en la cabecera del río. El agua de lluvia hace que los sulfuros piríticos del suelo se transformen en óxidos de hierro, que a su vez se enriquecen en metales nobles como oro y plata; este proceso origina un mineral que es conocido como Gossan, y es el responsable de la contaminación natural del río, ya que genera biolixiviados que son ricos en ácido sulfúrico y en trazas de metales pesados, como se expuso en el apartado anterior. A esta contaminación natural que tiene el río desde su nacimiento hay que añadir la contaminación debida a la acción humana, ya que la minería, y el acumulo de materiales en el exterior, formando escombreras, ha provocado que el agua se ponga en contacto con más cantidad de mineral. Por otro lado, la contaminación del río Odiel es de origen predominantemente antrópico, debido a que la actividad minera ha hecho que se ponga en contacto gran cantidad de mineral con el agua de lluvia, facilitando la actividad lixiviadora de una variedad de bacterias y, en consecuencia, el conocido fenómeno denominado "drenaje ácido de minas" (Evangelou y Zhang 1995; Edwards y cols. 2000; Green y cols. 2003; Sainz y cols. 2003; Grande y cols. 2005; Johnson y Hallberg 2005; Ganne y cols. 2006; Saria y cols. 2006; Sánchez España y cols. 2005; Nieto y cols. y cols. 2007; Cánovas y cols. 2007). Las aguas se canalizan en el río Odiel, que va acumulando y transportando materiales muy contaminantes de origen minero. De hecho, el primer tramo del cauce de este río es agua limpia, sin acidez ni metales, comenzando la contaminación tras atravesar las zonas mineras. Aunque en la actualidad ya no se encuentra ninguna explotación minera activa, en las escombreras el agua de lluvia sigue arrastrando minerales, incrementando la acidez de los ríos de la zona (Informe de Medio Ambiente, 1996).

Las condiciones extremas hacían pensar que la vida en las aguas del Tinto sería imposible pero, sin embargo, diversos estudios realizados han desvelado la existencia de microorganismos extremófilos, quimiolitotróficos, capaces de soportar las condiciones de pH extremo (de hecho generan esas condiciones). En este hábitat inhóspito se desarrollan bacterias extremófilas como (*Acidi*)*Thiobacillus ferrooxidans*, (*Acidi*)*Thiobacillus thiooxidans* o *Leptospirillum ferrooxidans* (López-Archilla y Amils 1999).

El río Odiel, como anteriormente se dijo, posee una parte no contaminada en su nacimiento, y allí, las comunidades de algas y bacterias presentes son similares a las que suelen aparecer en otros ríos (cianobacterias, algas pertenecientes al grupo de las Clorofitas como *Spirogyra* sp., *Cosmerium* sp., *Gongrosira* sp., *Scenedesmus* sp. y Baciliarofitas, como *Fragilaria* sp.). Una vez que el río se vuelve ácido, estas comunidades desaparecen, siendo sustituidas por las comunidades de bacterias extremófilas citadas anteriormente (López-Archilla y Amils 1999). En la parte ácida de ambos ríos también aparecen algas verdes filamentosas que no se encuentran en las zonas en las que los ríos son neutros (o en ríos no contaminados de la zona) (López-Archilla y Amils 1999). Las bacterias extremófilas, mediante su metabolismo, favorecen el incremento de la acidez, catalizando la reacción (2) anteriormente citada en el proceso de disolución de la pirita:

$$FeS_2 + 15/4\ O_2 + 7/2\ H_2O \rightarrow Fe(OH)_3 + 2H_2SO_4$$

Pero no sólo microorganismos son capaces de adaptarse a dichas condiciones, también organismos pluricelulares sobreviven aquí, como es el caso de *E. andevalensis*, un brezo endémico de la provincia de la Faja Pirítica Ibérica.

11. *Erica andevalensis*, un ejemplo singular de una planta adaptada a condiciones extremas. Antecedentes al estudio de esta especie

E. andevalensis Cabezudo y Rivera (Fig. 12 y 13) es un brezo que aparece en las orillas de los ríos Tinto y Odiel contaminados por metales pesados y con extrema acidez, descritos en la sección anterior, así como en las escombreras de las minas de la provincia de Huelva. En estos hábitats, el resto de especies no son capaces de establecerse, o si lo consiguen presentan un crecimiento muy lento, pudiéndose observar algunos ejemplares de corte raquítico.

E. andevalensis fue descrita por primera vez en 1980 por Cabezudo y Rivera. Sin embargo, la primera vez que se tuvo constancia de la existencia de la especie fue en 1974, cuando se recolectó en la zona minera del Andévalo una planta que no estaba descrita en la provincia de Huelva y que presentaba características que la hacían estar próxima tanto a *E. tetralix* L. como a *E. mackaiana* L. aunque también poseía características que permitían diferenciarla de éstas dos (Cabezudo y Rivera 1980). Unos años después la especie fue descrita en la zona portuguesa (Capelo y cols. 1998).

Figura 12. Detalle de las flores de *E. andevalensis*

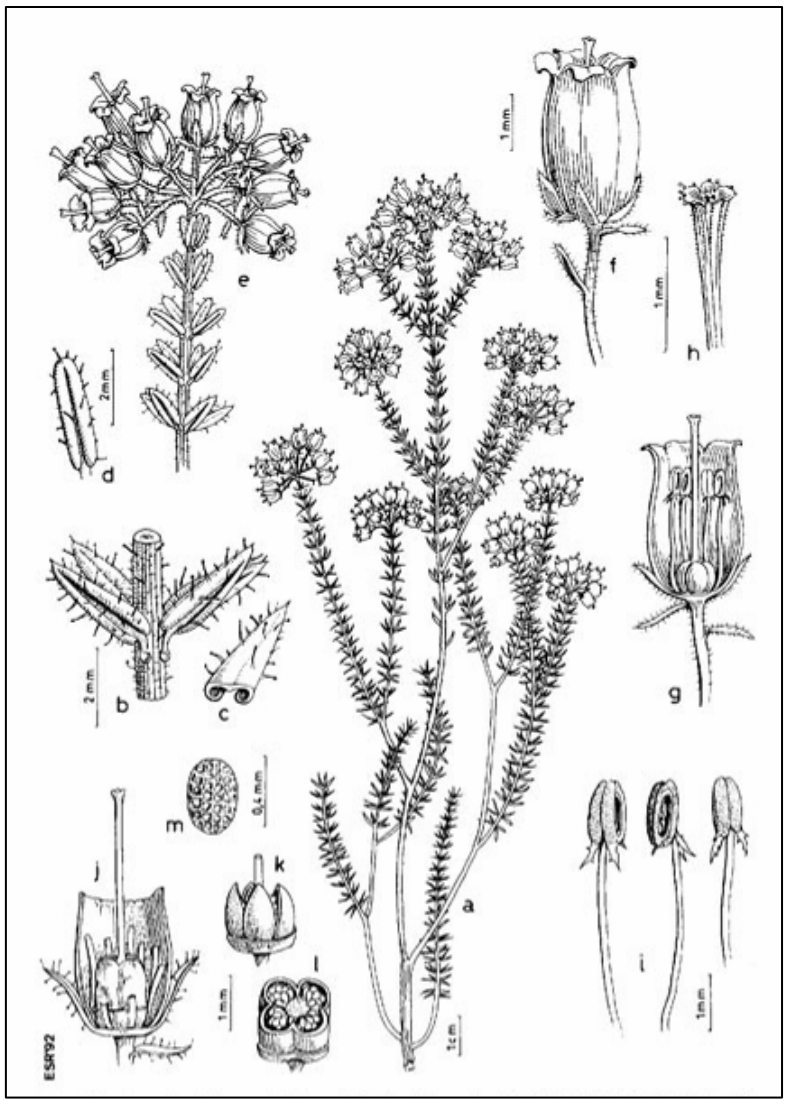


Figura 13. Lámina de *E. andevalensis* (Tomada de Villar 1993)

Una de las principales características de esta especie es que siempre aparece en suelos alterados (Nelson y cols. 1985; Aparicio 1999), motivo por el que la especie es considerada un edafoendemismo (Aparicio y García-Martín 1996, Asensi y cols. 1999). Un edafoendemismo se define como una planta que se desarrolla bajo unas condiciones fisico-químicas particulares del suelo (Macnair 1997).

Cuando la especie fue descrita por primera vez, su hábitat se enmarcó en las escombreras y alrededores de las minas de pirita de la comarca de Andévalo, en la provincia de Huelva (Cabezudo y Rivera 1980). Nelson y colaboradores (1985) describieron el hábitat de la especie formado por poblaciones monoespecíficas en las escombreras y en las cercanías de las minas, pero consideraron que éste no era el hábitat natural de la especie por ser consecuencia de la actividad humana llevada a cabo desde siglos atrás. En sitios mineros, la especiación y aparición de nuevas especies se produce a gran velocidad (Nabors Murray 2005).

E. andevalensis crece además en la ribera del río Odiel, en un sustrato de grava y arena. Crece donde el aporte de agua es abundante la mayor parte del año, con variaciones en la concentración de sales que se produce en verano, debido a la evaporación. En estas riveras está descrito que aparecen otras especies como *Arbutus unedo, Cistus ladanifer, Quercus coccifera, Erica scoparia, Erica australis, Pyrus pyraster, Juncus acutifolius* subsp. *rugosus* (Nelson y cols. 1985; Cabezudo y cols. 1989). Además, la especie aparece en las cunetas de algunas carreteras, pudiendo estar el origen de estas poblaciones en el transporte de las semillas por los camiones al entrar y salir de las minas, así como en la utilización de los escombros de minas para rellenos de carreteras (Nelson y cols. 1985).

La distribución de la especie no parece estar condicionada por la concentración de ninguno de los metales presentes, frente a los cuales se comporta como una especie tolerante (Aparicio 1999). Sin embargo, no hay ninguna cita de

la especie creciendo fuera de suelos ácidos, pobres y tóxicos (Aparicio y García-Martín 1996); esto no significa que la especie esté amenazada, sino mas bien que su supervivencia depende del mantenimiento de este hábitat (Aparicio y García-Martín 1996; Aparicio 1999). Si este hábitat desapareciese, probablemente también lo haría *E. andevalensis*. Buján y cols. (2007) a raíz del inicio de trabajos de restauración de zonas mineras de Huelva por la Agencia de Medio Ambiente, han llevado a cabo un estudio sobre las condiciones edáficas en las que la especie alcanza un desarrollo óptimo. En la mayoría de las poblaciones se pueden encontrar individuos de distintas edades, lo que es un claro indicador de que en la población existe un buen reclutamiento y una dinámica poblacional activa (Aparicio y García-Martín 1996).

La fenología floral abarca desde principios de verano hasta finales de otoño, estando en flor cuando ya el resto de especies de la zona están en fruto (Nelson y cols. 1985), fructificando entre 30 y 50 días después de la floración (Aparicio 1999). *E. andevalensis* posee parámetros característicos de especies entomófilas (Arroyo y Herrera 1988), conclusión a la que también llegaron Aparicio y García-Martín (1996) cuando realizaron un estudio sobre la biología de la reproducción de la especie tanto en condiciones naturales como en condiciones de invernadero. Este estudio puso de manifiesto que es una especie autocompatible, aunque no se autopoliniza en la naturaleza, y que utiliza como agentes polinizadores *Apis mellifera, Anthidium* sp., *Andrena* sp. y *Lusandra* sp. Tiene flores bastante duraderas y con baja cantidad de néctar, con una tasa de fertilización dependiente de las poblaciones, de las plantas e incluso de las inflorescencias, llegando en campo a un 85%. Las semillas presentan una dormancia fisiológica cuando son liberadas, que se rompe masivamente tras el frío y la humedad del invierno. La tasa de germinación es mayor si las semillas se someten a un tratamiento de frío y humedad, que simula el paso del invierno. Además, si tras 5 ó 6 semanas desde el fin del periodo de dormancia, las semillas no han germinado, no lo harán hasta el año siguiente, cuando hayan vuelto a pasar un periodo frío y húmedo. Este requerimiento de frío se entiende por un lado como

un mecanismo de defensa ante una germinación masiva en otoño (Thompson y Booth 1993) así como un mecanismo para tener una reserva de semillas permanente (Aparicio 1995). A partir de los estudios realizados, este autor propone una longevidad para las semillas de *E. andevalensis* de unos 3-4 años (Aparicio 1995).

El pequeño tamaño de las semillas, su dormancia y su longevidad son características de especies que viven en medios contaminados y con disponibilidad fluctuante de agua, condiciones que se cumplen en *E. andevalensis* (Aparicio 1995).

Con anterioridad a la descripción de *E. andevalensis*, en campo fue confundida con *E. tetralix* y *E. mackaiana* (Cabezudo y Rivera 1980). Un estudio realizado sobre la morfología de las semillas de estas tres especies puso de manifiesto que se trata de tres especies diferentes (Fagúndez e Izco 2004).

La peculiaridad del hábitat en el que crece *E. andevalensis* ha llevado a la realización de diversos estudios sobre la posible acumulación de metales por la planta, así como de las partes de la planta en la que se acumulan estos metales y los mecanismos por los cuales los metales entran en la planta; por otro lado, también se han estudiado los metales que la planta excluye y los mecanismos empleados para esta exclusión. Nelson y colaboradores (1985) describieron el hábitat de *E. andevalensis* y analizaron el contenido de metales pesados presentes en los suelos en los que la especie aparecía, pero no en el interior de las plantas. En 1992 se estudió por primera vez el contenido de metales en las plantas de la zona minera de Huelva, teniendo en cuenta la distancia a zonas contaminadas. Los principales resultados indicaron que la especie crecía en un suelo extremadamente ácido y con baja disponibilidad de nutrientes, junto con elevadas concentraciones de Cu y Pb. Estos metales también aparecieron en las plantas estudiadas a concentraciones elevadas. *E. andevalensis* acumula en hojas manganeso, y a menor concentración Cu, Fe y Al, excluyendo Zn (Soldevilla y cols. 1992).

En 1999, Asensi y cols. realizaron un nuevo estudio para establecer la tolerancia al cobre de *E. andevalensis*. No se encontró correlación entre el contenido de Cu en el suelo y el hallado en el interior de la planta. Este resultado era previsible, pues se trata de un elemento esencial en la nutrición de las plantas y las concentraciones internas tienden a ser constantes. *E. andevalensis* tiene una elevada tolerancia al Cu, aunque no se puede considerar que sea hiperacumuladora de dicho metal. La asimilación de este metal sigue el mismo patrón que el de las plantas que lo excluyen, dejando de asimilarlo una vez que se sobrepasa un determinado umbral. Este estudio puso de manifiesto que la especie no puede ser empleada como un indicador en prospecciones biogeoquímicas, ya que el contenido en Cu no está relacionado con la cantidad total del metal que existe en el suelo, aunque sí que es un buen indicador geobotánico, pues su crecimiento está restringido en medios en los que la concentración de este metal es elevada (Asensi y cols. 1999). En 2002, Heras y cols. estudiaron en qué regiones de la planta se acumulan los metales, analizando el contenido de metales tanto en suelos como en tejidos de la misma. *E. andevalensis* mostró una respuesta diferente en función del metal considerado.

Recientemente, Rodríguez y cols. (2007) vuelven a confirmar que *E. andevalensis* contiene niveles bajos de Zn, a pesar de que se encuentre a elevadas concentraciones en el suelo y que contiene elevados niveles de Mn, además de poseer una gran tolerancia al Fe. Este estudio vuelve a apoyar la propuesta del uso de *E. andevalensis* para la revegetación de suelos de minas, ya que es capaz de instalarse en suelos desnudos, ácidos y con elevada concentración de metales, haciendo que el suelo se vaya enriqueciendo en nutrientes y materia orgánica, estabilizando el desarrollo del resto de la comunidad vegetal. En 2008, Abreu y cols. realizaron un nuevo estudio que puso de manifiesto la capacidad de *E. andevalensis* para colonizar suelos ácidos y contaminados por metales en las Minas de São Domingos (Portugal). Los resultados obtenidos vuelven a destacar a la especie como acumuladora de Mn, además de ser tolerante al Al. Este estudio

destaca la capacidad de *E. andevalensis* y *E. australis* para la recuperación de las zonas de minas de azufre, aunque destaca que estas especies tienen un comportamiento diferente, no apareciendo nunca juntas como sucede en la zona española.

Por otro lado, se ha identificado en las raíces de *E. andevalensis* una micorriza *Hymenoscyphus ericae*, que acumula Fe y Al en sus tejidos (Turnau y cols. 2007). Estudios sobre la microbiología del suelo donde se desarrolla *E. andevalensis* han llevado a Mirete y cols. (2007) a estudiar la rizosfera de *E. andevalensis* en la zona del Río Tinto y han identificado una comunidad de bacterias resistentes al Ni.

Por primera vez, en 2007, se han realizado estudios con *E. andevalensis* cultivada en laboratorio (Bandeira de Albuquerque M, 2007, Tesis Doctoral) a partir de semillas y en cultivo hidropónico con tratamientos con Cu. Junto con esto, se ha realizado un estudio de proximidad genética de las poblaciones del campo y los resultados obtenidos muestran que no hay diferencias entre las poblaciones de la mina de São Domingos. Además se establecen dos poblaciones diferentes genéticamente en la zona española, las poblaciones de minas y las de orillas de ríos.

Además de estudios de acumulación de metales desde el punto de vista de estabilización de suelos mineros, *E. andevalensis* ha sido estudiada desde el punto de vista del interés en farmacología, ya que contiene flavonoides, tripernoides y ácidos fenólicos que pueden ser útiles para el tratamiento de úlceras, como agentes con actividad citotóxica y como antimicrobianos, respectivamente (Toro Sainz y cols. 1987; Aumente Rubio y cols. 1988; Toro y cols. 1988; Reyes y cols. 1996; Reyes Ruiz y cols. 1996; Martín Cordero y cols. 2001).

E. andevalensis, además del interés que ha despertado por su capacidad para sobrevivir en suelos ácidos y con elevadas concentraciones de metales, ha sido

propuesta por diversos autores como una especie de interés en jardinería, por el color intenso y llamativo de sus flores, que además se mantienen durante el verano (Cabezudo y Rivera. 1980; Valdés y cols. 1987; Villar 1993; Aparicio 1999).

OBJETIVOS

OBJETIVOS

EI principal objetivo de esta tesis es mejorar la comprensión de los mecanismos de tolerancia a metales pesados de *Erica andevalensis* a nivel metabólico y fisiológico.

Para alcanzar este objetivo principal, la tesis se ha dividido en varios objetivos parciales, que se enumeran a continuación:

1- Elección de poblaciones silvestres de *Erica andevalensis* representativas de los distintos medios en los que la especie crece y análisis del contenido de metales tanto en las hojas de la planta como en los suelos donde se desarrolla.

2- Estudio de los mecanismos de respuesta antioxidante de *Erica andevalensis*, a nivel de metabolitos (ascorbato, glutatión, pigmentos fotosintéticos, fenoles), enzimas (ascorbato peroxidasa, superóxido reductasa, catalasa, glutatión reductasa) o marcadores directos de estrés oxidativo, como grado de peroxidación lipídica en las poblaciones silvestres. Debido a la ausencia de poblaciones de *E. andevalensis* en medios no contaminados, en estos estudio se han incluido poblaciones de *E. australis* y de *E. arborea* con fines comparativos.

3- Cultivo de plantas de *E. andevalensis* en el laboratorio, utilizando tanto técnicas de micropropagación como de germinación de semillas bajo condiciones controladas a efectos de obtener ejemplares no expuestos a contaminación metálica.

4- Estudio de la influencia de factores extrínsecos e intrínsecos sobre la tasa de germinación de *Erica andevalensis*, al objeto de evaluar su posible aplicación en programas de conservación de la especie, así como para la obtención de la misma en condiciones controladas de laboratorio para futuros estudios.

5.-Análisis del efecto del cadmio, como ejemplo de un metal no esencial y extremadamente tóxico, en los niveles de ácido ascórbico y glutatión en ejemplares de *Erica andevalensis* cultivados en condiciones controladas de laboratorio.

The main objective of this Thesis is to improve the understanding of the heavy metal tolerance mechanisms of *Erica andevalensis* in a metabolic and physiologic level.

To reach this objective, the Thesis has been divided in several partial objectives:

1- Select representative wild populations of *Erica andevalensis* in the different habitats where the plant grows and analyze the metal content as much in plant leaves as in the soils where the plant live.

2- Study the antioxidant response mechanisms of *Erica andevalensis*, in a metabolite level (ascorbic acid, glutathione, photosynthetic pigments, phenols), enzimes (ascorbate peroxidase, superoxide reductase, catalase, gluathione reductase) or direct oxidative stress markers, like lipid peroxidation in the wild populations. Due to the lack of *E. andevalensis* populations growing in non-polluted soils, these studies included *E. australis* and *E. arborea* populations for comparative purposes.

3- Culture of *E. andevalensis* under laboratory conditions, making use as much micropropagation techniques as seed germination under controlled conditions, with the aim to get plants non-exposed to metal pollution.

4- Study of the influence of extrinsic and intrinsic factors in the germination rate of *E. andevalensis*, with the aim to evaluate the possible application in conservation programs, likewise to obtain plants growing under controlled conditions for future experiments

5- Analysis of Cd effect in the levels of ascorbic acid and glutathione in *E. andevalensis* plants cultured under laboratory controlled conditions, as an example of non-esential and highly toxic metal.

RESULTADOS

RESUMEN DE RESULTADOS

En cumplimiento de la normativa de la Universidad de Huelva en materia de Doctorado, donde se regula el procedimiento de presentación, autorización y evaluación de la Tesis doctoral artículo 53, en el que se expone: "*La tesis doctoral deberá ser redactada y presentada en castellano, excepto si se pretende optar a la mención europea en el título de Doctorado, en cuyo caso habrá de ajustarse a lo establecido en el artículo 58 de este reglamento. En cualquier caso, la tesis deberá incluir un resumen de al menos 25 páginas en castellano*". Por este motivo, a continuación se ha incluido un resumen de los principales resultados obtenidos. Debe notarse que se trata simplemente de un resumen donde se ha evitado la redundancia con los resultados presentados en los respectivos capítulos, tratando de no exceder demasiado la longitud de lo que se pretende un resumen.

Capítulo 1. Distribución de *Erica andevalensis.* Criterios de selección de zonas de muestreo y descripción de la zona de estudio

Distribution of *Erica andevalensis.* Criteria for sampling points selection and study area description

En este capítulo se han realizado muestreos de campo con el objetivo de analizar la distribución de *Erica andevalensis*, para posteriormente poder seleccionar zonas representativas donde la especie se desarrolla para realizar la toma de muestras. Junto con *E. andevalensis*, se seleccionaron distintas poblaciones de *E. australis* y de *E. arborea* que fueron incluidas en el estudio. *E. australis* crece tanto compartiendo hábitat con *E. andevalensis* cuando las condiciones del medio no son extremas, como en suelos que no están afectados por la contaminación minera. Por otro lado, *E. arborea* sólo se encuentra creciendo en suelos sin contaminación. En las zonas seleccionadas (Fig. 1) se analizó el contenido en metales en el suelo (totales y biodisponibles) y en las hojas de las especies y poblaciones seleccionadas (Tabla 1).

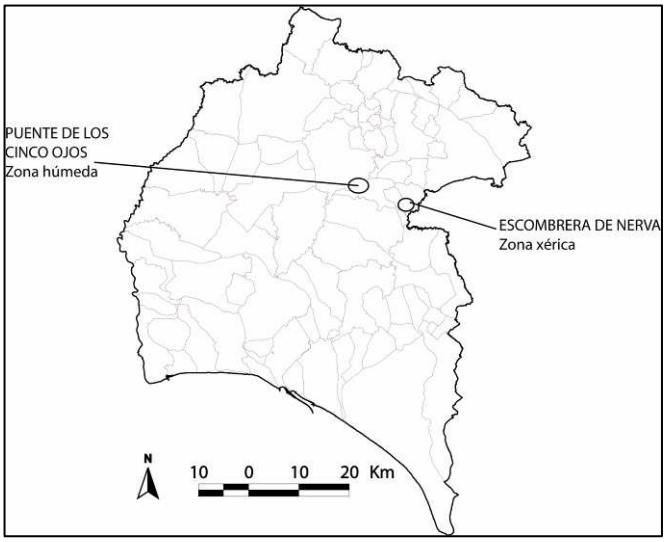


Figura 1: Mapa de la provincia de Huelva, donde se localizan las zonas de muestreo

Tabla 1

Contenido de metales en suelo y planta expresados en μg g^{-1}. Los datos muestran la media ± desviación estándar, n = 3.

	As	Cd	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Zn
Contenido total de metales en suelo										
Ladera	10,0 ± 1,0	0,387 ± 0,021	4,94 ± 0,21	21,0 ± 0,8	11900 ± 200	500 ± 10	0,508 ± 0,031	4,4 ± 0,2	47,2 ± 2,1	30,1 ± 1,1
Bancal	193 ± 10	0,578 ± 0,035	48,0 ± 2,4	250 ± 2	66100 ± 1100	1100 ± 10	3,70 ± 0,19	20,0 ± 2,5	164 ± 8	150 ± 1
Orilla	202 ± 10	4,10 ± 0,21	51,0 ± 2,6	1295 ± 47	130500 ± 4600	790 ± 20	3,40 ± 0,17	27,0 ± 1,4	179 ± 9	857 ± 10
Escombrera	518 ± 26	0,498 ± 0,023	6,86 ± 0,31	130 ± 5	21000 ± 300	70 ± 5	1,20 ± 0,06	5,7 ± 0,3	632 ± 35	50,0 ± 1,9
Contenido de metales biodisponibles en suelo										
Ladera	0,152 ± 0,022	<0,015	0,053 ± 0,004	7,01 ± 0,23	155 ± 1	231 ± 13	0,044 ± 0,006	0,181 ± 0,021	5,12 ± 1,79	4,40 ± 0,98
Bancal	0,487 ± 0,128	<0,015	0,080 ± 0,008	52,9 ± 2,3	459 ± 43	20,9 ± 1,2	0,026 ± 0,005	0,182 ± 0,011	8,14 ± 0,78	6,75 ± 0,13
Orilla	0,066 ± 0,005	1,08 ± 0,08	0,19 ± 0,01	443 ± 11	2447 ± 90	685 ± 16	< 0,005	3,07 ± 0,12	0,411 ± 0,011	347 ± 12
Escombrera	3,08 ± 0,79	<0,015	0,097 ± 0,015	35,7 ± 4,0	282 ± 10	9,81 ± 0,31	< 0,005	0,135± 0,021	3,15 ± 0,92	1,71 ± 0,04
Contenido de metales en las plantas										
E. arborea (Ladera)	1,4 ± 0,1	0,09 ± 0,01	2,7 ± 0,1	5,10 ± 0,21	260 ± 10	720 ± 20	0,29 ± 0,01	2,22 ± 0,11	1,32 ± 0,11	26,09 ± 0,02
E. australis (Escombr)	9,0 ± 0,5	0,16 ± 0,01	2,1 ± 0,1	2,43 ± 0,28	270 ± 10	270 ± 11	0,34 ± 0,02	2,21 ± 0,10	4,61 ± 0,20	27,86 ± 1,98
E. australis (Ladera)	1,2 ± 0,1	0,13 ± 0,01	1,7 ± 0,1	4,10 ± 0,37	270 ± 8	200 ± 10	0,11 ± 0,01	1,71 ± 0,11	1,07 ± 0,01	18,31 ± 0,97
E. australis (Bancal)	1,6 ± 0,1	0,10 ± 0,01	2,4 ± 0,1	4,60 ± 0,28	200 ± 5	250 ± 10	0,16 ± 0,01	2,51 ± 0,11	0,83 ± 0,03	58,76 ± 1,45
E. andevalensis (Escombrera)	3,7 ± 0,2	0,11 ± 0,01	2,9 ± 0,1	1,53 ± 0,05	430 ± 10	1100 ± 50	0,47 ± 0,02	2,96 ± 0,13	6,31 ± 0,30	15,64 ± 0,94
E. andevalensis (Bancal)	4,4 ± 0,2	< 0,015	3,8 ± 0,2	10, 00 ± 0,35	1550 ± 40	900 ± 80	0,19 ± 0,01	3,68 ± 0,11	5,08 ± 0,32	35,23 ± 0,98
E. andevalensis (Orilla)	2,5 ± 0,1	0,11 ± 0,01	1,7 ± 0,1	7,90 ± 0,21	850 ± 30	790 ± 20	0,20 ± 0,01	2,84 ± 0,11	2,06 ± 0,11	146 ± 3

Además se han analizado los datos climáticos (temperatura media, precipitación y niveles de radiación) a partir de los datos publicados en el atlas digital de Ninyerola y cols. (2005). Las zonas seleccionadas para la toma de muestras están próximas geográficamente y no se encontraron grandes diferencias en cuanto al clima se refiere.

Finalmente se realizó un estudio del tamaño de las hojas de *E. andevalensis*, en las tres poblaciones estudiadas, comparando los resultados obtenidos en primavera y en otoño. Los resultados que de este estudio se obtuvieron fueron que las hojas en la zona de la escombrera son más pequeñas, tanto en primavera como en otoño, diferencia que puede ser explicada por la disponibilidad de agua del lugar.

Capítulo 2. Composición de fenoles en *Erica sp*. expuestas a distintos grados de contaminación por metales en el suroeste de la Faja Pirítica Ibérica

Phenolics composition in *Erica* sp. diferentially exposed to metal pollution in the Iberian Southwestern Pyritic Belt

El objetivo de este trabajo fue analizar la composición fenólica en hojas de *E. andevalensis* creciendo en distintos hábitats, caracterizados por una diferente disponibilidad de agua y contenido metálico del suelo. Los fenoles son un grupo de compuestos que tienen tendencia a quelar metales, principalmente Cu y Fe (Jung y cols. 2003). Estos dos metales citados anteriormente son de los más abundantes en los suelos donde *E. andevalensis* se localiza (Soldevilla y cols. 1992; Asensi y cols. 1999; Heras y cols. 2002; Rodríguez y cols. 2007). Para ello se analizaron tres poblaciones de *E. andevalensis* (creciendo en una escombrera, orilla y terraza del río Odiel). Con el objeto de realizar comparaciones, el estudio incluyó poblaciones de *E. australis* (creciendo en la escombrera y terraza del río, junto a *E. andevalensis* y otra creciendo en la ladera de una colina) y *E. arborea* (creciendo en la colina junto a *E. australis*) (Tabla 2).

Tabla 2
Descripción de las diferentes poblaciones estudiadas, con las abreviaturas empleadas y una breve descripción del ambiente en el que vive cada población.

Especie	Abreviatura	Sitio de muestreo	pH suelo	Exposición a metales
E, andevalensis	AB	Orilla río	3,28 ± 0,06	Muy alta
E, andevalensis	AT	Terraza fluvial	3,75 ± 0,04	Alta
E, andevalensis	AM	Escombrera mina	4,38 ± 0,04	Alta
E, australis	UT	Terraza fluvial	3,75 ± 0,04	Alta
E, australis	US	Ladera colina	5,51 ± 0,06	Baja
E, australis	UM	Escombrera mina	4,38 ± 0,04	Alta
E, arborea	RS	Ladera colina	5,51 ± 0,06	Baja

Los resultados del contenido total de fenoles, así como de la actividad antioxidante revelaron que *E. andevalensis* es la especie que presenta un menor contenido de ambos parámetros (Tabla 3). Por otro lado, *E. australis* resultó ser la especie con un mayor contenido en fenoles totales, así como con unos mayores valores de actividad antioxidante, ocupando *E. arborea* una posición intermedia.

Tabla 3
Contenido de fenoles totales y actividad antioxidante de *Erica spp*. de diferentes localidades.

Especies y localización	Fenoles totales (FT)[a] (μg_{GAE}/mg peso seco)	Actividad antioxidante (AA)[a] (μmol_{TE}/mg peso seco)	(AA/FT)*100[a] (%)
Erica arborea (RS)	81,37 ± 8,63*	0,54 ± 0,08*	0,67 ± 0,05**
Erica australis (US)	104,87 ± 15,52**	0,70 ± 0,11**	0,68 ± 0,04**
Erica australis (UT)	113,48 ± 15,82**	0,74 ± 0,10**	0,64 ± 0,08**
Erica australis (UM)	112,39 ± 12,42**	0,68 ± 0,07**	0,60 ± 0,04**
Erica andevalensis (AB)	31,79 ± 9,43***	0,28 ± 0,07***	0,84 ± 0,10*
Erica andevalensis (AT)	33,61 ± 7,41***	0,29 ± 0,06***	0,83 ± 0,10*
Erica andevalensis (AM)	37,15 ± 13,20***	0,34 ± 0,08***	0,97 ± 0,41*

[a] Los resultados muestran la media ± desviación estándar de la medida por duplicado de 7 muestras.
[b] Los asteriscos indican diferencias estadísticas con un nivel de significación de $p< 0.05$.

Se realizó un análisis pormenorizado del contenido de compuestos fenólicos, mediante técnicas de separación cromatográficas (HPLC). El análisis de los distintos compuestos fenólicos mostró diferencias no sólo cuantitativas, sino también cualitativas como se muestra en la tabla 4.

Tabla 4
Concentración de los compuestos fenólicos identificados mediante HPLC en las diferentes poblaciones de las plantas estudiadas (µg compuesto/mg peso seco)[a].

COMPUESTO	Código	**RS**	**US**	**UT**	**UM**	**AB**	**AT**	**AM**
Ácidos fenólicos								
Ácido elágico	E	1,894±0,579*,b	2,173±0,781*	2,400±0,680*	1,898±0,658*	0,751±0,122**	0,809±0,059**	0,762±0,073**
Ácido vanillico	VA	0,304±0,255*	0,078±0,019**	0,060±0,025**	0,063±0,019**	0,045±0,008**	0,041±0,004**	0,049±0,007**
Derivado del ácido cinámico	CinD	NDc	ND	ND	ND	0,091±0,049*,**	0,076±0,023*	0,111±0,022**
Ácido *m*-coumarico	*m*-Cu	0,136±0,008*	0,089±0,020**	0,205±0,081***	0,074±0,013**	ND	ND	ND
Ácido cafeico	Caf	1,510±0,769	ND	ND	ND	ND	ND	ND
Derivado del ácido cafeico	Caf D	ND	0,298±0,136	0,319±0,179	0,481±0,273	ND	ND	ND
Derivado 2 ác, *p*-coumarico	*p*-C2	ND	0,221±0,099	0,886±0,028	0,168±0,072	ND	ND	ND
Derivado 1 ác, *p*- coumarico	*p*-C1	ND	ND	ND	ND	0,791±0,318	0,639±0,217	0,582±0,096
Flavonoides								
Catequina	C	0,547±0,392*	0,286±0,116**	0,243±0,085**	0,186±0,067**	0,218±0,117**	ND	ND
Epicatequina	EP	0,466±0,235**	2,494±0,606*	2,042±0,293*	2,079±0,544*	0,257±0,064**	0,450±0,231**	0,662±0,138**
Derivado 1 catequina	C1	0,260±0,145	0,276±0,161	0,326±0,155	0,301±0,189	ND	ND	ND
Derivado 2 catequina	C2	ND	0,240±0,076*	0,207±0,046*	0,144±0,029**	ND	ND	ND
Derivado 3 catequina	C3	0,510±0,157	0,566±0,143	0,659±0,312	0,762±0,395	ND	ND	ND
Derivado 4 catequina	C4	1,631±1,048*	0,596±0,164**	0,543±0,114**	0,656±0,292**	ND	ND	ND
Derivado 5 catequina	C5	0,598±0,077	ND	ND	ND	ND	ND	ND
Derivado 6 catequina	C6	0,258±0,062	0,717±0,357	0,573±0,296	0,478±0,133	ND	ND	ND
Derivado 7 catequina	C7	ND	0,273±0,096	0,217±0,033	0,273±0,096	ND	ND	ND
Derivado 8 catequina	C8	2,645±0,843*	0,564±0,119**	0,590±0,172**	ND	ND	ND	ND
Rutina	R	1,628±0,570*	2,060±0,857*	2,644±1,180**	1,772±0,564**	0,536±0,091***	0,620±0,081***	0,593±0,079***
Derivado 1 rutina	R1	1,318±0,599*	2,989±1,194**	4,390±1,445***	2,945±0,911**	1,304±0,396*	1,051±0,299*	1,106±0,217*
Derivado 2 rutina	R2	1,347±0,350*	2,362±0,847**	4,102±1,487***	2,868±0,875**	ND	ND	ND
Derivado 3 rutina	R3	3,511±1,775*	0,673±0,284**	0,952±0,642**	0,774±0,210**	3,565±1,624*	3,244±0,734*	2,276±0,598*
Derivado 4 rutina	R4	0,466±0,147	0,691±0,311	1,056±0,385	0,740±0,155	ND	ND	ND
Kaempferol	K	0,491±0,155*,**	0,557±0,114*	1,009±0,297**	0,652±0,121*,**	0,868±0,218*,**	0,936±0,154*,**	0,768±0,087*,**
Mirecitina	M	11,445±1,477*	0,935±0,077**	0,992±0,208**	1,035±0,195**	0,833±0,061**	0,800±0,026**	0,745±0,025**

[a] Los resultados muestran la media ± desviación estándar de la medida por duplicado de 7 muestras.
[b] Los asteriscos muestran las diferencias estadísticas con un nivel de significación de $p < 0.05$.
[c] ND = no detectado.

Los resultados obtenidos revelaron que la especie que crece siempre en los suelos más contaminados, *E. andevalensis*, es la que posee un contenido menor de fenoles, así como una menor riqueza de estos. Sin embargo, al medir la cantidad total de fenoles, pueden enmascararse las diferencias en la composición, así como las diferentes capacidades que los compuestos de forma individualizada presentan frente a la defensa a metales. Se encontró que *E. andevalensis* es la especie con una mayor concentración de un derivado del ácido cinámico, así como un derivado del ácido *p*-coumárico.

Una interpretación alternativa de los resultados puede deducirse a partir de los datos de Sakihama y cols. (2002) y Sakihama y Yamasake (2002), trabajos en los que se demuestra que metales no divalentes como el Al pueden llevar a la formación de radicales fenoxil. Estudios previos han revelado un alto contenido de Al en tejidos de *E. andevalensis*, no encontrado ni en *E. australis* ni *E. arborea*. Por tanto, un mecanismo de defensa de *E. andevalensis* frente a este metal podría ser la reducción de la concentración interna de fenoles para evitar el estrés oxidativo causado por los radicales fenoxil.

La conclusión de este trabajo es que *E. australis* es la especie que posee la mayor actividad antioxidante junto con los mayores niveles de fenoles, pudiendo ser estos compuestos la clave para que esta planta sobreviva en gran variedad de ambientes. Cuando todos los resultados se analizan de forma conjunta, se puede decir que las diferencias encontradas están más regidas por factores genéticos que por adaptaciones de las especies a los medios en los que viven.

Capítulo 3. Mecanismos antioxidantes en *Erica spp*. expuestas a distintos grados de contaminación por metales en el sur de la Península Ibérica

Antioxidant mechanisms in *Erica spp*. differentially exposed to metal pollution in South Iberian Peninsula

La Faja Pirítica constituye un borde natural dadas las peculiares características de acidez y contenido en metales pesados en sus suelos, metales que cuando se encuentran en elevadas concentraciones, pueden ser tóxicos para las plantas. Éstas han desarrollado mecanismos para controlar la entrada de metales, pero sí los metales superan las barreras y entran en las células pueden generar especies reactivas del oxígeno (EROs) y por tanto estrés oxidativo (Clijsters y cols. 1999; Prasad y cols. 1999; Briat y cols. 1999). Bajo condiciones fisiológicas normales en las células se generan también EROs y por ello las plantas han desarrollado mecanismos antioxidantes tanto enzimáticos (SOD, catalasa, APX, POD) como no enzimáticos (ácido ascórbico (AsA)) (Inzé y Van Montagu 1995; Arrigoni y De Tullio 2002).

El objetivo de este trabajo fue analizar los niveles de enzimas y metabolitos antioxidantes, así como peroxidación lipídica como marcador de estrés oxidativo en distintas poblaciones de *E. andevalensis*, *E. australis* y *E. arborea* expuestas a distintos niveles de metales para poder elucidar el daño oxidativo y los mecanismos antioxidantes desplegados en función del grado de contaminación y acidez de los diversos tipos de suelos seleccionados (orilla > bancal = escombrera > ladera) donde se desarrollan estas poblaciones de Ericaceas.

Tabla 5

Resultados de los niveles de antioxidantes, enzimas antioxidantes, marcadores de estrés oxidativo y pigmentos en las distintas poblaciones estudiadas de *E. arborea*, *E. australis* y *E. andevalensis.*

	E. arborea (Ladera)	*E. australis* (Ladera)	*E. australis* (Bancal río)	*E. australis* (Escombrera)	*E. andevalensis* (Orilla río)	*E. andevalensis* (Bancal río)	*E. andevalensis* (Escombrera)
Proteínas µg prot mg^{-1} PS	112,64 ± 3,87	96,17 ± 1,51 ^{a}	101,32 ± 11,52 ^{a,b}	134,64 ± 4,80 ^{b}	16,75 ± 3,13 $^{\alpha}$	41,06 ± 4,13 $^{\beta}$	43,97 ± 2,75 $^{\beta}$
Ác. ascórbico nmol mg^{-1} PF	46,21 ± 1,39	68,46 ± 1,84 ^{a}	72,00 ± 2,07 ^{a}	74,62 ± 3,39 ^{a}	39,43 ± 2,24 $^{\alpha}$	43,85 ± 2,02 $^{\alpha}$	45,70 ± 3,00 $^{\alpha}$
CATALASA U mg^{-1} prot	23,96 ± 2,30	24,30 ± 1,73 ^{b}	13,13 ± 1,00 ^{a}	23,69 ± 1,88 ^{b}	26,00 ± 3,17 $^{\alpha}$	22,36 ± 1,45 $^{\alpha}$	24,41 ± 1,85 $^{\alpha}$
SOD U mg^{-1} prot	44,80 ± 3,22	40,60 ± 2,87 ^{a}	42,30 ± 1,83 ^{a}	50,58 ± 2,00 ^{b}	45,91 ± 6,50 $^{\alpha}$	46,89 ± 3,12 $^{\alpha}$	57,78 ± 3,01 $^{\alpha}$
GPX U mg^{-1} prot	1,53 ± 0,28	2,48 ± 0,21 ^{b}	1,82 ± 0,06 ^{b}	1,98 ± 0,21 ^{a,b}	4,12 ± 0,46 $^{\alpha}$	1,41 ± 0,16 $^{\beta}$	1,15 ± 0,13 $^{\alpha}$
APX U mg^{-1} prot	292,54 ± 25,88	226,96 ± 25,77 ^{a}	267,97 ± 45,18 ^{a}	185,48 ± 35,53 ^{a}	801,01 ± 57,70 $^{\alpha}$	446,11 ± 71,70 $^{\beta}$	435,65 ± 42,23 $^{\alpha}$
MDA nmol mg^{-1} PF	0,064 ± 0,007	0,114 ± 0,012 ^{c}	0,057 ± 0,004 ^{b}	0,047 ± 0,005 ^{a}	0,057 ± 0,006 $^{\gamma}$	0,033 ± 0,002 $^{\beta}$	0,018 ± 0,001 $^{\alpha}$
R-OOH nmol mg^{-1} PF	8,74 ± 0,90	10,57 ± 0,82 ^{b}	10,18 ± 0,63 ^{b}	8,45 ± 0,40 ^{a}	55,23 ± 7,07 $^{\beta}$	54,23 ± 10,87 $^{\beta}$	30,73 ± 3,62 $^{\alpha}$
H_2O_2 nmol mg^{-1} PF	0,085 ± 0,009	0,071 ± 0,005 ^{a}	0,137 ± 0,018 ^{b}	0,098 ± 0,009 ^{b}	0,048 ± 0,002 $^{\alpha}$	0,061 ± 0,004 $^{\beta}$	0,059 ± 0,003 $^{\beta}$
Clorofila A µg mg^{-1} PF	0,889 ± 0,049	1,217 ± 0,061 ^{b}	0,980 ± 0,039 ^{a}	0,973 ± 0,037 ^{a}	1,195 ± 0,057 $^{\beta}$	1,111 ± 0,045 $^{\beta}$	0,946 ± 0,025 $^{\alpha}$
Clorofila B µg mg^{-1} PF	0,349 ± 0,028	0,413 ± 0,028 ^{b}	0,334 ± 0,029 ^{a,b}	0,282 ± 0,011 ^{a}	0,431 ± 0,022 $^{\alpha,\beta}$	0,382 ± 0,024 $^{\beta}$	0,334 ± 0,022 $^{\alpha}$
Carotenos µg mg^{-1} PF	0,298 ± 0,017	0,424 ± 0,015 ^{b}	0,404 ± 0,017 ^{a,b}	0,368 ± 0,015 ^{a}	0,408 ± 0,021 $^{\alpha}$	0,381 ± 0,014 $^{\alpha}$	0,355 ± 0,008 $^{\alpha}$

Las siglas utilizadas en la columna de parámetros son: PS peso seco; PF peso fresco; SOD superóxido dismutasa; POD guaiacol peroxidasa; APX ascorbato peroxidasa; MDA malondealdehido; R-OOH hidroperoxidos. Los datos representan la media ± error estándar de 7 muestras, analizadas por duplicado. El análisis estadístico (ANOVA y post-hoc test Duncan o Dunnett T3 (según homogenidad o no de varianzas) se indica para las distintas poblaciones dentro de la misma especie, denotando con distinta letra dentro de cada fila (a, b ó c, en el caso de *E. australis* y α, β o γ en el caso de *E. andevalensis*) cuando hay diferencias significativas ($p<0.05$).

Los principales resultados obtenidos en este estudio (Tabla 5) demostraron que *E. andevalensis* posee un contenido de proteínas solubles menor que el resto de las especies estudiadas, y en la población de la orilla del río es donde el contenido es el menor. Las poblaciones estudiadas están sometidas a una exposición crónica a metales, hecho ampliamente descrito como causante de estrés oxidativo (De Vos y cols. 1992; Gallego y cols. 1996; Schützendübel y Polle 2002). Se ha encontrado que *E. andevalensis* y *E. australis* combaten el estrés oxidativo producido por los metales con diferentes mecanismos. En el caso de *E. andevalensis*, la defensa frente al estrés oxidativo es llevada a cabo mediante un aumento de la actividad de las peroxidasas, principalmente APX, que se incrementa significativamente en las plantas de la orilla del río, donde la acumulación de metales en las hojas es mayor, llegando a niveles de 800 U mg^{-1} proteína frente a aproximadamente 270 U mg^{-1} proteína en *E. australis*; por otro lado, *E. australis* posee elevados niveles de AsA en todas las poblaciones estudiadas, independientemente de que crezcan en suelos afectados o no por los metales. Los niveles de AsA pueden ser utilizados como un marcador de estrés oxidativo (Foyer y cols.1991; Foyer 1993; Kaempfenkel y cols. 1995). Cuando se analizaron marcadores de daños producidos por el estrés oxidativo, como es el caso de la peroxidación lipídica (expresada como la concentración de malondialdehído, MDA) los resultados indicaron que la población de *E. andevalensis* de la orilla del río, que es la que está expuesta a una mayor concentración de metales y más acidez, es la que presenta mayores daños, sin embargo, la planta posee mecanismos para recuperarse, no viéndose afectada la actividad fotosintética, ya que no se observan diferencias, con respecto a las otras dos poblaciones de *E. andevalensis*, cuando se calcula la proporción entre clorofila *a* y *b*. La clorofila *b* es más sensible a la presencia de metales, disminuyendo su concentración en presencia de estos (MacFarlane y Burchett 2001). *E. australis* presenta menor concentración de pigmentos fotosintéticos cuando crece en la escombrera y el bancal, posiblemente debido al efecto de los metales, sin embargo, sorprendentemente, los niveles de MDA en la ladera son los mayores. Del mismo modo *E. arborea* también posee elevados niveles de MDA lo que podría explicarse por ser poblaciones (o especies en el caso de la última) que no tienen mecanismos

antioxidantes tan eficientes para defenderse de los metales, al no estar adaptadas a medios contaminados.

Capítulo 4. Fitoquelatinas, glutatión y compuestos tiolicos no proteicos en *Erica andevalensis* y *Erica australis* del SO español

Phytochelatins, glutathione and non-protein thiol compounds in *Erica andevalensis* and *Erica australis* from SW Spain

Está ampliamente descrito que la síntesis de fitoquelatinas (PCs) es inducida por la presencia de metales, sin que se hayan descrito otras funciones para estas moléculas diferentes de la detoxificación (Cobbett 2000; Cobbett y Goldsbrough 2002; Rauser 2005). Por otro lado, el glutatión (GSH), sustrato para la síntesis de PCs, desempeña también funciones de neutralización de radicales (Dixon y cols. 1998). Dado que *Erica andevalensis* es una especie que vive siempre en suelos con alto contenido en metales pesados, se han cuantificado los niveles de GSH y PCs, junto con los tioles no proteicos y los niveles de ácido ascórbico (AsA) para seguir profundizando en los mecanismos que esta especie posee para sobrevivir en ambientes extremos. De nuevo, como en capítulos anteriores, se han incluido poblaciones de *E. australis* (del bancal del río Odiel y de la ladera) a efectos comparativos.

