Universidad Autónoma de Madrid
Departamento de Química Agrícola

COMPORTAMIENTO *DE Silene vulgaris* (Moench.) Garcke FRENTE A LA EXPOSICIÓN A CROMO. EVALUACIÓN DE SU POSIBLE USO EN RECUPERACIÓN DE SUELOS

BEHAVIOR OF *Silene vulgaris* (Moench.) Garcke AGAINST EXPOSURE TO CHROMIUM. EVALUATION OF POTENTIAL USE IN SOIL REMEDIATION

TESIS DOCTORAL
ANA ELENA PRADAS DEL REAL
Madrid, 2013

DEPARTAMENTO DE QUÍMICA AGRÍCOLA

COMPORTAMIENTO DE *Silene vulgaris* (Moench.) Garcke FRENTE A LA EXPOSICIÓN A CROMO. EVALUACIÓN DE SU POSIBLE USO EN RECUPERACIÓN DE SUELOS

BEHAVIOR OF *Silene vulgaris* (Moench.) Garcke AGAINST EXPOSURE TO CHROMIUM. EVALUATION OF POTENTIAL USE IN SOIL REMEDIATION

Memoria presentada para optar al Grado de Doctor en Ciencias dentro del Programa de "Química Agrícola"

Madrid, Junio 2013

La doctoranda:

Fdo: Ana Elena Pradas del Real

Las Directoras: **La Tutora UAM:**

Dra. Araceli Pérez-Sanz
Investigadora IMIDRA

Dra. Mª Carmen Lobo Bedmar
Investigadora IMIDRA

Dra. Elvira Estaban
Profesora titular UAM

Este trabajo ha sido financiado por:

-Este trabajo ha sido realizado en el Instituto Madrileño de Investigación y Desarrollo Agrario y Alimentario (IMIDRA) gracias a la financiación de la beca predoctoral de Ana Elena Pradas del Real.

-Instituto Nacional de Investigación Agraria (INIA) a través del proyecto: Utilización de plantas silvestres y autóctonas para la recuperación sostenible de suelos contaminados (RTA2009-00150-00-00)

-Consejería de Educación, Comunidad de Madrid a través del Programa EIADES: Evaluación de Impacto ambiental y recuperación del medio natural en emplazamientos contaminados

EUROPEAN UNION
EUROPEAN
SOCIAL FUND

-Laboratorio de Ciencias de la Rizosfera de La Universidad de Kentucky (USA)

Agradecimientos/Aknowledgements

Esta tesis es el fruto de 4 años y medio de trabajo. Su realización no hubiera sido posible sin la participación y el apoyo de muchas personas. Quiero agradecer a todos aquellos que de forma directa o indirecta, me han prestado ayuda técnica o simplemente me han animado y acompañado durante todo este proceso. Sin todos ellos esto no hubiera sido posible

En primer lugar quiero agradecer a mis directoras el respaldo y la confianza brindada todo este tiempo. A Araceli por estar siempre disponible y cercana, trabajando conmigo codo con codo, por enseñarme todo lo que estaba en su mano, por el esfuerzo invertido. Más allá de la ciencia, por su psicología y su cariño, por ser mucho más que una directora. A Carmen quiero agradecerle la oportunidad que me dio al incorporarme a su equipo de trabajo, su apoyo y su confianza. Que siempre haya sacado un momento para para solucionar los muchos contratiempos que han ido surgiendo y hacerlo siempre con una palabra amable.

A todos mis compañeros de El Encín por fomentar un clima de trabajo agradable y amistoso, por los ánimos en estos últimos meses, por ayudarme en el laboratorio y por los buenos momentos charlando en los pasillos. A Pilar por ayudarme a entender las collejas y las cámaras de cultivo. A Miguel por cuidar las parcelas de collejas en el campo y en el invernadero y por ayudarme en el esquejado. A Ana, MªBel y Mari Rosi por ayudarme con los análisis de suelo. A Águeda por el SPAD. A Héctor, Lili y Paloma por su ayuda y por las charlas en la sala de becarios. Y de forma especial a Álvaro, MªÁngeles y Mar por haberme acompañado desde el principio con su amistad y sus palabras.

Entre los compañeros que he tenido la oportunidad de conocer en este tiempo "El equipo Encín" ha tenido un papel importantísimo para mantenerme cuerda, porque hay cosas que solo nosotras entendemos... Blanca porque además de estar siempre dispuesta a echar una mano es "curativa" en sí misma, por su dulzura y su bondad y Marta por ser todo alegría y palabras sabias .Qué suerte haberos tenido de compañeras de despacho, de penas y alegrías.

No puedo dejar de agradecer la ayuda prestada por todos los estudiantes que han pasado por "El Encín" estos años, por su ayuda inconmensurable sembrando plantas, moviendo bandejas, llenando bidones de solución nutritiva, abriendo los botecitos en la absorción atómica…. Sin vosotros no habría podido con todo y, sobre todo, los días y el laboratorio hubieran sido mucho más tristes sin vuestra compañía: Nerea, Ainhoa, Lourdes, Álvaro, Carol, Leti, María, Tamara, Ana, Adali, Javi, Marion y muchos más…gracias, gracias, gracias.

Thanks to people of University of Kentucky, especially to David McNear for giving me the oportunity to join his group and to work in the synchrotron, for being so enthusiastic and for helping me on my arrival and welcoming me into his home. To Joe Kupper for showing me everything in the lab, for all his help and encouragement, for “free food” and for his every “Keep on, Ana, you can do it” that I still have by email.

Gracias a los buenos amigos, que incluso estando lejos, me han animado siempre y me han ayudado a salir de todos los baches: Eva y Yiyi, mis chicas de la Uni, Agus, Pepe, Elisa…y gracias a “Friends For Ever & Company” por ser una constante en mi vida y un auténtico privilegio.

Quiero recordar a mi perrita Luna, que estuvo todas las horas de mi vida que he pasado estudiando desde los 9 años hasta hace muy poco, que me han traído hasta aquí, sentadita a mi lado, y han sido muchas…

Por último tengo que agradecer la realización de esta tesis a mi familia, por ayudarme en todo y aguantar mi mala leche, por haberme apoyado siempre en todas mis decisiones A mi abuelo Pepe, que siempre me dijo que yo era la “number one”. A mi hermano Diego por estar siempre tan orgulloso de mí, por sus regalitos y sus abrazos en el momento más oportuno. A mi padre por enseñarme a estar calmada y a quedarme con lo bueno que pueda aportarme cada persona, por enseñarme a tomar mis propias decisiones. Y en especial tengo que dar las gracias a mi madre, psicóloga, cocinera, secretaria, confidente… sin ella no habría conseguido nada de lo que he hecho hasta ahora. Por darme todo el tiempo que tenía y el que no tenía, por haberse ocupado de todo para que yo sólo me ocupara de esto.

Gracias

A mis padres

"*I am the master of my fate:*

I am the captain of my soul"

Nelson Mandela

Poema Invictus de William Ernest Henley

Resumen

Las propiedades anticorrosivas del Cr hacen que su uso en las industrias metalúrgica, química y refractaria sea cada vez más generalizado. Como resultado de la emisión atmosférica de estas industrias y del vertido incontrolado de residuos sólidos y líquidos procedentes de las mismas, los problemas de contaminación por Cr constituyen un problema ambiental cada vez más importante. Las dos especies químicas de Cr más abundantes en el medio ambiente son Cr(III) y Cr(VI). El Cr(VI) es altamente oxidante y muy soluble mientras que el Cr(III) suele encontrarse insoluble y es entre 10 y 100 veces menos tóxico. En concreto, la contaminación del suelo con Cr supone un grave riesgo ambiental y para la salud humana, tanto por contacto directo con el Cr como por el riesgo de que éste lixivie a las aguas subterráneas o sea absorbido por las plantas y pase a la cadena trófica. Además la contaminación del suelo con Cr puede suponer importantes pérdidas económicas. Esto hace necesario el desarrollo de nuevas tecnologías para su eliminación. Una alternativa ambientalmente sostenible y de bajo coste es el uso de plantas y sus microorganismos asociados para la descontaminación ambiental a lo que se denomina fitorremediación o fitotecnología.

Para la implantación adecuada de estas tecnologías es necesario un amplio conocimiento de las relaciones entre el metal y la planta, así como de su respuesta en el suelo. En este sentido existe menos información disponible para el Cr en comparación con otros metales y se han estudiado pocas especies vegetales para ser aplicadas en emplazamientos contaminados con Cr. El objetivo de esta tesis ha sido estudiar el comportamiento de *Silene vulgaris* frente a la exposición a Cr para evaluar su posible uso en emplazamientos contaminados con Cr. *S. vulgaris* es una metalofita facultativa que es considerada una especie interesante en fitorremediación de metales pesados pero no existe información disponible acerca de su respuesta a Cr. Se realizaron ensayos de exposición a Cr en hidroponía y en hidroponía soportada en perlita y un último ensayo en suelos contaminados con Cr. Como material vegetal se usaron tanto plántulas desarrolladas a partir de esquejes que constituyen clones de genotipos homogéneos como plántulas desarrolladas a partir de semillas de una misma población.

En *S. vulgaris* el Cr se acumuló preferentemente en la raíz y solo fue traslocado a la parte aérea en niveles próximos a la toxicidad lo que supone un primer mecanismo de protección frente al Cr. Las plantas acumularon más Cr en presencia de Cr(VI) que de Cr(III) debido a los distintos mecanismos de entrada en la raíz de cada especie química. En base al crecimiento de la raíz los genotipos más tolerantes a Cr(VI) mostraron EC_{100} entre 200 y 1200 µM y los más sensibles entre 200 y 1000 µM. Las plántulas desarrolladas a partir de semillas mostraron rangos de tolerancia menores, entre 30 y 100 µM Cr(VI). *S. vulgaris* mostró distintos mecanismos que la hacen tolerante al Cr. Por un lado los genotipos más tolerantes aumentaron las concentraciones de nutrientes, especialmente Fe, Ca y Mg para mantener su homeostasis y sintetizar moléculas antioxidantes y metabolitos esenciales. La exudación también se ha mostrado como un mecanismo controlado de tolerancia a Cr en *S. vulgaris*, ya que está directamente relacionada con el nivel de acumulación en planta pero no con el estrés oxidativo. Mediante HPLC-MS se identificaron la quercetina y la apiína como los principales polifenoles en los exudados de *S. vulgaris*. Mediante cromatografía iónica se determinaron las concentraciones de ácidos orgánicos en planta y en los exudados. Ácido cítrico y málico fueron los que mostraron una mayor respuesta al Cr, disminuyeron en los exudados y aumentaron en raíz lo que indicó su importante papel en la detoxificación del Cr en planta.

Para estudiar la localización y la especiación del Cr en los tejidos, se empleó microscopía de rayos X con fuente sincrotrón. Los resultados revelaron la capacidad de S. *vulgaris* para reducir Cr(VI) a la especie menos tóxica Cr(III), ya que todo el Cr en planta se encontró como Cr(III) aunque las plantas hubieran sido expuestas a Cr(VI). Los mapas de fluorescencia de rayos X mostraron que el Cr se acumuló principalmente en las paredes celulares y en el ápice de las raíces, cuando la capacidad de retención de las raíces fue superada, el Cr fue trasportado a través del xilema del tallo y acumulado en la pared celular de los márgenes de las hojas. Los análisis de µ-XANES confirmaron el papel de los ácidos orgánicos en la acumulación y el transporte de Cr en la planta.

Mediante SEM y DRIFTS- FTIR se identificaron gránulos de almidón en las hojas de plantas tratadas con dosis altas de Cr(VI) atribuibles al desajuste entre el

carbono utilizado para el crecimiento y carbono asimilado por fotosíntesis. Esto indica que la planta fue capaz de preservar la función fotosíntética mediante la retención del Cr en la raíz y los márgenes de la hoja pero la energía empleada en gestionar el exceso de Cr hizo que disminuya su biomasa. Los análisis de DRIFTS- FTIR confirmaron el papel de los polisacáridos y proteínas de la pared celular para enlazar el Cr y evitar su presencia en el citoplasma. Asimismo estos análisis revelaron síntesis de compuestos aromáticos en la hoja, posiblemente antioxidantes, y la ausencia de daño en las membranas.

Por último se realizó un ensayo en el que las plantas de *S. vulgaris* se cultivaron en suelos contaminados con Cr(III) o con Cr(VI) durante seis meses. Los resultados mostraron que S. *vulgaris* es capaz de desarrollarse en suelos con distintas características físico-químicas y contaminación moderada de Cr. El desarrollo de la cubierta vegetal de *S. vulgaris* aportó C al suelo procedente de la exudación de compuestos orgánicos. Este aporte de C incrementó el pH de los suelos y solubilizó parte del Cr que no se encontraba disponible incrementando la absorción por las plantas. Además estimuló la actividad de los microorganismos de suelo, como se mostró al incrementarse los valores de la actividad deshidrogenasa, contribuyendo a la recuperación de los suelos.

La capacidad de *S. vulgaris* para reducir Cr(VI) a Cr(III), acumular el Cr principalmente en la raíz y desarrollarse y aportar C en suelos contaminados con Cr, hace que esta especie pueda ser considerada interesante para la revegetación y recuperación de suelos contaminados con Cr.

Summary

Chromium, due to its anticorrosive properties, is widely employed in metallurgical, chemical and refractory industries. Atmospheric emissions and improper disposal of liquid and solid wastes from these industries makes Cr pollution an increasing environmental concern. The two most common chemical species of Cr in the environment are Cr(VI) and Cr(III). Cr(VI) is highly oxidizing and soluble while Cr(III) is normally insoluble and between 10 and 100 times less toxic than Cr(VI).

Particularly, soil pollution with Cr is a serious environmental and human health hazard both by direct contact with Cr and by the possibility of Cr to be leached to the ground water or to be taken up by plant thus being transferred to the food chain. Moreover, soil pollution with Cr can lead to major economic losses. Therefore, there is a need of developing new technologies for Cr removal. Phytoremediation or phytotechnology could be a green and low cost alternative to this purpose. This technology is based on the ability of plants and their associated microorganisms for environmental clean-up.

A deep knowledge of metal-plant interactions and plant response in soil is required to the proper implementation of these technologies. Less information about Cr-plant interactions is available regarding other heavy metals and less species have been evaluated for their application in Cr polluted sites. The objective of this thesis has been to study the response of *Silene vulgaris* to Cr exposition in order to evaluate their potential to be used in Cr polluted sites. *S. vulgaris* is a facultative methalophyte, it is considered an interesting species in heavy metal phytoremediation but no information is available about its response to Cr. Hydroponics and hydroponics with perlite essays as well as a pot experiment have been carried out. Both cuttings from homogeneous genotypes and seedlings developed from seeds of the same population have been used as plant material.

In *S. vulgaris* Cr was mainly accumulated in roots and only translocated to shoots near toxicity levels which is the first protection mechanism to Cr. Plants

accumulated more Cr in presence of Cr(VI) than of Cr(III) due to the different uptake mechanisms of each specie. Based on root growth, the most tolerant genotypes showed EC_{100} between 200 and 1200 µM and the less tolerant between 200 y 1000 µM. Seedlings developed from seeds showed lower tolerance levels, between 30 and 100 µM Cr(VI). *S. vulgaris* showed different tolerance mechanism to Cr. In one hand, the most tolerant genotypes increased their nutrient levels (especially Fe, Ca and Mg) to keep homeostasis balance and to synthesize antioxidant and essential metabolites. Exudation has also been identified as a possible tolerance mechanism to Cr as it is correlated with Cr accumulation in plant tissues but not with oxidative stress. Using HPLC-MS, quercetin and apiin have been identified as major polyphenols in *S. vulgaris* roots exudates. Concentrations of organics acids in both plant tissues and root exudates have been determined by ionic chromatography. Citric and malic acid showed the highest response to Cr exposition, their concentrations decreased in root exudates and increased in roots showing their role in Cr detoxification pathway in plant tissues.

With the aim of studying Cr distribution and speciation in plant tissues, micro synchrotron X-ray spectroscopy has been applied. Results from these studies showed the ability of *S. vulgaris* to reduce Cr(VI) to the less toxic Cr(III) as in plants growth in Cr(VI), all Cr found in plant tissues was Cr(III). Oxidation state maps showed Cr accumulated mainly in the roots tips and in roots cell wall. Once the retention capacity of roots was exceeded, Cr was transported through the steam xylem to the cell wall of leaf margins. µ-XANES analysis confirmed the role of organic acids in Cr accumulation and transport in the plant.

Starch granules have been identified in leaves of Cr(VI) treated plants using SEM and DRIFTS- FTIR. This could be attributed to an impairment between C assimilated in photosynthesis and C used in plant growth. This indicates that *S. vulgaris* plants were able to preserve photosynthetic function by Cr retention in roots and leaves margins but the energy invested in the managing of Cr excess made their biomass to decrease. DRIFTS- FTIR analysis confirmed the role of polysaccharides and proteins of the cell wall in Cr binding to avoid its presence in the cytoplasm. Moreover these data

showed synthesis of aromatic compounds in leaves, possibly antioxidant molecules, and also the absence of membrane damage.

Last in a pot experiment, plants were grown for six months in soils polluted with Cr(III) or Cr(VI). Results from this study showed that *S. vulgaris* was able to growth in soils with different physic-chemical characteristics and moderate levels of Cr pollution. The development of *S. vulgaris* cover provided C to soil from root exudation. The C input increased soil pH and solubilised part of the Cr that was not available hence increasing plants absorption. Moreover it stimulated soil microbial activity, as shown in dehydrogenase activity, contributing to soil recovery.

The ability of *S. vulgaris* to reduce Cr(VI) to Cr(III), to accumulate Cr mainly in roots and to develop and provide C to Cr polluted soils, makes it and interesting species to be applied in the revegetation and recovery of Cr polluted sites.

Índice/ Index

List of abbreviations

- **ANA:** 1-aftil acetic acid
- **API:** Application Programming Interface
- **ATSDR:** United States Agency for Toxic Substances and Disease Registry
- **BCF:** Bioconcentration Factor
- **BHT:** Buthylated hydroxytoluene
- **BOCM:** Boletín Oficial de la Comunidad de Madrid
- **CERCLA:** Comprehensive Environmental Response and Liability Act
- **DNA:** Deoxyribonucleic acid
- **DO:** Diario Oficial de la Unión Europea
- **DRIFTS-FTIR:** Diffusive Reflectance Infrared Fourier Transform Spectroscopy
- **D.W:** Dry Weight
- **EC:** Electric Conductivity
- **EC_{100}:** Efective Concentration 100%
- **EDDHA:** Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
- **EDTA:** Ethylenediaminetetraacetic acid
- **EPA:** United States Environmental Protection Agency
- **ESI:** Electrospray ionization
- **F.W:** Fresh Weight
- **GAE:** Gallic acid Equivalents
- **GLM:** General Lineal Model
- **GTF:** Glucose Tolerance Factor
- **HBED:** N,N'-di (2-hydroxybenzyl) ethylenediamine-N,N'- diacetic acid
- **HPLC-DAD:** High-Performance Liquid Chromatography with Diode Array Detection
- **IARC:** International Agency for Research on Cancer
- **INTF:** Iodonitrotetrazolium Formazan
- **LCF:** Linear Combination Fitting
- **MDA:** Malondyaldehyde
- **MES:** 2-morpholinoethanesulfonic acid monohydrate
- **OM:** Organic Matter
- **PCA:** Principal Component Analysis
- **PVC:** Polyvinyl Chloride
- **Q-LC/MS:** Quadrupole Liquid Chromatography Mass Spectrometry
- **ROS:** Reactive Oxygen Species
- **SE:** Standard Error
- **SEM:** Scanning Electron Microscopy
- **SPAD:** Soil-Plant Analysis Development Values
- **TBA:** Thiobarbituric acid
- **TCA:** Trichloroacetic acid
- **TF:** Transfer Factor
- **TPC:** Total Phenolic Content

- **XAS:** X-ray Absorption Spectroscopy
- **μ-SXRF:** Micro Synchrotron X-Ray Fluorescence
- **μ-XANES:** Micro X-Ray Absorption Near Edge Structure
- **μ-XAFS:** Micro X-Ray Absorption Fine Structure

List of tables

Table 7.8.
Concentration of organic acids (mg·kg^{-1} D.W.) in shoots and roots of *S. vulgaris*.

List of figures

Introducción

1.1. EL CROMO

1.1.1. Generalidades

El cromo es un metal de transición perteneciente al grupo VIB de la tabla periódica, con número atómico 24 y peso molecular 51.9961 $g \cdot mol^{-1}$. Recibe su nombre del griego "chrôma" (color) debido a la variedad de colores que presentan sus compuestos químicos (Gomez y Callao, 2006). Es el séptimo elemento disponible más abundante de la Tierra y el veinteavo más abundante de la corteza terrestre. El cromo presenta varios estados de oxidación desde -2 a +6 pero en condiciones ambientales normales las dos especies químicas más abundantes son +3 y +6 (Fendorf, 1995). La presencia de una u otra especie química está determinada por el pH y las condiciones redox del medio (Kotas y Stasicka, 2000).

El Cr se extrae en la naturaleza a partir del mineral cromita (Cr(III)), un óxido de magnesio, hierro, cromo y aluminio [$(Mg,Fe)(Al,Cr,Fe)_2O_4$] que se encuentra principalmente en rocas ultramáficas. La concentración de óxido de cromo (Cr_2O_3) en el mineral se presenta entre un 15 y un 65 % (Motzer, 2004). La forma hexavalente del Cr es poco frecuente en la naturaleza, solo aparece en algunas zonas muy localizadas por lo que su presencia en el medio ambiente suele atribuirse a fuentes antropogénicas. También pueden encontrase en el medio ambiente pequeñas cantidades de cromo metálico (0) procedente de uso industrial ya que se le conoce como el "aditivo supremo" por las propiedades anticorrosivas y resistentes que confiere a las aleaciones metálicas (Gomez y Callao, 2006).

1.1.2. Toxicidad

En el medio ambiente la toxicidad y biodisponibilidad del Cr vienen determinadas por la especie química. La especie más tóxica de Cr es el Cr(VI), se trata de una especie mucho más soluble que el Cr(III) y, por lo tanto mas biodisponible. Tiene un elevado poder oxidante por lo que es clasificado como carcinogénico de Grupo A por la EPA (Agencia de Protección ambiental de Estados Unidos) (USEPA, 1998) y la IARC (Agencia Internacional para el Estudio del Cáncer) (IARC, 2006).

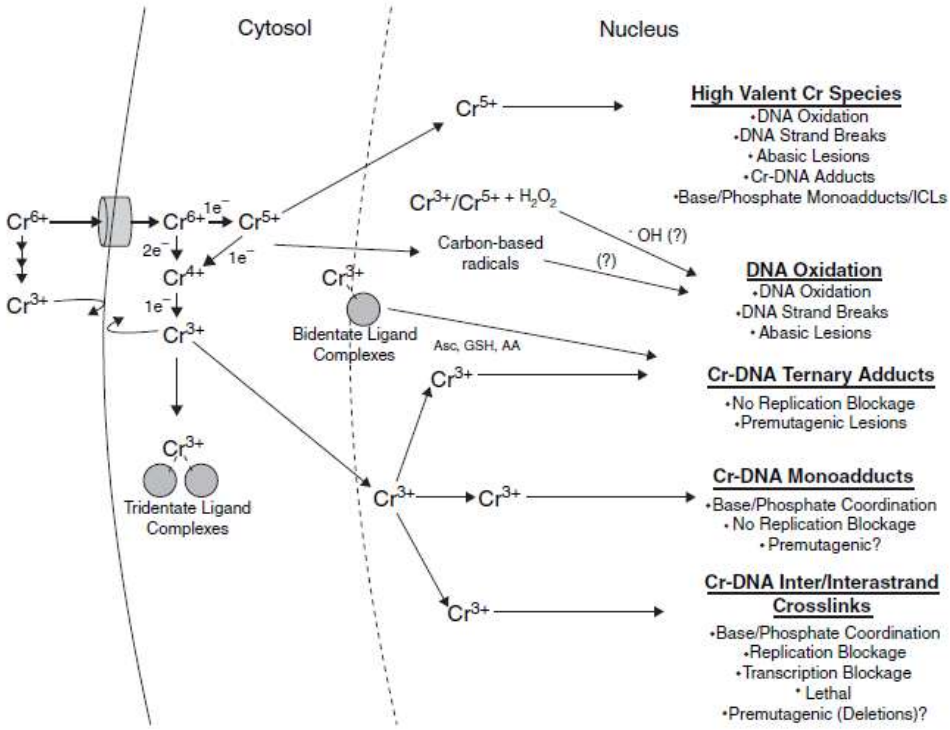


Figura 1: Resumen de los daños producidos por el Cr sobre el ADN de las células (Shanker *y col*., 2005)**.**

El Cr es tóxico para todos los organismos vivos, su toxicidad está relacionada con la producción de especies reactivas de oxígeno (ROS, radical superóxido, peroxido de hidrógeno y radicales hidroxilo) en las células que generan estrés oxidativo, dañan las membranas celulares y causan daños en el DNA. En la figura 1 se resumen los daños causados por el Cr en el DNA celular. El Cr(VI) es un oxidante fuerte con un alto potencial redox. En las células, el Cr(VI) es reducido a Cr(III) por la glutatión reductasa en presencia de NADPH (Shanker, 2008). En este proceso se producen reacciones de tipo Fenton en la célula que dan lugar a especies intermedias Cr (V/IV) y a la

producción de radicales hidroxilo(Stohs y Bagchi, 1995). El Cr (III) también puede ser reducido a Cr(II) que reacciona con peróxido de hidrógeno liberando radicales hidroxilo (Ozawa y Hanaki, 1990). Sin embargo, este proceso tiene lugar a concentraciones mucho más altas a las necesarias para la producción de radicales libres en presencia de Cr(VI). Por eso se establece que el Cr(VI) es entre 10 y 100 veces más tóxico que el Cr(III) (Deflora *y col.*, 1990).

El Cr(III) se ha considerado como un componente esencial del metabolismo de los lípidos y la glucosa en los mamíferos, ya que parece formar parte del factor de tolerancia a la glucosa (GTF) necesario para la síntesis de insulina (ATSDR, 2012). Sin embargo su papel como micronutriente no ha sido confirmado y es cuestionado ya que no se ha identificado en qué tipo de biomolécula se encuentra. En el caso de los humanos no hay casos registrados de problemas de salud asociados con deficiencias de Cr, por el contrario parece que ciertos complementos nutricionales de Cr(III) pueden inducir cáncer (Levina, 2008; Costa, 2006).

1.1.3. Usos industriales del Cromo

A nivel mundial, en 2010 y 2011 la cantidad de cromo extraída de las minas de cromita fue de 23,700,000 y 24,000,000 toneladas cúbicas, respectivamente. Las reservas mundiales de cromita se estiman en más de 480,000,000 toneladas cúbicas localizadas principalmente en Kazajistán (220,000,000), el sur de África (200,000,000), India (54,000,000) y Estados Unidos (620,000). El 90% del cromo extraído es utilizado en aplicaciones metalúrgicas, un 5% en aplicaciones químicas y el otro 5% restante en aplicaciones refractarias (Dhal *y col.*, 2013). En la industria metalúrgica el Cr se emplea principalmente en el recubrimiento de superficies metálicas mediante galvanoplastia debido a sus excepcionales propiedades anticorrosivas a temperatura ambiente. De hecho, su aplicación más importante es la manufactura del acero inoxidable ya que es precisamente el recubrimiento de cromo lo que le hace inoxidable (Barnhart, 1997). Las piezas metálicas comerciales contienen entre un 0.5 y un 30% de cromo. El Cr también forma parte de aleaciones de níquel, cobalto, aluminio, titanio o cobre que se emplea en la industria automovilística, en la aviación y en la construcción de trenes de alta velocidad (Bielicka *y col.*, 2005).

En la industria química la mayoría del cromo se dedica a la producción de pigmentos para producción de pinturas y tintes. También se aplica en el curtido de cuero, la industria textil y la preservación de maderas. En cuento a la industria de materiales refractarios, el cromo se emplea principalmente en la elaboración de materiales de construcción como ladrillos, cerámicas y cemento. El cromo confiere a estos materiales mayor estabilidad y resistencia térmica (Bielicka *y col.,* 2005).

1.1.4. El Cromo en el medio ambiente

Como se muestra en la figura 2, el Cr se encuentra en todos los compartimentos ambientales, su emisión desde fuentes antropogénicas ha incrementado las concentraciones naturales en los mismos, lo que en algunos casos, supone un riesgo para la salud humana y de los ecosistemas.

La atmósfera es unos de los principales medios de dispersión del Cr emitido por las industrias. En función de la meteorología, la topografía, la vegetación y el tamaño de partícula, el Cr emitido se deposita en suelos y aguas superficiales a mayor o menor distancia de la fuente emisora. Una vez en el suelo, las características del mismo la especie química de Cr van a determinar la evolución del metal, como se comentará más adelante. En el suelo, el Cr no se transporta a grandes distancias pero generalmente si puede lixiviarse y contaminar las aguas subterráneas. Desde el suelo, el Cr puede pasar a los vegetales y transmitirse a la cadena trófica. En ríos el Cr se transporta en forma de partículas en suspensión. En mares y océanos, el Cr procede tanto de la deposición atmosférica directa, como de los ríos y puede ser absorbido por los organismos vivos y retenido en sedimentos, donde se producen reacciones redox de interconversión entre Cr(VI) y Cr(III) (Bielicka *y col.,* 2005).

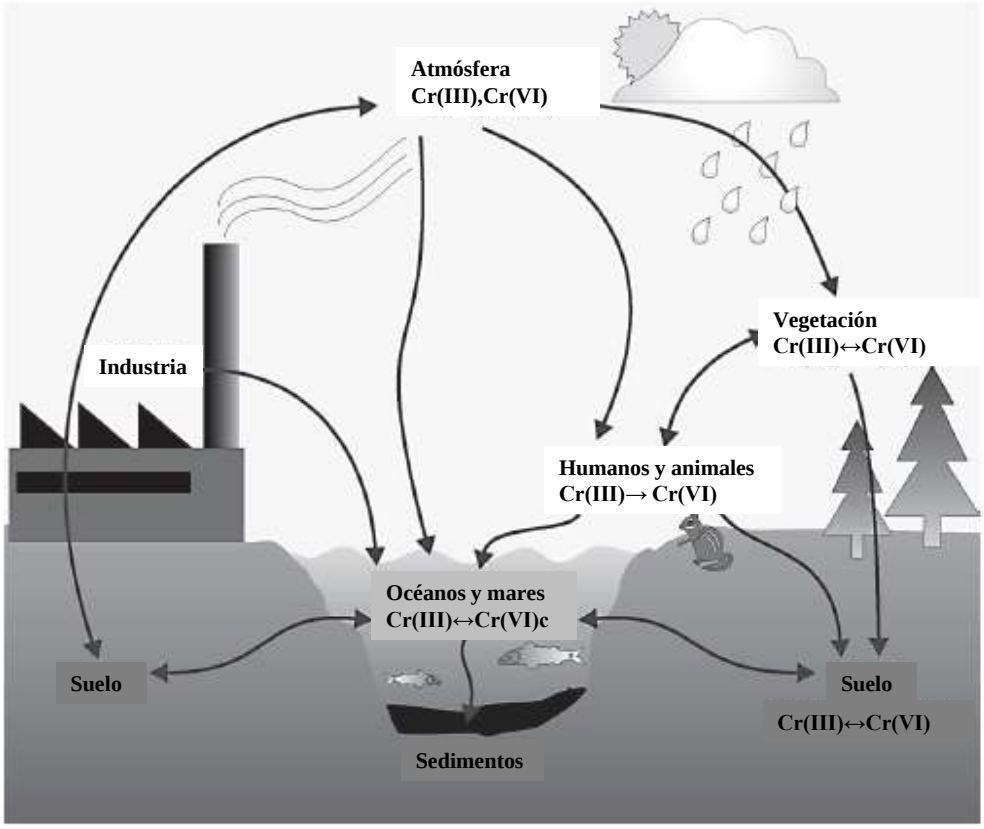


Figura 2: Circulación del Cr de origen antrópico en el medio ambiente.

En la tabla 1, se muestran las concentraciones de Cr en distintos compartimentos ambientales. Las concentraciones naturales de este elemento dependen en suelos de la naturaleza de la roca madre. Las mayores concentraciones se alcanzan en suelos ultramáficos (125 $g \cdot kg^{-1}$). En el agua dulce las concentraciones varían de 0.1 a 117 $\mu g \cdot L^{-1}$ mientras que en agua de mar el rango va de 0.2 a 50 $\mu g \cdot L^{-1}$. En la atmosfera la concentración de Cr varía de forma muy significativa según el punto de muestreo desde valores de $5.0 \cdot 10^{-6}$ $\mu g \cdot m^{-3}$ en áreas remotas no contaminadas a 0.03 $\mu g \cdot m^{-3}$ en muestras tomadas en áreas urbanas (Shanker *y col.*, 2005) .

Los valores máximos de Cr que no constituyen un riesgo para la salud del ecosistema en agua dulce son de 1 $\mu g \cdot L^{-1}$ y 8 $\mu g \cdot L^{-1}$ para Cr(III) y Cr(VI) respectivamente y de 1 $\mu g \cdot L^{-1}$ y 50 $\mu g \cdot L^{-1}$ para agua marina. Los valores máximos recomendados en agua de riego son de 8 $\mu g \cdot L^{-1}$ para Cr(VI) y de 5 $\mu g \cdot L^{-1}$ para Cr(III)

(Zayed y Terry, 2003). Para el agua de consumo humano el máximo se establece en 50 $\mu g \cdot L^{-1}$(ATSDR, 2012).

Tabla 1: Concentraciones de Cromo en el medio ambiente (Shanker *y col.*, 2005).

Tipo de muestra	Concentración
Suelos naturales	5–1000 $mg \cdot kg^{-1}$
	5–3000 $mg \cdot kg^{-1}$
	5–1500 $mg \cdot kg^{-1}$
	30–300 $mg \cdot kg^{-1}$
Suelos serpentínicos	634–125,000 $mg \cdot kg^{-1}$
Media de suelos mundial	200 $mg \cdot kg^{-1}$ (media)
	100–300 $mg \cdot kg^{-1}$
	10–150 $mg \cdot kg^{-1}$ (media 40 $mg \cdot kg^{-1}$)
Suelos Estados Unidos	25–85 $mg \cdot kg^{-1}$ (media 37 $mg \cdot kg^{-1}$)
	media 57 $mg \cdot kg^{-1}$
Suelos Canadá	100–5000 $mg \cdot kg^{-1}$ (media 43 $mg \cdot kg^{-1}$)
Suelos Japón	87 $mg \cdot kg^{-1}$ (media)
Suelos Suecia	74 $mg \cdot kg^{-1}$ (media)
Sedimentos	0–31,000 $mg \cdot kg^{-1}$
Agua dulce	0–117 $\mu g\ L^{-1}$ (media 9.7 $\mu g \cdot L^{-1}$)
Agua de mar	0–0.5 $\mu g \cdot L^{-1}$
Aire	1–545,000 $ng \cdot m^{-3}$
	100 $ng \cdot m^{-3}$ (media)
Plantas	0.006–18 $mg \cdot kg^{-1}$
Animales	0.03–1.6 $mg \cdot kg^{-1}$

En sedimentos, la concentración mínima de Cr que puede producir efectos tóxicos sobre el ecosistema (Threshold Effect Levels ,TEL) es de 37.7 $mg \cdot kg^{-1}$ (Evans y Gottgens, 2007). La Agencia de protección Ambiental de Estados Unidos (EPA) establece como niveles mínimos de intervención, concentraciones de Cr(VI) de 29 $mg \cdot kg^{-1}$ en suelos residenciales y 56 $mg \cdot kg^{-1}$ en suelos industriales, no se establecen niveles de intervención específicos para Cr total o Cr(III) (EPA, 2013). La legislación de la comunidad de Madrid establece distintas concentraciones permisibles en función de la actividad a la que esté destinado el suelo. Los valores de referencia de Cr total establecidos son de 2,300 mg kg^{-1} en suelos de uso industrial, de 230 $mg \cdot kg^{-1}$ suelos de uso urbano y 90 $mg \cdot kg^{-1}$ en suelos con otros usos (Orden 2270/2006, de 11 de agosto).

1.1.5. Problemas ambientales

El uso generalizado del Cr en la industria hace que la disposición de los residuos procedentes, tanto de su extracción minera, como de su uso en los distintos procesos industriales constituya un problema ambiental de problemática creciente. Durante el siglo veinte, el Cr, junto con el Ni, fueron los metales pesados que más aumentaron sus deposiciones en el medio ambiente desde fuentes antropogénicas y especialmente a partir de 1980 (Han *y col.*, 2002). Aproximadamente el 35% del Cr que llega al medio ambiente por causas antropogénicas es Cr(VI) (Dhal *y col.*, 2013). Sus principales fuentes de emisión son la minería de este metal, procesos de cromado de piezas, industria química del cromo y la evaporación procedente de torres de refrigeración industriales.

La minería del cromo supone un grave problema ambiental tanto por los efluentes líquidos contaminados, como por las escombreras de residuos sólidos que se generan. Estas últimas constituyen áreas a cielo abierto que quedan estériles debidos a la baja concentración de nutrientes, el pH, la baja capacidad de retención de agua y la presencia de metales pesados que impiden el desarrollo de una cubierta vegetal (Dhal *y col.*, 2011). Los residuos procedentes de la minería de Cr suponen un problema de contaminación ambiental en diversos puntos de Estados Unidos, Reino Unido, China, Japón y la India entre otros. Este residuo procedente de la minería es una mezcla de carbonato de sodio, cenizas, cal y cromato de sodio soluble. Se estima una deposición mundial de estos residuos en el medio de entre 2 y 3 millones de toneladas al año. Además, los hornos de fundición situados en muchas de estas minas también suponen un importante problema ya que emiten cenizas con altos contenidos en Cr. Se ha calculado que una sola de estas chimeneas puede emitir aproximadamente 54.6 toneladas de Cr al año (Dhal *y col.*, 2013).

Otras fuentes de emisión de Cr a la atmósfera son la industria del ferrocromo y la combustión del carbón, que emiten anualmente 12,300 y 520 toneladas de Cr respectivamente (Mohanty y Patra, 2011).

La industria del curtido de cuero es una de las causes principales de aporte de Cr al medio. Anualmente se vierten unas 50,000 toneladas de residuos sólidos ricos en

Cr procedentes de esta actividad. Más de 50,000 ha de suelos están contaminados con este tipo de residuos en India y Bangladesh. Además se trata de residuos muy persistentes en el medio ambiente. En un estudio realizado en suelos de un antiguo área de vertido de residuos de curtiduría en Adelaida (Australia) se encontraron concentraciones de Cr total de entre 70,000 y 100,000 $mg \cdot kg^{-1}$ 20 años después del cese de los vertidos (Kamaludeen *y col.*, 2003). Durante el proceso de curtido de cuero solo el 60% del Cr empleado reacciona con la piel y el resto acaba formando parte de los lodos procedentes del tratamiento de aguas residuales de las plantas, de los que constituye entre un 1 y 4% y que generalmente son depositados en vertederos (Lee y Pandey, 2012). Además, los efluentes líquidos que salen de estas curtidurías contienen entre 100 y 200 $mg \cdot L^{-1}$ de Cr (Sundaramoorthy *y col.*, 2010). Sólo en India se vierten cada año entre 12,000 y 20,000 toneladas de cromo procedente del proceso de curtido de cuero en forma de lodos o efluentes compuestos con concentraciones de entre 1,800 y 20,000 ppm de Cr que son depositados sin tratamiento previo.

Otra fuente importe de vertido incontrolado de Cr al medio ambiente son las aguas residuales urbanas. El vertido se produce principalmente en zonas urbanas cercanas a polígonos industriales donde los colectores de aguas residuales no posibilitan la separación de los dos tipos de efluentes. Los sistemas convencionales de depuración de aguas residuales cuentan con sistemas muy deficientes de eliminación de metales pesados y, por lo tanto de cromo. Esto da lugar al vertido de estos metales pesados a la cuenca receptora. Arauzo *y col.* (2002) estimaron un vertido anual de 2.2 toneladas de cromo desde el efluente de la depuradora de Arganda del Rey al río Jarama (Comunidad de Madrid, España), del cual un 60% era Cr(VI) (Arauzo *y col.*, 2002). En un estudio posterior, este mismo equipo encontró concentraciones de Cr en el agua del río de un orden de magnitud superior a los límites establecidos por la legislación. Asimismo, los niveles de contaminación por cromo en los sedimentos y en el agua intersticial a orillas del cauce resultaron muy elevados, constituyendo importantes reservorios de cromo que podrían actuar como fuentes potenciales para la dispersión del mismo (Arauzo *y col.*, 2003). Además de las aguas residuales, los lodos producidos en estas plantas de depuración también pueden contener altas concentraciones de Cr, lo

que supone un riesgo si son aplicados como fertilizantes en zonas agrarias (Ayari *y col.*, 2008; Mahvi, 2008,).

En los procesos de galvanoplastia y acabado metálico se generan gran cantidad de lodos tóxicos que generalmente son depositados en vertederos. Este tipo de residuos tienen una elevada peligrosidad ya que contienen entre un 7 y un 11% de Cr, aproximadamente entre un 3 y un 5% de Fe y cantidades variables de otros metales como Ni, Co, Zn, Mo, V o Cu (Lee y Pandey, 2012).

Además de las fuentes de contaminación industriales, en zonas agrícolas la aplicación sucesiva de fertilizantes fosfatados puede incrementar los niveles de Cr y otros metales pesados en los suelos, ya que éstos forman parte de las rocas de las que se extraen estos fertilizantes (He *y col.*, 2005).

El Cromo es el segundo contaminante inorgánico más frecuente en las aguas subterráneas de Estados Unidos (Blowes, 2002). Este metal lleva apareciendo en la lista de los 20 contaminantes prioritarios del programa "Superfund" de la EPA durante los últimos 15 años (EPA, 2013) y ha sido detectado en cantidades preocupantes en 722 de los emplazamientos registrados por este programa. Además de las fuentes industriales, en Estados Unidos, también se ha detectado contaminación por Cr asociada a zonas de almacenamiento de residuos nucleares donde se utiliza dicromato de sodio para prevenir la corrosión en las tuberías, por las que circula el agua de refrigeración (Fruchter *y col.*, 2000a). Por eso, el Cr es considerado contaminante de interés prioritario por la EPA y aparece en la lista de contaminantes prioritarios CERCLA de la Agencia para el Registro de Sustancias Tóxicas y Enfermedades (ATSDR) (Jennings, 2013).

En la Unión Europa, el uso de Cr en equipos electrónicos y eléctricos fue restringido mediante la directiva 2002/95/EC, conocida como la Directiva de Restricción de Sustancias Peligrosas (Dir 2002/95/Parlamento y Consejo Europeo de 13 de enero de2003). Pero ha recibido una menor atención por parte de las autoridades europeas que en los Estados Unidos, ya que no ha sido incluido en la lista de 33 sustancias tóxicas prioritarias establecidas en el anexo II de la Directiva 2008/105/EC

(Dir 2008/105/ Parlamento Europeo, de 16 diciembre de 2008). Sin embargo, un estudio realizado por Harmens *y col*. (2007) donde se analizaron la concentración de metales pesados en musgos para monitorizar la evolución de las deposiciones atmosféricas de metales en Europa entre los años 1990 y 2000, reveló un importante incremento en los niveles de Cr en los musgos recolectados en Islandia, Reino Unido, Italia, Lituania, Eslovaquia y España lo que indicó un aumento de emisiones de Cr en estos países (Harmens *y col.,* 2007). En 2010, los estados miembros han informado de un aumento de las emisiones de metales pesados y contaminantes orgánicos persistentes en comparación con 2009. Entre ellos destaca un aumento del 12,6% en Cr. Estos incrementos se debieron en parte a las emisiones crecientes de los hogares y de ciertos sectores industriales (European Environment Agency, 2010).

En España el principal aporte de origen antrópico de Cr al suelo se produce por deposición atmosférica en las proximidades de las fábricas de tratamiento de hierro y acero (López-Arias y Grau, 2005). En el año 2001 la industria española emitió 80Mt de Cr a la atmosfera, lo que supuso el 40% del Cr total emitido por la Unión Europea en ese año (García-Pérez *y col.,* 2007). En 2011, Vilavert *y col*. (2012) llevaron a cabo un análisis de elementos traza en suelos muestreados en los alrededores de una planta incineradora en España, los datos recogidos se compararon con los niveles determinados en 1998. Los niveles de Cr en estos suelos presentaron un aumento significativo respecto a los tomados en 1998, lo que pone de manifiesto el importante aporte que suponen las deposiciones atmosféricas en el contenido de Cr en suelos (Vilavert *y col.,* 2012). Otras fuentes de aporte importante de Cr al suelo en España son la aplicación de lodos de depurados y los residuos procedentes de la industria de curtidos (López-Arias y Grau, 2005).

Tanto en España (López-Arias y Grau 2005) como en Europa (Lado *y col.*, 2008) la concentración de Cr en suelos está fuertemente correlacionada con la presencia de Ni. En un estudio realizado por López *y col.*(2012) en los sedimentos del río Ebro, las concentraciones de ambos metales superaron los niveles de toxicidad establecidos en la literatura que pueden suponer un riesgo para el ecosistema y la salud humana. La presencia de Cr en aguas de riego, suelos y sedimentos puede constituir un importante

riesgo ambiental y para la salud humana. En la provincia de Tarragona (Cataluña) se analizaron muestras de suelo y plantas (*Beta vulgaris*) que demostraron que la elevada presencia de industrias petroquímicas en la zona incrementó los niveles de As, Cr y V en las muestras (Nadal *y col.*, 2004).

1.2. EL CROMO EN EL SUELO

1.2.1. El ciclo del Cr en el medio ambiente

Como ya se comentó anteriormente los dos estados de oxidación más abundantes de Cr en el medio ambiente son Cr(III) y Cr(VI). En la mayoría de los suelos (pH 7-10), el Cr se encuentra principalmente como $Cr(OH)_3$ insoluble en el suelo (Kotas, 2000). En función del pH del suelo se produce la hidrólisis de esta de forma (Fendorf, 1995), en suelos ácidos (pH<4) predomina el $Cr(H_2O)_6^{+3}$, mientras que a pH<5.5 predomina $CrOH^+$. Ambas especies son fuertemente absorbidas por las arcillas y los ácidos húmicos del suelo. Por lo tanto existe poco riesgo de que el Cr(III) en cualquiera de sus formas sea lixiviado a las aguas subterráneas o absorbido por las plantas. El Cr(VI) se encuentra principalmente como CrO_4^{2-} en suelos neutros y alcalinos y como $HCrO_4^-$ en pH<6 (Kotas y Stasicka, 2000). Ambas especies son repelidas por las cargas negativas de las arcillas del suelo y se encuentran, por lo tanto, muy solubles, móviles y disponibles. Su elevada solubilidad junto con su mayor toxicidad hacen que la presencia de Cr(VI) comprometa de forma importante la calidad de los suelos (Fendorf, 1995).

Ambas especies de Cr pueden estar sometidas a reacciones de inter conversión redox. Si el Cr se encontrara en equilibrio termodinámico con la atmósfera, el O_2 oxidaría todo Cr(III) y el Cr(VI) sería la única especie de Cr disponible. Afortunadamente el proceso de decromificación hace que la especie predominante de Cr en la naturaleza sea Cr(III) (Bartlett, 1991). En la figura 3 se muestra el ciclo del Cr y el proceso de decromificación en la naturaleza. Una vez en el suelo, el Cr(VI) es reducido a Cr(III), principalmente por la acción de materia orgánica del suelo que actúa como donadora de electrones (ecuación 1). Los ácidos orgánicos, el Fe(II) y los sulfuros también pueden actuar como agentes reductores (Fendorf, 1995). En el proceso

contrario, el Cr(III) puede ser solubilizado por ácidos orgánicos, principalmente ácido cítrico, y ser oxidado de nuevo a Cr(VI) por los óxidos de manganeso presentes en el suelo (Kotas y Stasicka, 2000) (ecuación 2).

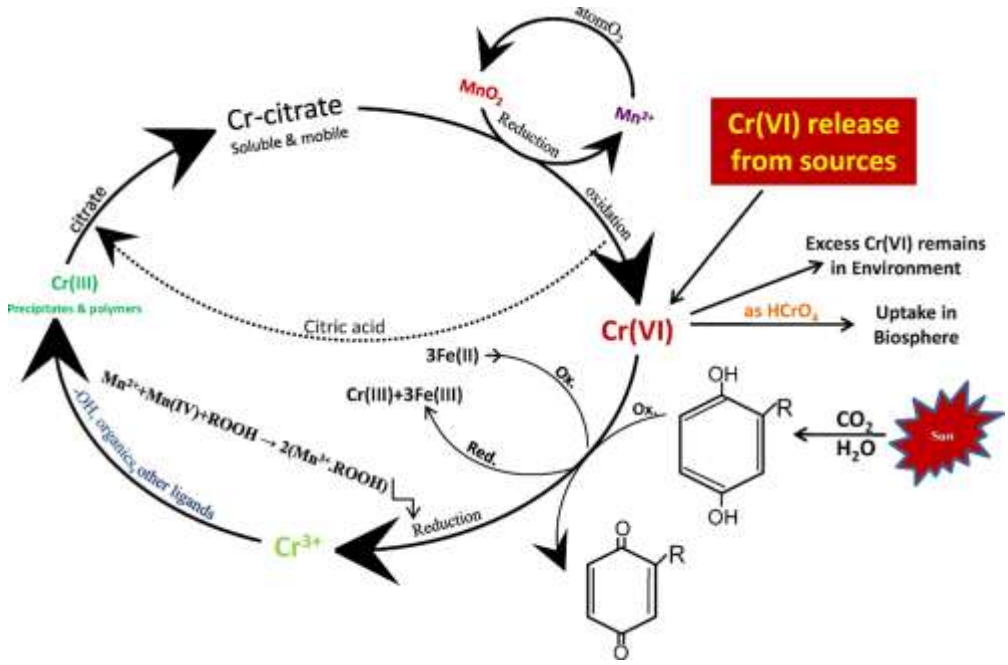


Figura 3: Ciclo natural del Cr (Dhal *y col.*, 2013)

Ambos procesos son termodinámicamente espontáneos y pueden ocurrir al mismo tiempo. Que predomine uno u otro depende del pH del suelo y de la presencia de agentes reductores y oxidantes. Sin embargo, el alto potencial de reducción de la pareja Cr(VI)/Cr(III) hace que el proceso de reducción predomine sobre el de oxidación, especialmente en medios ácidos (Unceta *y col.*, 2010). En los suelos, se pueden encontrar pequeñas cantidades de Cr(VI) fruto de la oxidación natural del Cr(III), pero la presencia de grandes cantidades de Cr(VI) se debe a contaminación de origen antropogénico.

$$C_6H_6O_2+CrO_4^{2-}+2H_2O \rightarrow 0.5Cr_2O_3+1.5C_6H_4O_2+2.5H_2O \quad (1)$$

$$Cr^{+3}+1.5MnO_2+H_2O \rightarrow HCrO_4^{-}+1.5Mn+2+H^{+} \quad (2)$$

Aunque estos procesos redox son los que tiene una mayor influencia a la hora de determinar la presencia de una u otra especie de Cr en el suelo, los procesos de precipitación-disolución y adsorción-desorción también son importantes a la hora de estudiar la dinámica del Cr.

Como se mencionó anteriormente, en principio el Cr(III) se va encontrar mayoritariamente precipitado en el suelo. La presencia de ácidos orgánicos procedentes de la exudación de las plantas puede dar lugar a complejos solubles de Cr(III) que pueden ser fácilmente oxidados a Cr(VI) o absorbidos por las plantas (Puzon *y col.*, 2008; Srivastava *y col.*, 1999,).

En suelos alcalinos el Cr(VI) se encuentra principalmente en forma soluble. En condiciones neutras y ácidas, el $HCrO_4^-$ y el CrO_4^{-2} puede ser absorbidos por los grupos hidroxilo positivos de los óxidos de Fe y Al y de las arcillas del suelo (Bartlett 1991, Rai *y col.*, 1987). La presencia de carbono inorgánico disuelto y de fosfatos en el suelo puede competir con el $HCrO_4^-$ y el CrO_4^{-2}, reduciendo su adsorción en los suelos (Stewart *y col.*, 2003). A medida que aumenta el pH, aumenta la adsorción de Cr(III) en el suelo y disminuye la de Cr(VI), de forma que a pH por encima de 8.5, todo el Cr(VI) se encuentra soluble (Zayed y Terry, 2003).

Por ultimo, la presencia de los microorganismos y las plantas también va a influir de forma significativa la dinámica del Cr en el suelo, ya que existen organismos que pueden llevar a cabo la reducción de Cr(VI) a Cr(III) (Cervantes *y col.*, 2001) y podrían afectar a las características del suelo determinantes en la dinámica del Cr como son el pH, el contenido en materia orgánica y el contenido de los ácidos orgánicos.

Los suelos tienen una capacidad limitada para llevar a cabo el proceso de decromificación o de adsorber e inmovilizar Cr(VI). Si el aporte de esta especie desde fuentes industriales y minería sobrepasa la capacidad del suelo, el exceso de Cr(VI) puede permanecer en el medio ambiente o ser absorbido por la biota, constituyendo un importante problema medioambiental y de salud pública. El papel de la materia orgánica del suelo es fundamental en el proceso de reducción de Cr(VI), ya que actúa

directamente como donador de electrones e indirectamente mediante la creación de condiciones reductoras al favorecer el crecimiento de los microorganismos del suelo (Losi *y col.,* 1994). El vertido de Cr(VI) es especialmente peligroso en suelos con bajo contenido en materia orgánica y pH básicos en los que el proceso de reducción no se ve favorecido.