Los resultados obtenidos en este estudio (Tabla 6) ponen de manifiesto que a pesar de ser plantas que crecen en suelos con alto contenido en metales y ser capaces de acumular metales en sus tejidos aéreos, las PCs no son la clave en la defensa frente a los metales en las especies del género Erica estudiadas. Los datos confirman los resultados obtenidos en el capítulo anterior sobre el menor contenido en AsA en *E. andevalensis* comparada con *E. australis*, con independencia de donde se encuentre esta última (suelos con mayor o menor contenido en metales), así como unos niveles más elevados de peroxidación lipídica en *E. australis* cuando crece en la ladera. *E. andevalensis* presentó niveles más bajos que *E.*

australis en la concentración de tioles, parámetro que no mostró diferencias en las poblaciones de *E. australis* estudiadas.

Tabla 6
Resumen de los niveles de tioles, glutatión, ácido ascórbico, capacidad antioxidante, clorofilas y fitoquelatinas.

	E. andevalensis (bancal)	*E. australis* (bancal)	*E. australis* (ladera)
Tioles (nmol GSH g^{-1} PF)	1007,7 ± 126,8 ^{a}	1187,6 ± 117,7 ^{a,b}	1316,1 ± 51,5 ^{b}
AsA (total) (nmol g^{-1} PF)	8127,2 ± 1280,3 ^{a}	22468,6 ± 2070,3 ^{b}	18981,4 ± 839,3 ^{b}
GSH (total) (nmol g^{-1} PF)	351,4 ± 84,4 ^{a}	292,1 ± 19,9 ^{a}	183,2 ± 6,3 ^{a}
TAC (µmol TROLOX g^{-1} PF)	22,7 ± 1,3 ^{a}	42,6 ± 0,7 ^{b}	43,3 ± 5,4 ^{b}
Clorofila *a* (µg g^{-1} PF)	322,9 ± 22,3 ^{c}	178,8 ± 8,4 ^{a}	244,7 ± 22,8 ^{b}
Clorofila *b* (µg g^{-1} PF)	210,2 ± 12,5 ^{b}	112,0 ± 20,7 ^{a}	194,9 ± 5,6 ^{b}
PC 2 (nmol g^{-1} PF)	10,0 ± 2,5 ^{a}	No detectada	5,7 ± 4,2 ^{a}
PC 3 (nmol g^{-1} PF)	25,1 ± 1,6 ^{b}	13,1 ± 1,6 ^{a}	20,7 ± 2,5 ^{b}
MDA (nmol g^{-1} PF)	63,1 ± 7,4 ^{a}	49,4 ± 6,7 ^{a}	110,3 ± 9,4 ^{b}

En la tabla se muestran media ± error estándar de n = 5. Dentro de cada fila se asigna la misma letra cuando no existen diferencias significativas ($p < 0,05$) después de ANOVA y test de Duncan.

Se ha descrito que las reservas de GSH pueden reducirse en plantas expuestas a metales ya que se requiere para la síntesis de PCs (Cobbett 2000 b). Sin embargo, los datos obtenidos en este estudio muestran como los valores de PCs son bajos en todos los casos analizados. Además, *E. australis* en el bancal no posee PC 2, que sí que aparece cuando la especie se encuentra en la ladera (donde la concentración de metales es menor).

No se encontraron diferencias en la proporción entre la clorofila *a* y la *b*, proporción que se utiliza como un marcador de estrés oxidativo (Macfarlane y cols. 2001) y los menores valores de MDA (marcador de los niveles de peroxidación lipídica) de las poblaciones del bancal respecto de la ladera apuntan a que, a pesar del contenido en metales en los tejidos de las plantas, éstos deben estar inmovilizados o secuestrados por otro tipo de moléculas distintas de las estudiadas hasta el momento, que hacen que el metabolismo de la planta no se vea afectado.

Capítulo 5. Efecto de la composición del medio de cultivo en la micropropagación de *Erica andevalensis*, una especie tolerante de metales que crece en zonas mineras (SO España)

Effect of different media composition on the micropropagation of *Erica andevalensis*, a metal tolerant species growing in mining areas (SW Spain)

El objetivo del capítulo es la obtención de un protocolo de micropropagación, para poder profundizar tanto en aspectos bioquímicos como en los relacionados con la conservación de la especie. Es la primera vez que se describe un protocolo de propagación de *E. andevalensis*, que puede ser utilizado tanto en programas de conservación *ex-situ* de la planta como en la profundización de los estudios bioquímicos iniciados con plantas colectadas en el campo.

Para la micropropagación se utilizaron fragmentos de tallos de *E. andevalensis*, recolectados en la orilla del Odiel, a la altura de Gibraleón. El material vegetal se esterilizó e inoculó en medios solidificados con agar enriquecidos con los nutrientes descritos por Murashige-Skoog (MS), Lloyd-McCown (LL) o Vienmont (VB), como se muestra en la Tabla 7. Los tubos se mantuvieron en la cámara de crecimiento bajo unas condiciones concretas: 16 horas de luz, con una intensidad de 100 μmol m^{-2} s^{-1} y temperatura de 24/18 ºC día/noche.

Tabla 7
Medios de cultivo utilizados en la propagación de *Erica andevalensis*.

Código del medio	Sales generales	Modificación aplicada	Sacarosa (g l^{-1})	Agar (g l^{-1})	pH	Vitaminas (mg l^{-1})						Hormonas (mg l^{-1})	
						Mio-inositol	Tiamina	Glicina	Piridoxina	Ácido nicotínico	Ácido pantotéico	IAA	K
1	MS	½ NH_4NO_3 concentración	30	6	5,8	100	0,1	2	0,5	0,5	0,1	2	0,5
2	MS	½ NH_4NO_3 concentración	30	6	5,8	100	0,1	2	0,5	0,5	0,1	0,5	2
3	LL	-	30	6	5,8	100	0,1	2	0,5	0,5	0,1	2	0,5
4	LL	-	30	6	5,8	100	0,1	2	0,5	0,5	0,1	0,5	2
5	VB	-	20	8	5,6	-	-	-	-	-	-	-	-
6	MS	½ NH_4NO_3 concentración	30	6	5,8	100	0,1	-	-	-	-	2	0,5
7	MS	¼ NH_4NO_3 concentración	30	6	5,8	100	0,1	-	-	-	-	2	0,5

Las soluciones nutritivas utilizadas en nuestro protocolo de micropropagación han sido las descritas por Murashige y Skoog (MS) (1962), Lloyd y McCown (LL) (1980) y Viémont y Beaujard (VB) 1983, con la adición de sacarosa, agar, vitaminas y fitohormonas en las concentraciones indicadas. La concentración de nitrato de amonio en el medio original MS ha sido reducida como se indica. Los números en la columna de la izquierda son usados a lo largo de texto para hacer referencia a los distintos medios.

Se realizó un seguimiento de los explantes tomando datos cada dos días. El crecimiento se midió a partir de la formación de nuevos brotes en cada explante y la producción de nuevas hojas en éstos (cuantificadas como verticilos nuevos formados). En la Tabla 8 se muestran los parámetros de crecimiento después de 30 días. El medio 7 fue el más productivo de todos y fue el que se utilizó de base para los ensayos de enraizamiento. Las raíces aparecieron cuando al medio no se añadieron ni fitohormonas ni vitaminas. Los nuevos explantes inoculados en el medio de enraizamiento tienen una fase de latencia de 3 semanas, después de la cuál las raíces emergen. El porcentaje máximo de enraizamiento obtenido fue de 63.41 ± 6.89 % a los 37 días. Entre 11 y 15 días después de la aparición de las raíces los explantes empiezan a crecer en longitud, formando nuevos verticilos. Las nuevas plántulas se transplantaron a recipientes de plástico con perlita, que se regó con MS diluido a la mitad, y se cubrió con plástico transparente, para evitar la perdida de humedad. Pasados 15 días, en los cuales el plástico se agujerea gradualmente, las plantas se transplantan a macetas con substrato comercial, donde en unos 10 días comienzan a producir brotes laterales.

Tabla 8
Nuevos brotes y verticilos por explante y porcentaje de explantes con nuevos brotes fueron evaluados para cada medio de cultivo 30 días después del inicio del protocolo de micropropagación.

Medio	Número de brotes por explante	Número de verticilos por explante	% de explantes con nuevos brotes
1	2,7±0,9 $^{b\,c}$	8,1±4,5 $^{b\,c}$	100±0,0 ^{a}
2	3,0±1,0 ^{c}	10,3±5,3 ^{c}	100±0,0 ^{a}
3	2,3±1,4 ^{b}	6,0±4,7 ^{b}	95,2±8,3 ^{a}
4	2,8±1,0 $^{b\,c}$	9,8±4,6 $^{b\,c}$	100±0,0 ^{a}
5	1,4±1,1 ^{a}	3,5±3,9 ^{a}	77,8±25,5 ^{a}
6	2,6±1,0 $^{b\,c}$	8,1±4,1 ^{b}	100±0,0 ^{a}
7	3,6±1,2 ^{d}	16,9±8,3 ^{d}	100±0,0 ^{a}

Se muestra la media ± la desviación estándar. Para cada columna, los datos que comparten la misma letra no son diferentes con un nivel de significación de $p<0,05$ (Test the *t*-Student).

Capítulo 6. Efectos de los metales pesados y la acidez en la germinación de *Erica andevalensis*, una especie endémica del SO de la Península Ibérica

Effects of heavy metals and acid pH in gemination of *Erica andevalensis*, an endemic species from SW Iberian Peninsula

Es ampliamente sabido que los metales son tóxicos para las plantas a elevadas concentraciones. La germinación de las semillas es uno de los procesos fisiológicos más sensibles a la contaminación por metales, debido a la carencia de mecanismos de defensa (Liu y cols.2005).

El objetivo de este trabajo fue estudiar el efecto que la acidez (desde pH 1 a 5) y algunos de los metales pesados más abundantes en la zona, como Fe, Mn, Cu y Zn, tienen en la germinación de las semillas de *E. andevalensis*, así como en el desarrollo inicial de las plantas. El diseño experimental incluyó los rangos en los que la especie vive normalmente. Las concentraciones utilizadas de cada metal en el ensayo fueron 5, 50, 200 y 500 ppm (por lo que tanto en tablas como en gráficas se indica el metal seguido de un número, que se corresponde con la concentración). La hipótesis inicial fue que la germinación de esta especie está favorecida por la acidez o por la presencia de alguno de los metales que se encuentran en el suelo, en ausencia de micorrizas o bacterias, que jugarán un importante papel una vez que la planta se establezca (Turnau y cols. 2007; Mirete y cols. 2007). Para ello las semillas se esterilizaron y colocaron en placas de petri, con tres capas de papel de filtro y 5 ml de cada una de las soluciones de metales o de acidez a utilizar. Se pusieron 3 placas por cada tratamiento y 50 semillas en cada placa.

La germinación se vio favorecida a pH 2 (Fig. 2), así como por las concentraciones altas de Fe (200 y 500 ppm) (Fig. 3).

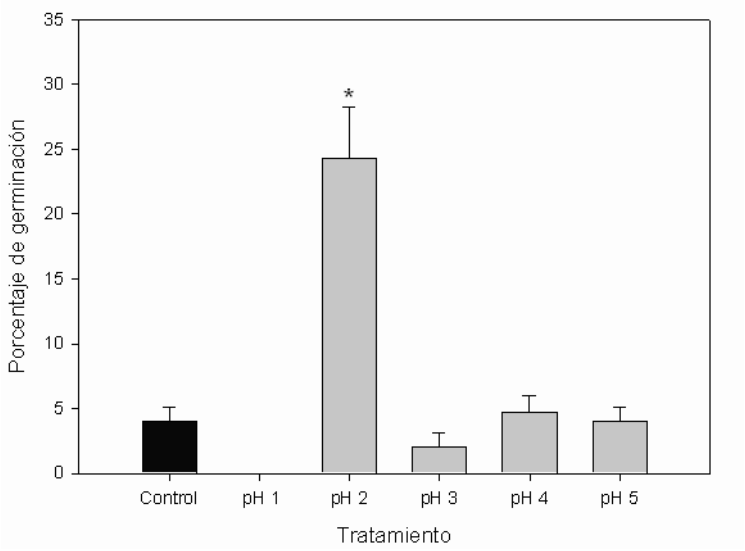


Figura 2. Porcentaje de germinación después de 55 días. Las barras indican la media ± el error estándar de 3 réplicas. Un asterisco encima de la barra señala diferencias significativas con respecto al control (p<0,05).

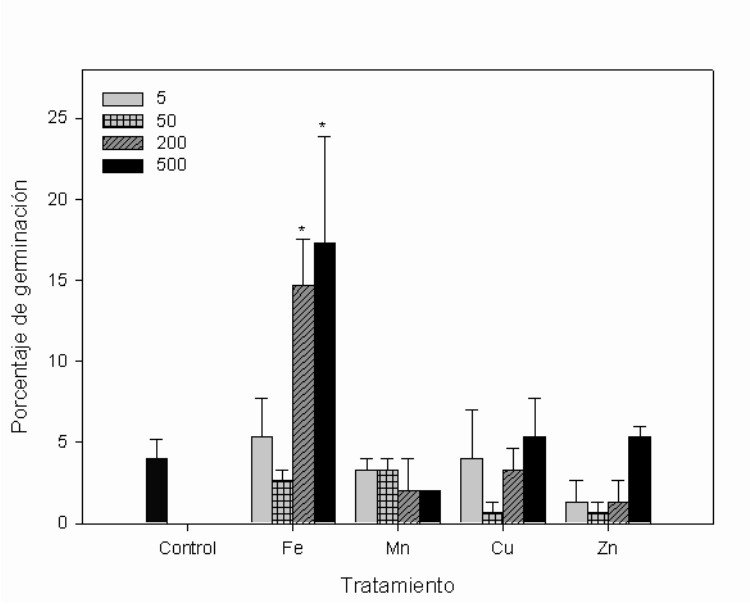


Figura 3. Porcentaje de germinación después de 55 días en los distintos tratamientos de metales. Las barras indican la media ± el error estándar de 3 réplicas. Un asterisco encima de la barra señala diferencias significativas con respecto al control (p<0,05).

Tabla 9
Resultados del análisis morfológico (media ± desviación estándar) de plantas de 10 días en los distintos tratamientos.

Tratamiento	n	Longitud hipocótilo (mm)	Longitud raíces (mm)	Número de raíces	Longitud cotiledón (mm)	Anchura cotiledón (mm)
Control	6	2,22 ± 0,57	1,65 ± 0,85	2,00 ± 0,63	0,617 ± 0,753	0,433 ± 0,052
pH 1	0	No desarrollado	No desarrollado	No desarrollado	No desarrollado	No desarrollado
pH 2	20	2,09 ± 0,25	3,65 ± 1,32 **	2,00 ± 0,56	0,745 ± 0,094 **	0,560 ± 0,114 **
pH 3	3	2,17 ± 0,45	1,33 ± 1,35	1,33 ± 1,15	0,670 ± 0,150	0,500 ± 0,170
pH 4	7	1,94 ± 0,53	1,14 ± 0,94	1,14 ± 0,90	0,543 ± 0,113	0,414 ± 0,107
pH 5	6	2,25 ± 0,70	1,83 ± 0,78	1,67 ± 0,52	0,617 ± 0,117	0,450 ± 0,105
Fe 5	8	2,16 ± 0,26	1,13 ± 0,36	2,25 ± 0,50	0,663 ± 0,092	0,475 ± 0,117
Fe 50	4	2,5 ± 0,47	1,85 ± 1,27	2,00 ± 0,00	0,800 ± 0,141	0,600 ± 0,141 *
Fe 200	19	2,13 ± 0,36	2,77 ± 2,45	1,84 ± 0,60	0,737 ± 0,138	0,511 ± 0,120 *
Fe 500	26	1,19 ± 0,40 ***	No desarrollado	No desarrollado	0,381 ± 0,247 **	0,212 ± 0,153***

Se muestran los datos de los tratamientos en los que la germinación mostró diferencias. Los distintos tratamientos han sido comparados con el control y los asteriscos muestran diferencias significativas según se indica: *** $p< 0.001$; ** $p< 0.01$; * $p<0.05$.

Se realizó un estudio del efecto de los tratamientos en el desarrollo inicial de las plantas. El crecimiento se estimulaba a pH 2, Fe 50, Fe 200 y Mn 50. Por otro lado se vió que 500 ppm de los metales utilizados inhibían el crecimiento de las plantas de *E. andevalensis* (Tabla 9).

Los resultados obtenidos en este trabajo confirman la hipótesis inicial de que la germinación de *E. andevalensis* está favorecida por la acidez, así como por elevadas concentraciones de Fe. Los otros metales incluidos en este estudio no favorecieron la germinación. Sólo a concentraciones muy elevadas, 500 ppm, se inhibió el crecimiento de las plantas germinadas en todos los casos.

Capítulo 7. Germinación y crecimiento de *Erica andevalensis*, una planta tolerante a metales del SO español. Efectos de la estraficación fría y el ácido giberélico

Germination and growth of *Erica andevalensis*, a metal tolerant plant from SW Spain. Effect of cold stratification and gibberellic acid (GA_3)

El ácido giberélico (GA_3) es una fitohormona que afecta al crecimiento de las plantas a nivel molecular, celular y orgánico, ya que regula la síntesis de lípidos de membrana, ARN y proteínas, síntesis y degradación de la pared celular, división, elongación y diferenciación de las células, gravitropismo, elongación de los tallos, expansión de la hoja o la germinación de semillas entre otras funciones (Kozlowski and Pallardi 1996; Schwechheimer 2008). Hasta el momento, poco se conoce de los factores que influyen en la germinación de *Erica andevalensis*. En 1995 Aparicio concluyó que las semillas tienen dormancia que se rompe tras un periodo de frío y humedad. Recientemente se ha descrito que la especie posee porcentajes bajos de germinación, y tras la aplicación de distintos tratamientos físicos y químicos se ha visto que el GA_3 induce una alta tasa de germinación, alcanzando porcentajes de hasta 41.6% con 400 ppm de dicha hormona.

El objetivo de este trabajo fue por un lado estudiar el efecto de distintos tratamientos de frío sobre la germinación de las semillas de *E. andevalensis*, y por otro lado analizar los efectos que el GA_3 tiene tanto en la germinación de semillas como en los estadios iniciales del desarrollo de esta especie, con el fin de poder aplicar o descartar el uso de dicha hormona en futuros estudio bioquímicos y programas de conservación.

Las semillas de *E. andevalensis* se sembraron en placas de petri con medio de agar enriquecido con las sales de Murashige y Skoog, vitaminas y sacarosa, y se mantuvieron en la cámara de germinación durante 17 días. Pasado este tiempo, se pasaron a tierra las plantas que habían germinado y las placas (con las semillas restantes) se sometieron a un segundo tratamiento de frío (14 días a 4 ºC), periodo tras el cual se volvieron a colocar en la cámara de germinación.

Los principales resultados obtenidos en este estudio han sido que la tasa de germinación aumenta cuando las semillas se mantienen en frío y oscuridad por un periodo de tiempo más prolongado (tratamiento de frío de 3 y 7 meses). En ambos casos, la tasa de germinación aumentó cuando las semillas se sometieron al segundo tratamiento de frío después de estar ya sembradas durante 17 días en placa de petri. Las plantas obtenidas de la primera tanda de germinación se sembraron en dos tipos de suelo: suelo normal y suelo de brezos, más ácido. Se obtuvo un mayor tamaño de los cotiledones, más hojas y mayor altura tras dos semanas de crecimiento en el suelo de brezos, por tanto se utilizó éste para los ensayos posteriores.

Los resultados obtenidos en este estudio (Tabla 10) confirmaron que el GA_3 tiene efectos beneficiosos en cuanto a la germinación de las semillas de *E. andevalesis*, sin embargo, una aplicación a altas concentraciones afecta al posterior desarrollo de la planta, aún en ausencia de dicha hormona, resultando plantas con hojas más estrechas. A concentraciones bajas la hormona afecta al contenido en proteínas y a la capacidad antioxidante total.

Tabla 10
Resumen de los resultados obtenidos del efecto del GA_3 en la germinación (%), desarrollo de *Erica andevalensis* después de 4 semanas sin estar expuestas a GA_3.

	Control	10 mg/L GA_3	100 mg/L GA_3
Germinación (% tras 40 días)	7,17 ± 2,57 [a]	26,23 ± 6,97 [b]	83,15 ± 14,79 [c]
Longitud hipocótilo (mm)	1,72 ± 0,061 [a]	3,68 ± 0,12 [b]	4,34 ± 0,10 [c]
Longitud cotiledón (mm)	0,465 ± 0,007 [b]	0,391 ± 0,008 [a]	0,426 ± 0,012 [a]
Ancho cotiledón (mm)	0,350 ± 0,018 [b]	0,262 ± 0,011 [a]	0,254 ± 0,010 [a]
Área cotiledón (mm)	0,139 ± 0,008 [b]	0,092 ± 0,005 [a]	0,097 ± 0,006 [a]
Longitud primera hoja (mm)	1,380 ± 0,051 [a]	1,434 ± 0,054 [a]	1,346 ± 0,061 [a]
Ancho primera hoja (mm)	0,851 ± 0,026 [b]	0,812 ± 0,035 [b]	0,574 ± 0,029 [a]
Área primera hoja (mm)	0,872 ± 0,057[b]	0,951 ± 0,061 [b]	0,675 ± 0,049 [a]
Número hojas	7,92 ± 0,424 [a]	7,800 ± 0,347 [a]	7,44 ± 0,404 [a]
Altura (mm)	4,879 ± 0,430 [b]	3,508 ± 0,234 [a]	4,461 ± 0,277 [b]
Biomasa (mg planta^{-1})	0,677 ± 0,075 [b]	0,535 ± 0,051 [a]	0,482 ± 0,024 [a]
Proteína (μg mg^{-1} PF)	454,27 ± 82,57 [a]	1433,93 ± 294,13 [b]	633,66 ± 163,42 [a]
TAC (nmol TROLOX mg^{-1} PF)	347,21 ± 158,24 [b]	48,36 ± 40,61 [a]	352,28 ± 26,64 [b]

En la tabla se muestra el porcentaje final de germinación después de 40 días, el tamaño de las plantas antes de ser transferidas a suelo (17 días después de la siembra en los distintos medios, expresado en el tamaño del hipocótilo y del cotiledón), el tamaño de las plantas después de 4 semanas creciendo en suelo (mostrando el tamaño de la primera hoja, número de hojas y altura total) y el resultado de los parámetros bioquímicos analizados. Cada dato representa la media ± error estándar (en el caso de datos morfológicos, donde n = 25) ó media ± desviación estándar (en el caso de la germinación, n= 3 placas por tratamiento; en el caso de proteínas y capacidad antioxidante total n = 4). En cada fila los números con distinta letra son estadísticamente diferentes ($p<0,05$).

Capítulo 8. Efectos del Cd en los niveles de ácido ascórbico y glutatión en *Erica andevalensis*, un brezo endémico que crece siempre asociado a zonas mineras (SO Península Ibérica)

Effects of Cd in ascorbic acid and glutathione levels in *Erica andevalensis*, an endemic heather growing always in mining areas (SW Iberian Peninsula)

El Cd es un metal tóxico, que se encuentra en los suelos en concentraciones trazas y para el que no se ha descrito ninguna función biológica en plantas (Marschener 1995; Schützendübel y cols. 2001; Kara and Zeytuluoglu 2007; Liu y cols. 2008). Este metal no induce estrés oxidativo a través de reacciones Fenton (Sandalio y cols. 2001; Schützendübel y cols. 2001; Semane y cols. 2007), sino que su toxicidad se debe a la reacción con los átomos de N y S de los aminoácidos (Clemens 2001). Además afecta a estructuras celulares como la membrana, alterando el transporte de electrones, inhibiendo/inactivando enzimas y produciendo alteraciones en el ADN (Smeets y cols. 2005). El glutatión (GSH) puede actuar como antioxidante, eliminando radicales y oxidándose a GSSG (Wu y cols. 2004). Se ha descrito que el Cd tiene una gran afinidad por los grupos tioles, por lo que puede producir un desequilibrio en las reservas de GSH, con el consiguiente desequilibrio del ciclo ácido ascórbico/glutation (AsA/GSH). Este ciclo es el más importante en cuanto al mantenimiento del estado redox de la célula (Semane y cols. 2007).

Los objetivos de este trabajo fueron estudiar los efectos que produce la exposición a Cd, estudiando la asimilación del metal y los niveles de AsA, GSH, proteínas y capacidad antioxidante total después de una exposición de 5 días a tres concentraciones distintas de Cd.

Las plantas germinadas en placa de petri con agar y transplantadas a suelo se sometieron a un tratamiento de 5 días con Cd, añadiendo al suelo 0,5 μM, 5 μM y 50 μM de dicho metal. Se analizó el contenido de Cd tanto en el suelo como en los tejidos aéreos de las plantas. El contenido de Cd en las plantas aumentó en función de la concentración de Cd añadida al suelo. Tras 5 días de tratamiento, se tomaron muestras y analizaron los niveles de ácido ascórbico (reducido, oxidado y total), glutatión (reducido, oxidado y total), proteínas y capacidad antioxidante total. El peso fresco producido por cada planta no varió en ninguno de los tratamientos. Los niveles de ácido ascórbico, junto con los niveles de proteínas, disminuyeron (con respecto al control) en las plantas expuestas a 0,5 μM de Cd, incrementándose en las concentraciones mayores ensayadas (Tabla 11). Por otro lado, el glutatión no varío bajo ninguna de las concentraciones utilizadas.

Los resultados obtenidos muestran que *E. andevalensis* es capaz de germinar y crecer en ausencia de metales, sin embargo, la acidez mejora el crecimiento. Las distintas concentraciones de Cd ensayadas no afectaron a la planta de forma severa, como se confirmó por la ausencia de daños macroscópicos o reducción de biomasa. A concentraciones pequeñas de metal la planta responde aumentando los niveles de ácido ascórbico y glutatión reducidos, pero cuando la concentración del metal se hace mayor, otros antioxidantes empiezan a desempeñar un papel importante, debido al aumento de la capacidad antioxidante total.

Tabla 11
Resumen de los resultados obtenidos tras una exposición a Cd de 5 días.

	Control	0,5 µM Cd	5 µM Cd	50 µM Cd
AsA RED (nmol g^{-1} PF)	2712,0 ± 115,3 ^{b}	2487,9 ± 32,6 ^{a}	2907,3 ± 137,7 ^{c}	2692,1 ± 178,5 ^{b}
AsA TOT (nmol g^{-1} PF)	3065,0 ± 321,0 ^{b}	2667,4 ± 109,8 ^{a}	3089,3 ± 140,0 ^{b}	2971,3 ± 154,5 ^{b}
DHA (nmol g^{-1} PF)	352,9 ± 212,1 ^{b}	242,2 ± 114,9 ^{a,b}	182,0 ± 44,0 ^{a}	279,2 ± 100,6 ^{a,b}
GSH (nmol g^{-1} PF)	645,3 ± 88,3 ^{a}	594,6 ± 57,1 ^{a}	619,2 ± 98,0 ^{a}	672,1 ± 113,1 ^{a}
GSH TOT (nmol g^{-1} PF)	719,4 ± 107,8 ^{a}	645,4 ± 54,4 ^{a}	671,3 ±117,8 ^{a}	754,3 ± 99,6 ^{a}
GSSG (nmol g^{-1} PF)	74,0 ± 36,2 ^{a}	63,2 ± 41,0 ^{a}	52,1 ± 26,0 ^{a}	82,1 ± 30,8 ^{a}
Proteínas (µg g^{-1} PF)	854,1 ± 131,0 ^{b,c}	300,8 ± 152,3 ^{a}	991,2 ± 166,9 ^{c}	650,3 ± 169,1 ^{b}
TAC (nmol TROLOX g^{-1} PF)	243,09 ± 49,39 ^{a}	357,10 ± 51,16 ^{a}	269,32 ± 119,55 ^{a}	613,03 ± 205,13 ^{b}

Los datos se indican como media ± desviación estándar. En cada fila, la misma letra se asigna cuando los valores son estadísticamente iguales ($p<0.05$). AsA RED Ácido ascórbico reducido; AsA TOT Ácido ascórbico total; DHA Ácido ascórbico oxidado; GSH glutatión reducido; GSH TOT glutatión total; GSSG glutatión oxidado; TAC Capacidad antioxidante total; PF peso fresco.

1. DISTRIBUTION OF *ERICA ANDEVALENSIS*. CRITERIA FOR SAMPLING POINTS SELECTION AND STUDY AREA DESCRIPTION

Distribución de *Erica andevalensis.* Criterios de selección de zonas de muestreo y descripción de la zona de estudio

Distribution of *Erica andevalensis.* Criteria for sampling points selection and study area description

INTRODUCTION

In the view that the objective of the present thesis is to analyze the defensive mechanisms of *Erica andevalensis* to cope with the excess of heavy metals present in the soils where the plant grows, field works were necesaries at the beginning with the aim to choose suitable areas to collect samples. These points were choosen to be representative of the different areas where *E. andevalensis* has been described. For this reason in first place a description and reevaluation of the distribution of the plant has been done and, afterwards to be focused on the selection of sampling points and biochemical analysis of the wild populations.

Fytosociologically *E. andevalensis* has been described in two different associations, depending on the abundance of water. In 1990, Cabezudo et al. described the association *Junco rugosi-Ericetum andevalensis* growing close to water streams and characterized by the presence of *Juncus acutiflorus* subsp. *rugosus*. Later, in 1993, Pérez Latorre et al. described a new subassociation localized in dry habitats that was called *Junco –Ericetum andevalensis ulicetosum*, with *Ulex eriocladus* as characteristic species. This subassociation was reevaluated and due to its wide distribution it was ascended to the association category, with the name: *Ulici eriocladi-Ericetum andevalensis* (Pérez Latorre et al. 1999). So *E. andevalensis* was included in two fytosociological associations: *Junco rugosi-Ericetum andevalensis* in the damp habitats and *Ulici ericladi-Ericetum andevalensis* in the xeric ones. Recently it has been described that in the water stream areas in the Iberian Pyritic Belt *E. andevalensis* belongs to the subassociation *Oenantho crocatae-Nerietum oleandri-Ericetosum andevalensis* and

the other two associations described above are considered as substitution stages of the mediterranean forest (Fuente et al. 2007). Furthermore, a recent study pointed out the genetic diversity of *E. andevalensis* populations concluding that *E. andevalensis* populations living on the bank of the Odiel River and the ones living on the Tailings have genetic differences, and that Spanish and Portuguese populations are also different (Bandeira de Albuquerque et al. 2008).

MATERIALS AND METHODS

Field works

The field works were done in the mining areas of the province of Huelva and Aznalcóllar (Spain) as well as in São Domingos mine (Portugal). The presence of *E. andevalensis* was recorded with a GPS and transferenced to maps with Arcview 3.2 (GIS software). The 1:10000 topographic map of Andalusia, the digital Ortophotography of Andalusia and the hydrologic shapes (Junta de Andalucia) were used as background to the cartography production.

Metals analysis and pH measurements

The metal analysis was performed starting from 3 different samples of each soil from the selected areas. The samples were dried at 50 ºC. For the total metal content, the samples were grounded and acid digested (HF, HNO_3 and HCl) and As, Cd, Co, Cr, Mo, Ni, Pb, Zn were quantified in ICP-MS (Hewlett Packard 4500) and Cu, Fe and Mn in ICP-OES (Yobinivon Ultima 2).

The pH of the soils was measured in a 1:2.5 soil / water suspension.

The bioavailable metals were analysed using 0.05 M EDTA (pH 7) solution, in the relation 1:25 (soil / solution) being mixed for 5 hours. After that the samples were centrifuged at 10000 g for 10 min and the supernatant was filtered (0.45 μm). As, Cd, Co, Cr, Mo, Ni, Pb, Zn were quantified in ICP-MS (Hewlett Packard 4500) and Cu, Fe and Mn by Atomic absorption (AAS).

The metal content in plant tissue was analysed after washing the stems (collected in the field, with around 20 cm lenght) with destilled water and drying at 70 ºC for 48 h. The leaves were excised from the branches and they were submitted to microwaves extraction (HNO_3, H_2O_2 and HCl), and the metal content was analysed in the same way as the total metal content in soils.

Climatic data analysis

Climatic data analysis was performed with the "Atlas digital" developed by Ninyerola et al. (2005). This atlas has a spatial resolution of 200 m. In each of the two selected sampling areas (river and tailings) 5 different points were selected from the atlas and the average precipitation, temperature and radiation levels were stimated. The statitistic analysis was done with ANOVA (SPSS Software).

Morphological parameters analysis of *Erica andevalensis*

The morphological analysis was focused on the size of the leaves in the three selected populations of *E. andevalensis*. Samples were collected in two different periods of the year (November and April). From each population, 22 different specimens were selected and one branch was taken. All the samples were taken with the same orientation from the Sun and from the up part of the plant. The

picked samples were fixed immediately in the field (fixative composition: formol-acetic acid-alcohol, in the proportion 1:1:8) and carried to the laboratory on cold condtions. The samples were kept at 4 ºC until the analysis (in the three days after the sampling). All the samples came from adult plants, 25-50 cm of height and 15-40 cm of width. In the laboratory 5 leaves, selected between 2-3 cm from the apical part, in length and width were measured.

The results were analysed with a general lineal model (GLM) in Statistica 6.0. In the analysis the dependent variables were the length and the width of the leaves and the categoric variables (independent) were the place of the collection and the season (autum or spring). The variance homogeneity was tested with Levene's test and post-hoc analysis (t-test) with $p<0.05$ was used to set the statitstical differences. In all cases $n = 110$ and in the graphics the mean value ± standard error of the mean are represented.

RESULTS AND DISCUSSION

E. andevalensis distribution results have been represented following the same format as the one used in Libro Rojo de la Flora Silvestre de Andalucía, in which *E. andevalensis* has been included in "endangered" category (Aparicio 1999). In this book the distribution follow the Odiel and the Tinto river courses, also including the main tributaries, once the mining areas are crossed (Figure 1).

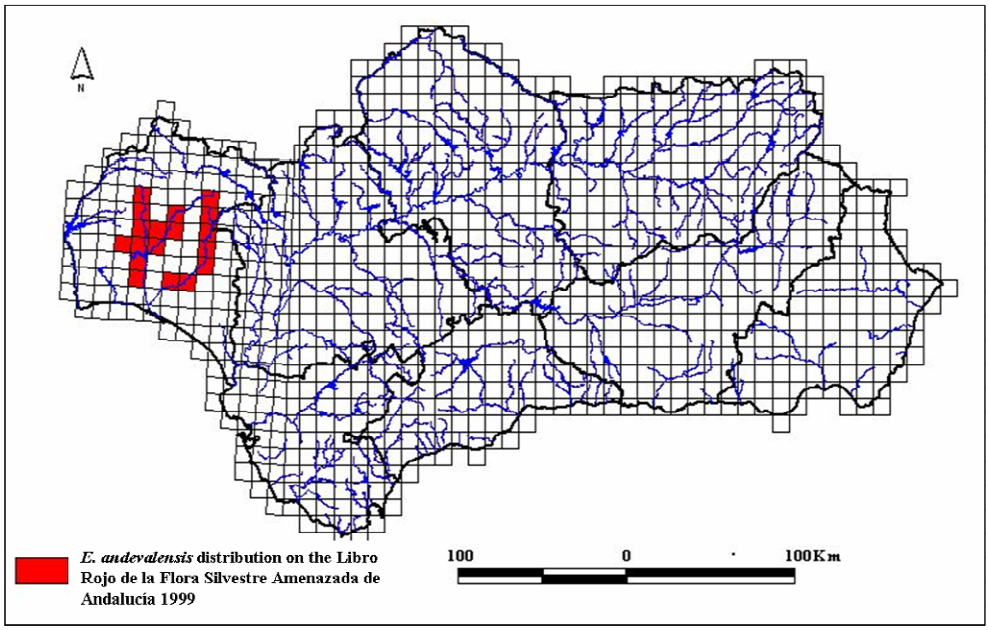


Figure 1: *E. andevalensis* distribution published in Libro Rojo de la Flora Silvestre de Andalucía (adapted from Aparicio 1999).

From the field works made by our research team a new map was produced, following the same illustration pattern as the previous published studies (Figure 2). As can be observed, *E. andevalensis* distribution in 2007 is larger than the one showed in Fig. 1, including some other mining areas in the province of Huelva, as well as the west of Sevilla and tributaries of Guadiana river and mining areas from Portugal.

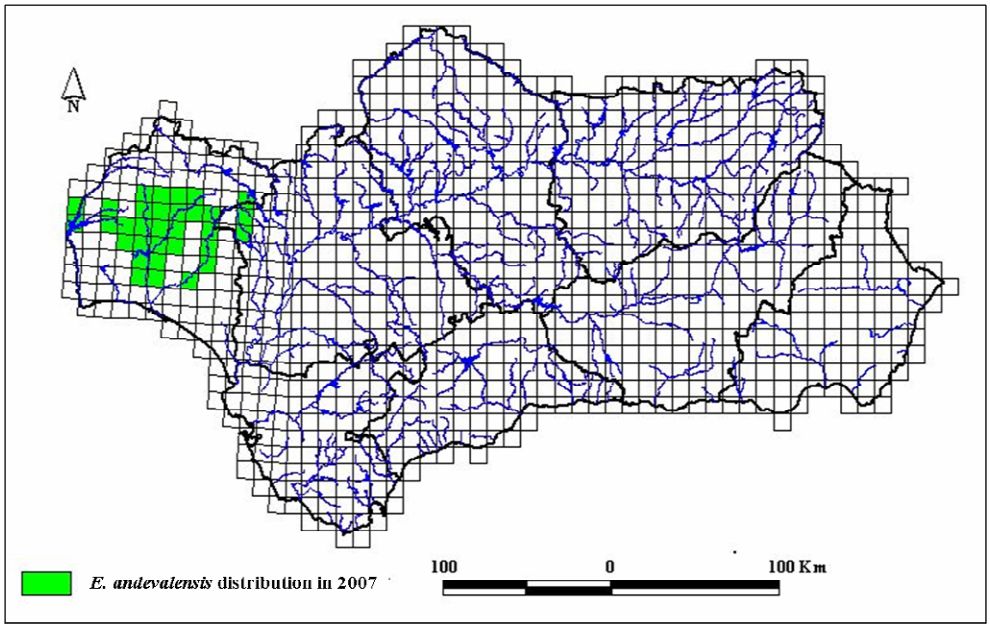


Figure 2: *E. andevalensis* distribution in 2007.

Taking into account the different places where *E. andevalensis* has been found, we have identified 6 large areas that are going to be commented next, from West to East. This classification has been made considering representative populations of *E. andevalensis*, but they have not discontinuity. A small map of the province of Huelva is showed together with each group of photos from the different areas, to indicate the general localization.

In the first area, placed in the Portuguese Pyritic Belt, in São Domingos Mine (Mertola), *E. andevalensis* grows associated to water streams that cross the mine, forming abundant populations, that become scattered in the tailings that are close to the river, associated in this second habitat with *E. australis* (Figures 3 A y B).

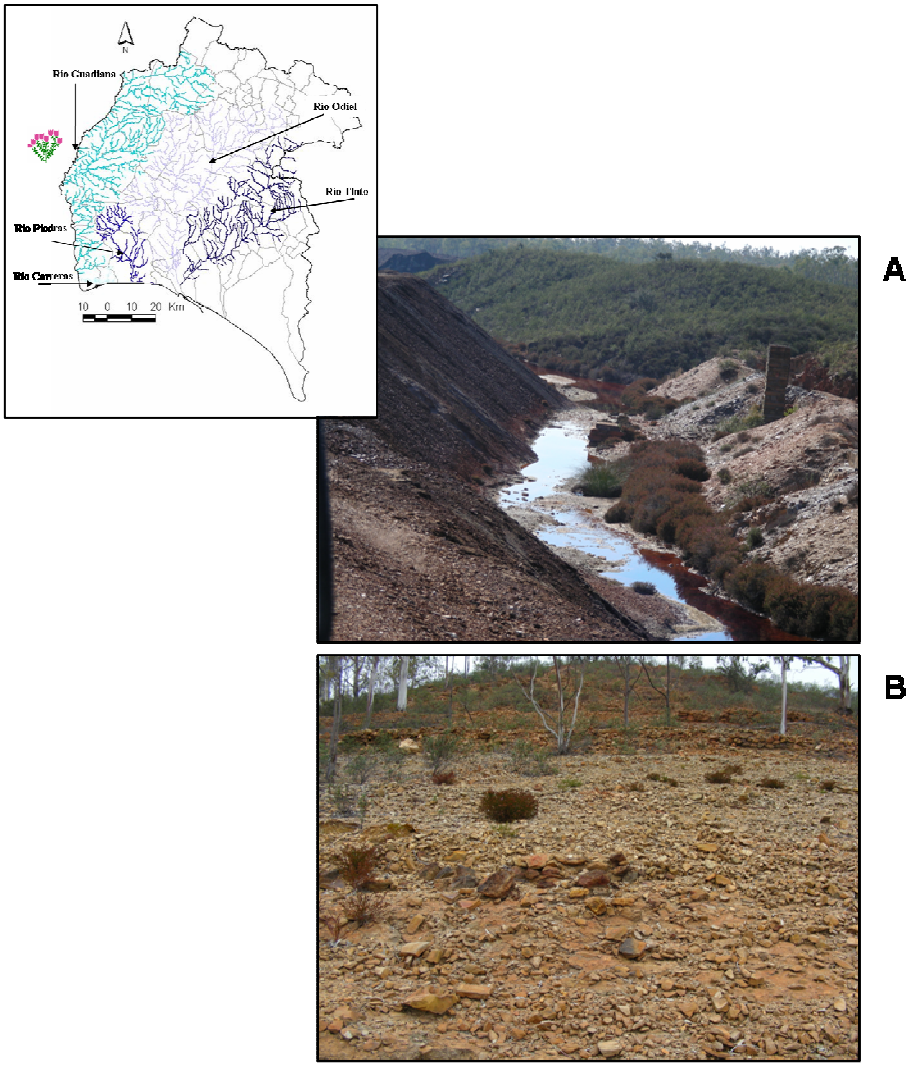


Figure 3. *E. andevalensis* in the bank of the river that crosses São Domingos mine (A) and growing in a tailings (B).

The second area is placed in the West of the province of Huelva, in the influence area of Lagunazo mine and Cobica river, once the river cross Lagunazo reservoir. In this area *E. andevalensis* is found in the banks of the water courses (Figure 4).

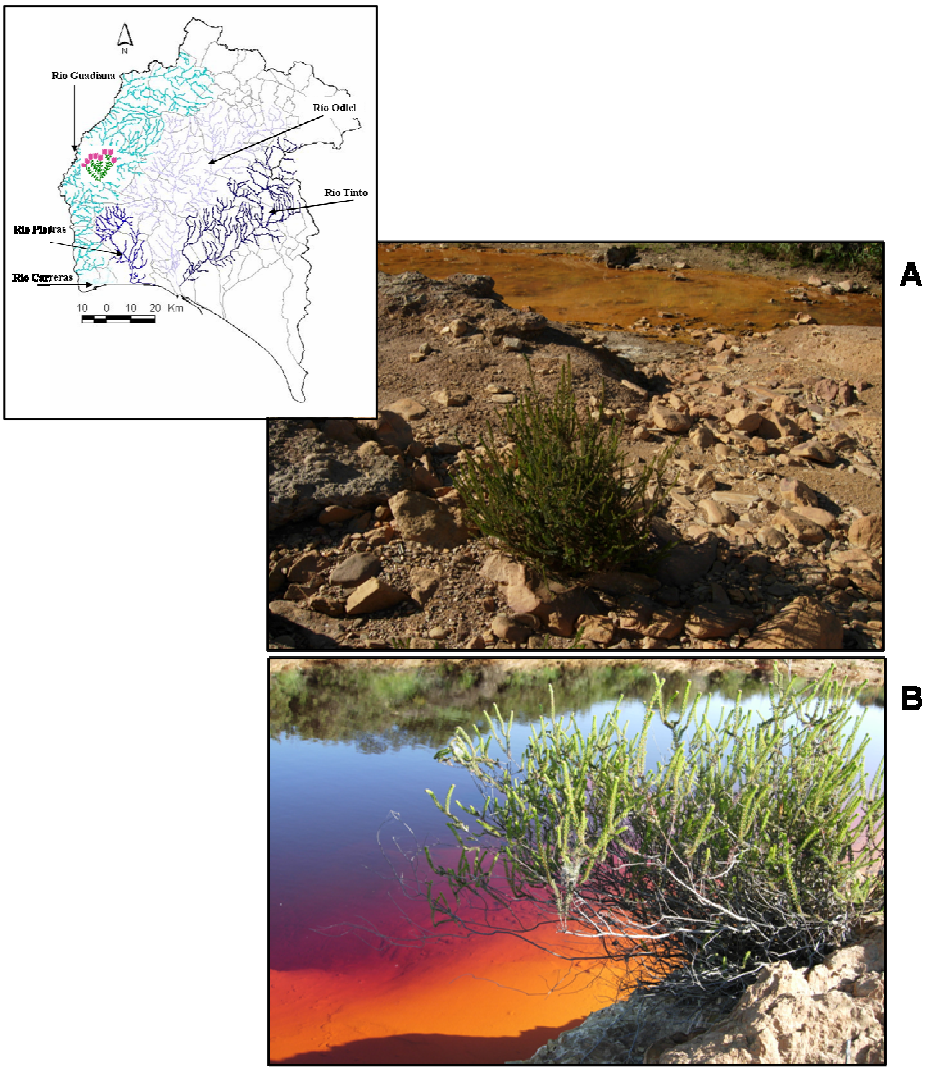


Figure 4. *E. andevalensis* in Cobica river (A and B). The red colour of the water can be apreciate, due to the mining pollution.

A third area is placed in Tharsis mines (Figure 5), with *E. andevalensis* growing in the tailings of the mines, as well as in the bank of water streams that crossed it. Agustinos river starts from Almagrera mine and receive water from Valdeoscuro stream, both of them with *E. andevalensis* in the banks. The plant is also present in the banks of Tiesa and Dehesa Boyal streams, and in La Almagrera, Sierra Bullones and Filon Norte mines.

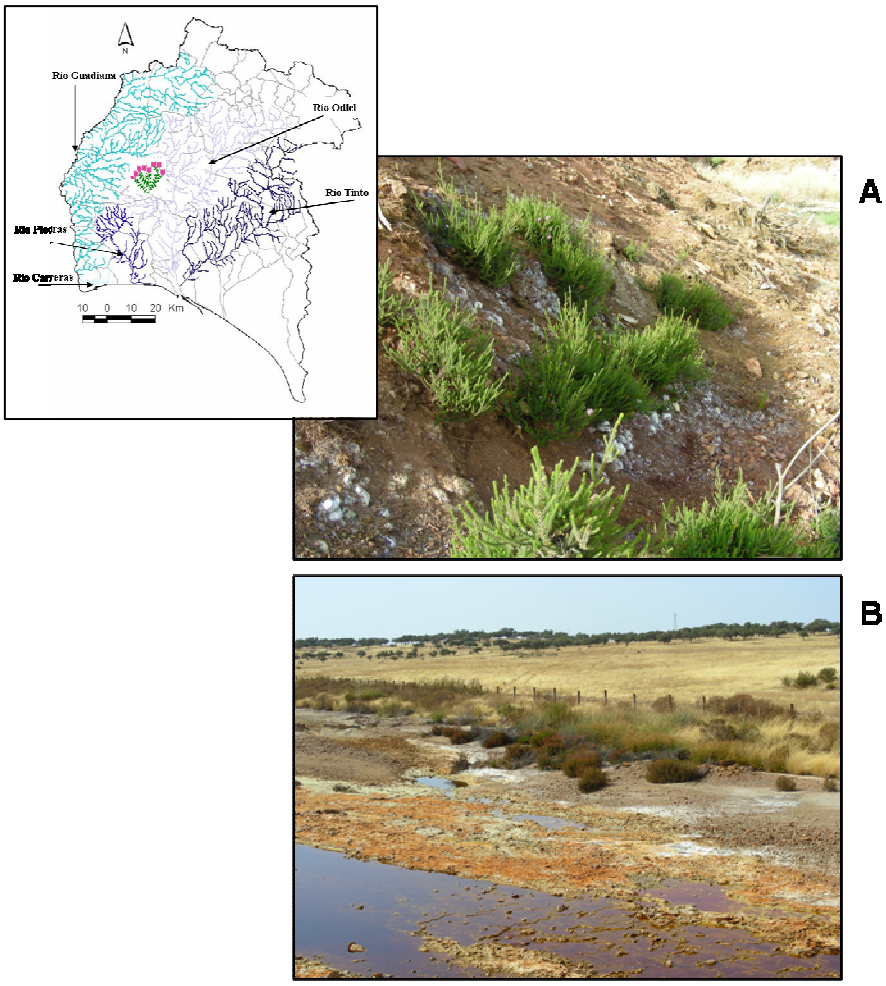


Figure 5. *E. andevalensis* growing in Tharsis mines area, in a tailings (A) and close to a water stream associated with mining wastes (B).