2.2. 2. Métodos tradicionales de descontaminación

Actualmente, en la remediación de suelos contaminados con Cr se aplican esencialmente técnicas físico-químicas, principalmente la excavación o el bombeo del material contaminado seguido de la adición de productos químicos que conducen a la reducción de Cr(VI) y a la posterior precipitación y/o sedimentación del Cr(III) producido.

En la revisión de Dhal *y col.* (2013) se exponen las principales técnicas aplicadas para la remediación de suelos contaminados con Cr. Entre las técnicas más innovadoras aplicadas destacan el uso de barreras permeables (Wanner *y col.,* 2011; Flury *y col.,* 2009), inyecciones de ditionito de Na (Fruchter *y col.,* 2000b) y aplicación de nanopartículas de Fe^0 (Du *y col.,* 2012). En la tabla 2, se resumen las principales ventajas y desventajas de los métodos tradicionales de descontaminación.

Entre los principales inconvenientes destacan su elevado precio y consumo energético, que mayoritariamente son aplicables en áreas contaminadas reducidas, la generación de residuos peligrosos y por último, que son técnicas muy agresivas que destruyen la estructura del suelo (Caliman *y col.,* 2011). Como consecuencia de estos inconvenientes, en los últimos años, se están desarrollado técnicas biológicas (biorremediación, fitorremediación, biosorción, bioxidoreducción) más respetuosas con el medio ambiente y más baratas que están cobrando mayor importancia.

Tabla 2: Ventajas y desventajas de los métodos tradicionales de remediación de suelos contaminados con Cr (Viti and Giovannetti, 2007).

Proceso	Ventajas	Desventajas
Excavación "off site"	Rápido. Apropiado para volúmenes pequeños de suelo. Elimina completamente el contaminante.	Caro. Riesgos para salud durante la excavación. El suelo eliminado necesita tratamiento. Destruye el emplazamiento pero no el contaminante.
Lavado	Reduce el volumen de suelo que necesita tratamiento. La solución de lavado puede ser reutilizada.	Requiere excavación del suelo. Riesgos para salud durante la excavación. Destruye el emplazamiento, pero no el contaminante. Genera efluentes líquidos contaminados. No es apropiado para todo tipo de suelos.
Lavado "*in situ*"	No requiere excavación, eficiente. Puede ser mejorada con electrocinética.	No destruye el contaminante. Genera efluentes líquidos contaminados.
solidificación/ estabilización "*Ex situ*"	Relativamente barata.	Riesgos para salud durante la excavación. No destruye el contaminante. Aumenta el volumen de material de desecho.
solidificación/estabilización "*In situ*"	Aplicable en emplazamientos con niveles freáticos cercanos a la superficie.	No elimina el contaminante. Es necesario reducir el Cr(VI) a Cr(III) antes del tratamiento para disminuir los riesgos de lixiviación.
Vitrificación "*in situ*" o "*ex situ*"	Reduce toxicidad y movilidad del contaminante. Reduce volumen de suelo contaminado. El producto final no es peligroso. Puede aplicarse "*in situ*".	Alta demanda energética y tecnológica. Requiere aditivos químicos.
Reducción química "*in situ*" o "*ex situ*"	Reduce la toxicidad y la movilidad del Cr(VI). Puede aplicarse "in *situ*".	No elimina el Cr(III) producido del suelo y éste puede volver a oxidarse. No es adecuado cuando es necesario reducir los niveles de Cr total. Requiere amplio conocimiento de las características físico-químicas del suelo. Proceso de reducción puede ser lento.

1.3. FITORREMEDIACIÓN COMO ALTERNATIVA

1.3.1. Fitorremediación

La fitorremediación se define como el uso de las plantas y sus microorganismos asociados para eliminar, contener, inactivar o degradar contaminantes ambientales o para revitalizar zonas contaminadas (Vangronsveld *y col.*, 2009). También suele recibir el nombre de fitotecnologías (Conesa *y col.*, 2012). Estas tecnologías engloban los siguientes procesos (Pilon-Smits, 2005; Vassilev *y col.*, 2004; Cunningham *y col.*, 1995):

- Fitoextracción: se basa en la capacidad de las plantas para absorber metales en las raíces y movilizarlos a la parte cosechable de la planta de tal forma que mediante continuos procesos de siembra y cosecha se van eliminando los metales del suelo. Las plantas ideales para ser usadas en este proceso son aquellas que acumulan grandes cantidades de metales en sus tejidos y que reciben el nombre de hiperacumuladoras.

- Fitoestabilización: es el uso de la plantas para reducir la biodisponibilidad, la toxicidad o la movilidad de los metales en los suelos. Los metales quedan retenidos en la raíz de las plantas.

- Rizofiltración: es el uso de las raíces de las plantas para eliminar contaminantes de efluentes líquidos.

- Fitovolatilización: consiste en la absorción de los contaminantes del suelo por las raíces de las plantas y su posterior eliminación a la atmósfera mediante la transpiración, en ocasiones transformados en especies químicas menos tóxicas.

En la tabla 3 se resumen las principales ventajas y limitaciones de esta tecnología que puede combinarse con otras técnicas para solventar algunas de estas limitaciones. Por ello recientemente se ha acuñado un nuevo concepto, el fitomanejo, que se define como la ingeniería o la manipulación del sistema suelo-planta para controlar el flujo de

los elementos traza o contaminantes en el medio. Se trata de un concepto más amplio que una tecnología aislada ya que abarca el conjunto procesos aplicables a un emplazamiento específico (Robinson *y col.*, 2009). Al contrario que la fitorremediación el objetivo del fitomanejo no es solo la recuperación de los suelos contaminados sino la revalorización económica del emplazamiento (Conesa *y col.*, 2012; Robinson *y col.*, 2009).

Independientemente del término que usemos, tanto la fitorremediación como las fitotecnologías o fitomanjeo se basan en la capacidad de las plantas para tolerar y acumular contaminantes. Para un desarrollo adecuado de estas tecnologías es necesario un profundo conocimiento de las relaciones específicas entre el contaminante que se quiere eliminar y la vegetación que se pretende sembrar.

Tabla 3: Ventajas y limitaciones de la fitorremediación

Ventajas	Limitaciones
Puede ser aplicada "*in situ*" reduciendo el riesgo de excavación de los suelos contaminados.	Necesario tener en cuenta las características del suelo y la toxicidad del contaminante.
Funciona con energía solar y fija CO_2.	Proceso lento.
Entre 10 y 100 veces más barata que las técnicas tradicionales.	Aplicable en zonas con contaminación moderada.
Mejor aceptación pública, no perturba la estructura del suelo y mejora el paisaje.	Depende de la climatología de la zona.
No requiere gran equipamiento ni consumo energético.	Limitado a la profundidad de las raíces ~50cm para herbáceas, ~3m para árboles.
Genera menos residuos y es menos ruidosa que las técnicas tradicionales.	Limitado a la fracción biodisponible de los contaminantes.
Reduce la erosión del suelo y la migración de los contaminantes y promueve la recuperación natural de los suelos.	Puede haber riesgo de transmisión de los contaminantes a la cadena trófica.
Puede combinarse con otras tecnologías.	La biomasa de las plantas procedentes de fitoextracción constituye un residuo que debe ser eliminado.

Las interacciones entre el Cr y las plantas han sido menos estudiadas en relación a otros metales pesados por dos causas principales: primero porque el Cr no cumple ninguna función en las plantas y segundo por la dificultad a la hora de determinar la especiación química del mismo, especialmente "*in vivo*" (Shanker *y col.*, 2009).

1.3.2. Toxicidad del Cr para las plantas

Como acabamos de indicar, el Cr no tiene una función biológica definida en las plantas, por lo tanto es tóxico y su acumulación en los tejidos impide el desarrollo normal de las mismas. La mayor o menor absorción de Cr depende de la especie química presente en el medio. Las plantas acumulan más Cr en presencia de Cr(VI) ya que esta especie es absorbida en las raíces a través de transportadores sulfato mediante un proceso metabólico activo. El Cr(III), sin embargo, entra en las raíces de forma pasiva por lo que su acumulación es menor (Skeffington *y col.*, 1976). Una vez en las raíces el Cr es retenido principalmente en la pared celular o en vacuolas de las células del córtex y la epidermis y sólo es movilizado a la parte aérea cuando se alcanzan grandes concentraciones de Cr en raíz (Hayat *y col.*, 2012; Shanker *y col.*, 2005,). Se ha visto que algunas plantas tiene la habilidad de reducir Cr(VI) a Cr(III) en la raíz, lo que supone un importante mecanismo de detoxificación (Lytle *y col.*,1998; Zayed *y col.*, 1998). Los ácidos orgánicos parecen estar relacionados con una mayor absorción de Cr en las raíces y con su posterior transporte a la parte aérea de las plantas y a los puntos de almacenamiento (vacuolas o pared celular) (Howe *y col.*, 2003; Srivastava *y col.*, 1999,).

La generación de radicales libres en la célula, así como, la interferencia en la absorción de nutrientes y el agua, constituyen las causas principales de la toxicidad que ejerce el Cr sobre las plantas, modificando sus procesos fisiológicos y metabólicos e impidiendo su correcto desarrollo (Singh, 2013; Hayat *y col.*, 2012; Panda y Choudhury, 2005).

A continuación se resumen los principales efectos que producen la acumulación de Cr sobre la planta.

- Retardo en el crecimiento: La acumulación de Cr afecta principalmente a la longitud de la raíz (Hayat *y col.*, 2012) y disminuye la biomasa total como se ha observado en *Sorghum vulgare* (Lopez-Luna *y col.*, 2009), *Amarantus viridis* (Zou *y col.*, 2006b) u *Oryza sativa* (Sundaramoorthy *y col.*, 2010), entre otras especies. El retardo en el crecimiento se debe a la inhibición de la división celular causada por los daños sobre el DNA de la raíz (Zou *y col.*, 2006a) que dificulta la normal asimilación de nutrientes y agua por la planta. Esto se traduce en inhibición del crecimiento de la parte aérea de las plantas expuestas a Cr como se ha visto para *Pisum sativum* (Pandey *y col.*, 2009), *Miscanthus sinensis* (Arduini *y col.*, 2006) *o Brassica oleracea* (Chatterjee y Chatterjee, 2000).

- Fotosíntesis*:* En plantas expuestas a Cr se ha descrito inhibición de la fotosíntesis (Subrahmanyam, 2008; Samantary, 2002; Nichols *y col.*, 2000,) aunque no está claro si se debe a la desorganización de la estructura de los cloroplastos, a la inhibición de la cadena transportadora de electrones o a la inhibición de las enzimas del ciclo de Calvin (Hayat *y col.*, 2012).

- Balance hídrico: La estabilidad del balance hídrico en las plantas depende del equilibrio entre la cantidad de agua absorbida por las raíces y la que se pierde por transpiración. Por un lado, el daño producido por el Cr en las raíces y la saturación de las mismas pueden disminuir la capacidad de las raíces para absorber agua. Por otro, parece que el Cr(VI) interfiere en el funcionamiento de las células guarda de los estomas, diminuyendo la conductancia de los mismos (Hayat *y col.*, 2012). En *Brassica oleracea*, la exposición a Cr disminuyó la transpiración y aumentó el contenido total de agua (Chatterjee y Chatterjee, 2000). Sin embargo disminuyó el turgor y generó plasmolisis en *Phaselous vulgaris* (Vazquez *y col.*, 1987).

- Balance de nutrientes: Debido a la similitud de su estructura química, el Cr puede competir con ciertos nutrientes esenciales por los canales de entrada a la raíz causando problemas en el desarrollo de la planta (Shanker *y col.,* 2005). En varios estudios se ha relacionado la acumulación de Cr con una disminución en los niveles de Zn, Fe, Ca, Mg, Mn o Cu (Mallick *y col.,* 2010; Gopal *y col.,* 2009; Liu *y col.,* 2008; Dube *y col.,* 2003) . Sin embargo, Zeng *y col*. (2010) relacionaron una mayor tolerancia a Cr en genotipos de arroz con una mayor capacidad para acumular micronutrientes (Zeng *y col.,* 2010). Resulta especialmente interesante la relación existente entre el Fe y el Cr. La exposición a Cr en plantas deficientes en Fe activa la actividad de la Fe(III) reductasa aumentando la fracción biológica de Fe(II) y causando estimulación en el crecimiento (Barcelo y Poschenrieder, 1997). También se ha demostrado que el Cr aumenta la biodisponibilidad del Fe para las plantas (Bonet *y col.,* 1991). Además se ha visto que las plantas con altos contenidos en Fe como *Spinacea oleracea* o *Brassica napa* son especialmente efectivas en la acumulación y translocación de Cr (Cary *y col.,* 1977).

- Estrés oxidativo: Como se describió anteriormente, el Cr y especialmente el Cr(VI) puede producir radicales libres en la célula. Éstos generan daños en las membranas celulares y el ADN e interfieren en la cadena transportadora de electrones en la mitocondria. El malondialdehido se ha utilizado en varios trabajos como indicador de peroxidación lipídica producida por estrés oxidativo en plantas expuestas a Cr. Entre otras especies, se han observado incrementos en el MDA en *Pisum sativum* (Pandey *y col.,* 2009), *Brassica juncea* y *Vigna radiata* (Diwan *y col.,* 2010) y *Oryza sativa* (Dubey *y col.,* 2010). El estrés oxidativo es mayor en plantas expuestas a Cr(VI) que a Cr(III) (Scoccianti *y col.,* 2008; Shanker y Pathmanabhan, 2004; Mei *y col.,* 2002) y las raíces parecen ser susceptibles de sufrir mayor estrés que los tallos (Pandey *y col.,* 2005; Shanker y Pathmanabhan, 2004,). Las plantas más tolerantes a Cr serán aquellas que produzcan menos radicales libres o que cuenten con los mecanismos antioxidantes necesarios para capturar los ROS e impedir que causen daños en la célula.

- Alteraciones metabólicas: El estrés por Cr puede alterar varios procesos metabólicos en la planta. Por un lado la inversión de energía en la síntesis de metabolitos relacionados con la tolerancia (glutatión, ácido ascórbico, metalotioneinas, ácidos orgánicos, etc) puede inhibir otros procesos metabólicos. Y por otro, el Cr puede dañar de forma directa la estructura de ciertas enzimas fundamentales en metabolismo de la planta como la nitrato reductasa, Fe(III) reductasa o la H^+ATPasa de la membrana celular (Singh, 2013; Hayat *y col.*, 2012; Shanker *y col.*, 2005).

Tabla 4: Especies terrestres estudiadas para uso en fitorremediación de Cr.

Especie	Medio de cultivo	Max Cr Parte aérea*	Referencia
Brassica juncea	Hidroponía	504	(Diwan *y col*., 2010)
Vigna radiata	Hidroponía	238	(Diwan *y col*., 2010)
Brassica napus	Suelo	32	(Brunetti *y col*.,. 2011)
Convulvulus arvensis	Hidroponía	2800	(Gardea-Torresdey *y col*., 2004)
Gynura pseudochina	Hidroponía	823	(Mongkhonsin *y col*., 2011)
Helianthus annuus	Arena	1356	(Davies *y col*., 2001)
Nopalea cochenillifera	*In vitro*	705	(Adki *y co*l., 2013)
Prosopis julifflora	arena y lodo	372	(Shukla *y col*., 2011)
Prosopis laevigata	Hidroponía	8,09	(Buendia-Gonzalez *y col*., 2010)
Pteris vittata	Suelo	1145	(Su *y col.*, 2005)
Salix matsudana	Hidroponía	29	(Yu *y col*., 2007)
Salix babylonica	Hidroponía	15	(Yu *y col*., 2007)
Salsola kali	Hidroponía	600	(Gardea-Torresdey *y col*., 2005)
Spartina argentinensis	Hidroponía	15,1	(Redondo-Gomez *y co*l., 2011)
Genipa americana	Hidroponía	45	(Barbosa *y col.*, 2007)
Glycine max	Hidroponía	1300	(Mei *y col*., 2002)

* ($mg \cdot kg^{-1}$PS)

La mayoría de los trabajos disponibles acerca de las interacciones Cr-planta, se centran en el estudio de especies con interés agronómico y su objetivo principal es evaluar cómo afecta el cromo al crecimiento especies y si los niveles de metal acumulados pueden constituir un riesgo para su consumo (Zeng *y col.*, 2011; Chand *y col.*, 2009; Dube *y col.*, 2009; di Toppi *y col.*, 2002; Zayed *y col.*, 1998). Como se muestra en la tabla 4, en comparación con otros metales, muy pocas especies terrestres

han sido evaluadas para su uso en fitorremediación de Cr. Además, la mayoría de los trabajos que han realizado en hidroponía y son poco extrapolables a las condiciones reales en un suelo contaminado. La concentración de Cr en los tejidos vegetales puede variar entre 0,006 y 18 $mg \cdot kg^{-1}$P.S., por lo general se encuentran entre 1-5 $mg\ kg^{-1}$P.S. (Zayed y Terry, 2003). Incluso las plantas recolectadas en suelos de serpentínicos no suelen mostrar contenidos de Cr superiores a 45 $mg \cdot kg^{-1}$P.S. y casi nunca superiores a 100 $mg \cdot kg^{-1}$P.S. (Barceló y Poschenrieder, 1997). Por ello para la implantación efectiva de procesos de fitorremediación o fitomanejo en emplazamientos contaminados con Cr se hace necesaria la búsqueda de nuevas especies capaces de tolerar y acumular Cr y de adaptarse y desarrollarse en las condiciones adversas que caracterizan a los suelos degradados.

1.3. SILENE VULGARIS

*Silene vulgaris (*Moench) Garcke, comúnmente llamada colleja, es una especie herbácea perenne perteneciente a la familia Caryophylaceae. Presenta una amplia distribución geográfica (figura 4) con poblaciones en Europa, Asia, Norte América, Norte de África y Australia (Global Biodiversity Information Facility, 2013). *S. vulgaris* es una metalífera facultativa ya que puede vivir tanto en suelos con altos contenidos en metales pesados como en suelos con niveles normales. Puede reproducirse sexualmente a partir de semillas o asexualmente por rizomas, lo que le permite rebrotar después de ser cosechada. *S. vulgaris* presenta una elevada variabilidad genética (Runyeon y Prentice 1997; Prentice y Giles, 1993) pero la resistencia a metales parece ser una característica propia de la especie (Jack *y col.,* 2005). Mediante cultivos hidropónicos se ha demostrado su tolerancia a varios metales pesados como Cu, As, Co, Ni, Cd, Zn y Hg (Sobrino-Plata *y col.,* 2013; Chardonnens *y col.,* 1999; Deknecht *y col.,* 1994; Harmens *y col.,* 1994; Schat y Tenbookum, 1992; Paliouris and Hutchinson 1991). La tolerancia de *S. vulgaris* a Zn se ha relacionado con la presencia de ácidos orgánicos en la planta (Harmens *y col.,* 1994) y con la estimulación del transporte del metal a través del tonoplasto (Verkleij *y col.,* 1998). Sin embargo la producción de fitoquelatinas se ha propuesto como mecanismo de resistencia a Cd aunque los resultados no fueron concluyentes (Deknecht *y col.,* 1994; Deknecht *y col.,*1992). En el

caso de plantas expuestas a Cu, la producción de fenoles parece estar relacionada con mecanismos de tolerancia a este metal (Kovacik *y col.*,2010). Estudios recientes sobre los cambios inducidos en los niveles de estrés oxidativo, procesos fotoquímicos y contenido en tioles muestran que *S. vulgaris* presenta distintas respuestas y puede contar distintos mecanismos de tolerancia en función del metal al que es expuesto (Sobrino-Plata *y col.*, 2013; Nadgorska-Socha *y col.*,2011; Kovacik *y col.*, 2010,) por lo que es necesario evaluar la respuesta de esta especie para cada metal por separado. Estudios en suelo muestran que *S. vulgaris* es capaz de crecer en suelos contaminados con Cd, Zn, Cu, Pb, As y Hg (Perez-Sanz *y col.*, 2012; Tang *y col.*, 2012; Carpena-Ruiz *y col.*, 2008; Ciarkowska y Hanus-Fajerska, 2008; Song *y col.*, 2004; Ernst y Nelissen, 2000) y produce un efecto positivo sobre la flora bacteriana de suelos contaminados contribuyendo a su recuperación (Martinez-Inigo *y col.*, 2009). Todo ello hace que *S. vulgaris* sea considerada como una especie interesante para la revegetación de suelos contaminados. No existe información disponible acerca de su capacidad para tolerar y acumular Cr.

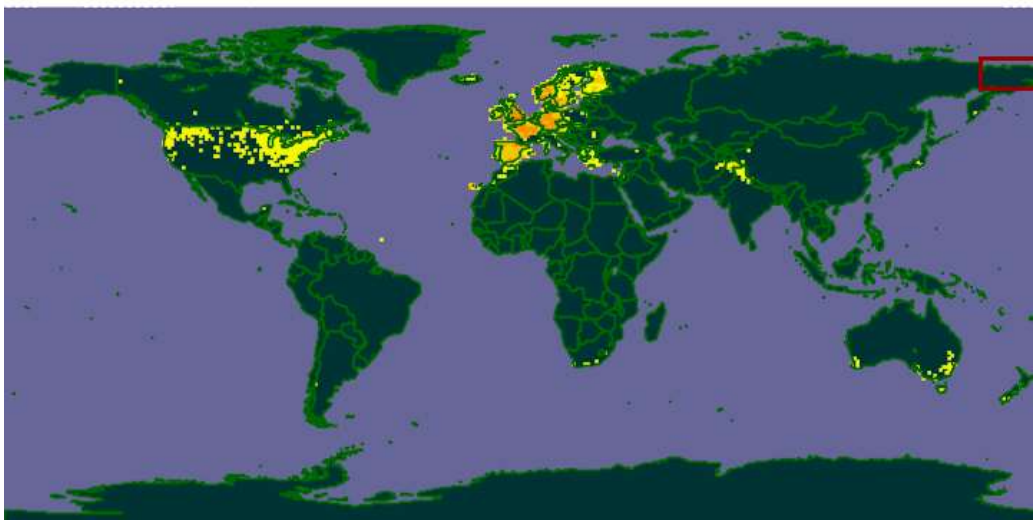


Figura 4: Distribución geográfica de *Silene vulgaris* (Global Biodiversity Information Facility, 2013).

1.4. REFERENCIAS

Adki, V.S., Jadhav, J.P., Bapat, V.A., 2013. Nopalea cochenillifera, a potential chromium (VI) hyperaccumulator plant. Environmental Science and Pollution Research 20, 1173-1180

Agency for Toxic Substances and Disease Registry (ATSDR), 2012. Toxicological profile for Chromium. http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=62&tid=17 2013.

Arauzo, M., Rivera, M., Valladolid, M., Noreña, C., Cedenilla, O., 2003. Contaminación por cromo en el agua intersticial, en el agua del cauce y en los sedimentos del río Jarama.

Arauzo, M., Valladolid, M., Noreña, C., Cedenilla, O., 2002. Limnología y contaminación: el lagunaje profundo como tratamiento ecológico de las aguas residuales urbanas y mixtas.

Arduini, I., Masoni, A., Ercoli, L., 2006. Effects of high chromium applications on miscanthus during the period of maximum growth. Environ. Exp. Bot. 58, 234-243

Ayari, F., Chairi, R., Kossai, R., 2008. Sequential extraction of heavy metals during composting of urban waste. Chinese Journal of Geochemistry 27, 121-125.

Barbosa, R.M.T., De Almeida, A.F., Mielke, M.S., Loguercio, L.L., Mangabeira, P.A., Gomes, F.P., 2007. A physiological analysis of *Genipa americana* L.: A potential phytoremediator tree for chromium polluted watersheds. Environ. Exp. Bot. 61, 264-271

Barcelo, J., Poschenrieder, C., 1997. Chromium in plants, in: Caneli, S., Tittarelli, F., Sequi, P. (Eds.). FrancoAngeli, Milan (Italy), pp. 102-129.

Barnhart, J., 1997. Occurrences, uses, and properties of chromium. Regulatory Toxicology and Pharmacology 26, S3-S7.

Bartlett, R.J., 1991. Chromium Cycling in Soils and Water - Links, Gaps, and Methods. Environ. Health Perspect. 92, 17-24.

Bielicka, A., Bojanowska, I., Wisniewski, A., 2005. Two faces of chromium - Pollutant and bioelement. Polish Journal of Environmental Studies 14, 5-10.

Blowes, D., 2002. Tracking hexavalent Cr in groundwater. Science(Washington) 295, 2024-2025.

Bonet, A., Poschereider, C., Barcelo, J., 1991. Chromium III ‑ iron interaction in Fe ‑ deficient and Fe ‑ sufficient bean plants. I. Growth and nutrient content. J. Plant Nutr. 14, 403; 403-414; 414.

Brunetti, G., Farrag, K., Soler-Rovira, P., Nigro, F., Senesi, N., 2011. Greenhouse and field studies on Cr, Cu, Pb and Zn phytoextraction by *Brassica napus* from contaminated soils in the Apulia region, Southern Italy. Geoderma 160, 517-523

Buendia-Gonzalez, L., Orozco-Villafuerte, J., Cruz-Sosa, F., Barrera-Diaz, C.E., Vernon-Carter, E.J., 2010. *Prosopis laevigata* a potential chromium (VI) and cadmium (II) hyperaccumulator desert plant. Bioresour. Technol. 101, 5862-5867

Caliman, F.A., Robu, B.M., Smaranda, C., Pavel, V.L., Gavrilescu, M., 2011. Soil and groundwater cleanup: benefits and limits of emerging technologies. Clean Technologies and Environmental Policy 13, 241-268

Carpena-Ruiz, R.O., Pérez Sanz, A., Vázquez, S., Moreno, E., Peñalosa, J., Hernández, L.E., Alarcón, R., García, P., Lobo, M.C., 2008. Respuesta de *Silene vulgaris* en Suelos Contaminados con Arsénico y Metales Pesados. Capacidad Fitorrecuperadora., in: Romero, R. (Ed.). Grupo Nutrición Mineral de Plantas-SEFV, Granada, pp. 485-501.

Cary, E.E., Allaway, W.H., Olson, O.E., 1977. Control of Chromium Concentrations in Food Plants .1. Absorption and Translocation of Chromium by Plants. J. Agric. Food Chem. 25, 300-304.

Cervantes, C., Campos-Garcia, J., Devars, S., Gutierrez-Corona, F., Loza-Tavera, H., Torres-Guzman, J.C., Moreno-Sanchez, R., 2001. Interactions of chromium with microorganisms and plants. FEMS Microbiol. Rev. 25, 335-347.

Chand, R., Watari, T., Inoue, K., Torikai, T., Yada, M., 2009. Evaluation of wheat straw and barley straw carbon for Cr(VI) adsorption. Separation and Purification Technology 65, 331-336

Chardonnens, A.N., Koevoets, P.L., van Zanten, A., Schat, H., Verkleij, J.A., 1999. Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. Plant Physiol. 120, 779-786.

Chatterjee, J., Chatterjee, C., 2000. Phytotoxicity of cobalt, chromium and copper in cauliflower. Environmental Pollution 109, 69-74.

Ciarkowska, K., Hanus-Fajerska, E., 2008. Remediation of Soil-Free Grounds Contaminated by Zinc, Lead and Cadmium with the Use of Metallophytes. Polish Journal of Environmental Studies 17, 707-712.

Comunidad de Madrid (Spain). Orden 2270/2006 de 11 de agosto por el que se establecen los. niveles genéricos de referencia de metales pesados y otros elementos traza en suelos contaminados de la Comunidad de Madrid, 28 agosto de 2006, num 204, pp. 29-30.

Conesa, H.M., Evangelou, M.W.H., Robinson, B.H., Schulin, R., 2012. A Critical View of Current State of Phytotechnologies to Remediate Soils: Still a Promising Tool? Scientific World Journal , 173829.

Cunningham, S.D., Berti, W.R., Huang, J.W., 1995. Phytoremediation of contaminated soils. Trends in Biotechnology 13, 393-397 doi: DOI: 10.1016/S0167-7799(00)88987-8.

Davies, F.T., Puryear, J.D., Newton, R.J., Egilla, J.N., Grossi, J.A.S., 2001. Mycorrhizal fungi enhance accumulation and tolerance of chromium in sunflower (*Helianthus annuus*). J. Plant Physiol. 158, 777-786.

Deflora, S., Bagnasco, M., Serra, D., Zanachi, P., 1990. Genotoxicity of Chromium Compounds - a Review. Mutat. Res. 238, 99-172 .

Deknecht, J.A., Vandillen, M., Koevoets, P.L.M., Schat, H., Verkleij, J.A.C., Ernst, W.H.O., 1994. Phytochelatins in Cadmium-Sensitive and Cadmium-Tolerant *Silene Vulgaris* - Chain-Length Distribution and Sulfide Incorporation. Plant Physiol. 104, 255-261.

Deknecht, J.A., Koevoets, P.L.M., Verkleij, J.A.C., Ernst, W.H.O., 1992. Evidence Against a Role for Phytochelatins in Naturally Selected Increased Cadmium Tolerance in *Silene-Vulgaris* (Moench) Garcke. New Phytol. 122, 681-688.

Dhal, B., Thatoi, H.N., Das, N.N., Pandey, B.D., 2013. Chemical and microbial remediation of hexavalent chromium from contaminated soil and mining/metallurgical solid waste: A review. J. Hazard. Mater. 250-251, 272-291

Dhal, B., Das, N.N., Pandey, B.D., Thatoi, H.N., 2011. Environmental Quality of the Boula-Nuasahi Chromite Mine Area in India. Mine Water and the Environment 30, 191-196

di Toppi, L.S., Fossati, F., Musetti, R., Mikerezi, I., Favali, M.A., 2002. Effects of hexavalent chromium on maize, tomato, and cauliflower plants. J. Plant Nutr. 25, 701-717.

Diwan, H., Khan, I., Ahmad, A., Iqbal, M., 2010. Induction of phytochelatins and antioxidant defence system in *Brassica juncea* and *Vigna radiata* in response to chromium treatments. Plant Growth Regulation 61, 97-107.

Du, J., Lu, J., Wu, Q., Jing, C., 2012. Reduction and immobilization of chromate in chromite ore processing residue with nanoscale zero-valent iron. J. Hazard. Mater. 215, 152-158.

Dube, B.K., Sinha, P., Chatterjee, C., 2009. Assessment of Disturbances in Growth and Physiology of Carrot Caused by Chromium Stress. J. Plant Nutr. 32, 479-488

Dube, B., Tewari, K., Chatterjee, J., Chatterjee, C., 2003. Excess chromium alters uptake and translocation of certain nutrients in citrullus. Chemosphere 53, 1147-1153

Dubey, S., Misra, P., Dwivedi, S., Chatterjee, S., Bag, S., Mantri, S., Asif, M., Rai, A., Kumar, S., Shri, M., 2010. Transcriptomic and metabolomic shifts in rice roots in response to Cr (VI) stress. BMC Genomics 11, 648.

EPA (United States Environmental Protection Agency), 2013. National Priorities List (NPL). http://www.epa.gov/superfund/sites/npl/ 2013.

Ernst, W.H.O., Nelissen, H.J.M., 2000. Life-cycle phases of a zinc and cadmium resistanta ecotype of *Silene vulgaris* in risk assessment of polymetallic soils. Environmental Pollution. 107, 329-338.

European Environment Agency, 2010. European Union emission inventory report 1990–2010 under the UNECE Convention on Long-range Transboundary Air Pollution (LRTAP). http://www.eea.europa.eu/publications/eu-emission-inventory-report-1990-2010/at_download/file 2013.

Evans, J.E., Gottgens, J.F., 2007. Contaminant stratigraphy of the Ballville reservoir, Sandusky River, NW Ohio: Implications for dam removal. J. Great Lakes Res. 33, 182-193.

Fendorf, S.E., 1995. Surface-Reactions of Chromium in Soils and Waters. Geoderma 67, 55-71

Flury, B., Frommer, J., Eggenberger, U., Mäder, U., Nachtegaal, M., Kretzschmar, R., 2009. Assessment of long-term performance and chromate reduction mechanisms in a field scale permeable reactive barrier. Environ. Sci. Technol. 43, 6786-6792.

Fruchter, J.,Williams, M., Vermeul, V., Szecsody, J., Martin, W. 2000a. In-situ redox manipulation for treatment of chromate in groundwater at the Hanford 100D area: Partmership for technology deployment.

Fruchter, J.b., Cole, C.R., Williams, M.D., Vermeul, V.R., Amonette, J.E., Szecsody, J.E., Istok, J.D., Humphrey, M., 2000b. Creation of a subsurface permeable treatment zone for aqueous chromate contamination using in situ redox manipulation. Ground Water Monitoring & Remediation 20, 66-77.

García-Pérez, J., Boldo, E., Ramis, R., Pollán, M., Pérez-Gómez, B., Aragonés, N., López-Abente, G., 2007. Description of industrial pollution in Spain. BMC Public Health 7, 40.

Gardea-Torresdey, J.L., de la Rosa, G., Peralta-Videa, J.R., Montes, M., Cruz-Jimenez, G., Cano-Aguilera, I., 2005. Differential uptake and transport of trivalent and flexavalent chromium by tumbleweed (*Salsola kali)*. Arch. Environ. Contam. Toxicol. 48, 225-232 doi: 10.1007/s00244-003-0162-x.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., Montes, M., de la Rosa, G., Corral-Diaz, B., 2004. Bioaccumulation of cadmium, chromium and copper by *Convolvulus arvensis* L.: impact on plant growth and uptake of nutritional elements. Bioresour. Technol. 92, 229-235 doi: 10.1016/j.biortech.2003.10.002.

Global Biodiversity Information Facility, 2013. Distribution map of *Silene vulgaris*. http://data.gbif.org/terms.htm?forwardUrl=http%3A%2F%2Fdata.gbif.org%2Fspecies%2F 2013.

Gomez, V., Callao, M.P., 2006. Chromium determination and speciation since 2000. Trac-Trends in Analytical Chemistry 25, 1006-1015

Gopal, R., Rizvi, A.H., Nautiyal, N., 2009. Chromium Alters Iron Nutrition and Water Relations of Spinach. J. Plant Nutr. 32, 1551-1559

Han, F., Banin, A., Su, Y., Monts, D., Plodinec, M., Kingery, W., Triplett, G., 2002. Industrial age anthropogenic inputs of heavy metals into the pedosphere. Naturwissenschaften 89, 497-504

Harmens, H., Norris, D.A., Koerber, G.R., Buse, A., Steinnes, E., Rühling, Å., 2007. Temporal trends in the concentration of arsenic, chromium, copper, iron, nickel, vanadium and zinc in mosses across Europe between 1990 and 2000. Atmos. Environ. 41, 6673-6687.

Harmens, H., Koevoets, P., Verkleij, J., Ernst, W., 1994. The Role of Low-Molecular-Weight Organic-Acids in the Mechanism of Increased Zinc Tolerance in *Silene Vulgaris* (Moench) Garcke. New Phytol. 126, 615-621

Hayat, S., Khalique, G., Irfan, M., Wani, A.S., Tripathi, B.N., Ahmad, A., 2012. Physiological changes induced by chromium stress in plants: an overview. Protoplasma 249, 599-611

He, Z.L.L., Yang, X.E., Stoffella, P.J., 2005. Trace elements in agroecosystems and impacts on the environment. Journal of Trace Elements in Medicine and Biology 19, 125-140 d

Howe, J., Loeppert, R., Derose, V., Hunter, D., Bertsch, P., 2003. Localization and speciation of chromium in subterranean clover using XRF, XANES, and EPR spectroscopy. Environ. Sci. Technol. 37, 4091-4097

IARC (International Agency forResearch on Cancer, 2006. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. http://monographs.iarc.fr/ 2013.

Jack, E.M., Anatasova, S., Verkleij, J.A.C., 2005. Callus induction and plant regeneration in the metallophyte *Silene vulgaris* (Caryophyllaceae). Plant Cell Tissue and Organ Culture 80, 25-31.

Jennings, A.A., 2013. Analysis of worldwide regulatory guidance values for the most commonly regulated elemental surface soil contamination. J. Environ. Manage. 118, 72-95

Kamaludeen, S.P., Megharaj, M., Juhasz, A.L., Sethunathan, N., Naidu, R., 2003. Chromium-Microorganism Interactions in Soils: Remediation Implications. Reviews of Environmental Contamination and Toxicology 178 178, 93-164.

Kotas, J., Stasicka, Z., 2000. Chromium occurrence in the environment and methods of its speciation. Environmental Pollution 107, 263-283.

Kovacik, J., Klejdus, B., Hedbavny, J., Backor, M., 2010. Tolerance of *Silene vulgaris* to Copper: Population-Related Comparison of Selected Physiological Parameters. Environ. Toxicol. 25, 581-592

Lado, L.R., Hengl, T., Reuter, H.I., 2008. Heavy metals in European soils: A geostatistical analysis of the FOREGS Geochemical database. Geoderma 148, 189-199.

Lee, J., Pandey, B.D., 2012a. Bio-processing of solid wastes and secondary resources for metal extraction–a review. Waste Manage. 32, 3-18.

iu, D., Zou, J., Wang, M., Jiang, W., 2008. Hexavalent chromium uptake and its effects on mineral uptake, antioxidant defence system and photosynthesis in *Amaranthus viridis* L. Bioresour. Technol. 99, 2628-2636.

Lopez, P., Dolz, J., Arbat, M., Armengol, J., 2012. Physical and chemical characterisation of superficial sediment of the Ribarroja Reservoir (River Ebro, NE Spain). Limnetica 31, 327-340.

López-Arias, M., Grau, J.M., 2005. Metales Pesados, Materia Orgánica y Otros Parámetros De La Capa Superficail De Los Suelos Agrícolas y De Pastos De La España Peninsular I:

Resultados Globales. Instituto Ncional de Investigación y Tecnología Agraria y Alimentaria., Madrid.

Lopez-Luna, J., Gonzalez-Chavez, M.C., Esparza-Garcia, F.J., Rodriguez-Vazquez, R., 2009. Toxicity assessment of soil amended with tannery sludge, trivalent chromium and hexavalent chromium, using wheat, oat and sorghum plants. J. Hazard. Mater. 163, 829-834

Losi, M., Amrhein, C., Frankenberger, W., 1994. Bioremediation of chromate-contaminated groundwater by reduction and precipitation in surface soils. J. Environ. Qual. 23, 1141-1150.

Lytle, C.M., Lytle, F.W., Yang, N., Qian, J.H., Hansen, D., Zayed, A., Terry, N., 1998. Reduction of Cr(VI) to Cr(III) by wetland plants: Potential for in situ heavy metal detoxification. Environ. Sci. Technol. 32, 3087-3093.

Mahvi, A., 2008. Application of agricultural fibers in pollution removal from aqueous solution. Int.J.Environ.Sci.Tech 5, 275-285.

Mallick, S., Sinam, G., Kumar Mishra, R., Sinha, S., 2010. Interactive effects of Cr and Fe treatments on plants growth, nutrition and oxidative status in *Zea mays* L. Ecotoxicol. Environ. Saf. 73, 987-995.

Martinez-Inigo, M.J., Perez-Sanz, A., Ortiz, I., Alonso, J., Alarcon, R., Garcia, P., Lobo, M.C., 2009. Bulk soil and rhizosphere bacterial community PCR-DGGE profiles and beta-galactosidase activity as indicators of biological quality in soils contaminated by heavy metals and cultivated with *Silene vulgaris* (Moench) Garcke. Chemosphere 75, 1376-1381

Mei, B.J., Puryear, J.D., Newton, R.J., 2002. Assessment of Cr tolerance and accumulation in selected plant species. Plant Soil 247, 223-231.

Mohanty, M., Patra, H.K., 2011. Attenuation of chromium toxicity by bioremediation technology, in: Anonymous . Springer, pp. 1-34.

Mongkhonsin, B., Nakbanpote, W., Nakai, I., Hokura, A., Jearanaikoon, N., 2011. Distribution and speciation of chromium accumulated in *Gynura pseudochina* (L.) DC. Environ. Exp. Bot. 74, 56-64

Motzer, W.E., 2004. Chemistry, geochemistry, and geology of chromium and chromium compounds. Chromium (VI) Handbook; Guertin, J.; Jacobs, JA; Avakian, CP, Eds , 23-91.

Nadal, M., Schuhmacher, M., Domingo, J., 2004. Metal pollution of soils and vegetation in an area with petrochemical industry. Sci. Total Environ. 321, 59-69.

Nadgorska-Socha, A., Kandziora-Ciupa, M., Ciepal, R., Walasek, K., 2011. Effects of Zn, Cd, Pb on Physiological Response of Silene vulgaris Plants from Selected Populations. Polish journal of environmental studies 20, 599- 604.

Nichols, P.B., Couch, J.D., Al-Hamdani, S.H., 2000. Selected physiological responses of *Salvinia minima* to different chromium concentrations. Aquat. Bot. 68, 313-319.

Ozawa, T., Hanaki, A., 1990. Spin-Trapping Studies on the Reactions of Cr(III) with Hydrogen-Peroxide in the Presence of Biological Reductants - is Cr(III) Nontoxic. Biochem. Int. 22, 343-352.

Paliouris, G., Hutchinson, T.C., 1991. Arsenic, cobalt and nickel tolerances in two populations of *Silene vulgaris* (Moench) Garcke fron Ontario, Canada. New Phytol 117, 449-459.

Panda, S., Choudhury, S., 2005. Chromium stress in plants. Brazilian Journal of Plant Physiology 17, 95-102.

Pandey, V., Dixit, V., Shyam, R., 2005. Antioxidative responses in relation to growth of mustard (*Brassica juncea*) and (*Pusa Jaikisan)* plants exposed to hexavalent chromium. Chemosphere 61, 40-47.

Pandey, V., Dixit, V., Shyam, R., 2009. Chromium (VI) induced changes in growth and root plasma membrane redox activities in pea plants. Protoplasma 235, 49-55.

Perez-Sanz, A., Millan, R., Jose Sierra, M., Alarcon, R., Garcia, P., Gil-Diaz, M., Vazquez, S., Carmen Lobo, M., 2012. Mercury uptake by *Silene vulgaris* grown on contaminated spiked soils. J. Environ. Manage. 95, 233-235.

Pilon-Smits, E., 2005. Phytoremediation. Annual Review of Plant Biology 56, 15-39

Prentice, H.C., Giles, B.E., 1993. Genetic Determination of Isozyme Variation in the Bladder Campions, Silene-Uniflora and *S. vulgaris*. Hereditas 118, 217-227.

Puzon, G.J., Tokala, R.K., Zhang, H., Yonge, D., Peyton, B.M., Xun, L., 2008. Mobility and recalcitrance of organo-chromium(III) complexes. Chemosphere 70, 2054-2059.

Rai, D., Sass, B.M., Moore, D.A., 1987. Chromium (III) hydrolysis constants and solubility of chromium (III) hydroxide. Inorg. Chem. 26, 345-349.

Redondo-Gomez, S., Mateos-Naranjo, E., Vecino-Bueno, I., Feldman, S.R., 2011. Accumulation and tolerance characteristics of chromium in a cordgrass Cr-hyperaccumulator, *Spartina argentinensis*. J. Hazard. Mater.

Robinson, B.H., Banuelos, G., Conesa, H.M., Evangelou, M.W.H., Schulin, R., 2009. The Phytomanagement of Trace Elements in Soil. Crit. Rev. Plant Sci. 28, 240-266

Runyeon, H., Prentice, H.C., 1997. Genetic differentiation in the bladder campions, *Silene vulgaris* and *S. uniflora* (Caryophyllaceae), in Sweden. Biol. J. Linn. Soc. 61, 559-584.

Samantary, S., 2002. Biochemical responses of Cr-tolerant and Cr-sensitive mung bean cultivars grown on varying levels of chromium. Chemosphere 47, 1065-1072.

Schat, H., Tenbookum, W.M., 1992. Genetic-Control of Copper Tolerance in *Silene. vulgaris*. Heredity 68, 219-229.

Scoccianti, V., Iacobucci, M., Paoletti, M.F., Fraternale, A., Speranza, A., 2008. Species-dependent chromium accumulation, lipid peroxidation, and glutathione levels in germinating kiwifruit pollen under Cr (III) and Cr (VI) stress. Chemosphere 73, 1042-1048.

Shanker, A.K., 2008. Mode of Action and Toxicity of Trace Elements, in: M. N. V. Prasad (Ed.). Wiley, Hoboken (New Jersey), pp. 525-555.

Shanker, A.K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S., 2005. Chromium toxicity in plants. Environ. Int. 31, 739-753.

Shanker, A.K., Pathmanabhan, G., 2004. Speciation dependant antioxidative response in roots and leaves of sorghum (*Sorghum bicolor* (L.) Moench cv CO 27) under Cr(III) and Cr(VI) stress. Plant Soil 265, 141-151.

Shanker, A.K., Djanaguiraman, M., Venkateswarlu, B., 2009. Chromium interactions in plants: current status and future strategies. Metallomics 1, 375-383

Shukla, O.P., Juwarkar, A.A., Singh, S.K., Khan, S., Rai, U.N., 2011. Growth responses and metal accumulation capabilities of woody plants during the phytoremediation of tannery sludge. Waste Manage. 31, 115-123.

Singh, H.P., 2013. Chromium toxicity and tolerance in plants. Environmental chemistry letters .

Skeffington, R.A., Shewry, P.R., Peterson, P.J., 1976. Chromium Uptake and Transport in Barley Seedlings (Hordeum-Vulgare-L). Planta 132, 209-214.

Sobrino-Plata, J.,Herreo, J.,Carrasco-Gil, S., Perez-Sanz, A., Lobo, C., Escobar, C., Millán, R., Hernández, L. 2013. Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically. RSC Advances 3, 4736.

Song, J., Zhao, F.J., Luo, Y.M., McGrath, S.P., Zhang, H., 2004. Copper uptake by *Elsholtzia splendens* and *Silene vulgaris* and assessment of copper phytoavailability in contaminated soils. Environmental Pollution 128, 307-315.

Srivastava, S., Prakash, S., Srivastava, M., 1999. Studies on mobilization of chromium with reference to its plant availability - Role of organic acids. Biometals 12, 201-207 .

Stewart, M.A., Jardine, P.M., Brandt, C.C., Barnett, M.O., Fendorf, S.E., McKay, L.D., Mehlhorn, T.L., Paul, K., 2003. Effects of contaminant concentration, aging, and soil properties on the bioaccessibility of Cr(III) and Cr(VI) in soil. Soil Sed. Contam. 12, 1-21.

Stohs, S., BagchiA, D., 1995. Oxidative Mechanisms in the Toxicity of Metal-Ions. Free Radical Biology and Medicine 18, 321-336 .

Su, Y., Han, F., Sridhar, B., Monts, D., 2005. Phytotoxicity and phytoaccumulation of trivalent and hexavalent chromium in brake fern. Environmental Toxicology and Chemistry 24, 2019-2026.

Subrahmanyam, D., 2008. Effects of chromium toxicity on leaf photosynthetic characteristics and oxidative changes in wheat (*Triticum aestivum* L.). Photosynthetica 46, 339-345.

Sundaramoorthy, P., Chidambaram, A., Ganesh, K.S., Unnikannan, P., Baskaran, L., 2010. Chromium stress in paddy: (i) Nutrient status of paddy under chromium stress; (ii) Phytoremediation of chromium by aquatic and terrestrial weeds. Comptes Rendus Biologies 333, 597-607 doi: 10.1016/j.crvi.2010.03.002.

Tang, Y.T., Cloquet, C., Sterckeman, T., Echevarria, G., Carignan, J., Qiu, R.L., Morel, J.L., 2012. Fractionation of stable zinc isotopes in the field-grown zinc hyperaccumulator Noccaea caerulescens and the zinc-tolerant plant *Silene vulgaris*. Environ. Sci. Technol. 46, 9972-9979 .

Unceta, N., Seby, F., Malherbe, J., Donard, O.F.X., 2010. Chromium speciation in solid matrices and regulation: a review. Analytical and Bioanalytical Chemistry 397, 1097-1111.

Unión Eropea. Directiva 2002/95/Parlamento Europeo y Consejo Europeo de 13 Enero 2003 sobre las restricciones a la utilización de determinadas sustancias en aparatos eléctricos y electrónicos.Diario Oficial de la Unión Europea, 13 de enero de 2003, num 37, pp19-23

Unión Europea. Directiva 2008/105/Parlamento Europeo de 16 de diciembre relativa a las normas de calidad ambiental en el ámbito de la política de aguas. Diario Oficial de Unión Europea, 24 de enero de 2008, num 384, pp 84-97

USEPA, 1998. Toxicological Review of Hexavalent Chromium. U.S. Goverment Printing Office, Washington.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environmental Science and Pollution Research 16, 765-794.

Vassilev, A., Schwitzguñebel, J., Thewys, T., Van der Lelie, D., Vangronsveld, J., 2004. The Use of Plants for Remediation of Metal Contaminated Soils. The Scientific Word Journal 4, 9-34.

Vazquez, D., Poschenrieder, C., Barcelo, J., 1987. Chromium-Vi Induced Structural and Ultrastructural-Changes in Bush Bean-Plants (*Phaseolus.vulgaris* L). Annals of Botany 59, 427-438.

Verkleij, J., Koevoets, P., Blake-Kalff, M., Chardonnens, A., 1998. Evidence for an important role of the tonoplast in the mechanism of naturally selected zinc tolerance in *Silene vulgaris*. J. Plant Physiol. 153, 188-191.

Vilavert, L., Nadal, M., Schuhmacher, M., Domingo, J.L., 2012. Concentrations of Metals in Soils in the Neighborhood of a Hazardous Waste Incinerator: Assessment of the Temporal Trends. Biol. Trace Elem. Res. 149, 435-442.

Viti, C., Giovannetti, L., 2007. Bioremediation of soils polluted with hexavalent chromium using bacteria: a challenge, in: Anonymous . Springer, pp. 57-76.

Wanner, C., Eggenberger, U., Mäder, U., 2011. Reactive transport modelling of Cr (VI) treatment by cast iron under fast flow conditions. Appl. Geochem. 26, 1513-1523.

Yu, X., Gu, J., Huang, S., 2007. Hexavalent chromium induced stress and metabolic responses in hybrid willows. Ecotoxicology 16, 299-309.

Zayed, A., Terry, N., 2003. Chromium in the environment: factors affecting biological remediation. Plant Soil 249, 139-156.

Zayed, A., Lytle, C.M., Qian, J.H., Terry, N., 1998. Chromium accumulation, translocation and chemical speciation in vegetable crops. Planta 206, 293-299.

Zeng, F., Zhou, W., Qiu, B., Ali, S., Wu, F., Zhang, G., 2011. Subcellular distribution and chemical forms of chromium in rice plants suffering from different levels of chromium toxicity. Journal of Plant Nutrition and Soil Science 174, 249-256.

Zeng, F., Ali, S., Qiu, B., Wu, F., Zhang, G., 2010. Effects of chromium stress on the subcellular distribution and chemical form of Ca, Mg, Fe, and Zn in two rice genotypes RID C-3348-2011. Journal of Plant Nutrition and Soil Science 173, 135-148.

Zou, J.H., Wang, M., Jiang, W.S., Liu, D.H., 2006a. Effects of hexavalent chromium (VI) on root growth and cell division in root tip cells of *Amaranthus viridis* L. Pak. J. Bot. 38, 673-681.

Zou, J., Wang, M., Jiang, W., Liu, D., 2006b. Chromium accumulation and its effects on other mineral elements in *Amaranthus viridis* L. Acta Biologica Cracoviensia Series Botanica 48, 7-12.

Justificación y objetivo

El Cromo es un metal pesado que se usa en multitud de procesos industriales como metalurgia, producción de pinturas y pigmentos, conservación de la madera, galvanoplastia y producción de papel. Como consecuencia, en los últimos años, su vertido al medio ambiente y, por lo tanto a los suelos, se ha convertido en un problema de interés prioritario. Las dos especies químicas más abundantes de cromo son el Cr(III) y el Cr(VI). A pesar de encontrarse sometidas a continuos procesos de interconversión, existen diferencias en su biodisponibilidad y toxicidad, siendo el Cr(VI) más soluble y 100 veces más toxico tanto para las plantas como para los animales y el ser humano. La fitorremediación se define como el uso de las plantas y sus microorganismos asociados para eliminar, contener, inactivar o degradar contaminantes ambientales o para revitalizar zonas contaminadas (Vangronsveld *y col.*, 2009). Se presenta como una alternativa a las técnicas físico-químicas tradicionales, aplicable *in situ*, más respetuosa con el medio ambiente y de menor coste. Al contrario que otros metales pesados, el estudio de las interacciones Cr-planta para su aplicación en fitorremediación ha recibido menor atención debido por una parte a que el Cr no cumple ninguna función biológica en las plantas y por otro a la dificultad de determinar la especie química del Cr presente en los tejidos.

Para una planificación efectiva de la estrategia de descontaminación del suelo, cada contaminante debe ser evaluado por separado, como consecuencia de la propia química de la planta y el suelo (Chaney *y col*., 1997). Además, numerosos autores destacan las ventajas de emplear especies o subespecies locales en los procesos de remediación de suelos contaminados que impidan la invasión del medio por especies alóctonas, que contribuyan a preservar el material genético de la zona y activen el proceso de sucesión natural (Ciarkowska and Hanus-Fajerska, 2008; Bradshaw, 1997;

Prach and Pysek, 2001). *Silene vulgaris* es una metalofita facultativa de amplia distribución cuya tolerancia y capacidad de absorción ha sido probada para varios metales. Por ello se considera una especie adecuada para la revegetación y recuperación de emplazamientos contaminados. No existe ningún trabajo que estudie la respuesta de esta especie frente al Cr.