The fourth area is placed in Odiel River (Figure 6). *E. andevalensis* starts to appear in Mina Concepcion, once the river has crossed the first mine. The plant is present along all the mining areas that are crossed by the river (from North to South) as Asperón, La Calañesa, Sotiel Coronada mines (Figure 7 A), Las Viñas, Tinto y Santa Rosa, La Morita, de la Gloria, Buitrón and Campanario mines (Figure 7 B), with populations more or less abundant, that disappears in Gibraleon, where the river have tide influence and no more *E. andevalensis* is seen.

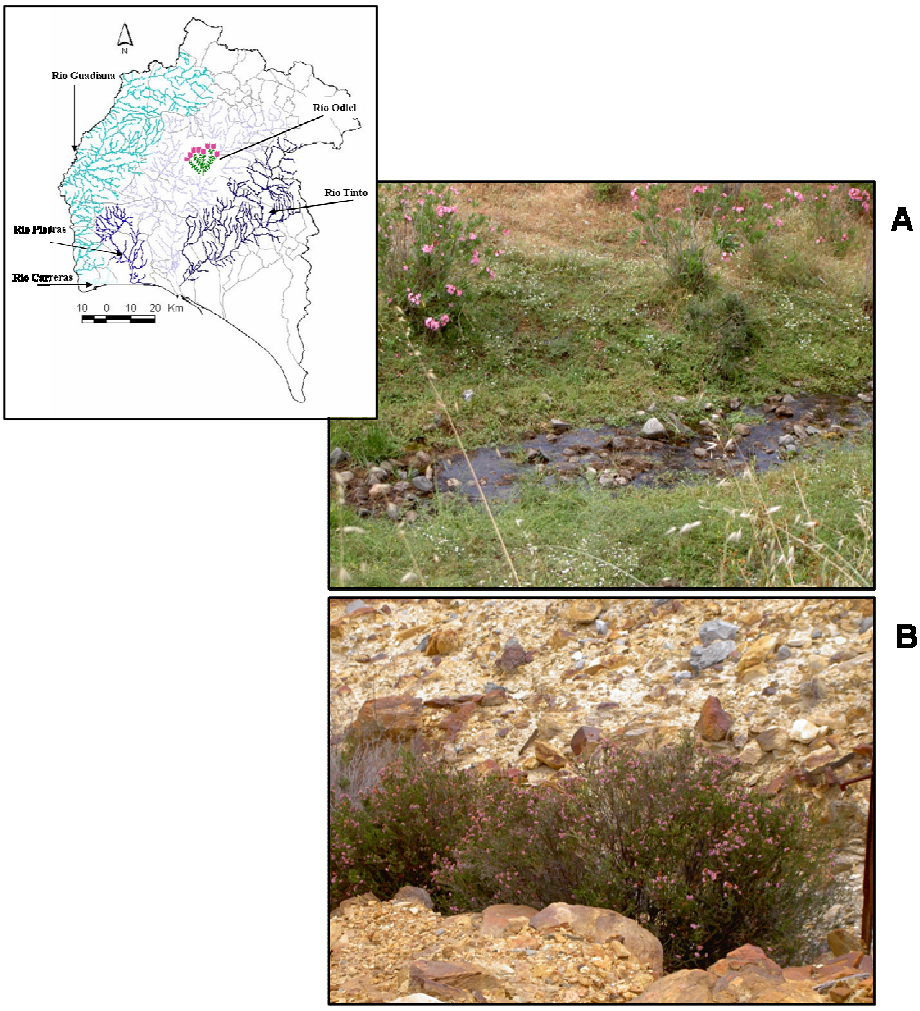


Figure 6. Appearance of Escalada stream, tributary of Odiel River, before crossing mining areas (A) and *E. andevalensis* in Mina Concepción (B).

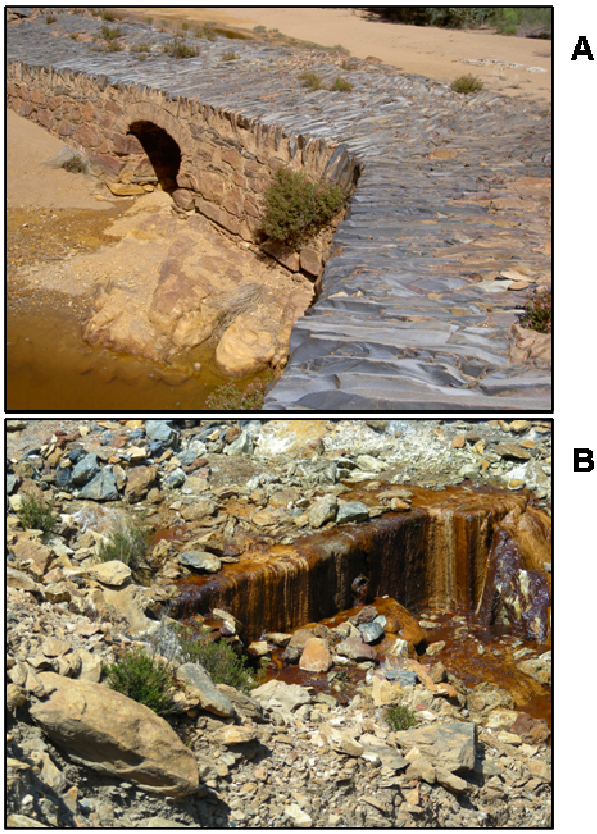


Figure 7. *E. andevalensis* in Sotiel Coronada mine (A) and in Mina Campanario (B).

The fifth area of *E. andevalensis* presence is placed in the Tinto river (Figure 8). The river source is placed in Peña del Hierro, hill formed by metal enriched soils and acid waters. *E. andevalensis* lives in the banks of the river until the union of the municipalities of Paterna del Campo, Niebla and La Palma del Condado. In this area, the plant is also present in the tailings of the main mines in the area.

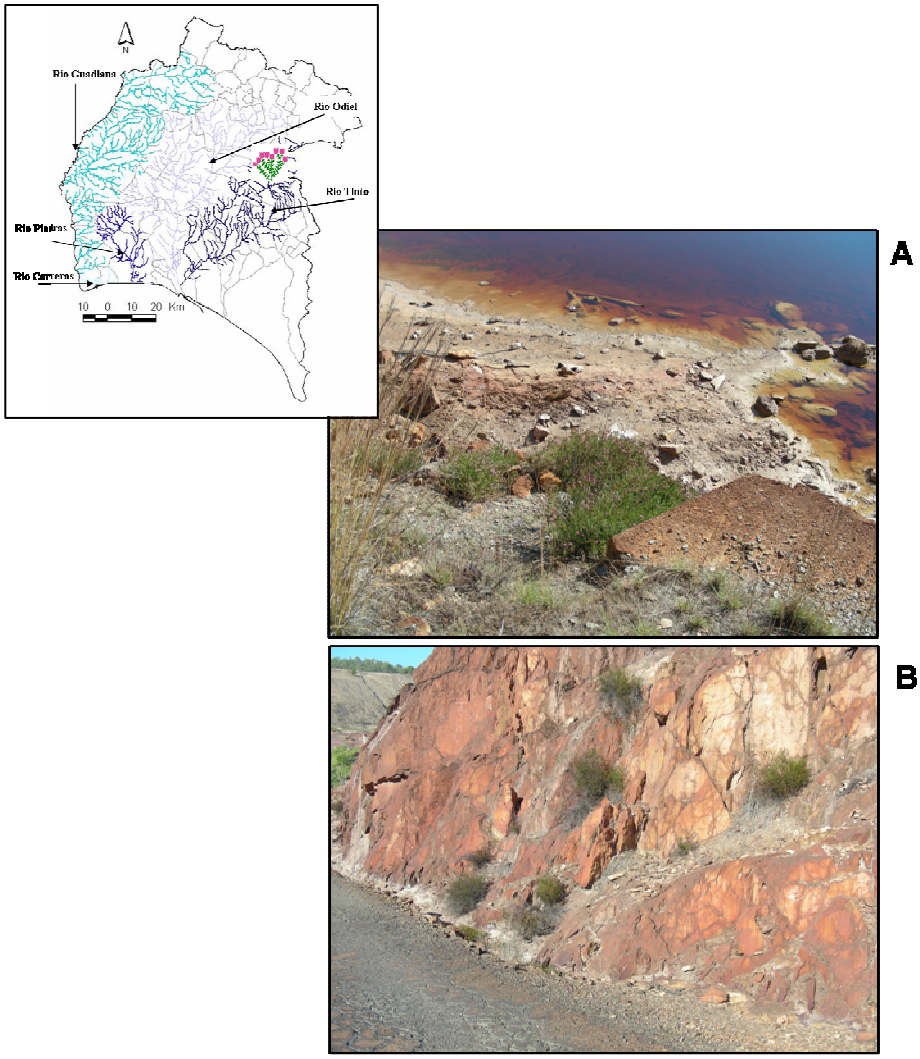


Figure 8. *E. andevalensis* in the bank of Marismillas Reservoir, close to a big tailing in Nerva (A) and *E. andevalensis* growing in a vertical wall close to the mine (B).

The last area is placed in Sevilla, in the municipalities of Castillo de las Guardas, El Madroño and Aznalcollar.

E. andevalensis is the species object of the present thesis, however, as may be observed in following chapters, in some studies *E. australis* and *E. arborea* has been included due to the lack of *E. andevalensis* populations growing in non polluted soils and these two other species has been used as a "control" to check the data coming from polluted soils. As much *E. australis* as *E. arborea* have well developed populations growing out of the influence of the mines.

Sampling areas selection

Once the distribution of *E. andevalensis* has been revised, together with a revision of the bibliographic references about the different habitats in which *E. andevalensis* has been described, representative points were selected to collect samples: one placed in a tailing (xeric habitat) and one placed around a river (damp habitat) (Figure 9). In these two places *E. andevalensis* share habitat with *E. australis*, a wide distributed heather, which is able to live in polluted soils, but not as extreme as some of the places where *E. andevalensis* can. The study included one more habitat, not affected by mining pollution, placed in a small hill (close to the Odiel river), where *E. australis* and *E. arborea* were studied. These data were considered as non exposed plants.

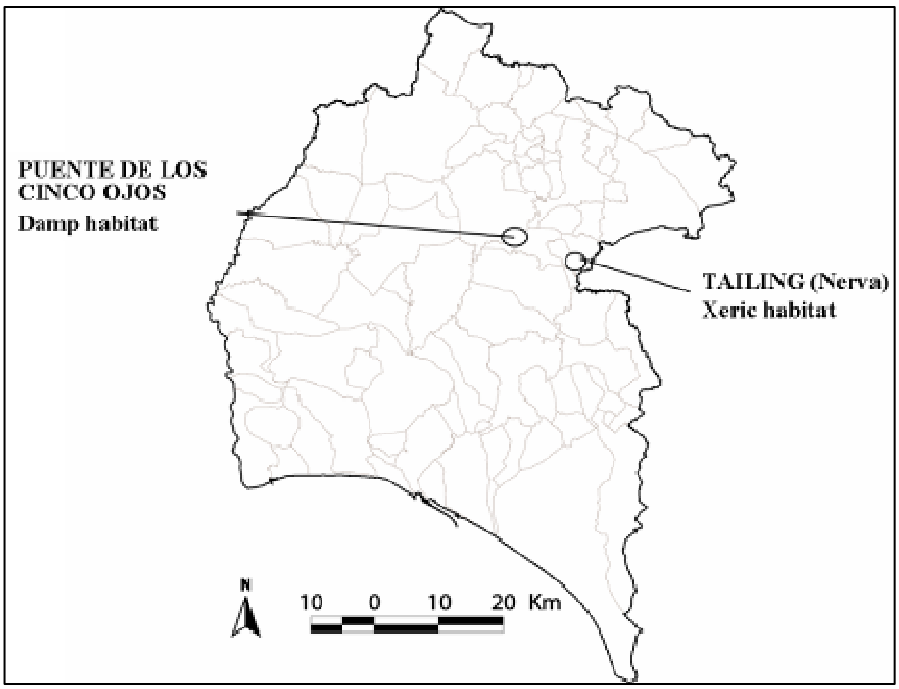


Figure 9. Map of the province of Huelva, with the situation of the sampling areas selected.

The sampling collection of *E. andevalensis* from a xeric habitat was in a Tailing close to Nerva town (37º 41' N - 6º 33' W), in a place called "Cerro del Muro", close to Marismillas reservoir, as can be observed in Figures 10 and 11. In this place, called mine or tailing site, *E. andevalensis* is growing in a dominant way, but sharing habitat with *E. australis*. The main characteristics of this place is that the plants are growing in an artificial hill, originated from mine wastes accumulation. The acidic soils and metal content are derived from the nature of the soils.

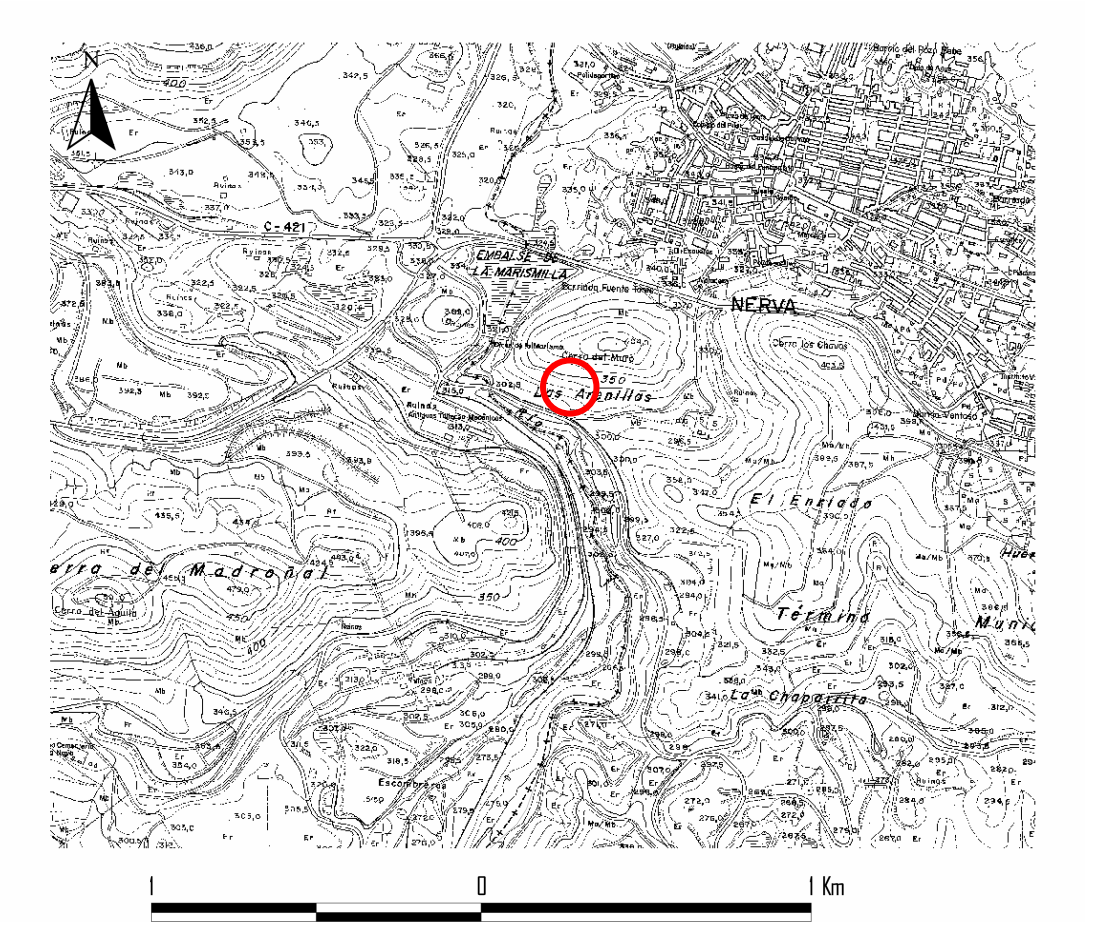


Figure 10. Topographic map (1:10 000). The sampling area is marked with a circle (addapted from Mapa Topográfico de Andalucía 1:10 000).

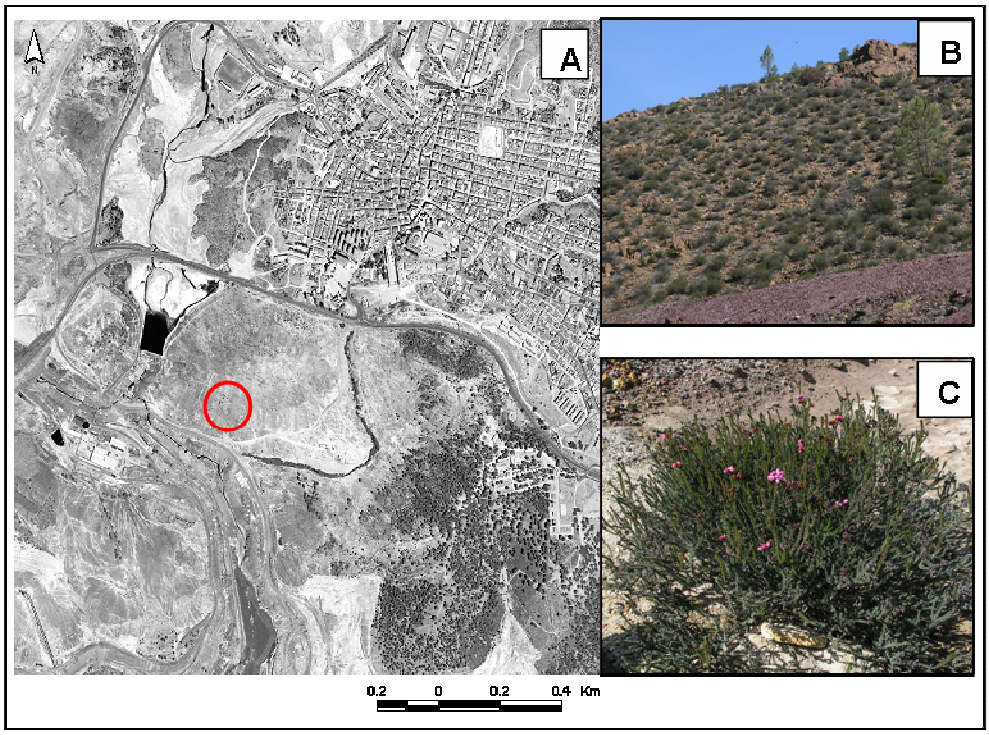


Figure 11. Aerial photography of the Tailings (A) (addapted from Ortofotografía Digital Andalucía), panoramic photographs of the aspect of the tailings (B) and detailed photo of one specimen of *E. andevalensis* (C).

The second sampling point selected, representative of the populations from damp sites, humid populations, was placed in the Odiel River, close to "Puente de los Cinco Ojos" (37º 43' N – 6º 42' W), a bridge placed between the municipalities of El Campillo and Almonaster la Real (Fig. 12). In this area, Odiel River cross a valley and it has crossed mining areas upstream, so the waters are acids (pH 2.8-3.5) and enriched in metals (50 mg/L Cu, 200 mg/L Fe, 80 mg/L Zn) (Montes-Botella and Tenorio 2003).

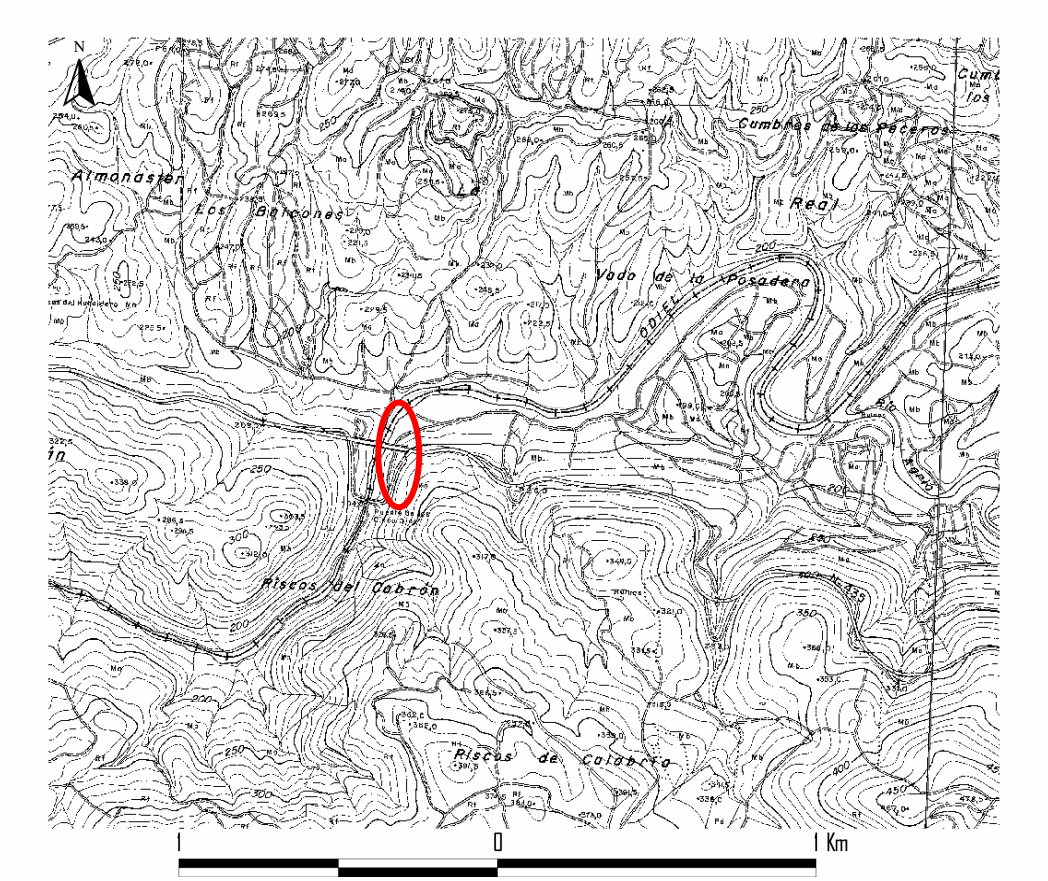


Figure 12. Topographic map (1:10 000). The sampling area is marked with a circle (addapted from Mapa Topográfico de Andalucía 1:10 000).

In this area (Figure 13), *E. andevalensis* is found growing in two different places: one growing in the bank of the river, in direct contact with the acid and enriched waters described above and that in flooding periods is submerged; the other one is placed on the terrace of the river, only submerged in great flood periods. In this second place, the same as in the tailings, *E. australis* appears. A third place was selected in this area, in the slope of a small hill, where *E. australis* and *E. arborea* grow, accompanied by the typical Mediterranean plants of the area.

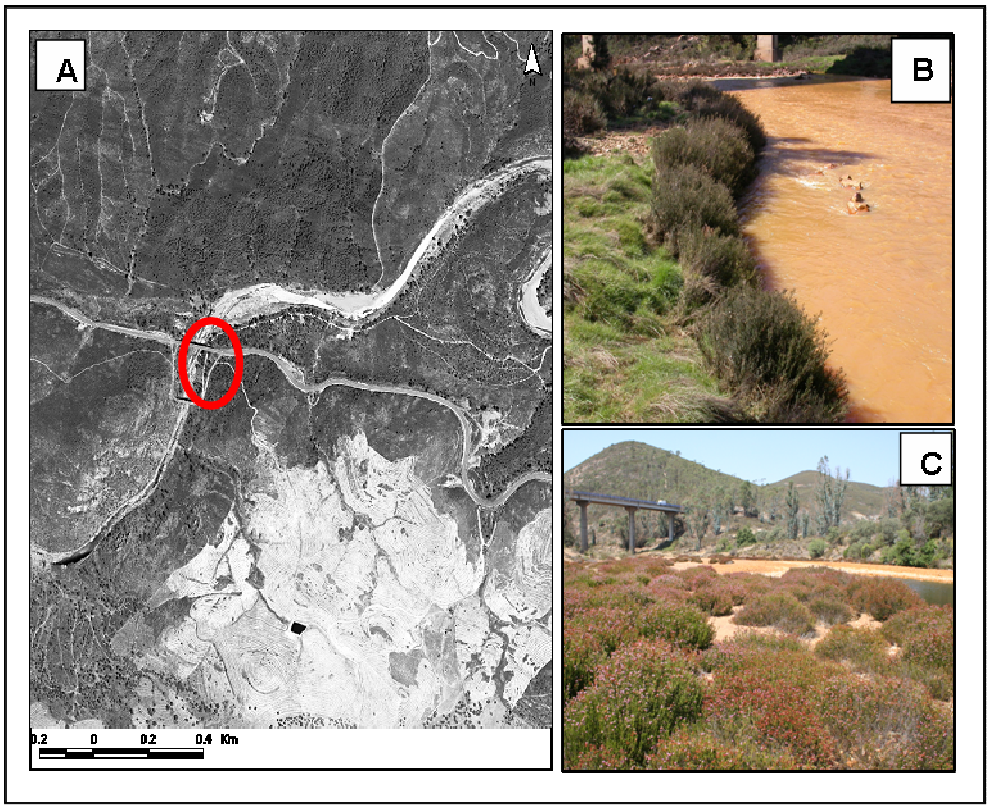


Figure 13. Aerial photography of the river area (A) (addapted from Ortofotografía Digital Andalucía), panoramic photograph of the aspect of the population from the bank (B) and panoramic photograph of the aspect of the population from the terrace (C).

Metal analysis

The pH levels in the analysed soils were: Bank 3.28 ± 0.06; Terrace 3.75 ± 0.04; Slope 5.51 ± 0.06; Tailings 4.38 ± 0.04. The results of the metal analysis in these soils, in total and bioavailable forms, and the metal content in leaves of the selected species (growing in the different locations) can be observed in Tables 1 and 2.

Table 1
Metal content in soil (total and bioavailable forms) (μg g^{-1}).

	As	Cd	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Zn
Total content										
Slope	10.0 ± 1.0	0.387 ± 0.021	4.94 ± 0.21	21.0 ± 0.8	11900 ± 200	500 ± 10	0.508 ± 0.031	4.4 ± 0.2	47.2 ± 2.1	30.1 ± 1.1
Terrace	193 ± 10	0.578 ± 0.035	48.0 ± 2.4	250 ± 2	66100 ± 1100	1100 ± 10	3.70 ± 0.19	20.0 ± 2.5	164 ± 8	150 ± 1
Bank	202 ± 10	4.10 ± 0.21	51.0 ± 2.6	1295 ± 47	130500 ± 4600	790 ± 20	3.40 ± 0.17	27.0 ± 1.4	179 ± 9	857 ± 10
Tailings	518 ± 26	0.498 ± 0.023	6.86 ± 0.31	130 ± 5	21000 ± 300	70 ± 5	1.20 ± 0.06	5.7 ± 0.3	632 ± 35	50.0 ± 1.9
Bioavailable content										
Slope	0.152 ± 0.022	<0.015	0.053 ± 0.004	7.01 ± 0.23	155 ± 1	231 ± 13	0.044 ± 0.006	0.181 ± 0.021	5.12 ± 1.79	4.40 ± 0.98
Terrace	0.487 ± 0.128	<0.015	0.080 ± 0.008	52.9 ± 2.3	459 ± 43	20.9 ± 1.2	0.026 ± 0.005	0.182 ± 0.011	8.14 ± 0.78	6.75 ± 0.13
Bank	0.066 ± 0.005	1.08 ± 0.08	0.19 ± 0.01	443 ± 11	2447 ± 90	685 ± 16	< 0.005	3.07 ± 0.12	0.411 ± 0.011	347 ± 12
Tailings	3.08 ± 0.79	<0.015	0.097 ± 0.015	35.7 ± 4.0	282 ± 10	9.81 ± 0.31	< 0.005	0.135± 0.021	3.15 ± 0.92	1.71 ± 0.04
Normal range of trace metals [a]		0.01 - 7	5 - 1000	2 - 200		200 - 2000		10 - 1000	2 - 200	10 - 300
Toxic concentration [b]		3 - 8	75 - 100	60 - 125		1500 - 3000		100	100 - 400	70 - 400

Each data is the mean ± starndard deviation , n = 3.
[a] Normal range of metal content (total form) in soils in μg g^{-1} (Swaine 1955).
[b] Range of concentration considered as toxic for plants in μg g^{-1} (Kabata Pendias and Pendias (2000)

Table 2.
Metal content in plant tissue (leaves) of the studied species (μg g^{-1}): *E. andevalensis*, *E. australis* and *E. arborea*.

Species /population	As	Cd	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Zn
E. arborea (Slope)	1.4 ± 0.1	0.09 ± 0.01	2.7 ± 0.1	5.10 ± 0.21	260 ± 10	720 ± 20	0.29 ± 0.01	2.22 ± 0.11	1.32 ± 0.11	26.09 ± 0.02
E. australis (Tailings)	9.0 ± 0.5	0.16 ± 0.01	2.1 ± 0.1	2.43 ± 0.28	270 ± 10	270 ± 11	0.34 ± 0.02	2.21 ± 0.10	4.61 ± 0.20	27.86 ± 1.98
E. australis (Slope)	1.2 ± 0.1	0.13 ± 0.01	1.7 ± 0.1	4.10 ± 0.37	270 ± 8	200 ± 10	0.11 ± 0.01	1.71 ± 0.11	1.07 ± 0.01	18.31 ± 0.97
E. australis (Terrace)	1.6 ± 0.1	0.10 ± 0.01	2.4 ± 0.1	4.60 ± 0.28	200 ± 5	250 ± 10	0.16 ± 0.01	2.51 ± 0.11	0.83 ± 0.03	58.76 ± 1.45
E. andevalensis (Tailings)	3.7 ± 0.2	0.11 ± 0.01	2.9 ± 0.1	1.53 ± 0.05	430 ± 10	1100 ± 50	0.47 ± 0.02	2.96 ± 0.13	6.31 ± 0.30	15.64 ± 0.94
E. andevalensis (Terrace)	4.4 ± 0.2	< 0.015	3.8 ± 0.2	10. 00 ± 0.35	1550 ± 40	900 ± 80	0.19 ± 0.01	3.68 ± 0.11	5.08 ± 0.32	35.23 ± 0.98
E. andevalensis (Bank)	2.5 ± 0.1	0.11 ± 0.01	1.7 ± 0.1	7.90 ± 0.21	850 ± 30	790 ± 20	0.20 ± 0.01	2.84 ± 0.11	2.06 ± 0.11	146 ± 3
Normal range in plants [a]	0.1	0.05	1.5	10	150	200	0.5	1.5	1	50
Range in plants [b]	0.02 - 7	0.1 - 2.4	0.2 - 1	4 - 15	140	15 - 100	1 - 10	1	1 - 13	8 - 100

Mean ± Standard deviation is showed, n = 3. .
[a] Markert 1994
[b] Shaw et al. 2004

As can be observed from Table 1, the metal content is the highest in the bank of the river (total and biodisponible forms), decreasing the concentration when the distance to the river is increased. The most abundant metals are Cd, Cu, Fe and Zn. In the tailings, due to the different origin of the soils, As and Pb stand out. The biodisponible metal content in soils was analysed in a EDTA extract, compound that is able to chelate metal ions in a similar way that they are in the soil (Kabata-Pendias and Pendias 2000).

The metal content in plant tissue (leaves), showed in Table 2, depends on the plant species and on the place where it is growing. In a general way the studied plants have high levels of Cr, Fe, Mn and Ni. The relation of metals with the physiological and biochemical effect produced will be analysed in Chapters 2, 3 and 4, besides the analysis of the phenolic compounds, antioxidant compounds and related enzymes and chelant molecules, such as phytochelatins.

Climatic data analysis

In relation with the climatic conditions, the selected areas are close to each other, as can be observed in Figure 9. Despite the fact that both areas has the pattern described for mediterranean climate, with mild and rainy winters and hot and dry summers, the data of average temperature, precipitation and radiation has been registered (Figure 14). The differences between both areas is low, even non-existing. In general the river area is damper than the tailings, but the last ones has higher radiation levels.

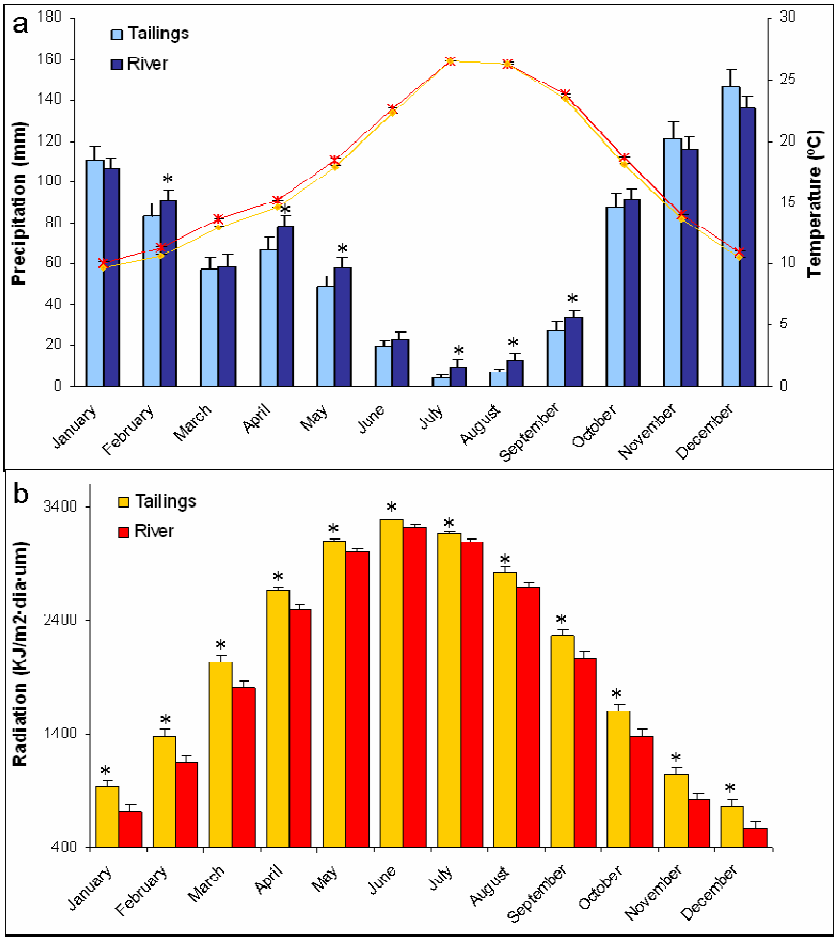


Figure 14: Average of precipitation and terperature (A) in the different study areas (tailings, blue and orange; river dark blue and red), and radiation levels (B) (tailings in orange; river in red). In all cases n = 5 for each mensual value. It is represented the mean ± Standard deviation. In A, an asterisk (*) jeans statitistical diferences ($p < 0,05$) in value of average precipitation. The temperatura is higher in the river than the tailings in all the months ($p < 0,05$), except in July, when no differences were found. In B, an asterisk (*) means statistical differences ($p < 0,05$) for each month between the two areas.

Morphological analysis of *Erica andevalensis* populations

The results of the morphological analysis of the size of the leaves of *E. andevalensis* showed that as much in length as in width, the place where the plants grows is the most important variable among all the variables introduced in the analysis, as SS and *p* values indicate (Tables 3 and 4)

Table 3
Results of the GLM analysis of *E. andevalensis* leave lenght. SS: sum of squares. The model showed a $R^2_{ajusted}$= 0,54 (F=154,90) and *p*< 0,01 was chosen.

	SS	**Freedon grades**	**F**	***p***
Intercept	5909.439	1	50095.63	< 0.001
Place	87.655	2	371.53	< 0.001
Season	1.428	1	12.11	< 0.001
Place*Sesaon	2.280	2	9.66	< 0.001
Error	77.148	654		

In the case of the width of the leaves, not only the place, but also de season had a high influence in the analysis, among all the variables introduced in it (Table 4).

Table 4
Results of the GLM analysis of *E. andevalensis* leave width. SS: sum of squares. The model showed $R^2_{ajusted}$= 0,25 (F=44,92) and *p*< 0,01 was chosen

	SS	**Freedon grades**	**F**	***p***
Intercept	946.8034	1	22226.98	< 0.001
Place	5.7366	2	67.34	< 0.001
Season	3.3612	1	78.91	< 0.001
Place*Sesaon	0.4703	2	5.52	< 0.001
Error	27.8585	654		

As can be observed in Figure 15, the plants growing in the tailing have shorter and thinner leaves than the river populations.

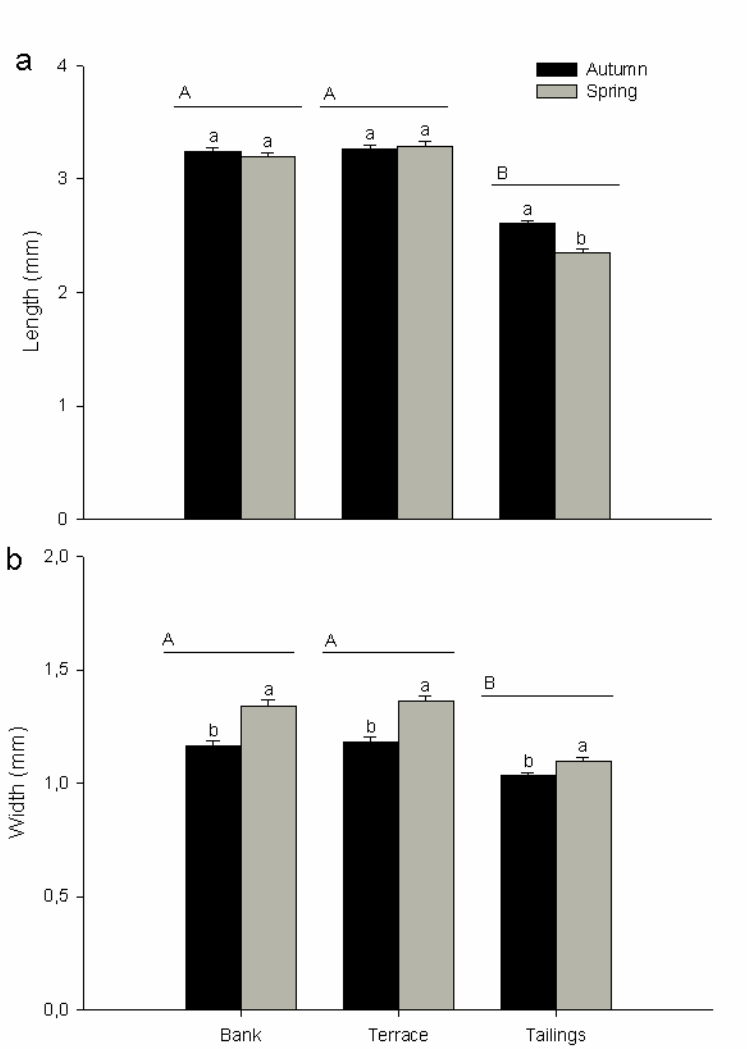


Figura 15: Length (mm) (a) and width (mm) (b) of *E. andevalensis* leaves. n = 110, and mean ± standard error of the mean is showed. Capital letters placed on top of each pair of bars (A, B) means significant differences between the studied places. Lower-case letters, placed on top of each bar, represent significant differences in the diferent seasons for each place. In all cases $p < 0.05$.

From the morphological study of the size of the leaves, differences were found not only in the place where the plants are growing, but also in the different seasons. In the tailings, the leaves are widther in spring than in autum. It is widely described that plants, under hydric stress situations reduce the size of the leaves, minimizing in this way the evapotranspiration lost (Sánchez-Díaz y Aguirreolea

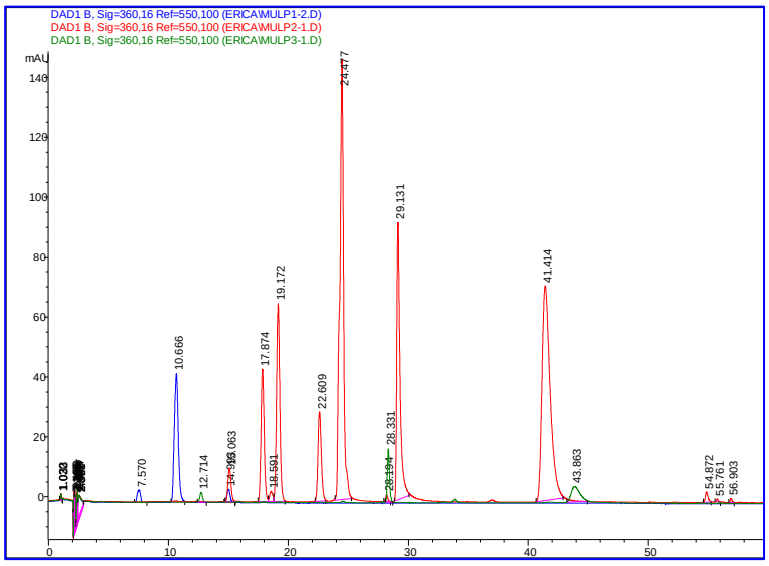
2000). In the two sampled areas, the differences found in the precipitation levels are very low. However, the water availability is different. The plants growing on the tailings should have a lower water availability than the ones living on the river area. The tailings are an antropic formation, poor in soil, constituted by disgregued rocks and big size materials from the extraction works in the mine, that have form a small hill. In this kind of formations, the water is poorly retained and percolation and runoff are high. These process resulted in a high dependency between water availability in the ground and rain pattern. This lower water availability can be reflected in a small size of the leaves of *E. andevalensis* when plants growing on the tailings are compared to the ones living close to the river.

The precipitation levels in April are smaller than the ones registered on November. However, the plants in April has been exposed to a longer period with continuity in water availability (all the winter) compared to November. This fact is reflected in the size of the leaves on the river, where the water is never a limitant factor, and the highest size is registered in spring. On the tailings, the soil have a lower retention capacity, and rain is more abundant in October and November than in March and April and this difference can be enough to explain the higher size of the leaves in autumn.

In this first chapter the distribution of *E. andevalensis* has been reevaluated and representative areas has been selected among the different habitats where the plant grows. It is a species growing in soils with high content of metals, and it posses high levels of Fe and Mn in its tissues, but without reaching levels to be considered as hyperaccumulator. There is almost no differences between the selected areas, so no differences in the biochemistry state of the plant is expected when the biochemical analysis was carried out. On the other hand, the differences found in the leaves size between tailing site and river (Terrace and Bank) can be related with the water availability and also with the genetic differences described between damp and xeric areas in *E. andevalensis* (Bandeira de Albuquerque et al. 2008).

2. PHENOLICS COMPOSITION IN *ERICA sp.* DIFFERENTIALLY EXPOSED TO METAL POLLUTION IN THE IBERIAN SOUTHWESTERN PYRITIC BELT

Composición de fenoles en *Erica sp*. expuestas a distintos grados de contaminación por metales en el suroeste de la Faja Pirítica Ibérica



(Published in Bioresource Technology (2009) 100: 446-451)

Phenolics composition in *Erica* sp. differentially exposed to metal pollution in the Iberian Southwestern Pyritic Belt

ABSTRACT

The phenolic composition of different populations of *Erica andevalensis, E. australis* and *E. arborea* was analyzed according to the different degree of exposition to metals in soils. *Erica andevalensis* populations, highly exposed to metal pollution, had the lowest total phenol content and the lowest antioxidant activity measured as trolox equivalents. The HPLC analysis of leaf extracts revealed that phenolic composition in all analyzed populations of *E. australis* and *E.arborea* was very similar, although wild populations were differentially exposed to metal pollutions. However, *E. andevalensis* showed a phenolic profile characterized by the absence of many phenolic compounds quantified in the other species, although two compounds derived from cinnamic and coumaric acids were exclusively observed in this species.

KEYWORDS

Erica andevalensis; phenolic compounds; flavonoids; antioxidant activity; acid mine drainage (AMD)

INTRODUCTION

Erica andevalensis Cabezudo & Rivera is an endemic species of South-Western Iberian Peninsula, which only grows on the spoil heaps and outskirts of the ancient pyrites mines and on the Odiel and Tinto river banks, whose main courses and their streams cross the mining areas (Cabezudo and Rivera 1980; Nelson et al. 1985; Rodríguez et al. 2007). The species has been described in similar mining areas in Beja region (Portugal), also located at the Iberian Pyritic Belt (Capelo 1998; Abreu et al. 2008). *E. andevalensis* flowers in soils and sediments highly polluted by a broad range of metals like iron > aluminium > lead > zinc > cadmium, and toxic metalloids as arsenic. (Soldevilla el al. 1992; Asensi et al. 1999; Heras et al. 2002; Abreu et al. 2008; Rodríguez et al. 2007; Turnau et al. 2007). Moreover, this heather grows under a very acid pH with values as low as 3, a common condition in other lands suffering acid mine drainage (AMD). *E. andevalensis* is also important to colonise mine areas where it may transform sterile substrates and immobilizes metals *in situ* (Abreu et al. 2008). The singularity of this species and its restricted flowering territory has addressed the inclusion of *E. andevalensis* in the list of endangered species (Aparicio 1999) thinking that cleaning and recovery of acid mine soils would lead to the extinction of such an odd species.

Curiously, in spite of the ability of *E. andevalensis* to survive successfully in extreme conditions, its tolerance mechanisms to metals and acidity have not yet been completely elucidated. The existence of metal-accumulating mycorrhizae (Turnau et al. 2007) and bacteria in the rhizosphere of *E. andevalensis* (Mirete et al. 2007) probably contribute to prevent metal uptake by this species. Both fungi and bacteria may contribute to reduce heavy metal mobility and availability to the plants through release of chelating agents, acidification, phosphate solubilization and redox changes, together of direct metal uptake and accumulation or sorption by external wall surfaces (Urrutia 1997; Hildebrandt et al. 2007; Yan-de Jing et al.

2007). In such a way, phenolics may act as reducing agents, hydrogen donors and singlet oxygen quenchers, then preventing the evolution of oxidant free radical and reactive species derived from metal catalysis by Fenton-like reactions (Jung et al. 2003; Lopes et al. 1999).

Moreover, phenolics have a high tendency to chelate heavy metals, especially copper and iron (Jung et al. 2003), the most prevelant metal pollutants in the pyritic lands where *E. andevalensis* survives (Soldevilla et al. 1992; Asensi et al. 1999; Heras et al. 2002; Rodríguez et al. 2007). The protective role of plant phenolic compounds could explain the modulation of their levels depending on stressing conditions in the environment (Dixon and Pavia 1995; Rice-Evans et al. 1997; Michalak 2006). However, quite recently the interest in the function of individual phenolic compounds against oxidative stress derived from heavy metals exposition increased (Tung et al. 2007).

Phenolic compounds are abundant in Ericaceae species (Lebreton and Bayet 2002) where they seem to have alelopatic functions (Ballester et al. 1975). In *E. andevalensis*, a variety of phenols and flavonoids have been isolated and proved to have antimicrobial (Toro et al. 1987) and antiulcer activities (Reyes et al. 1996; Reyes Ruiz et al. 1996). These reports focused the pharmacological interest of phenol-enriched extracts of this species, but no data on the antioxidant role of phenols in *E. andevalensis* have ever been reported, in spite of the prooxidant conditions in soils leading by the high levels of pollutant metals in environment.

In this paper, we have analyzed a variety of phenolic compounds in *E. andevalensis*. Taking into consideration that this species forms different populations depending on water content in soils (Cabezudo and Rivera 1980; Cabezudo et al. 1989; Perez Latorre and Cabezudo 1999), we have selected representative samples from plants living near river courses and on xeric mine spoil heaps. For comparative purpose, we have also measured phenolic composition in *E. australis* and *E. arborea*, two heathers living near *E. andevalensis*, but subjected

to different degrees of metal exposition in soils (Valdes 1987; Núñez et al. 2003). *E. arborea* has a wide distribution in non polluted soils in the hills of South Spain, flowering solely in non-polluted, acid soils (Núñez et al. 2003). *E. australis* on the other hand can live together with *E. andevalensis* in high metal-polluted soils, but also in less polluted habitats near *E. arborea*. It has been demonstrated that both *E. andevalensis* and *E. australis* accumulate heavy metals in leaves (Joaquim et al. 2003; Abreu et al. 2008), thus these heathers may develop oxidative stress by Fenton-like reaction catalyzed by metals. Thus, a comparative study of these species may contribute to understanding the composition and role of phenolic compounds in wild heather growing under the influence of iron/copper sulphurs in the Iberian Pyritic Belt.