El objetivo general de esta tesis doctoral es evaluar la respuesta de *Silene vulgaris* frente a la exposición a cromo para incorporar su uso en la remediación de emplazamientos contaminados con este metal. Para la consecución de este objetivo general se plantean los siguientes objetivos específicos:

1. Determinar la absorción de Cr y su distribución en la planta en función de la especiación química y disponibilidad del metal.
2. Determinar el rango de tolerancia de *S. vulgaris* a Cr.
3. Evaluar los efectos tóxicos del Cr en la planta a distintos niveles:
    a. Sobre los parámetros de desarrollo de la planta: biomasa, estado de clorofilas y balance nutritivo, que puedan comprometer la viabilidad de su uso en fitorremediación? o recuperación de suelos contaminados?
    b. Sobre los niveles de estrés oxidativo, especialmente en la exposición a Cr(VI), ya que al tratarse de una especie de elevado potencial redox, sus reacciones en la célula dan lugar a la liberación de radicales libres que dañan las membranas celulares.
    c. Sobre los cambios bioquímicos y estructurales en plantas expuestas a Cr(III) y Cr(VI).
4. Estudiar los mecanismos de entrada, transporte y almacenamiento de Cr en la planta mediante el estudio de la localización de Cr en la raíz, el tallo y las hojas. Así como, dilucidar los posibles mecanismos de la planta frente a la detoxificación de Cr(VI), especialmente si *S. vulgaris* es capaz de llevar a cabo la reducción de Cr(VI) a la especie menos tóxica, Cr(III).
5. Evaluar el papel de los exudados de la raíz de *S. vulgaris* bajo estrés por Cr, ya que estos compuestos pueden actuar sobre la biodisponibilidad de los metales en el suelo y sobre la microflora del mismo.

6. Por último, evaluar el crecimiento de *S. vulgaris* en suelos contaminados con Cromo, su influencia en la dinámica del Cr(VI) y su evolución en las distintas fracciones dependiendo de las propiedades físico-químicas del suelo así como sobre los parámetros de calidad del suelo.

2.2. REFERENCIAS

Bradshaw, A., 1997. Restoration of mined lands - using natural processes. Ecol. Eng. 8, 255-269.

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J.M., 1997. Phytoremediation of soil metals. Curr. Opin. Biotechnol. 8, 279-284.

Ciarkowska, K., Hanus-Fajerska, E., 2008. Remediation of Soil-Free Grounds Contaminated by Zinc, Lead and Cadmium with the Use of Metallophytes. Polish Journal of Environmental Studies 17, 707-712.

Prach, K., Pysek, P., 2001. Using spontaneous succession for restoration of human-disturbed habitats: Experience from Central Europe. Ecol. Eng. 17, 55-62.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environmental Science and Pollution Research 16, 765-794.

Effect of genotype, Cr(VI) and Cr(III) on plant growth and micronutrient status in *Silene vulgaris*

Abstract

The objective of this work was to study the uptake and influence on plant growth of Cr(VI) and Cr(III) in six genotypes of S*ilene vulgaris* (four hermaphrodites and two females) from different sites of Madrid (Spain). Plants were treated for 12 days with 60µM of Cr(III) or Cr(VI) in semihydroponics. Dry weigth, Soil-Plant Analysis Development values (SPAD) and micronutrient and total Cr concentrations were determined. Metal uptake was higher in presence of Cr(VI) than of Cr(III) and poorly translocated to the shoots. In both cases *S. vulgaris* did not show visual toxicity symptoms, biomass reduction, or differences among SPAD values as consequence of Cr additions. However genotypes SV36 and SV38 showed Fe and Mn imbalance. *S. vulgaris* presented high diversity at genotypic level.

Keywords: *bladder campion, metal pollution, metal speciation, tolerance, nutrient balance.*

3.1. BACKGROUND

Once Chromium enters the environment, its toxicity is determined to a large extent by its chemical form, which is also responsible for its mobility and biavailability (Kotas and Stasicka, 2000). The two most common and stable chemical species of chromium in the environment are Cr(III) and Cr(VI). The active-redox Cr(VI) is more water soluble and more bioavailable than Cr(III), and it has been classified as carcinogen of Group A by the EPA (USEPA, 1998). The role of Cr(III) as essential anion in mammals and plants is under question because it seems to be also toxic at higher doses than Cr(VI) (Levina and Lay, 2008).

In plants growth in non-contaminated soils, chromium concentrations are usually less than 1 $mg \cdot kg^{-1}$ dry weight, rarely exceed 5 $mg \cdot kg^{-1}$ and is typically in order of 0.02-0.2 $mg \cdot kg^{-1}$ dry weight (Pezennec, 2007). To date, high concentrations of Cr have been detected in different species as *Brassica oleracea* (Zayed *et al*., 1998), *Brassica juncea* (Kumar *et al*., 1995) and *Arabidopsis thaliana* (Salt *et al*., 1998). Accumulators have been reported in Gramineae family such as *Leersia hexandra* (Zhang *et al*., 2007) or *Miscanthus sinensis* (Arduini *et al*., 2006). High accumulations of Cr have been described in other species as *Salsola kali* (Gardea-Torresdey *et al*., 2005), *Convulvulus arvensis* (Gardea-Torresdey *et al*., 2004), *Polygonum hydropiperoides* (Qian *et al*., 1999) *Glycine max* and *Helianthus annuus* (Mei *et al*., 2002), *Azolla caroliana* (Bennicelli *et al*., 2004) and *Propopsis spp* (Aldrich *et al*., 2003). Genotypic differences in chromium uptake have been found in *Indian mustard* (Diwan *et al*., 2008) and rice (Zeng *et al*., 2010). In general, plants retain more Cr(VI) than Cr(III) (Zayed *et al*., 1998) with some exceptions as *Azolla caroliana* (Bennicelli *et al*., 2004), *Leersia hexandra* (Zhang *et al*., 2007) or *Glycine max* (Mei *et al*., 2002).

Biomass reduction and leaf chlorosis are the first toxicity symptoms showed by plants when they grow in high metal dosage and hence in chromium. Among other reasons, this is caused by an imbalance in micronutrient status (Shanker *et al*., 2005). The Fe accumulation was reduced in leaves in presence of Cr(III) or Cr(VI) in *Brassica oleracea* (Pandey and Sharma, 2003), *Spinacia oleracea* (Chatterjee and Chatterjee,

2000) or *Zea mays* (Mallick, 2010). Concentrations of other micronutrients such as, Cu, Zn and specially Mn, were greatly affected.

Silene vulgaris, the bladder campion, is a perennial weed widely distributed in Europe, North America, Asia and North Africa. It occupies a great variability of habitats including metalliferous soils. Flower phenology and insect pollination lead to a predominantly outcrossing habit, therefore it exhibit a high level of genetic variability (Prentice and Giles, 1993). Spanish natural populations of *S. vulgaris* have been characterized on morphological traits and molecular markers Alarcón *et al.*, 2008) showing a high genetic diversity. This species has a gynodioecious mating system characterized by the co-ocurrence of female and hermaphrodites individuals within populations (Taylor *et al*., 1999). Individuals of these populations are scattered along road sides and agricultural fields; thus their ecology is likely to be affected by anthropogenic factors associated with roadside maintenance as well as natural processes (Olson *et al*., 2005).

Theoretical studies suggest that because females cannot gain fitness through pollen, they must increase their investment in seed quantity and/or quality in order to coexist with hermaphrodites (Charlesworth and Laporte, 1998). This increase in female fitness has been observed in most gynodioecious species, but the few studies that investigated physiological differences between genders in gynodioecious species have produced inconsistent results (Caruso *et al*., 2003; Schultz, 2003; Poot *et al*., 1996).

The tolerance of many *S. vulgaris* ecotypes has been proved for most of heavy metal and their combinations, especially Cd, Cu, Fe, Mn, Pb, Zn and Hg (Ciarkowska and Hanus-Fajerska, 2008; Ernst and Nelissen, 2000; Paliouris and Hutchinson, 1991). Data from these studies make *S. vulgaris* to be considered an interesting species in the early stages of revegetation and soil restoration. Regards to genotypes, Cu has been the only one studied to evaluate differences to metal uptake among genotypes of *S. vulgaris* (Price and Abrahams, 1994). There is no information about physiological traits and heavy metal tolerance between genders of *S. vulgaris* and about the ability of this species to tolerate and accumulate chromium.

The objectives of this study were: a) to assess whether the presence of low concentrations of Cr(III) or Cr(VI) in nutrient solution led to differences in Cr uptake by *S. vulgaris* at genotype level; b) to evaluate the effectiveness of the genotypes using the classical approach of wild plants efficiency based on micronutrient status and state of chlorophylls.

3.2. MATERIALS AND METHODS

3.2.1. Plant material and growth conditions

Six genotypes of *S. vulgaris* were chosen from different populations of Madrid, Spain (Table 1). A permanent 10 x 1 m^2 plot (divided into 1 m^2 quadrats) was established to vegetative propagation in Alcalá de Henares, Madrid (Spain). Hermaphrodites and female individuals were easily discriminated by watching the presence or absence of mature anthers. Cuttings of each juvenile growth of mature genotype were collected in March. The base of the cuttings was dipped in hormone Inavarplanté 1(indol-3-butyric acid (A,B) 0.1%, 1-naftil acetic acid (ANA) 0.1%, ziram 4%) for five minutes, and transferred into floating polystyrene trays. Twenty five cuttings per tray were induced to root in tap water, and set in a growth chamber under controlled environmental conditions (temperature 20ºC/16ºC, 164.527 $\mu molE \cdot m^{-2} \cdot s^{-1}$, 16/8 hour photoperiod). They were allowed to root for 3 weeks until their roots reached a length of 2.0 (±0.5) cm (Wierzbicka and Panufnik, 1998). Then, four cuttings of each genotype were transferred into polyethylene containers of 4L (Figure 1.a and 1.b) provided with a polystyrene floating plate with modified half strength Hoagland´s nutrient solution (3 mM KNO_3, 2 mM $Na(NO_3)_2 \cdot 4H_2O$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 50 µM NaCl, 25 µM H_3BO_3, 2 µM $ZnSO_4 \cdot 7H_2O$, 2 µM $MnSO_4 \cdot H_2O$, 0.1 µM $CuSO_4 \cdot 5H_2O$, 0.5 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 20 µM Fe(EDDHA). Plants were acclimated for 2 weeks by a progressive increase of nutrient solution concentration. Afterwards plants were randomly selected to be treated as follows (a) Control, no Cr addition; (b) 60 µM Cr(VI) as $K_2Cr_2O_7$ (ACS grade Aldrich) and (c) 60 µM Cr(III) as $CrCl_3 \cdot 6\ H_2O$ (RT grade Aldrich). The applied dosages of chromium were chosen according to Zhang (2007) and Zayed (1998) and hormesis responses determined by Gardea-Torresday (2005).

Table 1. Geographical location and sex of *S. vulgaris* genotype

Genotype	Site (Madrid)	Sex	Altitude (m)	Latitude	Longitude	Lithology
SV19	Cadalso de los vidrios	F	808	401808	42638	Granite
SV21	Rozas de Puerto Real	H	867	401842	42933	Granite
SV27	Pinilla del Valle	H	1096	405562	34840	Limestone
SV30	Orusco	H	665	401712	31239	Limestone
SV36	Brea del Tajo	H	738	401350	30636	Loam
SV38	Valdequemada	F	860	403044	41812	Arkose

(F) Female; (H) Hermaphrodite.

Four trays, with four cuttings of each genotype in each tray, were used as independent replicate of each treatment. The pH of the solutions was buffered with 2 mM of MES and adjusted to 5.5 with KOH. Nutrient solution was replenished daily and completely changed every 3 days. Aliquots (20 mL) of nutrient solution were collected before and after each change to check pH and the oxidation state of chromium. Plants were treated for 12 days.

The concentration of Cr(VI) in nutrient solution was determined after changing the solution by UV-VIS light spectrophotometer (Thermo Spectronic Helios Alpha) (EPA method 7196A). The total chromium concentration was measured in the samples of nutrient solution previously acidified and then, by Atomic Absorption Spectrophotometry (VARIAN fast sequential, model AA240FS). The total concentration of Cr(III) was calculated by subtracting the concentration of Cr(VI) from the total chromium concentration. Results from Cr speciation analysis indicated the transformation among chromium species was not significant during the experiment.

3.2.2. Plant analysis

SPAD Index (Soil-Plant Analysis Development) was measured to estimate the chlorophyll state (Figure 1.c). This is a green colour index related to chlorophyll content. The average of six determinations per leaf was recorded in the four plants of each genotype per tray using a Minolta Chlorophyll Meter SPAD-502. Plants were harvested. The roots and aerial parts were separated and washed thoroughly with MilliQ water. A sample was made with the four plants of the same genotype per tray, thus four independent replicates per treatment and genotype. The vegetal material was dried in a forced air oven for 48 hours at 70ºC. Subsequently, the dry weights were recorded and aerial parts and roots were ground separately. Dried samples (0.25 mg) were digested in an Anton Paar Microwave Reaction System 3000 by adding 6 mL of HNO_3 65% and 2 mL H_2O_2 33%. After cooling, the digests were filtered (Whatman filter paper nº541) and brought up to a volume of 25 mL. Total concentrations of Cr, Fe, Mn and Zn were measured by Flame Atomic absorption spectrophotometer (VARIAN fast sequential model AA240FS) and Cu concentration by Sequential ICP-AES LIBERTY AX. Tobacco leaves were used as certified reference materials (CTA-VL2, tobacco leaves).

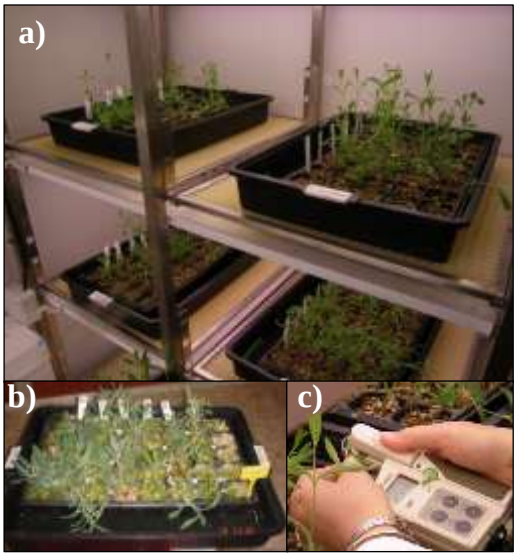


Figure 1: Experimental conditions; a) hydroponic system in the phytotron chamber; b) detail of experimental trays; c) SPAD measures sampling

3.2.3. Parameters of chromium accumulation in the plant

The following indexes were calculated to compare Cr uptake and translocation to the shoot as result of the different Chromium treatments. The translocation factor (TF) is defined as the ratio between the total metal concentration of shoots and roots. It shows the plant ability to translocate heavy metals from the roots to the harvestable aerial part (Mattina *et al*., 2003):

$$TF = \frac{C_{aerial}}{C_{root}} \quad (1)$$

The bioconcentration factor (BCF) or phytoextraction rate was described as the heavy metal concentration in total plant divided by heavy metal concentration in nutrient solution (Kumar *et al*., 1995).

$$BCF = \frac{C_{plant}}{C_{solution}} \quad (2)$$

Where C_{aerial} and C_{root} are the total chromium concentration in aerial part and roots respectively and $C_{solution}$ is the chromium concentration in the nutrient solution.

3.2.4. Statistical treatment

Statistical analysis were performed used the statistical package SPSS version 16.0. Data from all variables considered were analysed by General Lineal Model (GLM) with dose of Chromium (no chromium, 60µM Cr(III) or 60µM Cr(VI)) and *S. vulgaris* genotype (SV19, SV21, SV27, SV30, SV36 and SV38) as experimental factors at $\alpha = 0.05$ using the F-test. The statistical values *(F)* and empirical *p*-values of both factors and their interaction for all variables considered are presented in table 2. GLM was followed by a post hoc Duncan test to assess the significance of differences among treatments and among genotypes for each parameter. The results were shown within the tables and figures as lower case letters for differences among doses of chromium and with capital letters for differences among genotypes. Values given in the tables and figures indicate mean values ± standard error (S.E.).

3.3. RESULTS

3.3.1. Chromium Uptake

Figure 2 gives the total chromium concentration in the studied genotypes of *S. vulgaris*. Chromium uptake was strongly affected by the source of chromium in nutrient solution (p=0.000), but not by the genotype. Chromium was mainly accumulated in the roots of the genotypes exposed to Cr(VI) and Cr(III). The highest chromium concentrations were achieved in roots of plants growth with Cr(VI) with values between 374 and 481 mg Cr·kg^{-1}D.W. Concentration of Cr in the shoots of *S. vulgaris* genotypes only increased in treatments with Cr(VI), which ranged between 6 and 20 mg Cr·kg^{-1}D.W. The translocation factor (TF) presented very low values for all the genotypes, independently of Cr status in the nutrient solution (Table 3).

Table 2: Testing of general hypothesis in General Lineal Model (GLM) for variables studied in *S. vulgaris* at α = 0.05 (df – degrees of freedom , *F* – statistical value, *p* – empirical significance level).

Variable	Dose			Genotype			Dose x Genotype		
	df	***F***	***P***	**df**	***F***	***p***	**df**	***F***	***p***
TF	2	9.785	0.000***	5	0.833	0.533	10	0.602	0.804
BC	2	35.946	0.000***	5	0.223	0.313	10	0.815	0.616
DW shoots	2	1.963	0.144	5	4.050	0.002**	10	1.601	0.113
DW roots	2	0.476	0.622	5	6.653	0.000***	10	0.668	0.753
SPAD	2	2.853	0.066	5	7.493	0.000***	10	0.581	0.822
[Cr] shoots	2	31.100	0.000***	5	1.286	0.284	10	1.547	0.150
[Cr] roots	2	162.403	0.000***	5	1.197	0.324	10	0.395	0.943
[Fe] shoots	2	6.593	0.003**	5	0.653	0.660	10	0.826	0.606
[Fe] roots	2	6.478	0.003**	5	1.610	0.175	10	3.248	0.003**
[Zn] shoots	2	4.127	0.022*	5	4.517	0.002**	10	0.736	0.687
[Zn] roots	2	0.318	0.729	5	4.661	0.001**	10	2.166	0.036*
[Cu] shoots	2	6.744	0.002**	5	5.476	0.000***	10	0.639	0.773
[Cu] roots	2	4.898	0.012*	5	3.834	0.005**	10	2.072	0.045*
[Mn] shoots	2	17.181	0.000***	5	6.707	0.000***	10	0.209	0.995
[Mn] roots	2	4.053	0.024*	5	4.463	0.002**	10	1.637	0.124

The bioconcentration factor (BCF), which evaluates the plant ability to take up the metals from the nutrient solution, is given in Table 3 for each genotype at the end of the experiment. The highest values were achieved when Cr(VI) was added to the nutrient solution. This factor decreased by 80% for all the genotypes treated with

Cr(III) compared to Cr(VI). Related to genotypes, there are no significant differences in chromium concentrations or BCF values. Thought not statistically significant, there is a tendency of female individuals to accumulate less Cr in their tissues than hermaphrodites. The genotype SV21 seemed to be the most efficient in Cr uptake, what was also supported because it showed the highest bioconcentration factor.

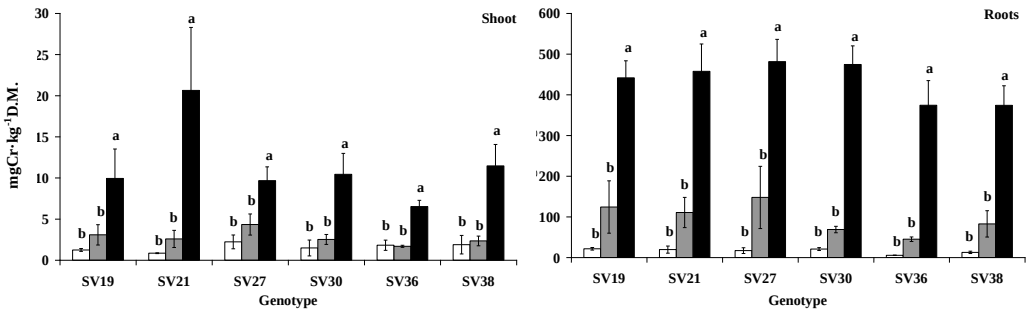


Figure 2: Total chromium concentrations (mg Cr·kg^{-1}D.W.) in *S. vulgaris* genotypes. Control, white bars; Cr(III) treatment, grey bars and Cr(VI) treatment, black bars. Different letters means significant differences in total chromium content between treatments (Duncan's test p<0.05, mean ± SE, n=4).

Table 3. Translocation factor (TF) and bioconcentration factor (BCF) of *S. vulgaris* genotypes after 12 days of treatment with 60µM of Cr(III) or Cr(VI)

Genotype		SV 19	SV21	SV27	SV30	SV36	SV38
TF	**Cr(III)**	0.015 ± 0.005^{b}	0.021 ± 0.003^{b}	0.031 ± 0.005^{b}	0.04 ± 0.02^{b}	0.04 ± 0.008^{b}	0.06 ± 0.03^{b}
	Cr(VI)	0.03 ± 0.01^{a}	0.04 ± 0.01^{a}	0.021 ± 0.004^{a}	0.026 ± 0.006^{a}	0.018 ± 0.002^{a}	0.03 ± 0.005^{a}
BC	**Cr(III)**	3 ±2	4 ±2^{b}	10 ±5^{b}	2.6 ±0.3^{b}	2.6 ±0.7^{b}	3.6 ±1.2^{b}
	Cr(VI)	13 ±1	31 ±14^{a}	23 ±2^{a}	13 ±2^{a}	20 ±4^{a}	21 ±3^{a}

Different lowercase letters means significant differences between chromium treatments. (Duncan's test p<0.05, mean ± SE, n=4).

3.3.2. Plant growth and SPAD Index

Table 4 presents the dry weight for roots and shoots of the *S. vulgaris* genotypes after 12 days of exposure to the different treatments. Significant differences were mainly due to genotypes (p=0.002 for shoots dry weights and p=0.000 for roots) rather than to chromium treatments. As regards treatment with Cr(VI), Duncan test

showed that the hermaphrodite genotype SV36 presented the highest dry weight in shoots and roots while the female genotypes (SV38 and SV19) showed growth inhibition.

Table 4. Dry weigth (mg·plant^{-1}) of *S. vulgaris* genotypes after 12 days. Control (no Chromium), 60µM Cr(III) and 60µM Cr(VI).

	Aerial part			Roots		
	Control	**Cr(III)**	**Cr(VI)**	**Control**	**Cr(III)**	**Cr(VI)**
SV19	332±48 ^{ABC}	408±42 ^{B}	275±51 ^{AB}	21±5 ^{C}	25±5 ^{B}	22±5 ^{B}
SV21	337±77 ^{ABC}	586±126 ^{A}	359±76 ^{AB}	53±15 ^{AB}	45±13 ^{B}	62±28 ^{AB}
SV27	353±56 ^{AB}	335±70 ^{BC}	415±84 ^{B}	35±9 ^{AB}	97±39 ^{A}	57±17 ^{AB}
SV30	251±50 ^{BC}	371±37 ^{B}	348±76 ^{AB}	17±3 ^{C}	33±6 ^{B}	33±10 ^{B}
SV36	439±53 ^{A}	447±58 ^{AB}	454±60 ^{A}	73±23 ^{A}	73±19 ^{AB}	79±12 ^{A}
SV38	171±41 ^{C}	186±23 ^{C}	202±27 ^{C}	24±6 ^{C}	21±3 ^{B}	31±6 ^{B}

Significant differences among genotypes are indicated by different capital letters (Duncan's test $p<0.05$, mean ± SE, n=4).

Table 5. SPAD values of *S. vulgaris* genotypes after 12 days of treatment with 60µM Cr(III) or Cr(VI).

Genotype	**SPAD Index**		
	Control	**Cr(III)**	**Cr(VI)**
SV19	34.125^{B}	36.150 ^{AB}	31.275^{C}
SV21	42.325^{A}	45.925^{A}	37.713^{AB}
SV27	40.525^{AB}	38.47^{AB}	39.400^{A}
SV30	40.925^{AB}	44.167^{AB}	38.763 ^{A}
SV36	40.525 ^{AB}	43.175^{AB}	41.950 ^{A}
SV38	34.250^{B}	33.000^{B}	31.887^{BC}

Significant differences among genotypes are indicated by different capital letters, (Duncan's test $p<0.05$, mean ± SE, n=4).

To compare the effect of chromium treatments on Chlorophyll content, SPAD values were measured for each genotype of *S. vulgaris* after 12 days of treatment started. The results are given in Table 5. In this setting, there were no significant differences in treatments (p=0.066) but once again there were among genotypes (p=0.000). The leaves of female genotypes (SV19 and SV38) displayed the lowest SPAD values. Note that this trend is not the same as biomass yield, where the maximum and minimum values were kept for the same genotypes regardless of treatment. The SPAD index values showed differences among genotypes due to the effect of Cr(VI). The females (SV19 and SV38) presented the lowest SPAD when plants were treated with Cr(VI).

3.3.3. Micronutrient concentration

Figures 3, 4, 5 and 6 gives the concentrations of Fe, Zn, Cu and Mn, respectively, in shoots and roots of *S. vulgaris* genotypes. Iron concentrations in shoots of genotypes SV36 and SV38 have decreased by 55% and 68% respectively, due to Cr(VI) in the nutrient solution. This fact was accompanied by increasing of Fe concentration in roots and significant to SV36 genotype, in which Fe increased by 160% over the control. Genotypes SV38, SV30, and SV36 treated with Cr(VI) have reduced by 40% Mn concentration in aerial part compared to controls. These genotypes showed increments in Mn concentrations of roots, but not statistically significant except for genotype SV30 (up to 30% more than control). Zinc and copper concentration in roots and aerial part are not consistent to predict if there is any influence of Cr uptake.

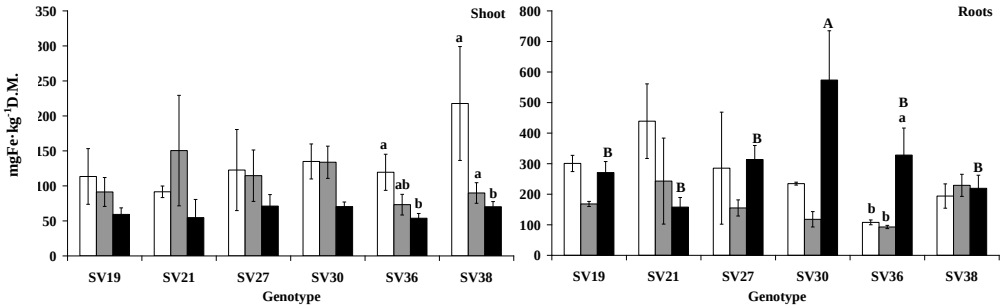


Figure 3. Total iron concentrations (mg Fe·kg^{-1}D.W.) in *S. vulgaris* genotypes. Control, white bars; Cr(III) treatment, grey bars and Cr(VI) treatment, black bars. Significant differences among Cr treatments for each genotype are indicated by lowercase letters, among genotypes are indicated by capital letters (Duncan's test $p<0.05$, mean ± SE, n=4).

It should be noted that differences in micronutrient concentrations were found among genotypes. But, there was a trend showed that hermaphrodite individuals had micronutrient concentrations lower than females. This is the case of hermaphrodite SV36 which presented the lowest micronutrient concentrations, significant only for Zn and Mn concentration when plants were treated with Cr(VI). Maximum micronutrient concentrations were reached by females and both hermaphrodites SV30 and SV27, specially for SV30 that achieved the highest concentrations of Zn, Cu and Fe when plants were treated with Cr(VI). Further studies are necessary to confirm this trend.

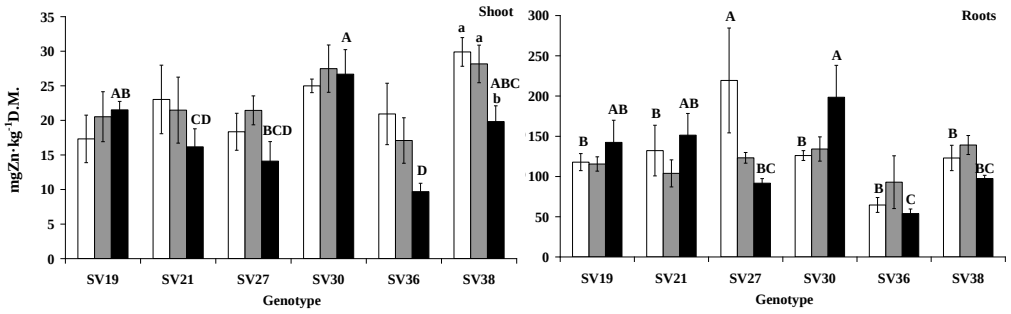


Figure 4. Total zinc concentrations (mg Zn·kg^{-1}D.W.) in *S. vulgaris* genotypes. Control, white bars; Cr(III) treatment, grey bars and Cr(VI) treatment, black bars. Significant differences among Cr treatments for each genotype are indicated by lowercase letters, among genotypes are indicated by capital letters (Duncan's test $p<0.05$, mean ± SE, n=4)

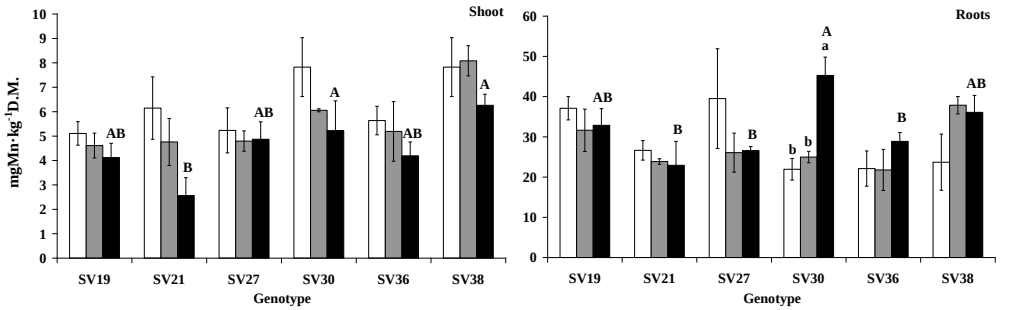


Figure 5. Total copper concentrations (mg Cu·kg^{-1}D.W.) in *S. vulgaris* genotypes. Control, white bars; Cr(III) treatment, grey bars and Cr(VI) treatment, black bars. Significant differences among Cr treatments for each genotype are indicated by lowercase letters, among genotypes are indicated by capital letters (Duncan's test $p<0.05$, mean ± SE, n=4).

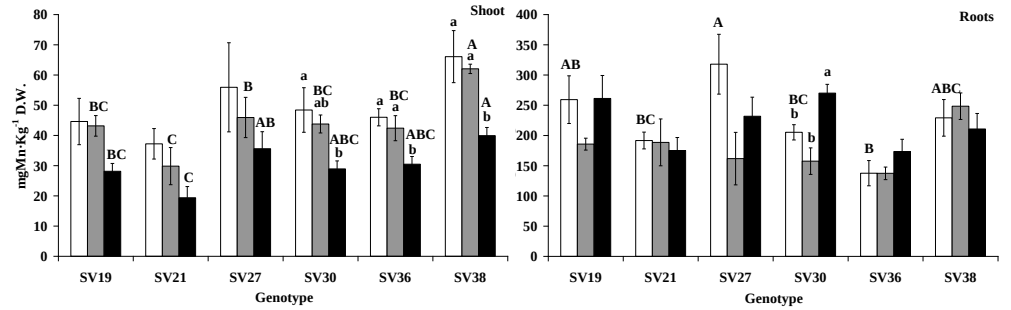


Figure 6. Total manganese concentrations (mg Mn·kg.$^{-1}$D.W.) in *S. vulgaris* genotypes. Control, white bars; Cr(III) treatment, grey bars and Cr(VI) treatment, black bars. Significant differences among Cr treatments for each genotype are indicated by lowercase letters, among genotypes are indicated by capital letters (Duncan's test $p<0.05$, mean ± SE, n=4).

3.4. DISCUSSION

The genotypes of *S. vulgaris* grown in nutrient solution did not show any visual or physiological toxicity symptoms after being treated with 60µM of Cr(III) or Cr(VI) during 12 days. With the exception of chromium uptake, that was higher in the plants treated with Cr(VI) than with Cr(III), the main differences in the plant development were found related to the genotypes more than to the Cr forms in the nutrient solution.

Total chromium concentrations in dry tissues of *S. vulgaris* exposed to Cr(III) and Cr(VI) were in the same range as other species treated with similar doses and time of exposure as *Helianthus annuus* and *Glycine max* (Mei *et al*., 2002), *Salsola cali* (Gardea-Torresdey *et al*., 2005) or *Vigna radiata* (Shanker *et al*., 2004).

As it could be seen in chromium tissue concentrations and bioconcentration factor, all genotypes of *S. vulgaris* studied here, presented a significantly greater uptake of Cr(VI) than Cr(III). These differences have already been explained based on different Cr uptake mechanism by the plant. Skeffington *et al*. (1976) carried out inhibitor studies with barley seedlings which demonstrated that Cr(III) and Cr(VI) do not share a common uptake mechanism. Cr(VI) is actively taken up in metabolically driven processes in contrast to Cr(III), which is passively taken up and retained by cation exchange sites of the cell wall. This fact explained why plants take up more Cr(VI) than Cr(III). Later it was confirmed by other authors (Gardea-Torresdey *et al*., 2005; Zayed *et al*., 1998).

The six genotypes of *S.vulgaris* presented translocation factors of chromium less than 1 in all treatments. Chromium was mainly accumulated in roots and poorly translocated to aerial parts as it was previously reported by (Sharma *et al*., 1995; Mcgrath, 1982;). The authors proposed that poor translocation of Cr to the shoots could be due to sequestration of Cr in the vacuoles of the root cells to render it non-toxic. Furthermore, Han *et al*. (2004) and Arduini *et al*. (2006) showed that chromium is only translocated at toxic levels far away of the dose applied in this study.

Among the effects caused by chromium toxicity to the plant, it should be highlighted the detriment on dry weight in both root and shoots and leaf chlorosis. None of these symptoms were shown in *S. vulgaris* genotypes at the applied dose. It is remarkable that previous studies found biomass decreases in *Zea mays*, *Licopersicom esculentum* and *Brassica oleracea* (di Toppi *et al*., 2002) treated with Cr(VI) at 5 $mg \cdot L^{-1}$ and in *Sorghum bicolour* at 50 μM (Shanker and Pathmanabhan, 2004). Similar doses also affected the chlorophyll contents of other species like *Vigna radiata* (Samantary, 2002), *Salvinia maritima* (Nichols *et al*., 2000) and *Zea mays* (Sharma *et al*., 2003).

Chromium, due to its structural similarity with some essential elements, can affect mineral nutrition of plants in a complex way. Competition mechanisms for transport bindings in the plant resulting in a decrease in micronutrient uptake and translocation have also been described (Shanker *et al*., 2005). Results from Cr(VI) treatment showed Mn decrease in the shoots of genotypes SV30, SV36 and SV38. They also showed a decrease of Fe in SV36 and SV38, which in case of SV36 is accompanied by a greater accumulation of Fe in roots. These results agree with those of Gopal *et al*. (2009) and Gardea-Torresdey *et al*. (2004) that reported decreases of Mn and Fe contents in stems and leaves of cauliflower and spinach respectively. Gardea-Torresdey *et al*. (2004) found that Fe was significantly concentrated in the root of Cr(VI)-treated plants of *Convultus arvensis* as in genotype SV36. It must be taken into account that treatment with Cr(VI) increased the differences in micronutrient concentrations among genotypes. No effect has been found related to Cr(III) treatment.

The imbalance in Fe and Mn found in Cr(VI) treatment does not happen in all genotypes and is not translated in biomass or SPAD values reductions, suggesting that *S. vulgaris* present a relatively high tolerance to Cr compared with other species treated with similar doses. The lack of toxicity symptoms could be related to the low dosage applied (close to hormesis). It should be taking into account that this experiment was conduced using Cr concentrations far less than those that cause toxicity because the objective was to evaluate the influence of the Cr speciation in the genotype development at environment concentrations.

Based on results obtained, there is a high level of variability of *S. vulgaris*. Each genotype studied presented differences in all parameters studied in this work except in chromium uptake. It could be difficult to choice the most efficient genotype in a possible treatment of chromium contaminated sites. Considering results of biomass and micronutrients concentrations, the common definition of "efficiency" for wild plants could be used. This efficiency is defined as the quantity of dry matter produced per g of nutrient and it is simply the inverse of tissue concentration (Small, 1972). Given this definition, hermaphrodite genotypes and especially SV21 and SV36 would be more efficient than females because they present higher biomass and lower nutrient concentrations especially in Cr(VI) treatment. Tissue concentration may be affected by processes like luxury consumption or substantial storage. Therefore a more useful measure of efficiency might be respiration, photosynthetic or net assimilation rate (Arduini *et al*., 2006; Shanker *et al*., 2004).

In relation to SPAD values, like a measure of the state of chlorophylls, the hermaphrodites, and especially SV21, could be considered more efficient too because they presented higher values than females. In general, hermaphrodite genotypes showed similar behaviour in chromium uptake and tolerance but SV21 seems to be the best candidate to be used in future assays. SV21 presented the highest bioaccumulation coefficient (BCF) and it was the genotype that showed the highest concentrations of chromium in aerial part without any alteration in its micronutrient balance. On the other hand female genotypes were less efficient in all parameters studied here, especially SV38. Both, SV38 and SV19 seem to have different behaviour. SV38 translocated more chromium than other genotypes in relation with its poor uptake. This leads to a decrease in the levels of Fe, Mn and Zn in the aerial part. On the other hand SV19 did not accumulate great amounts of chromium and it not showed any micronutrients imbalance probably due to an exclusory mechanism. Some plants simply avoid toxicity by preventing uptake of the metal as have been reported by De Vos *et al*. (1991) in copper treated *Silene cucubalus* plants.

Comparison of physiological traits between genders is scarce for gynodioecious species, and the results are generally inconsistent, with some suggesting that females

have higher photosynthetic rates (Caruso *et al*., 2003) and others suggesting the opposite (Schultz, 2003). At least three physiological mechanisms might underlie such sex differences. All require a limited pool of resources shared between the developing reproductive structures and supporting leaf tissue. As metal uptake is controlled by many physiological traits it was expected to find sex differences in chromium uptake, but further studies will be necessary to elucidate the mechanisms by which the gender may influence metal uptake in *S. vulgaris.*

Uptake and effects of Cr are related with the dose and time of exposure, more studies are necessary to better understand tolerance and chromium uptake of *S. vulgaris* and to select the most effective genotype in field. To our knowledge, this is the first report on the relative good performance of hermaphrodite and female *S. vulga*ris genotypes in Cr uptake and physiological traits. *S. vulgaris* uptakes mainly the hexavalent form of chromium and accumulates it mainly in roots. This species presents relative high tolerance to Cr at the applied dose. This work confirms the high variability in physiological traits of *S. vulgaris* even in genotypes from neighbouring areas. It seems that the treatment with hexavalent chromium increases the differences among genotypes hence, the use of cuttings from a homogeneous genotype seems to be an adequate method for the study of metal behaviour in this species.

3.5. REFERENCES

Alarcón, R., Pérez Sanz, A., Lobo, M.C., García, P, 2008. Estudio de la variabilidad genética en poblaciones de *Silene vulgaris* (Moench) Garcke, mediante marcadores SSR, In Actas Horticultura. Comunicaciones Técnicas Sociedad Española de Ciencias Hortícolas.

Aldrich, M.V., Gardea-Torresdey, J.L., Peralta-Videa, J.R., Parsons, J.G., 2003. Uptake and reduction of Cr(VI) to Cr(III) by mesquite (*Prosopis spp*.): Chromate-plant interaction in hydroponics and solid media studied using XAS. Environ. Sci. Technol. 37, 1859-1864.

Arduini, I., Masoni, A., Ercoli, L., 2006. Effects of high chromium applications on miscanthus during the period of maximum growth. Environ. Exp. Bot. 58, 234-243.

Bennicelli, R., Stepniewska, Z., Banach, A., Szajnocha, K., Ostrowski, J., 2004. The ability of *Azolla caroliniana* to remove heavy metals (Hg(II), Cr(III), Cr(VI)) from municipal waste water. Chemosphere 55, 141-146.

Caruso, C., Maherali, H., Jackson, R., 2003. Gender-specific floral and physiological traits: implications for the maintenance of females in gynodioecious Lobelia siphilitica. Oecologia 135, 524-531.

Charlesworth, D., Laporte, V., 1998. The male-sterility polymorphism of *Silene vulgaris*: Analysis of genetic data from two populations and comparison with *Thymus vulgaris*. Genetics 150, 1267-1282.

Chatterjee, J., Chatterjee, C., 2000. Phytotoxicity of cobalt, chromium and copper in cauliflower. Environmental Pollution 109, 69-74.

Ciarkowska, K., Hanus-Fajerska, E., 2008. Remediation of Soil-Free Grounds Contaminated by Zinc, Lead and Cadmium with the Use of Metallophytes. Polish Journal of Environmental Studies 17, 707-712.

di Toppi, L.S., Fossati, F., Musetti, R., Mikerezi, I., Favali, M.A., 2002. Effects of hexavalent chromium on maize, tomato, and cauliflower plants. J. Plant Nutr. 25, 701-717.

Diwan, H., Ahmad, A., Iqbal, M., 2008. Genotypic variation in the phytoremediation potential of Indian mustard for chromium. Environ. Manage. 41, 734-741.

Ernst, W.H.O., Nelissen, H.J.M., 2000. Life-cycle phases of a zinc and cadmium resistanta ecotype of *Silene vulgaris* in risk assessment of polymetallic soils. Environmental Pollution. 107, 329-338.

Gardea-Torresdey, J.L., de la Rosa, G., Peralta-Videa, J.R., Montes, M., Cruz-Jimenez, G., Cano-Aguilera, I., 2005. Differential uptake and transport of trivalent and flexavalent chromium by tumbleweed (*Salsola kali*). Arch. Environ. Contam. Toxicol. 48, 225-232.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., Montes, M., de la Rosa, G., Corral-Diaz, B., 2004. Bioaccumulation of cadmium, chromium and copper by *Convolvulus arvensis* L.: impact on plant growth and uptake of nutritional elements. Bioresour. Technol. 92, 229-235.

Gopal, R., Rizvi, A.H., Nautiyal, N., 2009. Chromium Alters Iron Nutrition and Water Relations of Spinach. J. Plant Nutr. 32, 1551-1559.

Kotas, J., Stasicka, Z., 2000. Chromium occurrence in the environment and methods of its speciation. Environmental Pollution 107, 263-283.

Kumar, P.B.A.N., Dushenkov, V., Motto, H., Raskin, I., 1995. Phytoextraction - the use of Plants to Remove Heavy-Metals from Soils. Environ. Sci. Technol. 29, 1232-1238.

Levina, A., Lay, P.A., 2008. Chemical properties and toxicity of chromium(III) nutritional supplements. Chem. Res. Toxicol. 21, 563-571.

Mattina, M.I., Lannucci-Berger, W., Musante, C., White, J.C., 2003. Concurrent plant uptake of heavy metals and persistent organic pollutants from soil. Environmental Pollution 124, 375-378 .

McGrath, S.P., 1982. The Uptake and Translocation of Tri-Valent and Hexa-Valent Chromium and Effects on the Growth of Oat in Flowing Nutrient Solution and in Soil. New Phytol. 92, 381-390.

Mei, B.J., Puryear, J.D., Newton, R.J., 2002. Assessment of Cr tolerance and accumulation in selected plant species. Plant Soil 247, 223-231.

Nichols, P.B., Couch, J.D., Al-Hamdani, S.H., 2000. Selected physiological responses of Salvinia minima to different chromium concentrations. Aquat. Bot. 68, 313-319.

Olson, M., McCauley, D., Taylor, D., 2005. Genetics and adaptation in structured populations: sex ratio evolution in *Silene vulgaris*. Genetica 123, 49-62.

Paliouris, G., Hutchinson, T.C., 1991. Arsenic, cobalt and nickel tolerances in two populations of *Silene vulgaris* (Moench) Garcke fron Ontario, Canada. New Phytol 117, 449-459.

Pandey, N., Sharma, C.P., 2003. Chromium interference in iron nutrition and water relations of cabbage. Environ. Exp. Bot. 49, 195-200.

Prentice, H.C., Giles, B.E., 1993. Genetic Determination of Isozyme Variation in the Bladder Campions, *Silene-uniflora* and *S. vulgaris*. Hereditas 118, 217-227.

Price, C.G., Abrahams, P.W., 1994. Copper Tolerance in a Population of *Silene vulgaris* Ssp Maritima (A-And-D Love) at Dolfrwynog Bog Near Dolgellau, North Wales. Environ. Geochem. Health 16, 27-30.

Qian, J.H., Zayed, A., Zhu, Y.L., Yu, M., Terry, N., 1999. Phytoaccumulation of trace elements by wetland plants: III. Uptake and accumulation of ten trace elements by twelve plant species. J. Environ. Qual. 28, 1448-1455.

Salt, D.E., Smith, R.D., Raskin, I., 1998. Phytoremediation. Annual Review of Plant Physiology & Plant Molecular Biology 49, 643.

Samantary, S., 2002. Biochemical responses of Cr-tolerant and Cr-sensitive mung bean cultivars grown on varying levels of chromium. Chemosphere 47, 1065-1072.

Schultz, S., 2003. Sexual dimorphism in gynodioecious *Sidalcea hirtipes* (Malvaceae). I. Seed, fruit, and ecophysiology. Int. J. Plant Sci. 164, 165-173.

Shanker, A.K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S., 2005. Chromium toxicity in plants. Environ. Int. 31, 739-753.

Shanker, A.K., Djanaguiraman, M., Sudhagar, R., Chandrashekar, C.N., Pathmanabhan, G., 2004. Differential antioxidative response of ascorbate glutathione pathway enzymes and metabolites to chromium speciation stress in green gram (*Vigna radiata* (L.) R.Wilczek. cv CO 4) roots. Plant Science 166, 1035-1043.

Shanker, A.K., Pathmanabhan, G., 2004. Speciation dependant antioxidative response in roots and leaves of sorghum (*Sorghum bicolor* (L.) Moench cv CO 27) under Cr(III) and Cr(VI) stress. Plant Soil 265, 141-151.

Sharma, D.C., Sharma, C., Tripathi, R.D., 2003. Phytotoxic lesions of chromium in maize. Chemosphere 51, 63-68.

Sharma, D.C., Chatterjee, C., Sharma, C.P., 1995. Chromium Accumulation and its Effects on Wheat (*Triticum-Aestivum* L Cv Hd-2204) Metabolism. Plant Science 111, 145-151.

Small, E., 1972. Photosynthetic rates in relation to nitrogen recycling as an adaptation to nutrient deficiency in peat bog plants. Canadian Journal of Botany 50, 2227-2266.

Taylor, D.R., Trimble, S., McCauley, D.E., 1999. Ecological Genetics of Gynodioecy in *Silene vulgaris:* Relative Fitness of Females and Hermaphrodites during the Colonization Process. Evolution 53, 745-751.

USEPA, 1998. Toxicological Review of Hexavalent Chromium. U.S. Goverment Printing Office, Washington.

Wierzbicka, M., Panufnik, D., 1998. The adaptation of *Silene vulgaris* to growth on a calamine waste heap (S. Poland). Environmental Pollution 101, 415-426.

Zayed, A., Lytle, C.M., Qian, J.H., Terry, N., 1998. Chromium accumulation, translocation and chemical speciation in vegetable crops. Planta 206, 293-299.

Zeng, F., Qiu, B., Ali, S., Zhang, G., 2010. Genotypic Differences in Nutrient Uptake and Accumulation in Rice Under Chromium Stress. J. Plant Nutr. 33, 518-528.

Zhang, X., Liu, J., Huang, H., Chen, J., Zhu, Y., Wang, D., 2007. Chromium accumulation by the hyperaccumulator plant *Leersia hexandra* Swartz. Chemosphere 67, 1138-1143.

Genotypic response to Cr(VI) of *Silene vulgaris* grown in hydroponics

Abstract

The effects of Cr(VI) have been evaluated in six genotypes of *Silene vulgaris* grown in hydroponics using Cr(VI) concentrations from 10 to 1200 µM. The plant experiment based on root growth has shown the tolerance range to Cr(VI) between 200 and 1200 µM for the most tolerant genotypes. Chromium uptake and plant development data have been recorded in semihydroponic conditions. The genotypes showed differences under Cr(VI) stress, especially at medium doses (10-100 µM) where hormetic effects have been found in dry matter, state of chlorophylls and water content. It seems that *S. vulgaris* avoid Cr stress by controlling its nutrient balance. Genotype SV21 might be the best candidate to be used in polluted soils.

Keywords: *metal tolerance, metallophyte, micronutrient status, soil pollution*

4.1. BACKGROUND

Cr(VI) is the most toxic form of chromium in the environment. Due to its high solubility in water, Cr(VI) enters easily in living cells where its redox-cycle generates reactive oxygen species (ROS), which may cause severe oxidative injuries to cell wall, plasma membrane and nucleic acid, reasons why Cr(VI) is classified as human carcinogen (USEPA, 1998). Thus, there is an increased need to develop efficient technologies for removing Cr (VI) from the environment.

Plants used in contaminated sites may evolve tolerance mechanism which allows them to resist and tolerate toxic concentrations of metals. Some quantitative genetic variation in this ability may exist among populations and organisms in the same population (Pollard *et al*., 2002). Metal tolerance and accumulation ability are not only related to the original soil in which plant has been developed. Plants can be original from contaminated areas (natural or anthropogenic) or agricultural lands. In some hyperaccumulators as *Thlaspi caerulescens,* it has been described that plants from non-methalliferous soils usually show higher metal accumulations than methalliferous ecotypes (Dechamps *et al*., 2005; Escarre *et al*., 2000). Ideal candidates to be applied in contaminated sites should be native from the region to avoid the introduction of non-native and potentially invasive plant, to preserve the genetic resources of the area and to activate the natural process of succession (Prach and Pysek, 2001). This is especially important in the Mediterranean area where the severe environmental conditions of polluted soils are enhanced by the prolonged drought (Escarre *et al*., 2000).

A basic constitutive level of metal tolerance is ubiquitous to *Silene vulgaris* (Jack *et al*., 2005), however the screening of adapted genotypes is required to its application in the mediaterran region for phytoremediation purposes, specially for Cr(VI).

The objectives of this work were to establish the tolerance range of six genotypes of *S. vulgaris* from Madrid region (Spain) to Cr(VI) and to study the differences among genotypes to Cr uptake and plant development. All these as the first

approach to evaluate the potential of these genotypes to be employed in the recovery of Cr(VI) contaminated sites.

4.2. MATERIALS AND METHODS

4.2.1. Plant material and growth conditions

Six genotypes of *S. vulgaris* were chosen from different populations of Madrid, Spain with different soil characteristics: SV19 (Cadalso de los Vidrios, granitic soil), SV21 (Rozas de Puerto Real, granitic soil), SV27 (Pinilla del Valle, limestone soil), SV30 (Orusco, limestone soil), SV36 (Brea de Tajo, loamy soil), SV38 (Valdemaqueda, arkosic soil). They were vegetative propagated in Alcalá de Henares, Madrid (Spain). At the beginning of autumn, cuttings of each juvenile growth of mature genotype were collected and dipped in hormone Inavarplanté 1(indol-3-butyric acid (A,B) 0.1%, 1-naftil acetic acid (ANA) 0.1%, ziram 4%). Cuttings were induced to root in tap water, and set in a growth chamber under controlled environmental conditions (temperature 20ºC/16ºC, 164.527 µmol photons m^2s^{-1}, 16/8 hours photoperiod). They were allowed to root for 3 weeks until their roots reached a length of 2.0 (±0.5) cm (Wierzbicka and Panufnik, 1998).

4.2.2. Tolerance test

In order to evaluate the range of *S. vulgaris* tolerance to Cr(VI), a test was carried out as previously reported by Schat and Ten Bookum (1992). In summary, the tolerance test was based on the root growth of plants subjected to increasing metal concentrations supplied at 200 µM Cr(VI) steps as $K_2Cr_2O_7$ in the nutrient solution. The minimum chromium concentration to completely inhibit growth [EC_{100}] was taken as a measure of tolerance.

4.2.3. Hydroponic assay

In March, cuttings of each genotype of *S. vulgaris* were rooting as described above and transferred into a hydroponic system with half strength Hoagland nutrient solution in a phytotron chamber. Plants were acclimated for 4 weeks. Afterwards, plants of each genotype were randomly selected and treated for 2 weeks based on the Cr(VI)

concentration range obtained in the tolerance test. Cr(VI) was supplied as $K_2Cr_2O_7$ in the nutrient solution at the following doses: 0, 10, 100, 600 and 1300 µM. Four trays with 4 plants per genotype were used as independent replicate of each treatment. The pH and chromium speciation in the solutions were measured after each changing (USEPA, 1992).

4.2.4. Chemical speciation of nutrient solution

Estimations of the concentration of Cr(VI) ionic species in the different nutrient solutions were carried out with MINTEQA2 for windows (version 3.0 visual basic.NET 2005 compiled by Jon Petter Gustafsson).

4.2.5. Chlorophyll Content

Estimates of chlorophyll content were determined from intact leaves using a portable SPAD-502 (Minolta, Ltd., Osaka, Japan). The measurements were made *in situ* just before harvesting on the fourth leaf of the main stem of each plant. Ten measurements were made per plant, and the mean value of each leaf was calculated (per genotype and treatment).

4.2.6. Growth parameters

Once the plants were harvested, roots and shoots were separated. Fresh (F.W.) and dry (D.W.) weights and water content (%) were also determined in all vegetal samples. The experiment was run with 4 different batches of plants and 16 plants per treatment were taken for analysis in each experiment.

4.2.7. Analysis of Cr and mineral nutrient concentrations

Roots and shoots were washed thoroughly with distilled water. Plant samples were dried in a forced air oven for 48 hours at 70ºC until constant weight. Plant dried samples (~500 mg) were digested in an Anton Paar Microwave Reaction System 3000 by adding of 6 mL of HNO_3 65% and 2mL H_2O_2 33%. Final cooling digested were filtered (Whatman filter paper nº541) and made up to 25 mL with Milli-Q water. Total Concentration of Cr, Fe, Mn, Zn, Ca and Mg were measured by Flame Atomic absorption spectrophotometer (VARIAN fast sequential model AA240FS). Total Cu

concentration was determined by Sequential ICP-AES LIBERTY AX. Tobacco leaves were used as certified reference materials (CTA-VL2, tobacco leaves). The translocation factor (TF) and bioconcentration factor (BCF) for Cr were calculated as described by Mattina *et al*. (2003) and Kumar *et al*. (1995) respectively.

4.2.8 Statistical treatment

Data were analysed for treatments and genotypes by General Lineal Model (GLM) using the statistical package SPSS version 16.0. GLM was followed by a post hoc Duncan test to assess the significance of differences among treatments and among genotypes for each parameter. The results were shown within the tables and figures as lower case letters for differences among doses of Cr(VI) and with capital letters for differences among genotypes. Values given in the tables and figures indicate means ± SE (4 batches of plants with 4 replicates per treatment).

4.3. RESULTS

4.3.1. Tolerance Test

The Cr(VI) tolerance of *S. vulgaris* genotypes was determined based on the dose-response for root growth. The frequency distribution of EC_{100} for Cr(VI), as established in the sequential exposure test, is given in Figure 1. Genotypic differences in Cr(VI) tolerance have been detected with this test and allowed to identify two groups of genotypes. The first one consisted of genotypes SV19, SV21 and SV36 which were the most tolerant and showed the lowest EC_{100} values between 200 and 1200 µM. The another one was comprised of genotypes SV27, SV30 and SV38 which were the most sensitive to Cr (VI) with 100% of root growth inhibition at 1000 µM. Genotype SV19 was the most tolerant to Cr(VI) because the percentage of individuals grown at 1000 µM Cr(VI) was 28%. Finally, genotype SV30 showed the least tolerance to Cr (VI) with a 61% percentage of individuals that reached their EC_{100} at 200 µM.

4.3.2. Chemical speciation of Cr(VI) in nutrient solution

In the Cr(VI) treatments, the major Cr(VI) species predicted to occur in the nutrient solution was $HCrO_4^-$ accounting from 83.5 and 77.5 % of total Cr(VI) in the evaluated concentration range. Approximately 11% of total Cr(VI) was predicted to occur in the form of CrO_4^{-2} in all treatments. Also, 5% and 0.4 % of total Cr(VI) were predicted as $CaCrO_4$ (aq) and $CrO_3HPO_4^{2-}$ respectively. Finally, the species $Cr_2O_7^{-2}$ will be increased with the increment of Cr(VI) in nutrient solution and suppose a detriment of $HCrO_4^-$ in nutrient solution.

Table 1: Species Distribution of Cr(VI) in nutrient solution (%)

$Cr(VI)_{total}$ (µM)	0	10	100	600	1300
$HCrO_4^-$	0	83.05	82.61	80.31	77.46
CrO_4^{-2}	0	11.32	11.27	11.00	10.66
$CaCrO_4$ (aq)	0	5.10	5.05	4.83	4.55
$CrO_3HPO_4^{2-}$	0	0.38	0.38	0.37	0.36
$KCrO_4$-	0	0.08	0.08	0.08	0.08
$Cr_2O_7^{-2}$	0	0.06	0.59	3.36	6.80
$NH_4Cr_2O_7^-$	0	0.00	0.00	0.02	0.03
$KCr_2O_7^-$	0	0.00	0.00	0.04	0.07

4.3.3. Cr accumulation and concentration in genotypes

Table 2 gives total chromium concentration in shoots and roots (mg $Cr \cdot kg^{-1}$ DW), transfer factor (TF) and bioconcentration factor (BCF) of *S. vulgaris* plants grown with increasing doses of Cr (VI). Significant changes in Cr concentrations, TF and BCF have been detected with the dose applied. Cr concentration increased in a dose-dependent manner in both shoots and roots. In all cases, *S. vulgaris* showed a typical excluder behaviour as concentration in roots was much higher than in shoots, as also TF values under 1. However, the difference in Cr accumulation between shoot and root decreased with increasing Cr exposure thus TF slightly increased with the applied dose. On the other hand, the plant efficiency to accumulate Cr in the tissues, calculated as the BCF, decreased at high exposures. Genotypic differences have been found in shoot Cr concentrations, TF and BCF but not in root Cr concentrations. The differences were statistically significant ($p<0.05$) at 100 and 600 µM (just at 600 µM for BCF), especially in shoot concentration where the interaction between dose and genotype was also significant. The genotype SV21 reached the greatest values to total Cr concentration in shoots, TF and BCF. The lowest values were showed by SV36.

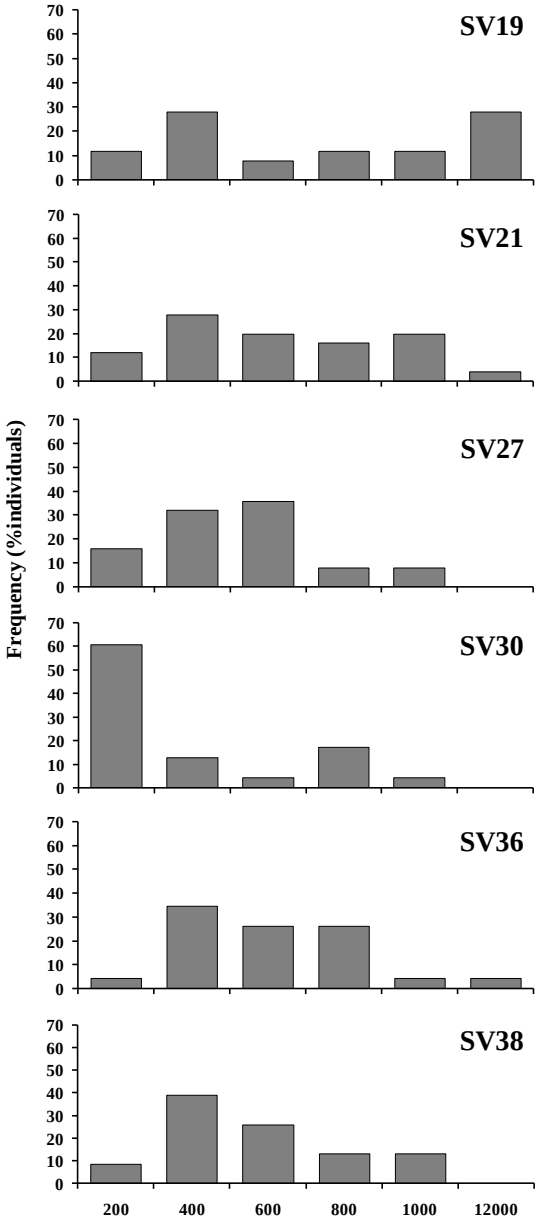


Figure 1: EC_{100} frequency distribution values for the effect of Cr(VI) on root growth for six genotypes of *S. vulgaris* as established in a sequential exposure test using concentration steps of 200 µM Cr(VI).

The response of total Cr accumulated for each genotype ($mgCr \cdot plant^{-1}$) is presented in figure 2. Differences among genotypes have been found for both shoots and roots. In shoots (Fig 2.a), the total Cr accumulated was linearly increased and the maximum accumulation has not been reached at the evaluated doses. On the opposite, the chromium accumulated in root (per plant) peaked at 100 or 600 µM depending to

the genotype to decrease at 1300 µM. Genotype SV21 achieved the highest total Cr concentration in both shoots and roots.

Table 2: Total chromium concentration in dry tissues (mg Cr·kg^{-1} D.W.) of *S. vulgaris* genotypes treated 2 weeks with different doses of Cr(VI). Transfer factor (TF) and bioconcentration factor (BCF) of total Chromium in the plants.

		Shoots	Roots	TF	BCF
0µM	**SV19**	1.87^{NS}	1.56^{NS}		
	SV21	1.51	2.55		
	SV27	1.52	1.65		
	SV30	1.84	0.85		
	SV36	1.87	3.03		
	SV38	1.96	1.36		
10µM	**SV19**	5.46^{NS}	425^{NS}	0.01^{NS}	13.2^{NS}
	SV21	6.06	368	0.02	16.7
	SV27	4.52	418	0.01	18.5
	SV30	5.28	474	0.01	18.4
	SV36	3.42	272	0.01	12.4
	SV38	4.11	349	0.01	21.6
100µM	**SV19**	84.9^{BC}	1572^{NS}	0.05^{BC}	8.3^{B}
	SV21	227^{A}	1916	0.12^{A}	17.6^{A}
	SV27	82.9^{BC}	1617	0.05^{BC}	13.4^{AB}
	SV30	64.5^{BC}	1483	0.04^{BC}	10.0^{B}
	SV36	33.5^{C}	1365	0.02^{C}	11.1^{B}
	SV38	98.3^{B}	1460	0.07^{AB}	13.4^{AB}
600µM	**SV19**	1526^{A}	2352^{NS}	0.65^{A}	10.3^{NS}
	SV21	1396^{A}	2773	0.50^{AB}	9.6
	SV27	1046^{AB}	2625	0.40^{BC}	7.7
	SV30	1054^{AB}	2695	0.39^{BC}	9.9
	SV36	432^{B}	2642	0.16^{C}	4.6
	SV38	1016^{AB}	2748	0.37^{BC}	8.1
1300µM	**SV19**	2625^{NS}	4348^{NS}	0.60^{NS}	8.1^{NS}
	SV21	2897	4172	0.69	8.1
	SV27	2680	5497	0.49	8.1
	SV30	1606	5263	0.31	5.8
	SV36	1534	2673	0.57	4.0
	SV38	2591	2965	0.87	8.9
Dose		***	**	*	***
Genotype		***	ns	*	**
DosexGenotype		**	ns	ns	ns

Data are means of 16 replications (4 batches of plants with 16 replicates per treatment). Data followed by the same letter are not significantly different (Duncan´s test at the $p<0.05$ level D:dose; G:Genotype)

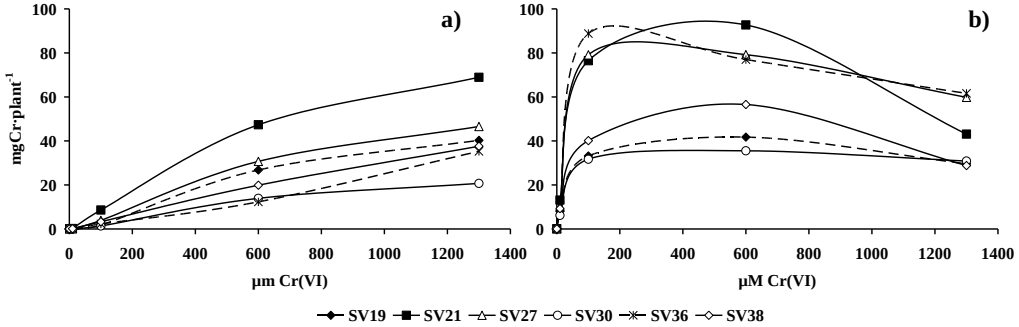
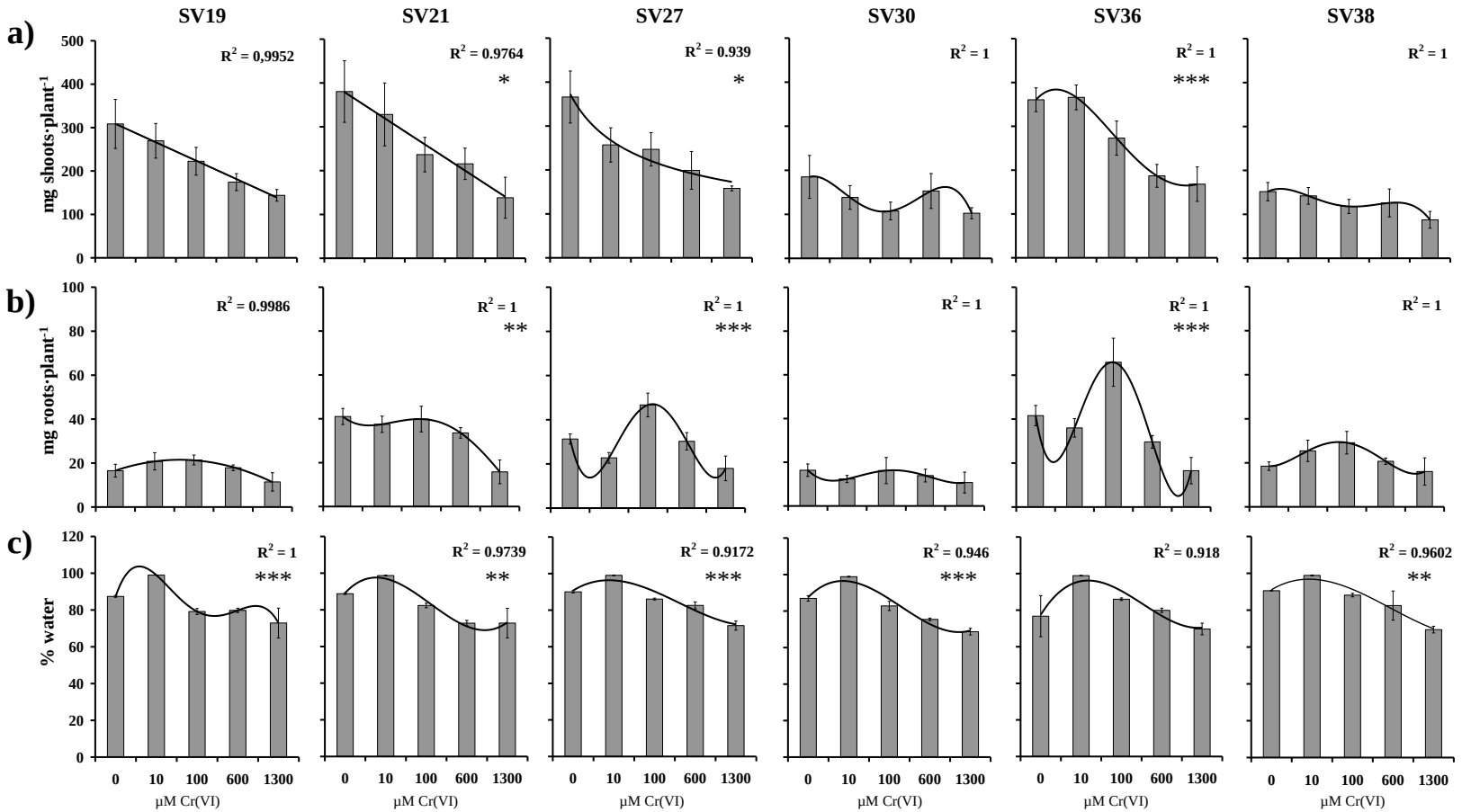


Figure.2: Total chromium (mgCr·plant^{-1}) in shoots (a) and roots (b) of *S. vulgaris* genotypes treated 2 weeks with Cr(VI). Data are means (4 batches of plants with 16 replicates per treatment).

4.3.4. Effects of Cr(VI) on growth

The Cr(VI) influence on plant development is given in figure 3. The dry weight was markedly affected in plants grown with Cr(VI). The behaviour was different for each genotype, especially in root dry mass. The different responses of each genotype grown by increasing Cr(VI) concentrations were represented by the corresponding line of best fit (R^2>0.9000). In shoots (Fig 3.a), the dry weight significantly decreased by the applied dose for genotypes SV21, SV27 and SV36. The first one showed a negative correlation, meanwhile both SV27 and SV36 displayed an exponential decay and a fourth-order polynomial trend line, respectively. The effect of chromium in roots dry mass (Fig 3.b) showed also a significant interaction between dose and genotype (p<0.001). Except to genotype SV21, whose dry mass decreased in a dose-dependent manner, the other genotypes tended to increase the biomass at medium doses. This increase was significant for SV27 and SV36 that reached a peak at 100 µM Cr(VI). Genotypic differences have been found in both root and shoots especially at low and medium doses where SV21, SV27 and SV36 showed greater dry mass than SV30, SV38 and SV19. There were no differences among genotypes at 600 µM Cr(VI) in shoots, nor at 1300 µM Cr(VI) in both root and shoots. The percentage of water (%) in *S. vulgaris* leaves is also given in figure 3. No significant differences have been found at genotypic level but with the applied dose. A similar behaviour was acquired by studied genotypes. The water content rose from 10% at 10 µM to drop by 20% at 1300 µM.

Figure 3: Growth parameters of *S. vulgaris* genotypes in absence and in presence of different doses of Cr (VI): a) shoot dry weight (mg·plant^{-1}) b) root dry weights (mg·plant^{-1}) c) shoot water content (%). Data are means ± SE (4 batches of plants with 16 replicates per treatment); * p<0.05, ** p<0.005, *** p<0.001)



4.3.4. Effects of Cr(VI) on Chlorophyll Content

State of chlorophylls, calculated as SPAD index, is presented in figure 4. Chlorophyll state for each genotype was significantly influenced by Cr(VI) concentration in nutrient solution. In genotypes SV19, SV21 and SV30, SPAD index values smoothly fell by increasing of Cr(VI) concentration, but genotypes SV27, SV36 and SV38 raised this index at 100 µM (10 µM for SV38) to fell down from 600 to 1200 µM. Significant differences in SPAD index among genotypes have also been detected at 0, 100 and 600 µM. Finally, the genotype SV30 reached the greatest values and the genotype SV38 the lowest one.

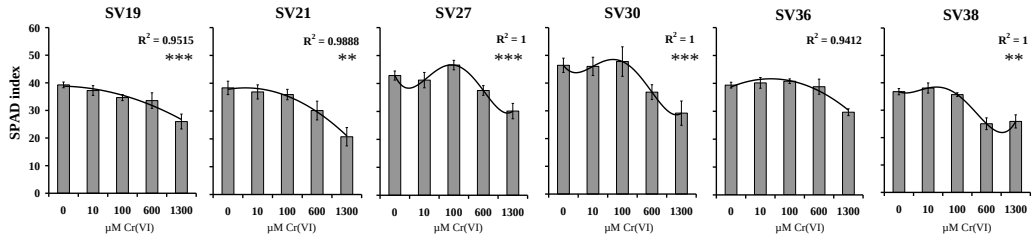


Figure 4: SPAD Index and water content in leaves of *S. vulgaris* genotypes treated 2 weeks with different doses of Cr(VI). Data are means ± SE (3 batches of plants with 16 replicates per treatment. Stars indicate differences among doses for each genotype (* $p<0.05$, ** $p<0.005$, *** $p<0.001$).

4.3.5. Effect of Cr(VI) on plant mineral concentrations

Table 3 shows micronutrient concentrations ($mg \cdot kg^{-1}$ D.W.), Ca (%) and Mg (%) content in shoots and roots of *S. vulgaris* grown in Cr(VI) solutions. In general, exposition to Cr(VI) altered all the evaluated nutrients in shoots and roots except to Cu. Significant differences in Fe, Mn and Ca concentration were found among genotypes in both shoots and roots. Zn concentration showed genotypic differences in shoots and Mg concentrations in roots. Though not statistically significant in all genotypes and nutrient studied, the common response of *S. vulgaris* to Cr(VI) exposure was to decrease nutrient concentration at 10 and 100 µM Cr(VI) and increase at 600 and 1300 µM. However two different trends have been found in the cases in which the interaction between genotype and dose was significant.

Table 3. Concentrations (mg·kg^{-1} D.W.) of micronutrients (Fe, Zn, Cu, Mn) and Ca and Mg contents (%) of *S. vulgaris* genotypes treated 2 weeks with different doses of Cr(VI).

		Fe		Zn		Cu		Mn		Ca		Mg	
	Dose (µM)	(mg·kg^{-1}D.W.)		(mg·kg^{-1}D.W.)		(mg·kg^{-1}D.W.)		(mg·kg^{-1}D.W.)		(%)		(%)	
		Shoots	Root	Shoots	Root	Shoots	Root	Shoots	Root	Shoots	Root	Shoots	Root
SV19	**0**	82.4^{b}	365^{ns}	47.7^{a}	224^{ns}	3.63^{ns}	19.2^{ns}	28.0^{a}	99^{ns}	0.55^{a}	0.92^{c}	3.41^{a}	0.39^{ns}
	10	61.4^{b}	382	34.3^{a}	204	3.71	17.8	30.2^{a}	111	0.53^{a}	1.01^{bc}	3.27^{a}	0.46
	100	67.3^{b}	563	18.8^{b}	176	2.39	17.7	16.8^{b}	108	0.10^{b}	1.15^{bc}	1.90^{b}	0.30
	600	156.8^{a}	314	34.6^{a}	194	4.12	17.2	31.2^{a}	91	0.22^{ab}	1.76^{ab}	2.93^{a}	0.30
	1300	197.0^{a}	457	39.5^{a}	199	4.92	13.8	29.6^{a}	63	0.28^{ab}	2.35^{a}	3.44^{a}	1.03
SV21	**0**	81.9^{c}	865^{ns}	41.4^{ns}	248^{ns}	3.81^{ns}	18.9^{ns}	32.4^{ns}	513^{ns}	0.25^{ns}	0.71^{b}	3.87	0.52^{b}
	10	65.1^{c}	804	45.6	202	4.60	17.9	32.6	332	0.23	0.54^{b}	3.85	0.53^{b}
	100	139.6^{b}	713	29.1	219	4.00	16.0	23.8	417	0.09	0.80^{b}	2.73	0.39^{ab}
	600	174.9^{a}	916	41.4	246	4.83	15.0	29.4	359	0.14	1.76^{a}	3.07	1.54^{ab}
	1300	196.2^{a}	872	56.3	255	4.86	13.6	34.3	195	0.22	1.94^{a}	3.38	0.34^{b}
SV27	**0**	81.0^{c}	326^{ns}	46.3^{ns}	237^{ab}	3.69^{ns}	14.9^{ns}	35.2^{ns}	346^{ab}	0.18^{ns}	0.98^{a}	3.50^{a}	0.36^{a}
	10	62.2^{c}	353	49.2	279^{ab}	3.98	18.4	40.1	192^{c}	0.12	0.89^{a}	3.36^{ab}	0.04^{a}
	100	71.2^{c}	387	32.7	180^{b}	3.10	15.1	26.7	407^{a}	0.12	0.62^{a}	2.47^{b}	0.29^{b}
	600	122.4^{b}	387	36.5	205^{b}	4.46	14.4	28.4	243^{abc}	0.08	1.53^{b}	2.43^{b}	0.20^{a}
	1300	180.8^{a}	524	50.4	227^{ab}	5.13	11.7	36.0	157^{c}	0.12	1.62^{b}	3.34^{ab}	0.23^{a}
SV30	**0**	93.1^{b}	243^{ns}	43.6^{ns}	194^{ns}	4.18^{ns}	16.5^{ns}	32.5^{ns}	124^{ns}	0.10^{ns}	0.80^{ns}	2.84^{ns}	0.29^{ns}
	10	88.1^{b}	411	48.9	318	4.47	24.2	52.1	63	0.22	1.36	3.26	2.04
	100	85.8^{b}	167	23.6	156	2.99	14.7	23.3	130	0.10	0.81	2.52	0.75
	600	172.1^{a}	199	38.9	187	5.43	17.6	34.5	73	0.12	1.87	2.96	0.60
	1300	154.6^{a}	323	37.7	197	3.96	13.8	34.3	78	0.12	1.46	2.75	0.45
SV36	**0**	73.4^{c}	626^{ns}	36.3^{ab}	222^{a}	2.78^{ns}	16.7^{ns}	33.6^{a}	235^{a}	0.25^{ab}	0.95^{b}	3.74^{b}	0.54
	10	58.2^{cd}	1332	38.6^{b}	234^{a}	3.97	17.3	38.1^{a}	410^{b}	0.35^{a}	0.54^{c}	4.86^{a}	0.01
	100	44.1^{d}	612	21.0^{c}	113^{b}	3.76	12.3	21.6^{b}	417^{b}	0.15^{b}	0.48^{c}	2.59^{c}	0.96
	600	96.2^{b}	883	28.8^{bc}	176^{ab}	3.50	14.3	28.1^{ab}	220^{a}	0.12^{b}	0.93^{b}	2.80^{bc}	0.58
	1300	132.3^{a}	964	39.5^{a}	185^{a}	3.83	13.6	35.8^{a}	160^{a}	0.18^{b}	1.24^{a}	3.46^{bc}	0.61
SV38	**0**	102.8^{bc}	456^{a}	54.6^{ns}	252^{a}	5.70^{ns}	22.6^{ns}	45.2^{a}	215^{a}	0.11^{ns}	1.02^{b}	3.16^{ns}	0.77^{a}
	10	74.0^{c}	260^{bc}	52.3	244^{a}	4.61	26.3	44.8^{a}	287^{a}	0.16	0.74^{b}	3.47	0.83^{a}
	100	93.4^{bc}	170^{c}	34.8	146^{b}	4.01	18.1	28.8^{b}	269^{a}	0.09	0.90^{b}	2.81	0.39^{b}
	600	134.8^{bc}	394^{ab}	41.8	205^{a}	4.37	16.9	32.6^{ab}	177^{ab}	0.10	1.92^{ab}	2.69	0.45^{ab}
	1300	183.5^{a}	322^{abc}	64.5	235^{a}	5.42	19.7	45.3^{a}	58^{b}	0.19	2.70^{a}	3.16	0.30^{ab}
	D	***	ns	***	***	ns	ns	*	*	***	***		***
	G	***	***	***	ns	ns	ns	***	***	***	***		***
	DxG	*	ns	ns	ns	ns	ns	ns	***	*	ns		***

Values are means ± SE (3 batches of plants with 16 replicates per treatment. Lower case letters indicate differences among doses (Duncan test, p<0.05: Ns, non significant, * p<0.05, **p<0.005, ***p<0.001; D:dose; G:Genotype)

Genotypes SV19, SV21, SV27 and SV30 and, on the other hand, SV36, significantly increased Fe and Ca concentration with increasing Cr(VI) in nutrient solution. Fe/Mn antagonism has no detected between in shoots. The Fe increase was not translated in Mn decrease. In roots of *S. vulgaris* genotypes, Cr(VI) exposure also

induced two different responses in relation to Mn and Mg concentration. The Mn concentration of SV27 decreased and showed an overcompensation at high dose, meanwhile genotypes SV36 and SV38 exhibited the opposite trend to reach the highest concentration between 10 and 100 µM Cr(VI). Mg concentration did not decrease in roots of genotype SV21 with Cr(VI) exposure, but it significantly increased at 600 µM over control. In both genotypes, SV27 and SV38, Mg concentration decreased at 100 µM Cr(VI) and reached similar level as control plants growth at 600 and 1300 µM. In brief, nutrient concentrations in *S. vulgaris* were notably affected by Cr(VI) exposure but not all genotypes studied shared a common response to this stress. Genotypes SV30 and SV21 presented lower nutrient imbalance than SV27, SV38 and SV36. Cr(VI) concentrations at 100 µM seem to be an inflexion point in the nutrient balance of *S. vulgaris*, it is the dose in which nutrient concentrations reached a minimum that is was compensated at high doses.

4.4. DISCUSSION

In the present study, the response of six genotypes of *S. vulgaris* from Madrid Region (Spain) has been studied into the hexavalent chromium tolerance range (to 1200 µM) and just above this level. In order to characterize each genotype, the study has been focused on their ability to accumulate and translocate chromium as well as on the effects produced on the main chromium toxicity targets: biomass, photosynthesis, water status and nutrients balance. Relevant differences have been found among genotypes, especially at doses of Cr(VI) between 10 and 100 µM.

The tolerance range of *S. vulgaris* genotypes was established based on root growth. The main advantage of this test sequence is that provides information about the quantitative variation in genotypes tolerance (Schat and Tenbookum, 1992). In general, it is established that Cr-sensitive cultivars accumulate higher chromium concentrations than the Cr-tolerant ones (Samantary, 2002), thus data from tolerance should be compared with those of Cr accumulation. This rule could be applied to genotype SV19 that showed the highest tolerance with low concentration of Cr in shoot and roots and the lowest bioconcentration factor (BCF). It also could explain why genotype SV38 is

one of the less tolerant, as it showed high bioconcentration factor and high Cr accumulation in root and shoots. However this general point could not be applied to genotype SV21, which showed the highest Cr uptake being the second genotype in tolerance.

Total Cr concentration in *S. vulgaris* tissues increased with the applied dose. But, as shown by BCF decreasing in a Cr(VI) dose depended manner, *S. vulgaris* seems to be more efficient to remove chromium at low doses than at high ones and above certain threshold levels. According to Hayat *et al*. (2012), the mechanism of Cr uptake and translocation in plants differs with lapse of time, chromium speciation and concentration. At low dosage, Cr(VI) is taken up actively by energy metabolism mechanisms involving carriers of essential anions such as sulphate (Skeffington *et al*., 1976). Chromium concentrations at 10 or 100 µM of Cr(VI) in the nutrient solution produced an improvement in the development parameters considered in this study. The stimulation response induced at low doses of toxic substances is called hormesis and represents an overcompensation response to an initial disruption in homeostasis (Calabrese, 1999). At toxic levels, passive transports seem to be involved in the uptake mechanism. It is well documented that the first defence mechanisms to avoid Cr stress in plants is metal exclusion by vacuolar sequestration in roots (Mcgrath, 1982), thus, Cr can be only translocated to the shoots at toxic levels due to the breakdown of the restriction barriers (Arduini *et al*., 2006). This could explain why *S. vulgaris* showed low TF values and higher Cr concentration in roots than in shoots and why Cr translocation increased when plants were grown at 600 µM, near the maximum level of tolerance.

Biomass reduction is one of the first targets of heavy metal toxicity. Genotypic differences have been found in both shoot and roots of plants treated with different Cr(VI) concentration but the plant tissues have presented different responses. Thought it was not linear in all genotypes, the tendency was to decrease biomass in shoots as consequence of poor uptake and distribution of nutrients and water. At toxic levels (600 and 1300 µM), Cr is translocated from root to aerial part and led to a linear increase of total Cr uptake in shoots. However, a stimulation of root dry mass was displayed at

medium doses followed by decreasing at high Cr(VI) concentration. The stimulatory effect has also been observed in *Salsola kali* (Gardea-Torresdey *et al*., 2005) and *Arabidopsis thaliana* (Castro *et al*., 2007) and seems to be related with the enhancement of lateral root. This makes that in roots, unlike the shoots, the maximum total Cr content per plant was reached just below the toxicity threshold, at 100 and 600 µM Cr(VI), and then started to decrease due to root biomass reduction. Despite all genotypes followed this behaviour, high significant differences have been found in root growth at genotype level as showed by the interaction between genotype and dose, corroborating that root studies are the best way to detect genotypic differences in *S. vulgaris*.

Chromium stress induces inhibition of photosynthesis, but it is not clear if it is caused by disorganization of chloroplast ultraestructure or by inhibition of electron transport and enzyme activities (Hayat *et al*., 2012). In this work, SPAD index values have been taken as reference of the state of chlorophylls. All genotypes showed SPAD index reduction by Cr(VI) application. That agrees with previous results in *Triticum aestivum* (Subrahmanyam, 2008) or *Lemna gibar* (Ali *et al*., 2006). But then again, genotypes SV27, SV30, SV36 and SV38 showed an increase in SPAD index values at low Cr(VI) doses. The hormesis effect related to chlorophyll content has also been observed in *Phaselous vulgaris* (Vazquez *et al*., 1987) and *Eichicoria crapisses* (Mishra *et al*., 2009) treated with Cr(VI). Light microscopy studies carried out by Vázquez (1987) on leaves of *Phaseolus vulgaris* showed that plants treated with Cr(VI) had much smaller cells and more reduced intercellular spaces than controls while chloroplast remained less affected. That causes the leaf area decreases but increases chlorophyll content per unit leaf area, which could explain the increase in reported SPAD values at low doses of Cr(VI).

Similar hormesis effect like in root dry mass and SPAD index values have been detected in water content of *S. vulgaris,* followed by a decrease from 100 µM Cr(VI). Decreased on water content may be the result of decreasing water uptake or enhancing water loss produced by cell membrane damage caused by Cr(VI) stress. Electron

microscopy studies carried out by Vazquez *et al*. (1987) with *Phaselus vulgaris* and by Bassi *et al*. (1990) with *Lemna minor* and *Pistia stratiote* showed plasmolysis of peripheral cells in Cr treated plants leading to water imbalance.

It has been largely described that Cr stress alters mineral nutrition in plants but different results have been described (Hayat *et al*., 2012). From our results, it is clear that mineral nutrition in *S. vulgaris* is influenced by Cr(VI) concentration, with the exception of Cu. Low Cr(VI) concentrations (10 and 100 µM) resulted in reducing nutrient concentration in both shoots and roots. The results are in consonance with the findings of other authors that explain this effect on based as structural similarity of Cr with essential elements and as Cr competition with nutrient to reduce their uptake and transport (Shanker *et al*., 2005; Barceló *et al*., 1985). At high Cr(VI) concentrations (600 and 1300 µM), nutrients of *S. vulgaris* genotypes treated with Cr(VI) reached similar concentration than those of controls. At these doses, biomass reduction may be a protection mechanism against nutrient deficiency. Nutrient status of *S. vulgaris* have shown to be strongly genotypic dependent to Cr(VI) stress, thus, there are some excetions.

The first exception is related to iron concentration. In general, Cr phytotoxicity is mainly associated with Fe metabolism impairment. Some authors reported Fe uptake decreased under Cr stress (Pandey and Sharma, 2003) while others found Fe concentration increased in root and decreased in shoot due to inhibition of translocation (Gardea-Torresdey *et al*., 2004). The six genotypes of *S. vulgaris* increased Fe concentration in shoots at 600 and 1300 µM Cr(VI), regards to controls and no significant effect on root concentration. These results agree with the findings of Liu *et al*. (2008) in *Amarantthus viridis*. The author explained the unusual high Fe concentration under excess Cr supply due to the damage produced on the selective mechanisms for control of inorganic uptake. Cary *et al*. (1977) also reported Cr accumulation in plant tissues accompanied by Fe accumulation. Shiv *et al*. (2007) described the amelioration effect of Fe on *Zea mays* treated with Cr. So as Fe is an integral cofactor of many enzymes implicated in antioxidative system of cells, its accumulation in plant could be related with Cr stress alleviation.

Another exception to the general behaviour showed in nutrient status by *S. vulgaris* is related to Mn concentration. The genotypes SV36, SV38 and SV19 showed an increase of Mn concentration over control at 10 and 100 µM, probably due to the inhibition of translocation. The increase of Ca concentration showed in both shoot and roots by some genotypes is in consonance with the findings in rice (Zeng *et al*., 2010) and in *Salsola kali* (Gardea-Torresday *et al*., 2005). These authors suggested that Ca could alleviate Cr toxicity as it may be transported into the vacuoles of cells in order to regulate cell homeostasis. In the same way, the increase found in Mg concentration in shoots of SV36 and in roots of SV21 could be also related with Cr toxicity alleviation, as Mg plays several important roles in plant metabolism such as component of the chlorophyll molecule and of many enzymes, including glutathione synthase but also, in homeostasis regulation (Shaul, 2002).

Genotypic differences in heavy metal tolerance related to nutrient balance have been previously documented by Mariano *et al*. (2005) and Zeng *et al*. (2010). The authors reported that the potential of certain genotypes to tolerate high heavy metal concentration is due to their ability to keep a low disturbed mineral nutrition suggesting that tolerant genotypes are able to absorb and store more nutrients than sensitive ones to alleviate heavy metal stress. This ability is related both with the capacity to maintain metabolic nutrient uptake process as to develope a root system that will be able to explore the medium and take up the nutrients.

Thus, taking into account the parameters studied here, genotype SV21 seems to be the best candidate to be used in soil recuperation processes in the Mediterranean region as it showed high tolerance related to root length and it presented the highest accumulation levels and transfer factor. All these, by keeping the highest dry weight in both shoot and roots, probably thanks to its ability to accumulate high micronutrient concentration to alleviate Cr toxicity.

4.5. REFERENCES

Ali, N.A., Dewez, D., Didur, O., Popovic, R., 2006. Inhibition of photosystem II photochemistry by Cr is caused by the alteration of both D1 protein and oxygen evolving complex. Photosynthesis Res. 89, 81-87.

Arduini, I., Masoni, A., Ercoli, L., 2006. Effects of high chromium applications on miscanthus during the period of maximum growth. Environ. Exp. Bot. 58, 234-243.

Barcelo, J., Poschereider, C., GUNSE, B., 1985. Effect of Chromium-VI on Mineral Element Composition of Bush Beans. J. Plant Nutr. 8, 211-217.

Calabrese, E., 1999. Evidence that hormesis represents an "overcompensation" response to a disruption in homeostasis. Ecotoxicol. Environ. Saf. 42, 135-137.

Castro, R.O., Trujillo, M.M., López Bucio, J., Cervantes, C., Dubrovsky, J., 2007. Effects of dichromate on growth and root system architecture of *Arabidopsis thaliana* seedlings. Plant Science 172, 684-691.

Dechamps, C., Roosens, N., Hotte, C., Meerts, P., 2005. Growth and mineral element composition in two ecotypes of *Thlaspi caerulescens* on Cd contaminated soil. Plant Soil 273, 327-335.

Escarre, J., Lefebvre, C., Gruber, W., Leblanc, M., Lepart, J., Riviere, Y., Delay, B., 2000. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. New Phytol. 145, 429-437.

Gardea-Torresdey, J.L., de la Rosa, G., Peralta-Videa, J.R., Montes, M., Cruz-Jimenez, G., Cano-Aguilera, I., 2005. Differential uptake and transport of trivalent and flexavalent chromium by tumbleweed (*Salsola kali*). Arch. Environ. Contam. Toxicol. 48, 225-232.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., Montes, M., de la Rosa, G., Corral-Diaz, B., 2004. Bioaccumulation of cadmium, chromium and copper by *Convolvulus arvensis* L.: impact on plant growth and uptake of nutritional elements. Bioresour. Technol. 92, 229-235.

Hayat, S., Khalique, G., Irfan, M., Wani, A.S., Tripathi, B.N., Ahmad, A., 2012. Physiological changes induced by chromium stress in plants: an overview. Protoplasma 249, 599-611.

Jack, E.M., Anatasova, S., Verkleij, J.A.C., 2005. Callus induction and plant regeneration in the metallophyte *Silene vulgaris* (Caryophyllaceae). Plant Cell Tissue and Organ Culture 80, 25-31.

Mcgrath, S.P., 1982. The Uptake and Translocation of Tri-Valent and Hexa-Valent Chromium and Effects on the Growth of Oat in Flowing Nutrient Solution and in Soil. New Phytol. 92, 381-390.

Mishra, K., Gupta, K., Rai, U.N., 2009. Bioconcentration and phytotoxicity of chromium in *Eichhornia crassipes*. J. Environ. Biol. 30, 521-526.

Pandey, N., Sharma, C.P., 2003. Chromium interference in iron nutrition and water relations of cabbage. Environ. Exp. Bot. 49, 195-200.

Pollard, A.J., Powell, K.D., Harper, F.A., Smith, J.A.C., 2002. The Genetic Basis of Metal Hyeraccumulation in Plants. Critical Reviews in Plant Sciences 21, 539-566.

Prach, K., Pysek, P., 2001. Using spontaneous succession for restoration of human-disturbed habitats: Experience from Central Europe. Ecol. Eng. 17, 55-62.

Samantary, S., 2002. Biochemical responses of Cr-tolerant and Cr-sensitive mung bean cultivars grown on varying levels of chromium. Chemosphere 47, 1065-1072.

Schat, H., Tenbookum, W.M., 1992. Genetic-Control of Copper Tolerance in *Silene vulgaris.* Heredity 68, 219-229.

Shanker, A.K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S., 2005. Chromium toxicity in plants. Environ. Int. 31, 739-753.

Shaul, O., 2002. Magnesium transport and function in plants: the tip of the iceberg. Biometals 15, 309-323.

Skeffington, R.A., Shewry, P.R., Peterson, P.J., 1976. Chromium Uptake and Transport in Barley Seedlings (*Hordeum vulgare*-L). Planta 132, 209-214.

Subrahmanyam, D., 2008. Effects of chromium toxicity on leaf photosynthetic characteristics and oxidative changes in wheat (*Triticum aestivum* L.). Photosynthetica 46, 339-345.

USEPA, 1998. Toxicological Review of Hexavalent Chromium. U.S. Goverment Printing Office, Washington.

USEPA, 1992. Method 7196a. Chromium hexavalent (colorimetric).

Vazquez, D., Poschenrieder, C., Barcelo, J., 1987. Chromium-Vi Induced Structural and Ultrastructural-Changes in Bush Bean-Plants (*Phaseolus vulgaris* L). Annals of Botany 59, 427-438.

Wierzbicka, M., Panufnik, D., 1998. The adaptation of *Silene vulgaris* to growth on a calamine waste heap (S. Poland). Environmental Pollution 101, 415-426.

Zeng, F., Ali, S., Qiu, B., Wu, F., Zhang, G., 2010. Effects of chromium stress on the subcellular distribution and chemical form of Ca, Mg, Fe, and Zn in two rice genotypes RID C-3348-2011. Journal of Plant Nutrition and Soil Science 173, 135-148.

Capítulo 5/Chapter 5

Influence of Cr speciation in exudation and organic acid balance in *Silene vulgaris*

Abstract

The objective of this study was to study the effect of Chromium (Cr) stress on the changes of root exudate composition and Cr accumulation in *Silene vulgaris* genotypes differing in Cr tolerance. Two experiments were performed in hydroponics to achive this objective. The first one was carried out with two genotypes of the metalophyte *S. vulgaris* having a different tolerance to Cr(VI) to study the mechanism by which Cr influenced root exudation. Detriment on plant growth has been related with oxidative stress measured as MDA. Cr accumulation was correlated to exudates release rate and seemed to induce changes in their composition. The tolerant genotype SV21 showed less biomass reduction and oxidative stress than the sensitive SV38 but higher exudation rates, suggesting that exudation could be one of the mechanisms implicated in Cr tolerance. A second experiment was done with a tolerant population of *S. vulgaris* to further study the composition of root exudates and the balance of organic acids in plant-root system. Results indicate a hormesis response to chromium applied doses especially for exudation rate, total polyphenols, quercetin and formic, lactic and acetic acid in root exudates. Increments in the concentration of apiin in roots exudates seem to be related with protection against Cr toxicity. Citric and malic acids seem to play a role in Cr detoxification in roots. These results indicate that exudation might be a regulated mechanism of protection under Cr exposition in *S. vulgaris*. The response to Cr stress takes place early in plant tissues than in root exudates.

Keywords: *metal tolerance, oxidative stress, exudation, Silene vulgaris.*

5.1. BACKGROUND

The poor translocation of Cr from roots to shoots is a major hurdle in using plants and trees as soil clean-up techniques. Mycorrhizae and organic acids have been reported to play an important role in phytoremediation of Cr-contaminated soils by enhancing Cr uptake and increasing translocation to shoots (Davies *et al,*. 2001; Chen *et al,*. 1994). An important reason for enhanced accumulation of Cr in the root may be the presence of organic acids in the root exudates which form complexes with Cr, thereby making them available for the uptake by roots.

It is well known that plants modify the chemistry and biology of the surrounding soil (rhizosphere) through releasing of exudates in order to enhance their adaptation to a particular environment (Wenzel, 2009). Under a range of environmental stress, as metal exposition, exudation rates could be incremented (Rengel, 2002). Two possible explanations have been given to this phenomenon: the first one is based on the lost of cytoplasm constituents by passive diffusion due to the loss of membrane integrity caused by metal toxicity (Jones *et al*., 2003); the second one suggests that plants can exert some control in exudation to increase their tolerance to heavy metals. However, the mechanism by which tolerance is enhanced is not clear and it has been related with different processes such as the ability of exuded organic molecules to exclude heavy metals through chelation in the rhizosphere or in the apoplastic space (Meier *et al.*, 2012), the reduced toxicity of organically bound forms compared with metals in ionic's forms (Drzewiecka *et al*., 2012); the maintenance of charge balance in roots (Hinsinger *et al*., 2003) or the increase in nutrient uptake (Alford *et al*., 2010). On the other hand, more than with tolerance, exudation has also been related with hyperacumulation in some species (Bao *et al*., 2011; Tu *et al*., 2004) based on the mobilising effect of root exudates (Wenzel, 2009). Exudates consist primarily of carbon compounds, mainly amino acids, low-molecular-weight carboxylic acids, sugars and flavonoid-type phenolics (Kidd *et al*., 2009).

Polyphenols represents one of the most ubiquitous and numerous plants metabolites. They are characterized by the presence of large number of phenol

structural units formed by at least one aromatic ring (C6) bearing to one or more hydroxyl groups. There is evidence of induction of phenolic metabolism in plants under environmental stress including heavy metals exposition (Michalak, 2006). Thought phenolics have been demonstrated to act as antioxidants by means of free radical scavengers (Laguerre, 2007) and to have a high tendency to chelate heavy metals (Michalak, 2006) , their role in metal detoxification has been poorly investigated.

Organic acids are low-molecular weight CHO containing compounds which are characterised by the presence of one or more carboxyl groups that confer them negative charge, thereby allowing the complexation of metal cations in solution and the displacement of anions from the soil matrix (Jones, 1998). Plants growing on metal polluted soils just can regulate metal uptake by rhizosphere processes but cannot prevent it, so, after metal uptake, transport and detoxification in storage sites are required. This is achieved by chelation, transport, trafficking, and sequestration by organo-ligands (Clemens, 2001) among which organic acids have a remarkable role. For this reason organic acids, and specially citric and malic, have been studied not only due to their potential to chelate heavy metals in the rhizosphere (Meier *et al*., 2012; Magdziak *et al*., 2011) but also because they participate in root-shoot transport *(Xu et al*., 2012; Olko *et al*., 2008) and in vacuolar sequestration *(Sarret et al., 2009; Kupper et al*., 2004). Moreover soluble low molecular weight organic molecules, such as organic acids, have also shown to be effective reductants of Cr(VI) leading to soluble organo-Cr(III) complexes (Fendorf, 1995) and become bioavailable (Luo *et al*., 2010) representing an integral part of the natural cycling of chromium (Puzon *et al*., 2005).

Composition and exudation rate depend on the plant species, metal exposure and dose. While exudation induced by exposition to Al or Cu (Meier *et al*., 2012; Jung *et al*., 2003; Pineros *et al*., 2002; Wang *et al*., 2006) have been well documented, data from Cr stressed plants is scarce.

The objective of this study was to evaluate the influence of Cr on nature of root exudates in *Silene vulgaris.* The work was focused by measuring concentrations of both

organic acids and phenolics to have knowledge of organic acid balance in the plant-root system and to propose a feasible root exudation mechanism.

5.2. MATERIALS AND METHODS

5.2.1. Plant material and growth conditions

For the first experiment, two genotypes of *S. vulgaris* from Madrid (Spain) were chosen according to their tolerance range and their different response to Cr(VI). The tolerant genotype SV21 came from Rozas de Puerto Real and showed an EC_{100} between 200 and 1200 µM Cr(VI). The sensitive genotype SV38 was obtained from Valdemaqueda and presented an EC_{100} between 200 and 1000 µM Cr(VI). Clones from each genotype were vegetative propagated in field (Alcalá de Henares, Madrid, Spain) in a permanent 10 x 10 m^2 plot (divided into 1 m^2 quadrats). Cuttings of each genotype were collected and rooted in a mixture of peat for three months in a greenhouse. Then, they were thoroughly washed with MilliQ water and transferred into a hydroponic system (four cuttings of each genotype per tray) with vermiculite and 1L of half-strength modified Hoagland nutrient solution (Sobrino-Plata *et al*., 2013): 3 mM KNO_3, 2 mM $Na(NO_3)_2 \cdot 4H_2O$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 50 µM NaCl, 25 µM H_3BO_3, 2 µM $ZnSO_4 \cdot 7H_2O$, 2 µM $MnSO_4 \cdot H_2O$, 0.1 µM $CuSO_4 \cdot 5H_2O$, 0.5 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 20 µM FeEDTA into a phytotron chamber (photoperiod 16h/8h, temperature 20ºC/16ºC, 164.527 µmol photons $m^{-2}s^{-1}$). The pH of the solutions was buffered with 2 mM of MES and adjusted to 5.5 with KOH. Seedlings were acclimated 2 weeks before metal exposure with progressive increase in the nutrient solution concentration. Afterwards, plants were randomly selected to be treated for two weeks as follows (a) Control, no Cr addition; (b) 60 µM Cr(VI) c) 300 µM Cr(VI) and d) 300 µM Cr(III). Hexavalent chromium was provided as $K_2Cr_2O_7$ and trivalent chromium as $Cr(NO_3)_3 \cdot 9H_2O$.

In the second experiment seeds from a chromium tolerant population of *S. vulgaris* (EC_{100} for Cr(VI) between 30 and 100 µM, (see appendix I) from Rozas de Puerto Real (Madrid, Spain) were germinated in Petri dishes with 500 $mg \cdot L^{-1}$ of Giberelic acid in dark and room temperature. Then, plants were transferred into the

same hydroponic system as in the first experiment and acclimated for 4 weeks. Afterwards, plants were randomly selected to be treated for two weeks as follows (a) Control, no Cr addition; (b) 30 µM Cr(VI); c) 60 µM Cr(VI); d) 30 µM Cr(III) and e) 60 µM Cr(III); hexavalent chromium was provided as $K_2Cr_2O_7$ and trivalent as $Cr(NO_3)_3 \cdot 9H_2O$.

In both experiments four trays with four plants in each one were used as independent replicates of each treatment. Nutrient solution was replenished daily and completely changed every 3 days. Aliquots (20 mL) of nutrient solution were collected before and after each change to check pH and the oxidation state of chromium (EPA method 7196A) (USEPA, 1992). Results from Cr speciation analysis indicated that transformation among chromium species was not significant during the experiment.

5.2.2. Chemical speciation of nutrient solution

Estimations of the concentration of Cr(III) and Cr(VI) ionic species in the different nutrient solutions were carried out with MINTEQA2 from windows (version 3.0 visual basic.NET 2005 compiled by Jon Petter Gustafsson).

5.2.3. Collection and analysis of root exudates

After two weeks of chromium treatment, plants were washed with autoclaved MilliQ water and 20 mM of EDTA solution. Then, they were placed in sterile falcon tubes with 40 mL of autoclaved MilliQ water to collect root exudates (Tu *et al*., 2004a) (Figure 1). After 24 hours, extracts of root exudates were filtered through sterile PVC filters of 0.20µm and immediately frozen in liquid N_2. The process was carried out inside laminar flow cabinet using standard axenic techniques. The extracts were kept at -80ºC to be lyophilised in a Heto Drywinner 3. The dried lyophilised root exudates were dissolved in 5 mL of MeOH:H_2O (1:1) and analysed for organic acids, total polyphenols and flavonoids.

Figure 1: Collection of root exudates

5.2.4. Plant analysis

After collecting root exudates, roots and shoots were separated and washed thoroughly with MilliQ water. Fresh weight was recorded and subsamples of shoots (2 g) and roots (3 g) were frozen in liquid nitrogen and stored at -80ºC. The rest of the plant tissues were dried in a forced air oven for 48 hours at 70ºC. Dry weights were determined and samples (~30 mg) were digested by adding 0.5 mL of HNO_3 and 0.5 mL $HClO_4$, (Nitric acid 65% Suprapur®, Perchloric acid 70%, Suprapur®) (Zhao *et al*., 1994). Samples were left to stand overnight and heated at 130ºC for 2 hours. After cooling the digest was diluted in 15mL of MilliQ water. Total Cr concentrations were determined by Atomic Absorption Spectrometer (Zeman AA2407) equipped with graphite tube atomizer GTA 120.

5.2.5. Estimation of lipid peroxidation: malondyaldehyde (MDA)

Lipid peroxidation was evaluated as malondialdehyde (MDA) by the method of Reilly and Aust (1999) modified by Catalá *et al*., (2010). Fresh samples (0.1 g) of shoots and roots were homogenized on ice with 1 mL of MilliQ water and centrifuged at 16,000 g for 10 min. Supernatants were removed, and the pellets were resuspended in 500 µL of 0.01% buthylated hydroxytoluene (BHT) in ethanol 80%. Then 900 µl of TBA (2.57×10^{-2} M), TCA (9.18×10^{-1} M), and HCl (3.20 M) were added to each sample. Then samples were vortexed and incubated in a water bath at 70°C for 30 minutes. Afterwards, samples were cooled on ice and centrifuged at 16,000 g for 10 min. The absorbance of supernatants was measured at 532 nm. Absorbance at 600 nm was subtracted to this measure to eliminate the interferences of soluble sugars in the

samples. Both absorbances were determined by UV-VIS light spectrophotometer (Thermo Spectronic Helios Alpha).

5.2.6. Assessment of total phenolic content (TPC)

The concentration of total polyphenols (TPC) in the exudates was determined in accordance with the Folin-Ciocalteu micromethod as previously described by Schmidt, *et al*., 2005. Briefly, samples (10 µL) were added to a 96-well microtiter plate (Sarstedt) at an adequate dilution in triplicate. To start the reaction, 150 µL of aqueous Folin-Ciocalteu (Sigma-Aldrich, Missouri, USA) solution (14 mL water to 1 mL of Foline Ciocalteu reagent) was added to each well. After 3 min, 50 µL of $NaHCO_3$ solution (2 mL of saturated $NaHCO_3$ to 3 mL of water) was added to each well and the plate was placed in the dark at room temperature for 2h. Absorbance was measured at 725nm using a BioTek Synergy HT Multi-Mode microplate reader (BioTek Instruments Inc., Vermont, USA), and the data were acquired and processed using BioTek's Gen5 software (BioTek Instruments Inc.). Gallic acid (Sigma-Aldrich) was used as the standard for a calibration curve. TPC was expressed as milligrams of gallic acid equivalents per liter (mg $GAE \cdot L^{-1}$).