MATERIALS AND METHODS

Plant material was collected in two different areas of the Pyritic Belt in Huelva (Spain), the first one near the Odiel river (37º43'N 6º42'W) and the second one in a bare spoil heaps (37º41'N 6º33'W). Table 1 summarizes some characteristics of soils and metal pollution degree of the *Erica* sp. populations analyzed in this report. Abbreviations used in tables and figures are also indicated in table 1.

The most metal-polluted areas are the river banks where water mobilizes metals from mine exploitations but also the mine spoil heaps, while the lower metal-polluted soils are represented by the wet slopes where a lot of vegetation is found. The river terrace is representative of an intermediate condition since polluted-water floods terrace by river swollen after intense rains.

Mature, healthy leaves of the selected species were collected in January 2006, and they were immediately immersed in liquid nitrogen and stored at -80ºC.

Leaves were air dried during three days before being extracted (Salminen et al. 2004). Seven samples of each population (i.e. 49 samples) were taken. Each sample was twice extracted and analyzed. Leaves homogenization was carried out into liquid N_2 followed by a 30 % methanol extraction step. The total content of phenolic compounds was assayed spectrophotometrically following the method described by Grubešic et al. (2005). Measurements were carried out in duplicate and total phenolics were expressed as micrograms of gallic acid equivalents (GAE) per milligram of dry weight (DW), after a calibration curve was obtained using gallic acid as standard (Sigma, St. Louis, MO, USA).

Table 1
Description of the different populations studied, with the abbreviation code used, and a brief description of the environment to which each plant population is subjected

Species	Abbreviation	Sampling area	Soil pH	Metal exposition
E. andevalensis	AB	River bank	3.28±0.06	Very high
E. andevalensis	AT	River terrace	3.75±0.04	High
E. andevalensis	AM	Mine spoil heap	4.38±0.04	High
E. australis	UT	River terrace	3.75±0.04	High
E. australis	US	Land slope	5.51±0.06	Low
E. australis	UM	Mine spoil heap	4.38±0.04	High
E. arborea	RS	Land slope	5.51±0.06	Low

The qualitative metal exposition is based on quantitative data from other authors cited in the text and our own soils metal analyses (not published).

Individual phenolic compounds in leaf extracts were analyzed by high performance liquid chromatography (HPLC) with direct injection of 50 µl of a 45µm filtered, methanol-extracted sample. An Agilent 1100 series (Palo Alto, CA, USA) chromatograph equipped with a diode array detector was used. A gradient of solvent A (water:acetic acid, 98:2 v/v) and solvent B (water:acetonitrile:acetic acid,

58:40:2 v/v/v) was applied to a reversed phase Nova-pack C18 column (300 x 3.9 mm I.D., particle size 5 μm) as follows: 0–25 min, 45% B linear; 25–45 min, 45% B isocratic; 45–60 min, 100% B linear; 60–70 min, washing and reequilibration of the column. The flow rate was 1.0 mL/min and the temperature was set at 20 ºC. Detection was performed by scanning from 200 to 700 nm. Identification of individual compounds was carried out by comparing their retention times and spectra with those of original standards. Standards were obtained from Merck (Darmstadt, Germany), Fluka (Buch, Switzerland) and Sigma (St. Louis, MO, USA). Quantitative determinations were carried out with standard external calibration method. Wavelengths used for quantification were 260nm for ellagic and vanillic acid, 280 nm for benzoic acids and tyrosol, 320 nm for cinnamic acids and their tartaric esters, and 360 nm for flavonols. Identification of unknown phenolic compounds not included as standards was based on retention times and spectra obtained from the literature. These compounds were assayed by assuming that their molar absorptivities were the same as those of their corresponding free standard molecules.

The antioxidant activity of the extracts was determined using 2,2-diphenyl-1- picryl-hydrazyl radical (DPPH·) method described by Brand-Williams (1995), with some modifications (Leong and Shui 2002; Wong et al. 2006). An aliquot fraction (40 μl) of the methanolic plant extract was added to 3 ml of 0.1 mM DPPH in 30% methanol. The mixtures were vortexed and then left to stand at room temperature for 30 minutes. The antioxidant capacity based on DPPH·scavenging activity was expressed as μmol trolox equivalent (TE) per milligram of dry weight (DW) plant material. Methanolic solutions of 10 to 0.625 mM trolox were used to make the standard curve. The degree of radical scavenging activity was calculated as described by Ardestani and Yazdanparast (2007) by using the ratio $(A_{time=0} - A_{time=30\ min})/A_{time=0}$. Kinetics of reduction of DPPH·in the presence of trolox or plant methanolic extracts was followed by reading absorbance decrease at 515 nm every 20 s during 16 min. A thirty percent methanol solution was chosen for background correction.

A Beckman Coulter DU 800 Spectrophotometer with 1 cm of pathway was used for all absorbance measurements.

Statistical analyses were achieved with one-way analysis of the variance (ANOVA). When the *p* value indicated significant differences among populations ($p<0.05$), post hoc comparisons were carried out with the Student-Newman-Keuls test, using SPSS 12.0. Principal component analysis (PCA) and analysis of correlation were carried out using Statistica 6.0.

RESULTS

The content of total phenolics and the antioxidant activity of the plant extracts are showed in Table 2. In both cases, results showed three statistically different groups, which matched up with the three species studied.

Table 2
Content of total phenolic compounds and antioxidant activity of *Erica* sp. from different populations.

Plant population and sampling areas	Total phenolic compounds (TP)[a] (μg_{GAE}/mg DW)	Antioxidant activity (AA)[a] (μmol_{TE}/mg DW)	(AA/TP)*100[a] (%)
E. arborea/ slope	81.37 ± 8.63*[,b]	0.54 ± 0.08*	0.67 ± 0.05**
E. australis/ slope	104.87 ± 15.52**	0.70 ± 0.11**	0.68 ± 0.04**
E. australis/ terrace	113.48 ± 15.82**	0.74 ± 0.10**	0.64 ± 0.08**
E. australis/ mine	112.39 ± 12.42**	0.68 ± 0.07**	0.60 ± 0.04**
E. andevalensis/ bank	31.79 ± 9.43***	0.28 ± 0.07***	0.84 ± 0.10*
E. andevalensis/ terrace	33.61 ± 7.41***	0.29 ± 0.06***	0.83 ± 0.10*
E. andevalensis/ mine	37.15 ± 13.20***	0.34 ± 0.08***	0.97 ± 0.41*

[a] Results are the mean ± standard deviation of two replicates of 7 different samples.
[b] Asterisk show the statistical differences of the values at $p < 0.05$ probability level.

The highest phenolic concentrations were found in *Erica australis* and the lowest in *Erica andevalensis.* In parallel, the highest antioxidant activity was observed in *E. australis* and the lowest in *E. andevalensis*. *E. arborea* extracts displayed intermediate values. The total phenolic concentrations did not explain the different antioxidant activity of the different species, as shown by the ratio between the antioxidant activities to the total phenolic concentrations in the seven analysed populations. However, this ratio is similar in samples from the same species and between *E. australis* and *E. arborea*, while the ratio observed in *E. andevalensis* populations was significantly higher than observed in the other species (Table 2).

When all samples were analyzed individually, without grouping in populations, a high linear correlation between the antioxidant activity and the total phenolic levels could be observed as it is shown in Fig. 1A. The correlation analysis was also performed separating data from the three investigated Erica *sp*. As shown in Fig 1B, *E. arborea* showed a higher correlation between the antioxidant activity and the total phenolics, compared to *E. australis* and *E. andevalensis.* This lower correlation was, mainly due to samples of the terrace, in the case of *E. australis*, and of spoils heaps in the case of *E. andevalensis* (results not shown). From data observed in Fig. 1B, it was possible to classify the species by their antioxidant activity or by their phenolics levels, from high to low, as *E. australis* > *E. arborea* > *E. andevalensis*.

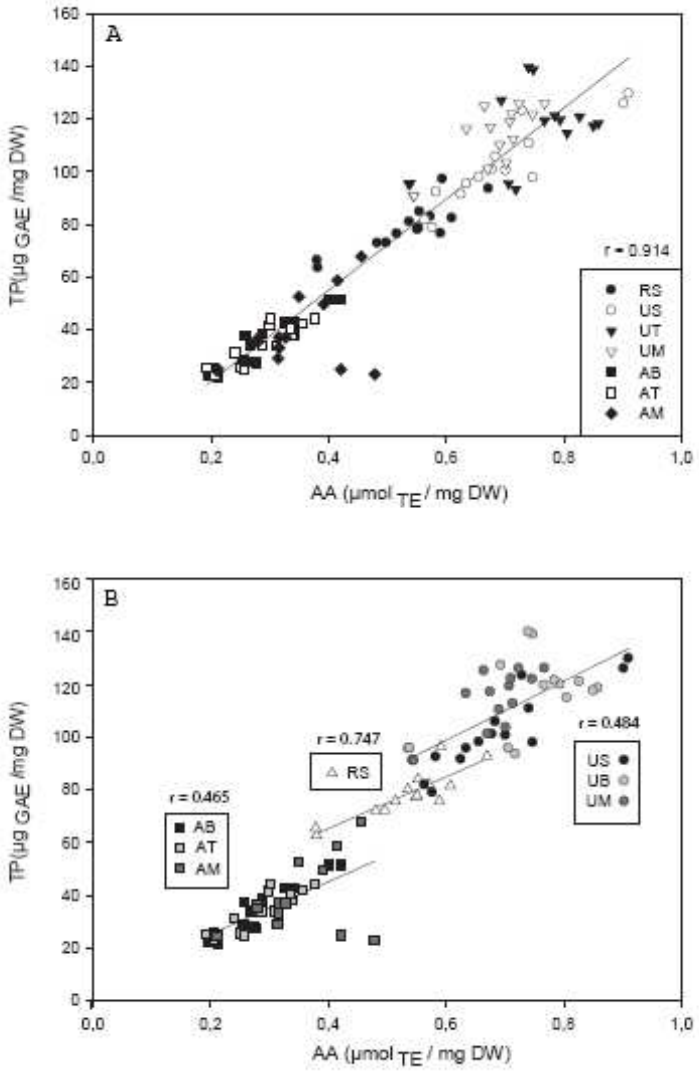


Figure 1. Lineal correlation between antioxidant activity (AA) and the total phenolic compounds concentration (TP). In A, the correlation between the antioxidant activity and the total phenolic compounds concentration in all populations of the investigated species is shown. In B, results of the same correlation analysis are shown for *E. arborea*, *E. australis* and *E. andevalensis* populations, respectively. Two samples per plant were twice analyzed (n=98) as indicated in Materials and Methods. Abbreviations of plant populations as in the Table 1.

As indicated in Materials and Methods, the antioxidative capacity was measured kineticly as the DPPH·reduction by trolox. As shown in Fig. 2, the addition of Trolox to an oxidized DPPH solution resulted in a rapid production of DPPH reaching a constant value of 86% of reduced DDPH at about 7 min from the onset of the experiment. However, the addition of leaves extracts from the seven populations of *Erica* sp. to the oxidized DPPH·solutions rendered biphasic kinetics of production of reduced DPPH with an initial rapid but low reduction of DPPH in the first 2 min, and a second phase of slowly and continuous DPPH·reduction without reaching stabilization at the end of the experimental period and even lower to that observed with Trolox.

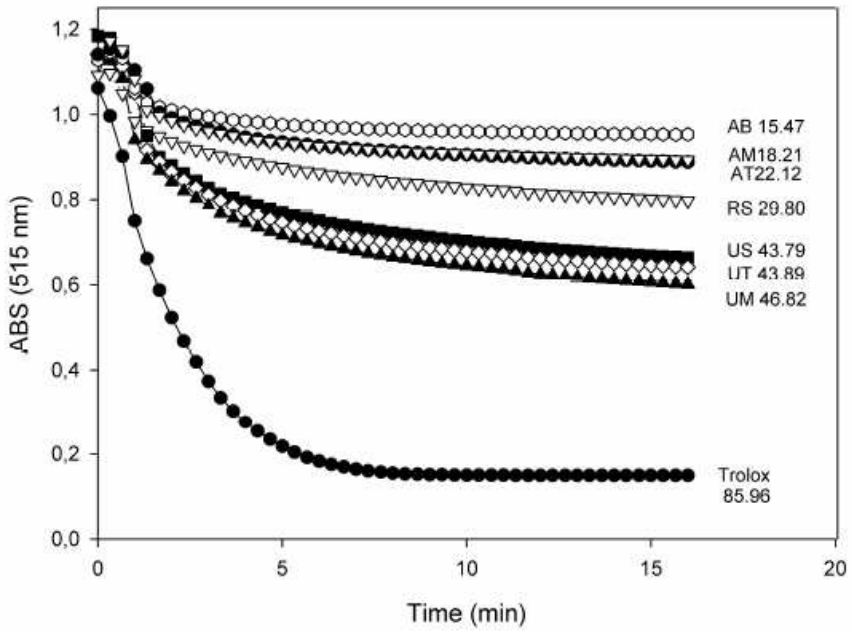


Figure 2. DPPH radical scavenging capacity of extracts of different Ericaceae population. At 0 time, Trolox, used as reference, or sample extracts were added to a solution of oxidized DPPH· radical and formation of reduced DPPH was followed spectrophotometrically by 16 min. Numbers at the end of the kinetics curves indicate the percentage degree of DPPH reduction as indicated in Material and Methods. Abbreviations as in the Table 1

The profile of different phenolic compounds indicated both qualitative and quantitative differences between *Erica* sp. although some patterns might be established (Table 3). From a qualitative point of view, *E. arborea* and *E. australis* shared many phenolic compounds, although some differences were observed. In contrast, *E. andevalensis* showed a different profile lacking many phenolics compounds observed in the other two species and exclusively containing two derivates from cinnamic and coumaric acids. Quantitatively, *E. andevalensis* had the lowest concentration of almost all studied compounds, with some exceptions such as rutin derivate 3.

The composition of the different phenolic compounds was also compared to the habitat the samples were taken from. For *E. andevalensis* no significant differences were found between the three populations analyzed. For *E. australis* the degree of metal-pollution is very different between the slope plants and those flowering in habitats exposed to AMD. However, no significant differences were found in the phenolic composition within these three populations of *E. australis.* However, the phenolic composition in *E. australis* differed considerable from that of *E. andevalensis* taken from the same habitat (*i.e*. river terrace or spoil heaps). When comparing *E. australis* and *E. arborea* from slopes, a similar qualitative profile of phenolic compounds but a number of significant differences in many specific phenolics, particularly in the case of myricetin, were observed.

Table 3

Concentration of individual phenolic compounds identified by HPLC in the different plant populations studied

COMPOUND	CODE	RS	US	UT	UM	AB	AT	AM
PHENOLIC ACIDS								
Ellagic acid	**E**	1.894±0.579^{*,b}	2.173±0.781*	2.400±0.680*	1.898±0.658*	0.751±0.122**	0.809±0.059**	0.762±0.073**
Vanillic acid	**VA**	0.304±0.255*	0.078±0.019**	0.060±0.025**	0.063±0.019**	0.045±0.008**	0.041±0.004**	0.049±0.007**
Cinnamic acid derivate	**CinD**	ND^{c}	ND	ND	ND	0.091±0.049*,**	0.076±0.023*	0.111±0.022**
***m*-Cumaric acid**	***m*-Cu**	0.136±0.008*	0.089±0.020**	0.205±0.081***	0.074±0.013**	ND	ND	ND
Caffeic acid	**Caf**	1.510±0.769	ND	ND	ND	ND	ND	ND
Caffeic acid derivate	**Caf D**	ND	0.298±0.136	0.319±0.179	0.481±0.273	ND	ND	ND
***p*-Cumaric acid derivate 2**	***p*-C2**	ND	0.221±0.099	0.886±0.028	0.168±0.072	ND	ND	ND
***p*-Cumaric acid derivate 1**	***p*-C1**	ND	ND	ND	ND	0.791±0.318	0,639±0,217	0.582±0.096
FLAVONOIDS								
Catechin	**C**	0.547±0.392*	0.286±0.116**	0.243±0.085**	0.186±0.067**	0.218±0.117**	ND	ND
Epicatechin	**EP**	0.466±0.235**	2.494±0.606*	2.042±0.293*	2.079±0.544*	0.257±0.064**	0.450±0.231**	0.662±0.138**
Catechin derivate 1	**C1**	0.260±0.145	0.276±0.161	0.326±0.155	0.301±0.189	ND	ND	ND
Catechin derivate 2	**C2**	ND	0.240±0.076*	0.207±0.046*	0.144±0.029**	ND	ND	ND
Catechin derivate 3	**C3**	0.510±0.157	0.566±0.143	0.659±0.312	0.762±0.395	ND	ND	ND
Catechin derivate 4	**C4**	1.631±1.048*	0.596±0.164**	0.543±0.114**	0.656±0.292**	ND	ND	ND
Catechin derivate 5	**C5**	0.598±0.077	ND	ND	ND	ND	ND	ND
Catechin derivate 6	**C6**	0.258±0.062	0.717±0.357	0.573±0.296	0.478±0.133	ND	ND	ND
Catechin derivate 7	**C7**	ND	0.273±0.096	0.217±0.033	0.273±0.096	ND	ND	ND
Catechin derivate 8	**C8**	2.645±0.843*	0.564±0.119**	0.590±0.172**	ND	ND	ND	ND
Rutin	**R**	1.628±0.570*	2.060±0.857*	2.644±1.180**	1.772±0.564**	0.536±0.091***	0.620±0.081***	0.593±0.079***
Rutin derivate 1	**R1**	1.318±0.599*	2.989±1.194**	4.390±1.445***	2.945±0.911**	1.304±0.396*	1.051±0.299*	1.106±0.217*
Rutin derivate 2	**R2**	1.347±0.350*	2.362±0.847**	4.102±1.487***	2.868±0.875**	ND	ND	ND
Rutin derivate 3	**R3**	3.511±1.775*	0.673±0.284**	0.952±0.642**	0.774±0.210**	3.565±1.624*	3.244±0.734*	2.276±0.598*
Rutin derivate 4	**R4**	0.466±0.147	0.691±0.311	1.056±0.385	0.740±0.155	ND	ND	ND
Kaempferol	**K**	0.491±0.155*,**	0.557±0.114*	1.009±0.297**	0.652±0.121*,**	0.868±0.218*,**	0.936±0.154*,**	0.768±0.087*,**
Myricetin	**M**	11.445±1.477*	0.935±0.077**	0.992±0.208**	1.035±0.195**	0.833±0.061**	0.800±0.026**	0.745±0.025**

[a] Results are the mean of two replicates of seven different samples. All data are expressed as lg/mg DW.

[b] Asterisk show the statistical differences of the values at $p < 0.05$ probability level in each row. No asterisk in a row means no statistical differences at $p < 0.05$ probability level.

[c] ND = not detected.

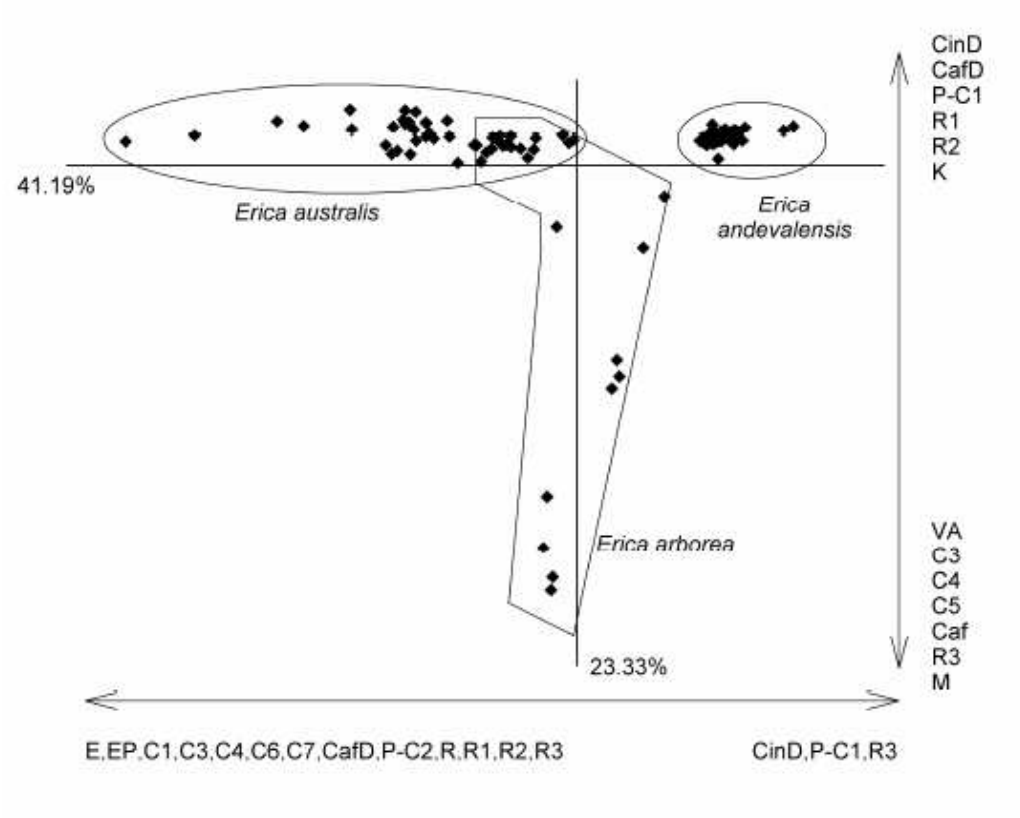


Figure 3. Principal component analysis of the individual phenolic compounds identified by HPLC. The axis 1, horizontal, explains 41.19% of the variance, and the second axis explains 23.33%. The arrows at the edges of the figures (down and right) show the different phenolic compounds which are responsible for the grouping of the different species. Abbreviations of phenolic compounds as shown in the table 3.

To facilitate the comprehension of all data showed in table 3, a principal component analysis (PCA) of the phenolic concentrations in all samples was performed (Fig.3). *E. andevalensis* data formed an independent and homogenous group, characterized by the presence of cinnamic acid derivate, p-coumaric acid derivate 1 and rutin derivate 3. *E. australis* appeared on the opposite part of the axis 1, and it was characterized by ellagic acid, epicatechin, the catechin derivates 1, 3, 4, 6 and 7, caffeic acid derivate, p-coumaric acid derivate 2 and rutin and rutin derivates, except derivate 3. *E. arborea* held an intermediate position, but closer to *E. australis* than to *E. andevalensis*. However, *E. arborea* had a scattered

distribution mainly in axis 2. Because of the different pattern shown by *E. arborea*, the PCA was repeated only with data derived from *E. andevalensis* and *E. australis* extracts, but no modification of the points distribution, with respect to those showed in Fig. 3 was observed (data not shown).

DISCUSSION

Erica andevalensis is an extreme species that only grows in very acidic, metal-polluted areas exposed to severe AMD in the Iberian Pyritic Belt (Cabezudo and Rivera 1980; Nelson et al. 1985; Soldevilla et al. 1992; Heras et al. 2002; Rodríguez et al. 2007). It is expected to have evolved strategies to cope with the high level of metals in its environment such as defence mechanisms against oxidative stress induced by metal-catalysed reactions (Briat and Lebrun 1999; Clijster et al. 1999; Michalak 2006; Okamoto et al. 2000). Since prevention of cell oxidative damage in plants involves a variety of low and high molecular mass compounds, as ascorbic acid, glutathione, carotenoids, reductases, peroxidases, etc. (Smirnoff 1998; Mittler 2002; Inzé and Van Montagu 1995; Clijsters et al. 1999), it could be predicted that one of the strategies that these plants exposed to continuous substrate stressors in the Iberian Pyritic Belt have evolved is the synthesis of specific antioxidant protectors. For instance, we previously reported that *E. andevalensis* possesses an efficient ascorbate peroxidase that may partially counteract oxidative damage (Márquez-García 2006). To study the possible role of phenolic compounds as reductive agents to prevent oxidative damage derived from metal exposition in *E. andevalensis*, we have performed a detailed analysis of the phenolic composition and antioxidant activities of *E. andevalensis* extracts. Results were compared to those obtained from *E. australis* (growing in soils exposed and not exposed to AMD) and *E. arborea* (growing in not polluted soil).

The total phenolic concentration in the *Erica* sp. is slightly higher than

observed in aromatic Greek species as *Capparis spinosa*, *Castanea vulgaris*, *Geranium purpureum*, *Nepeta cataria*, *Origanum dictamnus*, *Phytolacca americana*, *Ruta graveolens* and *Styrax officinalis* (Proestos et al. 2006) and similar to that observed in *Plantago* species (Grubešic et al. 2005), a variety of Iranian medicinal plants as *Mellilotus officinalis*, *Equisetum maximum*, *Plantago major*, *Adiantum capillus-veneris* and *Urtica dioica* (Pourmorad et al. 2006) and in endemic *Tanacetum* sp. from Turkey (Tepe and Sokmen 2007). Our results agree with the correlation that the higher phenolic concentration correspond to a higher antioxidant activity described in other plant species (Pourmorad et al. 2006 and Tepe and Sokmen 2007). This may suggest that antioxidative activity is depending directly on specific levels of phenolics more than on the adaptation of one species to different habitats.

Surprisingly, our results are controversial since in spite of the oxidative stressing environmental conditions derived from the exposition to high metals concentration in acid soils, *E. andevalensis* has the lowest antioxidant activity, the lowest total phenolic levels and the simpliest composition and lowest concentration of individual phenolic compounds, with some exceptions, compared to *E. arborea* and *E. australis*. Thus, the above hypothesis may be initially rejected since *E. andevalensis* adaptation to acid, metal-polluted soils, can not be explained by its total antioxidant activity nor the phenolics variety and concentration. Measuring overall phenolic concentrations could mask the changes and possible roles of individual compounds in the antioxidative response. Thus, qualitative differences between the populations of *E. andevalensis* with respect to those of *E. arborea* and *E. australis* were investigated to try to explain the peculiar distribution of the former species in the acidic, high metal-polluted environment. In this respect, more studies will be designed to identify and analyse the physiological role of the cinnamic acid derivate and the p-coumaric acid derivate 1 in *E. andevalensis*, two phenolics exclusively present in *E. andevalensis* and not in *E. australis* and *E. arborea.*

However, there is an alternative interpretation of our results based mainly on data published by Sakihama et al. (2002) and Sakihama and Yamasaki (2002). These authors demonstrated that a non-divalent metal as Al, then unable to catalyse Fenton-like reactions enhances the phenolic-induced lipid peroxidation by a spin-stabilizing effect of the phenoxyl radical (Kalyanaraman 1990). Thus, the phenoxyl radical produced through antioxidative reactions is a potential prooxidant by synergistic interactions with heavy metals. For instance, our previous data showed that Al concentration in leaves of *E. andevalensis* reached about 1,000 ppm, more than 3 times the concentrations measured in *E. australis* and *E. arborea*. Considering that Al is taken up easily by plants at acid pH (Kabata-Pendias and Pendias 2000; Kochian et al. 2004), a suggestive hypothesis to understand our results could be that *E. andevalensis* adaptation to its harse environment, was possible by a reduction of phenolics variety and concentration in such a way that prooxidant effects of phenolic compounds were prevented.

Our results also show that *E. australis* possesses the greatest antioxidant activity together with the highest phenolic levels, suggesting that these compounds may be one of the reasons to explain its success to flowering in many environmental conditions. Moreover, *E. arborea* contains more phenolics and more antioxidant activity than *E. andevalensis*. This fact could be in relation with the observation described by Kraus et al. (2004) about plants growing in poor soils tend to contain high concentration of phenolic. Taken together the results presented here suggest that total phenolic concentration in *E. arborea*, *E. australis* and *E. andevalensis* depends mainly on constitutive genetic differences more than on the different environmental conditions affecting the physiology of the studied species.

Acknowlodgements

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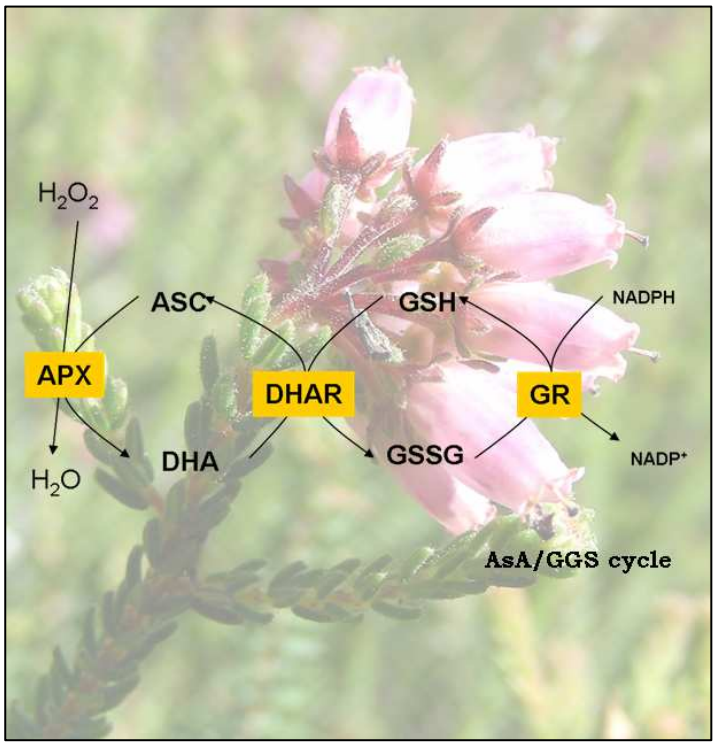
3. ANTIOXIDANT MECHANISMS IN *ERICA* SPP. DIFFERENTIALLY EXPOSED TO METAL POLLUTION IN SOUTH IBERIAN PENINSULA

Mecanismos antioxidantes en *Erica* spp. expuestas a distintos grados de contaminación por metales en el sur de la Península Ibérica



ANTIOXIDANT MECHANISMS IN *ERICA spp*. DIFFERENTIALLY EXPOSED TO METAL POLLUTION IN SOUTH IBERIAN PENINSULA

ABSTRACT

The Iberian Pyritic Belt is a geological formation characterized by a high content of heavy metal and very acidic soils. Different populations of *Erica andevalensis* Cabezudo & Rivera, *Erica australis* L. and *Erica arborea* L. have been studied when they grow in soils with different metal composition. The aim of the present work was to investigate the antioxidant mechanisms that the different species have developed to cope with the metals and reactive oxygen species (ROS) produced in the cells. *E. andevalensis*, an endemic heather always growing associated with acid and heavy metal enriched soils, have developed an efficient defence system based on ascorbate peroxidase (APX) reaching high values. On the other hand, *E. australis* antioxidant system consists of high levels of ascorbic acid (AsA). *E. arborea*, species never growing in soils affected directly by mining activities, did not show any special enhancement in the analysed parameters.

KEYWORDS

Erica andevalensis; oxidative stress; ascorbic acid; heavy metals; Iberian Pyritic Belt.

INTRODUCTION

The so-called “Iberian Pyritic Belt” is the largest accumulation of massive sulphide on Earth, stretching from the North of Seville (Spain) to the South of Lisbon (Portugal). Pyrite, its main constituent, has been exploited for over 5000 years (Nelson and Lamothe 1993; van Geen et al. 1999; Davis et al. 2000, Sainz et al. 2003; Sánchez España et al. 2005). Pyrite mines, as in general mining areas, form a significant burden on the natural environment, where endemic plant species appear to be adapted to the particular soil conditions. Typically, water, oxygen and sulphur-oxidizing bacteria leading to the acidification and heavy metal pollution of soil water and nearby rivers. This phenomenon is known as acid mine drainage or AMD (Davis et al. 2000; Sainz et al. 2005; Ferreira da Silva et al. 2005). The province of Huelva, located at SW Spain, is crossed by two rivers, the Odiel and the Tinto, both affected by AMD and showing high concentration of heavy metals, mainly Fe, Mn, Zn, Cu and Pb (Nelson and Lamothe 1993; van Geen et al. 1999; Sainz et al. 2003; Braungardt et al. 2003; Montes-Botella and Tenorio 2003).

Metals, essentials or not, are toxic for plants when they are present in high concentrations. Plants possess mechanisms to avoid the excess uptake and also to cope with metals when the internal concentration in the cells is too high and interferes with the normal cell metabolism. When metals enter the cells they can react catalyzing the oxygen reduction with the subsequent formation of reactive oxygen species (ROS), which results in the production of oxidative stress that can injure cells by inducing lipid peroxidation, protein oxidation, enzyme inhibition and DNA damage (Clijsters et al. 1999; Prasad et al. 1999; Briat and Lebrun 1999; Dat et al. 2000; Briat 2002). Plants have evolved different antioxidant mechanisms consisting of enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase, or small metabolites such as ascorbic acid to cope with them (Inzé and van Montagu 1995; Arrigoni and de Tullio 2002).

In some of the most inhospitable environments of the province of Huelva, such as the Odiel and the Tinto River banks and terraces and onto the tailings piles, with soils pH as low as 2.5 and elevated concentrations of Fe, Cu or Zn (Soldevilla et al. 1992; Asensi et al. 1999; Rodríguez et al. 2007) an endemic heather, *Erica andevalensis* Cabezudo and Rivera (1980), is able to grow while no other species can do it. In Portugal, this remarkable species has also been encountered in the Sao Domingos Mines, a polluted site showing similar characteristics as the site described in this study (Capelo et al. 1998; Turnau et al. 2007; Abreu et al. 2008). Surprisingly, *E. andevalensis* has never been seen growing out of the influence of metals and acid soils, but in mildly polluted sites another heather *Erica australis* L. can be found (Valdés et al. 1987; Soldevilla et al. 1992; Abreu et al. 2008).

The aim of the present work was to investigate the antioxidant mechanisms that different populations of *E. andevalensis* possess to survive in different highly polluted and acid soils. This study included *E. australis*, due to its ability to survive in polluted soils sharing habitat with *E. andevalensis* when the conditions become less extreme and in non-mining affected soils. A third species, never growing in polluted mining soils, *E. arborea*, has also been included for comparison purposes. Total and EDTA-extractable metal content in soils, metal content in leaves, analyzed in the chapter 1 will be referred, as well as antioxidant enzymes activity and antioxidants contents in leaves of all the three heathers, have been thoroughly analyzed in order to establish a possible mechanism to explain the metal tolerance.

MATERIAL AND METHODS

Plant and soil sampling

This study was carried out with samples collected in January 2006 in two different points of the Pyritic Belt, based in the different metal composition of the soils (Chapter 1). From the first point, located at the Odiel River (37º 43' N – 6º 42' W), samples from 5 different populations were collected. Here the pollution degree gradually increases from the river bank, were only *E. andevalensis* could be sampled because no other species withstands the extreme conditions, to the river terrace, were *E. australis* appears sharing habitat with *E. andevalensis*, and to the slope of a hill, located nearby (but elevated above) were *E. australis* grows together with other typical Mediterranean bushes, including *E. arborea*, also included in our study. The second point of the Pyritic Belt was chosen on a mine spoil heap, near Nerva village (37º 41' N - 6º 33' W). On this site, *E. andevalensis* and *E. australis* were found growing in a small hill resulting from the mine extraction accumulated- material.

Samples were taken from plants of the same size and with the same orientation to the sun and samples from 7 different individual plants were collected from each population for all biochemical analysis. The leaves were immediately frozen in liquid N_2 in the field, and stored at -80ºC in the laboratory for further analysis.

Water content and soluble proteins

The water content was calculated drying the leaves samples at 60ºC until constant weight was reached. Protein concentration was measured following the method described by Bradford (1976) (Fig. 1).

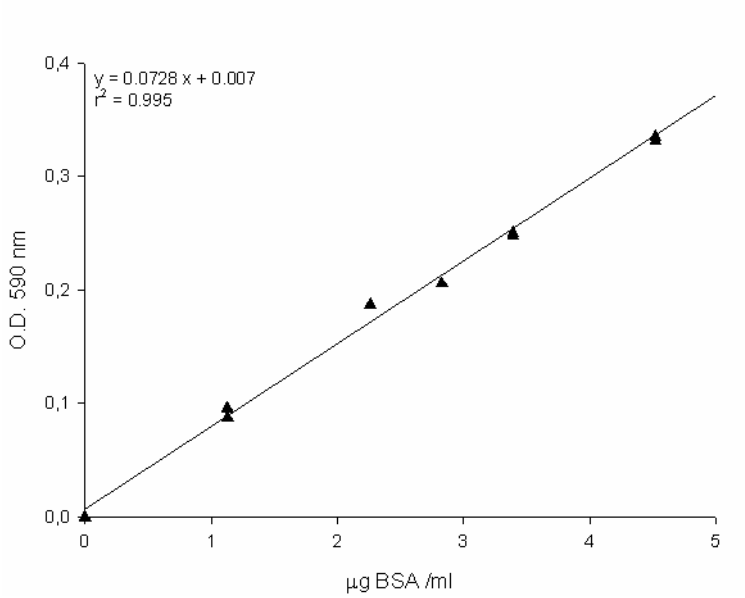


Figure 1. Calibration curve of protein, using BSA as standard.

Total ascorbic acid measurements

Ascorbic acid (AsA) in leaves was quantified using the bypiridyl method (Knörzer et al. 1996). In the extractions, the frozen material was grounded in a pre-cooled mortar and liquid N_2. When a homogenous powder was obtained 1 mL of 5 % meta-phosphoric acid was added. The homogenate was then centrifuged at 10,000 g at 4º C for 30 min. The resulting supernatant was kept at -80º C until analysis. For the analysis, the samples were taken out and keep in ice. An aliquot fraction of 20 µL was pipetted and 105 µL of 5 % meta-phosphoric acid were added. The solution was neutralized with 25 µL of 1.5 M triethanolamine, and buffered with 150 µL of 150 mM sodium phosphate buffer (pH 7.4). Seventy five microlitres of 10 mM dithiothreitol (DTT) were added and the samples were incubated for 15 min in the dark at room temperature. This step reduced all the possible oxidized forms of ascorbic acid, and the total form is obtained. Afterwards

75 µL of 0.5 N-ethylendiamine (NEM) were added to stop the reaction and successively, followed by vortex after each addition, 300 µL 10 % trichloroacetic acid, 300 µL 44 % ortophosphoric acid, 300 µL 4 % 2,2-bypiridyl (dissolved in 70 % ethanol) and 150 µL 3 % iron chloride (III) were added. For the measurement of the reduced form of ascorbic acid present in the samples 150 µL of deionised water was added instead of the DTT and NEM and the rest of protocol continue in the same way as described above. The mixture was incubated 1 hour at 37 º C and the absorbance was measured at 525 nm. The oxidized form of ascorbic acid (DHA) was calculated subtracting the reduced form to the total concentration. The values were compared with a calibration curve with reduced ascorbic acid (Fig. 2).

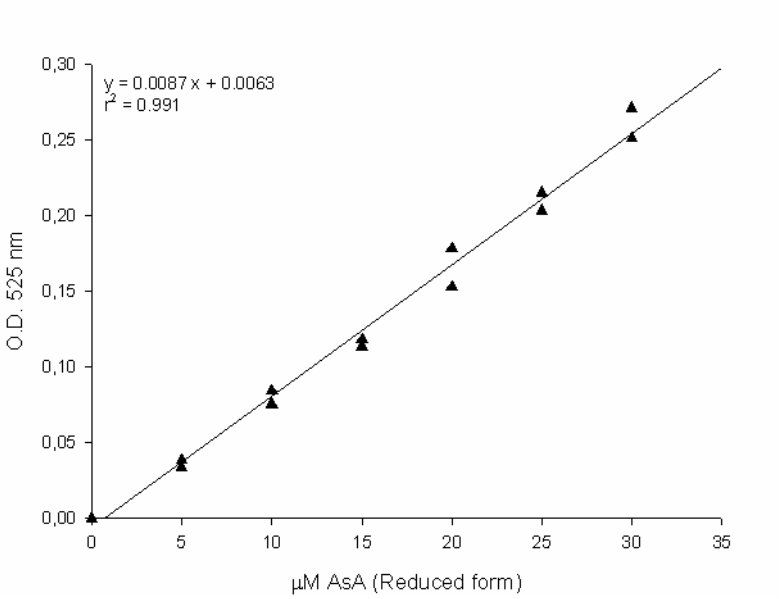


Figure 2: Calibration curve of ascorbic acid.

Enzymatic activities

Leaves collected at the field were frozen in liquid N_2 until needed. For analysis, leaves were grounded with a porcelain mortar and pestle. The extract was prepared with 1 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA, 1

% (v / v) phenylmethanesulphonylfluoride, 10 mM DTT, 3 % insoluble polyvinyl pyrrolidone and 5 mM ascorbic acid (only for ascorbate peroxidase, and then without DTT). The homogenized material was centrifuged for 20 min at 10,000 g, using the supernatant for determining the enzymatic activities. For activities determination about 30 μg of protein were added to the reaction mixture. Unless otherwise stated, one unit was defined as the consumption of one micromole of substrate per minute.

The catalase activity (CAT; E.C. 1.11.1.6) was determined using the method described by Aebi (1983). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5) and 10 mM H_2O_2. The reaction was started with the addition of the sample, and the decomposition for H_2O_2 was followed at 240 nm (molar extinction coefficient = 43.6 mM^{-1} cm^{-1}).

The superoxide dismutase activity (SOD; E.C. 1.15.1.1) was measured following the method described by McCord and Fridovich (1969). The dismutation of superoxide was assayed measuring the inhibition of xanthine oxidase-dependent reduction of nitroblue tetrazolium (NBT). The reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM EDTA, 50 μL 10 mM xanthine, 50 μL 1 mM NBT and 0.04 units of xanthine oxidase. The reduction of NBT was followed at 530 nm. One unit of SOD was defined as the amount of enzyme that inhibits the rate of absorbance increase by 50 %.

The guaiacol peroxidase activity (POD; E.C. 1.11.1.7) was measured according to Zheng and Van Huystee (1992), with some modifications. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.03 % H_2O_2, 0.01 % guaiacol and about 30 μg of protein. The total peroxidase activity was determined by spectrophotometry following the rate of formation of tetraguaiacol at 470 nm (molar extinction coefficient = 26.6 mM^{-1} cm^{-1}).

The ascorbate peroxidase activity (APX; E.C. 1.11.1.11) was determined according to the method described by Asada (1984), with some modifications (Amako et al. 1994). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM ascorbic acid, 10 mM H_2O_2 and about 30 μg of protein. The reaction was started with the addition of hydrogen peroxide (1 mM) and the rate of oxidation of ascorbic acid was followed at 290 nm (molar extinction coefficient = 2.80 mM^{-1} cm^{-1}).

Lipid peroxidation measurements

As described by Hodges et al. (1999), lipid peroxidation was estimated from the amount of malondialdehyde (MDA). Frozen leaves were homogenized in 80 % ethanol in a mortar, with liquid N_2. The extract was centrifuged at 3,000 g for 10 min and the supernatant was used. The method made a correction of the interferences by subtracting absorbance values of samples without thiobarbituric acid (TBA). A 1 mL of diluted sample was added to a 1 mL of either i) –TBA solution comprised of 20 % (w / v) trichloroacetic acid and 0.01 % butylated hydroxytoluene, or (ii) +TBA solution containing the above plus 0.65 % TBA. Samples mixed and heated at 95 ºC for 25 min and then cooled and centrifuged at 3,000 g for 10 min. The absorbance was read at 440, 532 and 600 nm and the following equations were applied:

$$[(Abs\ 532_{+TBA})-(Abs\ 600_{+TBA})-(Abs\ 532_{-TBA} - Abs\ 600_{-TBA})] = A$$

$$[(Abs\ 440_{+TBA} - Abs\ 600_{+TBA})\cdot 0.0571] = B$$

$$MDA\ equivalents\ (nmol\ mL^{-1}) = [(A - B)/157]\cdot 10^3$$

Hydroperoxide and hydrogen peroxide measurements

Hydroperoxide concentration (R-OOH) was estimated following the method described by Gay et al. (1999) with the modifications of Gay and Gebicki (2000) in order to increase the sensitivity of the method. Leaves (0.1 g) were homogenized in 1 mL 10 mM potassium phosphate buffer (pH 7) in a mortar with liquid N_2. The extract was centrifuged at 1,000 g for 5 min. The measure was carried out adding 100 µL supernatant to a 900 µL of a mixture in 25 mM sulphuric acid composed by 100 µM xylenol orange, 250 µM iron (II) sulphate and 100 mM sorbitol. The mixture was kept 45 min at room temperature. Absorbance was measured at 560 nm. A calibration standard curve was used for calculations (Fig. 3).

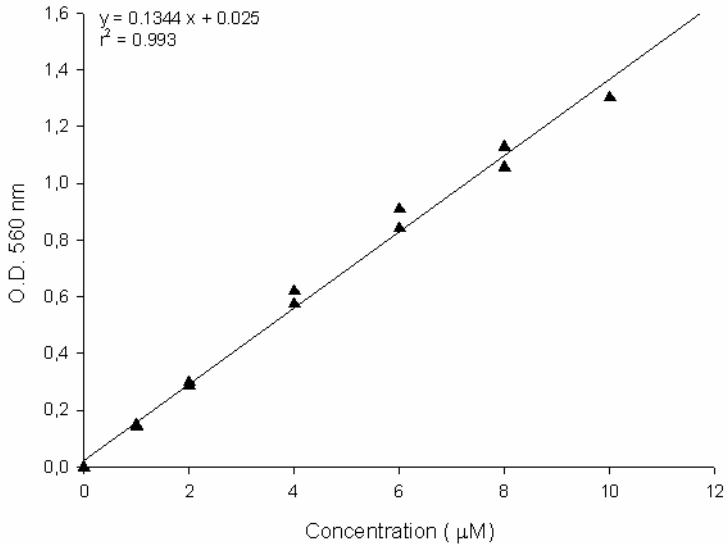


Figure 3: Calibration curve of R-OOH

Internal H_2O_2 was determined following the method described by Jena and Choudhuri (1981) adapted by Lin and Kao (1998). Frozen leaves (0.05 g) were ground in 50 mM potassium phosphate buffer (pH 6.5) in a mortar with liquid N_2.

The homogenate was centrifuged at 6,000 g for 25 min. The supernatant (0.75 mL) was added to a 0.25 mL of reaction mixture consisting of 0.1 % titanium sulphate in 20 % H_2SO_4 (v / v). The mixture was centrifuged at 6,000 g for 15 min and absorbance was measured at 410 nm. A calibrated standard curve was used for calculations (Fig. 4).

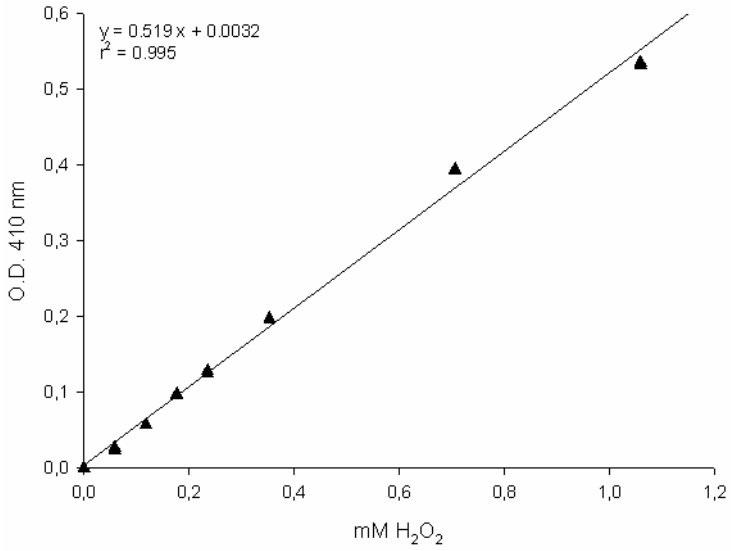


Figure 4: Calibration curve of H_2O_2.