5.2.7. Polyphenols analysis

Polyphenols were quantified by HPLC-DAD using an Agilent 1200 series liquid chromatograph with a quaternary pump and a photodiode array detector. Briefly, after centrifugation (5000 g for 5 min) supernatant filtered (pore size 0.45 µM) samples were injected into the HPLC system. HPLC chromatographic separation was carried out using an Ultrabase C18 column (5 µm; 4.6 mm × 150 mm). The mobile phase consisted of acetic acid 2.5% (A), HPLC-grade acetonitrile (B), ultrapure water (C), and acetic acid 2.5% HPLC-grade acetonitrile (90:10) (D) at a flow rate of 0.5 $mL \cdot min^{-1}$. Starting with 100% A, the gradient was the following: from 100% A to 100% D in 3 min, from 100% D to 1% B in 4 min, isocratically 1% B in 3 min, from 1% B to 15% B in 20 min, from 15% B to 60% B in 5 min, and isocratically 60% B in 5 min. Detection

wavelengths were 280, 330, and 370 nm. The peaks were identified by comparing their retention times and UV spectra with those of authentic standards.

In order to confirm the identity of the recorded phenolic compounds and to detect new compounds that could not be detected with HPLC-DAD, additional analysis were performed by HPLC with mass spectrometry detection. Mass spectrometry was performed using an Agilent 1100 series liquid chromatograph equipped with an API source and employing an ESI (electrospray ionization) interface. The HPLC system was connected to a photodiode array detector and a simple quadruple G1946D Q-LC/MS. Sheath as well as auxiliary gas was a mixture of helium and nitrogen. The capillary voltage was 3 V and the capillary temperature 180°C. Solvents and gradient used were the same as described above. The MS detector was programmed to perform a series of consecutive scans: full scan from m/z 150 to 1500 and in addition MS2 spectra from the most abundant ion were recorded. Spectra were recorded in the positive ion mode. Standards of phenolic compounds were also injected to help in the identifications.

5.2.8. Determination of organic acids

Organic acids were extracted from plant samples as described by Arnetoli *et al*. (2008). Samples (1 g) of frozen fresh weight were homogenized in 10 mL of MilliQ water using a mortar and pestle in liquid nitrogen; then homogenates were centrifuged for 20 min at 10000 rpm at 4ºC. The supernatant was stored at -20ºC and filtered through PVC filters of 0.20 µm before analysis. Organic acids from plant samples and root exudates were measured by Ionic Chromatography (Dionex DX 500) using conductivity detector. Chromatographic conditions were as follows: sample loop volume: 25 µL; analytical column: IonPac ICE-AS6; eluent: 0.4 mM heptafluorobutyric Acid (flow rate: 1.0 $mL \cdot min^{-1}$); suppressor: MicroMembrane Anion-ICE; regenerant: 5mM tetrabutylammonium hydroxide (flow rate: 5 $mL \cdot min^{-1}$); analysis time: 20 min. Organic acids were identified by comparing the retention times of the samples against retention times of the standards. Calibration curves have been performed using Merck (purity=98%) reagents: oxalic acid from 50 to 280 $mg \cdot L^{-1}$; citric, malic and acetic acids

from 1 to 50 $mg \cdot L^{-1}$; lactic and succinic acids from 1 to 30 $mg \cdot L^{-1}$ and formic acid from 0.5 to 10 $mg \cdot L^{-1}$.

5.2.9. Statistical treatment

Data were analysed for treatments and genotypes by General Lineal Model followed (GLM) at $\alpha = 0.05$ using the F-test using the statistical package SPSS version 19.0. GLM was followed by a post hoc Duncan test to assess the significance of differences among treatments for each parameter. The results were shown within the tables and figures as lower case letters for differences among chromium treatments. Values given in the tables and figures indicate mean values (n=4). Variables from the experiments were also analysed through lineal regression to establish their dependence correspondence using Pearson correlation coefficient (r). Results of organic acids concentrations were evaluated by multidimensional statistical method PCA (principal analysis of main components).

5.3. RESULTS

5.3.1. Experiment I. Exudation of genotypes

5.3.1.a. Chemical speciation of Cr(III) and Cr(VI) in nutrient solution

The 100% of total Chromium ionic species were predicted to be dissolved in the nutrient solution at the evaluated pH. Table 1 gives the ionic species distribution in the nutrient solution according to MinteQA2. In the Cr(VI) treatments, the major Cr(VI) species predicted to occur in the nutrient solution was $HCrO_4^-$ accounting about 82% of total Cr(VI) in the evaluated concentration range. Approximately 11% of total Cr(VI) was predicted to occur in the form of CrO_4^{-2} in both treatments. Finally, 5% and 0.4 % of total Cr(VI) were predicted as $CaCrO_4$ (aq) and $Cr_2O_7^{-2}$ respectively in nutrient solution. Related to Cr(III) treatment, 89.5% of total Cr(III) forms aqueous complexes as $CrOH^{+2}$, $Cr_3(OH)_4^{+5}$, $Cr_2(OH)_2^{+4}$ and $Cr(OH)_2^{+1}$. Approximately, 6% of total Cr(III) is predicted to link EDTA as Cr-EDTA. Finally, 1.31% and 1.09% of total Cr(III) were predicted in nutrient solution as Cr^{+3} and $CrOHSO_{4(aq)}$ respectively.

Table 1 Species Distribution of Cr(III) and Cr(VI) in nutrient solution (%) at evaluated dosage

Cr(III)		Cr(VI)		
	300 µM		60 µM	300 µM
$CrOH^{+2}$	64.2	$HCrO_4^-$	82.81	81.66
$Cr_3(OH)_4^{+5}$	9.03	CrO_4^{-2}	11.30	11.16
$Cr_2(OH)_2^{+4}$	8.48	$CaCrO_4$ (aq)	5.073	4.961
$Cr(OH)_2^{+1}$	7.76	$Cr_2O_7^{-2}$	0.356	1.735
$CrEDTA^-$	6.61			
Cr^{+3}	1.31			
$CrOHSO_4$ (aq)	1.09			

5.3.1.b. Effect on biomass and stress parameters

Table 2 shows the toxic effect of Cr treatments on dry weights (mg D.W·plant^{-1}) and lipid peroxidation (µmol MDA·g^{-1} F.W.) in shoots and roots of *S. vulgaris* genotypes. The dose and chemical speciation of Cr in nutrient solution resulted in a significant increment of lipid peroxidation and a reduction of biomass especially shoots. The effect on root grown was only observed when plants were cultivated at high dosage of Cr(VI). Genotypic differences have been found in biomass and also in lipid peroxidation of roots.

Both genotypes showed a maximum shoot dry weight when growing at 300 µM Cr(III) and a detriment with both Cr(VI) treatments. Shoot dry weight also presented a significant interaction between dose and genotype thus SV21 showed a significant decrease by increasing Cr(VI) concentrations whereas SV38 showed a similar decrease at 60 and 300 µM Cr(VI). Root dry weights have been not influenced by Cr stress. The lipid peroxidation increased in both shoots and roots. As it is indicated by the non significant interaction between dose and genotype; in shoots both genotypes showed similar response to Cr oxidative stress. The genotypes displayed a significant increase in MDA level regards to control when growing at 300 µM Cr(VI) but the sensitive genotype SV38 also presented an increase in MDA at 60 µM Cr(VI). In roots of both genotypes, MDA values reached a pick at 300 µM Cr(VI), but SV38 showed higher MDA concentrations than SV21 even in control plants, which is the cause of the highly significant interaction between dose and genotype. Exposure to Cr(III) and 60 µM Cr(VI) did not increase the oxidative stress in roots of *S. vulgaris*. Significant negative

correlations have been found between dry weight and MDA in shoots (p<0.001) and roots (p<0.05) (Table 3)

Table 2: Biomass (mg D.W·plant^{-1}) and lipid peroxidation (µmol MDA·g^{-1}F.W.) of *S. vulgaris* genotypes

		Biomass		Lipid peroxidation	
		Shoot	**Root**	**Shoot**	**Root**
SV21	**Control**	222b	96.2ns	7.34b	2.87b
	300 µM Cr(III)	329^{a}	71.0	10.0^{ab}	2.93^{b}
	60 µM Cr(VI)	160^{b}	79.9	10.5^{ab}	3.00^{b}
	300 µM Cr(VI)	70.5^{c}	54.4	20.3^{a}	5.71^{a}
SV38	**Control**	105^{ab}	35.9^{ns}	6.70^{c}	12.3^{b}
	300 µM Cr(III)	130^{b}	30.7	9.39^{c}	12.0^{b}
	60 µM Cr(VI)	71.5^{b}	31.2	12.6^{b}	26.3^{b}
	300 µM Cr(VI)	70.4^{b}	28.0	24.6^{a}	13.3^{a}
Dose		***	**	***	***
Genotype		***	***	ns	***
Dose x genotype		***	ns	ns	***

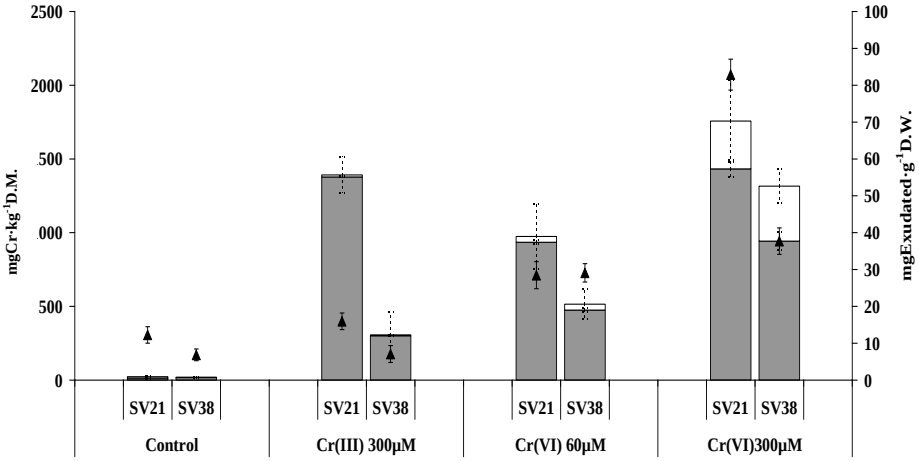
Significant differences among treatments are indicated by different letters (Duncan's test p<0.05, mean values, n=4).

Table 3: Correlation matrix of the variables studied

Variables	**Crshoot**	**CrRoot**	**shootDW**	**rootDW**	**MDAshoot**	**MDAroot**	**Exudation**	**C**	**N**	**H**	**S**
Crshoot[a]	…	,441*	-,450**	-,312	0,846**	,066	,766**	,728**	,828**	,735**	,724**
CrRoot[b]		…	-,022	-0,009	,412*	-,201	,435*	,470**	,419*	,469**	,359*
shootDW[c]			…	,640**	-,395*	-,553**	-,488**	-,508**	-,580**	-,509**	-,307
rootDW[d]				…	-,381*	-,696**	-,163	-,137	-,221	-,141	-,054
MDAshoot[e]					…	,301	,652**	,524**	,641**	,537**	,508**
MDAroot[f]						…	,047	-,049	,039	-,044	-,147
Exudation[g]							…	,917**	,887**	,917**	,749**
C[h]								…	,959**	,999**	,819**
N[i]									…	,965**	,866**
H[j]										…	,832**
S[k]											…

Pearson coefficients (ns=no significant *p<0.05; **p<0.01) chromium concentration (mg Cr·kg^{-1} D.W.) in [a] shoots and [b] roots; dry weigh (mgD.W·plant^{-1}) in [c] shoots and in [d] roots; lipid peroxidation (µmol MDA g^{-1} FW) of [e] shoots and [f] roots; [g] exudation rate (mg exudates· g^{-1} D.W.); elemental composition of root exudates (mg·g^{-1}D.W.) [h] C, [I] N, [j] H and [k] S.

5.3.1.c. Influence of Cr accumulation on exudation rate



	mgCr·kg^{-1}D.W. Shoots	mgCr·kg^{-1}D.W. Roots	mgExudated·g^{-1}D.W.
Dose	***	***	***
Genotype	ns	***	*
Dose x Genotype	ns	*	**

Figure 2: Chromium concentration in roots (grey bars) and shoots (white bars) and exudation rate (mg exudated·plant^{-1}) of two genotypes of *S. vulgaris* treated at different doses of Cr(III) or Cr(VI) for two weeks (means ± SE, n=4)

Figure 2 shows chromium concentration (mg Cr·kg^{-1} D.W.) in roots (grey bars) and shoots (white bars) as well as exudation rate (mg exudates·g^{-1}root D.W.) (solid triangles) in *S. vulgaris* genotypes under different Cr exposition. High correlation (p<0.01) have been found between Cr accumulation and exudation rate (Table 3). Metal concentration and exudation rate in *S. vulgaris* plants increased in a dose dependent manner in both shoots and roots. Chromium uptake and translocation were also dependent to metal speciation in nutrient solution. Both genotypes showed similar Cr concentration in shoots, which increased significantly in plants treated with 300 µM Cr(VI) regards to control plants. The highest Cr accumulation has been found in roots of all Cr treatments with a maximum at 300 µM Cr(VI). In both genotypes, root exudation increased with Cr(VI) treatments. When the genotypes were grown in nutrient solution with 300 µM Cr(III), root exudation did not significantly increased regards to control plants.

Genotypic differences were found between the two clones because of Cr exposure. The genotype SV21 presented significantly higher metal concentration in

roots and exudation production than SV38 as it is shown by the statistically significance between dose and genotype (figure 1).

5.3.1.d. Elemental composition of root exudates

Table 4 gives the elemental composition ($mg \cdot g^{-1}$ root D.W.) of root exudates from *S. vulgaris* genotypes after two weeks grown under different Cr exposures. The concentration of the four elements increased with Cr(VI) treatments. Positive correlations have been found between elemental concentration in roots exudates, Cr accumulation and levels of lipid peroxidation in plant tissues (Table 3). Genotype SV21 showed a greater increase of C and N concentration in Cr(VI) treatments regards to control meanwhile SV38 showed greater increases in H and S concentrations.

Table 4: Elemental composition ($mg \cdot g^{-1}$D.W) of root exudates of two genotypes of *S. vulgaris* treated with different doses of Cr(III) and Cr(VI) for two weeks

		C	N	H	S
SV21	**Control**	3.02^{bc}	0.63^{bc}	0.67^{bc}	0.04^{b}
	300µMCr(III)	1.85^{c}	0.43^{c}	0.36^{c}	0.01^{b}
	60µMCr(VI)	6.78^{b}	0.86^{b}	1.11^{b}	0.08^{b}
	300µMCr(VI)	23.1^{a}	2.09^{a}	1.59^{a}	0.20^{a}
SV38	**Control**	4.31^{c}	0.68^{c}	0.48^{c}	0.05^{b}
	300µMCr(III)	2.29^{c}	0.50^{c}	0.32^{c}	0.07^{b}
	60µMCr(VI)	7.33^{b}	0.99^{b}	1.08^{b}	0.12^{ab}
	300µMCr(VI)	10.4^{a}	1.47^{a}	3.31^{a}	0.36^{a}
	Dose	***	***	***	***
	Genotype	***	ns	**	*
	Dose x genotype	***	**	***	ns

Significant differences among treatments are indicated by different letters (Duncan's test $p<0.05$, mean values, n=4).

5.3.2. Experiment II. Population

5.3.2.a. Chemical speciation of Cr(III) and Cr(VI) in nutrient solution

	Cr(III)			Cr(VI)	
	30 µM	**60 µM**		**30 µM**	**60 µM**
Cr-EDTA^{-}	63.65	32.73	$HCrO_4^{-}$	82.95	82.81
$CrOH^{+2}$	30.21	55.48	CrO_4^{-2}	11.31	11.30
$Cr(OH)_2^{+1}$	3.68	6.747	$CaCrO_4$ (aq)	5.088	5.073
$Cr_2(OH)_2^{+4}$	0.091	1.243	$CrO_3HPO_4^{-2}$	0.382	0.381
Cr^{+3}	0.619	1.137	$Cr_2O_7^{-2}$	0.179	0.356
$CrOHSO_4$ (aq)	0.527	0.965			
Cr(OH)3 (aq)	0.463	0.851			
CrOHEDTA-2	0.445	0.229			

Table 5 Species distribution of Cr(III) and Cr(VI) in nutrient solution (%) at evaluated dosage

The 100% of total Chromium ionic species were predicted to be dissolved in the nutrient solution at the evaluated pH. Table 5 gives the ionic species distribution in the nutrient solution according to MinteQA2. Species distribution resulted to Cr(III) are strongly dependent to Cr(III) concentration in the nutrient solution. At 30 µM Cr(III), 63.65% of total Cr(III) is predicted to be as Cr-EDTA^{-} and aqueous complexes as $CrOH^{+2}$, $CrOH^{+2}$, Cr^{+3} and $Cr_2(OH)_2{}^{+4}$ are expected to achieve percentages of 30.21%, 3.68%, 0.619% and 0.091% respectively. When Cr(III) increased at 60 µM, $CrOH^{+2}$ is predicted to be the main species of total Cr(III) with a percentage of 55.48%. Secondly, Cr-EDTA^{-} would attain a percentage of 32.7% and the other aqueous complexes as $CrOH_2{}^{+1}$, $Cr_2(OH)_2{}^{+4}$ and Cr^{+3} are expected to reach percentages of 6.747%, 1.243% and 1.137% respectively. In the Cr(VI) treatments, the major Cr(VI) species predicted to occur in the nutrient solution was $HCrO_4^{-}$ accounting about 82% of total Cr(VI) in the evaluated concentration range. Approximately 11% of total Cr(VI) was predicted to occur in the form of CrO_4^{-2} in both treatments. Finally, 5% and 0.4% of total Cr(VI) were predicted as $CaCrO_4$ (aq) and $Cr_2O_7^{-2}$ respectively in nutrient solution.

5.3.2.b. Cr accumulation and root exudation

Table 6 presents chromium concentrations and biomass of the tolerant population SV21 treated for two weeks as follows: i) no chromium addition, ii) 30 µM Cr(III), iii) 60 µM Cr(III), iv) 30 µM Cr(VI) or v) 60 µM Cr(VI).

Table 6: *S. vulgaris* exposed to 30 and 60 µM of Cr(III) and Cr(VI) for two weeks: chromium concentration (mg Cr kg^{-1} DW); biomass (mg DW plant^{-1}); exudation rate (mg root exudate g^{-1} root DW); concentration in root exudates of total polyphenols (mg g^{-1} root DW); quercitin (µg g^{-1} root DW) and apiin (µg·g^{-1} root DW).

	Cr		Biomass		Root exudates			
	Shoots	**Roots**	**Shoots**	**Roots**	**Exudation rate**	**Total**	**Quercetin**	**Apiin**
Control	0.38^{c}	4.26^{b}	183^{ab}	15.6^{ns}	96.7^{a}	1.08^{a}	23.08^{a}	1.46^{b}
30µMCr(III)	0.84^{c}	16.9^{b}	225^{a}	25.1	83.6^{b}	0.48^{cd}	12.67^{bc}	1.54^{b}
60µMCr(III)	1.80^{c}	47.9^{b}	213^{a}	22.8	53.3^{a}	0.75^{d}	12.68^{bc}	1.60^{b}
30µMCr(VI)	17.1^{b}	622^{a}	139^{b}	20.4	35.6^{b}	0.34^{bc}	10.29^{c}	0.81^{b}
60µMCr(VI)	28.1^{a}	700^{a}	166^{ab}	20.9	98.4^{a}	0.93^{ab}	19.43^{ab}	2.97^{a}

Significant differences among treatments are indicated by different letters, (Duncan's test $p<0.05$, mean values, n=4).

Chromium concentrations in plants exposed to Cr(VI) were significantly higher than in control plants. Maximum values were achieved at 60 µM. Cr(III) treatments did not increase chromium concentration significantly. Shoot dry weights decreased under Cr(VI) exposure while root dry weights have not been significantly influenced by

chromium treatments. Table 5 also shows the exudation rate of these plants (mg exudated·g^{-1} root F.W.) and the concentration in root exudates of total polyphenols (mg·g^{-1}·root D.W.), quercetin (µg·g^{-1} D.W.) and apiin (µg·g^{-1}D.W.). Exudation rate diminished when plants were grown at 30 µM of both Cr(III) and Cr(VI). Total polyphenols and quercetin concentrations decreased with Cr(III) treatments and the low dose of Cr(VI) but increased and almost reach the same values of control plants at 60 µM Cr(VI). However concentration of apiin was not affected by Cr(III) and the low dose of Cr(VI) but increased twice the concentration of control when exposed to 60 µM Cr(VI). Table 7 displays the concentration (mg·g^{-1}·root F.W.) of organic acids considered in this study (oxalic, citric, malic, formic, lactic, acetic and succinic) in shoots, roots and root exudates of *S. vulgaris* after two weeks of exposition to different doses and species of Cr. The concentration of organic acids in plant tissues were: oxalic>citric>malic>acetic>succinic>formic. In root exudates organic acid concentration follow the same trend with the exception of succinic acid that is not detectable.

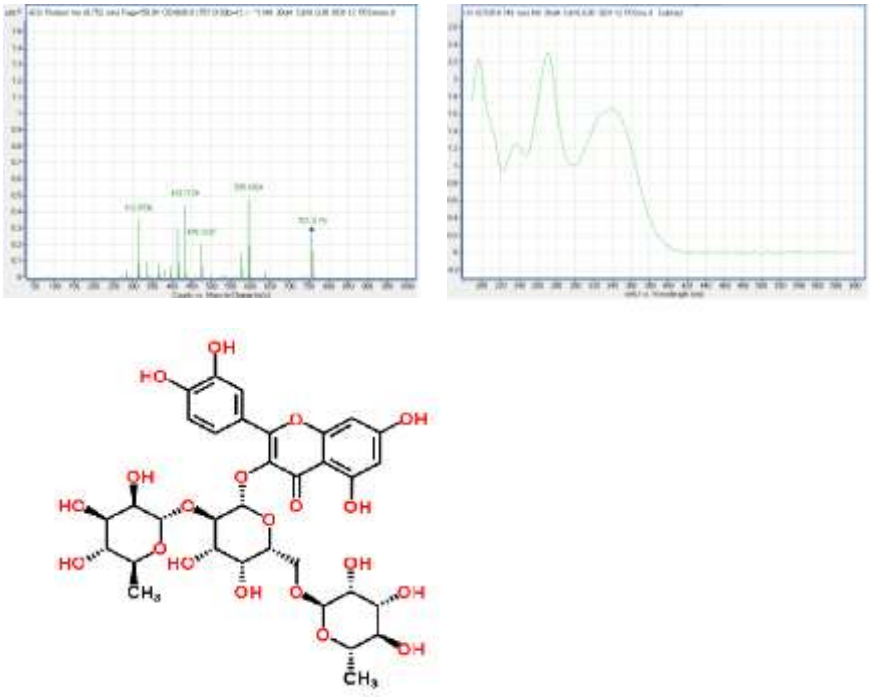


Figure 3: Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7t rihydroxy-4H-chromen-4-one. a) ms/ms spectra, b) uv/vis spectra, c) chemicals structure

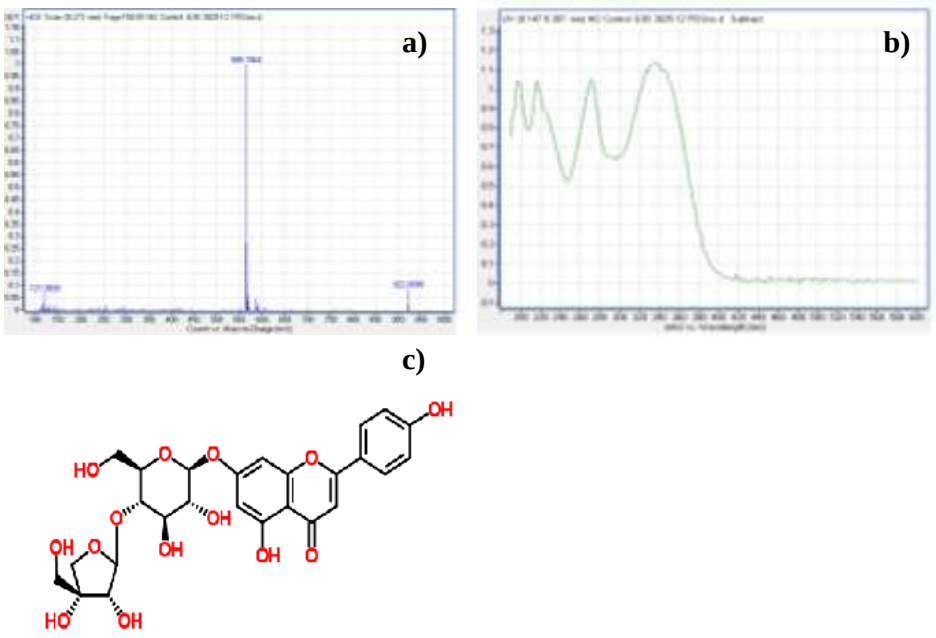


Figure 4: Apiin (Apigenin- 7-O-apiosyl-glucoside): a) ms/ms spectra, b) uv/vis spectra, c) chemicals structure.

5.3.2.c. Organic acids

Table 7 Organic acid concentration (mg·g^{-1} F.W.) in shoots, roots and exudates of *S. vulgaris* exposed to chromium for two weeks

		Control	Cr(III)		Cr(VI)	
			30µM	60µM	30µM	60µM
Oxalic	Shoots	6112^{b}	7377^{a}	6710^{ab}	4790^{c}	4450^{c}
	Roots	1920^{ab}	1859^{ab}	2072^{a}	1272^{b}	2227^{a}
	Exudates	9.58^{b}	11.89^{b}	7.63^{c}	4.30^{bc}	17.06^{a}
Citric	Shoots	69.1^{b}	100.1^{b}	82.7^{b}	226.1^{a}	281.2^{a}
	Roots	86.7^{b}	109.8^{b}	107.1^{b}	249.4^{a}	264.5^{a}
	Exudates	0.31^{ab}	0.41^{a}	0.13^{c}	0.08^{c}	0.25^{b}
Malic	Shoots	73.3^{b}	120.9^{b}	93.1^{b}	271.4^{a}	257.2^{a}
	Roots	108.3^{b}	94.4^{b}	102.2^{b}	211.8^{a}	217.8^{a}
	Exudates	0.42^{a}	0.38^{a}	0.02^{b}	0.04^{b}	0.09^{b}
Formic	Shoots	7.29^{ns}	9.42	9.65	6.97	5.08
	Roots	9.18^{b}	6.23^{b}	4.26^{b}	9.50^{b}	18.61^{a}
	Exudates	0.02^{c}	0.02^{c}	0.07^{a}	0.04^{bc}	0.04^{b}
Lactic	Shoots	39.2^{ab}	44.6^{a}	38.8^{ab}	26.8^{b}	5.3^{c}
	Roots	61.1^{b}	63.8^{b}	47.5^{b}	112.8^{a}	140.2^{a}
	Exudates	0.14^{b}	0.35^{a}	0.32^{a}	0.32^{a}	0.17^{b}
Acetic	Shoots	79.5^{b}	93.2^{b}	104.9^{b}	197.2^{a}	183.6^{a}
	Roots	78.3^{ns}	68.1	101.9	95.7	134.7
	Exudates	0.51^{b}	0.50^{b}	0.87^{a}	0.34^{b}	0.33^{b}
Succinic	Shoots	30.1^{a}	30.7^{a}	27.9^{a}	3.7^{b}	8.6^{b}
	Roots	14.0^{c}	29.6^{bc}	32.7^{bc}	53.8^{b}	109.9^{a}
	Exudates	nd	nd	nd	nd	nd

Significant differences among treatments are indicated by different letters (Duncan's test $p<0.05$, mean ± SE, n=4).

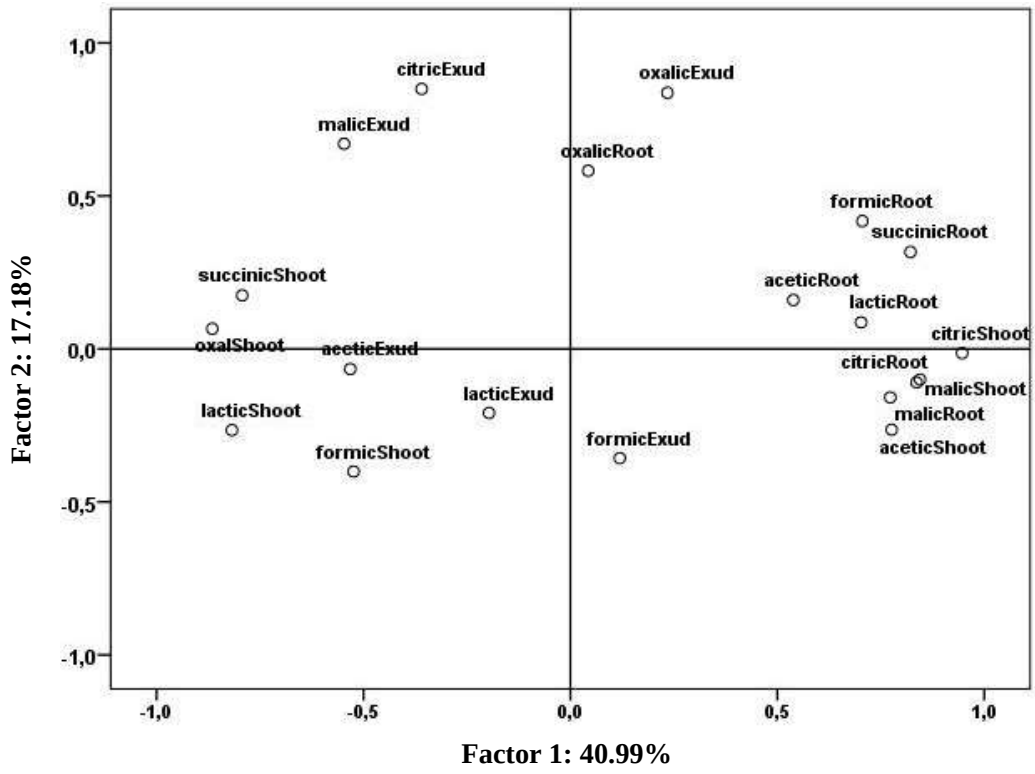


Figure 5: PCA centered score plot of organic acids content in plant tissues and root exudates of *S. vulgaris* after 2weeks of exposition for all treatments. Projection of the cases, where factors 1 and 2 explain 17.18% and 40.99% of total variability of the data.

The balance of organic acids in plant tissue and root exudates is quite complex as could be seen in the PCA (Figure 5) where no groups could be clearly discriminated, indicating that each organic acid had a different response to Cr treatments. In roots, organic acids increased their concentrations with Cr treatments as resulted from positive correlation found between chromium and organic acid concentration in roots. One exception is found in oxalic and acetic acids. Both concentrations did not significantly change due to chromium treatments.

Citric, malic and acetic acids also increased their concentration in shoots and they were positively correlated to shoot chromium concentration. Oxalic, lactic and succinic acids decreased when plants were grown in chromium as resulted a negative correlation with Cr concentration in shoots. On the other hand, oxalic and lactic acids experimented an increase at concentrations 30 µM Cr(III) (Table 7).

In root exudates, just malic acid showed negative correlation with chromium concentration in plant tissues. Other organic acids in root exudates were not correlated with Cr concentration in plant tissues thus organic acid concentration seemed to dependent on dose and speciation of chromium in nutrient solution. Oxalic acid is the only one that reached a maximum concentration at 60 μM Cr(VI). Both citric and malic acid showed a similar decrease in concentration at high dose of Cr(III) and Cr(VI). Formic and acetic acid reached a maximum at 60 μM Cr(III) and decreased to a similar concentrations than controls when grown at both doses of Cr(VI). Lactic acid concentration increased in root exudates from control plants and the low dose of Cr(VI) and showed similar concentration under 60 μM Cr(VI).

5.4. DISCUSSION

According to Shanker *el at*. (2005), chromium compounds are highly toxic to plants and are detrimental to their growth and development. The authors suggest Cr is toxic to most higher plants at 100 $\mu M \cdot kg^{-1}$ dry weight, although some crops are not affected by low Cr concentration ($3.8 \cdot 10^{-4}$ μM). Cr toxicity and chemical speciation relationships have been shown from our results. The development of shoots and roots of two genotypes of *S. vulgaris* did not decrease at 300 μM Cr(III), but dry mass did it. This fact and no effect on lipid peroxidation denote that 300 μM Cr(III) is in the hormesis range, far away from toxicity threshold. At same dose of Cr(VI) and even less, Cr caused a significant decrease in shoot biomass and increased MDA levels of both shoots and roots of *S. vulgaris* which shown Cr(VI) is more toxic than Cr(III). This is in agreement with previous findings in other plant species (Zhang *et al*., 2007; Shanker *et al*., 2004; Bennicelli *et al*., 2004). The toxicity differences found between the two Cr species have been explained based on plant uptake (Skeffington *et al*., 1976). Cr(VI) is actively taken up by sulphate carriers, meanwhile Cr(III) enters by passive mechanism and it is bound to cation exchange sites in the cell wall. That makes plants to accumulate more Cr in presence of Cr(VI) than Cr(III).

Cr toxicity is also related to enhance ROS production as shown the correlation between biomass detriment and MDA increment (table 3). Both species of Cr can

promote ROS generation in plant and cause oxidative stress, but Cr(III) concentrations between 10 and 100 times higher than of Cr(VI) are required to get the same grade of toxicity (Deflora *et al.*, 1990).

Shoots of both genotypes were more sensitive to Cr than roots. Data agrees with the findings of other authors (Zhang *et al*., 2007; Arduini *et al*., 2006; Pandey and Sharma, 2003) who suggested that root elongation decreased while hairy and lateral roots were stimulated at low doses of Cr. Thus, the root weight was constant when Cr doses were below the toxicity threshold (Suseela *et al*., 2002).

From the results found in the experiments shown here, it can be concluded that Cr accumulation was highly correlated with exudation rate. Due to the different uptake mechanisms explained above, the two genotypes of *S. vulgaris* accumulated more Cr in presence of Cr(VI) than Cr(III). In this study, there was no correlation between MDA levels in roots of *S. vulgaris* genotypes and exudation rate. Furthermore, tolerant genotype SV21 showed higher exudation rates than the sensitive one SV38. Both findings suggest that at applied doses of Cr, root exudation of *S. vulgaris* is related to the tolerance mechanism and not to the membrane damage.

Roots receive 30-60% of the net photosynthetic carbon, from which 10-20% is released by rhizodeposition (Singer *et al*., 2003) thus it is not surprising that C was the most abundant element in root exudates of *S. vulgaris.* Changes in exudation composition have been largely described under heavy metal stress when plants need to activate chelation mechanism to control metal bioavalability, transport and storage (Drzewiecka *et al*., 2012; Meier *et al*., 2012; Soudek *et al*., 2011). Baker *et al*. (2000) have been classified potential ligands into three major classes; oxygen donor ligands (eg, carboxylates: citrate, malate, oxalate), sulphur donor ligands (e.g. metallothioneins and phytochelatins), and nitrogen donor ligands (e.g. amino acids). Thus increases found in C, N, H and S concentrations in root exudates due to chromium treatments may be also related with a chelation protective mechanism. Metal tolerance is a genetically controlled characteristic (Macnair, 1993), probably for that reason, Cr accumulation caused more oxidative stress and hence, higher detriment of biomass in

the sensitive genotype SV38 than in the tolerant SV21. The exudation production and composition may be related which a worse defence machinery to Cr stress.

In order to further investigate Cr tolerance mechanisms in *S. vulgaris*, a second experiment was carried out to study the changes produced in exudation composition and organic acid balance. The tolerance population SV21 was grown in hydroponics with 30 µM Cr(III), 60 µM Cr(III), 30 µM Cr(VI) or 60 µM Cr(VI) in nutrient solution. Because of different plant development, plants obtained from seeds were more sensitive to Cr exposition than plants obtained from cuttings. Populations and clones showed similar tendencies: higher Cr accumulations when plants were exposed to Cr(VI) than Cr(III) and positive correlation between Cr(III) uptake and shoot biomass but no effect on root biomass. That means that Cr(III) applied doses are into the hormesis range during the second experiment. Also, Cr(VI) doses are very close to this range. Taken into account from the first experiment that S. vulgaris regulates its exudation rates as mechanism to undergo stress caused by Cr, it is not surprising that no increments in exudation rates and even decreases have been found when plants were grown with these doses of Cr.

Information about the role of polyphenols in root exudates under heavy metal stress is scarce. Their properties and results from previous studies (Jung *et al*., 2003; Kidd *et al*., 2001) prompted us to investigate their function in the exudation of *S. vulgaris* under Cr stress. Among polyphenols, flavonoids play an important role in rhizosphere processes such as the regulation of the interaction of roots with microorganism (Hassan and Mathesius, 2012) and plant nutrient adquisition (Cesco *et al*., 2012). From our results, total polyphenol concentrations decreased in root exudates from plant treated with Cr(III) and at low doses of Cr(VI). When plants were grown in nutrient solution with 60 µM Cr(VI), polyphenol composition in root exudates showed similar levels than those from control plants to achieve the highest concentration of total polyphenols. Similar results were found by Drzewiecka *et al*. (2012) in roots exudates of *Salix viniminalis* exposed to Ni. According to the authors, polyphenols are not only antioxidant molecules but also structural components. The drop of phenolic concentration at medium doses of metal may be due to rearrangement of plants

reactions that take place in hormesis response and it could be related with the lignification of the cell wall to immobilize metals. The two main flavonoids identified in *S. vulgaris* root exudates have been quercetin (Fig. 2) and apiin (Fig. 3). Quercetin is one of the most widely distributed flavonols in root exudates. It has been related with protection of root elongation in *Zea mays* against Al (Kidd *et al*., 2001). In root exudates of *S. vulgaris*, quercetin showed same behaviour as total polyphenolics which indicates that exudation is not related to protection of root elongation at the applied doses of Cr but with hormesis response. Apiin is a flavone, to our knowledge there is no information about its exudation in plants exposed to heavy metals. It has been demonstrated to be a potent free radical scavenger (Mikhaeil *et al*., 2004) and to have a strong ability to bind metal ions (Kasthuri and Rajendiran, 2009). From our results, an enhancement of apiin concentration was found in the root exudates of *S. vulgaris* grown at 60µM Cr(VI), thus exudation seems to be related with a protection mechanism against Cr. This mechanism could be activated just at the dose in which plants accumulated the greatest concentration of this metal.

Organic acids are well known compounds to be involved in heavy metal tolerance by transport and storage (Clemens, 2001). They have been identified as positive bio-reagents to accelerate metal absorption by root and the root-shoot transportation (Wu *et al*., 2010). XAS studies have been demonstrated that Cr in plant tissues is presented as Cr(III) form, independently from the source of Cr (Lytle *et al*., 1998; Zayed *et al*., 1998) and then it is bounded by organic acids (Aldrich *et al*., 2003). The concentration of organic acids in plant tissues of *S. vulgaris* have been correlated to Cr concentration, exception to formic acid in shoots and oxalic and acetic acids in roots. Oxalic, lactic and succinic acids showed negative correlations meanwhile malic, acetic and citric were negative correlated to Cr uptake. Concentration of citric and malic acids increased in shoot and root with increasing Cr concentrations. Both organic acids have been found to be the major complexants of Cr(III) in the xylem sap of maize (Juneja and Prakash, 2005). Citric and malic concentration have also increased in the Cd hyperaccumulator *Solanum nigrum* to reduce the stress produced and to improve Cd transport from roots to shoots (Xu *et al*., 2012). Increments in the levels of these two organic acids have also been found in *Heliantus annnus* when exposed to Al and Zn

(Saber *et al*., 1999). Oxalic, citric and malic acids were the most abundant acids in *S. vulgaris*. Harmens *et al*. (1994) studied changes in organic acids concentration in plants of *S. vulgaris* exposed to Zn. Oxalate was the most concentrated in *S. vulgaris* tissues, however, it seemed to be of no importance in chelating Zn, while citrate and malate did play a central role in Zn tolerance. The authors suggested that citrate could act as Zn chelator while malate was important to keep charge balance and as a pH-state anion. These findings suggest that citric and malic acids might be involved in tolerance mechanism to Cr in *S. vulgaris* while the role of oxalic is unclear at the applied doses of Cr.

The role of organic acids in root exudation of *S. vulgaris* is not easy to explain because there is no a partner to describe its behaviour. At the applied doses, exudation seems to be more related to dose and Cr speciation in nutrient solution or medium than to Cr concentration in plant tissues. There is one exception to malic acid. In the literature, oxalic, citric and malic acids have been very often studied in root exudates due to their high ability to bind heavy metals. Two different mechanisms have been identified in root exudation of plants exposed to heavy metals. Firstly, increments in organic acids concentration in root exudates have been described as exclusion mechanism to alleviate metal toxicity (Magdziak *et al*., 2011; Pineros *et al*., 2002; Zhu *et al*., 2011). Secondly, exudation has been related with hyperaccumulation. In this case the highest concentrations of organic acids were found at medium doses of metal exposition and the lowest concentrations were found at higher doses in which organic acids concentrations increase in root tissues (Bao *et al*., 2011; Tu *et al*., 2004b). From our results, exudation in *S. vulgaris* seems to be more related with accumulation than with exclusion. Citric and malic acids decrease in root exudates at high Cr concentration in dry tissues as they tend to accumulate in plant tissues. These data support the idea that citric and malic acid are actively involved to metal detoxification process into the plant. Formic, lactic and acetic acids showed peak of concentration at Cr doses in which plants could respond to hormesis rearrangement similar than total polyphenols and quercetin exuded as previously explained. Oxalic acid, that seems not no be related with internal tolerance to Cr, is the only organic acid studied that enhanced its concentration in roots exudates at the highest exposition to Cr(VI). Zeng *et*

al. (2008) obtained similar results in the study of organic acid exudation in rice genotypes exposed to Cr, they concluded that organic acids may be important in alleviating Cr toxicity but data were not enough to clearly describe the protection mechanism. Oxalic acid seems to play a role in Cr trafficking in the plant-rhizosphere system of *S. vulgaris* but further studies, probably by the exposition to higher doses of Cr(VI), are necessaries to clearly elucidate its role. On the other hand, Cr(III)-organic complexes have shown to be soluble in soils (Fendorf, 1995) and the application of organic acids on Cr polluted soils have been demonstrated to enhance the availability and accumulation of Cr by plants (Srivastava *et al*., 1999; Mandiwana, 2008). Thus an exclusion role of organic acids against Cr toxicity in *S. vulgaris* could be rejected. Furthermore, organic acids in soil solution have been proven to reduce Cr(VI) to the less toxic Cr(III) with subsequent formation of Cr(III)-organic complexes (Puzon, 2008). The mobility of the Cr(III)-organic complexes appeared to vary depending on the organic ligand. Multi-dentate ligands (Cr(III)-citrate and Cr(III)-malate) are more likely to form soluble complexes with Cr in comparison to uni-dentate ligands (Cr(III)-acetate, Cr(III)-lactate and Cr(III)-succinate) (Puzon, 2008). Therefore it is necessary to describe the organic acid exudation of plants in Cr polluted soils as they have influence in the oxidation state and mobility of Cr which is important in terms of plant uptake and risk assessment.

Results from this study support that exudation rate in *S. vulgaris* is strongly related to Cr concentration in dry tissues. Cr uptake in *S. vulgaris* is determined by the Cr oxidation state in nutrient solution. Exudation process does not seem to be due to passive transport or membrane integrity loss, but to be mediated by transporters which are either activated or induced by Cr. At the doses of Cr used in this assay, the composition of root exudates did not change very much. It might be suggested that the early response to Cr stress in *S. vulgaris* is related to organic acids concentration in plant tissues. Citric and malic acid seem to play a central role. It is essential to study the input of organic acids released from roots of plants grown in Cr polluted soils because they play a central role in the natural cycling of Cr.

5.5. REFERENCES

Aldrich, M.V., Gardea-Torresdey, J.L., Peralta-Videa, J.R., Parsons, J.G., 2003. Uptake and reduction of Cr(VI) to Cr(III) by mesquite (*Prosopis spp*.): Chromate-plant interaction in hydroponics and solid media studied using XAS. Environ. Sci. Technol. 37, 1859-1864.

Alford, E.R., Pilon-Smits, E.A.H., Paschke, M.W., 2010. Metallophytes: a view from the rhizosphere. Plant Soil 337, 33-50.

Arduini, I., Masoni, A., Ercoli, L., 2006. Effects of high chromium applications on miscanthus during the period of maximum growth. Environ. Exp. Bot. 58, 234-243.

Arnetoli, M., Vooijs, R., ten Bookum, W., Galardi, F., Gonnelli, C., Gabbrielli, R., Schat, H., Verkleij, J.A.C., 2008. Arsenate tolerance in *Silene paradoxa* does not rely on phytochelatin-dependent sequestration. Environmental Pollution 152, 585-591.

Baker, A., McGrath, S.P., Reeves, R.D., Simth, A.C., 2000. Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Pollutes Soils., in: Terry, N., Bañuelos, G. (Eds.). Lewis Publishers, pp. 85-107.

Bao, T., Sun, T., Sun, L., 2011. Low molecular weight organic acids in root exudates and cadmium accumulation in cadmium hyperaccumulator *Solanum nigrum* L. and non-hyperaccumulator *Solanum lycopersicum* L. African Journal of Biotechnology 10, 17180-17185.

Bennicelli, R., Stepniewska, Z., Banach, A., Szajnocha, K., Ostrowski, J., 2004. The ability of *Azolla caroliniana* to remove heavy metals (Hg(II), Cr(III), Cr(VI)) from municipal waste water. Chemosphere 55, 141-146.

Catalá, M., Gasulla, F., Pradas del Real, A.E., Garcia-Breijo, F., Reig-Arminana, J., Barreno, E., 2010. Fungal-associated NO is involved in the regulation of oxidative stress during rehydration in lichen symbiosis. BMC Microbiology 10, 297.

Cesco, S., Mimmo, T., Tonon, G., Tomasi, N., Pinton, R., Terzano, R., Neumann, G., Weisskopf, L., Renella, G., Landi, L., Nannipieri, P., 2012. Plant-borne flavonoids released into the rhizosphere: impact on soil bio-activities related to plant nutrition. A review. Biol. Fertility Soils 48, 123-149.

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486.

Deflora, S., Bagnasco, M., Serra, D., Zanachi, P., 1990. Genotoxicity of Chromium Compounds - a Review. Mutat. Res. 238, 99-172.

Drzewiecka, K., Mleczek, M., Gasecka, M., Magdziak, Z., Golinski, P., 2012. Changes in *Salix viminalis* L. cv. 'Cannabina' morphology and physiology in response to nickel ions - Hydroponic investigations. J. Hazard. Mater. 217, 429-438.

Fendorf, S.E., 1995. Surface-Reactions of Chromium in Soils and Waters. Geoderma 67, 55-71 doi: 10.1016/0016-7061(94)00062-F.

Harmens, H., Koevoets, P., Verkleij, J., Ernst, W., 1994. The Role of Low-Molecular-Weight Organic-Acids in the Mechanism of Increased Zinc Tolerance in *Silene vulgaris* (Moench) Garcke. New Phytol. 126, 615-621.

Hassan, S., Mathesius, U., 2012. The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. J. Exp. Bot. 63, 3429-3444.

Hinsinger, P., Plassard, C., Tang, C., Jaillard, B., 2003. Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: A review. Plant Soil 248, 43-59.

Jones, D.L., 1998. Organic acids in the rhizosphere - a critical review. Plant Soil 205, 25-44.

Jones, D., Dennis, P., Owen, A., van Hees, P., 2003. Organic acid behavior in soils - misconceptions and knowledge gaps. Plant Soil 248, 31-41.

Juneja, S., Prakash, S., 2005. The chemical form of trivalent chromium in xylem sap of maize (*Zea mays* L.). Chem. Spec. Bioavail. 17, 161-169.

Jung, C., Maeder, V., Funk, F., Frey, B., Sticher, H., Frossard, E., 2003. Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. Plant Soil 252, 301-312.

Kasthuri, J., Rajendiran, N., 2009. Functionalization of silver and gold nanoparticles using amino acid conjugated bile salts with tunable longitudinal plasmon resonance. Colloids and Surfaces B-Biointerfaces 73, 387-393.

Kidd, P.S., Llugany, M., Poschenrieder, C., Gunse, B., Barcelo, J., 2001. The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (*Zea mays* L.). J. Exp. Bot. 52, 1339-1352.

Kidd, P., Barcelo, J., Pilar Bernal, M., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clernente, R., Monterroso, C., 2009. Trace element behaviour at the root-soil interface: Implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.

Kupper, H., Mijovilovich, A., Meyer-Klaucke, W., Kroneck, P., 2004. Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy. Plant Physiol. 134, 748-757.

Luo, Z., Wadhawan, A., Bouwer, E.J., 2010. Sorption behavior of nine chromium (III) organic complexes in soil. International Journal of Environmental Science and Technology 7, 1-10.

Lytle, C.M., Lytle, F.W., Yang, N., Qian, J.H., Hansen, D., Zayed, A., Terry, N., 1998. Reduction of Cr(VI) to Cr(III) by wetland plants: Potential for in situ heavy metal detoxification. Environ. Sci. Technol. 32, 3087-3093.

Macnair, M.R., 1993. The genetics of metal tolerance in vascular plants. New Phytol. 124, 541-559 .

Magdziak, Z., Kozlowska, M., Kaczmarek, Z., Mleczek, M., Chadzinikolau, T., Drzewiecka, K., Golinski, P., 2011. Influence of Ca/Mg ratio on phytoextraction properties of Salix viminalis. II. Secretion of low molecular weight organic acids to the rhizosphere. Ecotoxicol. Environ. Saf. 74, 33-40.

Mandiwana, K.L., 2008. Rapid leaching of Cr(VI) in soil with Na_3PO_4 in the determination of hexavalent chromium by electrothermal atomic absorption spectrometry. Talanta 74, 736-740 .

Meier, S., Alvear, M., Borie, F., Aguilera, P., Ginocchio, R., Cornejo, P., 2012. Influence of copper on root exudate patterns in some metallophytes and agricultural plants. Ecotoxicol. Environ. Saf. 75, 8-15.

Michalak, A., 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies 15, 523-530.

Mikhaeil, B., Badria, F., Maatooq, G., Amer, M., 2004. Antioxidant and immunomodulatory constituents of henna leaves. Zeitschrift Fur Naturforschung C-a Journal of Biosciences 59, 468-476.

Olko, A., Abratowska, A., Zylkowska, J., Wierzbicka, M., Tukiendorf, A., 2008. *Armeria maritima* from a calamine heap - Initial studies on physiologic-metabolic adaptations to metal-enriched soil. Ecotoxicol. Environ. Saf. 69, 209-218.

Ozawa, T., Hanaki, A., 1990. Spin-Trapping Studies on the Reactions of Cr(III) with Hydrogen-Peroxide in the Presence of Biological Reductants - is Cr(III) Nontoxic. Biochem. Int. 22, 343-352.

Pandey, N., Sharma, C.P., 2003. Chromium interference in iron nutrition and water relations of cabbage. Environ. Exp. Bot. 49, 195-200.

Pineros, M., Magalhaes, J., Alves, V., Kochian, L., 2002. The physiology and biophysics of an aluminum tolerance mechanism based on root citrate exudation in maize. Plant Physiol. 129, 1194-1206.

Puzon, G., Roberts, A., Kramer, D., Xun, L., 2005. Formation of soluble organo-chromium(III) complexes after chromate reduction in the presence of cellular organics. Environ. Sci. Technol. 39, 2811-2817.

Relly, C.A., Aust, S.D., 1999. Measurent of Lipid Peroxidation, in: Wiley, J. (Ed.), pp. 1.

Rengel, Z., 2002. Genetic control of root exudation. Plant Soil 245, 59-70.

Saber, N., Abdel-Moneim, A., Barakat, S., 1999. Role of organic acids in sunflower tolerance to heavy metals. Biol. Plant. 42, 65-73.

Sarret, G., Willems, G., Isaure, M., Marcus, M.A., Fakra, S.C., Frerot, H., Pairis, S., Geoffroy, N., Manceau, A., Saumitou-Laprade, P., 2009. Zinc distribution and speciation in *Arabidopsis halleri* x *Arabidopsis lyrata* progenies presenting various zinc accumulation capacities RID A-1549-2010 RID A-2499-2009. New Phytol. 184, 581-595Schmidt, B.M., Erdman, J.W., Lila,

Schmidt, B.A.,Erdman J.W.,Lila, M.N. 2005. Effects of food processing on blueberry antiproliferation and antioxidant activity. J. Food Sci. 70, S389-S394.

Shanker, A.K., 2008. Mode of Action and Toxicity of Trace Elements, in: M. N. V. Prasad (Ed.). Wiley, Hoboken (New Jersey), pp. 525-555.

Shanker, A.K., Djanaguiraman, M., Sudhagar, R., Chandrashekar, C.N., Pathmanabhan, G., 2004. Differential antioxidative response of ascorbate glutathione pathway enzymes and metabolites to chromium speciation stress in green gram (*Vigna radiata* (L.) R.Wilczek. cv CO 4) roots. Plant Science 166, 1035-1043.

Singer, A.C., Crowley, D.E., Thompson, I.P., 2003. Secondary plant metabolites in phytoremediation and biotransformation. Trends in Biotechnology 21, 123-130.

Skeffington, R.A., Shewry, P.R., Peterson, P.J., 1976. Chromium Uptake and Transport in Barley Seedlings (*Hordeum vulgare*-L). Planta 132, 209-214.

Sobrino-Plata, J.,Herreo, J.,Carrasco-Gil, S., Perez-Sanz, A., Lobo, C., Escobar, C., Millán, R., Hernández, L. 2013. Specific stress responses to cadmium, arsenic and mercury appear in the metallophyte *Silene vulgaris* when grown hydroponically. RSC Advances 3, 4736.