Photosynthetic pigments

For the determination of carotenes and chlorophylls *a* and *b*, approximately 100 mg of fresh weight of leaves were used. Plant material was homogenized using acetone in a mortar, with liquid N_2. The extract was centrifuged at 10,000 g for 10 min (Arnon 1949). Finally, the mentioned pigments were determined by means of spectrophotometry from the supernatant (Lichtenthaler 1987).

$$\text{Chlorophyll a} = 11.24\ \text{Abs}_{661.6} - 2.04\ \text{Abs}_{644.8}$$

$$\text{Chlorophyll b} = 20.13 \cdot \text{Abs}_{644.8} - 4.19\ \text{Abs}_{661.6}$$

$$\text{Carotenes} = (1000 \cdot \text{Abs}_{470} - 190 \cdot \text{Chlor a} - 63.14 \cdot \text{Chlor b}) / 214$$

Statistical analysis

All spectrophotometric analyses were expressed as mean of 7 independent (measured twice and averaged) originated from 7 individual plants collected from each site ± SEM (standard error of the mean; $n = 7$). A general lineal model was employed to estimate species effects, site effects and allowing interactions. Data of two species (*E. australis* and *E. andevalensis*) for which we had enough information were analysed separately through an ANOVA. A different post-hoc analysis was used depending on the variances' homogeneity (Duncan or Dunett T3, respectively), as previously tested with a *Levene's* test. All statistical analyses were performed using SPSS.

RESULTS

The pH and metal content in soils, as well as the EDTA extractable metals and metal content in leaves are showed in Tables 1 and 2 in Chapter 1.

Despite the high metal content, none of the studied population of *E. andevalensis, E. australis* or *E. arborea* showed macroscopic injuries, and all of the populations seemed to be in a good state of health.

Total soluble protein content was different in every studied species. The smaller amounts were found in *E. andevalensis* (Fig. 5 A). Concerning each population, *E. australis* showed more protein growing in the Tailings than in the Slope ($p<0.05$), whereas *E. andevalensis* growing in the Bank showed the smallest protein content ($p<0.05$). Here, the data were expressed per dry weight, but similar results were found when expressed on fresh weight basis (data not shown). In Fig. 5 B, the relative water content is displayed. No significant differences were found

in *E. andevalensis* populations; however, *E. australis* accumulates more water in plants growing in the Tailings, followed by plants from the Terrace ($p<0.05$).

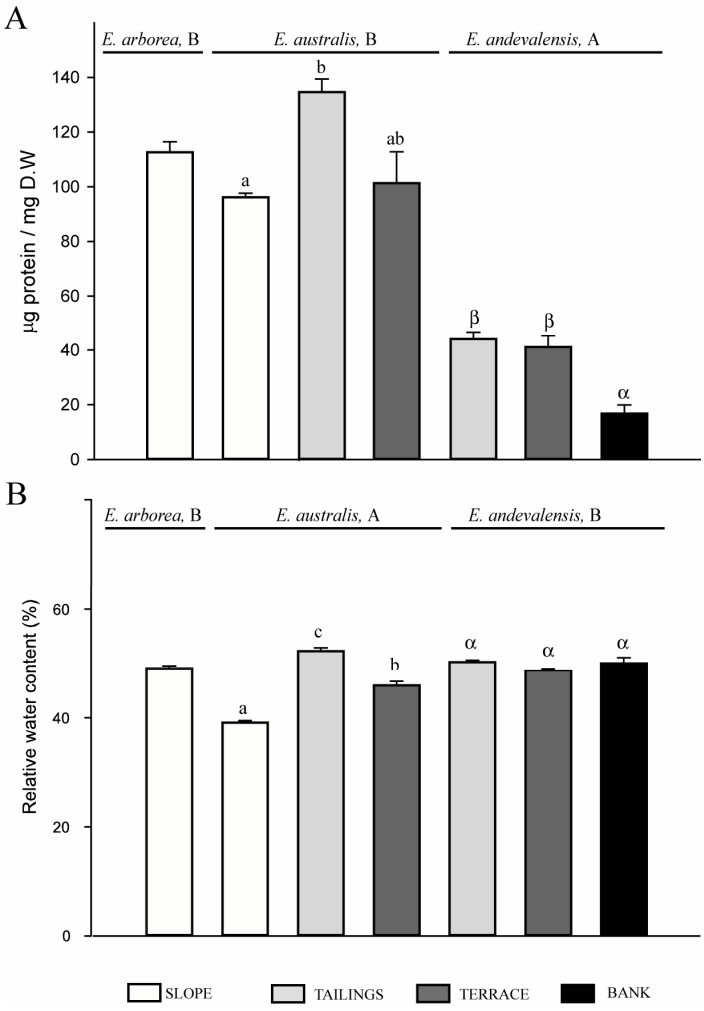


Figure 5. Protein content (A), and relative water content (%) (B) of the three *Erica* sp., sampled at four different locations of increasing heavy metal pollution (Control (white), Tailings pile (light grey), Terrace (dark grey) and Bank (black). Data represents means ± SEM (n = 7). Capital letters next to the species name on the top of the graph indicate statistical significant differences among species. Lower-case letters (a, b, c) or Greek letters (α, β, γ) indicate statistically differences for intra-species comparisons.

Ascorbic acid (AsA) content was measured using the bypiridyl method (Fig. 6), and significantly higher values were found in *E. australis* ($p<0.05$) than in the other two species, as much in the reduced as in the total forms. However, there were not significant differences among the populations of the same species. The ratio DHA/AsA reduced (Fig. 6 C) was in all the cases lower than 0.6, without significant differences among the studied species. However, differences appeared when *E. australis* was analyzing in the different soils studied, and plants growing in the Tailings were which possessed the highest ratio ($p<0.05$).

In relation with the antioxidant enzymes, SOD activity did not presente significant differences among the studied species. *E australis* from the Tailings presented the highest value when populations of this species were compared and in the case of *E. andevalensis* it was also the Tailings the place where this species activate more SOD (Fig. 7A), but without significant differences among *E. andevalensis* populations. The CAT activity (Fig. 7B) in *E. australis* was lower in the samples from the Terrace site than those collected in other locations. No differences ($p<0.05$) were found in *E. andevalensis* (Fig. 7A) populations. POD activity (Fig. 7C) was lower in *E. australis* populations growing in the Terrace when compared to the Slope location ($p<0.05$), but was higher in the Terrace specimens compared with those collected at the Bank. APX showed the highest activity, reaching values as high as 801.01 ± 57.70 U mg^{-1} protein in *E. andevalensis* growing in the Bank (Fig. 7D), statitistically different to the other populations of this species and, also higher than *E. australis* growing in the Terrace, which presented 267.97 ± 45.18 U mg^{-1} protein but without differences ($p<0.05$) among *E. australis* populations.

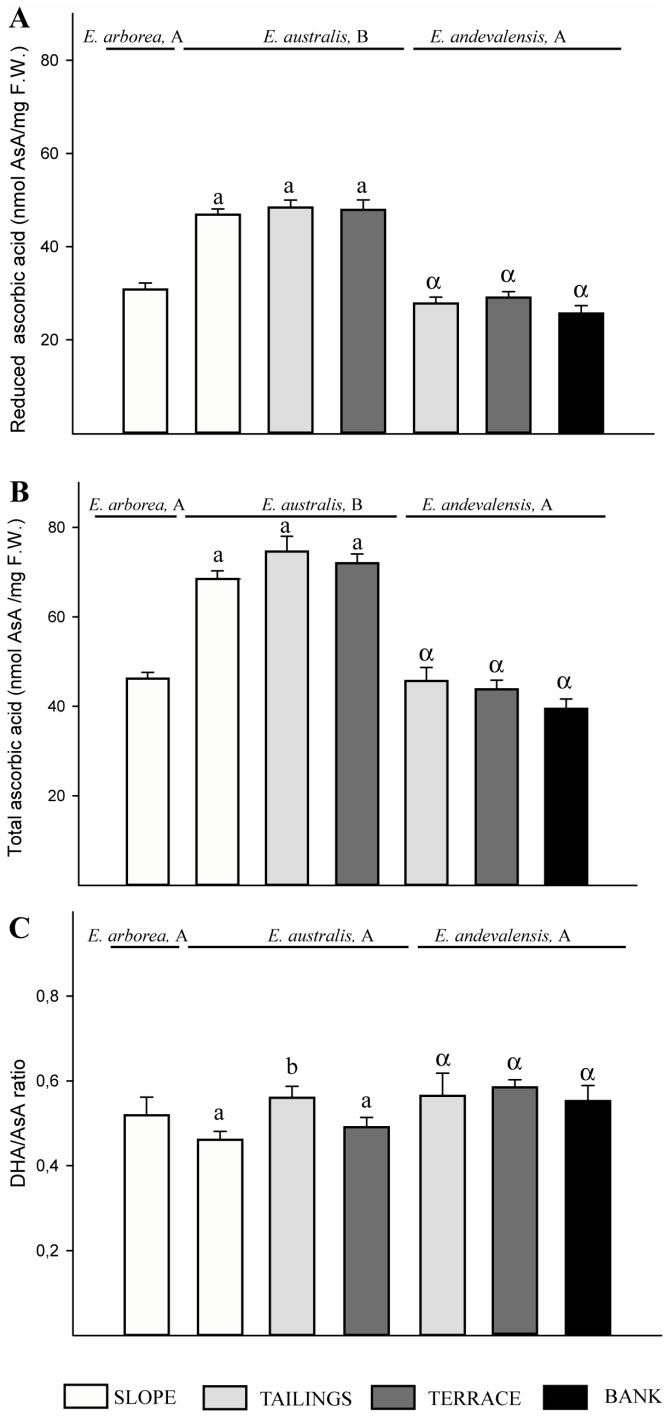


Figure 6. The concentration of AsA in the reduced and total forms (nmol AsA / mg F.W.) and DHA/AsA ratio of three *Erica* sp. sampled at four different locations. Data represent means ± SEM (n = 7). Legends and notes as in Fig. 5.

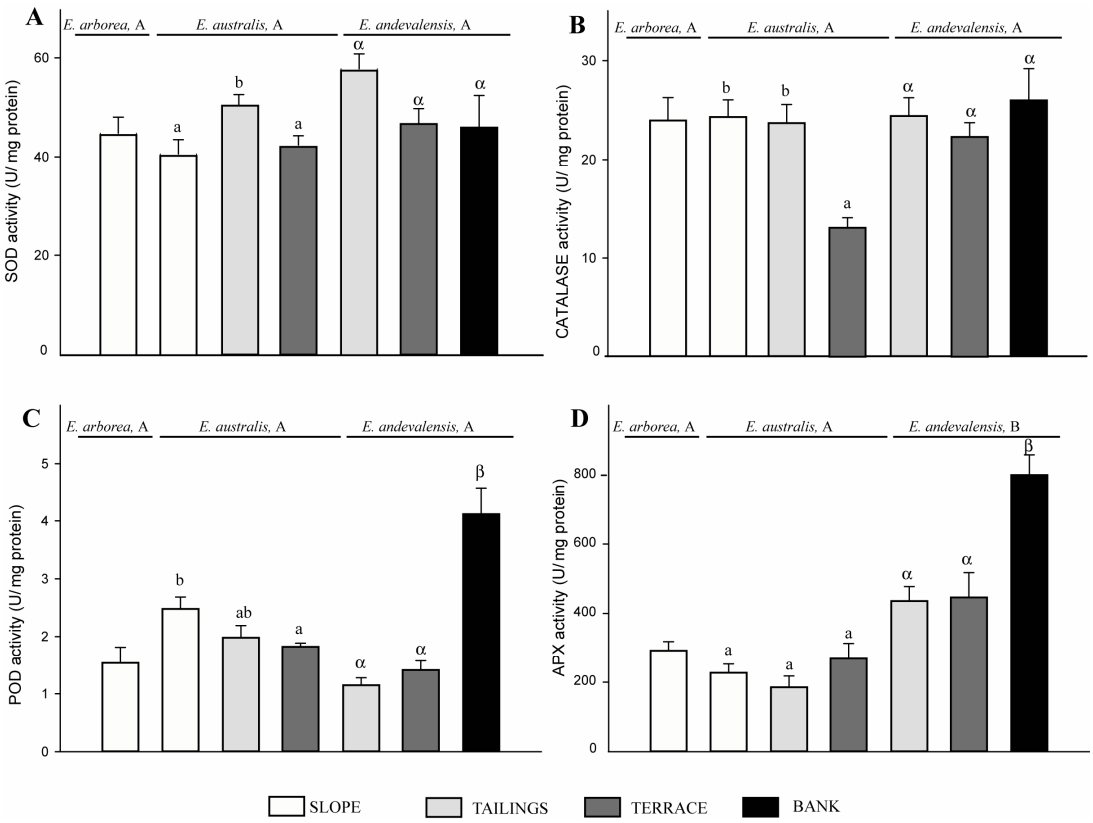


Figure 7. The activity of SOD (A), CAT (B), POD (C) and APX (D), of three *Erica* sp., sampled at four different locations. Data represent means ± SEM (n = 7). Legends and notes as in Fig. 5.

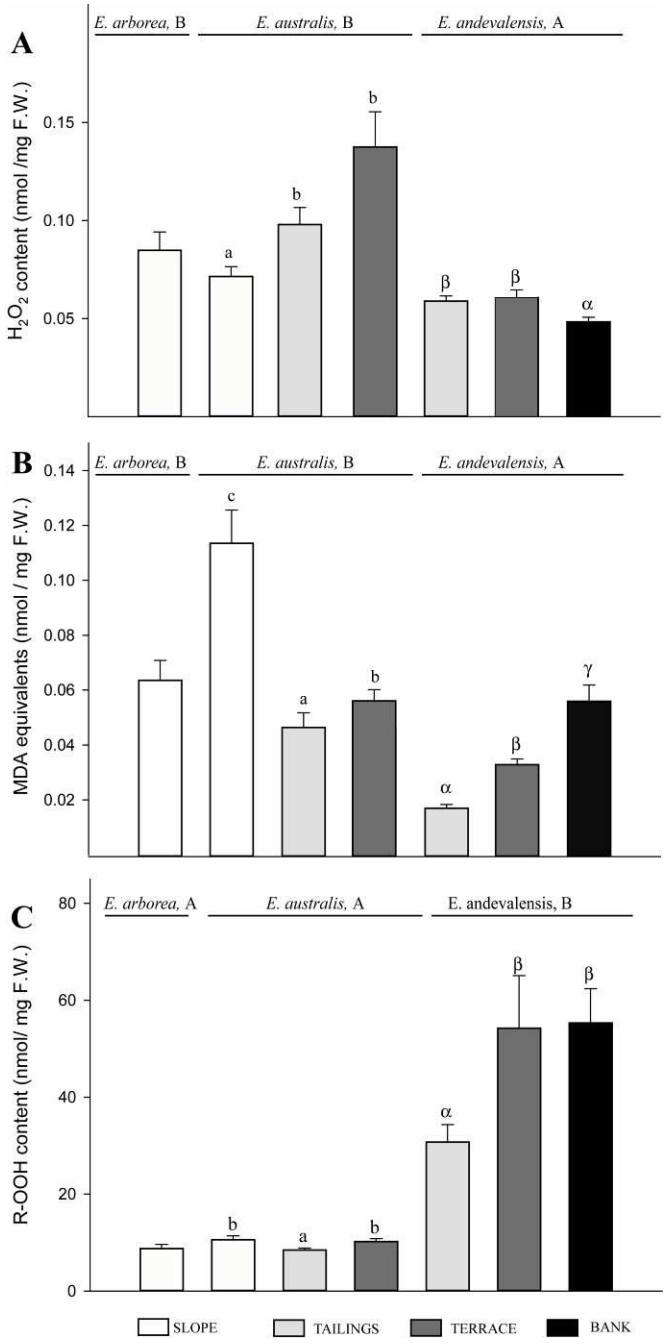


Figure 8. A) Hydrogen peroxide (H_2O_2) content in nmol/mg FW; B) Lipid peroxidation, expressed as MDA equivalents (nmol/mg FW); C) Hydroperoxides content in nmol/ mg F.W., of three *Erica* sp., sampled at four different locations. Data represent means ± SEM (n = 7). Legends and notes as in Fig. 5

Hydrogen peroxide (H_2O_2) content in leaves was determined and the results are displayed in (Fig. 8A). *E. andevalensis* showed significant lower levels ($p<0.05$) than the other two species, and the population growing in the Bank had about 21 % less content than the other two populations of *E. andevalensis* studied. MDA equivalents, used to measure the lipid peroxidation (Fig. 8B), showed also the lowest values in *E. andevalensis* ($p<0.05$) when the species were compared as a gruop. Looking inside each species, the Tailings showed the lowest MDA content in both species: *E. andevalensis* and *E. australis*. When hydroperoxide (R-OOH) accumulation was measured (Fig. 8C) *E. andevalensis* showed the highest value ($p<0.05$), especially in the Terrace and Bank populations.

In relation to pigment content, higher values of chlorophyll *a* and *b* were found in *E. australis* growing in the Slope than in the Tailings or Terrace ($p<0.05$) (Fig. 9 A and B). However, in *E. andevalensis* the pigment concentration was smaller in the Tailings than in the Bank ($p<0.05$). Finally, carotenes did not show any change in *E. andevalensis*, however *E. australis* showed a reduction from the Slope to the Tailings populations ($p<0.05$) (Fig. 9C).

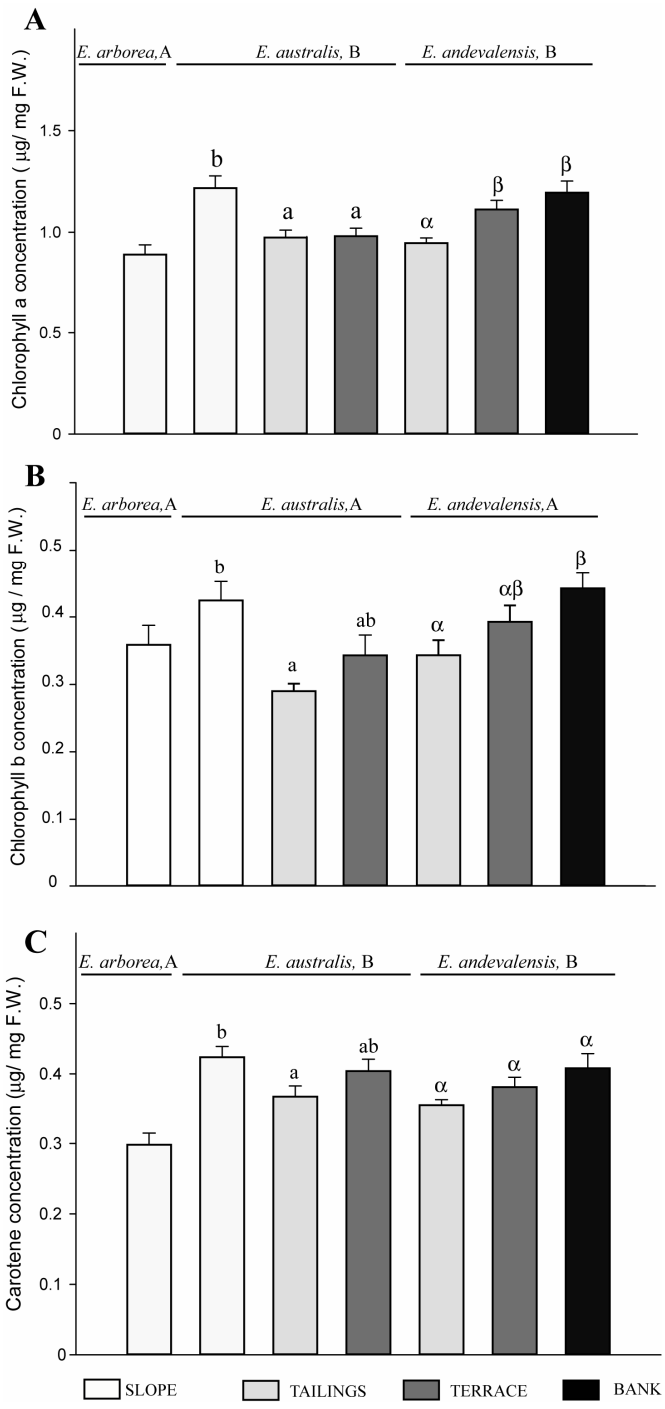


Figure 9. Concentration of chlorophyll a (A), chlorophyll b (B), and carotenes (C) of three *Erica* sp., sampled at four different locations. Data represent means ± SEM (n = 7). Legends and notes as in Fig. 5.

DISCUSSION

Heavy metal contamination in soil or sediments can constitute a powerful selective force in plant evolution (Antonovics 1971). In the Pyritic Belt metal richness and acid conditions could made possible that an endemic plant species, *Erica andevalensis,* had developed tolerance mechanisms to survive where no other species can do (Cabezudo and Rivera 1980, Nelson et al. 1985; Soldevilla et al. 1992; Heras et al. 2002; Rodríguez et al. 2007). In the present study, the exposure of plants to heavy metals has been chronic and the initial hypothesis was that both *E. andevalensis* and *E. australis* might have evolved cell defence mechanisms against ROS derived from metal-catalysed reactions as reported for other plants (Briat and Lebrun 1999; Clijster et al. 1999; Okamoto et al. 2000; Michalak 2006). In order to study the possible different heavy metals tolerance mechanisms in *E. andevalensis, E. australis* and *E. arborea*, soils metal content has been analysed to get a general picture about the metal abundance in the study area. In leaves, metal composition analysis together with biochemical (ascorbic acid, protein, hydrogen peroxide, hydroperoxide radicals, products from lipid peroxidation, pigments) and enzymatic assays (catalase, superoxide dismutase, ascorbate peroxidase and guaiacol peroxidase) have been carried out.

The metal content analysis showed that, in general, its concentration in the soils from the Odiel sampling point decrease as the distance to the river increased. This fact reinforces the notion that AMD is taking place at these sites and that the Odiel River is the main source of pollution, with high levels of Cu, Fe, Mn and Zn, as much in total as in biodisponible forms (Chapter 1, Table 1). On the other hand, the highest concentrations of As and Pb was found in the Tailings soils. The different metal composition in these two kinds of soils is due to the different origin that they have: soils close to the river water are enriched with the metals present in the soils where the river cross, whereas the Tailings are an artificial accumulation of wastes after the mineral extraction. What are the mechanisms that *E.*

andevalensis possess to survive in such soils have a growing interest nowadays, including the rhizosphere that have been studied from a metagenomic point of view, and genes resistant to Ni have been described (Mirette el al. 2007).

The metal content in plant tissue (Chapter 1, Table 2) showed that, in general, *E. arborea* and *E. australis* behave in a similar way, and that *E. andevalensis* only showed a strong difference with respect to the other populations in Zn content when growing in the Bank, where this metal presented the highest concentration. These species may have mechanisms to avoid metals uptake. Accumulating Fe and Al mycorrhizas have been described in roots of *E. andevalensis* (Turnau et al. 2007). However, we have found that Fe and Mn contents are the highest among the studied species and that their levels exceeded the concentration reported as normal range in plants (Shaw et al. 2004). Although Mn accumulation has been previously described in *E. andevalensis* (Soldevilla et al. 1992), we have found that, despite the previous studies suggesting *E. andevalensis* to be Zn excluder (Soldevilla et al. 1992), the Zn internal concentration in the Bank was 4-fold higher than the concentration found in plants from the Terrace. These data suggest that *E. andevalensis* control uptake mechanisms can be overcome due to the high concentration in soils (Chapter 1, Table 2).

We found that *E. andevalensis* growing in the Bank showed the lowest soluble protein content (Fig. 5 A), and that this effect was not related to higher water content (Fig. 5 B). Metals can affect the protein content in plants, but this effect has been shown to depend strongly on the metal used and its concentration (Ewais 1997).

The accumulation of Fe and Al in leaves of *E. andevalensis* (especially in the cuticle and upper epidermis) has been previously described (Turnau et al. 2007). On the other hand, the higher values found in *E. australis* and *E. arborea*

may be an effect of the antioxidative protection due to the increase of antioxidant enzymes, as has been previously suggested by Kovácik et al (2009).

As stated above, the analyzed plants were chronically exposed to heavy metals, and this fact is widely known to induce oxidative stress (De Vos et al. 1992; Gallego et al. 1996; Schützendübel and Polle 2002). Plants have developed barrier mechanisms to avoid the entrance of the metals (Macnair 1997); or even, once the metals have entry the cells, mechanisms focused in the metal inactivation to avoid reactive oxygen species (ROS) formation. To cope with ROS, plants mechanisms are focused on metal toxicity reduction with molecules such as ascorbic acid, or by enzymatic mechanisms (Stadtman 1991; Smirnoff 1996, 1998, 2000; Horemans et al. 2000).

The AsA concentration is generally used as an oxidative stress marker (Foyer et al 1991; Foyer 1993; Kaempfenkel et al. 1995). In our study, *E. australis* presented the highest levels of AsA, as much in the total as in the reduced form, without differences among the different population studied inside of each species. The AsA levels in *E. andevalensis* did not show differences among populations and in all the cases they were lower than in *E. australis.* Possibly, these findings are related to different strategies of tolerance/avoidance displayed by *E. australis* and *E. andevalensis*. On the other hand, *E. arborea* AsA levels did not show differences with *E. andevalensis*. Along this study, *E. arborea* antioxidant levels have been taken as a reference between the other two studied species. The activity of antioxidant enzymes is generally increased in plants exposed to stressful conditions and the increase is correlated with an increase in stress tolerance, preventing cell and tissue damage (Allen 1995; Shi et al. 2006).

When reacting with oxygen, divalent metals such as Fe (II and III) and Cu (I and II) produce the radical superoxide ($O_2\bullet^-$), starting an oxidative stress chain-reaction (Briat and Lebrum, 1999). Not only divalent metals, but also Cd (II) and Zn (II) have been described to cause oxidative stress (Schützendübel and Polle

2002).In our study, SOD activity was elevated in *E. australis* collected from the Tailings site compared with the Slope and Terrace ($p<0.05$) and slightly higher in *E. andevalensis* but without significant differences among the Terrace and Bank populations. This increased SOD activity is reflected in the smaller levels of MDA and hydroperoxides found in the populations of both species growing in this place (compared with other populations of the same specie). Catalase, an enzyme with a high reactivity capacity but low substrate affinity (Smirnoff 1998), showed a significant lower activity in *E. australis* growing in the Terrace. This population showed higher H_2O_2 concentration (Fig. 8 A) and higher values of MDA and ROOH (Fig. 9 B and C), increases that could be consequence of the higher Zn content in the leaves (Chapter 1, Table 2).

APX and POD are induced by heavy metals (Clijsters et al. 1999) and both consume H_2O_2 (Inze and Montagu 1995). POD showed a different behaviour in *E. australis* and *E. andevalensis*, decreasing the activity in the first one from the plants less exposed to metals to the most exposed, while in *E. andevalensis* growing in the Bank the activity was more than 2.5 fold higher than the plants from the Terrace. APX showed a high activity in all the studied populations, but it was *E. andevalensis* the species that possessed the highest activity and in the Bank site where the activity reached values as high as 840 U mg^{-1} protein. The Bank is the most extreme of the studied habitats and plants growing there should cope against the ROS enhancing the activity of peroxidase enzymes, mainly APX, reflected in a lower total ascorbic acid levels and H_2O_2 contents in all *E. andevalensis* populations (Fig. 6 and 8 A). The increase in peroxidase activity in response to heavy metals has been previously reported (Smirnoff 1996; Wecks and Clijsters 1997; Cuypers et al. 1999; Gupta et al. 1999).

When the effect of oxidative stress is analyzed through the level of MDA, usually taken as an index of lipid peroxidation (Zhang et al. 2007) *E. andevalensis* growing at the Bank, appeared as the population with the highest concentration in this species. We found that *E. andevalensis* and *E. australis* presented the same

pattern when the populations growing in the Tailings and in the Terrace were compared, showing the last one the highest values. This data, together with the higher Cr, Cu, Fe, Mn, Mo, Ni and Zn in the Terrace than in the Tailings suggest that plants in this site were affected by oxidative stress. Plants in the Slope showed the highest MDA levels, suggesting that the mechanisms displayed by these plants to cope with oxidative stress, were not so efficient than those evolved in populations growing in more polluted soils. The concentration of R-OOH in *E. andevalensis* was higher than in the other species; although no big differences in the MDA levels were found among all the species studied. *E. andevalensis* antioxidant system made possible that, despite the high concentration of hydroperoxides, lipid peroxidation did not increase significantly. This ability to eliminate hydroperoxides can be explained on the basis of the different antioxidant mechanisms analyzed in this study, and maybe also by the different phenolic composition found in *E. andevalensis* compared with *E. australis* and *E. arborea* (Márquez-García et al. 2009).

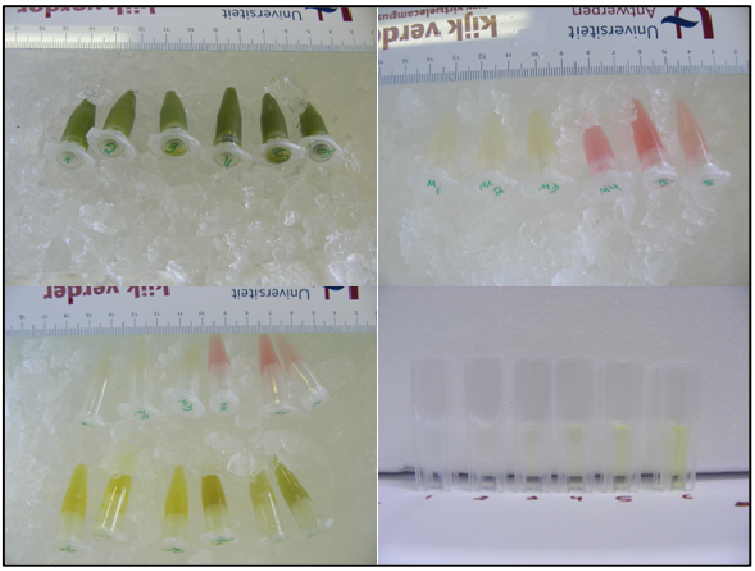
The studied species were not highly affected by the internal metal content, as can be demonstrated with the pigment contents (Fig. 9). Thus, it is possible to observe that *E. andevalensis* in the Bank had significantly more chlorophyll *a* and *b* than the populations growing in the Tailings. Indeed, it has been described in the literature that heavy metals can reduce the chlorophyll content, and this reduction can be used as an indicator of metals-induced stress (Dietz et al. 1999; Mysliwa-Kurdziel et al. 2004; Šimonova et al. 2007). The effects of the heavy metals were noticeable in *E. australis* when the populations from the Slope were compared to those growing in the Tailings or Terrace, in which chlorophylls *a* and *b* were reduced. It has been reported that chlorophyll *b* is more sensitive to the presence of heavy metals, suffering greater depletions (MacFarlane and Burchett 2001). When the ratio chlorophyll *a* / chlorophyll *b* were analyzed, no differences were found in *E. andevalensis;* however, *E. australis* showed a higher ratio in the population from the Tailings. These plants presented a higher internal concentration of As and Pb and those metals can be the responsible of the reduction in chlorophyll *b* and the

increase of SOD. Arsenic has been described to produce ROS (Hartley-Whitaker et al. 2001), however, the defence mechanisms of this plant avoid are efficiently working and no a higher lipid peroxidation was observed. No changes were observed among the different *E. andevalensis* studied populations, however, a reduction was found in *E. australis* from the Tailings.

In conclusion, we have found that *E. andevalensis* antioxidant system is characterized by high peroxidase activity, mainly APX, which increases its activity as internal metal content increases whereas *E. australis* mechanisms are focused in high ascorbic acid content. Both species seem to have efficient mechanisms to avoid metal uptake, as deduced from the slight differences in metal composition found when plants are growing in different conditions.

4. PHYTOCHELATINS, GLUTATHIONE AND NON-PROTEIN THIOL COMPOUNDS IN *ERICA ANDEVALENSIS* AND *ERICA AUSTRALIS* FROM SW SPAIN

Fitoquelatinas, glutatión y compuestos tiólicos no proteicos en *Erica andelavalensis* y *Erica australis* del SO español



Phytochelatins, glutathione and non-protein thiol compounds in *Erica andevalensis* and *Erica australis* from SW Spain

ABSTRACT

Phytochelatins, glutathione and non-protein thiol compounds has been analyzed in *Erica andevalensis* and *E. australis. E. andevalensis* did not present differences in glutathione levels compared with *E. australis* growing in the same habitat, but small ascorbic acid concentration in *E. andevalensis* were confirmed in this new experiment. Phytochelatins (PCs) levels have been measured due to their importance in heavy metal resistance in other plant species. The low levels of PCs found in both species, together with the lower concentration found in *E. australis* growing in the polluted soil compared to the plants growing in a non-polluted one, indicate that *Erica* heavy metal tolerance can not be explained by PCs neither the thiol compounds considered in the present study.

KEYWORDS

Ericaceae; Heavy metals; Ascorbic acid; Total antioxidant capacity

INTRODUCTION

The Iberian Pyritic Belt is a metal enriched area in SW Spain and Portugal (Davis et al. 2000; Sainz et al. 2003; Sánchez-España et al. 2005). In this soils Fe, Mn, Cu, Zn and Pb are found in high concentrations (van Geen et al. 1999; Sainz et al. 2003; Braungardt et al. 2003; Montes-Botella and Tenorio 2003). Plants have evolved mechanisms to control the uptake and accumulation of metals (essentials or not) that include chelation and sequestration of heavy metals by particular ligands, included phytochelatins (Cobbett 2000a; Cobbett and Goldsbrough 2002), to avoid the reaction of metals with oxygen, reaction that lead the increase of reactive oxygen species (ROS) that react with cellular components and produce oxidative stress (Briat 2002).

Phytochelatins (PCs) are a family of non-protein thiol peptides with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly, with n from 2 to 11, where in the C-terminal Gly can be exchanged for other amino acid (Rauser 1995; Zenk 1996; Cobbett and Goldsbrough 2002). Metals induce the synthesis of PC (Cobbett 2000a) and there is no direct evidence that these molecules had another functions outside the detoxification (Cobbett and Goldsbrough 2002).

Glutathione (GSH) has been confirmed to be the substrate for the synthesis of PC by different physiological, biochemical and genetic studies (Cobbett 2000a). Different functions has been attributed to GSH and reductans and radical scavenging are important ones (Dixon et al. 1998). The antioxidant properties of GSH depends on the oxidation of the SH group, giving rise to disulphide form (GSSG). The ratio reduced to oxidized form is maintained by glutathione reductase (GR) (Madhava Rao and Sresty 2000) and the proportion of reduced GSH is greater than 0.9 under non-stress conditions in plant tissue (Noctor et al. 1998). GSH is related with ascorbic acid (AsA) through the ascorbic acid-glutathione cycle as follows: the oxidized ascorbic acid (DHA) is reduced to AsA using

electrons coming from GSH (Foyer 1993; Foyer et al. 1994). The AsA is one of the most powerful antioxidants in plant cells (Smirnoff 1996; Horemans et al. 2000; Smirnoff 2000) and under physiological conditions the percentage of reduced form is close to 90% in leaves (Smirnoff 2000).

Erica andevalensis Cabezudo & Rivera (1980) is an endemic species from the Iberian Pyritic Belt, always growing in acid soils with high levels of heavy metals (Nelson et al. 1985; Soldevilla et al. 1992; Heras et al. 2002). The levels of antioxidant enzymes (SOD, catalase, peroxidases, APX), AsA and oxidative stress markers (MDA) has been previously analyzed in *E. andevalensis* and *E. australis* growing in soils with different metal composition in the soils (Chapter 3), with the conclusion that both species used differentially their antioxidant mechanisms: *E. andevalensis* enhanced the APX activity and *E. australis* had highest levels of AsA. As well, the phenolic composition has been analyzed in different populations of *E. andevalensis* and *E. australis* as a clue to the antioxidant properties of these compounds (Chapter 2) and different phenolic composition was found among both species. However, until now the levels of GSH and related enzymes have not been possible to assay by spectrophotometric techniques in these species.

In the present study the levels of GSH, using the HPLC with electrochemical detection method optimised in Antwerpen University, Plant Physiology Research Team, as well as the levels of non-protein thiols and phytochelatins has been analyzed for the first time in these species with the aim to go into the tolerance mechanisms in depth that *E. andevalensis* and *E. australis* possess to survive in such a extreme habitats, where no other species can survive.

MATERIALS AND METHODS

Plant material

In this study plant material was collected from the field in the Odiel river (close to "Puente de los Cinco Ojos", 37º 43' N – 6º 42' W), in February 2008. Two different sampling points were chosen: one affected by the river influence, and therefore the AMD process, in the Terrace of the river, where *E. andevalensis* and *E. australis* share habitat, and one placed close, but in a slope, with no direct relation with the river, called slope in the Chapter before, where only *E. australis* was sampled. These sampling points were defined in Chapter 1.

Leaves from 5 different individual plants from each plant species and site (population) were collected in February 2008 and immediately frozen in liquid N_2. Samples were kept frozen in dry-ice and sent to Antwerpen University (Plant Physiology research team) and stored at -80ºC until they were analysed.

Glutathione and ascorbic acid determination

Plant material was taken out of -80º C and fractions of around 100 mg were weighted and quickly grounded in liquid nitrogen in a pre-cooled mortar. At this stage it is essential to keep samples frozen to prevent oxidation. When a homogeneous powder was obtained, 1 ml of ice cold 6 % (w / v) meta-phosphoric acid was added. The samples were thawed on ice and the mixture was clarified by centrifugation at 12,000 *g* at 4°C for 15 min. The resulting supernatant was kept on ice until HPLC analysis. Antioxidants were separated on a 100 mm x 4.6 mm Polaris C18-A reversed phase HPLC column (3 µm particle size; 30°C; Varian, CA, USA) with an isocratic flow of 1 ml min^{-1} of the elution buffer (25 mM KPO_4-buffer, pH 3.00). The components were quantified using a home made

electrochemical detector with glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The purity and identity of the peaks was confirmed using a diode-array detector (SPD-M10AVP, Shimadzu, 's-Hertogenbosch, The Netherlands) which was placed on line with the electrochemical detector. The amount of oxidised DHA or GSH concentration was measured indirectly as the difference between the total concentrations of antioxidants in a DTT reduced fraction and the concentration in the sample prior to reduction. Reduction of the sample was obtained by incubation of an aliquot of the extract in 400 mM of Tris and 200 mM of DTT for 10 min in the dark. The pH of this mixture was checked to be between 6 and 7. After 15 min the pH was lowered again by fourfold dilution in elution buffer prior to HPLC analysis.

Non-protein thiol compounds determination

Non-protein thiol compounds were measured following the method described by Queval and Noctor (2007). Samples were ground in liquid nitrogen and extracted into 1 mL of 0.2 N HCl. The homogenate was centrifuged at 16,000 g for 10 min at 4 ºC. A supernatant aliquot of 0.5 mL was neutralized with 0.5 ml of 0.2 N NaOH in the presence of 50 µl of 0.2 M NaH_2PO_4 (pH 5.6). The final pH was between 5 and 6.

The method was described for plate reader assays and the volumes were adapted to spectrophotometric assays with a final volume of 1 mL. The assay contained 700 µL of 0.12 M NaH_2PO_4 (pH 7.5) 6 mM EDTA, 100 µL of 6 mM DTNB and 200 µL of neutralized extract. For standards, extract was replaced by 0, 10, 20, 50 and 100 nmol of GSH. These standards solutions were neutralized and treated in the same way that the samples (Fig. 1). Assays of extracts were corrected for the absorbance at 412 nm in both the absence of DTNB (cuvette with extract

but no DTNB) and the basal absorbance of DTNB (cuvette with DTNB but no extract or standard).

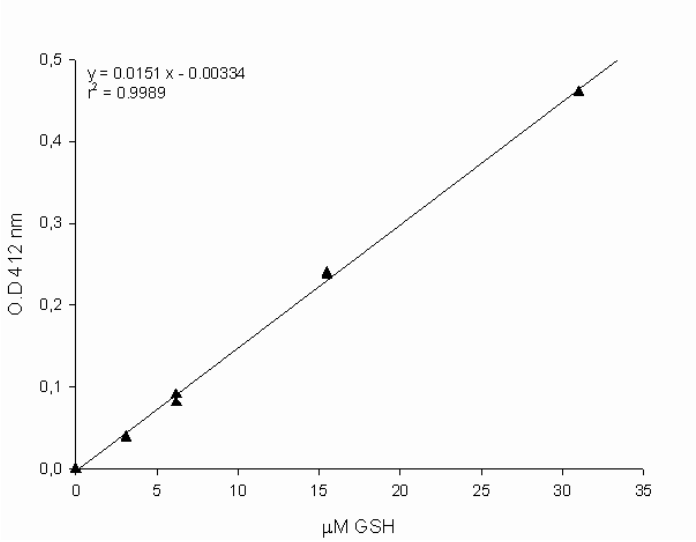


Figure 1. Calibration curve used in the measurement of non-protein thiols.

Phytochelatin measurements

Phytochelatin analysis was performed by Dr. Schat, Laboratory of Plant Ecology and Physiology, Free University, Amsterdam, The Netherlands as described by Sneller et al. (2000). Briefly leaves tissue was ground using a mortar and pestle and subsequently extracted with 1.98 ml of 6.3 mM diethylenetriaminepentaacetic acid with 0.1 % (v / v) trifluoroacetic acid at 4°C supplemented with 20 mL of 10mM N-acetyl cysteine as an internal standard. The suspension was centrifuged at 13,000 *g* for 10 min at 4 °C and the supernatant filtered through a Costar Spin-X centrifuge tube with a 0.22 mm nylon filter. The thiols in the extract were derivatized with 10 ml of 25 mM monobromobimane, together with 450 mL of 200 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid buffer at pH 8.2 and 6.3 mM diethylenetriaminepentaacetic acid. After 30 min at 45 °C the derivatitation was stopped by the addition of 300

mL of 1 M methanesulphonic acid. The PCs were separated on two tandemly arranged Nova-Pak C18 columns (6 nm, 4 µm, 3.9 x 150 mm, Waters, Milford, MA) at 37ºC, using a slightly concave gradient of 12–25% (v / v) methanol for 15 min and then a linear gradient from 25 to 50 % (v / v) methanol from 15 to 40 min. Fluorescence was monitored using a Waters 474 fluorescence detector. HPLC peaks were identified based on a *Silene vulgaris* sample in which the PC composition was established previously through amino acid analysis of the purified peak fraction (Sneller et al. 2000).

Lipid peroxidation measurements

Lipid peroxidation was measured following the protocol described by Hodges et al. (1999), in which the amount of malondialdehyde (MDA) was estimated. Frozen leaves were homogenized in 80 % ethanol in a mortar, with liquid N_2. The extract was centrifuged at 3,000 g for 10 min and the supernatant was used. The method made a correction of the interferences by subtracting absorbance values of samples without thiobarbituric acid (TBA). Briefly, a 1 mL of diluted sample was added to a 1 mL of either i) –TBA solution comprised of 20 % (w / v) trichloroacetic acid and 0.01 % butylated hydroxytoluene, or (ii) +TBA solution containing the above plus 0.65 % TBA. Samples mixed and heated at 95 ºC for 25 min and then cooled and centrifuged at 3,000 g for 10 min. The absorbance was read at 440, 532 and 600 nm and the following equations were applied:

$$[(\text{Abs } 532_{+TBA})-(\text{Abs } 600_{+TBA})-(\text{Abs } 532_{-TBA} - \text{Abs } 600_{-TBA})] = A$$

$$[(\text{Abs } 440_{+TBA} - \text{Abs } 600_{+TBA})\cdot 0.0571] = B$$

$$\text{MDA equivalents (nmol ml}^{-1}) = [(A - B)/157000]\cdot 10^6.$$

Total antioxidant capacity measurement

The total antioxidant activity was measured using the ferric reducing antioxidant power (FRAP) method described by Peñarrieta et al. (2008). Leaves were extracted in 80 % ethanol (v/v) and centrifuged at 3,000 g for 10 min. The method was adapted to plate reader and to 100 µL of plant extract, with the appropriate dilution, was added 100 µL of reagent. The reagent mixture consisted of 0.1M sodium acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM ferric chloride, in 10:1:1 (v/v/v) proportion. The absorbance was read at 600 nm after 10 min. A standard curve (Fig. 2) was made following the same protocol with Trolox from 0 to 100 µM. The data were expressed as µmol Trolox equivalents per gram of fresh weight.

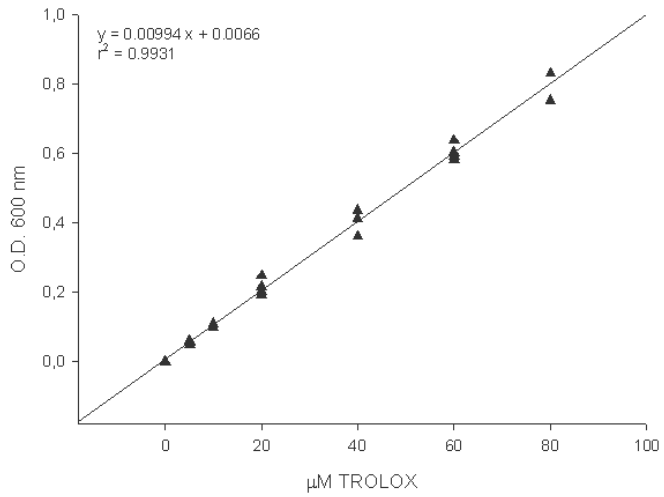


Figure 2. Calibration curve of total antioxidant capacity using Trolox as reference.

Pigments measurement

The content in chlorophyll *a*, *b* and total was measured in the remainder supernatant after the total antioxidant capacity was assayed. The quantification was made using the equations given by Lichtenthaler (1987) for ethanolic extracts.

$$\text{Chlor } a = 13.36 \cdot ABS_{665} - 5.15 \cdot ABS_{649}$$

$$\text{Chlor } b = 27.43 \cdot ABS_{649} - 8.12 \cdot ABS_{665}$$

$$\text{Chlor total} = 5.24 \cdot ABS_{665} + 22.24 \cdot ABS_{649}$$

Statistical analysis

The statistical analysis were analysed by ANOVA. A different post-hoc analysis was used depending on the variances' homogeneity (Duncan or Dunett T3, respectively), as previously tested with a *Levene's* test. All statistical analyses were performed using SPSS and *post hoc* Duncan test.

RESULTS

Some antioxidant mechanisms of *E. andevalensis* and *E. australis* have been previously analyzed (Chapter 3), together with the heavy metal content in both, soil and plant tissue (Chapter 1). In the present Chapter plants from the Terrace and Slope has been selected to go more deeply into the antioxidant mechanisms in *E. andevalensis* and *E. australis*. The values of AsA as much as in the reduced as in the total concentration (Fig. 3) showed lower values ($p<0.05$) in *E. andevalensis* when they were measured by HPLC.

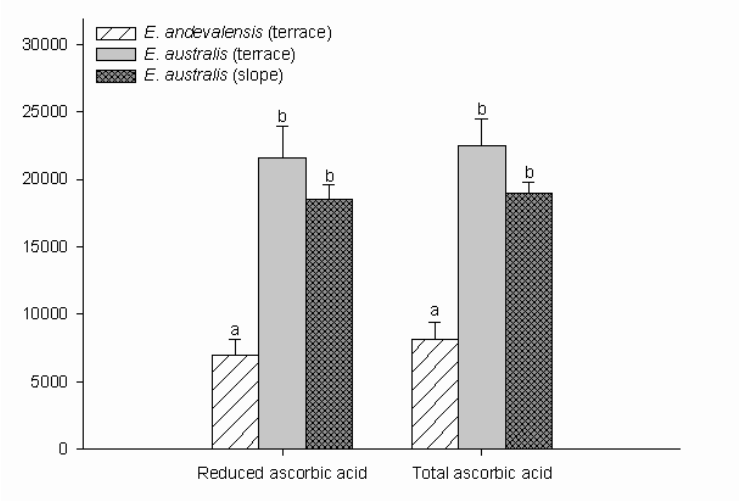


Figure 3. Levels of reduced and total ascorbic acid in the leaves of the *Erica* studied. Data represents means ± standard error of the mean, n= 5. A letter on top of a bar in the same parameter, indicate that the populations are statistically different ($p<0.05$) .

The non-protein thiols content was statistically higher in *E. australis* from the Slope than in *E. andevalensis* as can be observed in Fig. 4. In relation to glutathione, the reduced form was smaller in *E. australis* from the slope ($p<0.05$), but no other differences appeared. The glutathione levels were measured twice, in Antwerpen and in Amsterdam by different methods and no differences were found.

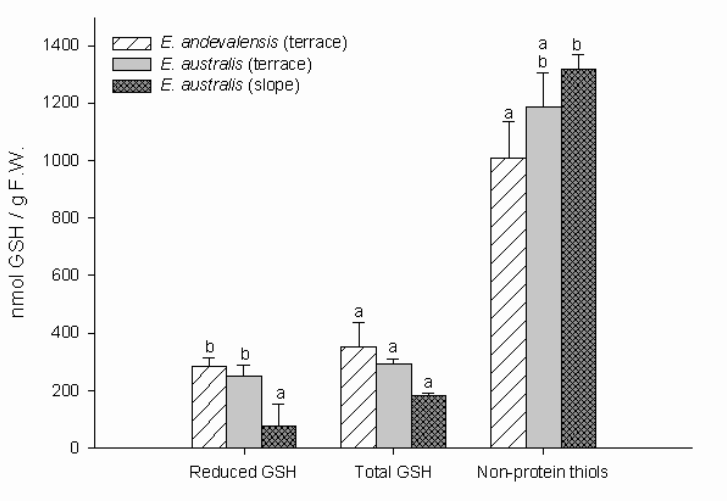


Figure 4. Levels of reduced and total glutathione (GSH) and non-protein thiols in the leaves of the *Erica* species studied. Data represents means ± standard error of the mean, n= 5. A letter on top of a bar in the same parameter, indicate that the populations are statistically different ($p<0.05$) .

AsA and GSH can act as antioxidant molecules, but they are not the only ones with this capacity. When the total antioxidant capacity was measured in an ethanolic extract, we found that *E. andevalensis* is the plant that showed the lowest one (Fig. 4), without differences between the two different populations of *E. australis*, but slightly small in *E. australis* from the terrace.

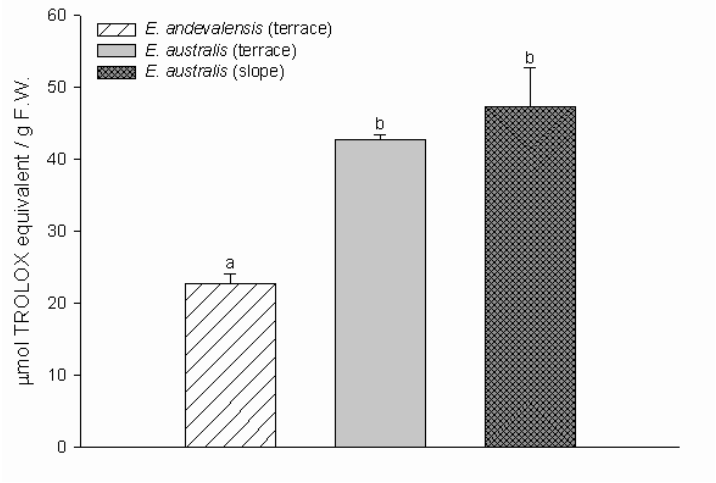


Figure 5. Total antioxidant capacity measured using TROLOX as reference in the leaves of *Erica* species studied is represented by the mean ± SEM (standard error of the mean), n= 5. Different letters on top of the bar means statistical differences $p<0.05$.

Results about phytochelatins showed that no PC2 was found in *E. australis* growing in the terrace, and PC 3 was smaller ($p<0.05$) in that population too. No differences were found in *E. andevalensis* and *E. australis* from the slope in the PC content.

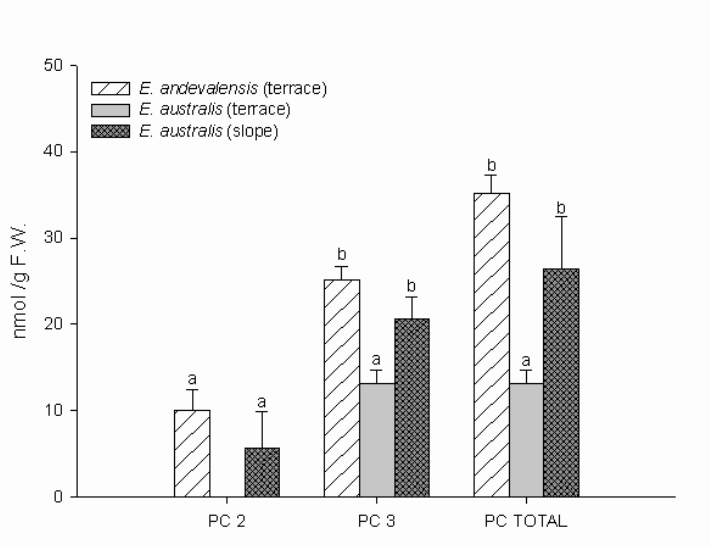


Figure 6. Phytochelatin (PC) content is represented in each bar as the mean ± SEM (standard error of the mean), n= 4. Different letters on top of the bar means statistical differences $p<0.05$

Chlorophyll *a* content was higher ($p<0.05$) in *E. andevalensis*. Chlorophyll *a*, *b* and total were smaller in *E. australis* from the terrace (Fig. 7).

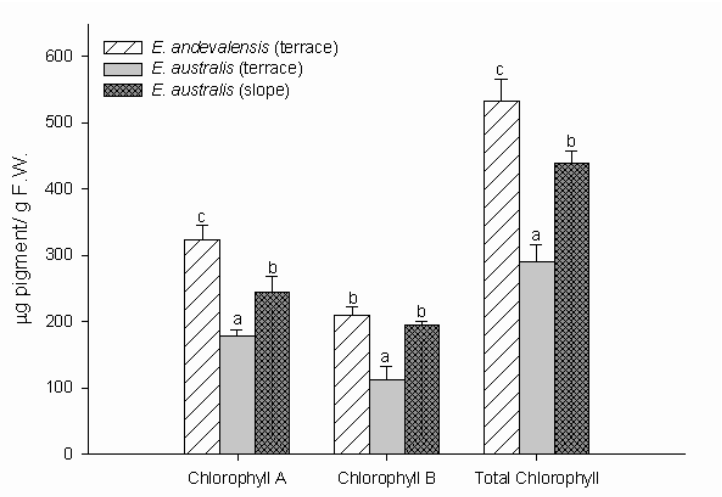


Figure 7. Levels of chlorophyll *a*, *b* and total is represented in each bar as the mean ± SEM (standard error of the mean), n= 5. Different letters on top of the bar means statistical differences $p<0.05$

The highest concentration of MDA ($p<0.05$) was found in *E. australis* from the slope (Fig. 8), without differences between *E. andevalensis* and *E. australis* in the terrace site.

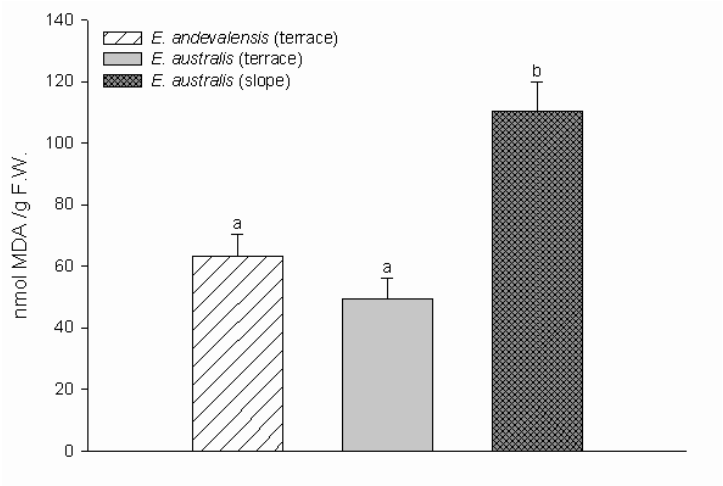


Figure 8. Lipid peroxidation, expressed as MDA equivalents (nmol/g F.W) is represented in each bar as the mean ± SEM (standard error of the mean), n= 5. Different letters on top of the bar means statistical differences $p<0.05$

DISCUSSION

The antioxidant mechanisms in *E. andevalensis* and *E. australis* had been previously analyzed (Chapter 3), with the aim to find out the clue of the resistance of *E. andevalensis* to the extreme habitats in which it always lives. Now, in this new study the interest was focused on the GSH and related molecules, due to the great importance described in heavy metal resistance in plants and the impossibility to be measured until now in *Erica* species. The levels of AsA were smaller in *E. andevalensis* ($p<0.05$) as much in the reduced as in the total forms, without differences between the two populations of *E. australis* sampled. This data

presented the same pattern that we obtained before with spectrophometric assays (Chapter 3), in which the levels of AsA in *E. andevalensis* were smaller than in *E. australis.* In this new measurement the final values have been smaller that the previous data, but the differences may be due to the different methods used in the quantification. Ascorbic acid is a key soluble antioxidant in plant cells against oxidative stress (Stadman 1991; Foyer 1993; Smirnoff 1996; Horemans et al. 2000). *E. andevalensis* presented the lowest AsA concentration and also the lowest total antioxidant capacity (Fig. 5), which was measured in an ethanolic extract and therefore include the liposoluble antioxidants. A low antioxidant capacity in *E. andevalensis* was already described before (Chapter 2). *E. andevalensis* is a species that always grows in soils with high levels of metals, so it is a species adapted to that situation (extreme and stressful for other plant species) and the constitutive antioxidant levels may be smaller than the levels showed by other plant species that grows in the same soils but that there are not as well adapted as *E. andevalensis*.

Several studies have pointed out the importance of thiols compounds in heavy metal defence in plants (Nagalakshmi and Prasad 2001; Mishra et al. 2008). We have found that the concentration of non-protein thiols in *E. andevalensis* was smaller ($p<0.05$) than the concentration found in *E. australis* from the Slope (Fig. 4). When the data of GSH in the reduced form were analyzed, significant differences were found between the populations growing in the Terrace and the Slope. Plants growing in the Terrace, where the metal concentration in both soils and plant tissues is bigger, presented a big concentration of reduced GSH without showing differences in the total form. When the ratio GSH/GSSG was calculated, no differences were found between the populations growing in the Terrace and a value of 0.4 resulted in *E. australis* growing in the Slope. This value, smaller than 0.9, is indicative that the plants there can be suffering from oxidative stress (Noctor et al. 1998). The stress state of this population was also reflected in the higher values of MDA content (Fig. 8). However, despite the bigger damage in the lipids compared with the other two populations sampled, the photosynthesis was not more affected in *E. australis* in the Slope than in the Terrace. As much chlorophyll

a as chlorophyll *b* were higher in the populations from the Slope. No differences were found in the ratio chlorophyll *a* and chlorophyll *b* in any of the populations, ratio that can be used as a marker of damage produced by metals due to the higher sensitivity of chlorophyll *b* (Macfarlane and Burchett 2001).

It has been described that in presence of heavy metals the GSH pool can be reduced, due to the synthesis of PCs (Cobbett 2000a). As was commented in the previous Chapter, plants growing in the Slope should be less adapted to the presence of heavy metals, and small concentrations present in the soil can produce more oxidative stress. However, when PCs were analysed, very low levels were found, with the only presence of PC 2 and PC 3 (Fig. 6). The concentration found is low compared with the level of non-protein thiols and with the levels described in other studies (Lee et al. 2003; Mishra et al. 2009). *E. andevalensis* PC levels were similar to *E. australis* from the Slope. A decline in the PCs levels after long treatments, due to plant adaptation and the activation of different tolerance mechanisms has been described (Landberg and Greger 2004). In our case, plants are exposed chronically to the metals present in the soils and despite these species are not considered hyperaccumulators, it has been described that plants growing in strongly metal-enriched soils do not have to be associated with enhanced PCs synthesis (Schat et al. 2002). In relation with that it has been suggested that Cu, Cd and Zn hypertolerant populations of *Silene vulgaris* possessed different sequestration mechanisms no related with PCs (De Knecht et al. 1995).

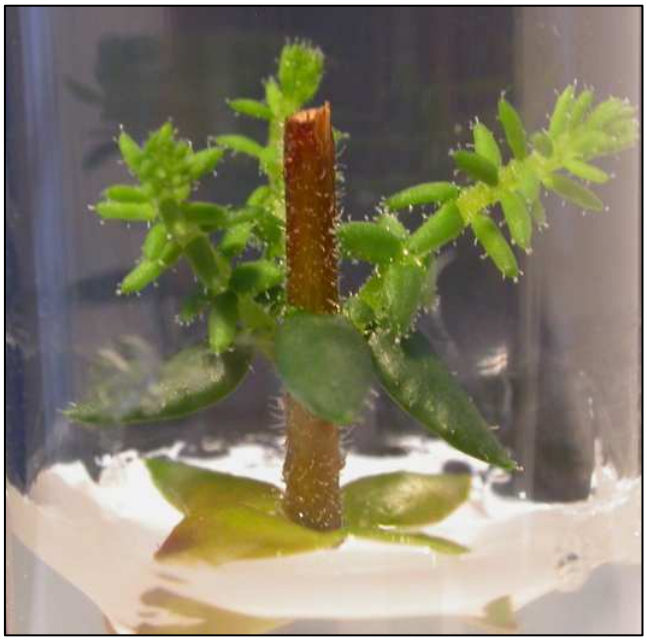
Some studies estimated PCs content subtracting the GSH levels to non-protein thiols (Bhargava et al. 2005; Seth et al. 2008). This estimation is a little tricky and not always true, as we can observe with our data. *Erica* species should contain a different kind of thiols compounds different to PC as can be deduced from the non-protein thiols, GSH and PCs levels. These undetermined compounds may be the clue in the heavy metal tolerance mechanisms in *E. andevalensis* and *E. australis*. When GHS is subtracted from thiols, the pattern and statistical differences were the same that the one found in thiols, with *E. andevalensis*

presenting the lowest value. It has been described that not all the metals induce PCs and Zn and Pb ions can be bound to a different low molecular weight compounds (Leopold et al. 1999).

In conclusion the data provided in this study confirm the lower levels of AsA and antioxidant capacity in *E. andevalensis* compared with *E. australis*. On the other hand we found that the non-protein thiols can play a role in heavy metal tolerance in *Erica* species, but no PC, which were found in very low concentrations. Other possible heavy metal tolerance mechanisms should be active in this species to avoid oxidative stress damages, which can be the objective of future experiments.

5. EFFECT OF DIFFERENT MEDIA COMPOSITION ON THE MICROPROPAGATION OF *ERICA ANDEVALENSIS*, A METAL TOLERANT SPECIES GROWING IN MINING AREAS (SW SPAIN)

Efecto de la composición del medio en la micropropagación de *Erica andevalensis*, una especie tolerante de metales que crece en zonas mineras (SO España)



(Aceptado en Acta Physiologiae Plantarum)

Effect of different media composition on the micropropagation of *Erica andevalensis*, a metal tolerant species growing in mining areas (SW Spain)

ABSTRACT

We describe a micropropagation protocol and acclimatization of *Erica andevalensis* Cabezudo & Rivera, using stem pieces collected from wild plants. This species always grows in soils enriched with metals such as Fe, Pb, Zn, and acid pH (2.5 – 4). Among the different media and hormone concentration tested, MS medium with ammonium nitrate concentration reduced to a quarter, 2 mg/L indole-acetic acid (IAA) and 0.5 mg/L kinetin was found to yield a better growht response in the plants. This medium produced an average of 3.6 ± 1.2 shoots and 16.9 ± 8.3 verticils per explant after 30 days of the onset of the culture. MS medium achieved rooting without phytohormones. Rooted plantlets were successfully transferred to plastic glasses with autoclaved perlite, watered with MS half-strength, and then to pots with commercial substrate after 2 weeks. New plants were produced in about 100 days. As far as we know, this is the first time that a replicable and complete micropropagation protocol for *E. andevalensis* has been developed. The plants looked healthy with no visual detectable phenotypic variations. The protocol provides a successful technique that could be used for the conservation of the species, as well as new researches on phenolics and triterpenoids found in plant extracts.

KEYWORDS

Acid mine drainage; endemic species; *in vitro* culture; heavy metals; plant conservation.

INTRODUCTION

Erica andevalensis Cabezudo & Rivera, described in 1980 in Spain, and recently in Portugal (Capelo et al. 1998), is a singular, endangered heather of the Iberian Pyritic Belt (SW Spain and Portugal). This species is considered to be vulnerable species in the IUCN list (IUCN 2001) and endangered by the regional government of Andalusia in Spain (Aparicio 1999). This heather only grows onto the tailings derived from the mining activities in the last 5000 years and along the Tinto and Odiel river banks that cross the mining areas (Aparicio and García-Martín 1996; Aparicio 1999). The very acidic soil in these mining areas contains levels of heavy metals as high as 77.8 g/kg Fe, 1301 mg/kg Cu, 600 mg/kg As, 2175 mg/kg Pb (Nelson et al. 1985; Soldevilla et al. 1992; Heras et al. 2002; Rodríguez et al. 2007; Abreu et al. 2008). *E andevalensis* is able to accumulate high concentrations of heavy metal in its tissues with levels as high as 1,745 mg/kg Al, 2,328 mg/kg Fe, 798 mg/kg Mn, 2 mg/kg Hg (Rodríguez et al. 2007, Turnau et al. 2007). The area where *E. andevalensis* grows successfully is seriously affected by acid mine drainage (AMD): a worldwide environmental problem (*eg*. Johnson and Hallberg 2005; Sainz et al. 2005; Akcil and Koldas 2006). The species has been proposed to be used as a tool to phytostabilizate metal contaminants in sulphide mine environments (Abreu et al. 2008) therefore, studies about conservation and physiology of this singular scrub could be very important from an environmental point of view and for *ex situ* conservation strategies (Dodds 1991; Frankel et al. 1995). Important antimitotic effect of triterpenoids extracted from *E. andevalensis* aerial parts were applied in antitumoral treatments as has been

described (Martín-Cordero et al. 2001). Likewise, an antiulcer activity (Reyes Ruiz et al. 1996) and antimicrobial activity (Toro Sainz et al. 1987) were observed for different extracts of *E. andevalensis.*

Recently, in a comparative study with other *Erica* sp, some phenolic compounds that derivate from *p*-coumaric acid, cinnamic acid and rutine have been identified only in *E. andevalensis*, possibly relating to the antioxidant mechanisms that the species possess to survive in such extreme habitat (Márquez-García et al. 2009). Since Fay (1992) has proposed *in vitro* propagation as an important tool for plant conservation, we have successfully developed a replicable and complete micropropagation procedure for *E. andevalensis*. As far as we know, this is the first report for the *in vitro* propagation of *E. andevalensis* and it can be applied to soil stabilization and in both pharmaceutical and biochemical research. The aim of this work is the production of plants in the laboratory to delve deeper into biochemistry studies related with the antimitotic, antiulcer and antimicrobial properties described for the plant, as well as the antioxidant properties derived from the special phenolic composition found. Fuuurthermore this protocol may be useful as an *ex situ* conservation tool for this endangered and law protected species or biochemical research.

MATERIALS AND METHODS

Sample collection and sterilization

Plant material, consisting of apical stem fragments of about 10 cm from mature and healthy *E. andevalensis*, was collected in the Odiel river bank (37º 38’ N; 6º 97’ W) (Fig. 1). Once in the laboratory, the stems were washed immediately with tap water.

The stems were sterilized in 70 % (v / v) ethanol for 45 sec and 25 % (v / v) of sodium hypochlorite for 20 min. The disinfectants were removed by 3 successive rinses in sterile water (5, 10 and 15 min respectively). Glassware, culture media and instruments were sterilized by autoclaving at 103 kPa at 121 ºC for 20 min.

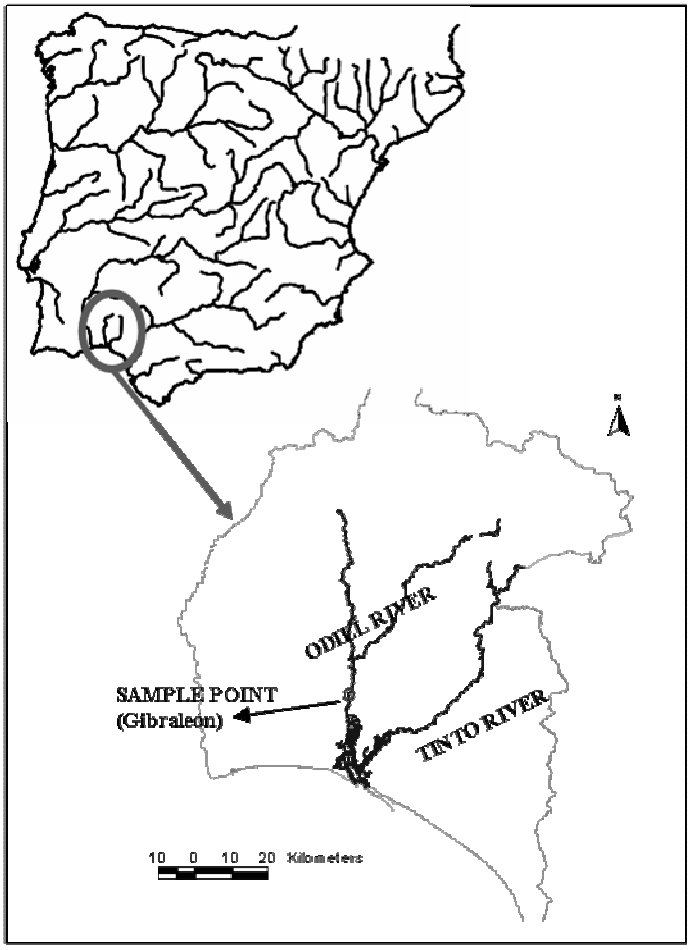


Figure 1. Localization of the sampling point in the bank of the Odiel river (close to Gibraleón village, Spain).

Shoot multiplication

After the stems washing, they were kept in sterilized water in the laminar flow chamber until they were inoculated in glass tubes (140 x 14 mm) containing 12 mL of semisolid solution of agar enriched with different culture media and covered with safety caps to prevent contamination, while facilitating aeration. The stems were cut into fragments about 10-15 mm, consisting of two verticils with 4 leaves in each one, as they have been described to propagate *Erica x darleyensis* (Viémont and Lambert 1994).

Tubes were kept in a plant growth chamber, with 16 h photoperiod with a light intensity of 100 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. Temperature was 24/18 ºC day/night following the photoperiod.

The culture media used consisted of the salt and vitamins solutions of Murashige and Skook (MS) (1962), Lloyd and McCown (LM) (1980) and Viémont and Beaujard (VB) (1983). Both MS and LM media contained sucrose as a carbon source and vitamins and hormones as indicated in Table 1. The VB medium was composed of a half-strength Knop's macronutrients and Berthelot micronutrients. (Viémont and Bejaurd 1983).

The fungi-contaminated explants were discarded during the first week of culture. Non contaminated explants were followed during 30 days by counting the appearance of new shoots and verticils.

Table 1
Culture media used in propagation of *Erica andevalensis*.

Media code	Basic salt medium	Modification applied	Sucrose (g/L)	Agar (g/L)	pH	Vitamins (mg/L)						Hormones (mg/L)	
						Myo-inositol	Thiamine	Glycine	Pyridoxin	Nicotinic acid	Pantoteic acid	IAA	K
1	MS	½ NH_4NO_3 concentration	30	6	5.8	100	0.1	2	0.5	0.5	0.1	2	0.5
2	MS	½ NH_4NO_3 concentration	30	6	5.8	100	0.1	2	0.5	0.5	0.1	0.5	2
3	LL	-	30	6	5.8	100	0.1	2	0.5	0.5	0.1	2	0.5
4	LL	-	30	6	5.8	100	0.1	2	0.5	0.5	0.1	0.5	2
5	VB	-	20	8	5.6	-	-	-	-	-	-	-	-
6	MS	½ NH_4NO_3 concentration	30	6	5.8	100	0.1	-	-	-	-	2	0.5
7	MS	¼ NH_4NO_3 concentration	30	6	5.8	100	0.1	-	-	-	-	2	0.5

Nutrient solutions used in our micropropagation procedure were as those described by Murashige and Skoog, 1962 (MS), Lloyd and McCown, 1980 (LL) and Viémont and Beaujard, 1983 (VB), with the addition of sucrose, agar, vitamins and phytohormones at the indicated concentrations. Ammonium nitrate concentration of the original MS medium was reduced as shown. Numbers in the left column are used along the text to refer to the media composition.

Root induction and acclimatization

Thirty days after the onset of the *in vitro* culture, the explants that were actively growing in Medium 7 were taken out. The new shoots produced were cut at the basal end and transferred individually to half strength MS medium with a quarter of ammonium nitrate supplemented with thiamine (0.1 mg/L), myo-inositol (100 mg/L), sucrose (30 g/L) and agar (6 g/L) as a basic medium, where non-phytohormones were added or we added 2 mg/L of IAA or 2 mg/L of kinetin to induce the root formation. Full strength MS with and without phytohormones was also tested. The new tubes were placed in the same plant growth chamber, under the same growing conditions used for shooting. Fifteen plants were used in the rooting experiments and the experiments were made three different times in each medium.

Forty-five days after rooting (*i.e.* 75 days from the onset of the *in vitro* cultures) plantlets that showed a good development of roots and elongation of the stem, were transferred to plastic containers (120 x 80 mm) containing autoclaved perlite, as a substrate. They were watered with MS half-strength solution, without vitamins, phytohormones and sucrose. The containers were kept in the plant growth chamber. Desiccation was avoided by using a transparent plastic cover (Baskaran and Jayabalan 2008). Four or five days after transferring the plantlets to the containers, some holes were made in the plastic cover to facilitate a progressive acclimatization to the environment. Plantlets were maintained for two weeks in the plastic containers until they were transferred to pots (15 x 15 cm) with commercial substrate (50% of dry matter, which contained 65 % of organic matter; pH 6.6). After growing for two weeks in the plant growth chamber, the plantlets were moved and exposed to outside conditions. In the figure 2 we show a scheme of all the protocol, with time programming.

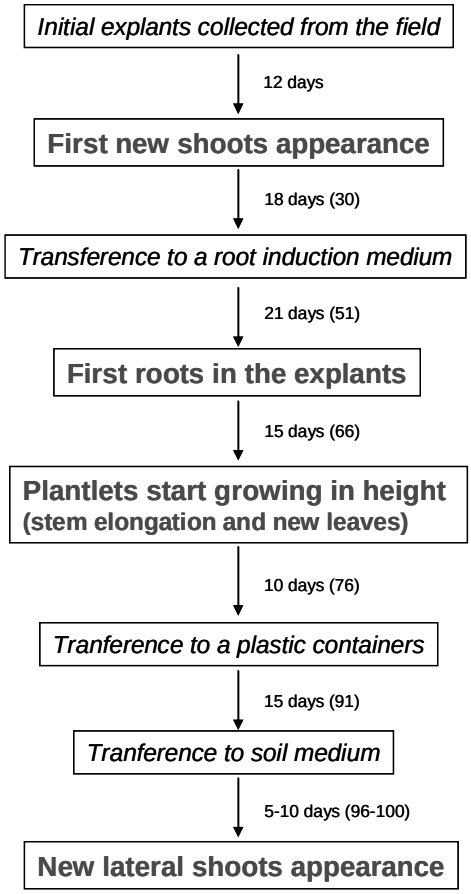


Figure 2. Scheme of the micropropagation protocol of *Erica andevalensis*

Statistical analysis

In the shooting phase, each medium was repeated three times. One stem was placed per tube and at least twenty tubes per medium were made in batches in different time periods. In each batch, the tubes containing the different culture media were labelled and mixed to avoid differences due to the time elapsed until all the tubes were inoculated.

In the rooting phase, 15 new shoots produced in the previous phase in Medium 7 were inoculated in the different rooting media. The statistical analysis was carried out with SPSS. Where indicated, the data were subjected to a Student's *t*-test, with a significance *p*-level of 0.05.

RESULTS AND DISCUSSION

Shoot multiplication

Stem explants were obtained from wild plants growing on an acidic, metal-polluted soil of the Odiel river banks and were immediately transferred to the different culture media indicated in Table 1. The ammonium nitrate concentration was reduced to a half or a quarter in MS media because explants began browning and no growth was observed (data not shown). This behaviour has been previously described by Dalal et al. (1991).

In the beginning of the culture fungi contamination of the explants was between 14 % and 65 % even after conventional sterilization procedures as exposed in "Materials and Methods" and consequently these explants were discarded from the experiment. After the first week, no more contamination in the explants was detected, and no fungi appeared in the observations that followed. A high percentage of contamination by fungi has been previously described when plants were taken directly from the countryside, for instance 100% of fungi contamination in sterilized stems from *Rhododendron ponticum* (Almeida et al. 2005) has been reported.

The rate of growth was measured by counting the number of new shoots and verticils per explant every two days for thirty days after the onset of the culture and the percentage of explants with new shoots. This is shown in Table 2. The

production of new shoots was neither affected by simplification in vitamins content of the MS media nor by reduction of the ammonium nitrate concentration. However, twenty days after the beginning of the culture, more than 90 % of explants showed active growth, except those explants growing in Medium 5, which only achieved 50 % of active explants.

Table 2
New shoots and verticils per explant and percentage of explants with new shoots were counted for each culture medium 30 days after the onset of the micropropagation procedure.

Medium	Number of shoots per explant	Number of verticils per explant	% of explants with new shoots
1	2.7±0.9 $^{b\,c}$	8.1±4.5 $^{b\,c}$	100±0.0 ^{a}
2	3.0±1.0 ^{c}	10.3±5.3 ^{c}	100±0.0 ^{a}
3	2.3±1.4 ^{b}	6.0±4.7 ^{b}	95.2±8.3 ^{a}
4	2.8±1.0 $^{b\,c}$	9.8±4.6 $^{b\,c}$	100±0.0 ^{a}
5	1.4±1.1 ^{a}	3.5±3.9 ^{a}	77.8±25.5 ^{a}
6	2.6±1.0 $^{b\,c}$	8.1±4.1 ^{b}	100±0.0 ^{a}
7	3.6±1.2 ^{d}	16.9±8.3 ^{d}	100±0.0 ^{a}

The means ± SD are shown. For each column, data sharing the same superscript lower case letter are no different from the significance level of $p<0.05$ (Student's *t*-test).

The most effective medium was Medium 7, with 3.6 ± 1.2 and 16.9 ± 8.3 shoots and leaves per explants respectively (Table 2), whereas Medium 5 showed the lowest values in both parameters.

The detailed analysis of the shooting phase showed three periods of growth under our experimental conditions (Fig. 3). First, a latency phase of about 10 days with no detected changes in explants. Secondly, an exponential emergence of new shoots for 10 days appeared in 80-90 % of the explants. Finally a stationary phase

with a slight number of new shoots emerged; although elongation and subsequent development of new verticils and leaves in the plantlets were produced without any lateral ramification.

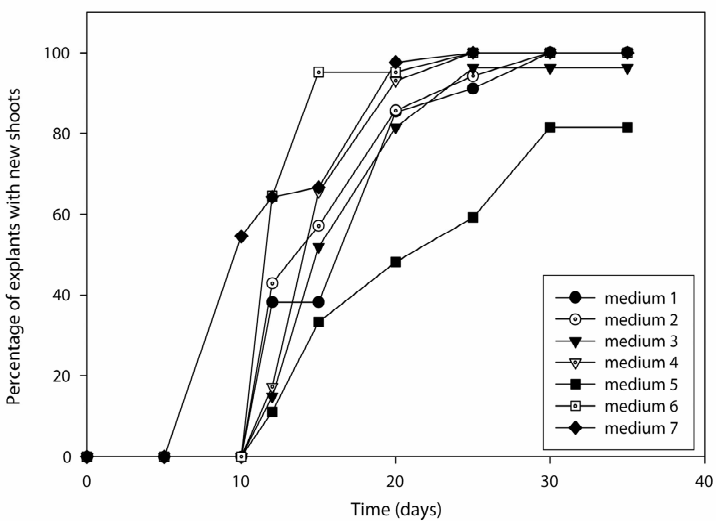


Figure 3. Kinetics of the shoots appearance. New shoots evolved in explants was registered from the onset of sowing up to day 35. The results are expressed as a percentage of overall cultures tubes, after discarding the fungi polluted cultures. In the inset, the symbol numbers correspond to media codes as in Table 1.

Root induction

After 30 days from the beginning of the *in vitro* culture, shoots produced in Medium 7 in the previous phase were removed and they were inoculated in a new culture medium. Root development was only achieved by using half-strength MS medium with a quarter of ammonium nitrate and supplemented with thiamine and myo-inositol as vitamins, as concentrations indicate in "Materials and Methods". When phytohormones were added to the culture media, roots did not sprout. No roots were produced when full salt MS medium was used or when plantlets were kept in Medium 5, contrary to what was described by Viémont and Lambert (1994) for *Erica x darleyensis*. This observation remained the same even after 70 days of culture. In fact, explants of *E. andevalensis* growing in the Viémont's medium

showed a slight shoot elongation without lateral ramification and with smaller leaves than the ones observed with the other culture media. The plantlets kept in the original medium did not show roots emergence, and therefore medium change was necessary.

After three weeks of latency, roots began to appear in the plantlets and the maximum rooting percentage came up to 63.41 ± 6.89 % on day 37, with no new root appearance in later days. Once roots were developed plantlets began to grow in length without lateral ramification, yet producing new verticils. The stem elongation and new verticils formation started between 11 and 15 days after the first root appearance.

Because the production of roots in an *in vitro* culture depends on many factors, specialized literature shows contradictory evidences. Therefore no general rules may be established for all *in vitro* cultures. For instance, *Lavandula dentata* achieved 100 % of rooting on MS phytohormone free medium (Echeverrigaray et al. 2005). Also using a MS phytohormone free medium, 75 % rooting induction was obtained by using a half-strength MS medium, in the propagation of *Tylophora indica* (Faisal and Anis 2005).

Acclimatization

Once the plantlets were propagated *in vitro*, after 75 days from the beginning of the cultures, they were temporarily transferred for two weeks to plastic containers with perlite as substrate and kept in the plant growth chamber. After that period, the plantlets produced were transferred to pots which contained commercial substrate. The pots were in the growth chamber for two weeks, and, after that time, they were exposed to outdoor environmental conditions. The survival of the plants transferred to pots was 64.85 ± 5.48 %. The start of lateral

ramification was observed 15 days after the change to pots and at the same time that stem elongation was taking place. The growth of plants was followed for one year. No apparent differences with natural plants growing in the acidic, polluted soils in mine locations were observed.

The different stages of the micropropagation of *E. andevalensis* are shown in Fig. 4.

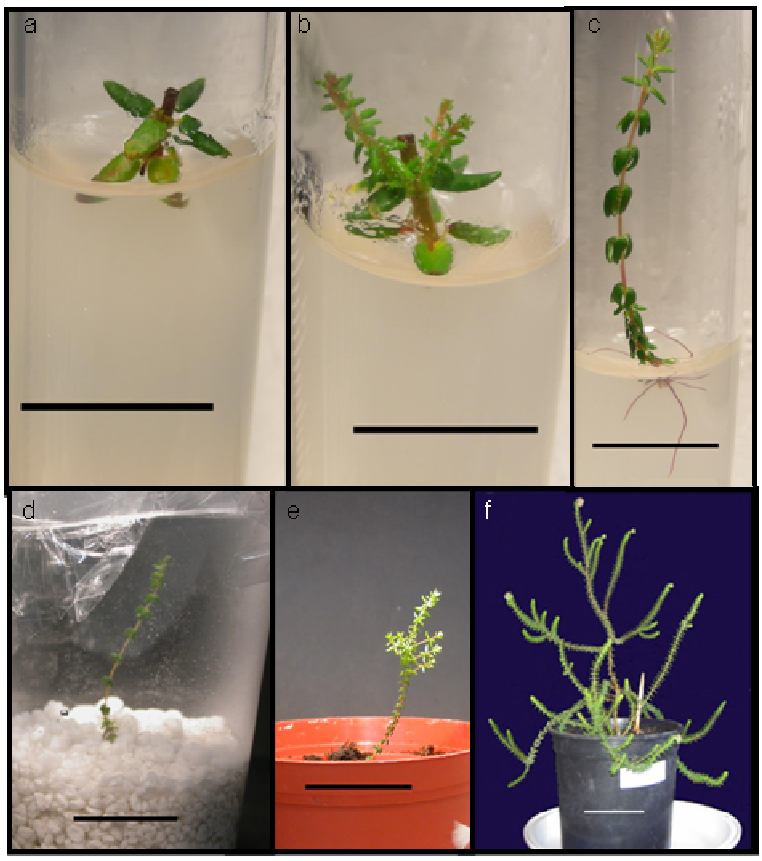


Figure 4. Sequence of the main stages in the micropropagation of *Erica andevalensis*. **a** Stem fragment inserted in the culture medium at the day 0 (bar 1 cm). **b** Explant with new shoots and verticils (day 20) (bar 1 cm). **c** Explant with elongated stem after roots development (day 70) (bar 1 cm). **d** *E. andevalensis* growing in a perlite substrate inside a plastic container covered with a transparent plastic film to prevent dessication (day 80) (bar 2 cm). **e** *E. andevalensis* growing in the plant growth chamber in a pot containing a commercial plant substrate (day 100) (bar 2 cm). **f** *E. andevalensis* growing outdoor in a pot containing a commercial plant substrate (bar 5 cm).

CONCLUSIONS

The overall objective of this study was to develop a system for the mass propagation and aseptic culture of *E. andevalensis*. New shoots were regenerated from pieces of stems collected in the field, achieving the best propagation with the medium containing the lowest ammonium nitrate concentration. Thiamine and myo-inositol were the only vitamins added. Phytohormones were necessary in the first phase for new shoot production (auxin in a higher concentration than cytokinin) and no phytohormones were required for the root induction. This is the first report on *in vitro* propagation of *E. andevalensis.* With this micropropagation procedure it would be possible to produce plant material that can then be used for indepth biochemistry studies related to the antimitotic, antiulcer and antimicrobiane properties, and also to the antioxidant properties derived from the special phenolic composition found in this species. Most importantly the plants produce can be used for an *ex situ* conservation tool for this endangered and law protected species.

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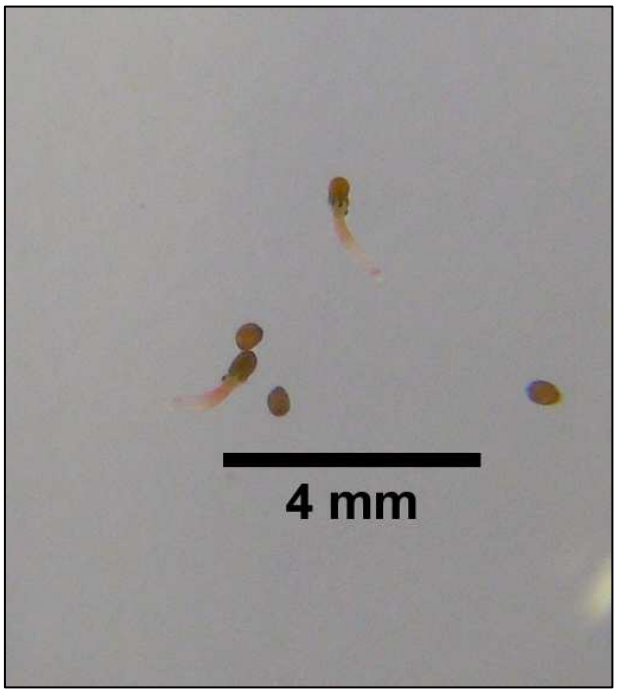
6. EFFECT OF HEAVY METALS AND ACID PH IN GERMINATION OF *ERICA ANDEVALENSIS*, AN ENDEMIC SPECIES FROM SW IBERIAN PENINSULA

Efectos de los metales pesados y la acidez en la germinación de *Erica andevalensis*, una especie endémica del SO de la Península Ibérica



Effect of heavy metals and acid pH in germination of *Erica andevalensis*, an endemic species from SW Iberian Peninsula.

ABSTRACT

Erica andevalensis Cabezudo & Rivera is an endangered heather from SW Iberian Peninsula growing always associated to mining areas of the Iberian Pyritic Belt. Laboratory experiments were carried out to test the effect of acid conditions (testing pH 1, 2, 3, 4 and 5) and different metals such as Fe, Mn, Cu and Zn at 5, 50, 200 and 500 mg/L on the seed germination and initial plant development. Germination was favoured significantly by pH 2 ($p < 0.05$) and 200 and 500 mg/L Fe ($p < 0.05$). No significant differences were found in the rest of the acidic or metal treatments compared with the control one. Anyway, the seedling growth was favoured by pH 2, 200 mg/L Fe.

A negative effect, and therefore toxic for *E. andevalensis* under the experimental conditions, was produced by 500 mg/L of all the tested metals. Seedling in these treatments showed smaller roots and smaller or non developed cotyledons. These results suggest that *E. andevalensis* not only resists extreme conditions but also its germination is favoured by them, colonizing habitats where no other species could survive.

KEYWORDS

Ericaceae; Iberian Pyritic Belt; Hypocotyls; Iron; Seeds

INTRODUCTION

Erica andevalensis is an endemic heather of the SW Spain and Portugal, described by Cabezudo and Rivera in 1980. This species is included in the endangered category by the Regional Government (Aparicio 1999). This heather is characterized by growing always in soils with acid pH (2.5-4.5) and withstands levels of heavy metal as high as 77.8 g/kg Fe, 1301 mg/kg Cu, 600 mg/kg As, 2175 mg/kg Pb (Nelson et al. 1985; Soldevilla et al. 1992; Heras et al. 2002; Rodríguez et al. 2007). The soils in this region, known as "Iberian Pyritic Belt", have been exploited since pre-Roman's time (5000 years ago) to extract copper, silver and gold (Hudson-Edwards et al. 1999). This area is the largest mass of sulphide and manganese ores in West Europe (Montes-Botella and Tenorio 2003). The result of all this mining activity is a landscape dotted with open-pit pyrite mines, as well as galleries in the underground with the waste tailings outside (Davis et al. 2000). The mining activity has increased the amount of mineral exposed to environmental conditions, giving rise to a high pollution in metals and acidity in the rivers and watercourses in the region. The presence of metals and acidity in the soils and waters for a long time has made possible the adaptation of some species of fungus and bacteria to such extreme conditions (López-Archilla et al. 2001). It is in these tailings, as well as in the banks and terraces of the rivers that cross the Pyritic Belt, Odiel and Tinto, where *E. andevalensis* lives. In the most extreme habitats no other plant species are able to live. When the conditions are temper, other heather, *Erica australis*, is commonly found growing together with *E. andevalensis* (Aparicio 1999). Little is known at the moment about the germination requirements of this peculiar species, which produces successfully high number of seeds, but it is always associated to acid and metal-enriched soils (Aparicio and García-Martín 1996). It has been described that the seeds have a dormancy period which is broken after submitting the seeds to a cold and wet treatment (Aparicio 1995). Recently, a new study (Rossini-Oliva et al. 2008) pointed out the effect of cold pre-treatment,

heat and acid conditions, gibberellic acid and nitrogen, with a significant increase in the germination after gibberellic acid treatment, reaching values close to 40 %.

It is widely described that heavy metals has toxic effects in plants at high concentrations. Seed germination is one of the most sensitive physiological processes in plants and it is more sensitive to metal pollution due to the lack of defence mechanisms (Liu et al. 2005). The literature related with heathers and germination is wide in the effects of fire (Cruz et al. 2003; Crosti et al. 2006), however, in the case of *E. andevalensis*, growing close to rivers or in mining tailings the fire has not a general role to explain germination.

The aim of the present work was to test the effects of the acidity (testing pH from 1 to 5) and some of the most abundant heavy metals in the area, such as Fe, Mn, Cu and Zn, which are essential for plants, but toxic at high concentrations, on the germination of *E. andevalensis* seeds. In the experimental design the concentrations tested included the range in which the species usually lives. The initial hypothesis to test was that *E. andevalensis* seed germination is influenced by the acid conditions as well as the presence of metals in the soil, without the presence of mycorrhiza or bacteria in the rhizosphere that has been also described (Turnau et al. 2007; Mirete et al. 2007), which will play an important role once the plant is settled.

MATERIALS AND METHODS

The seeds of *Erica andevalensis* were collected in a terrace of the Odiel River (37º 43’ N – 6º 42’ W) in autumn 2007, from mature fruits from different specimens. The seeds were cleaned and stored in paper envelops under dry conditions, room temperature and dark.

The viability of the seeds was checked selecting only the ones that sank in a distilled water pot, rejecting those seeds which float as being considered empty. *E. andevalensis* seeds has been described to have physiological dormancy, which is broken when the seeds are submitted to cold (4ºC) and imbibed conditions for 20 days (Aparicio 1995). After the cold pre-treatment, the seeds were air dried and stored at 4ºC for one month. The seeds were surface sterilized just before being used in 70 % ethanol (v / v) for 1 min, then rinsed in sterilized water and imbibed in 5 % (v / v) sodium hypochlorite for 10 min and finally they were rinsed three times with sterile water. Seeds were placed in petri dishes (9 cm diameter) with 3 layers of autoclaved filter paper, watered with 5 ml of the different treatments solutions and sealed with adhesive tape (Parafilm™) to avoid desiccation. Each plate contained 50 seeds and three dishes of each treatment were prepared. A total amount of 3,300 seeds were used.

The seeds were germinated under controlled-environment conditions with 12 / 12 h day/night and 29/24 ºC. The light was provided by fluorescent lamps that produce a photosynthetic photon flux density of 60 μmol m^{-2} s^{-1}. A seed was considered germinated when the radicle had the same size as the seed (between 0.2 and 0.3 mm). The germination was followed during 55 days, recording the germination in each plate every 2 or 3 days. Germination percentages were calculated for each plate.

Effect of pH

The seeds were watered with deionised water acidified with sulphuric acid, to get a final pH of 1, 2, 3, 4 and 5. The use of the sulphuric acid to adjust the pH of the water was justified by its presence in the river water suffering AMD (López-Archilla and Amils 1999; Montes-Botella and Tenorio 2003) and the toxicity produced by chloride in germination studies (León et al. 2005).

Effect of metals

The metal concentrations used were chosen taking into account the concentrations described as dissolved in the waters of the Odiel River (Montes-Botella and Tenorio, 2003) in the point were the seeds were collected (close to "Puente de los Cinco Ojos"). This study tested the effect of lower and higher metal concentrations in the germination process, including the range in which the plant survives.

Aqueous solutions of the metals were prepared at 5 mg/L, 50 mg/L, 200 mg/L and 500 mg/L. The metals used in the experiment were added as sulphates: $Fe_2(SO_4)_3$ $7H_2O$, $CuSO_4$ $5H_2O$, $MnSO_4$ H_2O and $ZnSO_4$ $7H_2O$ and they were dissolved in deionised water (final pH between 4.40 and 5.60). The chemical form of the metals added was decided according to the most abundant form in the environment where the plant usually lives (López-Archilla and Amils 1999; Montes-Botella and Tenorio 2003).

A control experiment was designed using only deionised water as previously described by Aparicio (1995).

Initial development of the germinated plants

Each new germinated seed was marked and kept in the plate during 10 days. The seedling development was analyzed after this time, measuring the length of hypocotyls and the main root, number of roots, length and width of the cotyledons.

Data analysis

The germination dynamics were analysed using the parameters t_0 (number of days until the first seed germinates) and t_{50} (number of days necessaries to reach the 50 % of the final germination percentage of germinated seeds) that had been previously described in *E. andevalensis* germination assay by Rossini-Oliva et al (2008).

The statistical analyses of the data were performed using SPSS. The normality of the data was tested and Kruskal-Wallis and Mann-Withney U tests were performed due to the non-normality of the data. The significance levels considered in each case are indicated in each figure or table.

RESULTS

Effect of pH

The effect of the pH in the time course of germination (Fig. 1) was stimulated at pH 2 since the beginning and along all the treatment, significantly different to the control ($p<0.05$). This treatment reached a final germination percentage of 24.28 ± 6.94 % of germinated seeds after 55 days (Fig. 2). No more seeds germinated after this day. Slight differences were found in the t_0 in this experiment (7 days for control and pH 2 and 9 days for the rest of treatments) and no differences were found when t_{50} was calculated. No germination took place at pH 1 and no differences compared with the control were found in the rest of pH tested.

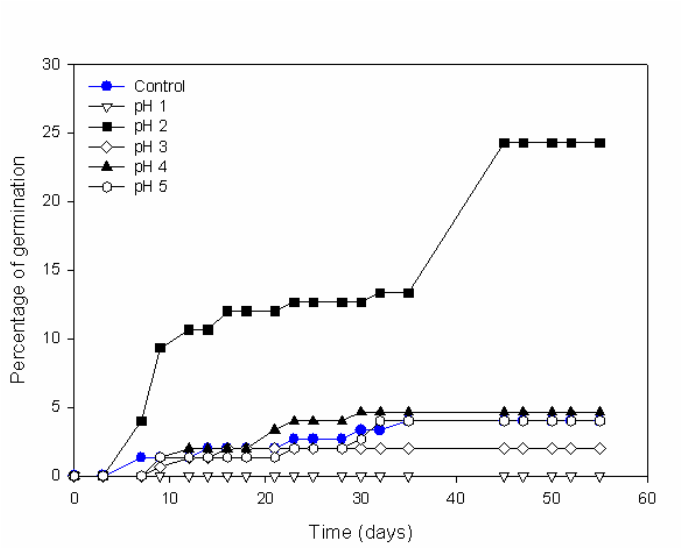


Figure 1. Cumulative percentage of germination for *E. andevalensis* seeds germinated under the different pH treatments for a 55 days period.