Soudek, P., Petrova, S., Vanek, T., 2011. Heavy metal uptake and stress responses of hydroponically cultivated garlic (*Allium sativum* L.). Environ. Exp. Bot. 74, 289-295.

Srivastava, S., Prakash, S., Srivastava, M.M., 1999. Chromium mobilization and plant availability - the impact of organic complexing ligands. Plant Soil 212, 203-208.

Stohs, S., Bagchi, D., 1995. Oxidative Mechanisms in the Toxicity of Metal-Ions. Free Radical Biology and Medicine 18, 321-336 .

Suseela, M., Sinha, S., Singh, S., Saxena, R., 2002. Accumulation of chromium and scanning electron microscopic studies in Scirpus lacustris L. treated with metal and tannery effluent. Bull. Environ. Contam. Toxicol. 68, 540-548..

Tu, S.X., Ma, L., Luongo, T., 2004a. Root exudates and arsenic accumulation in arsenic hyperaccumulating *Pteris vittata* and non-hyperaccumulating *Nephrolepis exaltata*. Plant Soil 258, 9-19.

USEPA, 1992. Method 7196a. Chromium hexavalent (colorimetric).

Wang, P., Bi, S., Wang, S., Ding, Q., 2006. Variation of wheat root exudates under aluminum stress. J. Agric. Food Chem. 54, 10040-10046.

Wenzel, W.W., 2009. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321, 385-408.

Wu, G., Kang, H., Zhang, X., Shao, H., Chu, L., Ruan, C., 2010. A critical review on the bio-removal of hazardous heavy metals from contaminated soils: Issues, progress, eco-environmental concerns and opportunities. J. Hazard. Mater. 174, 1-8.

Xu, J., Zhu, Y., Ge, Q., Li, Y., Sun, J., Zhang, Y., Liu, X., 2012. Comparative physiological responses of *Solanum nigrum* and *Solanum torvum* to cadmium stress. New Phytol. 196, 125-138 .

Zayed, A., Lytle, C.M., Qian, J.H., Terry, N., 1998. Chromium accumulation, translocation and chemical speciation in vegetable crops. Planta 206, 293-299.

Zeng, F., Chen, S., Miao, Y., Wu, F., Zhang, G., 2008. Changes of organic acid exudation and rhizosphere pH in rice plants under chromium stress. Environmental Pollution 155, 284-289.

Zhang, X., Liu, J., Huang, H., Chen, J., Zhu, Y., Wang, D., 2007. Chromium accumulation by the hyperaccumulator plant *Leersia hexandra* Swartz. Chemosphere 67, 1138-1143 .

Zhao, F., McGrath, S., Crosland, A., 1994. Comparison of 3 Wet Digestion Methods for the Determination of Plant Sulfur by Inductively-Coupled Plasma-Atomic Emission-Spectroscopy (Icpaes). Commun. Soil Sci. Plant Anal. 25, 407-418.

Zhu, X.F., Zheng, C., Hu, Y.T., Jiang, T., Liu, Y., Dong, N.Y., Yang, J.L., Zheng, S.J., 2011. Cadmium-induced oxalate secretion from root apex is associated with cadmium exclusion and resistance in *Lycopersicon esulentum*. Plant Cell and Environment 34, 1055-1064.

Elucidating insights into the chromium detoxification pathway in the multi-metal accumulator *Silene vulgaris*

Abstract

Phytoremediation could be a novel alternative to be applied in areas polluted with wastes from chromium industries. Cr(III) and Cr(VI) are the two most common oxidation states of Cr but Cr(VI) is much more available and toxic to living organisms than Cr(III). This study have been performed to investigate the ability of *Silene vulgaris* to uptake Cr(III) and Cr(VI) and the changes induced in plants making specially attention to asses the mechanism used by this species to tolerate high doses of Cr(VI). With this purpose a hydroponic study have been carried out and a combination of selective X-ray spectroscopic techniques, Scanning Electron and Light Microscopy and Diffusive Reflectance Infrared Fourier Transform Spectroscopy have been applied. *S. vulgaris* accumulated more Cr in presence of Cr(VI) inducing biomass reduction and phenotypic and chemical changes in plants. The tolerance of *S. vulgaris* to Cr(VI) implies the total reduction of Cr(VI) to Cr(III) in the fine lateral roots tips which makes this species interesting for *"in situ"* clean up of Cr polluted soils.

Keywords: *Chromium speciation, metal mapping, S. vulgaris, XAS, TEM, DRIFTS-FTIR.*

6.1. BACKGROUND

It is well recognised that metal speciation is more important than total concentration in terms of availability and toxicity in the environment. In the case of plants, metal speciation determinates the distribution of an element in plant tissues and ultimately the various mechanisms of detoxification, toxicity and availability. The chemical form and distribution of a metal in the plant is also important to determinate the risk assessment derived from animal/human consumption.

In this sense, the development of new nondestructive spectroscospy techniques, with minimal sample preparation is useful to research metal speciation and distribution in plants. Synchrotron X-ray fluorescence (SXRF) provides 2D images of elemental distributions that results in x –y pixels of quantitative concentrations of elements. This technique could be used in tandem with X-ray absorption spectroscopy (XAS) to speciate an element at points of interest within the 2D maps (Lombi *et al*., 2011a). The main advantages of these techniques are: i) they are elemental specific methods that allows the identification of an specific element; ii) samples require minimal preparation eliminating possible artefacts in the oxidation state and chemical bonding that can occur as a result of homogenization or extraction procedures; iii) the quantity of sample required is small (<1g); iv) they offers low detection limits (Lombi *et al*., 2011b; Lombi and Susini, 2009; Gardea-Torresdey *et al*., 2005b). All these characteristics allow the detection of metals in plants tissues in the forms in which they are actually present in the intact and functioning plant.

As it has been previously mentioned, Cr(VI) is the most oxidizing and toxic form of Cr. By using XAS, an important finding in the field of Cr speciation has been that some plants are able to upatake Cr(VI) and then reduce this toxic chemical species to another less toxic as Cr(III). (Montes-Holguin *et al*., 2006; Howe *et al*., 2003; Lytle *et al*., 1998). It is not clear, if all plants have this ability or if it is a specific characteristic of some of them. (Zayed *et al*., 1998). The use of plants showing the Cr(VI) reduction to Cr(III) as a detoxification mechanism may be of interest to address and implement "*in situ"* strategies of soil decontamination.

Scanning Electron Microscopy (SEM) is used for the anatomical study of plant samples. This technique provides excellent and high resolution images of biological ultrestructure. It can be used to study morphological effects of metals on plants and imaging highly detailed structure to correlate with elemental distribution obtained with SXRF (Lombi *et al*., 2011a).

Metal toxicity to plants could induce biochemical changes thus interfering with their normal growth and compromising their use in soil recuperation and revegetation processes. The use of Infrared Fourier Transform Spectroscopy (FTIR) is useful in the study of these induced changes (Dokken *et al*., 2005; Budevska *et al*., 2003). This technique also provides information about the identity of functional groups involved in metal binding (D'Souza *et al*., 2008; Sawalha *et al*., 2007).

The objectives of this study were: i) to compare Cr uptake and the phenotypic and biochemical changes induced in *S. vulgaris* when grown in Cr(III) or Cr(VI) supplemented media and ii) to describe the Cr(VI) tolerance pathway for *S. vulgaris.* To achieve these objectives, a combination of x-ray spectroscopic techniques, scanning electron and light microscopy and diffusive reflectance infrared Fourier transform spectroscopy have been used.

6.2. MATERIALS AND METHODS

6.2.1. Plant material and growth conditions

Seeds of a chromium tolerant population of *S. vulgaris* were collected from Rozas de Puerto Real (Madrid, Spain). Previous studies have shown an EC_{100} for this population between 30 and 100 µM (data not shown). Seeds were germinated in Petri dishes with 500 ppm of Giberelic acid in the dark at room temperature. Seeds were then transferred into polystyrene trays (16 plants per tray) filled with vermiculite and placed in a growth chamber (Conviron Adaptis 1000) set to a photoperiod of 16h/8h, light intensity of 164.53 µmol photons $m^{-2}s^{-1}$ and a day/night temperature 20ºC/16ºC,). Before metal exposure, plants were precultured by adding 1 L of a modified ¼ strength Hoagland's nutrient solution containing the following constituents: 3mM KNO_3, 2 mM

$Na(NO_3)_2 \cdot 4H_2O$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 50 μM NaCl, 25 μM H_3BO_3, 2 μM $ZnSO_4 \cdot 7H_2O$,2 μM $MnSO_4 \cdot H_2O$, 0.1 μM $CuSO_4 \cdot 5H_2O$, 0.5 μM $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 20 μM Fe(III)- HBED. At the end of the three weeks preculture period, the plants were then randomly selected to be treated as follows: a) control, no Cr addition, b) 30 μM Cr(III), c) 60 μM Cr(III), d) 30 μM Cr(VI) and d) 60 μM Cr(VI). Cr(III) was provided as $Cr(NO_3)_3 \cdot 9H_2O$ and Cr(VI) as $K_2Cr_2O_7$. Treatments were arranged in a randomized complete block statistical design (n = 3). Solutions were buffered with 2 mM of MES and pH was adjusted to 5.5 with KOH before use, they were replenished with distilled water daily and completely renewed twice a week.

6.2.2. Plant tissue analysis

Plants were harvested after 3 weeks of treatment. Roots and shoots were separated and washed thoroughly with water. Roots were also rinsed with 0.001 M of $CaCl_2$ to remove weakly sorbed metals. Samples were immediately frozen in liquid nitrogen and stored at -80ºC and then freeze-dried under vacuum for 48 hours at -80ºC. Plant dry weights were recorded and then the samples were ground and digested in MARS express (CEM, Matthews, North Carolina) microwave digester using method EPA 3052. After cooling, the digests were diluted to 50 ml with ultra-pure double-deionized water (18.2 Ω Millipore, Billerica, MA) and then analysed for Cr concentrations using an Agilent 7500 series ICP-MS (Santa Clara, Ca). After every 20^{th} sample, one sample duplicate, blank and standard reference material (NIST SRM 1573a Tomato) were digested and analyzed to account for matrix effects, carry over and metal recovery, respectively.

6.2.3. Statistical treatment

Statistical analysis was performed using the statistical package SPSS version 19.0. Data from dry weights and Cr accumulations were analysed by General Lineal Model (GLM) using chromium treatment as experimental factor at $\alpha = 0.05$ using the F-test. GLM was followed by a post hoc Duncan test to assess the significance of differences among treatments for each variable. The

results were shown within the tables as lower case letters. Values given in the tables indicate mean values (n=3) ± standard error (S.E.).

6.2.4. Synchrotron based X-ray spectroscopy (μ-SXRF, μ-XANES)

Chromium speciation and element distributions were determined using synchrotron micro x-ray fluorescence spectroscopy (μ-SXRF, μ-XAFS) on beamline 10.3.2 (1.9 GeV and 300 mA) at the Advanced Light Source, Lawrence Berkeley National Lab (ALS-LBNL, Berkeley, CA). Fresh plants from the 60 μM Cr(VI) treatment were transported to the beamline where, just prior to analysis, leaves, stems and roots were removed, rapidly frozen in liquid N_2 and mounted on a Peltier cold stage using silica vacuum grease. The stage was maintained -30ºC throughout the experiment. Prior to analysis the beamline was calibrated using metal foil standard. The sample stage was attached to an x,y,θ stepping stage positioned 45° to the incident x-ray beam. For collecting μ-SXRF maps, the beam energy was set to 11 keV such that the Kα fluorescence line intensities of Cr, Mn, Fe, Zn, Cu, Ni, Ti, As Mg, Ca, and K were detected using a 7-element Canberra Ultra LE-Ge detector (Meriden, CT, USA) positioned 90° to the incident beam. Analysis started by first collecting coarse fluorescence maps using beam sizes from 5 to 16 μm and step sizes between 20 and 5 μm depending on the area mapped and the resolution required. The dwell time was between 20 and 100 ms. Chromium oxidation state were initially collected to assess the distribution of Cr(VI) and Cr(III) in the plant tissues. Each oxidation state map was recorded at the energy of maximum absorbance for each Cr species and above the absorption edge of both (5984.00, 5993.00, 6006.00 keV, respectively). After several maps were collected in this fashion it was determined that only Cr(III) was present in the tissues and the subsequent maps were recorded at 11 keV.

Using the μSXRF maps, regions of interest ("hotspots") were identified and μXANES spectra were collected in these spots. The oxidation sate of Cr in the plants tissues was determined using μXANES using a quick-scanning mode which permitted the collection of a single spectrum in ~40 s allowing for the detection of any beam induced changes in the Cr oxidation state. On average, 5 spectra were collected at each

hotspot in fluorescence mode from 50 eV below to 150 eV above Cr k-edge (5989.02 keV). Prior to averaging each spectra was background subtracted and normalized prior to least squares linear combination fitting (LCF) using using Athena EXAFS analysis software (Ravel and Newville, 2005) The following Cr(III) standards used in the fits were prepared as stated in (Howe *et al*., 2003): Cr(III)-acetate, Cr(III)-citrate, Cr(III)-EDTA, Cr(III)-malate, Cr(III)-malonate, Cr(III)-nitrate, Cr(III)-oxalate and Cr(III)-tartrate. Standard spectra were not included in the fitting unless they decreased the NSS by 20% or more.

6.2.6. Scanning Electron and Light Microscopy

Light microscopy and Scanning Electron Microscopy (SEM) were carried out to locate Cr in plant tissues and to study phenotypic changes induced in Cr exposed plants. Fresh samples were examined in Nikon Eclipse 90i light microscope. Freeze-dried samples were cut with a surgical blade to obtain regular cross-sections of steam and leaves. Pieces were then mounted on a carbon stub, sputter coated with 2-3 nm of gold and analysed on a Model S-3200-N Hitachi scanning electron microscope (SEM)

.

6.2.7. Diffusive Reflectance Infrared Fouried Transform Spectroscopy (DRIFTS-FTIR)

Infrared spectroscopy was used to evaluate the plant structural or biochemical changes that may have occurred with Cr exposure. Infrared spectra from the mid-IR region (600 to 1800 cm^{-1}) provide the most information about the plant functional groups of organic and amino acids implicated in metal complex formation. A 5 % mixture of freeze dried leaves and roots from each of the treatments were prepared by grinding and thoroughly mixing with potassium bormide (KBr). Infrared spectra were recorded using a Thermo Scientific Nicolet 6700 FTIR equipped with a Smart collector diffuse reflectance accessor and MCT/A detector. Each sample spectra is an average of 254 spectra collected with 4 cm^{-1} resolution.

6.3. RESULTS AND DISCUSSION

6.3.1. Plant growth and Cr uptake

Dry mass reduction in shoots from plants grown in 30 µM Cr(III) and Cr(VI) are similar (~20%) (Table 1). There was a significant decrease in shoot biomass (~40%) in plants grown with 60 µM Cr(VI) and a significant increase (+20%) in shoot dry mass in plants grown in the 60 µM Cr(III). There is a similar reduction in root biomass (~24%) in plants grown with 30 and 60 µM Cr(III) and the lower dose of Cr(VI), however the 60 µM Cr(VI) treatment resulted in ~60% reduction in root biomass. Overall, Cr(VI) was the most inhibitory to whole plant (root+shoot) dry matter yield, as has been observed in other species (Shanker et *al*., 2005), while the 60 µM Cr(III) treatment resulted in a noticeable increase in shoot dry mass due to hormesis rearrangements as previously reported in shoots of *Phaselus vulgaris* (Bonet *et al*., 1991; Poschenrieder *et al*., 1991)

Table 1: percentage of dry weighs regards controls and total chromium concentration in dry tissues in shoots and roots of *S. vulgaris* exposed to 30 and 60 µM of Cr(III) and Cr(VI).

Treatment	% of Dry weights regards controls		µg Cr·g^{-1}DW	
	Shoot	Roots	Shoot	Root
Control	100%	100%	0.45±0.21^{c}	0.75±0.32^{d}
30µM Cr(III)	85%	70%	2.19±0.87^{c}	105±18^{cd}
60µM Cr(III)	120%	78%	4.32±0.94^{c}	186±49^{c}
30µM Cr(VI)	81%	79%	51.3±7.9^{b}	926±109^{b}
60µM Cr(VI)	59%	58%	73.5±10.4^{a}	1155±77^{a}

Different lowercase letters means significant differences between chromium treatments. (Duncan's test $p<0.05$, mean ± SE, n=3).

Total Cr concentrations were significantly greater in the shoots of plants grown with Cr(VI), especially at the higher dose, compared to those of the controls and plants grown in Cr(III) (Table 1). Plants grown in 30 and 60 µM Cr(III) had significantly more Cr in their roots than the control plants (Table 1). The highest concentrations in the roots were found in plants grown in Cr(VI), with significantly more in the 60 µM Cr(VI) treatment. Chromium has been mainly accumulated in roots and poorly translocated to the shoots in all treatments which has been described as a mechanism by plants to prevent Cr toxicity (Sharma *et al*., 2003; Mcgrath, 1982) likely due to the affinity of Cr to bind to root cell wall (Shanker *et al*., 2009; Zayed *et al*., 1998). The

more efficient uptake of Cr(VI) compared to Cr(III) in *S. vulgaris* results from the different mechanism used by the plant to take up Cr. Cr(VI) is actively taken up in metabolically driven processes involving the same transporters used for essential anions such as sulphate, in contrast to Cr(III), which is passively taken up (Skeffington *et al*., 1976).

6.3.2. Distribution and speciation of Cr on *S. vulgaris* tissues (XAS)

Oxidation state mapping revealed only Cr(III) was present in the roots from plants grown with Cr(VI), indicating that Cr(VI) was actively reduced to Cr(III) within the root system or the rhizosphere. No correlations were observed between Cr and the other elements analysed via μ-SXRF. Cr(III) was found throughout the root system particularly in the root tips (Figure 1.f and Figure 1.i) possibly eluding to a greater importance of root tips in the process of reducing and taking up Cr as previously stated in other species (Bluskov *et al*., 2005; Aldrich *et al*., 2003; Lytle *et al*., 1998). Many of the root tips appeared to be collapsed and in some cases curled (Figure 1.i and Figure 2). Several researchers have noted the same trend in plants grown with Cr(VI) *(Zou et al*., 2006; di Toppi *et al*., 2002; Liu *et al*., 1992). Root tip deformation is attributed to the direct contact of roots with the metal that causes a collapse of roots and subsequent inability to absorb water from the medium (Hayat *et al*., 2012). Moreover, the oxidizing power of Cr(VI) leads to the plasmolysis of root cells causing leakage of the cell content (Vazquez *et al*., 1987). Cr injury has been demonstrated to be concentration dependent (Han *et al*., 2004). The sensitive surface of roots meristematic zone is less suberized and hence more permeable to Cr thus it has been observed to be the most affected by Cr exposure showing irregular profile of cell walls and retraction of plasma membranes (di Toppi *et al*., 2002; Samantary, 2002). Both the direct effect on root structure and chromosomal aberrations induced by Cr in roots cells causes the inhibition cell division (Liu *et al*., 1992) and the extension of the cell cycle (Sundaramoorthy *et al*., 2010) which explain root growth retardation found in roots of 60μM Cr(VI) treated plants. The decrease on shoots dry mass observed in these plants is a secondary effect of the inability of roots to absorb nutrients and water. Moving up from the root tip, the Cr appears to outline the root cells where it is likely bound to the cell wall, as has been observed in other studies (Mangabeira *et al*., 2011; Howe *et al*.,

2003). Cr can also appear to be in the vascular tissues of the roots indicated by the pattern of Cr following the trajectory of the root (Figure 1.g and h). Cr uptake has been described to be across the root radius (Mangabeira *et al*., 2006), at low doses it is preferentially retained in the root cortex cells to avoid Cr toxicity, at higher concentrations Cr cross endodermis via symplast to be transported to the upper parts of the plant (Bluskov *et al*., 2005; Shanker *et al*., 2005).

Within the stem, Cr(III) was found in the xylem (Figure 1.d and Figure 1.e) but absent from the phloem and the other tissues outside of the vascular elements indicating that the stem is used for transport and not storage of Cr in *S. vulgaris*. Cr(III) mainly accumulated in the mesophyll (both palisade and spongy) of the leaf tissues and was absent from the other leaf tissues (Figure 1.c). In the leaf tips (Figure 1.a and b), Cr(III) clearly outlines leaf cells where it appears to be stored in the cell wall; a pattern that is especially evident in the leaf margins. Chromium did not appear to accumulate in the vascular system of the leaves (Figure 1.a and 1.b). Interestingly, there were discrete spots of Ca accumulated (blue in Figure 1.d) in some of the phloem tissues of the stem and within the leaf veins (blue in Figure 1.c and d). Increasing concentration of Ca in plants exposed to Cr(VI) over controls has been described in rice and *Salsola kali* (Zeng *et al*., 2010; Gardea-Torresdey *et al*., 2005a) and it has been related to homeostasis regulation to avoid Cr toxicity. In addition, the formation Ca-heavy metal grains have been proposed as a mechanism to chelate heavy metals to prevent their presence in the cytoplasm (Sarret *et al*., 2006 ; Choi and Harada, 2005). Further studies are required to clarify whether Ca play any protective role against Cr in *S. vulgaris.*

In the +6 oxidation state Cr is in 4-fold, tetrahedral coordination (e.g. CrO_4^{2-}) which is indicated by a diagnostic large pre-edge peak due to the 1s to 3d electron transition. When in the +3 oxidation state, Cr forms complexes with 6-fold, octahedral coordination, in which case the diagnostic pre-edge peak is notably smaller and is located at slightly lower energy (Zayed *et al*., 1998). In all of the XAS scans form our plant sample grown with Cr(VI) the pre-edge feature was similar to that of Cr(III) model compounds demonstrating that all Cr in *S. vulgaris* was present as Cr(III) (data not shown). The ability of plants to reduce Cr(VI) to the less toxic Cr(III) has been

observed in other species (Zhao *et al*., 2009; Howe *et al*., 2003; Zayed *et al*., 1998) and it is thought to represents an important detoxification pathway for plants. It seems that Cr reduction in *S. vulgaris* takes place within the root system or rhizosphere possibly catalysed by microbes or a plant membrane-bound reductase. The process by which reduction of Cr(VI) takes place in plants has not been yet fully elucidated. Specific chromate reductase has been isolated in bacteria (Opperman and van Heerden, 2008). Some researchers support that a similar enzyme may be present in plants (Shanker *et al*., 2009; Cervantes *et al*., 2001; Lytle *et al*., 1998). Shanker *et al.* (2009) found 45 percent of similarity of sequence between dihydrolipoyl dehydrgenase/oxidoreductase isolated from various plants and the chromate reductase isolated from bacteria. However, most of researchers consider that in plants Cr(VI) reduction may be mediated by Fe(III)-reductase enzymes (Singh, 2013; Santana *et al*., 2012; Zayed and Terry, 2003). This is supported by the fact that Cr supply to Fe-deficient plants increases the activity of root associated Fe(III)-reductase (Schmidt, 1996) and increase the availability of Fe to plants (Bonet *et al.,* 1991).

Best linear combination fits of the μ-XANES spectra from the roots were achieved using a combination of Cr(III)-nitrate, Cr(III)-acetate, Cr(III)-citrate and to a lesser extent Cr(III)-malonate and Cr(III)-Oxalate (Table 2). As stated by Aldrich (2003) nitrogen is main component of plant cell and structures thus the chromium distribution as Cr(III) nitrate is not surprising.

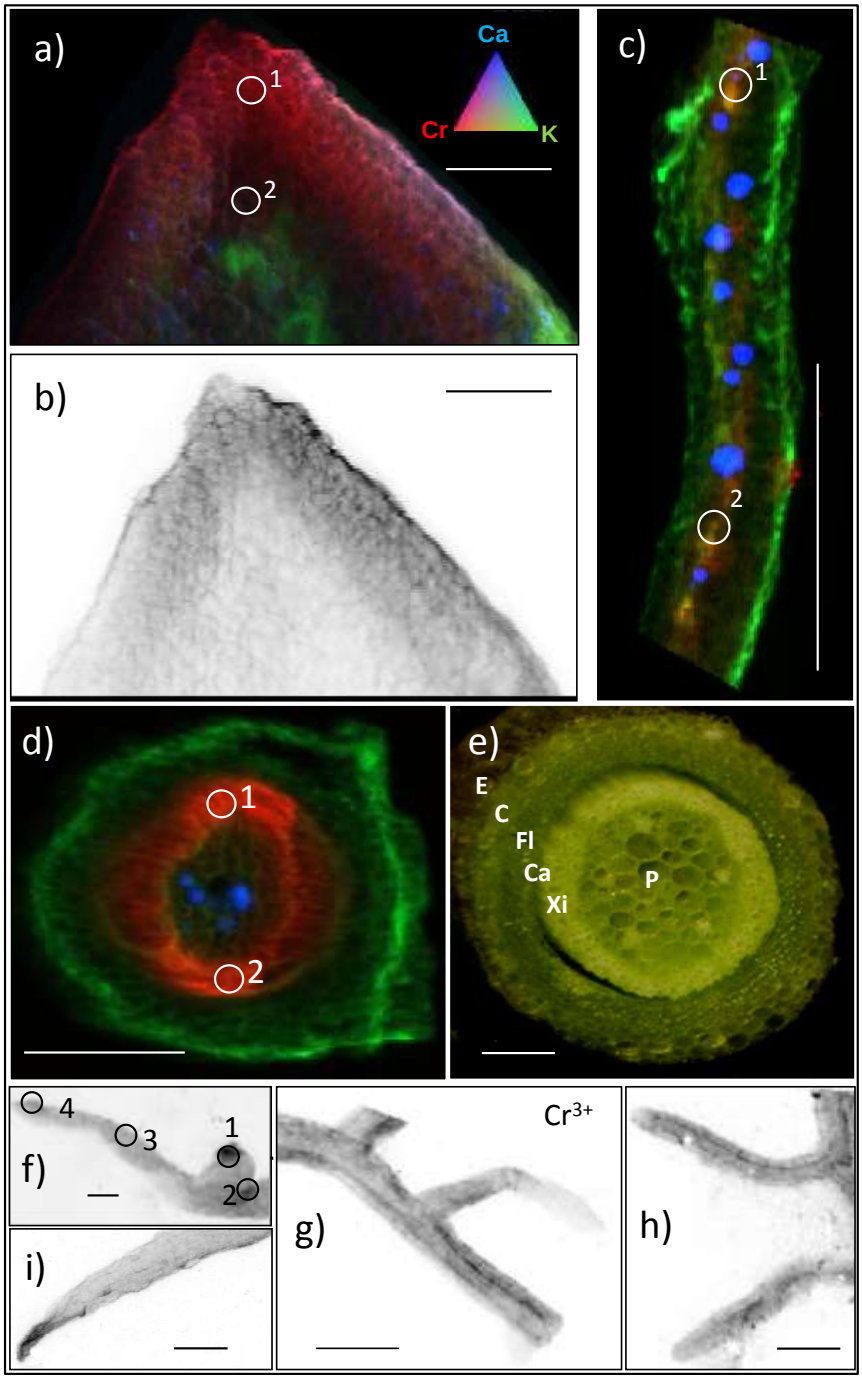


Figure 1: Micro synchrotron X-ray fluoresce (µSXRF) maps showing the distribution of Cr(III) (red), K(green) and Ca (blue) in a,b) leaf surface, c) leaf cross section, d)stem cross section, f) main roots and g) and h) fine lateral roots from *S. vulgaris* grown three weeks in 60 µM Cr(VI). Circles correspond to spots where µ-XANES spectra were collected. Light microscope image of stem cross-section e). E: epidermis, Pm: palysade mesophyll, Sm: spongy mesophyll, C: cortex, Ph: phloem, Ca: cambium, Xi: xylem and P: pith.

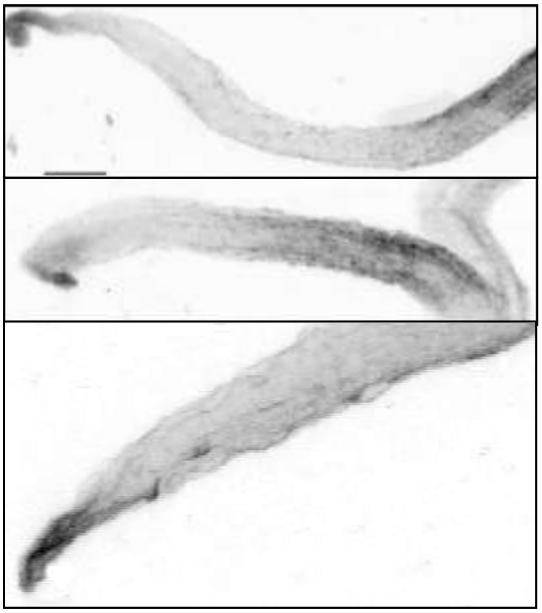


Figure 2: micro synchrotron x-ray florescence (µ-SXRF) micrographs showing the distribution of Cr^{3+} in the roots of *S. vulgaris* grown in 60 µM Cr(VI).

On the other hand, in plants organic acids are known to play an important role in metal tolerance, transport through the xylem and vacuolar sequestration (Clemens, 2001) and Cr(III) has been shown to form stable complexes with them *(Srivastava et al.*, 1999). Using electron parametric resonance (EPR) in Subterraneam Clover and Indian mustard exposed to Cr, Howe *et al*. (2003) demonstrated that different Cr(III) complexes may be predominant in different plant species. That explains why some authors found that Cr(III) oxalate is the predominant form of Cr in plant tissues (Bluskov *et al*., 2005; Lytle *et al*., 1998) while others found that it is Cr(III) acetate (Montes-Holguin *et al*., 2006; Aldrich *et al*., 2003). Citrate and malonate have been found to form Cr(III) complexes in roots and xylem of some plants (Juneja and Prakash, 2005; Lyon *et al*., 1969). Moreover citrate seems to be important in the transport of Zn through the xylem of *S. vulgaris* (Harmens *et al*., 1994). Thus, from our results it could be hypothesized that acetate play a role in chelating Cr in storage sites (roots and leaves) meanwhile citrate and malonate participate as Cr(III)-chelators in transport processes as they are found in roots and stems.

Table 2: Least squares Linear combination fits (LCF) for the µXANES spectra collected at points indicated in Figure 1

Sample	Cr(III)-Acetate	Cr(III)-Citrate	Cr(III)-Malonic	Cr(III)-Nitrate	Cr(III)-Oxalate	SUM (%)	NSS
	---------- % ----------						
LeafCross Spt1	57.3	0	0	0	41.5	99	1.55E-03
LeafTip1 Spot1	28.9	37.6	0	30.5	0	97	7.86E-05
LeafTip2 Spot1	28.8	44.4	0	19.8	17.5	111	4.26E-04
Leaf Avg.	38.3	27.3	0	16.8	19.7	102	
Stem3 Spot1	0	38	29.3	38.8	0	106	2.03E-04
Stem3 Spot2	0	31.3	21.7	40.7	0	94	3.22E-04
Stem2 Spot2	17.5	38.1	0	42.7	0	98	1.75E-04
Stem Avg.	5.8	35.8	17	40.7	0	99	
Root4 Spot1	0	27.1	16.4	54.9	0	98	1.04E-04
Root4 Spot3	26.7	0	34.4	41.7	0	103	1.02E-04
Root4 Spot4	36.3	30.1	0	37.1	0	104	1.20E-04
Root Avg.	24.1	21.6	12.7	42.4	0	101	
Overall Avg.	22.88	27.56	10.18	34.22	5.9	101	

NSS = Normalized Sum Square

Note: standards were not included unless they improved the fit by more than 10%

6.3.3. Phenotypic changes in leaves of *S. vulgaris* treated plants

There were evident differences between leaves from control and 60 µM Cr(VI) plants in both light microscopy and SEM (Figure.3). In light microscopy images, dense granular precipitates, absent in control and Cr(III) leaves, can be seen in the leaves of plants grown in Cr(VI) (Figure 2.c) which correlate with the large number of grains in the SEM micrograph (Figure 2. f). The granules may be starch grains as was also observed using SEM in leaves of *Phaselous vulgaris* (Vazquez *et al.*, 1987) and by Transmission Electron Microscopy (TEM) in the chloroplasts of *Spirodela polyrhiza* (Appenroth *et al.*, 2003) and *Lycopersicon esculetum* (di Toppi *et al.*, 2002) grown in Cr(VI).

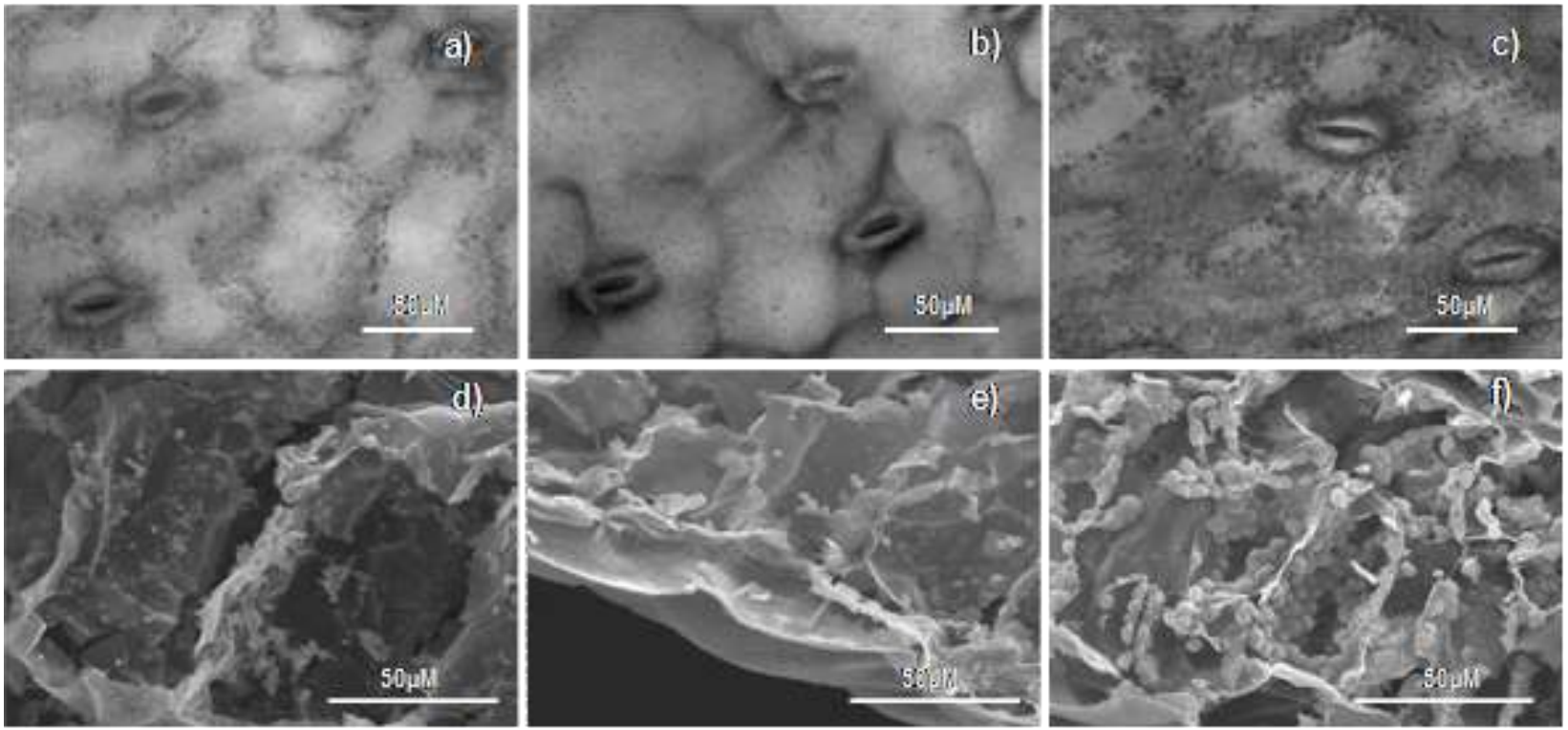


Figure 3: Light microscopy images of *S .vulgaris* fresh leaf surfaces from a) control, b) 60 µM Cr(III), and c) 60 µM Cr(VI) treated plants. Scanning electron micrographs of *S. vulgaris* freeze-dried leaf cross sections showing palisade mesophyll cells from: d) control plants, e) 60 µM Cr(III) treated plants , f) 60 µM Cr(VI) treated plants.

6.3.4. Biochemical effects of Cr in S. vulgaris (DRIFT- FTIR)

There was little change in the characteristic protein region (1750-1500 cm^{-1}) in the DRIFT spectra from both leaves (Figure 4.a) and roots (Figure 4.b) of *S. vulgaris* (Stewart, 1996). The major peaks at ~1650-60 cm^{-1} (C=O stretch of Amide I) and at ~1540-50 cm^{-1} (N-H bending vibrations of Amide II) were of similar intensity in all treatments providing evidence that no protein degradation had occurred (Dokken *et al*., 2005). The absorption band at 1710 cm^{-1}, corresponding to the conjugated aldehyde C=O stretch of fatty acids (Dokken *et al*., 2005) present in the cell membrane was not changed in the leaves or roots of plants grown with Cr(III) or Cr(VI) indicating that chromium treatments did not cause appreciable alteration of the cell membrane. Further, no change was seen in the ester peak at ~1740 cm^{-1} indicating no quinone-bearing structures formation from the oxidation of aromatics compounds in plant cell walls (Sundaramoorthy *et al*., 2010). The spectra of leaves with Cr show a shift to lower wavenumber in Amide I and II bands indicating Cr binding to proteins.

Changes in spectra corresponding to C-H bending vibrations of CH_3 and CH_2 groups (1350-1470 cm^{-1}), N-H deformation of amines (1180-1360 cm^{-1}) and nitro compounds (-NO_2) (1345-1385 cm^{-1}) (Morrison and Boyd, 1985) were detected in Cr treated plants. Spectra from leaves of control plants had bands at 1387 cm^{-1} and at 1325 cm^{-1}, the intensity of which diminished and peak position shifted to lower wavenumbers in plants treated with both dosages of Cr(III) and low concentrations of Cr(VI). The peaks virtually vanishes at high concentrations of Cr(VI). In the spectra from control roots, these bands were located at 1324 cm^{-1} and 1386 cm^{-1}, respectively. These bands also showed a downshift in all Cr treatments similar to that observed in the leaves, however the shift in the 60 μM Cr(VI) treatment was not as pronounced. The shift to lower wavenumbers of the absorption peak associated with N-H stretching found in Amides and Amines could be explained by alteration of the binding environment around nitrogen caused by metal binding with proteins (D'Souza *et al*., 2008).

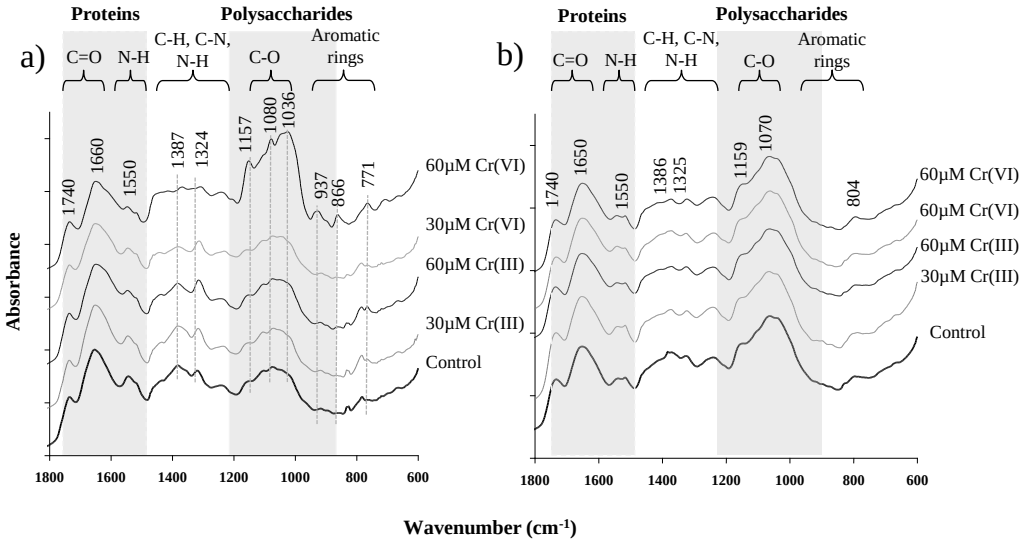


Figure 4: Diffuse reflectance infrared Fourier transform (DRIFT) spectra of a) leaves and b) roots of *S. vulgaris* treated 3weeks with different concentrations and forms of chromium.

The spectral region between 1200 cm^{-1} and 800 cm^{-1} is considered the fingerprint region of polysaccharides (e.g. pectin, cellulose, hemicellulose), which are key components making up the plant cell wall, and are dominated by a sequence of absorption bands due to C-O, C-C, C-O-C and C-O-P stretching and ring vibrations (Dokken *et al*., 2005). In the spectra from leaves, the peaks associated with the C-O stretching of starch, (region 1150-980 cm^{-1}) (Stewart, 1996) increased in intensity particularly in leaves of plants treated with 60 µM Cr(VI). This finding provides some indication that the granules we observed via light microscopy and SEM in the Cr(VI) treatments, but absent in the control and Cr(III) exposures (Figure 3 a-f), are in fact starch grains. Accumulation of starch in leaves due to Cr(VI) treatments has been previously reported in *Phyllanthus amarus* (Rai and Mehrotra, 2008), *Salvinia minima* (Nichols *et al*., 2000) and *Pea sativum* (Rodriguez *et al*., 2012). Apperonth *et al.* (2003) found that plants of *Spirodela polyrhiza* exposed to low chromate concentrations or for short periods increased their starch contents while increasing chromate concentration or extending the period of exposure resulted in decrease of starch content and in the disappearance of starch granules. On the other hand, Rodriguez *et al*. (2012) studied the effect of increasing Cr(VI) exposure on photosynthetic parameters and starch contents in *Pea sativum* concluding that biochemical processes are affected by lower concentrations of Cr(VI) while photochemical efficiency is compromised at

higher dosages. Thus starch accumulation in leaves of Cr treated plants has been attributed to an impartment between carbon utilization and assimilation that takes places at doses in which plant growth is retarded but photosynthesis is not yet inhibited. In roots, the region related to starch (1150-980 cm^{-1}, polysaccharide region) displays higher absorption than the leaves spectra, but no changes were observed due to Cr treatments.

New absorption bands in spectra of leaves from the plants grown in 60 µM Cr(VI) were identified at 1157 cm^{-1} and 1036 cm^{-1} (aromatic C-H deformation), 1080 cm^{-1} (C-P deformation of alcohols and ethers) (Kubo and Kadla, 2005) and at 937 cm^{-1} (glycosidic linkages) (Kacurakova *et al*., 2000) indicating synthesis or rearrangement of polysacharides.

The region from 870 to 675 cm^{-1} is characteristic of aromatic rings (Morrison and Boyd, 1985). In leaves, the band found at 784 cm^{-1} in control spectra shift to 771 cm^{-1} and increase in absorption and a new band appears at 866 cm^{-1}, both bands attributed to C-H out of plane bending of benzene rings (Ramamurthy, 2007). As no signals of protein integrity changes, cell membrane oxidation or nucleid acid damage have been found in this tissue, these aromatics could be related with the production of antioxidant compounds for the prevention of damage caused by excess of Cr. In roots, no changes have been found in this region.

Taking into account that the cell wall is a complex mixture of biopolymers mainly composed by cellulose/hemicellulose, pectins and structural cell wall proteins (Kacurakova *et al*., 2000), changes in protein and polysacharides region seems to be related with Cr binding to the cell wall in both roots and leaves. The overproduction of polysaccharides found in leaves of 60 µM Cr(VI) treated plants evidenced plant growth retardation and the pass of plants to storage activity.

The higher uptake of Cr in presence of Cr(VI) than of Cr(III) together with the higher oxidizing power of Cr(VI) relies on growth retardation, specially at 60 µM. However photosynthesis is not inhibited at this dose indicating that *S. vulgaris* plants

posses tolerance mechanisms that allows them to survive in presence of Cr(VI). The main process implicated in tolerance is the total reduction of Cr(VI) to the less toxic Cr(III) immediately after uptake in the roots tips. Then, protection of cell functionality is achieved by Cr binding to proteins and polysaccharides of the wall. Photosynthetic function is preserved by: i) Cr retention in the roots, ii) transport of Cr excess by organic acids to the storage sites in the cell wall of leaf margins and iii) synthesis of antioxidant compounds to preserve membrane integrity in leaves. High energy assigned for activation of these tolerance mechanisms under Cr(VI) exposition could deprive plants of its quota of energy required for its normal growth making them to focus in storage activity.

From the point of view of environmental clean- up, revegetation with *S. vulgaris* supposes a green alternative for *"in situ"* detoxification of Cr(VI) to the stable and less toxic Cr(III) with the subsequent reduction in environmental risk. Moreover the low quantity of Cr transferred to the above ground tissues of the plant, reduces the transfer of Cr to the food chain. That makes *S. vulgaris* an interesting species to be applied in the phytoestabilization of Cr polluted soils.

6.4. REFERENCES

Aldrich, M.V., Gardea-Torresdey, J.L., Peralta-Videa, J.R., Parsons, J.G., 2003. Uptake and reduction of Cr(VI) to Cr(III) by mesquite (*Prosopis spp*.): Chromate-plant interaction in hydroponics and solid media studied using XAS. Environ. Sci. Technol. 37, 1859-1864.

Appenroth, K., Keresztes, A., Sarvari, E., Jaglarz, A., Fischer, W., 2003. Multiple effects of chromate on S*pirodela polyrhiza*: Electron microscopy and biochemical investigations. Plant Biology 5, 315-323.

Bluskov, S., Arocena, J., Omotoso, O., Young, J., 2005. Uptake, distribution, and speciation of chromium in *Brassica juncea.* Int. J. Phytoremediation 7, 153-165.

Bonet, A., Poschenreider, C., Barcelo, J., 1991. Chromium III - iron interaction in Fe - deficient and Fe - sufficient bean plants. I. Growth and nutrient content. J. Plant Nutr. 14, 403-414.

Budevska, B.O., Sum, S.T., Jones, T.J., 2003. Application of Multivariate Curve Resolution for Analysis of FT-IR Microspectroscopic Images of *in Situ* Plant Tissue. Appl. Spectrosc. 57, 124-131.

Cervantes, C., Campos-Garcia, J., Devars, S., Gutierrez-Corona, F., Loza-Tavera, H., Torres-Guzman, J.C., Moreno-Sanchez, R., 2001. Interactions of chromium with microorganisms and plants. FEMS Microbiol. Rev. 25, 335-347.

Choi, Y.E., Harada, E., 2005. Roles of calcium and cadmium on Cd-containing intra- and extracellular formation of Ca crystals in tobacco. Journal of Plant Biology 48, 113-119.

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486.

di Toppi, L.S., Fossati, F., Musetti, R., Mikerezi, I., Favali, M.A., 2002. Effects of hexavalent chromium on maize, tomato, and cauliflower plants. J. Plant Nutr. 25, 701-717.

Dokken, K., Davis, L., Marinkovic, N., 2005. Use of infrared microspectroscopy in plant growth and development. Applied Spectroscopy Reviews 40, 301-326.

D'Souza, L., Devi, P., Shridhar, D.M.P., Naik, C.G., 2008. Use of Fourier Transform Infrared (FTIR) spectroscopy to study cadmium-induced changes in *Padina tetrastromatica* (Hauck). Analytical chemistry insights 3, 135-143.

Gardea-Torresdey, J.L., de la Rosa, G., Peralta-Videa, J.R., Montes, M., Cruz-Jimenez, G., Cano-Aguilera, I., 2005a. Differential uptake and transport of trivalent and flexavalent chromium by tumbleweed (*Salsola kali*). Arch. Environ. Contam. Toxicol. 48, 225-232.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., de la Rosa, G., Parsons, J.G., 2005b. Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coord. Chem. Rev. 249, 1797-1810.

Han, F.X.X., Sridhar, B.B.M., Monts, D.L., Su, Y., 2004. Phytoavailability and toxicity of trivalent and hexavalent chromium to *Brassica juncea*. New Phytol. 162, 489-499.

Harmens, H., Koevoets, P., Verkleij, J., Ernst, W., 1994. The Role of Low-Molecular-Weight Organic-Acids in the Mechanism of Increased Zinc Tolerance in *Silene vulgaris* (Moench) Garcke. New Phytol. 126, 615-621.

Hayat, S., Khalique, G., Irfan, M., Wani, A.S., Tripathi, B.N., Ahmad, A., 2012. Physiological changes induced by chromium stress in plants: an overview. Protoplasma 249, 599-611.

Howe, J.A., Loeppert, R.H., Derose, V.J., Hunter, D.B., Bertsch, P.M., 2003. Localization and speciation of chromium in subterranean clover using XRF, XANES, and EPR spectroscopy. Environ. Sci. Technol. 37, 4091-4097.

Juneja, S., Prakash, S., 2005. The chemical form of trivalent chromium in xylem sap of maize (*Zea may*s L.). Chem. Spec. Bioavail. 17, 161-169.

Kacurakova, M., Capek, P., Sasinkova, V., Wellner, N., Ebringerova, A., 2000. FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. Carbohydr. Polym. 43, 195-203.

Kubo, S., Kadla, J., 2005. Hydrogen bonding in lignin: A Fourier transform infrared model compound study. Biomacromolecules 6, 2815-2821.

Liu, D.H., Jiang, W.S., Li, M.X., 1992. Effects of Trivalent and Hexavalent Chromium on Root-Growth and Cell-Division of *Allium cepa*. Hereditas 117, 23-29.

Lombi, E., Scheckel, K.G., Kempson, I.M., 2011b. In situ analysis of metal(loid)s in plants: State of the art and artefacts. Environ. Exp. Bot. 72, 3-17.

Lombi, E., Susini, J., 2009. Synchrotron-based techniques for plant and soil science: opportunities, challenges and future perspectives. Plant Soil 320, 1-35.

Lyon, G., Peterson, P., Brooks, R., 1969. Chromium-51 Distribution in Tissues and Extracts of *Leptospermum scoparium*. Planta 88, 282-287.

Lytle, C.M., Lytle, F.W., Yang, N., Qian, J.H., Hansen, D., Zayed, A., Terry, N., 1998. Reduction of Cr(VI) to Cr(III) by wetland plants: Potential for in situ heavy metal detoxification. Environ. Sci. Technol. 32, 3087-3093.

Mangabeira, P., Gavrilov, K., de Almeida, A., Oliveira, A., Severo, M., Rosa, T., Silva, D., Labejof, L., Escaig, F., Levi-Setti, R., Mielke, M., Loustalot, F., Galle, P., 2006. Chromium localization in plant tissues of *Lycopersicum esculentum* Mill using ICP-MS and ion microscopy (SIMS). Appl. Surf. Sci. 252, 3488-3501.

Mangabeira, P.A., Ferreira, A.S., de Almeida, A.F., Fernandes, V.F., Lucena, E., Souza, V.L., dos Santos Junior, A.J., Oliveira, A.H., Grenier-Loustalot, M.F., Barbier, F., Silva, D.C., 2011. Compartmentalization and ultrastructural alterations induced by chromium in aquatic macrophytes. Biometals 24, 1017-1026.

Mcgrath, S.P., 1982. The Uptake and Translocation of Tri-Valent and Hexa-Valent Chromium and Effects on the Growth of Oat in Flowing Nutrient Solution and in Soil. New Phytol. 92, 381-390.

Montes-Holguin, M.O., Peralta-Videa, J.R., Meitzner, G., Martinez-Martinez, A., De la Rosa, G., Castillo-Michel, H.A., Gardea-Torresdey, J.L., 2006. Biochemical and spectroscopic studies of the response of *Convolvulus arvensis* L. to chromium(III) and chromium(VI) stress. Environmental Toxicology and Chemistry 25, 220-226.

Morrison, R.T., Boyd, R.N., 1985. Organic Chemistry. Fondo Educativo Interamericano, Mexico D.F.

Nichols, P.B., Couch, J.D., Al-Hamdani, S.H., 2000. Selected physiological responses of *Salvinia minima* to different chromium concentrations. Aquat. Bot. 68, 313-319.

Opperman, D.J., van Heerden, E., 2008. A membrane-associated protein with Cr(VI)-reducing activity from Thermus scotoductus SA-01. FEMS Microbiol. Lett. 280, 210-218.

Poschenrieder, C., Vazquez, M.D., Bonet, A., Barcelo, J., 1991. Chromium III - iron interaction in iron sufficient and iron deficient bean plants. II. Ultrastructural aspects. J. Plant Nutr. 14, 415-428.

Rai, V., Mehrotra, S., 2008. Chromium-induced changes in ultramorphology and secondary metabolites of *Phyllanthus amarus* Schum & Thonn. - an hepatoprotective plant. Environ. Monit. Assess. 147, 307-315.

Ramamurthy, S.K., 2007. Fourier Transfrom Infrared Spectroscopic analysis of a plant (*Calotropis gigantea* Linn) from an industrial village, Cuddalore Dt, Tamilnadu, India. Romanian Journal of Biophysics 17 No 4, 269-276.

Ravel, B., Newville, M., 2005. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. Journal of synchrotron radiation 12, 537-541.

Rodriguez, E., Santos, C., Azevedo, R., Moutinho-Pereira, J., Correia, C., Dias, M.C., 2012. Chromium (VI) induces toxicity at different photosynthetic levels in pea. Plant Physiology and Biochemistry 53, 94-100.

Samantary, S., 2002. Biochemical responses of Cr-tolerant and Cr-sensitive mung bean cultivars grown on varying levels of chromium. Chemosphere 47, 1065-1072.