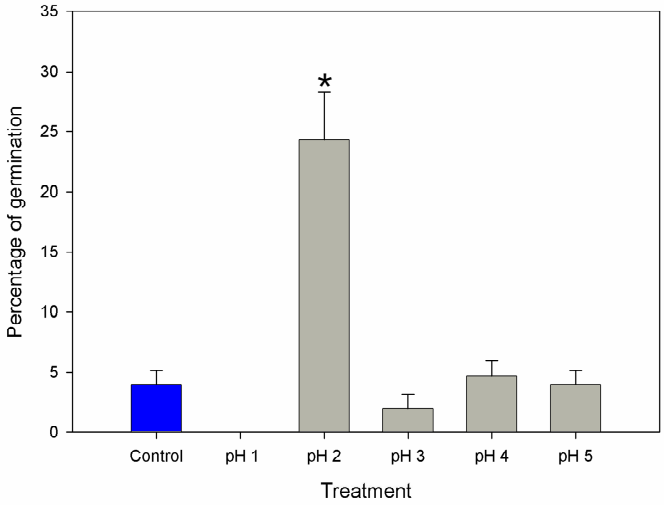


Figure 2. Final percentage of germination after 55 days of treatment. Mean ± standard error is represented. An asterisk on top of the bar (*) means a significant difference at $p < 0.05$, compared with the control.

Effect of metals

The tested metals had different effects in germination of *E. andevalensis* (Fig. 3 A-D). After 55 days, the germination percentage in the plates with 200 and 500 mg/L of Fe were significantly higher than the control ($p<0.05$) (Fig. 4). The concentrations tested for Mn, Cu and Zn did not affect the final seed germination percentage of *E. andevalensis* (Fig. 3 B, C and D). In general all the treatments started the germination between the days 7 and 9. The highest Mn concentration retarded the start of the germination ($t_{0\ =}$ 14) and a delay was also produced by 5 mg/L Zn and 50 mg/L Cu, which started the germination at 23 and 28 days, respectively. Only Fe stimulate the germination when it was applied in the highest concentrations (Fig. 3 A). However, any of the tested metals had effect in the time course of the germination when t_{50} was calculated.

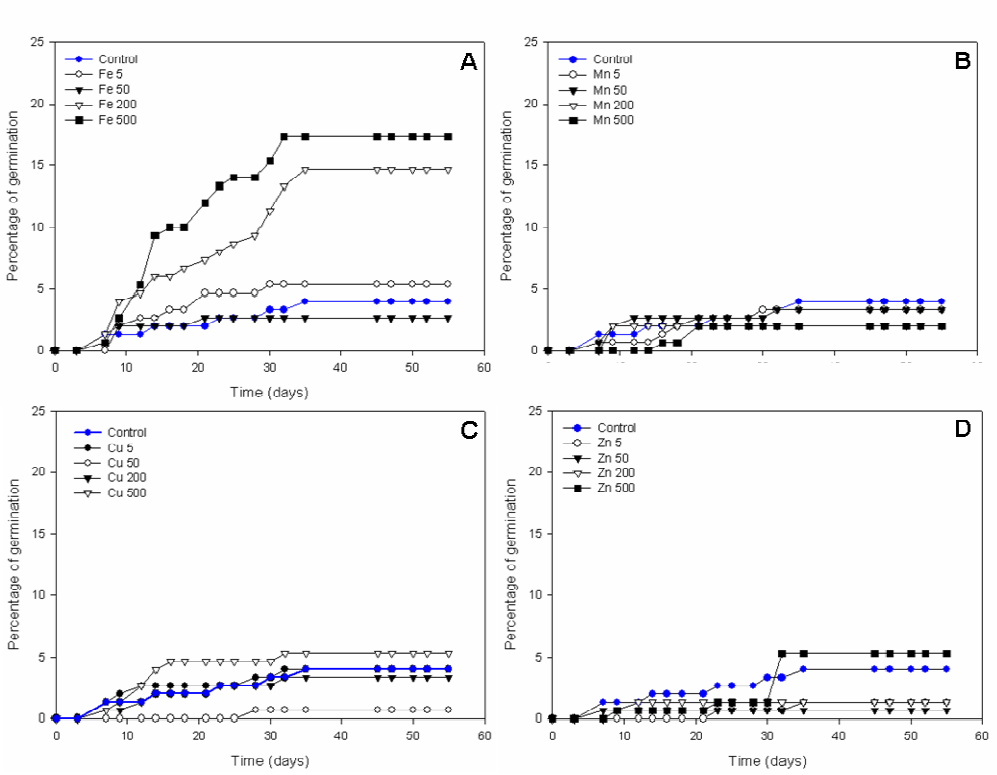


Figure 3. Effects of Fe (A), Mn (B), Cu (C) and Zn (D) in the cumulative percentage of germination for *E. andevalensis* seeds during 55 days.

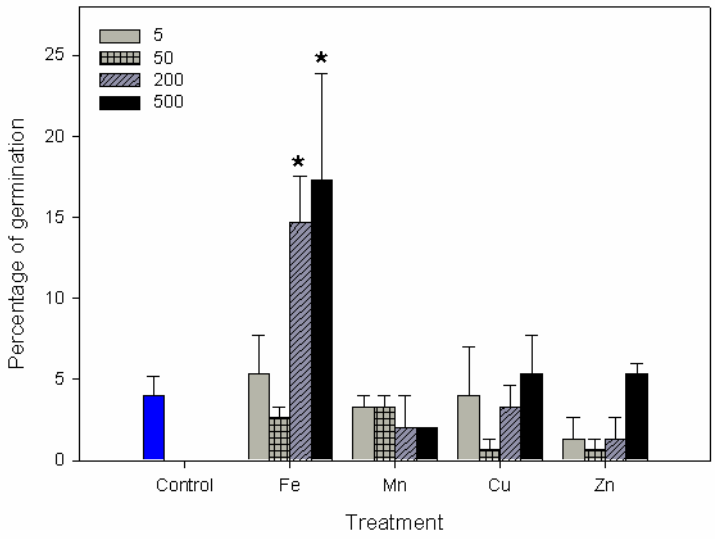


Figure 4. Final percentage of germination after 55 days of treatment. An asterisk on top of the barr (*) means a significant difference at $p < 0.05$, compared with the control.

Initial development of the germinated plants

The seedling development was analyzed in those treatments that experimented changes in the germination rates: pH and Fe (Table 1; Fig. 5). The pH 2 had not only a positive effect in the germination rate, but also the plants were larger, with longer roots and larger cotyledons, compared with the control ($p<0.05$). No differences were found in the plant size in the rest of pH levels tested.

No differences in size were found in plants treated with 5, 50 or 200 mg/L of Fe, except for the cotyledon width that was larger in 50 and 200 mg/L ($p<0.05$, compared with the control) (Table 1). High concentrations of Fe, 500 mg/L, resulted in toxic effects for the plants, due to the reduced hypocotyls length and cotyledons size and no development of roots. This toxic effect was also observed in the little number of seeds that germinated in 500 mg/L of Cu and Zn. In the case of Cu, any of the 8 germinated seeds were able to develop the roots and the cotyledons were included in the testa during the 10 days that the growth was followed. In the case of Zn, none of the 7 germinated seeds were able to develop roots.

Table 1
Results of the morphological analysis (mean ± standard deviation) of 10 days old plants in the different treatments are showed.

Treatment	**n**	**Hypocotyl lenght** (mm)	**Root lenght** (mm)	**Number of roots**	**Cotyledon lenght** (mm)	**Cotyledon width** (mm)
Control	6	2.22 ± 0.57	1.65 ± 0.85	2.00 ± 0.63	0.617 ± 0.753	0.433 ± 0.052
pH 1	0	No development	No development	No development	No development	No development
pH 2	20	2.09 ± 0.25	3.65 ± 1.32 **	2.00 ± 0.56	0.745 ± 0.094 **	0.560 ± 0.114 **
pH 3	3	2.17 ± 0.45	1.33 ± 1.35	1.33 ± 1.15	0.670 ± 0.150	0.500 ± 0.170
pH 4	7	1.94 ± 0.53	1.14 ± 0.94	1.14 ± 0.90	0.543 ± 0.113	0.414 ± 0.107
pH 5	6	2.25 ± 0.70	1.83 ± 0.78	1.67 ± 0.52	0.617 ± 0.117	0.450 ± 0.105
Fe 5	8	2.16 ± 0.26	1.13 ± 0.36	2.25 ± 0.50	0.663 ± 0.092	0.475 ± 0.117
Fe 50	4	2.5 ± 0.47	1.85 ± 1.27	2.00 ± 0.00	0.800 ± 0.141	0.600 ± 0.141 *
Fe 200	19	2.13 ± 0.36	2.77 ± 2.45	1.84 ± 0.60	0.737 ± 0.138	0.511 ± 0.120 *
Fe 500	26	1.19 ± 0.40 ***	No development	No development	0.381 ± 0.247 **	0.212 ± 0.153***

Data are showed for group of treatments that experimented a effect in the germination rate. The different treatments have been compared with the control and asterisks show statistical differences *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

DISCUSION

The results obtained in this study showed that *E. andevalensis* has a low germination percentage in *in vitro* conditions, as previously has been described by Aparicio (1995) and Rossini-Oliva et al. (2008). In these previous studies the final germination percentage was around 20 % after 20 days of cold and wet treatment of the seeds in the case of Aparicio (1995), and from 6 to 22 %, depending on different cold treatments applied to the seeds placed in water in the case of Rossini-Oliva et al. (2008). In the present study the germination percentage of the seeds placed in deionised water (control) was 4 ± 2 %. The seed sterilization may be partly responsible of the small value obtained in this control when it is compared with the previous studies. Moreover, the seeds used in the present experiment were submitted to a cold stratification (1 month) once the 20 days of cold and wet

treatment described by Aparicio (1995) was finished. Although no differences in the germination percentages were found by Rossini-Oliva et al. (2008) after 1 or 4 months of cold treatment. Nevertheless, all the seeds in the present experiment have been treated in the same way, and therefore the results obtained under the different treatments are comparable.

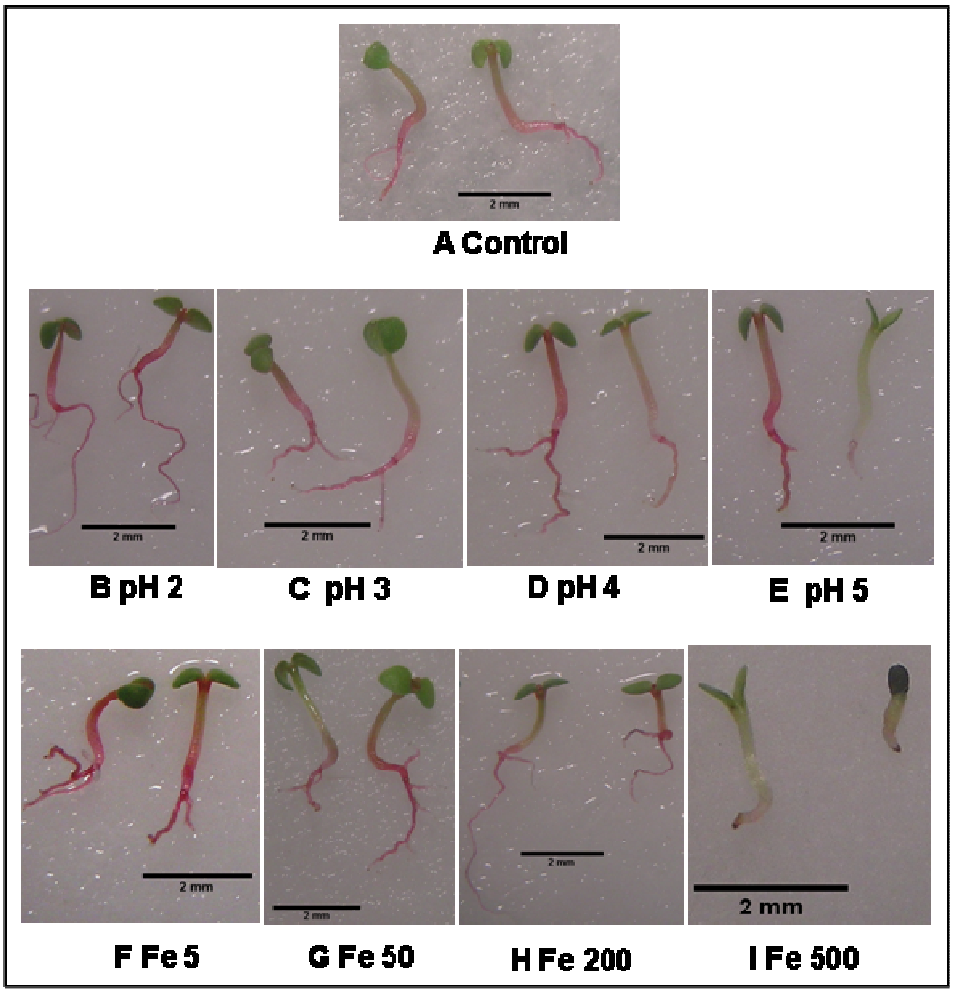


Figure 5. Seedling after 10 days of germination from pH and Fe treatments. A) Control; B) pH 2; C) pH 3; D) pH 4; E) pH 5; F) 5 mg/L Fe; G) 50 mg/L Fe; H) 200 mg/L Fe; I) 500 mg/L Fe. Bars 2 mm.

The acidity had a stimulatory effect in the germination of *E. andevalensis*. The seeds placed at pH 2 reached the highest germination percentages after 55 days. The most acidic treatment, pH 1, resulted to be too extreme and seeds were not able to stand up to it. As soon as they were placed in the plates with pH 1 solution, the covert were burned immediately, changing from brown to yellow, without any other change along the time. Not only in germination, but also in the initial plant development pH 2 treatments resulted beneficial to *E. andevalensis.* This positive effect was obvious by the longest roots and largest cotyledons (in length and in width). The pH of the Odiel river water at the sampling point is in the range of 2.8-3.4 (Montes-Botella and Tenorio 2003). This result, higher germination rate and larger plants in pH 2 treatment, supports our initial idea that *E. andevalensis* seeds germination can be influenced by acidity. The possible positive relation between pH and seed germination in *E. andevalensis* has been previously proposed by Aparicio (1999), however, in a very recent new study about different factors influencing the germination of *E. andevalensis*, the conclusions pointed that the pH did not have influence in the germination, after testing pH 3.5, adjusted with HCl (Rossini-Oliva et al. 2008). We consider that the data provided in that study is short of variables, testing only one pH point. We did not find any differences in pH 3 and 4 treated seeds compared with the control ($p<0.05$). However, a significant increase in germination and plant size was produced a pH 2. Therefore, the present study reveals that the ability of *E. andevalensis* to colonize extreme acid habitats is innate of the species, and it is already expressed in youthful stages. Furthermore, the acid pH preference seem to be independent of the presence of mycorrhiza, which are favourable to the heavy metal tolerance in *E. andevalensis* (Turnau et al. 2007).

Among the metals tested, Fe has been the only one with a stimulatory effect in the germination rate when it was added at high concentrations (200 and 500 mg/L). Despite the highest seed germination percentage obtained with 500 mg/L of Fe, this concentration resulted too high and it was not accompanied by a

good plant development (Table 1; Fig. 5 I) due to the small size of the plants observed.

The preference of *E. andevalensis* for the acid conditions and high levels of Fe is marked in its own germination. Therefore, before the competition with other species can prevent the colonization of different areas, the preferences of the *E. andevalensis* are the responsible of the restricted habitats with extreme conditions where the species lives.

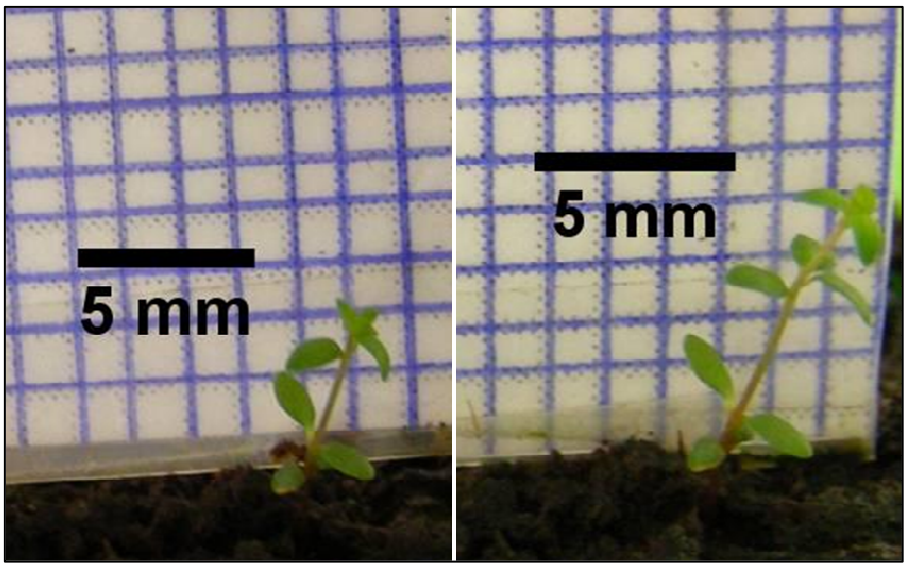
On the other hand, in this study Mn, Cu and Zn did not show any effect in the germination of *E. andevalensis*. Toxic symptoms appear at 500 mg/L in all the tested metals, with inhibition in the root formation, likewise the development of the cotyledons. Copper, abundant in the studied soils due to the relation with the mining activity in the area, it is very toxic at high concentrations for plants, coming up to inhibit the normal growth and development (Ahsan et al. 2007).

E. andevalensis has been proposed as a tool for recovery of the sulphide mining areas (Abreu et al. 2008) and the germination data presented in this study can be used to support this proposal, due to the beneficial effects of iron and acidity found in the germination process. *E. andevalensis* seeds should no present problems to germinate in soils highly affected by mining activities, undertaking the role of pioneer in the colonization of soils where other plant species are not able to survive.

The beneficial relation between acidity and Fe on the germination and initial development of *E. andevalensis* seeds could explain why this species has never been seen growing out of these conditions. *E. andevalensis* has adaptations and preferences to survive under conditions that are not favourable for most of the other species and therefore it has not developed competitive strategies to survive out of this extreme habitat. Once the extreme conditions disappear, *E. andevalensis* has not optimum for development and other species take the place of this one.

7. GERMINATION AND GROWTH OF *ERICA ANDEVALENSIS*, A METAL TOLERANT PLANT FROM SW SPAIN. EFFECTS OF COLD STRATIFICATION AND GIBBERELLIC ACID (GA_3)

Germinación y crecimiento de *Erica andevalensis*, una planta tolerante a metales del SO español. Efectos de la estratificación fría y del ácido giberélico (GA_3)



Germination and growth of *Erica andevalensis*, a metal tolerant plant from South-West Spain. Effects of cold stratification and gibberellic acid (GA_3)

ABSTRACT

Erica andevalensis seed germination was improved by an extended cold stratification period and by a second cold and wet treatment applied to the imbibed seeds 20 days after the initial sow. The growth of the germinated seeds was stimulated by transferring the seedlings to an acid soil. On the other hand, the effects of GA_3 in the germination of *E. andevalensis* have been studied. The addition of 10 or 100 mg/L of GA_3 resulted in a final percentages of germination of 26.23 ± 6.97 % and 83.15 ± 14.79 %, respectively. The no addition of GA_3 resulted in a 7.18 ± 2.57 % of germinated seeds. Seedlings were transferred to soils after 17 days in contact with the phytohormone and no more GA_3 was added. At this moment plants treated with GA_3 had elongated hypocotyls (2.1 and 2.5 times in 10 and 100 mg/L) but thinner and reduced cotyledon area. After 28 days of growing in soil, treated plants showed less biomass, thinner leaves (100 mg/L GA_3) and small height (10 mg/L GA_3). However, the plants treated with 10 mg/L possessed the highest protein content and the lowest total antioxidant capacity.

KEYWORDS

Ericaceae; Growth regulators; Hypocotyls; Pyritic Belt; Total antioxidant capacity

INTRODUCTION

Erica andevalensis Cabezudo & Rivera is an endemic species from SW Iberian Peninsula described in 1980 in Spain (Cabezudo and Rivera) and in 1998 in Portugal by Capelo et al. This heather restrictively grows in highly heavy-metal polluted soils with an acidic pH (Soldevilla et al. 1992; Nelson et al. 1993; Asensi et al. 1999; Rodríguez et al. 2007). It was catalogued as endangered by the local authorities and as vulnerable following the IUCN criteria (Aparicio 1999). Seeds of *E. andevalensis* have a dormancy which was shown to be broken after a cold and wet pre-treatment, however, germination rate remains low, reaching percentages close to 20 % after this pre-treatment (Aparicio 1995). Recently Rossini-Oliva et al (2008) tested different chemical and physical treatments to increase seed germination. The highest increase in germination percentages was observed in seeds sown in agar medium enriched with gibberellic acid (GA_3). Gibberellic acid (GA_3) is a phytohormone that affects plant growth at molecular, cellular, organic levels such as regulating synthesis of membrane phospholipids, RNA and protein synthesis, cell wall synthesis and loose, division, elongation and differentiation of cells, gravitropism, stem elongation, leaf expansion, flower initiation and seed germination (Kozlowski and Pallardi 1996; Schwechheimer 2008). Highest germination percentage increase (41.6 %), measured as appearance of the radicle, was found in seeds imbibed in the presence of 400 ppm of GA_3 to the germination media, but no cold and wet pre-treatment were applied to the seeds (Rossini-Oliva et al. 2008).

The objectives of the present work were to establish *E. andevalensis* growth under controlled laboratory conditions and to analyze the effects of cold treatments in the germination of seeds and the effect of GA_3 in the germination and initial stages of plant development in *Erica andevalensis.* Results may be considered for the possible application of this phytohormone treatment in future

conservation programs as well as new studies on the biochemical characteristics of the species.

MATERIAL AND METHODS

Erica andevalensis seeds were collected from the field in the autumn of 2007 picking mature fruits and cleaning the seeds in the laboratory. To avoid the use of empty seeds, they were placed in a glass with distilled water and only the ones sinking were chosen as viable seeds. The seeds were submitted to a cold and wet pre-treatment to break the dormancy as has been described by Aparicio (1995), and stored, after that, in cold and dry conditions for 3 and 7 months (hereafter called C3 and C7 respectively).

Prior to the experiments seeds were surface sterilized by submerging them 60 sec in ethanol (70 % v / v) and 10 min in sodium hypochlorite (5% v / v). Finally they were washed 3 times with sterilized water. To germinate sterilised seeds were sown in petri dishes of 9 cm diameter containing 0.8 % agar medium with half strength MS salts and 30 g/L of sucrose. The pH of the media was adjusted to 5.8. Vitamins (10 mg/L myoinositol, 0.1 mg/L nicotinic acid, 0.1 mg/L pyridoxine and 1 mg/L thiamine) were added sterilized by filtration through a 0.45 μm filter to have cooled down medium. The petri dishes were sealed with elastic tape (Parafilm^{TM}) and placed in a growth chamber with 26 ºC 16/8 day-night photoperiod and irradiance of 121.53 ± 3.46 μmol m^{-2} s^{-1}.

Effect of cold stratification

To test the effect of cold stratification, seeds were kept under cold and dry conditions after the cold and wet pre-treatment of the seeds for 3 and 7 months (C3 and C7). Before the pre-treatment, seeds were kept at room temperature. Between 150 and 200 seeds were placed in each plate, and 15 and 10 plates from C3 and C7 were sown respectively. The germination was followed every 2 to 3 days and the germination was expressed as percentages. After 17 days the seedlings were transferred to a soil substrate and the plates with the remaining non-germinating seeds were submitted to an additional cold treatment of 14 days at 4º C (second cold and wet treatment). After this second cold treatment, the plates were placed again in the initial growth chamber for another 27 days and the germination was followed as described.

The seedlings transferred to soil were placed in a growth chamber programmed at 22 ºC and 10 hours light, with 49.60 ± 1.17 μmol $m^{-2}s^{-1}$ provided by fluorescent tubes. Two kinds of soils were tested: normal substrate (Tref, substrate for professional horticuluture, pH between 4.2 and 6.1) and a more acidic soil commercially labelled as "heather soil" (DCM, with a pH between 3.5 and 5). Plants coming from the first germination batch were sown in both soils. Plants were placed in small pots (from 16 to 25 plants per pot) with 50 g of soil. They were watered with tap water twice a week. Photos were taken to analyse plant size.

Effect of GA_3

To test the effect of GA_3 two different concentrations of the phytohormone were added to the agar medium prior to autoclavation, to get a final concentration of 10 and 100 mg/L in the media. Three plates were prepared of each concentration (including a control without phytohormone) and around 500 seeds were used per

treatment. Seeds coming form 3 months of cold stratification (3C) were used in this experiment.

The germination was followed every 2-3 days. The germination data were expressed as percentages. After 17 days pictures were taken from the plates and the hypocotyls and cotyledons were measured in 25 plants from each treatment. These plants were transferred to 5 cm x 5 cm pots filled with 50 g of acidic soil (special for heathers, DCM) to further following of the development of the plants. Plants were placed in a plant growth chamber with the same conditions as described above. The growth of the plants in soil was followed for 4 weeks. After four weeks, the plants were harvest and used for biochemical analysis.

Protein content was measured as described by Bradford (1976) in 2 samples (and 3 replications) of each treatment were done. (Fig.1).

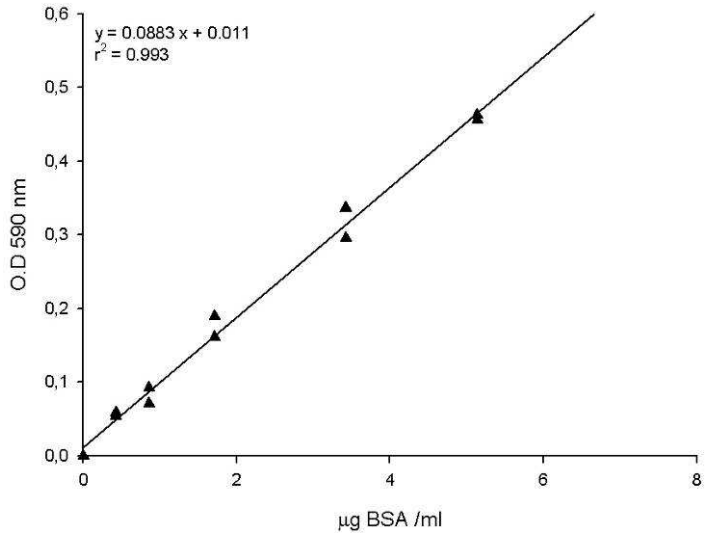


Figure 1. Calibration curve of protein, using BSA as standard.

The total antioxidant capacity was measured in two samples of each treatment (and 3 replications were made) using the FRAP method described by Peñarrieta et al. (2008). Briefly, the Fe^{3+}-TPTZ complex is reduced to blue Fe^{2+}-TPTZ complex by electron donating substances under acidic conditions. The FRAP reagent was a mixture of 100 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM ferric chloride (10:1:1). The method was adapted to a plate reader and 100 µl of reagent was added to 100 µl of sample (previously diluted when it is necessary). The absorbance was measured at 600 nm after 10 min of incubation at room temperature. The final absorbance of the samples were compared with a standard curve made using Trolox (0-100 µM) (Fig. 2). Trolox stock solution was made in methanol and all the subsequent dilutions were made in potassium phosphate buffer. The data were expressed as µmol Trolox equivalents per gram of fresh weight. The total antioxidant capacity in the buffer soluble fraction was obtained.

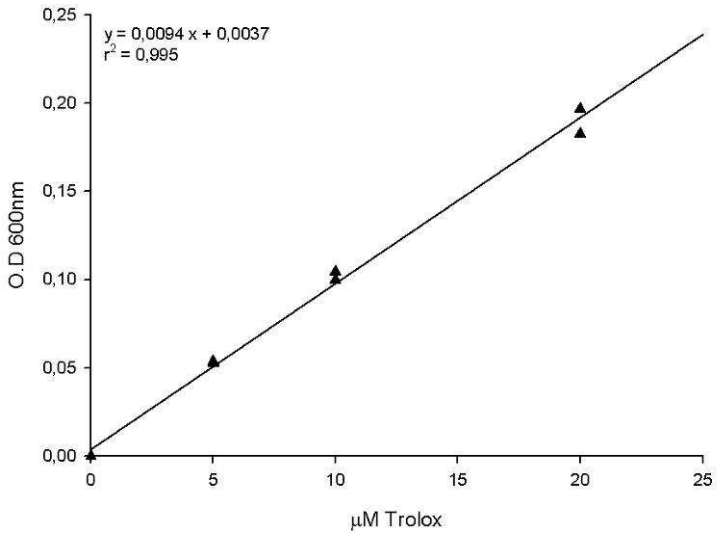


Figure 2. Calibration curve of the total antioxidant capacity, using TROLOX dissolved in methanol and buffer as standard.

Data analysis

The dynamics of the germination was analyzed by the parameters t_0 (number of days necessary to start the germination, taken as the emergence of the radicle) and t_{50} (number of days necessary to reach 50% of the total seed germination). These parameters have been described by Rossini-Oliva et al. (2008) in a previous study about *E. andevalensis* seed germination.

The size of the plants was analyzed from photos by using ImageJ Software.

The statistical analysis was carried out using SPSS and non parametric test, Kruskal-Wallis Test and Man Whitney U, with a significance level of 0.05, due to the non-normality of the data.

RESULTS AND DISCUSSION

Effect of cold stratification

Seed germination was strongly influenced by the length of the stratification period they were submitted previous to sowing (Fig. 3). In our experiments seeds were stored at 4°C for either 3 (3C) or 7 (7C) months. After 20 days the germination percentage reached values of maximum 3 % in 3C seeds compared to 21 % germination reached in 7C seeds (Fig 3). After a second cold treatment, germination percentage increased to 37 % and 71 % in 3C and 7C, respectively.

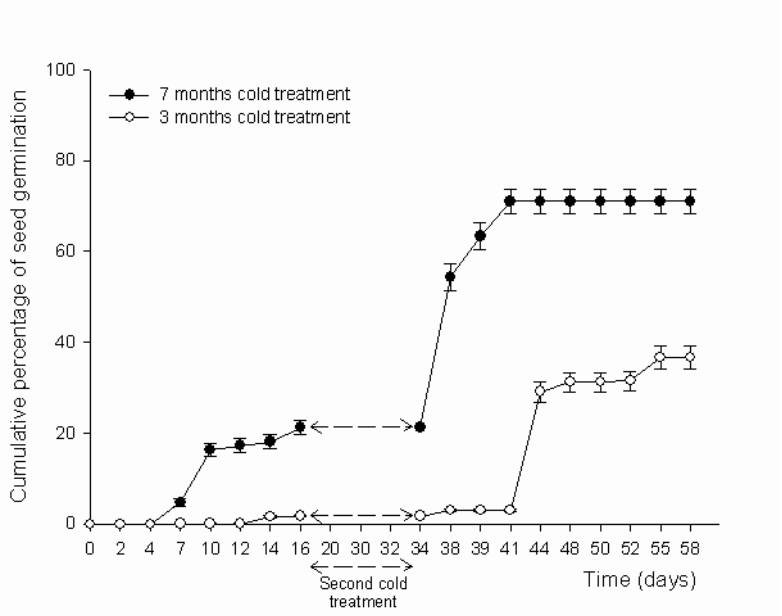


Figure 3. Time course of the germination rate of the seeds after 7 or 3 months of cold treatment. Mean value ± standard error is showed.

These results indicate that seed germination was dependent on the length of the stratification period. This result is not according to previous studies in which no differences were found in the germination of *E. andevalensi*s with seeds stored for 1 and 4 months in cold (Rossini-Oliva et al. 2008). However, the cold periods used in the work of Rossini-Oliva et al (2008) were different to ours. In addition it is shown here that germination percentage benefited from a second cold treatment, as has previously been described in *Erica andevalensis* seeds (Aparicio 1995).

At day 17 just prior to the second cold treatment, all viable seedlings were transferred from the plates to pots containing either normal soil (Tref) or heather soil (DCM) as described in material and methods. Two weeks after transfer, plants growing in the heather soil were significant ($p<0.001$) bigger than the others (Table 1). Therefore in subsequent experiments only the acidic heather soil was used. Acidic soils are the natural habitat of *E. andevalensis* (Soldevilla et al. 1992; Aparicio 1999). Here we tested both a basic and a more acidic soil found that this

species is able to grow in absence of acidity, but growth rate is clearly reduced, as indicate Table 1. The dependency of *E. andevalensis* for acid conditions maybe due to a better nutrient uptake in acidic conditions.

Table 1

Number of verticils, leaves, length of the cotyledon and total height of *E. andevalensis* plants after 2 weeks growing in different substrates.

	Number of verticils	Number of leaves	Cotyledon length (mm)	Total height (mm)
Normal soil	1.72 ± 0.06^{a}	3.70 ± 0.11^{a}	0.55 ± 0.01^{a}	1.68 ± 0.06^{a}
Heather soil	2.11 ± 0.07^{b}	4.44 ± 0.14^{b}	0.63 ± 0.01^{b}	2.01 ± 0.06^{b}

Mean ± standard error from 53 and 55 plants growing in normal and heather soil, respectively, are showed. Different superscript letter in each column means significant differences ($p<0.001$) after a Kruskal-Wallis test for number of verticils and number of leaves and ANOVA for cotyledon length and total height.

Effect of GA_3

Seed germination is one of the most sensitive physiological processes in plants life cycle (Lui et al. 2005). Although little is known at the moment on germination and initial plant development in *E. andevalensis*, GA_3 is an important endogenous growth regulator with several effects on plant growth and development (Chuanren et al. 2004) and beneficial effects have been described in a recently in the germination of *E. andevalensis* (considered as radicle appearance). However, and to minimize the possible effects that this phytohormone could have in plant development, low concentrations of GA_3 were chosen in this study. A similar experimental set up was recently used to describe germination characteristics of *Scrophularia marilandica* L. (Nurse and Cavers 2008).

Highest germination rates (83.15 ± 14.79 after 33 days) were found in the plates containing the highest GA_3 concentration (Fig. 4). After 33 days seeds

germination ceased in all tested conditions (Fig. 4). The effect of GA_3 on germination is strong reaching germination percentages 4 and 12 times higher than the control when 10 and 100 mg/L GA_3 were added to the medium. However, the different treatments did not affect the parameters t_0 neither t_{50}. As such our data are contrasting to the results obtained by Rossini-Oliva et al. (2008), who showed a decrease in t_0 and t_{50} when GA_3 concentration was increased, testing 0, 25, 50, 100, 200 and 400 mg/L. However, it is not excluded that these differences can be due to differences in the experimental set-up between these two studies.

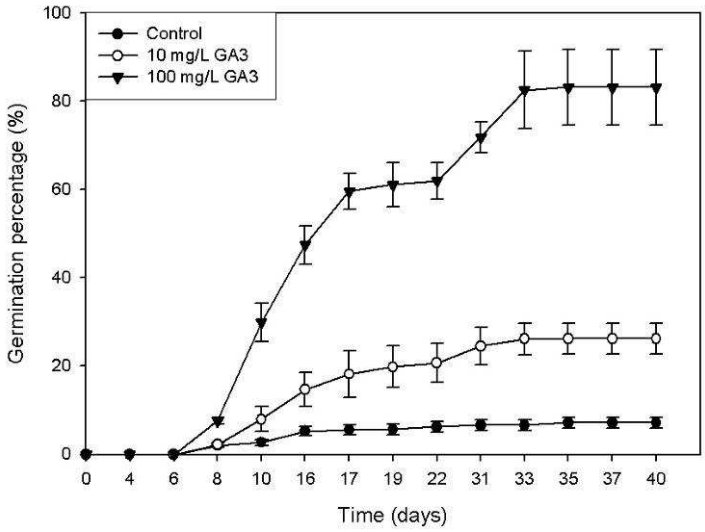


Figure 4. Percentage of accumulate number of germinated seeds of *E. andevalensis.* Each value represents mean ± standard error n=4. Significant differences (p<0.05) were found among all the treatments.

The biometric parameters used in this study to analyse the effects of GA_3 were the length, width and area of the cotyledons and the first leaf, the total height of the plants, the number of leaves produced and the fresh weigh per plant. The protein content and the total antioxidant capacity were selected as biochemical parameters. The results showed that GA_3 affect to plant biometric parameters, not only when the phytohormone is present, but also 28 days after its application,

resulting in smaller leaves in the highest concentration tested (Fig. 6 C) as it will be commented.

In the initial developmental stages (17 days after the germination), GA_3 treatments affected hypocotyls length (Fig. 5 and 6) with significant differences ($p<0.05$) among all treatments. This effect was previously described in other species, such as *Aquilegia nivalis, Lagostis cashmeriana* and *Meconopsis latifolia* (Dar et al. 2009), *Scrophularia marilandica* (Nurse and Cavers 2008). However, in general, all these studies only studied the effect of the phytohormone on the promotion of germination, without looking further at biometric or physiological parameters of the seedlings. After 17 days of growth in the petri dishes, seedlings were transferred to soil, in absence of GA_3 and the size of the plants were followed for the next 4 weeks. When the seedlings were transferred to pots, those ones that had germinated in the presence of GA_3 (100 mg/L) had smaller cotyledons compared to the control (Fig. 7 A, B and C (point time 0)). Transfer to soil diminished the effect on the size of the cotyledons in length and area and after seven days in soil no significant differences among the different treatments were presented (Fig. 7 A and C). However, at day 14 the control had cotyledons significantly ($p<0.05$) shorter than the GA_3 treated plants in both concentrations tested (Fig. 7 A) and the plants treated with 100 mg/L GA_3 had thinner cotyledons ($p<0.05$) (Fig. 7 B). The combination between length and width made that at day 14 the cotyledons from plants with 10 mg/L GA_3 treatment were significantly bigger ($p<0.05$) than the rest of treatments (Fig. 7 C). From the day 14 the cotyledons reduced their size, more markedly in 10 mg/L treatment (Fig. 7 C).

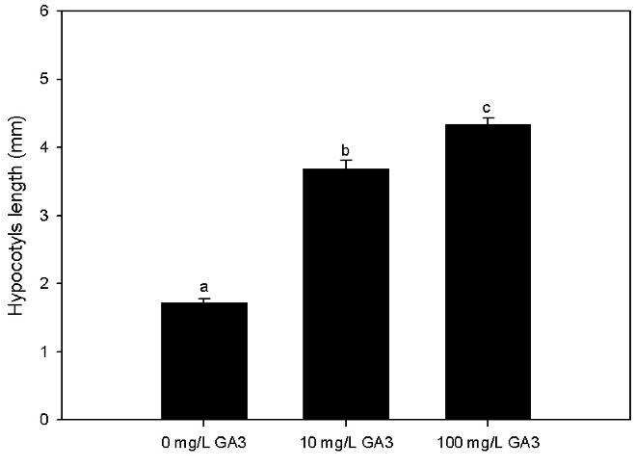


Figure 5. Length (± S.E.) of the hypocotyls resulting from the different treatments after 17 days. For each treatment n=25. Different letters means statistical differences ($p<0.05$).

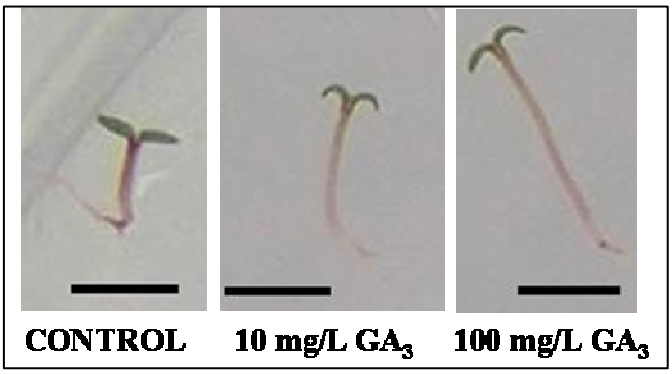


Figure 6. Hypocotyls of *E. andevalensis* after 17 days in plates with no GA3 (control) 10 or 100 mg/L GA_3. Bar = 2 mm.

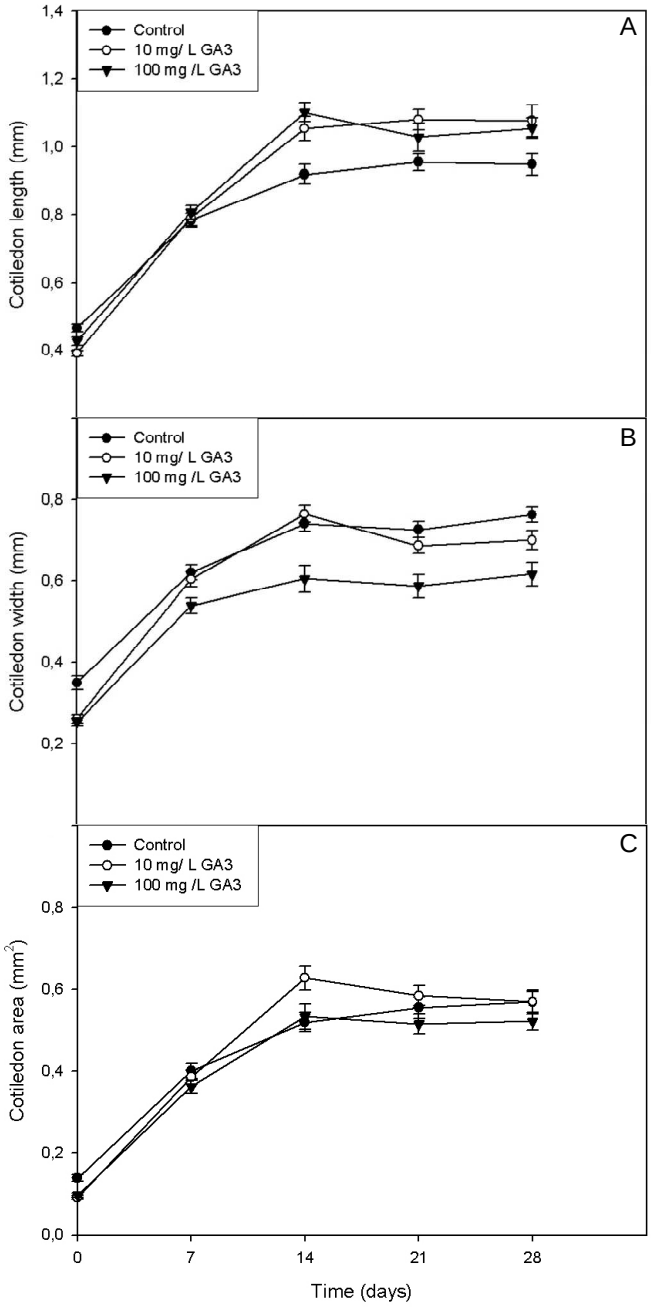


Figure 7. Length (A), width (B) and area (C) (± S.E.) of cotyledon from the different treatments along the time after planting in soil. For each point n=25.

The growth of the first leaf showed that the different GA_3 concentrations applied to the seed germination media did not have any effect in the length of it once the plants were growing in soil (Fig. 8 A). The width, as it was described above for the case of the cotyledons, was affected by the highest GA_3 concentration applied (100 mg/L), making that the plants produce narrower leaves ($p<0.05$) from the day 14 onwards (Fig. 8 B). The area of the leaf (Fig. 8 C) was significantly bigger ($p<0.05$) at the days 7 and 14 in the control. From day 21 no differences were found in the area of the first leaf between the control and 10 mg/L of GA_3, remaining smaller ($p<0.05$) the leaf in plants coming form 100 mg/L GA_3 due to the small width of the leaf (Fig. 8 C).

The growth of the plants in height (Fig. 9 A) showed differences since the day 14, reflected in the fact that plants coming from 10 mg/L of GA_3 treatment were significant smaller ($p<0.05$) than the others. From the day 21, no differences in plant height were found between control and 100 mg/L GA_3 (Fig. 9 A). The number of leaves (Fig. 9 B) seems not to be affected by GA_3 treatment, since the only significant difference ($p<0.05$) was found at day 14, when the numbers of leaves in the control was bigger.

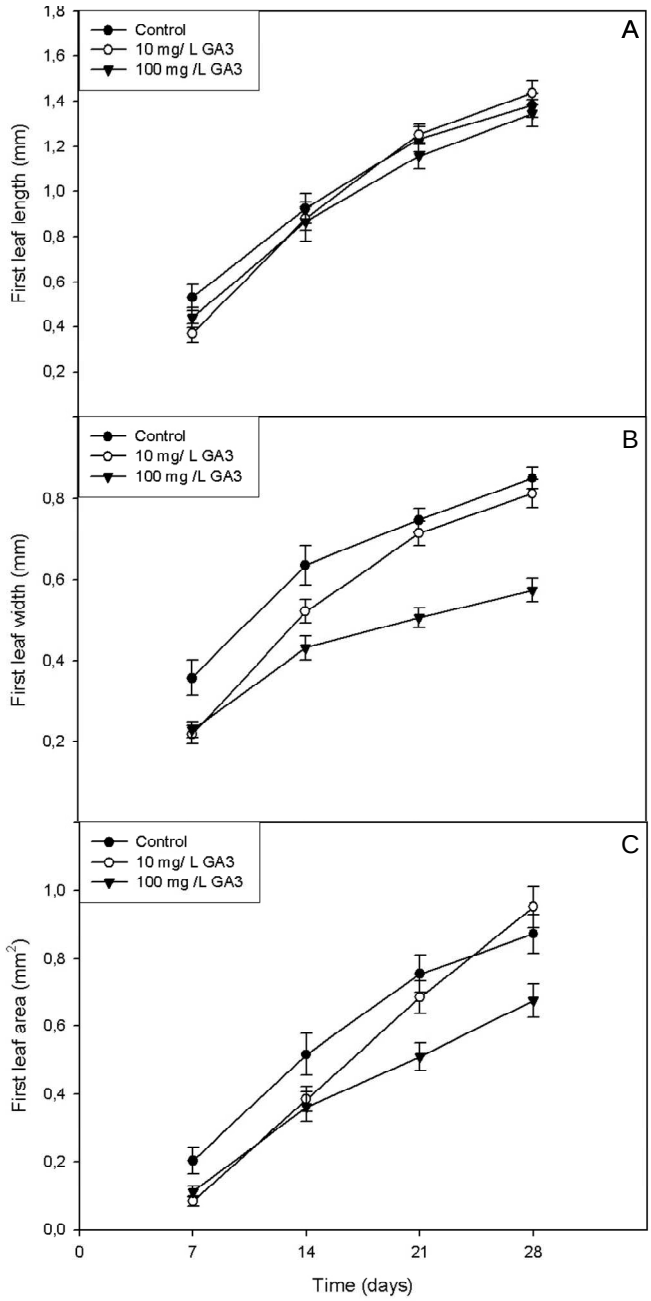


Figure 8. Length (A), width (B) and area (C) (± S.E.) of the first leaf from the different treatments along the time after planting in soil. For each point n=25.

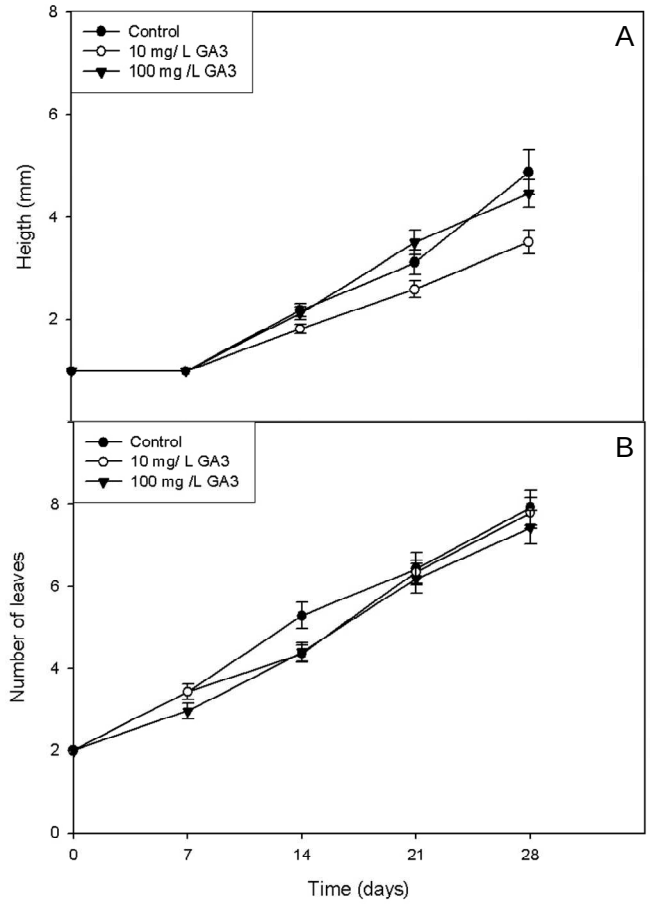


Figure 9. Growth expressed as the total height (mm) (A) and the number of leaves (B) for the different treatments along the time after planting in soil. For each point n=25.

After 28 days of growing in soil, the biomass produced per plant and the protein content was measured. Plants that were germinated in the presence of GA_3 had smaller biomass per plant compared to the control (Fig. 10 A). A reduction in dry weight in shoots and roots has been previously described in *Glycine max* when GA_3 was added at 0.05 mM (Saeidi-Sar et al. 2007). In the present study the biomass reduction can possibly be explained by the smaller size of the leaves in 100 mg/L GA_3 and the smaller plant height in 10 mg/L GA_3 treatment. However, we have found that the lower biomass did not correspond with the soluble protein

content, which was the highest in 10 mg/L GA_3 (Fig. 10 B). An increase in protein content related with an increase in N assimilation and in plant size has been proposed in okra plants (*Abelmoschus esculentus*) (Ilias et al. 2007). However, we have found that in *E. andevalensis* the protein content increased at 10 mg/L GA_3 but it was reduced, without significant differences with the control, in plants coming form 100 mg/L GA_3 in the germination media. A similar pattern was described for *Rosa canina* (Atalay and Kadioglu 2001) but no explanation was given for this data.

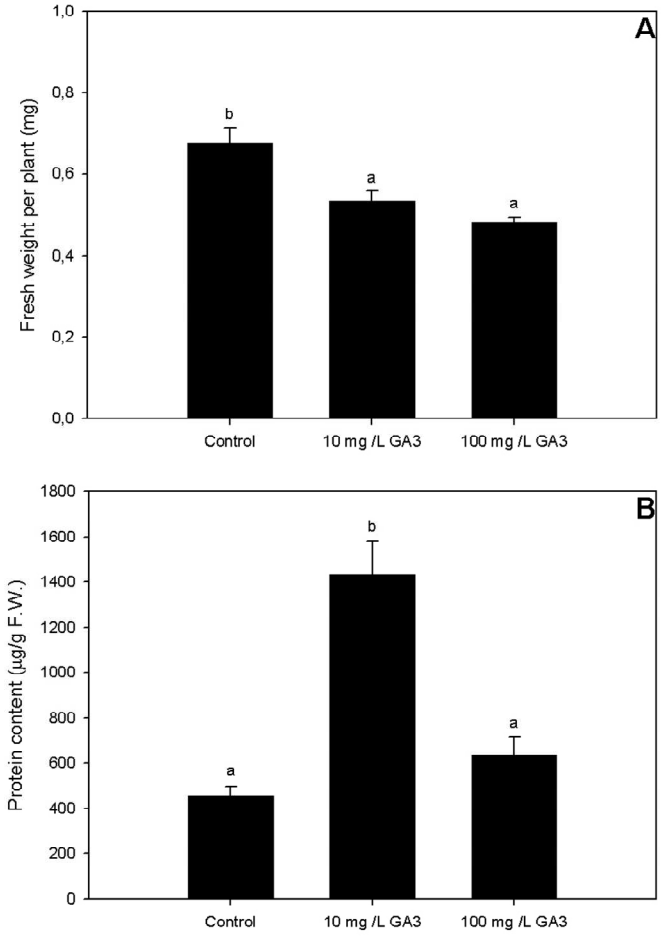


Figure 10. Fresh weight per plant and protein content in *E. andevalensis* plants coming from different germination media after 28 days growing in soil. n= 2 in A and 6 in B for each bar. Different letters indicate significant difference (Kruskal Wallis and Man Whitney U test) ($p<0.05$)

GA_3 is known to have effects on gene transcription (Chaudhry et al. 2006) and a treatment at the beginning of the plant development can have long-lasting effects as we could observe in the smaller size of leaves after 28 days without phytohormone, as well as the smaller biomass per plant produced.

The total antioxidant capacity gives the concentration of electron donating substances that are able to reduce the Fe^{3+}-TPTZ complex that are present in the sample (Peñarrieta et al. 2008). We have found that this capacity was the smallest in plants treated with 10 mg/L of GA_3 (Fig. 11). Little is known about the relation between GA_3 and reactive oxygen species (ROS) (Ducic et al. 2003/4). It has been described that GA_3 reduce the oxidized glutathione (GSSG) levels, without affect the total amount of glutathione in cell cultures of *Catharanthus roseus* (Ohlsson and Berglund 2001). However, in our case, the smallest concentration of GA_3 had the opposite effect: a reduction in the amount of antioxidant compounds, with a recovery, with no differences to the control, when higher concentrations of GA_3 were applied (Fig. 11).

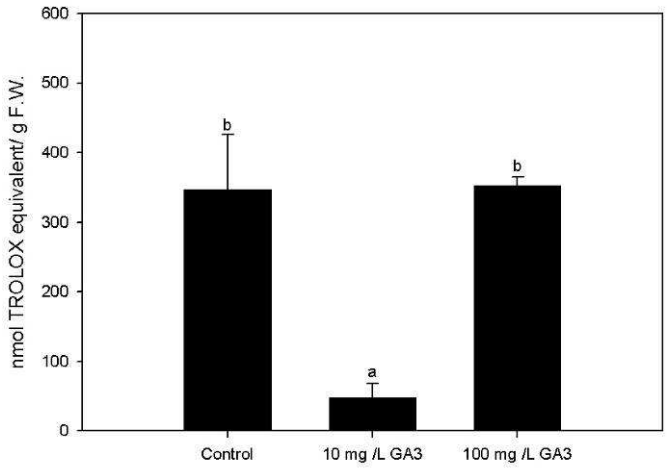


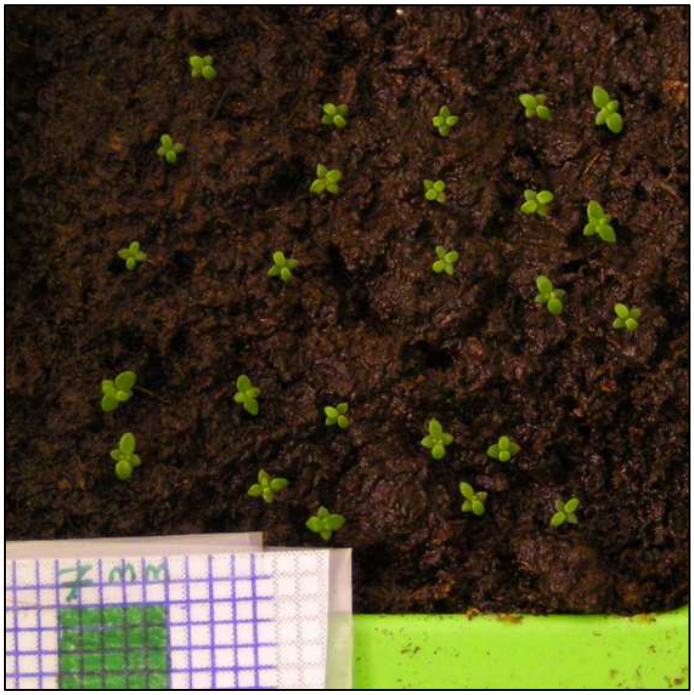
Figure 11. Total antioxidant capacity (TAC) nmol TROLOX equivalent/ g F.W. in *E. andevalensis* plants coming from different germination media after 28 days growing in soil. n= 6 in each bar. Different letters indicate significant difference (Kruskal Wallis and Man Whitney U test) ($p<0.05$)

CONCLUSIONS

We found *E. andevalensis* seed germination was influenced by the length of the cold treatment to which the seeds were submitted. On the other hand, GA_3, added at 10 and 100 mg/L, increase the germination in *E. andevalensis*; however, this phytohormone had effects on the growth of the plant. The effects remain even 28 days after the exposure to GA_3. When hight concentrations were added the development of the plant is affected and small concentrations severely interfered with protein content and TAC.

8. EFFECTS OF CADMIUM IN ASCORBIC ACID AND GLUTATHIONE LEVELS IN *ERICA ANDEVALENSIS*, AN ENDEMIC HEATHER GROWING EXCLUSIVELY IN MINING AREAS (SW IBERIAN PENINSULA)

Efectos del cadmio en los niveles de ascorbato y glutatión en *Erica andevalensis*, un brezo endémico que crece únicamente asociado a zonas mineras (SO Península Ibérica)



Effects of Cd in ascorbic acid and glutathione levels in *Erica andevalensis*, an endemic heather growing exclusively in mining areas (SW Iberian Peninsula)

ABSTRACT

Erica andevalensis Cabezudo & Rivera is an endemic heather restrictively growing in soils affected by mining activities in the Iberian Pyritic Belt (SW Iberian Peninsula). This species has been difficult to grow in controlled non-polluted conditions in the past. In this report, the response of *E. andevalensis* to Cd was assessed by treating 5 weeks old plants, with three different concentration of Cd (0.5, 5 and 50 μM). After 5 days the contents of Cd, protein, ascorbic acid, glutathione and total antioxidant capacity were measured. No changes were observed in glutathione under any of the tested Cd concentrations; however the ascorbic acid level increased when 0.5 and 5 μM was applied and the total antioxidant capacity was significant higher when plants were exposed to 50 μM of Cd. As the increase in total antioxidant capacity is not explained by a change in glutathione nor ascorbic acid levels these result indicate the importance of other antioxidative molecules in the response of *E. andevalensis* towards enhanced Cd levels.

KEYWORDS

Ericaceae; Pyritic Belt; Heavy metals; Total antioxidant capacity

INTRODUCTION

The Iberian Pyritic Belt is the largest accumulation of massive sulphide on the Earth, stretching from the North of Seville to the South of Lisbon. Pyrite, its main constituent, has been exploited for over 5000 years (Nelson and Lamothe 1993; van Geen et al. 1999; Davis et al. 2000, Sainz et al. 2003; Sánchez España et al. 2005). In this area, high concentrations of Fe, Mn, Zn, Cu has been described (Nelson and Lamothe 1993; van Geen et al. 1999; Sainz et al. 2003; Braungardt et al. 2003; Montes-Botella and Tenorio 2003), however Cd has received little attention due to its low abundance. In the Tinto River Cd concentrations ranges are from 0.16 to 0.65 ppm (Rodríguez et al. 2007) and from 0.5 to 4 in the Odiel River and Tailings soils (Chapter 1, Table 1). The legal limit of Cd in soils in Spain is set in 1 ppm (RD 1310/1990).

Erica andevalensis Cabezudo & Rivera is an endemic heather characterized by the capacity of growing in soils affected by mining waste in the Iberian Pyritic Belt (Cabezudo and Rivera 1980; Nelson 1985; Aparicio 1999).

Cd is a toxic metal found in soils at trace concentrations. Although there are plants known to be able to hyperaccumulate this metal in their tissues such as *Thlaspi caerulescens*, *Arabidopsis halleri* and *Sedum alfredii* (Liu et al. 2008), Cd is without biological functions in higher plants (Marschener 1995; Schützendübel et al. 2001; Kara and Zeytuluoglu 2007; Liu et al. 2008). Cd is not a transition metal and therefore it may not induce oxidative stress via Fenton-type reactions such as Cu or Fe (Sandalio et al. 2001; Schützendübel et al. 2001; Semane et al. 2007), however it is highly toxic due to its reactivity with S and N atoms in amino acids (Clemens 2001). It is also known to affect cellular structures and produce membrane damage, disruption of the electron transport, inhibition/activation of enzymes and alteration of DNA (Smeets et al. 2005).

Glutathione (GSH) is the major non-protein thiol in plants with many functions in plant metabolism (Schützendübel et al. 2001), and one of the major scavengers of peroxides (May et al. 1998). It can act as antioxidant, by scavenging radicals, resulting in the oxidation of GSH to glutathione disulfide (GSSG) (Wu et al. 2004). Cd has been described to have a high affinity for thiols groups, and can therefore produce an imbalance of the available GSH pool, with the consequent disturbance of the ascorbic acid AsA-GSH cycle that maintains the cellular redox homeostasis controlling the synthesis, consumption and recycling of AsA and GSH (Semane et al. 2007).

The objectives of the present work were to study the effects of a short acute exposure to Cd on ascorbic acid and glutathione levels in the leaves, as well as in the total antioxidant capacity of this heather.

MATERIALS AND METHODS

Erica andevalensis seeds were collected from the field in the autumn of 2007. They were kept in paper bags under dry conditions. Seeds were submitted to a cold and wet pre-treatment for 20 days, to break the physiological dormancy (Aparicio 1995). After that period, seeds were dried and kept at 4 º C for 3 months.

Germination of the seeds was performed in agar medium with half strength MS salts and pH adjusted to 5.8. After pH adjustment, 30 g/L of agar was added. Vitamins (10 mg/L myoinositol, 0.1mg/L nicotinic acid, 0.1 mg/L pyridoxine and 1 mg/L thiamine) sterilized by filtration were added. Medium was poured in petri dishes of 9 cm diameter. Seeds were sterilized in a sterile eppendorf tube with ethanol (70 % v / v) for 60 sec and with sodium hypochlorite (5 % v / v) 10 min. Subsequently seeds were washed 3 times with sterilized water and sow in the

dishes previously prepared. Two or three drops of seeds were placed in each plate (from 150-200 seeds). The plates were placed in a growth chamber at 26 ºC 16/8 day-night photoperiod and 121.53 ± 3.46 μmol m^{-2} s^{-1} of light. The germination was followed every 2-3 days. After 20 days, the plants were transferred to a soil substrate and they were watered 2 times a week during 2 weeks with tap water.

Previous to the Cd exposure, watering was stopped when plants were 15 days old (in soil) and 6 days later Cd was supplied as 15 ml of Cd solution (or deionised water to the control plants) applied on top of the soil. Cd was added as $3CdSO_4 8H_2O$ (Merck). Three concentrations of Cd were applied: 0.5 μM, 5 μM and 50 μM, equivalents to 0.05, 0.5 and 5 ppm total Cd content in soil. 500 plants per group were studied. After 5 days plants were harvested weighted and kept at room temperature for Cd analysis or snap frozen in liquid N_2 and stored at -80ºC until biochemical analysis.

Ascorbic acid and glutathione (reduced and total forms) measurement

Frozen plant material, stored in fractions of 20–25 mg (30-40 plants per sample) was quickly grounded in liquid nitrogen in a pre-cooled mortar. At this stage it is essential to keep samples frozen to prevent oxidation. When a homogeneous powder was obtained, 250 μl of ice cold 6 % (w / v) meta-phosphoric acid was added. The samples were thawed on ice and the mixture was clarified by centrifugation at 12 000 *g* at 4° C for 15 min. The resulting supernatant was kept on ice until HPLC analysis. Antioxidants were separated on a 100 mm x 4.6 mm Polaris C18-A reversed phase HPLC column (3 μm particle size; 30°C; Varian, CA, USA) with an isocratic flow of 1 $mL.min^{-1}$ of the elution buffer (25 mM KPO_4-buffer, pH 3.00). The components were quantified using a home made electrochemical detector with glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The purity and identity of the peaks was confirmed

using a diode array detector (SPD-M10AVP, Shimadzu, Hertogenbosch, Netherlands) which was placed on line with the electrochemical detector. The amount of oxidised DHA or GSH concentration was measured indirectly as the difference between the total concentrations of antioxidants in a DTT reduced fraction and the concentration in the sample prior to reduction. Reduction of the sample was obtained by incubation of an aliquot of the extract in 400 mM of Tris and 200 mM of DTT for 10 min in the dark. The pH of this mixture was checked to be between 6 and 7. After 15 min the pH was lowered again by fourfold dilution in elution buffer prior to HPLC analysis. Three samples from each treatment were taken and each sample was measured in triplicate.

Cd measurements in plant tissue and soil

Cd concentrations were measured as described in Blust *et al.* (1988) with some modifications. Briefly 5 weeks old plants were exposed to Cd ($3CdSO_4 8H_2O$) during 5 days and the plant material was collected in eppendorf tubes, taking 50 mg of plant tissue per analysis, and three samples per Cd concentration. As quality control, three preparation blanks and three references samples of olive leaves (BCR 062, EU Institute for Reference Materials and Measurements, Geel, Belgium) were included. For each sample, 250 µL of highly purified concentrated HNO_3 was added then placed in a fumes-hood to pre-digest during 24h at room temperature. Further digestion was carried-out by sequential heating in a microwave oven: 3 times 2 min at 100W, followed by 3 times 2 min at 150 W and 2 times 2 min at 250W. Then 20 µL of H_2O_2 was added and the solutions were left for 30 minutes before giving them an additional 2 times 2 min microwave treatment at 350 W. After digestion, the solutions were diluted by adding 2 mL MilliQ water and Cd concentrations in the solutions were determined using an ICP-MS (Model 810, Varian Inc, Australia). Yttrium was added as an internal standard to correct for the influence of sample matrix. The recovery of Cd in the reference samples was within the acceptable range of 10 % of the certified values. As a background

experiment plants without $3CdSO_4 8H_2O$ were also sampled and the Cd concentration was determined. No significant levels of Cd could be determined in these controls.

The Cd content in soil was measured analyzing the amount of Cd in the soluble fraction. Soil was dried in oven at 60º C and 21 ml of water was added to 3 g of soil. The tubes were mixed during one hour and centrifuged. The supernatant was collected and the Cd was determined using an ICP-MS (Model 810, Varian Icn, Australia).

Soluble protein, total antioxidant capacity and pigment measurement

Frozen plant material, stored in fractions of 10 – 15 mg (25-35 plants per sample) was grounded in a pre-cooled mortar with 250 μL of potassium phosphate buffer (144 mM, pH 7). The homogenate was centrifuge at 10,000 g for 15 min at 4ºC and the supernatant collected and kept on ice or frozen at -80ºC until analysis and the pellet was kept on ice in dark. Two samples of each treatment were taken and each sample was analyzed in triplicate.

Protein levels were measured as described by Bradford (1976) and using BSA as a standard (Fig. 1).

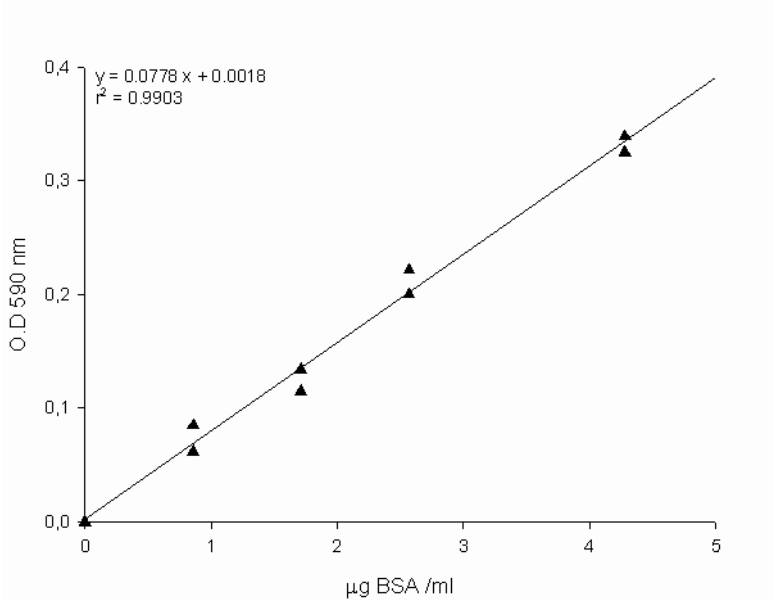


Figure 1. Calibration curve of protein, using BSA as standard.

The total antioxidant capacity was measured using the FRAP method described by Peñarrieta et al. (2008). Briefly, the Fe^{3+}-TPTZ complex is reduced to blue Fe^{2+}-TPTZ complex by electron donating substances under acidic conditions. The FRAP reagent was a mixture of 100 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM ferric chloride (10:1:1). The method was adapted to a plate reader and 100 µL of reagent was added to 100 µL of sample (previously diluted when necessary). The absorbance was measured at 600 nm after 10 min of incubation at room temperature. The final absorbance of the samples were compared with a standard curve (Fig. 2) made using Trolox (0-100 µM). Trolox stock solution was made in methanol and all the subsequent dilutions were made in potassium phosphate buffer. The data were expressed as µmol Trolox equivalents per gram fresh weight. The total antioxidant capacity in the buffer soluble fraction was obtained.

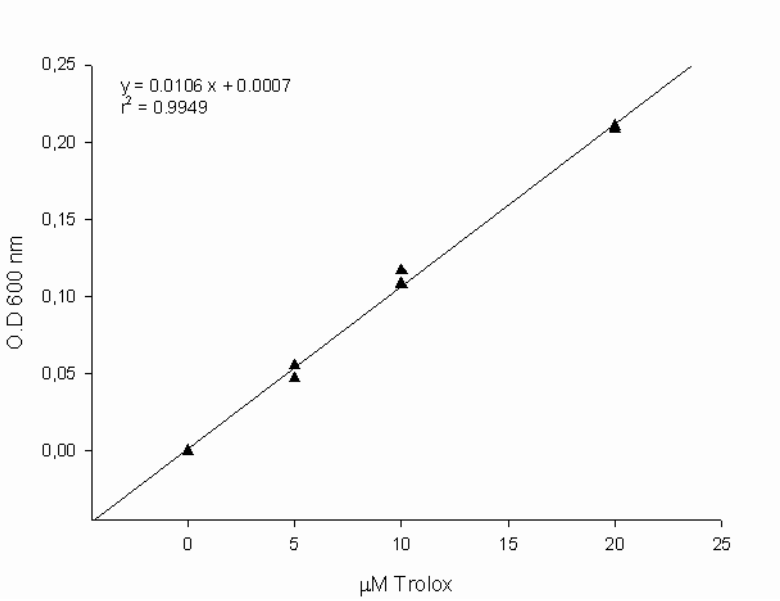


Figure 2. Calibration curve of the total antioxidant capacity, using TROLOX dissolved in methanol and buffer as standard.

Pigments were measured dissolving the pellet obtained after the centrifugation in 1 ml of 80 % (v / v) acetone and mixing well until the entire pellet was discoloured. Samples were centrifuged at 4º C at 10000*g* for 10 min. The absorbance was read at 661.6, 644.8 and 470 nm and the equations given by Lichtenthaler (1987) were applied.

Chlorophyll a: $Ca = 11.24\ A_{661.6} - 2.04\ A_{644.8}$

Chlorophyll b: $Cb = 20.13\ A_{644.8} - 4{,}19\ A_{661.6}$

Chlorophyll total: $Ct = 7.05\ A_{661.6} + 18{,}09\ A_{644.8}$

Carotenes = $(1000 \cdot A_{470} - 1.90Ca - 63.14Cb) /214$

Statistics

All measurements were made in triplicate. Means ± standards error are showed. The normality of the data was checked with Shapiro-Wilk test and

ANOVA or Kruskal-Wallis was used to analyze the differences in the data, depending on the normality of the data.

RESULTS

To test the response of greenhouse plants grown on non-polluted soils to heavy metals, 5 weeks old plants were exposed to different Cd concentrations. After 5 days Cd extractable content in soils was measured and the concentration increased together with the amount of Cd added (Table 1) to each soil. The bioaccumulation factor is defined as the ratio of the metal concentration in the plant tissue to that in the soil (Sun et al. 2009). As it is showed in Table 1, plants were able to accumulate Cd from 4.74 to 12.34 times.

Table 1
Cd added to the soil and extractable after 5 days.

Label	Cd added (µM $3CdSO_4 \cdot 8H_2O$)	Extractable Cd per gram of soil (µg/g)	Bioaccumulation factor
Control	0	0.006 ± 0.000	4.54
0.5 µM	0.5	0.013 ± 0.002	7.47
5 µM	5	0.062 ± 0.019	7.96
50 µM	50	0.267 ± 0.144	12.34

After 5 days growing under Cd treatment the internal content of the metal was increased together with the amount present in the soil (Fig. 3) although no visual differences were appreciated in the plants, neither in the biomass produced per plant (Fig. 4) nor the pigment content (Fig. 5). However soluble protein content of the plants was affected by the treatment (Fig. 6). The protein content decreased when plants were exposed to 0.5 µM Cd, however no differences ($p<0.05$) were found with the two other tested concentrations compared with the control.

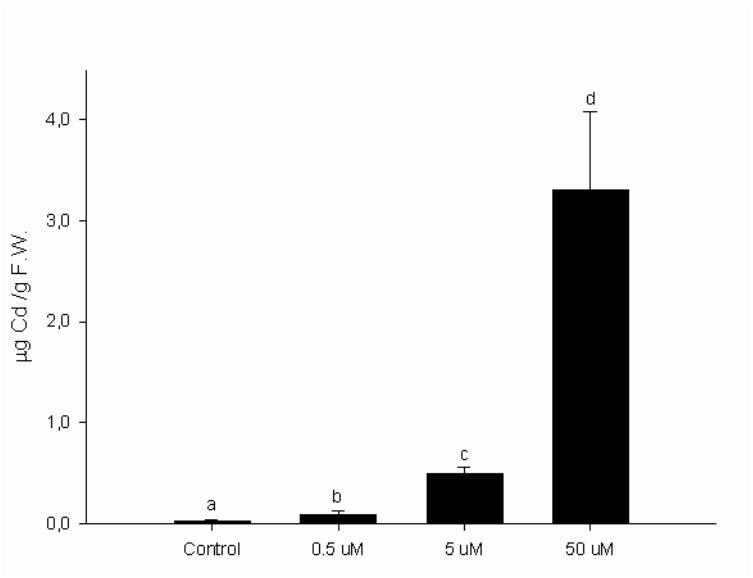


Figure 3. Cd content in plant tissue, expressed as µg Cd/ g tissue. For each bar n=3, coming from 60 - 90 plants necessaries to reach 50 mg of plant tissue. Means ± standards error are represented and different letters of top of the bars indicate significant differences ($p<0.05$) after a Kruskal Wallis and Mann Withney U test.

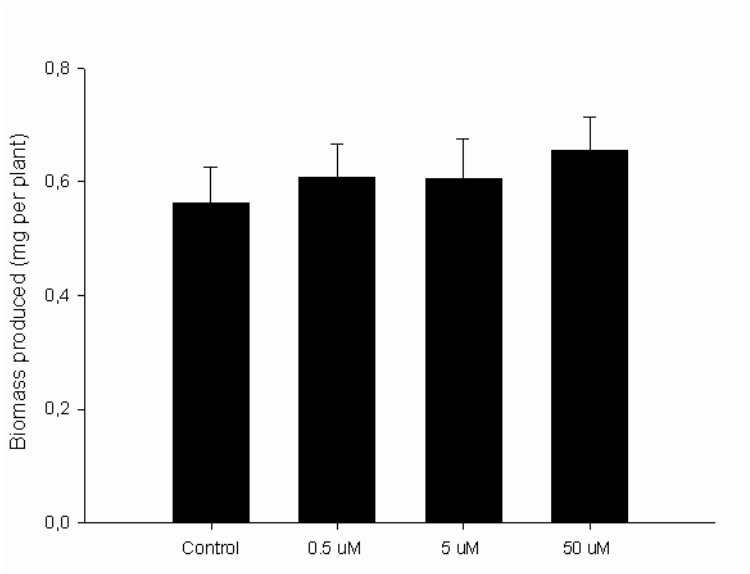


Figure 4. Biomass produced per plant, calculated from the weight and number of plants taken from each treatment for the metal and biochemical analysis. No significance differences were found ($p<0.05$) after ANOVA.

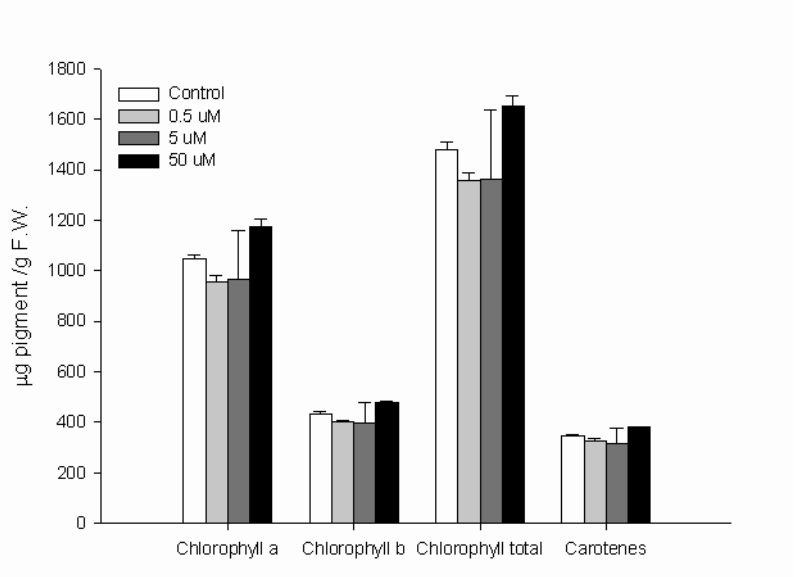


Figure 5. Chlorophyll *a*, *b*, total chlorophylls and carotenes were measured (µg pigment/ g F.W.) after 5 days under Cd treatment. For each bar mean ± standard error is represented of n = 2 (30 - 35 plants). No significant differences were found ($p<0.05$) after a Kruskal-Wallis test.

Related to soluble protein levels, only the lowest Cd concentration tested significantly decreased protein content (Fig. 6). The protein content was affected by Cd in *E. andevalensis;* however, not all the concentrations produced the same effect. Low concentrations of Cd reduced the protein content, but an increase was registered when higher metal concentrations were added.

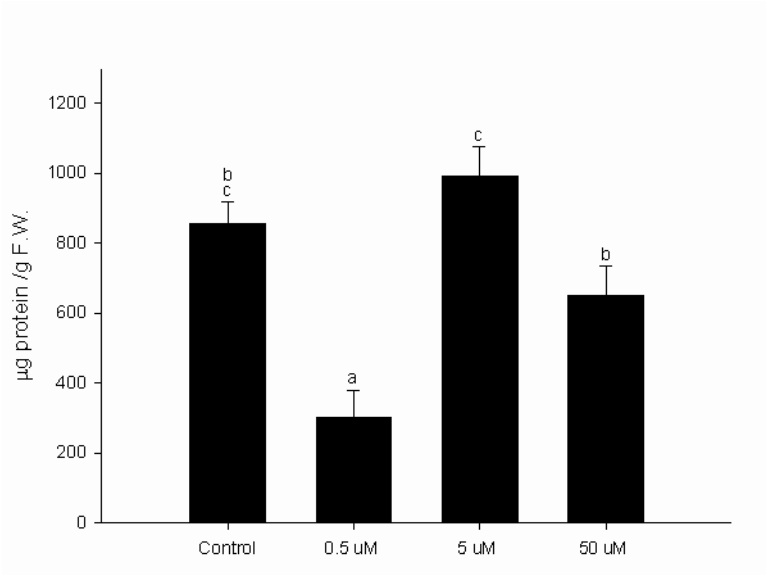


Figure 6. Soluble protein content expressed as µg protein / g F.W. For each bar mean ± standard error is represented of n = 2 (30 - 35 plants) and each measurement was done in triplicate. Different letters on top of the bars means significant differences ($p<0.01$) after ANOVA and Duncan test.

As shown in Fig. 7 ascorbic acid levels were affected by Cd in leaves of treated plants. Both reduced as well as total ascorbic acid concentration were significantly lower in 0.5 µM Cd exposed plants and increased at 5 µM. At the highest tested concentration ascorbic acid levels did however not change compared with the control. The amount of the oxidized form of ascorbic acid (dehydroascorbate) was significant lower when 5 µM was added (Fig. 9). Surprisingly, no significative changes were observed in glutathione levels in any of the treatments (Fig. 8).

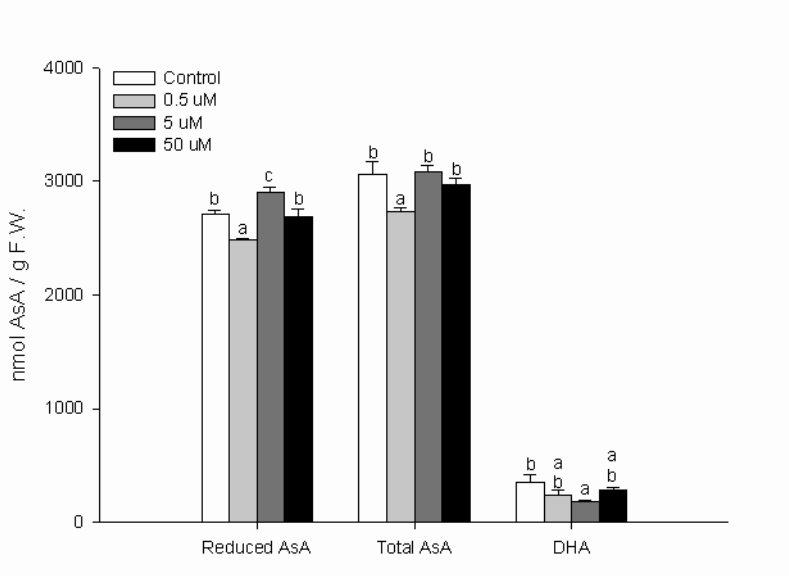


Figure 7. Ascorbic acid (nmol/ g F.W.) content in the reduced, total and oxidized (DHA) forms are represented. For each bar mean ± standard error is represented of n = 3 (30 - 45 plants) and each measurement was done in triplicate. Different letters on top of the bars from the same parameter means significant differences ($p<0.01$) after ANOVA and Duncan test.

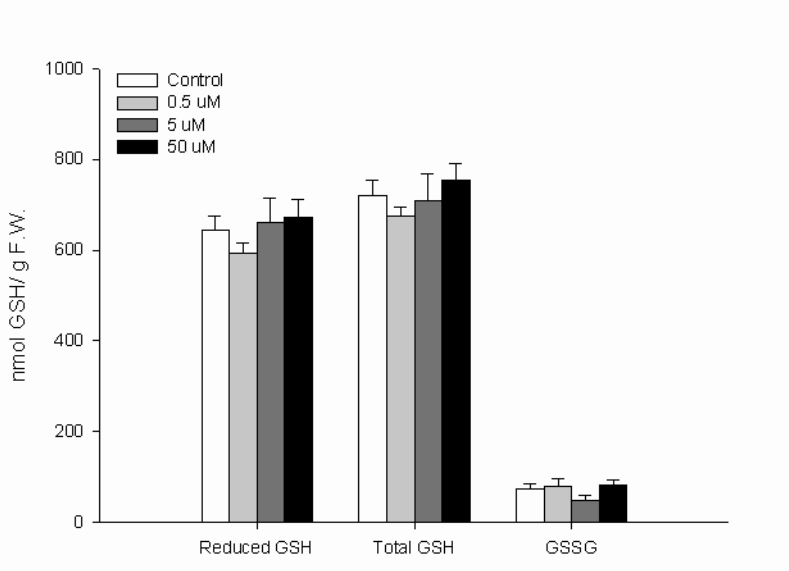


Figure 8. Glutathione (nmol/ g F.W.) content in the reduced, total and oxidized (GSSG) forms are represented. For each bar mean ± standard error is represented of n = 3 (30 - 45 plants) and each measurement was done in triplicate. No significant differences were found ($p<0.05$).

When the ratios between reduced and oxidized forms were calculated (Fig. 9) the same pattern can be observed in the ascorbic acid and glutathione. The ratio increased with the small concentrations of Cd added, but a decrease is observed with 50 μM.

In addition to ascorbic acid and glutathione levels the total antioxidant capacity of the plants was measured. As shown in Fig. 10, only plants treated with 50 μM of Cd showed an increase in this activity ($p<0.05$) (Fig.10).

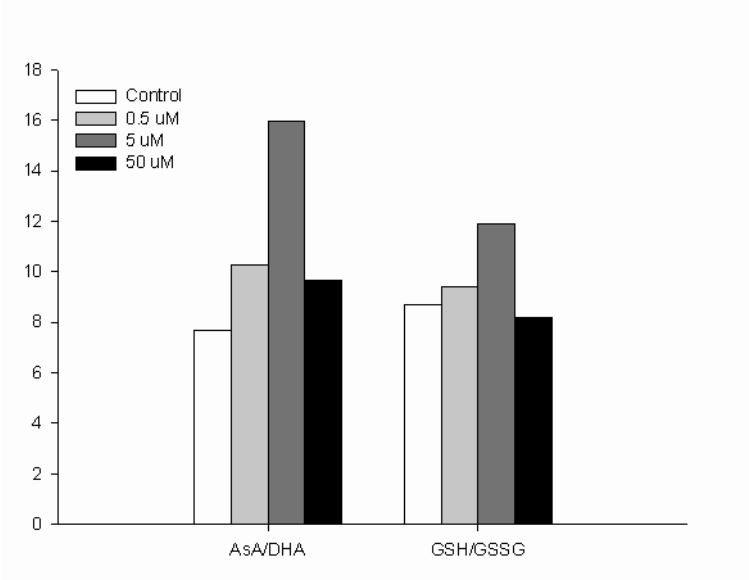


Figure 9. AsA/ DHA and GSH/GSSG ratio in plant tissue of *E. andevalensis* after 5 days of exposure to different Cd concentrations are calculated from the observed values in Fig. 7 and 8.

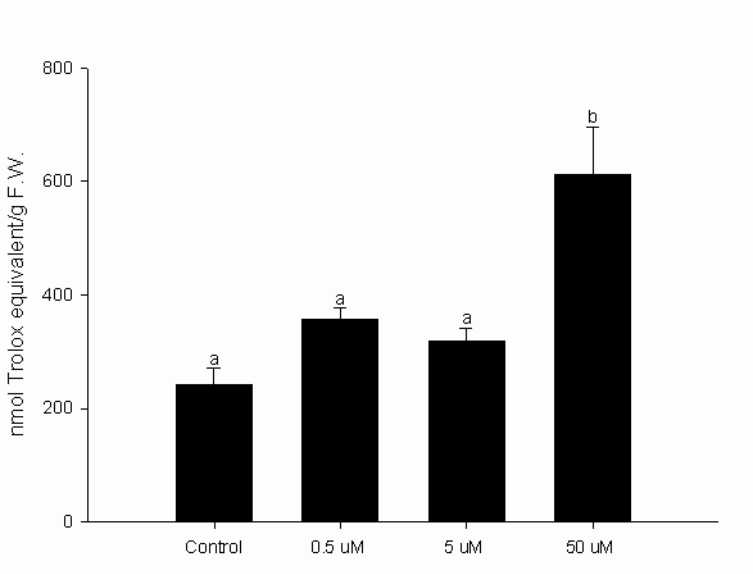


Figure 10. Total antioxidant capacity (nmol TROLOX equivalents/g F.W.) after the different Cd treatments are represented. For each bar mean ± standard error is represented of n = 2 (30 - 35 plants) and each measurement was done in triplicate. Different letters on top of the bars means significant differences ($p<0.05$) after ANOVA and Duncan test.

DISCUSSION

The aim of this work was to culture *E. andevalensis* under controlled environmental conditions and to evaluate the effects of different Cd concentrations on the AsA and GSH levels, together with the protein, total antioxidant capacity and pigments.

Acidic soils are the natural habitat of *E. andevalensis* (Soldevilla et al. 1992; Aparicio 1999) and the growth is favoured in this kind of soils, as it was exposed in chapter 7.

If we compare with natural conditions, the lowest concentration tested is within the range of natural levels present in soils. The Cd content in *E. andevalensis* tissues increased with the increase of the available Cd in the

substrate, reaching a maximum bioaccumulation factor of 12 times with the highest amount of Cd available in soil. The bioaccumulation factor increased following a potential equation with the availability of the metal in soil. *E. andevalensis* is able to accumulate Cd depending on the concentration available in the substrate and it may have internal mechanisms to immobilize or to cope with the excess of metal in the cells. An increased level of Cd in plant leaves has been also registered in other studies. In these studies, the increase of the metal in the soil or nutritive solution leads to a metal accumulation in plant tissue (Sandalio et al. 2001; Wu et al. 2004). However, to make comparations is not really possible, due to the different conditions set in each experiment.

No visible signs of Cd toxicity were evident after 5 days under any of the concentrations tested, as evidenced by chlorophyll levels in contrast to other species as *Brassica juncea* and *Vigna radiata* (Šimonová et al. 2007), *Phaseolus vulgaris* (Cahoui et al. 1997) that were affected after being treated under similar conditions. We did not find significant differences in biomass levels, although the biomass of Cd treated plants seemed slightly bigger than the control (Fig. 4). It has been described that Cd exposure restricts biomass production due to physiological disturbances (Smeets et al. 2005; Han et al. 2007). In the Cd/Zn hyperaccumulator *Thlapsi caerulescens* however, biomass increased (Roosens et al. 2003; Liu et al. 2008) indicating that tolerant Cd species can grow in the presence of the metal (Sun et al. 2009). Our results therefore give an indication that *E. andevalensis* in addition to its tolerance to Mn (Soldevilla et al.1992) is also able to withstand Cd. Growth inhibition and decrease in chlorophyll content has been suggested to be included in a phytotoxicity index for Cd exposure (Šimonová et al. 2007), and due to the no changes observed in these two parameters it is possible to conclude that the Cd concentrations tested were not toxic for *E. andevalensis*. The concentrations tested in this experiment were quite low with the aim to expose the plant to natural occurring contaminations. Some biochemical changes were induced by the Cd treatment. The protein increase may be due to the synthesis of antioxidant

enzymes. Increase of peroxidases, mainly ascorbate peroxidase, has been seen in *E. andevalensis* exposed to increased levels of metals (Chapter 3).

The total AsA levels seemed to follow the pattern observed in protein content with the smallest Cd concentration reducing AsA but not dehydroascorbate levels, resulting in a lower AsA/DHA ratio. The addition of 5 μM Cd increased the reduced and total forms of AsA, which indicates that the antioxidant mechanisms are actively working. Therefore *E. andevalensis* seems to counteract the presence of Cd by increasing ascorbic acid synthesis as has been previously described in plants treated with metals (Cuypers et al. 2001). As evidenced by measuring the total antioxidative capacity (TAC), in addition to ascorbic acid and glutathione, it seems to be another important soluble antioxidant, especially in the response to higher Cd concentrations. This increase in the TAC can be interpreted as an activation of different antioxidant mechanisms, which together with AsA/GSH cycle may act to cope with the effects caused by Cd, as the possible reactive oxygen species (ROS) generated (Foyer and Noctor 2005). Among the wide variety of possible antioxidants, phenolics have been described to be increased under Cd treatment in scots pine (Schützendübel et al. 2001).

The AsA/GSH cycle is considered to be of great importance in controlling the cellular redox status (Smeets et al. 2005). In spite of the observed changes in the AsA levels, no changes were observed under any of the Cd concentrations tested in the GSH level, although the ratio GSH/GSSG had the same pattern as AsA/DHA. This resemblance between AsA/DHA and GSH/GSSG may be due to the connection of both redox couples in Halliwell-Asada cycle (Foyer 1993; Foyer et al. 1994). GSH has also an important role in the synthesis of phytochelatins (PCs). PCs have been extensively studied in relation with Cd exposition (Rauser 2001; Cobbett 2000a) and some studies indicated that Cd treatments decrease GSH levels and increase PCs in *Arabidopsis thaliana* (Semane et al. 2007). However our previous studies have demonstrated that, in its natural habitat, PCs concentrations of *E. andevalensis* are very low (see Chapter 4). Of course, we have to be careful

with this conclusions as only one time point (5 days) was monitored in this study and it is conceivable that at this time plant could already have recovered from the initial possible glutathione depletion (Schützendübel et al. 2001).

In conclusion, our results suggest that the different Cd concentration tested in *E. andevalensis* did not seriously affect this species as confirmed by the absence of macroscopic injuries neither biomass or pigment reduction. At low concentrations of metal added, the plant responds increasing the reduced/oxidized forms of AsA and GSH, but when the metal concentration is higher, other yet unidentified antioxidants seem to play an important role, since total antioxidant capacity was increased significantly.

CONCLUSIONES

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1- *Erica andevalensis* es una planta tolerante y adaptada a las condiciones del medio en el que vive (ácido y con elevado contenido en metales) sin mostrar síntomas de estar afectada por estrés oxidativo, como indican la ausencia de daños macroscópicos y los marcadores de estrés utilizados: pigmentos y peroxidación lipídica. El efecto del exceso de metales puede ser contrarrestado con la activación de enzimas con actividad peroxidasa, principalmente ascorbato peroxidasa y otros antioxidantes distintos al ácido ascórbico y glutatión, aunque otros mecanismos no pueden ser descartados, como la exclusión de metales.

Erica andevalensis is a tolerant species and adapted to environmental conditions in which it lives (acid and metal enriched soils) without showing signs of being affected by oxidative stress, as indicated by the absence of macroscopy damage and oxidative stress markers used: pigments and lipid peroxidation. The effect of excess metals could be countered with the activation of enzymes with peroxidase activity, mainly ascorbate peroxidase and other antioxidant molecules different to ascorbic acid and glutathione, although other mechanisms can not be discarded, as metal exclusion.

2- Los niveles constitutivos de ácido ascórbico en *Erica andevalensis* son mas bajos que los encontrados en otras *Ericas*, lo cual puede ser indicativo de su adaptación al medio. Del mismo modo *E. andevalensis* posee una baja concentración de compuestos fenólicos, tanto medidos de forma global como individualizada, independientemente del medio en el que la especie vive, destacando la presencia de derivados de los ácidos *p*-coumárico y cinámico, no presentes en las otras Ericas incluidas en el estudio.

Constitutive levels of ascorbic acid in *E. andevalensis* are much lower than the levels found in other *Erica* species, which may be indicative of their adaptation to the environment. Similarly, *E. andevalensis* has a low concentration of phenolic compounds, as much in global as individual analysis, independently of the environment in which the species live, highlighting the presence of *p*-coumaric and cinnamic acid derivates, which are not present in the other *Erica* species included in the study.

3- Las fitoquelatinas, compuestos inducidos por la presencia de metales en gran diversidad de especies vegetales, parecen no estar implicadas en la tolerancia a los metales en *Erica andevalensis*, dada la baja concentración en la que se encuentran en ejemplares recolectados en el campo. Por otra parte, los niveles de GSH no se alteran cuando la planta se expone a Cd.

Phytochelatins, compounds induced by the presence of metals in a large variety of plant species, appear not to be involved in metal tolerance in *E. andevalensis*, due to the low concentration measured in plants collected from the field. On the other hand, the GHS levels are not altered when the plant is exposed to Cd.

4- Por primera vez se han obtenido ejemplares de *E. andevalensis* a partir de técnicas de micropropagación y sorprendemente, las plantas obtenidas se desarrollan de forma normal en sustratos no contaminados.

This is the first time that *E. andevalensis* has been cultivated using micropropagation techniques and surprisingly, the obtained plants are able to develop in a natural way in non-polluted substrates.

5- Las preferencias de *E. andevalensis* por la acidez y alto contenido en Fe están marcadas en su propia germinación. Probablemente son las preferencias de *E. andevalensis* las responsables de su restringida distribución y de su éxito competitivo. Sin embargo en condiciones de laboratorio se ha comprobado que la planta es capaz de crecer y desarrollarse en ausencia de concentraciones elevadas de metales y de acidez extrema.

E. andevalensis preferences by acidity and high content of Fe are marked in its own germination. *E. andevalensis* preferencies are probable responsible for its restringed distribution and its competitive success. However under laboratory conditions the plant is able to grow and develop in the absence of high metal content and extreme acidity.

6- El ácido giberélico es una buena opción para aumentar la germinación de *E. andevalensis*, y por tanto podría ser usado para el cultivo de plantas en el laboratorio, sin embargo, concentraciones elevadas afectan al desarrollo de la planta y deben ser descartadas. Con el objetivo de cultivar plantas en el laboratorio, la tasa de germinación de las semillas puede incrementarse aumentando el tiempo de conservación en frío de éstas o aplicando un segundo tratamiento de frío y humedad.

Gibberellic acid is a good option to increase the germination of *E. andevalensis,* and therefore could be used for growing plants in the laboratory; however, high concentrations affect the plant growth and must be discarded. With the aim to cultivate plants in the laboratory, the germination rate can be increased by an increase in the time of the cold storage of seeds or giving to the seeds a second cold and wet treatment.

7- *Erica andevalensis* es capaz de acumular Cd en sus tejidos aéreos en mayor concentración de la presente en el suelo, lo que indica que la planta posee mecanismos de adquisición e inmovilización de Cd, sin que su estado vegetativo se vea seriamente afectado, aunque se incrementó la capacidad antioxidante total.

E. andevalensis is able to accumulate Cd in its aerial tissues in a greater concentration than the present in the substrate, which is indicative of the acquisition and inmobilization Cd mechanisms, whithout affecting seriously the vegetative state, although the total antioxidant capacity was increased.

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