Santana, K.B., de Almeida, A.F., Souza, V.L., Mangabeira, P.A.O., Silva, D.d.C., Gomes, F.P., Dutruch, L., Loguercio, L.L., 2012. Physiological analyses of *Genipa americana* L. reveals a tree with ability as phytostabilizer and rhizofilterer of chromium ions for phytoremediation of polluted watersheds. Environ. Exp. Bot. 80, 35-42.

Sarret, G., Harada, E., Choi, Y., Isaure, M., Geoffroy, N., Fakra, S., Marcus, M.A., Birschwilks, M., Clemens, S., Manceau, A., 2006. Trichomes of tobacco excrete zinc as zinc-substituted calcium carbonate and other zinc-containing compounds. Plant Physiol. 141, 1021-1034.

Sawalha, M.F., Peralta-Videa, J.R., Saupe, G.B., Dokken, K.M., Gardea-Torresdey, J.L., 2007. Using FTIR to corroborate the identity of functional groups involved in the binding of Cd and Cr to saltbush (*Atriplex canescens*) biomass. Chemosphere 66, 1424-1430.

Schmidt, W., 1996. Influence of chromium(lll) on root-associated Fe(lll) reductase in L. J. Exp. Bot. 47, 805; 805-810; 810.

Shanker, A.K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S., 2005. Chromium toxicity in plants. Environ. Int. 31, 739-753.

Shanker, A.K., Djanaguiraman, M., Venkateswarlu, B., 2009. Chromium interactions in plants: current status and future strategies. Metallomics 1, 375-383.

Sharma, D.C., Sharma, C., Tripathi, R.D., 2003. Phytotoxic lesions of chromium in maize. Chemosphere 51, 63-68.

Skeffington, R.A., Shewry, P.R., Peterson, P.J., 1976. Chromium Uptake and Transport in Barley Seedlings (*Hordeum vulgare*-L). Planta 132, 209-214.

Singh, H.P., 2013. Chromium toxicity and tolerance in plants. Environmental chemistry letters .

Srivastava, S., Prakash, S., Srivastava, M., 1999. Studies on mobilization of chromium with reference to its plant availability - Role of organic acids. Biometals 12, 201-207.

Stewart, D., 1996. Fourier transform infrared microspectroscopy of plant tissues. Appl. Spectrosc. 50, 357-365.

Sundaramoorthy, P., Chidambaram, A., Ganesh, K.S., Unnikannan, P., Baskaran, L., 2010. Chromium stress in paddy: (i) Nutrient status of paddy under chromium stress; (ii) Phytoremediation of chromium by aquatic and terrestrial weeds. Comptes Rendus Biologies 333, 597-607.

Vazquez, D., Poschenrieder, C., Barcelo, J., 1987. Chromium-VI Induced Structural and Ultrastructural-Changes in Bush Bean-Plants (*Phaseolus vulgaris* L). Annals of Botany 59, 427-438.

Zayed, A., Terry, N., 2003. Chromium in the environment: factors affecting biological remediation. Plant Soil 249, 139-156.

Zayed, A., Lytle, C.M., Qian, J.H., Terry, N., 1998. Chromium accumulation, translocation and chemical speciation in vegetable crops. Planta 206, 293-299.

Zeng, F., Qiu, B., Ali, S., Zhang, G., 2010. Genotypic Differences in Nutrient Uptake and Accumulation in Rice Under Chromium Stress. J. Plant Nutr. 33, 518-528.

Zhao, Y., Parsons, J.G., Peralta-Videa, J.R., Lopez-Moreno, M.L., Gardea-Torresdey, J.L., 2009. Use of synchrotron- and plasma-based spectroscopic techniques to determine the uptake and biotransformation of chromium(III) and chromium(VI) by *Parkinsonia aculeata*. Metallomics 1, 330-338 .

Zou, J.H., Wang, M., Jiang, W.S., Liu, D.H., 2006. Effects of hexavalent chromium (VI) on root growth and cell division in root tip cells of *Amaranthus viridis* L. Pak. J. Bot. 38, 673-681.

Assessing of *Silene vulgaris* to recover recovery chromium polluted soils: a pot experiment

Abstract

A pot experiment was carried out to evaluate the response of *S. vulgaris* to two different soils polluted with chromium in order to assess its application in remediation processes. The study has been performed on two soils that mainly differed from pH and OM contents. Chromium rich sludge and $K_2Cr_2O_7$ solution have been used as sources of pollution. It has been demonstrated that Cr spills are more dangerous in alkaline soils and poor in OM where Cr was found soluble and in the toxic form, Cr(VI). In acid soils with high content in OM, Cr was less available and it was detected in the less toxic specie, Cr(III). Sludge pollution decreased pH, specially in the acid soil, and enhanced EC resulting in plant growth retardation. Pollution with $K_2Cr_2O_7$ was more toxic in the alkaline soil and poor in OM. In this soil, no Cr(VI) was reduced to Cr(III) and plants were not able to growth. Sludge application increases biological activity of soils due to its content in OM but $K_2Cr_2O_7$ drastically decreased it. *S. vulgaris* cover increased the biological activity of soil, especially the dehydrogenase activity. The exudation of carbon compounds (mainly organic acids) from roots supposes a source of C for microorganism growth, increases soil pH and participates in Cr(VI) reduction and Cr(III) solubility. The *S. vulgaris* ability to growth in soils with different characteristics and polluted with different Cr sources makes this species a good candidate for the recovery of Cr polluted sites.

Keywords: Remediation, Chromium, *Silene vulgaris*, metal availability, biological indicators, rhizosphere, soil

7.1. BACKGROUND

Soil contamination has become an important environmental concern worldwide since it supposes a risk to human and ecosystem health and a detriment to soil productivity with the consequent socioeconomic impact. More than real clean up of contaminated soils, re-vegetation performs some important functions such as protecting contaminated soil from wind and air erosion, reducing water percolation to prevent leaching of contaminants, reducing direct exposure of soil to living organism and enhancing biodiversity (Vassilev *et al*., 2004). Particularly, plants modify the volume of soils around roots that is influenced by the activity of living plants roots, named as rhizosphere (Hinsinger *et al*., 2003). Plant-induced modifications are mainly produced by the exudation of a variety of organic compounds (carboxylate anions, phenolics, carbohydrates, amino acids, enzymes, other proteins, etc.) and inorganic ions (protons, phosphate, other nutrients, etc.) to change the chemistry and biology of the rhizosphere and enhance their adaptation to a particular environment (Rengel, 2002). Main changes produced in rhizosphere are related with metal availability, pH, pCO_2, pO_2, redox potential, organic ligand concentration and microbial biomass (Kidd *et al*., 2009).

Literature about chromium-plants interactions in Cr polluted soils is scarce and available studies are focused on Cr phytotoxicity (Lopez-Luna *et al*., 2009; Chen *et al*., 2008; Ali *et al*., 2006) and metal accumulation (Revathi *et al*., 2011; Bluskov *et al*., 2005; Turgut *et al*., 2004;) rather than plant growth effect in soils.

Silene vulgaris is a facultative methalophyte with a vigour growth by producing seeds and rhizomes. It is a perennial specie able to regrow after harvesting. This plant have been proven to growth in soils polluted with Cd, Zn, Cu, Pb, As and Hg (Perez-Sanz *et al*., 2012; Carpena-Ruiz *et al.*, 2008; Ciarkowska and Hanus-Fajerska, 2008; Song *et al*., 2004; Ernst and Nelissen, 2000) and to reduce heavy metal toxicity on soil bacteria from multipolluted soils (Martinez-Inigo *et al*., 2009). Previous hydroponics studies have demonstrated the capacity of this specie to tolerate high levels of Cr, using different mechanism to avoid Cr toxicity.

The objective of this study is to evaluate the feasibility of using *S. vulgaris* to remediate soils with different physic-chemical characteristics and polluted with different sources of Cr. This evaluation comprises the study of: i) plant growth and phytotoxicity, ii) the influence of plant rhizosphere on Cr chemistry, iii) the effect of plant cover on physic-chemical characteristics of soils and iii) monitoring of soil functionality by the use of biological indicators.

7.2. MATERIALS AND METHODS

7.2.1. Greenhouse experiment

7.2.1.a. Soil consolidation

A pot experiment using a complete factorial design was performed with two soil with different characteristics (table 1); three levels of pollution: (i) no pollution, ii) $K_2Cr_2O_7$ to simulate a Cr(VI) spilling and iii) Cr rich sludge spilling; and three vegetation treatments: i) no plant, ii) *S. vulgaris* genotype SV21 (tolerant) and iii) *S. vulgaris* genotype SV38 (sensitive). Levels of each factor were replicated 3 times.

Plastic pots were filled with 17 kg of the corresponding soil. Soils were collected from the top layer (0-30 cm) of two agricultural soils located in Alcalá de Henares (Soil A) and El Escorial (soil B) in Madrid (Spain). Immediately after collection, the soil was air-dried and sieved (< 5 mm). Soils were analyzed according to the procedures described in material and methods 2.3 and were selected based on their differences in pH and organic matter (table 1).

Degradation of soil caused by Chromium industrial activities was simulated by spilling $K_2Cr_2O_4$ solution (1000 mg·Cr·L^{-1}) or a Cr rich sludge (1050 mg·Cr·kg^{-1}) to reach a final concentration of chromium in pots of 100 mg·kg^{-1}soil. This Cr concentration corresponds to a 10% increase regards to the regulatory value established by Madrid Regional Government for agricultural purposes (Orden 2270/2006, de 11 de agosto). The sludge (1.5 kg) was thoroughly mixed with 3kg of soil in top layer of pots. Sludge main characteristics were determined according to material and methods 2.3 (table 2). Control pots were left uncontaminated. The soil was brought to 50% water

holding capacity and maintained by addition of deionized water for three months (from 1 September to 1 December).

7.2.1.b Plant experiment

Based on previous studies two genotypes of *S. vulgaris* originated from Madrid region (Spain) were chosen based on their tolerance to Cr(VI). Genotype SV21 (from Rozas de Puerto Real; EC_{100}: 200-1200 µM Cr(VI)) and genotype SV38 (from Valdemaqueda; EC_{100}: 200-1000 µM Cr(VI)). Clones of each genotype were vegetative propagated in field (Alcalá de Henares, Madrid, Spain) in a permanent 10 x 10 m^2 plot (divided into 1 m^2 quadrats). Cuttings of each genotype were collected in September 2011 and rooted in a mixture of soil/peat for three months. After soil consolidation, 10 cuttings of the corresponding genotype were transplanted to pots (01/12/2011). Plants were grown during two vegetative periods from December 2011 to May 2012. In parallel, soil in unplanted pots was exposed to metal contamination at the applied dose. Pot irrigation was scheduled based on Watermark sensor readings (Model 200SS, Irrometer moisture sensors, Irrometer Company, Inc., P.O. Box 2424, Riverside, CA 92516) to prevent the soil at the 20 cm depth from drying beyond 60 kPa soil water tension). The total amount of water applied in each irrigation cycle was based on the normal rainfall in Mediterranean conditions. Plant experiment was conducted in a greenhouse. No artificial light was provided, and air temperature averaged 21ºC with high and low daily means of 27 and 15ºC. Relative humidity was maintained near 60%. Plants were harvested at the end of Mars (H1) and roots were retrieved just before full physiological maturity at the end of the second vegetative period in May (H2). At the end of the experiment (H2) full plant, and soil samples were collected. Soil samples were air dried and sieved at < 2 mm. For the analysis of biological parameters, soils (aprox 10 g) were stored at 4ºC until analysis. Shoots were also washed with tap water thoroughly and rinsed twice with Millipore water. Roots were washed with tap water, shaken in Millipore water three times for three cycles of five minutes in an ultrasonic bath, dried with filter paper and weighed. Approximately, 2 grams of fresh samples were frozen in liquid N_2 and stored at -80ºC. The rest of the plant material was dried in a forced air oven for 48 hours at 70ºC, after then, dry weights were recorded.

Table 1: soil characteristics

	Encín	**Escorial**
Classification	Haplic calcisol	eutric cambisol
Particle size fraction		
sand (%)	21.25	75.5
silt (%)	37.5	10
clay (%)	41.25	14.5
USDA Texture	Clay	Sandy Loam
pH	8.1	6.62
carbonates (%)	5.6	0.6
E.C. ($dS \cdot m^{-1}$)	0.22	0.65
N (%)	0.1	0.13
P ($mg \cdot kg^{-1}$)	21	55
OM (%)	0.63	2.67
Exchangeable cations ($mg \cdot kg^{-1}$)		
Ca	3862	1437
Mg	448	131
Na	3.1	8.15
K	286	193
Metals ($mg \cdot kg^{-1}$)		
Cr	31	29
Fe	25384	19272
Pb	26	26
Cd	0.22	0.24
Cu	20	15
Zn	17.26	87.12
Mn	229	238
Ni	27	12
MnO_2	46	91
Fe_2O_3	0.45	0.36

Table 2: Sludge characteristic

Chemical characteristic							
pH	E.C. ($dS \cdot m^{-1}$)	O.M. (%)					
6.69	1.61	39.5					
Exchangeable cations ($mg \cdot kg^{-1}$)							
Ca	Mg	Na	K				
9897	599	440	448				
Metal s ($mg \cdot kg^{-1}$)							
Cr	Fe	Pb	Cd	Cu	Zn	Mn	Ni
1050	89970	940	1.2	778	934	1608	352

7.2.2. Plant analysis

7.2.2.a. Trace element Analysis

Vegetal dry samples were mill grounded and after a dry digestion in a muffle furnace (480 ºC) the ashes were digested using HCl, according to Gárate *et al*. (1984). After cooling, the digests were filtered (Whatman filter paper nº 541) and diluted with MilliQ water to 50 mL. Total concentrations of Cr, Cd, Fe, Cu, Mn, Zn, Pb, Ni, Mg, Ca and K were measured by Flame Atomic absorption spectrophotometer (VARIAN fast sequential model AA240FS). Bush branches and Leaves (DC73348GSV-1) were used as reference material.

7.2.2.b. Estimation of oxidative stress, malondyaldehyde (MDA)

Lipid peroxidation was evaluated as malondialdehyde (MDA) by the method of Reilly and Aust (1999) modified by Catalá *et al*. (2010). Fresh samples (0.1 g) of shoots and roots were homogenized on ice with 1mL of deionised water and centrifuged at 13000 rpm for 10 min. Supernatants were removed, and the pellets were resuspended in 500 µL of 0.01% buthylated hydroxytoluene (BHT) in ethanol 80%. Then 900 µL of TBA (2.57×10^{-2} M), TCA (9.18×10^{-1} M), and HCl (3.20 M) were added to each samples. Samples were vortexed and incubated in a water bath at 70°C for 30 min. Then, the samples were cooled on ice and centrifuged at 13000 rpm for 10 min. The absorbance of supernatants was measured at 532 nm. Absorbance at 600 nm was subtracted to this measure to eliminate the interferences of soluble sugars in the samples. Both absorbances were determined by UV-VIS light spectrophotometer (Thermo Spectronic Helios Alpha).

7.2.2.c. Extraction and determination of organic acids

Organic acids were extracted from fresh plant tissues as described by Arnetoli *et al*. (2008) samples (1 g) of frozen fresh tissues were homogenized in 10 mL of MilliQ water using a mortar and pestle in liquid nitrogen; then homogenates were centrifuged for 20 min at 10000 rpm at 4ºC. The supernatant was stored at -20ºC and filtered through a 0.20 µM before analysis. Organic acids (oxalic, citric, malic, formic, lactic, acetic and succinic) were measured by Ionic Chromatography (Dionex DX 500)

using a conductivity detector. Chromatographic conditions were as follows: sample loop volume, 25 µL); analytical column, IonPac ICE-AS6; eluent, 0.4 mM heptafluorobutyric Acid; (flow rate, 1.0 $mL \cdot min^{-1}$); suppressor, MicroMembrane Anion-ICE; regenerant, 5 mM tetrabutylammonium hydroxide (flow rate: 5 $mL \cdot min^{-1}$); analysis time, 20 min. Organic acids were identified by comparing the retention times of the samples against retention times of the standards. Calibration curves have been performed using Merck (purity=98%) reagents from 50 to 280 $mg \cdot L^{-1}$ for oxalic acid; from 1 to 50 $mg \cdot L^{-1}$ for citric, malic and acetic acids; from 1 to 30 $mg \cdot L^{-1}$ for lactic and succinic and from 0.5 to 10 $mg \cdot L^{-1}$ for formic acid.

7.2.3. Soil analysis

7.2.3.a. Soil characterisation

Soil samples were air dried, disaggregated and sieved (< 2 mm). Then, dried samples (500 mg) were digested in an Anton Paar Microwave Reaction System 3000 by adding of 2 mL of HCl 37% and 6 mL HNO_3 69%. After cooling, the digest were filtered to (Whatman filter paper nº541) and brought up to a volume of 50 mL. Total concentrations of Cr, Fe, Cu, Mn, Zn, Pb, Ni were measured by Flame Atomic absorption spectrophotometer (VARIAN fast sequential model AA240FS) and Cd concentration by Sequential ICP-AES LIBERTY AX.

The soil properties were analyzed according to the official Spanish methodology for soil analysis (MAPA, 1994), and results are shown in Table 1. In brief, the electrical conductivity (EC) and pH were measured in at a 1:2.5 soil-to-water ratio; the organic matter and total nitrogen content were determined using the Walkley–Black and Kjeldahl methods, respectively; the percentage of carbonates was measured using the Bernard calcimeter; available phosphorus was evaluated using sodium bicarbonate at a pH of 8.5; available nutrients were extracted with 0.1 N NH_4 (aq) 0.1 N and assessed using atomic absorption spectrometry (AAS) (AA 240 FS, Varian, Victoria, Australia). The soil texture was analyzed using a Bouyoucos densimeter. Oxides of Fe and Mn were determined using the Sodium Dithionite-Citrate-Bicarbonate (DCB) method (Mehra and Jackson, 1960) and hydroxylamine hydrochloride ($NH_2OH \cdot HCl$ respectively.

7.2.3.b. Respiration and enzyme activity

For the analysis of biological parameters soils were stored at 4ºC until analysis. Glucose-induced soil respiration was determined by the method of Fernandez *et al.* (2004) based on monitoring of CO_2 produced at 30ºC for 24h of soil incubation (5 g) in presence of KOH (0.2%) by using the µ-Trac 4200 system (SY-LAB, GmbH P.O.Box 47, A-3002 Pukersdorf, Austria). Dehydrogenase activity was measured according to (Trasar-Cespeda *et al.*, 2003). This method is based on the spectrophotometric determination at 490 nm of the iodonitrotetrazolium formazan (INTF) produced by the reduction of 2-p-iodofenil-3-p-nitrophenyl-5-phenyl-tetrazolium (INT) after 1 h of soil incubation in dark (1 g at 40ºC and pH 7.5). The *β*-galactosidase activity was measured according to Eivazi and Tabai (1988). Briefly, soil (1 g) was incubated for 1 h at 37ºC and pH 6 in presence of toluene and the enzyme sustrate *p*-Nitrophenyl-β-D-galactopyranoside. The *p*-nitrophenol produced by the enzymatic hydrolysis of this substrate is determined by UV-VIS light spectrophotometry at 400 nm. In both determinations, UV-VIS light spectrophotometer Thermo Spectronic Helios Alpha was used.

7.2.4. Soil solution analysis

One Rhizon soil moisture sampler (Rhizon SMS 19.21, length: 10 cm, diameter: 2.5 mm, pore diameter:0.1 µm) was placed vertically in each pot at a distance < 1 mm from roots (Dessureault-Rompre *et al*., 2010) to extract about 30 mL of pore water solution from the rhizosphere. Two samples were collected before transplanting (5 and 20 October) and another two afterwards (19 April and 10 May). Electric conductivity (EC) and pH were measured immediately after collecting samples. Total trace element concentrations, including Cr, were determined by Atomic Absorption Spectrometer (Zeman AA2407) equipped with graphite tube atomizer GTA 120. Determination of Cr(VI) in pore water samples was carried out by colorimetric EPA method 7196A (USEPA, 1992) using UV-VIS light spectrophotometer (Thermo Spectronic Helios Alpha. The detection limit of this method is 0.5 mg $Cr(VI)\cdot L^{-1}$.

Water-soluble carbon species in pore water solution were quantified in a Shimadzu TOC-5000 carbon analyzer. The apparatus was calibrated with stock

solutions of potassium hydrogen phthalate for water-soluble total carbon (WSTC) determination and with stock solutions of sodium hydrogen carbonate and sodium carbonate for water-soluble inorganic carbon (WSIC) determination. The water-soluble organic carbon (WSOC) content in the sample was determined by the difference between the WSTC and the WSIC.

7.2.5 Statistical treatment

Data were analysed by General Lineal Model (GLM) using the statistical package SPSS version 19.0. Experimental factors were: sample time (T), soil (S), pollution (Po) and plant (Pl). Three-way ANOVA was applied for analysis of the same sample time. GLM was followed by a post hoc Duncan test to assess the significance of differences among pollution and plant treatments for each variable. Values given in tables and figures indicate mean values (n=3) ± standard error (S.E.).

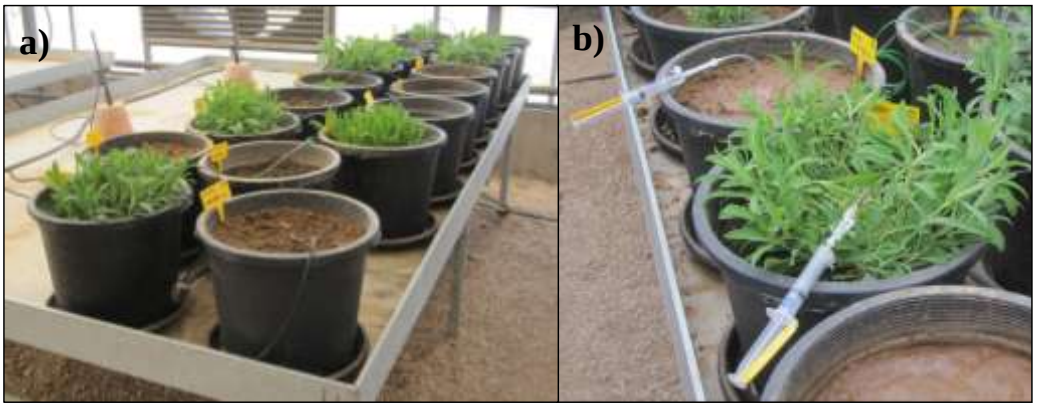


Figure 1: Pot experiment in the greenhouse; a) pots distribution; b) detail of soil solution sampling

7.3. RESULTS

7.3.1. Soil parameters

7.3.1.a. Trace element concentration

Table 1 shows the mean of total metals concentration in original soils ($mg \cdot kg^{-1}$). All concentrations were in accordance with the references levels established in the legislation with the exception of Ni concentration in Encin treatments and Cd and Zn concentrations in Escorial treatments. In this cases, metal were slightly higher than reference values but still under established levels for agricultural soil use (Orden 2270/2006 de 11 de agosto).

Total metal concentrations in soil after consolidation are displayed in table 3. This table shows total trace element concentrations (Cr, Cu, Pb, Zn, Mn, Ni, Fe and Cd) in soils ($mg \cdot kg^{-1}$) and, also, in soil solutions ($\mu g \cdot L^{-1}$). The last one represents the most available fraction. Only Cr and Zn concentrations exceeded the levels established by the legislation for agricultural land use (Orden 2270/2006 de 11 de agosto).

Similar total Cr levels have been reached by sludge application and by spiking $K_2Cr_2O_7$ solution in both soils (112-164 $mg \cdot kg^{-1}$). However sludge application did not increase Cr concentration in soil solution. Pots polluted with $K_2Cr_2O_7$ solution significantly increased Cr concentration in pore water, especially in Encín soil. Cr(VI) was just detectable in soils polluted with $K_2Cr_2O_7$ solution and represented 100% of total Cr detected in soil solution.

Sludge application increased the concentrations of the tested heavy metals but some differences have been found between soil and concentrations in pore water. Concentrations of Cu, Zn, Ni and Cd in both soil as soil solution have been increased due to sludge supplement. Pb, Mn and Fe showed different behaviour. Related to Pb, total concentration increased in both soils, but it not did in soil solution samples from Encín treatments. Manganese increased its available concentration in both soils, though the total concentration did not significantly in Escorial treatments. Finally, soluble Fe regards total concentration was lower than the other studied metals and it did not

increase in Encín treatments. Metal concentration in pore water solution was strongly influenced by soil characteristic and pollution, as shown in table 4 the statistically significant interaction between these variables (p<0.05). The metal availability in soils enriched Cr sludge was greater in pots with soil from Escorial than from the Encin one.

7.3.1.b. Physic-Chemical characteristics

Soil physic-chemical characteristics (pH, Electric conductivity (EC), carbonates, nitrogen, organic matter and phosphorous) before and after the two vegetative periods of plant growth are shown in table 4. Changes produced on studied characteristics were statistically different depending on the presence or absence of vegetal cover and independently of *S. vulgaris* genotype grown in the pots, thus table 4 shows differences between planted and unplanted soils.

Except to carbonates percentage, all evaluated parameters were significantly (p<0.001) influenced by pollution. Sludge application slightly acidified both soils, but pH tend to increase over time in Encín soil. Electrical conductivity increased in all polluted soils, but specially in thoses with sludge applications. This parameter decreased over time, in soils from pots with plants. Significant increases (p<0.001) in concentrations of nitrogen, organic matter and phosphorus were detected in the pots with soils treated by sludge

Table 3: Total Heavy metal concentration in soil ($mg \cdot kg^{-1}$) and in soil pore water ($\mu g \cdot L^{-1}$) at the beginning (t0) and at the end of the experiment (tf).

Soil	Pollution	Cr		Cu		Pb		Zn		Mn		Ni		Fe		Cd	
		total	***solution***	***total***	***solution***	***Total***	***solution***	***total***	***solution***	***total***	***solution***	***total***	***solution***	***total***	***solution***	***total***	***solution***
Escorial	**Control**	32.4^{b}	5.5^{b}	11.1^{b}	9.4^{b}	17.8^{b}	17.3^{ab}	79.2^{b}	9.1^{b}	317^{ns}	7.8^{b}	19.7^{b}	24.5^{b}	38133^{b}	20.9^{ns}	0.25^{b}	0.03^{b}
	Sludge	121^{a}	9.4^{b}	50.1^{a}	15.0^{a}	68.6^{a}	29.8^{a}	143^{a}	617.1^{a}	334	750^{a}	43.1^{a}	69.3^{a}	52810^{a}	22.7	0.36^{a}	0.08^{a}
	$K_2Cr_2O_7$	112^{a}	35.3^{a}	11.5^{b}	12.7^{ab}	18.2^{b}	7.7^{b}	79.0^{b}	10.9^{b}	302	17.4^{b}	20.5^{b}	26.9^{b}	39189^{b}	27.8	0.24^{b}	0.01^{b}
Encin	**Control**	32.8^{b}	7.5^{b}	18.2^{b}	12.4^{b}	19.2^{b}	15.8^{ns}	68.3^{b}	10.0^{ab}	379^{b}	6.5^{b}	27.7^{b}	26.3^{b}	44673^{b}	8.9^{b}	0.26^{b}	0.01^{b}
	Sludge	136^{a}	6.2^{b}	50.4^{a}	22.4^{a}	63.3^{a}	32.3	115^{a}	17.0^{a}	348^{a}	9.9^{a}	50.5^{a}	38.5^{a}	52470^{a}	17.4^{a}	0.36^{a}	0.02^{a}
	$K_2Cr_2O_7$	164^{a}	135151^{a}	18.4^{b}	14.7^{b}	18.7^{b}	26.1	68.2^{b}	9.3^{b}	373^{b}	8.2^{b}	27.1^{b}	18.2^{b}	42671^{b}	9.0^{b}	0.29^{b}	0.01^{b}
three way ANOVA																	
	Soil (S)	**	*	*	**	ns	ns	***	***	***	***	***	**	*	***	ns	***
	Pollution (Po)	***	*	***	***	***	ns	***	***	ns	***	***	***	***	ns	***	***
	S x Po	ns	*	ns	ns	ns	ns	ns	***	ns	***	ns	**	ns	ns	ns	**

Values are means (n=3). Different lowercase letters indicate significant differences among planted and unplanted pots. Different capital letters indicates differences among sampling time (Duncan's test $p<0.05$).Three-way ANOVA's results indicates the statistical influence of the factors: soil (S), pollution (Po). Soil factor includes two levels: Escorial (A) and Encín (B); Pollution factor has three levels: no pollution (control), sludge and spiked $K_2Cr_2O_7$ solution. (ns= non significant; *p<0.05; **p<0.01; ***p<0.001).

Table 4: Chemical characteristic of soils at beginning (*t0*) and at the end of the experiment (*tf*).

Soil	Pollution		pH		EC (dS·m^{-1})		$CaCO_3$ (%)		N (%)		O.M (%)		P (mg·kg^{-1})	
			t0	*tf*	*t0*	*tf*	*t0*	*tf*	*t0*	*tf*	*t0*	*tf*	*t0*	*tf*
A	**Control**	**U**	7.00	7.15	0.34	0.41^{a}	⋯	⋯	0.11	0.15	1.88	2.82	51.0^{A}	63.0^{B}
		P	7.16	7.22	0.4^{A}	$0.13^{b,B}$	0.50	0.80	0.12	0.14	2.21	2.76	57.8	61.3
	Sludge	**U**	5.43^{B}	5.74^{A}	2.28^{A}	1.88^{B}	⋯	⋯	0.31	0.30	4.14	4.42	$76.^{A}$	240^{B}
		P	5.49^{B}	5.80^{A}	1.83^{A}	1.12^{B}	⋯	⋯	0.29	0.27	3.58	4.29	149^{A}	220^{B}
	$K_2Cr_2O_7$	**U**	7.02	7.20	0.42	0.79^{a}	⋯	⋯	0.13	0.17	2.34	2.86	62.0	65.3
		P	7.03^{B}	7.33^{A}	0.38^{A}	$0.13^{b,B}$	⋯	0.60	0.10	0.15	2.00^{B}	2.68^{A}	58.3	59.3
B	**Control**	**U**	8.13	$7.92^{b,B}$	0.79	0.68^{a}	4.07	4.07	0.12	0.12^{a}	1.27	1.33	20.7	18.3
		P	7.94^{B}	8.47^{aA}	0.60^{A}	$0.30^{b,B}$	3.92	4.18	0.11	0.10^{b}	1.32	1.49	18.7^{B}	14.7^{A}
	Sludge	**U**	7.52	7.51^{b}	3.04^{A}	1.64^{B}	3.42	3.77	0.28	0.20	3.11	2.50	92.3	154
		P	7.53^{B}	$7.70^{a,A}$	2.91^{A}	1.41^{B}	3.73	3.62	0.30^{A}	0.22^{B}	2.98	2.67	125	132
	$K_2Cr_2O_7$	**U**	8.12	8.27	0.82	0.79	4.07	3.97	0.11	0.11	1.37	1.57	18.3	19.0
						three way ANOVA								
	Soil (S)		***		***		***		***		***		***	
	Pollution (Po)		***		***		ns		***		***		***	
	Plant (Pl)		*		*		ns		ns		ns		ns	
	S x Po		***		ns		ns		*		ns		ns	
	S x Pl		ns		ns		ns		*		ns		ns	
	Po x Pl		ns		ns		ns		ns		ns		ns	
	S x Po x Pl		ns		ns		ns		*		ns		ns	

Values are means (n=3). Different lowercase letters indicate significant differences among planted and unplanted pots. Different capital letters indicates differences among samples time (Duncan's test $p<0.05$). Three-way ANOVA's results indicates the statistical influence of the factors: soil (S), pollution (Po) and plant (Pl) and their interactions. Soil factor includes two levels: Escorial (A) and Encín (B); Pollution factor has three levels: no pollution (control), sludge and spiked $K_2Cr_2O_7$ solution and Plant factor includes two levels: planted (P) and unplanted (U). (ns= non significant; *p<0.05; **p<0.01; ***p<0.001)

7.3.1.c. Biological indicators

Soil respiration (mg $CO_2 \cdot 100g^{-1}$ soil) and the enzyme activity dehydrogenase (µmol INTF$\cdot g^{-1}$soil$\cdot h^{-1}$) and *β*-galactosidase (µmol *p*-nitrofenol$\cdot g^{-1}\cdot$soil$\cdot h^{-1}$) have been determined as bioindicators of soil quality. Results are given in figure 2, 3 and 4, respectively. Soil from Escorial presented higher biological activity than the Encín one. As seen by the significant interaction between soil characteristics and pollution, no differences have been found in "Escorial soil" due to pollution but in "Encin soil". Sludge application increased soil respiration and dehydrogenase activity regards to unpolluted soils, while $K_2Cr_2O_7$ reduced it in treaments. The presence of *S. vulgaris* cover increased: soil respiration in non polluted Encin soils and dehydrogenase activity in unpolluted Escorial soils and in sludge polluted pots of both soils. *β*-galactosidase activity was not significantly affected by soil pollution and just an increase in sludge Escorial polluted soil have been found due to plant growth

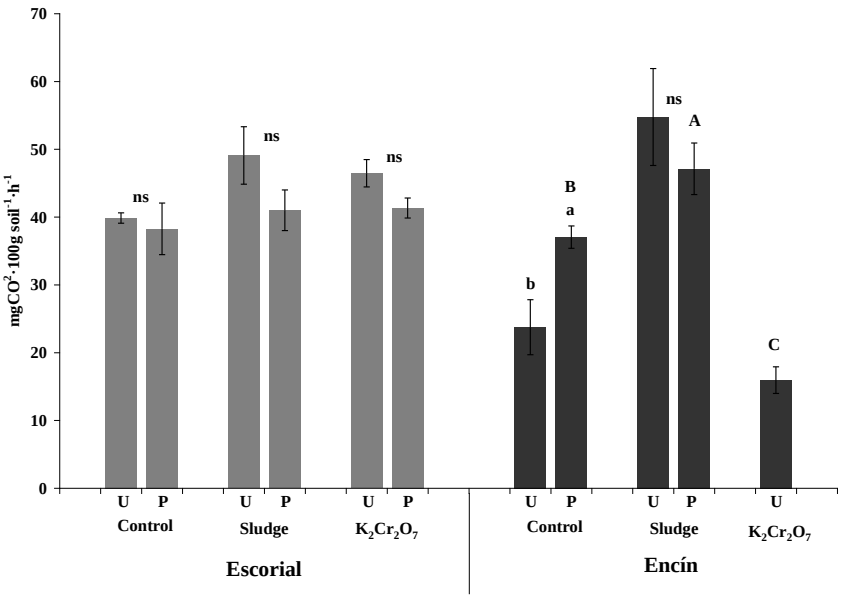


Figure 2: Soil respiration (mg $CO_2 \cdot 100g^{-1}$ soil) in unplanted (U) and planted (P)pots at the end of the experiment. Lowercase letter indicates– differences among planted and unplanted pots of the same treatment and capital letters indicates differences among pollutions treatments in pots with the same plant treatment (Duncan´s test at the $p<0.05$ level). Mean values ±SE (n=3

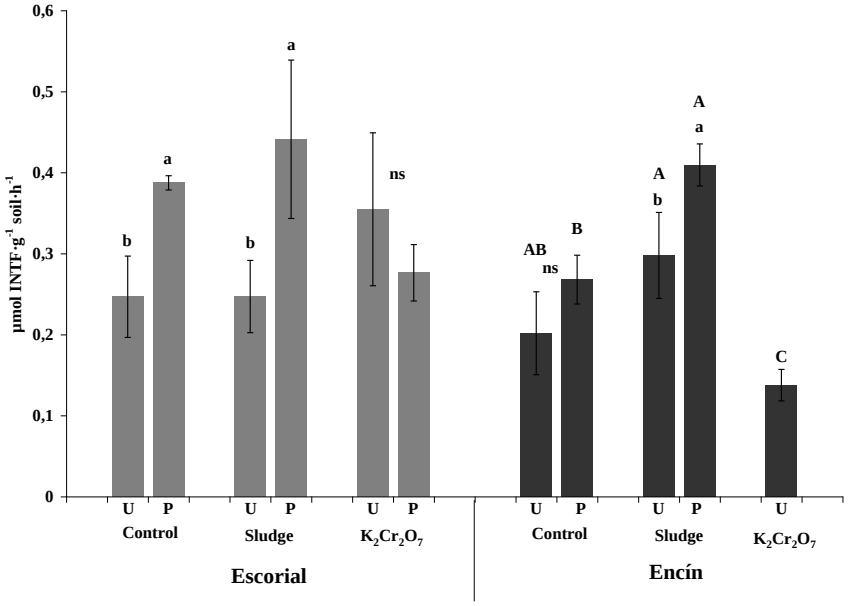


Figure 3: Soil dehydrogenase activity (µmol INTF·g^{-1}soil·h^{-1}) in unplanted (U) and planted (P)pots at the end of the experiment. Lowercase letter indicates differences among planted and unplanted pots of the same treatment and capital letters indicates differences among pollutions treatments in pots with the same plant treatment (Duncan´s test at the p<0.05 level). Mean values ±SE (n=3).

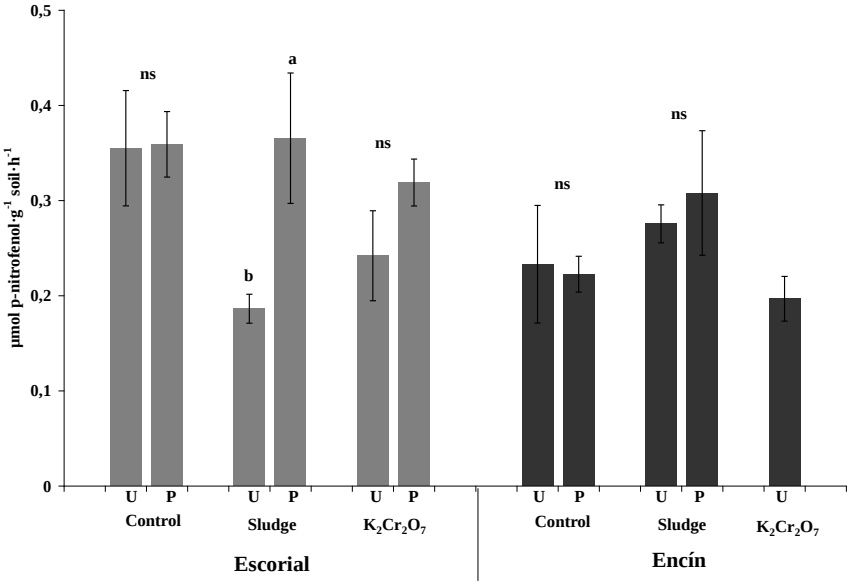


Figure 4: Soil β-galactosidase (µmol p-nitrofenol·g^{-1}·soil·h^{-1}) in unplanted (U) and planted (P)pots at the end of the experiment. Lowercase letter indicates differences among planted and unplanted pots of the same treatment and capital letters indicates differences among pollutions treatments in pots with the same plant treatment (Duncan´s test at the p<0.05 level). Mean values ±SE (n=3).

7.3.2. Plant analysis

7.3.2.a. Heavy metal accumulation

Heavy metal concentration in shoots and roots ($mg \cdot kg^{-1}$D.W.) of *S. vulgaris* after two vegetative periods are shown in tables 5 and 6, respectively. Cr concentration increased in roots of plants grown in pots polluted with both sources of chromium, especially in $K_2Cr_2O_7$ spiked pots. Shoots collected in the first harvest showed increased of Cr content in genotype SV21, and in the second harvest, shoots of both genotypes increased their Cr concentrations when plants were grown in Escorial soil.

The sludge application to soil induces plant accumulation of Cu, Pb, Zn, Ni and Cd, especially in plants grown in Escorial soil. However, Mn tends to decrease in plants grown in sludge polluted soils. Iron concentration in shoots of plants is significantly influenced by Cr pollution as it increased in plants grown in $K_2Cr_2O_7$ spiked soils and it decreased in plants grown in sludge enriched soils. In roots, Fe concentrations diminished in both polluted soils.

7.3.2.b. Phytotoxicity

Table 7 gives dry weights (mg D.W$\cdot plant^{-1}$) and MDA levels (µmol MDA$\cdot g^{-1}$ F.W.) as phytotoxicity indicators for *S. vulgaris* genotypes. In Encin soil polluted with $K_2Cr_2O_7$ plants died two weeks after transplanting. Plant growth was higher in Escorial than in Encín soil. However sludge application reduced root dry weight in both soils. Shoots dry mass showed significant interaction between pollution and plant. No changes have been found in the dry mass of genotype SV21 due to pollution treatments but an increase in pots with sludge in Encin soil. Genotype SV38, however reduced its shoots dry weights when grown in sludge enriched pots. 5Genotype SV21 showed significant higher shoot dry weight than SV38. Figure 5 shows representative pictures of shoots and root developed in the different polluted soils. Note that the most significative effect of pollution is root growth reduction in soils polluted with sludge. However good vegetative cover is developed in all treatments.

MDA levels in plant tissues were independent of soil type. Sludge application produced an enhance in the MDA levels in roots in both genotypes. Genotype SV38 also increased the MDA levels of their roots when grown in $K_2Cr_2O_7$ spiked pots. However no MDA induction due to soil pollution has been found in shoots of *S. vulgaris* plants.

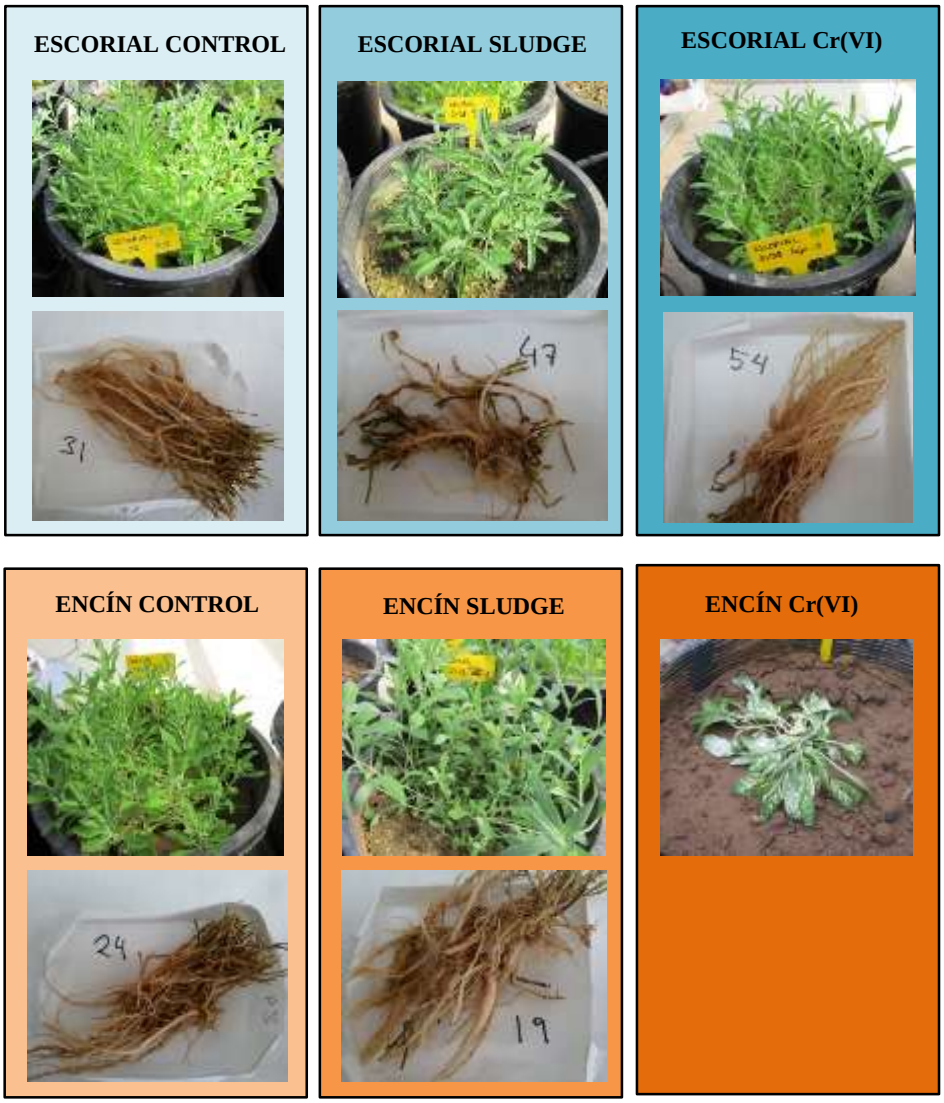


Figure 5: Representative shoots and roots developed in the different polluted soils

Table 5: Trace element concentration in shoot of *S. vulgaris* genotypes (mg·kg^{-1} D.W.)

			Cr		Cu		Pb		Zn		Mn		Ni		Fe		Cd	
Soil	**Genotype**	**Pollution**	***H1***	***H2***	***H1***	***H2***	***H1***	***H2***	***H1***	***H2***	***H1***	***H2***	***H1***	***H2***	***H1***	***H2***	***H1***	***H2***
Escorial	**SV21**	**Control**	1.68^{b}	3.82^{b}	6.22^{ns}	4.39^{ab}	1.29^{b}	1.77^{ns}	37.9^{b}	39.9^{ab}	101^{a}	101^{a}	1.34^{b}	4.01^{ns}	64.2^{b}	54.5^{ns}	0.66^{ns}	0.51^{b}
		Sludge	2.29^{ab}	3.09^{b}	6.89	4.79^{a}	2.82^{a}	2.95	68.6^{a}	51.0^{a}	50.8^{b}	33.9^{b}	7.53^{a}	1.80	68.9^{b}	63.6	0.62	1.40^{a}
		$K_2Cr_2O_7$	5.58^{a}	8.50^{a}	6.65	3.83^{b}	1.61^{ab}	2.93	37.7^{b}	30.3^{b}	113^{a}	74.7^{a}	1.68^{b}	5.27	95.2^{a}	52.0	0.68	1.21^{a}
	SV38	**Control**	3.94^{ns}	3.25^{b}	9.09^{ns}	6.48^{b}	3.01^{b}	2.29^{ns}	41.5^{b}	30.5^{b}	50.8^{ns}	64.7^{ns}	2.05^{b}	1.83^{ns}	112^{ns}	80.2^{a}	0.25^{ns}	0.44^{b}
		Sludge	3.81	5.00^{a}	8.90	5.90^{b}	4.66^{a}	2.91	74.4^{a}	59.6^{a}	50.4	47.2	7.73^{a}	2.46	100	70.2^{b}	0.65	1.21^{a}
		$K_2Cr_2O_7$	5.89	6.22^{a}	9.28	7.29^{a}	3.07^{b}	3.28	44.6^{b}	30.6^{b}	74.5	55.9	2.19^{b}	2.93	167	76.1^{ab}	0.72	0.98^{a}
Encín	**SV21**	**Control**	2.47^{b}	3.55^{ns}	8.44^{ns}	4.53^{ns}	3.04^{ns}	3.08^{b}	50.8^{ns}	28.3^{ns}	97.9^{ns}	68.9^{a}	3.19^{ns}	6.04^{ns}	91.8^{a}	55.9^{ns}	0.81^{b}	0.54^{b}
		Sludge	4.97^{a}	3.92	7.02	5.18	3.29	4.59^{a}	49.5	27.8	46.1	29.7^{b}	2.52	4.78	58.0^{b}	62.2	1.37^{a}	1.52^{a}
	SV38	**Control**	3.58^{ns}	3.49^{ns}	8.79^{ns}	6.50^{ns}	3.35^{b}	2.23^{ns}	85.2^{a}	33.3^{ns}	51.7^{ns}	60.6^{a}	3.74^{ns}	2.78^{ns}	123^{ns}	63.3^{ns}	0.48^{b}	0.92^{b}
		Sludge	8.77	2.91	9.50	4.45	3.96^{a}	5.20	58.3^{b}	30.1	30.6	29.0^{b}	3.63	2.91	107	72.9	0.78^{a}	1.03^{a}
	three way ANOVA																	
		Soil (S)	*	ns	ns	ns	*	**	ns	***	ns	**	**	ns	ns	ns	***	ns
		Pollution (Po)	*	***	ns	ns	***	**	***	**	**	***	***	ns	**	ns	**	***
		Plant (Pl)	*	*	***	***	***	ns	***	ns	***	ns	ns	ns	***	**	**	ns
		S x Po	*	ns	ns	ns	**	ns	***	**	ns	ns	***	*	ns	ns	ns	ns
		S x Pl	ns	ns	ns	ns	**	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns
		Po x Pl	ns	**	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
		S x Po x Pl	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns

H1: First harvest; H2: second harvest. Values are means (n=3). Different lowercase letters indicate significant differences among treatments. Different capital letters indicates differences among harvests (Duncan's test $p<0.05$). Three-way ANOVA's results indicates the statistical influence of the factors: soil (S), pollution (Po) and plant (Pl) and their interactions. Soil factor includes two levels: Escorial and Encín; Pollution

Table6: Trace element concentration in roots of *S. vulgaris* genotypes (mg·kg^{-1} D.W.)

			Cr	Cu	Pb	Zn	Mn	Ni	Fe	Cd
Soil	**Genotype**	**Pollution**	***H2***	***H2***	***H2***	***H2***	***H2***	***H2***	***H2***	***H2***
Escorial	**SV21**	**Control**	4.26^{b}	5.69^{b}	5.39^{ns}	67.0^{b}	71.3^{a}	8.14^{b}	1142^{ns}	0.50^{b}
		Sludge	23.5^{ab}	13.2^{a}	5.12	97.2^{a}	45.2^{b}	35.7^{a}	1336	0.74^{a}
		$K_2Cr_2O_7$	46.2^{a}	5.00^{b}	5.57	57.1^{b}	70.1^{a}	3.97^{b}	1229	0.44^{b}
	SV38	**Control**	9.85^{c}	6.45^{b}	2.78^{ns}	47.2^{ns}	79.0^{ns}	18.9^{a}	1627^{a}	0.29^{b}
		Sludge	31.6^{b}	20.1^{a}	5.27	81.9	52.3	15.7^{a}	1268^{a}	0.72^{a}
		$K_2Cr_2O_7$	71.3^{a}	7.19^{b}	4.93	48.8	97.2	8.65^{b}	860^{b}	0.34^{b}
Encín	**SV21**	**Control**	16.1^{b}	5.71^{ns}	4.75^{ns}	40.4^{ns}	57.5^{ns}	3.52^{b}	444^{a}	0.76^{b}
		Sludge	25.9^{a}	10.1	7.44	41.7	32.6	5.95^{a}	398^{b}	1.13^{a}
	SV38	**Control**	2.95^{b}	8.58^{ns}	4.38^{ns}	51.0^{ns}	68.7^{ns}	3.53^{b}	646^{ns}	0.19^{b}
		Sludge	126^{a}	20.7	9.63	50.2	43.0	11.0^{a}	592	0.52^{a}
three way ANOVA										
Soil (S)			**	ns	ns	**	ns	***	***	*
Pollution (Po)			***	**	*	**	**	***	***	***
Plant (Pl)			***	*	ns	ns	ns	***	**	***
S x Po			**	ns	ns	*	ns	ns	ns	***
S x Pl			*	ns	ns	ns	ns	**	ns	ns
Po x Pl			**	ns	ns	ns	ns	***	***	ns
S x Po x Pl			**	ns	ns	ns	ns	***	ns	ns

H1: First harvest; H2: second harvest. Values are means (n=3). Different lowercase letters indicate significant differences among treatments. Different capital letters indicates differences among harvests (Duncan's test $p<0.05$). Three-way ANOVA's results indicates the statistical influence of the factors: soil (S), pollution (Po) and plant (Pl) and their interactions. Soil factor includes two levels: Escorial and Encín; Pollution factor has three levels: no pollution (control), sludge and spiked $K_2Cr_2O_7$ solution and Plant factor includes two levels: SV21 and SV38. (ns= non significant; *p<0.05; **p<0.01; ***p<0.001).

Table 7: Phytotoxic effe**ct** of treatment in *S. vulgaris* genotypes: biomass (mg D.W·plant^{-1}) and lipid peroxidation (µmol MDA·g^{-1}F.W.).

			Biomass (g D.W·plant^{-1})			Oxidative stress (µmol MDA· g^{-1} F.W.)		
Soil	**Genotype**	**Pollution**	**Shoots**		**Roots**	**Shoots**		**Roots**
			H1	***H2***	***H2***	***H1***	***H2***	***H2***
Escorial	**SV21**	**Control**	29.20^{ns}	10.45^{ns}	8.83^{a}	7.63^{ns}	4.56^{ns}	2.34^{b}
		Sludge	33.39	15.25	1.73^{b}	4.34	5.54	8.76^{a}
		$K_2Cr_2O_7$	28.69	8.42	7.03^{a}	6.19	3.71	4.12^{b}
	SV38	**Control**	16.88^{a}	10.89^{a}	8.17^{a}	3.13^{ns}	4.64^{ns}	4.52^{b}
		Sludge	13.92^{b}	3.63^{b}	0.67^{b}	5.59	6.26	11.30^{a}
		$K_2Cr_2O_7$	20.06^{a}	8.82^{a}	5.91^{a}	4.74	3.29	10.69^{a}
Encín	**SV21**	**Control**	16.24^{b}	13.85^{ns}	6.47^{a}	4.55^{ns}	6.84^{ns}	3.35^{b}
		Sludge	28.22^{a}	12.08	2.19^{b}	5.18	6.49	6.48^{a}
	SV38	**Control**	10.52^{a}	11.36^{a}	6.55^{a}	3.10^{ns}	4.17^{ns}	13.45^{a}
		Sludge	3.31^{b}	3.53^{b}	1.07^{b}	4.41	5.33	6.35^{b}
	three way ANOVA							
		Soil (S)	***	***	**	ns	ns	ns
		Pollution (Po)	**	**	***	ns	ns	*
		Plant (Pl)	***	***	ns	**	**	ns
		S x Po	**	**	**	ns	ns	ns
		S x Pl	ns	ns	ns	ns	ns	ns
		Po x Pl	***	***	ns	*	*	*
		S x Po x Pl	ns	ns	ns	ns	ns	ns

H1: First harvest; H2: second harvest. Values are means (n=3). Different lowercase letters indicate significant differences among treatments. Different capital letters indicates differences among harvests (Duncan's test p<0.05). Three-way ANOVA's results indicates the statistical influence of the factors: soil (S), pollution (Po) and plant (Pl) and their interactions. Soil factor includes two levels: Escorial and Encín; Pollution factor has three levels: no pollution (control), sludge and spiked $K_2Cr_2O_7$ solution and Plant factor includes two levels: SV21 and SV38. (ns= non significant; *p<0.05; **p<0.01; ***p<0.001).

7.3.2.c. Organic acid content in plant tissues

Table 8 shows concentration of organic acids in plant tissues (mg·kg^{-1}D.W.). Soil characteristics significantly (p<0.05) influenced concentrations of citric, malic and succinic acids in shoots and roots and concentrations of formic, lactic and acetic acids in roots. Treatment applied to soil induced changes in the concentrations of oxalic, citric, malic, acetic and succinic and of citric, malic of roots. In the first harvest, the concentration of oxalic, citric, malic and succinic decreased in shoots of plants grown in polluted soils. Related to the second harvest, the concentration of these organic acids reached similar concentrations in control plants than in thoses grown in $K_2Cr_2O_7$ spiked soils. Differences in the organic acid concentrations have been found in oxalic and

citric acid between the two harvests that tended to increase in the second one. Malic showed the opposite trend.

In roots, concentrations of citric and malic acids were the most affected by factors of the study, as seen by the statistically significant (p<0.01) interaction among soil caracteristics, pollution and genotype. In Escorial soil, the genotype SV21 increased its concentration of citric acid when plants were grown in $K_2Cr_2O_7$ spiked soils. No effect was found on malic acid concentration. Genotype SV38 decreased citric acid concentration when grown in Escorial soils polluted with both sources of Cr and malic acid also decreased in plants from sludge enriched pots. In Encin soil, genotype SV21 decreased its concentrations of citric and malic due to sludge treatment, by contrast SV38 did not shown any change in citric and malic concentration due to pollution.

7.3.3. Changes in soil solution

Figure 4 displays the evolution of pore water pH along the essay. Samples have been collected before (5 and 20 October) and after plant transplanting (19 April and 10 May). The two soils used in the study showed high significant differences (p<0.001) in this variable being the values found in Escorial soil more acid than in Encín one. Significant differences (p<0.001) have also been found in pots due to pollution source. The solution pH decreased with sludge applications, this parameter increased in $K_2Cr_2O_7$ solution. In both soils, pH tended to increase with time. The presence of *S. vulgaris* genotypes basified soil solution in Escorial pots spiked with $K_2Cr_2O_7$ and in control and sludge polluted pots of Encín soil.

Electric conductivity (EC) ($dS \cdot m^{-1}$) of pore water is shown in figure 5. Encín soil showed significantly higher EC than Escorial soil. Pollution increased EC of both soils; this increment was greater with sludge application. In Escorial soil, the presence of *S. vulgaris* decreased EC in control and $K_2Cr_2O_7$ spiked pots but in pots polluted with sludge, EC increased in pots with cover of genotype SV38. In Encín soil, the presence of vegetation increased EC in both control and sludge pots.

Table 8: Concentration of organic acids (mg·Kg D.W.$^{-1}$) in shoots and roots of *S. vulgaris*.

			oxalic acid			citric acid			malic acid			formic acid			lactic acid			acetic acid			succinic acid		
Soil		**Pollution**	**Shoots**		**Roots**	**Shoots**		**Roots**	**Shoots**		**Roots**	**Shoots**		**Roots**	**Shoots**		**Roots**	**Shoots**		**Roots**	**Shoots**		**Roots**
			H1	***H2***		***H1***	***H2***		***H1***	***H2***		***H1***	***H2***		***H1***	***H2***		***H1***	***H2***		***H1***	***H2***	
A	**SV21**	**Control**	12275^{a}	12984^{a}	2133^{a}	304^{a}	345^{a}	219^{b}	$1979^{a,A}$	$651^{a,B}$	90.0	2.02	5.63	11.6^{a}	40.0^{a}	21.0	6.71^{b}	$253^{a,A}$	$155^{a,B}$	99.2	$148^{a,A}$	$98.3^{a,B}$	7.9
		Sludge	10039^{b}	1022^{b}	3900^{ab}	89^{b}	67^{b}	117^{b}	$319^{b,A}$	$125^{b,B}$	70.2	2.98	3.57	4.35^{b}	17.7^{b}	11.2	28.7^{b}	101^{b}	75.5^{b}	78.5	62.8^{b}	43.5^{b}	19.2
		$K_2Cr_2O_7$	$5274^{c,B}$	$11235^{ab,A}$	5656^{a}	$67^{b,B}$	$333^{a,A}$	351^{a}	$756^{c,A}$	$497^{a,B}$	148	4.36	2.60	13.7^{a}	18.3^{b}	26.1	71.8^{a}	93.4^{b}	127^{ab}	99.2	54.5^{b}	72.4^{ab}	17.8
	SV38	**Control**	$14066^{a,A}$	$11840^{a,B}$	3176	222^{a}	201^{a}	433^{a}	731^{a}	319^{a}	303^{a}	1.58	3.38	3.2^{b}	$43.2^{a,A}$	14.0^{B}	19.7	140^{a}	103	81.5	60.5^{a}	41.2^{a}	10.6^{b}
		Sludge	$2944^{b,B}$	$8576^{b,A}$	3406	55^{b}	71^{b}	166^{b}	212^{b}	101^{b}	63.0^{b}	3.21	2.18	5.6^{ab}	15.6^{b}	28.6	35.6	41.8^{b}	39.5	118	9.23^{c}	9.92^{b}	8.67^{b}
		$K_2Cr_2O_7$	$4986^{b,B}$	$11328^{a,A}$	3073	$72^{b,B}$	$217^{a,A}$	213^{b}	$370^{ab,A}$	$217^{ab,B}$	268^{a}	2.98	4.90	7.8^{a}	11.2^{b}	14.6	27.7	51.4^{b}	91.4	86.1	30.3^{b}	27.1^{ab}	22.7^{a}
B	**SV21**	**Control**	5210^{B}	10999^{A}	3457	60	85	680^{a}	438	336	315^{a}	2.16	2.13	13.6	10.0	19.8	55.9	58.1	102	167	32.5	41.8^{a}	33.4
		Sludge	10648	10446	4506	91	58	423^{b}	438^{A}	135^{B}	83.0^{b}	1.87	1,81	7.91	16.8	26.7	45.6	35.5	39.5	133	35.8	35.3^{b}	9.55
	SV38	**Control**	7582^{B}	10863^{A}	3550	90	119	344	264	118	230	2.32	1.31^{b}	20.5	15.2	9.75	80.1	38.9	41.5	113	14.8	10.9	17.2
		Sludge	8699^{B}	10203^{A}	4097	178	190	414	347	181	183	2.86	2.25^{a}	16.1	31.4	43.9	56.7	82.8	76.6	155	31.2	23.7	31.9
	three way ANOVA																						
		Soil (S)	ns		ns	*		***	**		*	ns		**	ns		***	ns		**	**		*
		Pollution (Po)	*		ns	***		**	***		**	ns		ns	ns		ns	*		ns	*		ns
		Plant (Pl)	ns		ns	ns		*	***		*	ns		ns	ns		ns	ns		ns	***		ns
		S x Po	*		ns	***		ns	***		ns	ns		ns	ns		*	ns		ns	*		ns
		S x Pl	ns		ns	**		***	ns		ns	ns		*	ns		ns	ns		ns	ns		ns
		SxPoxPl	ns		ns	ns		**	ns		**	ns		*	ns		ns	ns		ns	ns		*

H1: First harvest; H2: second harvest. A: Escorial soil; B: Encín soil. Values are means (n=3). Different lowercase letters indicate significant differences among treatments. Different capital letters indicates differences among harvests (Duncan's test $p<0.05$). Three-way ANOVA's results indicates the statistical influence of the factors: soil (S), pollution (Po) and plant (Pl) and their interactions. Soil factor includes two levels: Escorial and Encín; Pollution factor has three levels: no pollution (control), sludge and spiked $K_2Cr_2O_7$ solution and Plant factor includes two levels: SV21 and SV38. (ns= non significant; *p<0.05; **p<0.01; ***p<0.001).

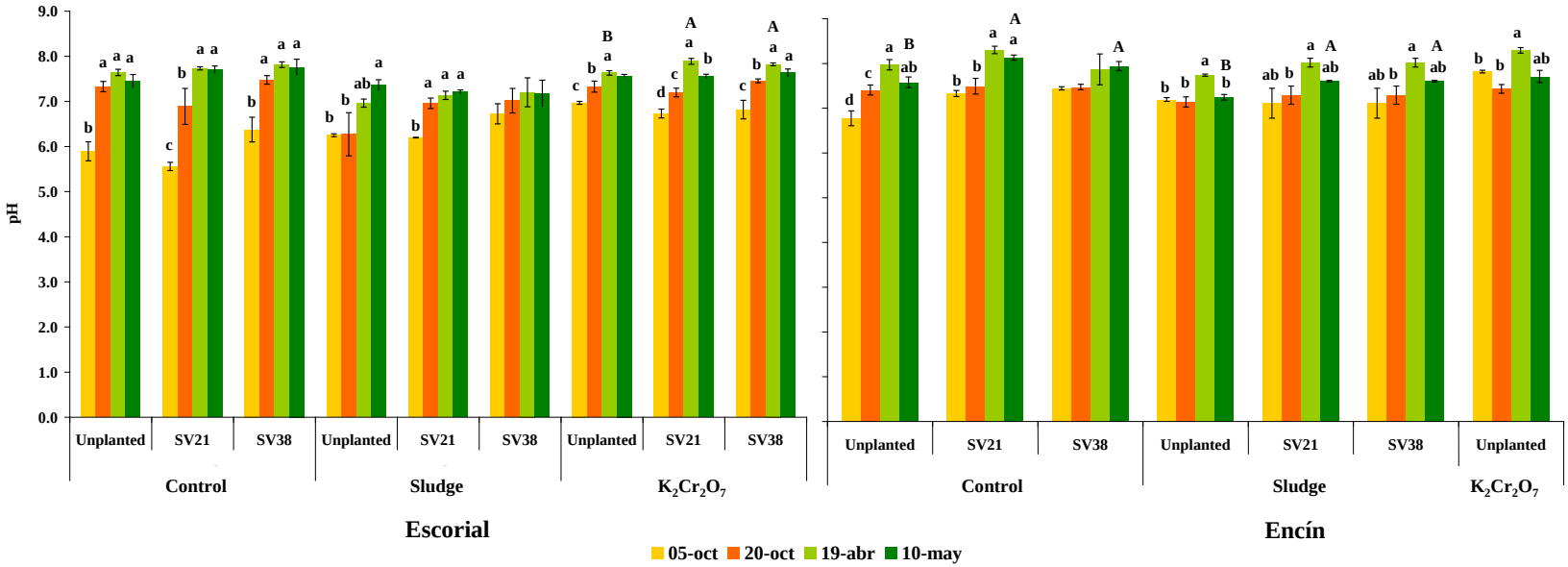


Figure 6: pH monitoring of soil solution from cluster roots. Lowercase letter indicate differences among samples time in the same treatment, Capital letters indicate differences among plant treatment in the pollution treatment. (Duncan´s test at the $p<0.05$ level). Mean values ±SE (n=3).

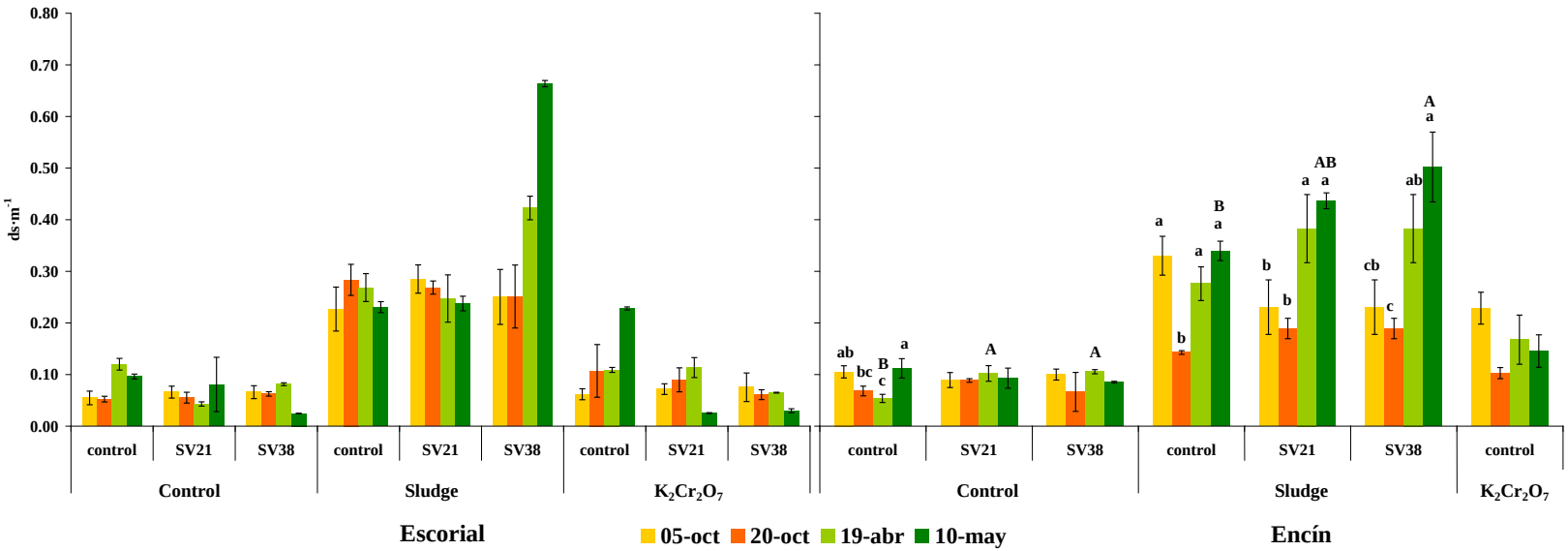


Figure 7: Monitoring of electric conductivity (dS·m^{-1}) of soil solution from cluster roots. Lowercase letter indicate differences among samples time in the same treatment, Capital letters indicate differences among plant treatment in the pollution treatment. (Duncan´s test at the p<0.05 level). Mean values ±SE (n=3).

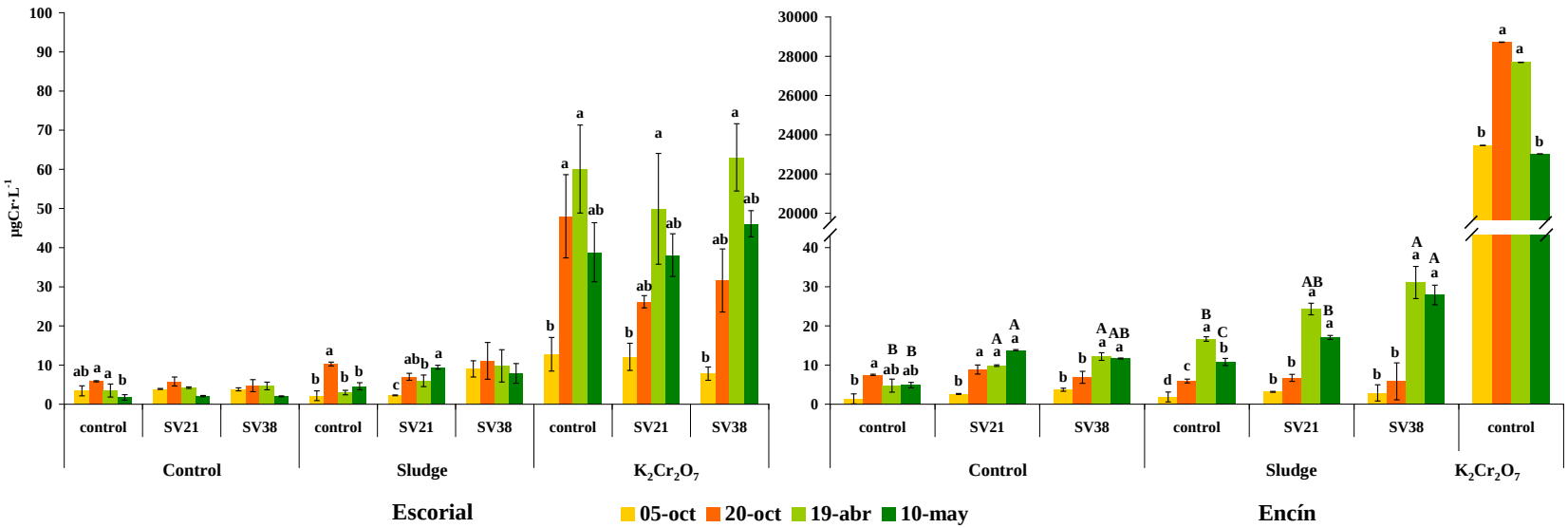


Figure 8: Monitoring of Cr in soil solution from cluster roots(µgCr·L^{-1}). Lowercase letter indicate differences among samples time in the same treatment, Capital letters indicate differences among plant treatment in the pollution treatment. (Duncan´s test at the p<0.05 level). Mean values ±SE (n=3).

Concentration of total Cr in soil solution is given in figure 5. Soil characteristics strongly influenced Cr solubility ($p<0.001$) thus Encín soil showed higher concentration of dissolved Cr than Escorial. Only Cr input into soils from $K_2Cr_2O_7$ solution enhance Cr concentration in pore water regards control soil before plant transplanting. By contrast, the presence of *S. vulgaris* cover increased Cr concentration in sludge polluted pots. 100% of all dissolved Cr found in Encín soil spiked with $K_2Cr_2O_7$ solution was Cr(VI). Soluble Cr in Escorial pots spiked with $K_2Cr_2O_7$ solution correspond to Cr(VI) in the first sample (5 October) time but was not detectable in other samples times. No Cr(VI) was detectable in soil solution of the other treatments by method applied.

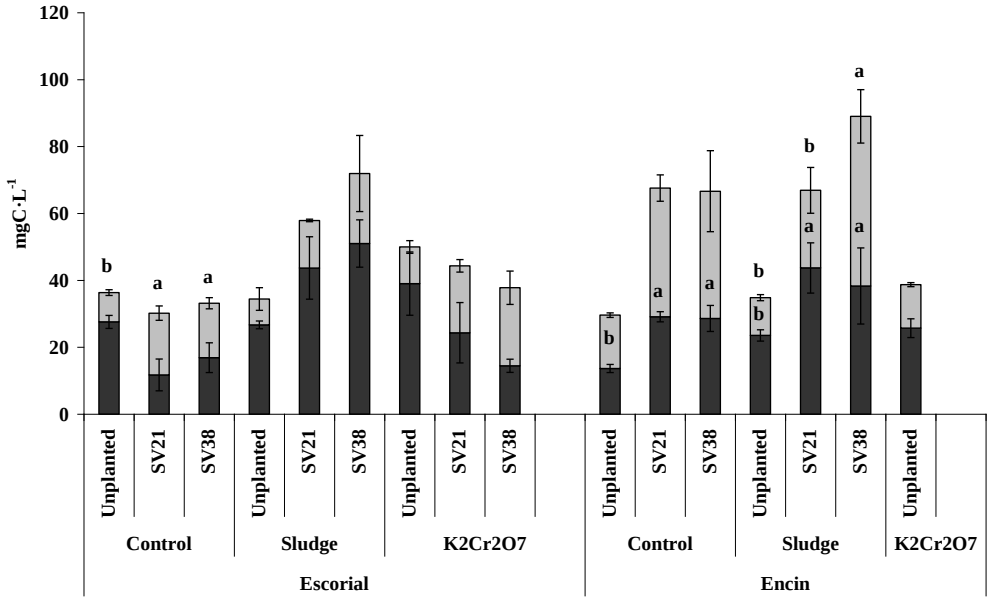


Figure 9: Water soluble organic carbon (black bars) and water soluble inorganic carbon (grey bars) in pore water of cluster roots (mgC·L^{-1}) at the end of the experiment. Lowercase letters indicate differences among plant coverin pot with the same pollution treatment. Duncan´s test at the $p<0.05$ level). Mean values ±SE (n=3).

Figure 9 shows water soluble total carbon (WSTC, mg C·L^{-1}) which includes water soluble inorganic carbon (WSIC, mg C·L^{-1}) and water soluble organic carbon (WSOC, mg C·L^{-1}) in pore water samples at the end of the experiment. Soil type significantly ($p<0.001$) influences WSIC but not WSOC. Encin soil showed higher WSIC than Escorial. The application of sludge increases WSOC in both soils, by contrast no effects due to pollution have been recorded in WSIC. The presence of vegetal cover tended to increase WSOC values being statistically significant ($p<0.05$) in

Encin soil. WSIC was also enhanced by *S. vulgaris*, this influence is significant in Encín and control pots of Escorial soil.

7.4. DISCUSSION

Total metal concentrations in soils are usually not good indicators of environmental hazard because they do not provide any information about the risk of metal to be leachated to the groundwater or to be taken up by plants and thus transferred to the food chain. Soil characteristics influence metal speciation and hence their soluble and bioavailable fractions that are much more interesting than the total ones in terms of risk assessment.

This fact is of special importance in the case of Chromium as its solubility and toxicity strongly depend on its chemical form. The two most common Cr species, trivalent and hexavalent, could interconvert by redox reactions. However, once a source of Cr pollution is discharged into soils, kinetic limitations based on electron symmetry constraints in the electron transfer process stabilize the existing Cr oxidation state (either trivalent or hexavalent Cr) unless a suitable redox couple directly complexes with the Cr species (Fendorf, 1995). Organic matter and pH of soils are the key factors that will determinate Cr speciation and availability in soils.

After soil consolidation, total Cr concentration reached similar levels independently of Cr pollution source (sludge or $K_2Cr_2O_7$) and soil characteristics. This is because both Cr(III) and Cr(VI) species are included in this variable. By contrast, significant differences have been found among Cr concentrations in soil solution. Cr in soil solution corresponded just with soluble fractions and included the soluble organic complexes of Cr(VI) and Cr(III). Due to the acid pH and the high content in organic matter of sludge, most of Cr provided may be precipitated as $Cr(OH)_3$, no soluble Cr have been detected in soils polluted with sludge. The increases in concentrations of soluble Cr have only been found in soils directly spiked with Cr(VI) as $K_2Cr_2O_7$ solution. Speciation analysis has confirmed that soluble Cr found in this soils correspond mainly to Cr(VI). However, as expected given the high differences in pH

and organic matter between the two soils, high differences have been found in Cr(VI) availability. Manganese and iron oxides seem not to be differential factors between the two soils and redox reactions because these components are unlikely. In Escorial soil, acid and rich in OM, Cr(VI) have been reduced to Cr(III). In alkaline and poor soil from Encín, high amounts of Cr(VI) have been detected in pore water with the consequent higher environmental risk.

Sludge applications have increased the concentration of total heavy metals in both soils, however Cr and Zn overcome the limits imposed by legislation (Orden 2270/2006, de 11 de agosto). Sludge amended soil also resulted in increasing heavy metals in soil solution. On contrary to Cr, most of heavy metals increased their solubility in soils under acid conditions, that makes that in Escorial soil, the increments of metals concentrations in soil solution due to sludge application were higher than in Encin soil. This fact corroborates the importance to determinate the bioavailable and soluble fractions of metals as they could strongly differ in soils even when total metal concentrations are in same range.

Soil characteristics at the beginning of the experiment (to) are given in table 4 that shows differences between the soils objective of study. Main differences are related to pH and OM content. Spiked soils with $K_2Cr_2O_7$ solution lead to a slight increase in EC regard to controls, due to the supplement of K^+ from $K_2Cr_2O_7$ solution. Therefore, pH also increased because Cr(VI) enhanced nitrification process (oxidation of ammoniacal N to nitrate N) and proton release into soils (Bolan *et al*., 2003). Sludge application, improved soil fertility by increasing the contents of N, OM and P. Sludge addition also incremented EC in both soils regards to control and $K_2Cr_2O_7$ spiked soils due to high concentration in exchangeable cations and metals. Soil pH also decreased, especially in treatment with Escorial soil. This decrease in pH was previously reported (Planquart *et al*., 1999; Navas *et al*., 1998) and it may be related to organic acid release during sludge decomposition as resulting influence in metal mobility. (Singh and Agrawal, 2008)

Two genotypes of *S. vulgaris* have been grown for two vegetative periods (6 months) in these soils to study plant-soil interactions. The chromium accumulation in plant depended on available Cr which was determined by Cr speciation and soil characteristics. *S. vulgaris* genotypes accumulated more Cr in plants grown in Encín soil than in Escorial due to Cr concentration increased in available fractions in Encín samples. In general, plants accumulated more Cr in soil spiked with $K_2Cr_2O_7$ because Cr(VI) is more soluble and hence bioavailable and it enters in plant roots by metabolically active mechanisms (Skeffington *et al*., 1976) meanwhile Cr(III) from sludge amended is less soluble and does not posses any specific uptake mechanism in plants. In fact, Cr(VI) was so available in Encín soil that plants were not able to growth in $K_2Cr_2O_7$ spiked pots. Thought non significant greater soluble Cr fraction regards controls has been found in sludge rich soils, plants from these soils have accumulated significantly more Cr than plants from control pots. That may be explained because *S. vulgaris* exudates organic acids into the rhizosphere that forms soluble Cr(III)-organic complexes increasing Cr uptake as shown in chapter 5.

In studied soils, Cr was mainly accumulated in roots as it has been described in other species grown in soils polluted with different Cr sources (Lopez-Luna *et al*., 2009; Bluskov *et al*., 2005; Turgut *et al*., 2004). Normal ranges of Cr concentration in plant are between 0.006 and 18 $mg \cdot kg^{-1}$D.W., usually in the order of 1-5 $mg \cdot kg^{-1}$D.W. (Zayed and Terry, 2003). Even plants adapted to serpentine soils rarely show Cr contents higher than 45 $mg \cdot kg^{-1}$D.W. and seldom if ever higher than 100 $mg \cdot kg^{-1}$D.W. (Barceló and Poschenrieder, 1997). Thus, in general, plant to soil concentration ratios are small, reducing the possibility to apply phytoextraction process in Cr polluted areas. McGrath *et al*. (2006) established the threshold value of bioconcentration factor below 10 for a phytoextraction process to be feasible. Though *S. vulgaris* showed Cr concentrations in shoots slight higher than usual values when plants grown in Cr polluted soils, genotypes used in this study showed bioconcentration factors for Cr between 0.018 and 0.075. That indicates that this specie could not be applied for phytoextraction processes but in the phytoestabilisation of Cr polluted sites. The low transfer factor for Cr and the other metals considered, lower than 1, means a low risk of

heavy metals to be transferred through the food chain supporting the potential use of this specie in phytoestabilitation processes.

Sludge pollution increased the concentrations of Pb, Zn, Ni and Cd in plant tissues but these values did not overcome the normal concentrations described for plants (Gardea-Torresdey *et al*., 2005). Changes in Mn and Fe concentrations, however, seem to be caused by physiological responses under Cr toxicity as have been described in chapters 3 and 4.

Plant development, measured as dry mass and oxidative stress determined as levels of MDA, have been taken as parameters to evaluate plant toxicity to soil pollution. Dry mass has been shown to be more sensitive to pollution than MDA. Specially, genotype SV38 decreased dry mass when grown in pots polluted with sludge. Roots were more affected by pollution toxicity than shoots as they showed both dry mass reduction and increments in MDA in plant from sludge amended pots. The higher toxicity found in plants grown in sludge amended pots than in $K_2Cr_2O_7$ spiked pots means that phytoxicity is not related with Cr accumulation in dry tissues. Though, the application of sludge provides N, P, nutrients and organic matter to soils it also increases salt concentrations in soils, as seen in EC increments in both soils and soil solution samples. Salinity is considered a major abiotic stress to plants. High salt levels disrupts homeostasis in water potential and ion distribution (Zhu, 2001) leading to increments in oxidative stress of roots and hence to root growth retardation in *S. vulgaris*. Moreover *S. vulgaris* has been proven to be sensitive to low pH (Carpena-Ruiz *et al.,* 2008). In studies to asses the toxicity of Cr from different sources using wheat, oat and sorghum plants, Lopez-Luna *et al*. (2009) concluded that the study of root growth was the best variable to estimate Cr toxicity. The absence of growth retardation and oxidative stress in roots of *S. vulgaris* grown in Escorial soils spiked with $K_2Cr_2O_7$ makes *S. vulgaris* to be considered a relatively tolerant specie able to develop a root system and growth in soils with moderate available fractions of Cr.

Organic acids play an important role in heavy metal detoxification by participating in metal chelation, transport and storage (Clemens, 2001). Most crops

species present organic acid concentrations between 1 and 17 $mg \cdot kg^{-1}$D.M. (Boominathan and Doran, 2003). *S. vulgaris* showed higher constitutive levels of organic acids than theses species, similar to that of hypeeracumulator plants (Boominathan and Doran, 2003) which confers this specie a mechanism to manage the excess of Cr. Depending on heavy metal and plant, the release of organic acids into the rhizosphere has been related with two antagonism process: metal exclusion or hyperaccumulation (Wenzel, 2009; Barceló and Poschenrieder, 2002). The concentration of organic acids in *S. vulgaris* tissues decreased when plants grown in sludge amended soils, especially in the acid soil from Escorial. In this case, significant increments of heavy metals and WSOC concentrations have been found in soil solution samples from cluster root. That indicates that organic acid decrease in plant tissues may be related to root exudation in order to chelate heavy metals in the rhizosphere. Similar chelation process have been previously proposed to diminish toxicity of Cu (Meier *et al.*, 2012), Pb, Zn (Magdziak *et al.*, 2011), Ni (Drzewiecka *et al*., 2012) and Cd (Zhu *et al.*, 2011) in different plant species. From results in chapter 5, citric and malic acid seems to be the most important organic acids in Cr accumulation and detoxification in *S. vulgaris*. Plants from pots spiked with $K_2Cr_2O_7$ solution tended to accumulate citric and malic acid in roots confirming their role in Cr accumulation, chelation and storage.

Not only soil characteristic and pollution have influenced in plant development and trace element concentration in plant, but plant growth in turn has influenced soil characteristics.

The pH of soils tended to increase over time especially in pots where *S. vulgaris* formed cover. Several mechanisms have been described by which root plants modify the pH of the surrounding soil, namely: cation-anion exchange balance, organic anion release, root exudation and respiration and redox-coupled processes (Hinsinger *et al*., 2003). Related to cation-anion balance, plants could counterbalance the excess of negative charges from the uptake of anions CrO_4^{2-} and $HCrO_4^-$ by releasing equivalent amounts of OH^- or HCO^-_3 into the rhizosphere, thereby increasing rhizosphere pH. Besides this, Cr stress have been reported to cause strong inhibition of H^+ and K^+ uptake which could also result in pH increases (Zaccheo *et al*., 1985). Moreover Cr

toxicity could inhibit ATPase activity, a key component of the pH-stat system, decreasing proton exclusion (Astolfi *et al*., 2003). In other hand, the realise of organic acids by roots could dissolve $CaCO_3$ increasing the pH (Hinsinger *et al.,* 2003; Miyasaka *et al*., 1989) that could explains why in the carbonate soil of Encín, the effect of plant cover on pH is greater than in Escorial.

In recent years biological and biochemical indicators have been frequently used to asses the efficiency of phytoremediation process (Epelde *et al.,* 2012; Epelde *et al*., 2009; Moreno-Jimenez *et al.,* 2009;) due to their sensitive and capacity to provide information that integrates many environmental factors (Alkorta *et al.,* 2003). In particular, enzyme activities are of especial importance in the evaluation of soil functionality because of their major contribution to organic matter degradation in soils (Trasar-Cepeda *et al*., 2000).

In this study biological activity (respiration rate) as well as dehydrogenase and β-galactosidase activities have been determined. It is widely accepted that biological activity reduction in soils is strongly dependent on the available fraction of metals and hence on soil characteristics (Giller *et al.,* 2009). Escorial soil showed higher values of respiration and enzymatic activities than Encín soil due to high content in OM, which masks the effect of metal contamination in this soil. Due to the input of OM, soils spiked with sludge showed an increase in biological activity over controls. However, soils spiked with $K_2Cr_2O_7$ solution reduced biological activity in Encín soil. These results are according to other authors (Epelde *et al.,* 2009; Martinez-Inigo *et al.,* 2009; Mench *et al.,* 2006) by which biological indicators have been negatively correlated with labile metal pools and supported the idea that available metal fractions in soils have more environmental relevance than total concentration. Among evaluated biological parameters, dehydrogenase activity has been demonstrated to be the most sensitive to both pollution and plant treatment. In fact, dehydrogenase activity is the most widely studied enzyme indicator of soil biological activity because it represents just the activity of viable cells and because it strongly depends on the source of pollution and soil characteristics (Trasar-Cepeda *et al.,* 2000). Moreover dehydrogenase activity is also correlated to improvement on soil physical characteristics due to the beneficial effect on

soil microorganism activity. The presence of *S. vulgaris* in pots has been found to increase the activity of this enzyme. Increases on dehydrogenase activity due to vegetation cover have been previously reported in polluted soils (Belen Hinojosa *et al*., 2010; Epelde *et al.,* 2009; Hinojosa *et al.,* 2004). This enhancement in activity has been atributed to plant root exudation (amino acids, organic acids, sugars and flavonids) and other rhizodepositions (plant mucilages, root lysates) that suppose a source of carbon that is metabolised by microorganism to growth leading to soil quality recovery. The development of plant roots also increased soil porosity and hence O_2 concentrations in soils which contributes to growth of soil microorganisms.

Soil solutions from cluster roots have been collected to study the influence of plant rhizosphere on soil chemistry. As seen in data from bulk soil, each soil presented differences in pH, EC and available Cr. At the beginning of the experiment, Escorial soil showed lower pH than the Encín one, however, pH increased over the time, especially in soils with *S. vulgaris* cover. Both soils reached similar pH levels at the end of the experiment. Considerable controversy exists in the literature regarding the effect of plant growth on the pH at the root interface that depends on plant, pollutant and soil characteristics (Blossfeld *et al.,* 2011; Kim *et al*., 2010; Gonzaga *et al.,* 2006). Several mechanisms by which *S. vulgaris* could increase soil pH have been proposed above. In the study of the rhizosphere of *Brassicaea juncea* and *Heliantus annus*, Kim *et al*. (2010) found similar results than in this study. The author proposed that pH increase is due to increments in WSOC resulted from organic acid exudation. This effect is greater in alkaline than in acidic soils. Significant WSOC increases have been found in the rhizosphere solutions of *S. vulgaris* grown in Encín soil suggesting that the process by which the presence of *S. vulgaris* induces changes in pH is mainly governed by root exudation of carbon compounds.

The presence of plant in pots increased EC of soils regards unplanted pots as result of solutes exudation by plant roots, thus EC was positively correlated with WSOC in soil solution.

The monitoring of Cr available in soil solution from cluster roots showed the same profile as described for soil solution at the beginning of the experiment. Available Cr is defined by soil characteristics, mainly pH and OM and its concentration is greater in Encín soil than in Escorial. Available Cr in sludge polluted soils is correlated to soluble Cr(III) complexes meanwhile in $K_2Cr_2O_7$ treatment, Cr is found totally as Cr(VI) form. As has been discussed, plants increased pH and WSOC which caused an increase in the available Cr fraction in pots of Encín soil polluted with sludge. Organic acid have been shown to form soluble Cr(III)-organic acid complexes (Srivastava *et al*., 1999) enhancing Cr accumulation in roots (Hayat *et al*., 2012). Thus WSOC increases of *S. vulgaris* rhizosphere may be related to the exudation of organic acids by plants to enhance Cr availability and hence Cr uptake.

S. vulgaris cover increases water soluble total carbon (WSTC) in soil solutions regard to unplanted pots. That means both an increase in water soluble organic carbon (WSOC) as in water soluble inorganic carbon (WSIC). As it has been described, the enhancement in WSOC increases Cr solubility but it also has been demonstrated to reduce Cr(VI) to Cr(III) (Bolan *et al.,* 2003). Although, total soluble Cr does not change over time, no Cr(VI) was detectable in Escorial soils polluted with $K_2Cr_2O_7$ at the end of the experiment. This data indicated that the reduction of Cr(VI) to Cr(III) have been carried out by WSOC with the consequent environmental risk reduction. Moreover WSOC serves as a source of carbon for the growth of microorganisms, as has been reflected in the enhancement of the hydrogenase activity, some of which could also reduce Cr(VI) to Cr(III) (Cervantes *et al.,* 2001). The organic carbon mineralisation by microorganisms leads to an increase in WSIC which in turn enhances Cr solubility by competition for adsorption sites.

The main carbon fraction of water soluble organic carbon (WSOC) in soil solution corresponds to humic fractions which are determined by soil characteristics. Low molecular weight organic acids from biotic sources comprise less than 10% of total WSOC in soil solutions (Strobel, 2001). For that reason, in soil with high OM content as Escorial, the input of carbon from plant exudation is not significant, and it is in the soil with low OM content as Encín.

This work outline the environmental relevance to determinate available fractions of metals in polluted soils because they strongly determinates toxicity to plants and soil microorganism as well as soil ability to recover its quality. In the case of Cr spills, the chemical form of Cr in the pollution source is of special importance but also soil characteristic. Soils with low content in OM and alkaline pH are specially vulnerable to Cr(VI) pollution.

The ultimate goal of any heavy metal remediation process must be not only to remove the heavy metals from the soil but also to restore soil quality. Forming a vegetation cover in polluted soils improves soil structure, reduces soil erosion and pollutant mobilization. *S. vulgaris* has been able to grow in polluted soils with moderate concentrations of available Cr, even in soils with different characteristics and not only the tolerant genoptypes but also the sensitive one. Forming of *S. vulgaris* cover is a source of carbon that produces changes in both rhizosphere and bulk soil which is of special importance in soils with low OM contents. The carbon input from root exudates is mainly attributed to organic acid exudation and it is of especial relevance to Cr(VI) reduction and Cr(III) solubility by direct reaction and by its influence in soil pH. The Carbon provided by root exudation also promotes soil microorganism growth to enhance the process of soil quality recovery. All these reasons make *S. vulgaris* a good candidate to the revegetation and recovery of chromium polluted soils.

7.5. REFERENCES

Ali, N.A., Dewez, D., Didur, O., Popovic, R., 2006. Inhibition of photosystem II photochemistry by Cr is caused by the alteration of both D1 protein and oxygen evolving complex. Photosynthesis Res. 89, 81-87.

Alkorta, I., Aizpurua, A., Riga, P., Albizu, I., Amezaga, I., Garbisu, C., 2003. Soil enzyme activities as biological indicators of soil health. Rev. Environ. Health 18, 65-73.

Arnetoli, M., Montegrossi, G., Buccianti, A., Gonnelli, C., 2008. Determination of organic acids in plants of *Silene paradoxa* L. by HPLC. J. Agric. Food Chem. 56, 789-795.

Astolfi, S., Zuchi, S., Chiani, A., Passera, C., 2003. In vivo and *in vitro* effects of cadmium on H(+)ATPase activity of plasma membrane vesicles from oat (*Avena sativa* L.) roots. J. Plant Physiol. 160, 387-393 .

Barcelo, J., Poschenrieder, C., 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48, 75-92.

Barcelo, J., Poschenrieder, C., 1997. Chromium in plants, in: Caneli, S., Tittarelli, F., Sequi, P. (Eds.). FrancoAngeli, Milan (Italy), pp. 102-129.

Belen Hinojosa, M., Carreira, J.A., Garcia-Ruiz, R., Rodriguez-Maroto, J.M., Daniell, T.J., Griffiths, B.S., 2010. Plant treatment, pollutant load, and soil type effects in rhizosphere ecology of trace element polluted soils. Ecotoxicol. Environ. Saf. 73, 970-981.

Blossfeld, S., Gansert, D., Thiele, B., Kuhn, A.J., Loesch, R., 2011. The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of Juncus spp. Soil Biology & Biochemistry 43, 1186-1197 .

Bluskov, S., Arocena, J., Omotoso, O., Young, J., 2005. Uptake, distribution, and speciation of chromium in *Brassica juncea.* Int. J. Phytoremediation 7, 153-165.

Bolan, N.S., Adriano, D.C., Natesa, R., Koo, B.J., 2003. Effects of organic amendments on the reduction and phytoavailability of chromate in mineral soil. J. Environ. Qual. 32, 120-128.

Boominathan, R., Doran, P.M., 2003. Organic acid complexation, heavy metal distribution and the effect of ATPase inhibition in hairy roots of hyperaccumulator plant species. J. Biotechnol. 101, 131-146.

Carpena-Ruiz, R.O., Pérez Sanz, A., Vázquez, S., Moreno, E., Peñalosa, J., Hernández, L.E., Alarcón, R., García, P., Lobo, M.C., 2008. Respuesta de *Silene vulgaris* en Suelos Contaminados con Arsénico y Metales Pesados. Capacidad Fitorrecuperadora., in: Romero, R. (Ed.). Grupo Nutrición Mineral de Plantas-SEFV, Granada, pp. 485-501.

Catalá, M., Gasulla, F., Pradas del Real, A.E., Garcia-Breijo, F., Reig-Arminana, J., Barreno, E., 2010. Fungal-associated NO is involved in the regulation of oxidative stress during rehydration in lichen symbiosis. BMC Microbiology 10, 297.

Cervantes, C., Campos-Garcia, J., Devars, S., Gutierrez-Corona, F., Loza-Tavera, H., Torres-Guzman, J.C., Moreno-Sanchez, R., 2001. Interactions of chromium with microorganisms and plants. FEMS Microbiol. Rev. 25, 335-347.

Chao, T.T., 1972. Selective dissolution of manganese oxides from soils and sediments with acidified hydroxylamine hydrochloride. Soil. Sci. Soc. America 36, 764-768.

Chen, C., Lee, D., Juan, K., Lin, T., 2008. Phytotoxicity of soil trivalent chromium to wheat seedlings evaluated by chelating resin extraction method. Soil Sci. 173, 638-648.

Ciarkowska, K., Hanus-Fajerska, E., 2008. Remediation of Soil-Free Grounds Contaminated by Zinc, Lead and Cadmium with the Use of Metallophytes. Polish Journal of Environmental Studies 17, 707-712.

Comunidad de Madrid (Spain). Orden 2270/2006 de 11 de agosto por el que se establecen los. niveles genéricos de referencia de metales pesados y otros elementos traza en suelos contaminados de la Comunidad de Madrid, 28 agosto de 2006, num 204, pp. 29-30

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486.

Dessureault-Rompre, J., Luster, J., Schulin, R., Tercier-Waeber, M., Nowack, B., 2010. Decrease of labile Zn and Cd in the rhizosphere of hyperaccumulating *Thlaspi caerulescens* with time. Environmental Pollution 158, 1955-1962 .

Drzewiecka, K., Mleczek, M., Gasecka, M., Magdziak, Z., Golinski, P., 2012. Changes in *Salix viminalis* L. cv. 'Cannabina' morphology and physiology in response to nickel ions - Hydroponic investigations. J. Hazard. Mater. 217, 429-438 .

Eivazi, F., Tabatabai, M., 1990. Factors affecting glucosidase and galactosidase activities in soils. Soil Biol. Biochem. 22, 891-897.

Epelde, L., Becerril, J.M., Blanco, F., Kowalchuk, G.A., Garbisu, C., 2012. Links between pseudometallophytes and rhizosphere microbial communities in a metalliferous soil. Pedobiologia 55, 219-225.

Epelde, L., Mijangos, I., Becerril, J.M., Garbisu, C., 2009. Soil microbial community as bioindicator of the recovery of soil functioning derived from metal phytoextraction with sorghum. Soil Biology & Biochemistry 41, 1788-1794.

Ernst, W.H.O., Nelissen, H.J.M., 2000. Life-cycle phases of a zinc and cadmium resistanta ecotype of *Silene vulgaris* in risk assessment of polymetallic soils. Environmental Pollution. 107, 329-338.

Fendorf, S.E., 1995. Surface-Reactions of Chromium in Soils and Waters. Geoderma 67, 55-71 .

Fernandez, C., Alonso, C., Babın, M., Pro, J., Carbonell, G., Tarazona, J., 2004. Ecotoxicological assessment of doxycycline in aged pig manure using multispecies soil systems. Sci. Total Environ. 323, 63-69.

Gárate, A., Ramón, A.M., Carpena, R.O., ., 1984. Influence of boron on manganese and other nutrients in sap. Anales de Edafología y Agrobiología 43, 1467-1477.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., de la Rosa, G., Parsons, J.G., 2005. Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coord. Chem. Rev. 249, 1797-1810.

Giller, K.E., Witter, E., McGrath, S.P., 2009. Heavy metals and soil microbes. Soil Biology & Biochemistry 41, 2031-2037.

Gonzaga, M.I.S., Santos, J.A.G., Ma, L.Q., 2006. Arsenic chemistry in the rhizosphere of *Pteris vittata* L. and *Nephrolepis exaltata* L. Environmental Pollution 143, 254-260.

Hayat, S., Khalique, G., Irfan, M., Wani, A.S., Tripathi, B.N., Ahmad, A., 2012. Physiological changes induced by chromium stress in plants: an overview. Protoplasma 249, 599-611.

Hinojosa, M.B., Carreira, J.A., Garcia-Ruiz, R., Dick, R.P., 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. Soil Biology & Biochemistry 36, 1559-1568.

Hinsinger, P., Plassard, C., Tang, C., Jaillard, B., 2003. Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: A review. Plant Soil 248, 43-59.

Kidd, P., Barcelo, J., Pilar Bernal, M., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clernente, R., Monterroso, C., 2009. Trace element behaviour at the root-soil interface: Implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.

Kim, K.R., Owens, G., Naidu, R., 2010. Effect of Root-Induced Chemical Changes on Dynamics and Plant Uptake of Heavy Metals in Rhizosphere Soils. Pedosphere 20, 494-504.

Lopez-Luna, J., Gonzalez-Chavez, M.C., Esparza-Garcia, F.J., Rodriguez-Vazquez, R., 2009. Toxicity assessment of soil amended with tannery sludge, trivalent chromium and hexavalent chromium, using wheat, oat and sorghum plants. J. Hazard. Mater. 163, 829-834.

Magdziak, Z., Kozlowska, M., Kaczmarek, Z., Mleczek, M., Chadzinikolau, T., Drzewiecka, K., Golinski, P., 2011. Influence of Ca/Mg ratio on phytoextraction properties of *Salix viminalis*. II. Secretion of low molecular weight organic acids to the rhizosphere. Ecotoxicol. Environ. Saf. 74, 33-40.

Martinez-Inigo, M.J., Perez-Sanz, A., Ortiz, I., Alonso, J., Alarcon, R., Garcia, P., Lobo, M.C., 2009. Bulk soil and rhizosphere bacterial community PCR-DGGE profiles and beta-galactosidase activity as indicators of biological quality in soils contaminated by heavy metals and cultivated with *Silene vulgaris* (Moench) Garcke. Chemosphere 75, 1376-1381.

McGrath, S.P., Lombi, E., Gray, C.W., Caille, N., Dunham, S.J., Zhao, F.J., 2006. Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri*. Environmental Pollution 141, 115-125.

Mehra, O., Jackson, M., 1960. Iron oxide removal from soils and clays by a dithionite-citrate system buffered with sodium bicarbonate. 5, 317-327.

Meier, S., Alvear, M., Borie, F., Aguilera, P., Ginocchio, R., Cornejo, P., 2012. Influence of copper on root exudate patterns in some metallophytes and agricultural plants. Ecotoxicol. Environ. Saf. 75, 8-15.

Mench, M., Renella, G., Gelsomino, A., Landi, L., Nannipieri, P., 2006. Biochemical parameters and bacterial species richness in soils contaminated by sludge-borne metals and remediated with inorganic soil amendments. Environmental Pollution 144, 24-31.

M.A.P.A. (Ministerio de Agricultura), 1994. Métodos Oficiales de Análisis, Ed: Secretaría General Técnica.Vol. III, Spain

Miyasaka, S.C., Kochian, L.V., Shaff, J.E., Foy, C.D., 1989. Mechanisms of Aluminum Tolerance in Wheat - an Investigation of Genotypic Differences in Rhizosphere Ph, K^+, and H^+ Transport, and Root-Cell Membrane-Potentials. Plant Physiol. 91, 1188-1196.

Moreno-Jimenez, E., Peñalosa, J.M., Manzano, R., Carpena-Ruiz, R.O., Gamarra, R., Esteban, E., 2009. Heavy metals distribution in soils surrounding an abandoned mine in NW Madrid (Spain) and their transference to wild flora. J. Hazard. Mater. 162, 854-859.

Navas, A., Bermudez, F., Machin, J., 1998. Influence of sewage sludge application on physical and chemical properties of Gypsisols. Geoderma 87, 123-135.

Perez-Sanz, A., Millan, R., Jose Sierra, M., Alarcon, R., Garcia, P., Gil-Diaz, M., Vazquez, S., Carmen Lobo, M., 2012. Mercury uptake by *Silene vulgaris* grown on contaminated spiked soils. J. Environ. Manage. 95, S233-S237.

Planquart, P., Bonin, G., Prone, A., Massiani, C., 1999. Distribution, movement and plant availability of trace metals in soils amended with sewage sludge composts: application to low metal loadings. Sci. Total Environ. 241, 161-179.

Relly, C.A., Aust, S.D., 1999. Measurent of Lipid Peroxidation, in: Wiley, J. (Ed.), pp. 1.

Rengel, Z., 2002. Genetic control of root exudation. Plant Soil 245, 59-70.

Revathi, K., Haribabu, T.E., Sudha, P.N., 2011. Phytoremediation of Chromium contaminated soil using Sorghum plant. International Journal of Environmental Sciences 2, 417-28.

Singh, R.P., Agrawal, M., 2008. Potential benefits and risks of land application of sewage sludge. Waste Manage. 28, 347-358.

Skeffington, R.A., Shewry, P.R., Peterson, P.J., 1976. Chromium Uptake and Transport in Barley Seedlings (*Hordeum vulgar*e-L). Planta 132, 209-214.

Song, J., Zhao, F.J., Luo, Y.M., McGrath, S.P., Zhang, H., 2004. Copper uptake by *Elsholtzia splendens* and *Silene vulgaris* and assessment of copper phytoavailability in contaminated soils. Environmental Pollution 128, 307-315.

Srivastava, S., Prakash, S., Srivastava, M.M., 1999. Chromium mobilization and plant availability - the impact of organic complexing ligands. Plant Soil 212, 203-208.

Strobel, B.W., 2001. Influence of vegetation on low-molecular-weight carboxylic acids in soil solution - a review. Geoderma 99, 169-198.

Trasar-Cepeda, C., Leiros, M.C., Seoane, S., Gil-Sotres, F., 2000. Limitations of soil enzymes as indicators of soil pollution. Soil Biology & Biochemistry 32, 1867-1875.

Trasar-Cepeda C, F. Gil-Sotres, M.C. Leirós de la Peña, Determinación de la actividad deshidrogenasa del suelo. In: Técnicas de Análisis de parámetros bioquímicos de suelos (eds. C. García, F. Gil-Sotres, M.T. Hernández, C. Trasar-Cepeda), pp 210-227, Mundi-Prensa, Madrid, 2003.

Turgut, C., Pepe, M.K., Cutright, T.J., 2004. The effect of EDTA and citric acid on phytoremediation of Cd, Cr, and Ni from soil using *Helianthus annuus*. Environmental Pollution 131, 147-154.

USEPA, 1992. Method 7196a. Chromium hexavalent (colorimetric).

Vassilev, A., Schwitzguñebel, J., Thewys, T., Van der Lelie, D., Vangronsveld, J., 2004. The Use of Plants for Remediation of Metal Contaminated Soils. The Scientific Word Journal 4, 9-34.

Wenzel, W.W., 2009. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321, 385-408 .

Zaccheo, P., Cocucci, M., Cocucci, S., 1985. Effects of Cr on Proton Extrusion, Potassium Uptake and Transmembrane Electric-Potential in Maize Root Segments. Plant Cell and Environment 8, 721-726.

Zayed, A., Terry, N., 2003. Chromium in the environment: factors affecting biological remediation. Plant Soil 249, 139-156.

Zhu, J.K., 2001. Plant salt tolerance. Trends Plant Sci. 6, 66-71.

Zhu, X.F., Zheng, C., Hu, Y.T., Jiang, T., Liu, Y., Dong, N.Y., Yang, J.L., Zheng, S.J., 2011. Cadmium-induced oxalate secretion from root apex is associated with cadmium exclusion and resistance in Lycopersicon esulentum. Plant Cell and Environment 34, 1055-1064.

Discusión general y conclusiones

8.1. DISCUSIÓN

Desde la revolución industrial el uso de metales pesados y su vertido incontrolado al medio ha sido la causa de importantes problemas medioambientales. Entre ellos destaca la degradación de los suelos y en concreto la contaminación, que supone tanto un riesgo para el correcto funcionamiento de los ecosistemas como un problema grave de salud pública. Además, la degradación de los suelos supone importantes pérdidas económicas ya que puede comprometer su uso para fines agrícolas e incluso para albergar poblaciones. El cromo ha sido uno de los metales pesados cuyo uso industrial ha crecido más en los últimos años. Gracias a sus propiedades anticorrosivas, tiene multitud de aplicaciones en la industria metalúrgica, química y refractaria. Como consecuencia del creciente vertido de Cr al medio ambiente, se hace necesario el desarrollo de nuevas tecnologías para su eliminación, entre ellas la fitorremediación puede ser una alternativa barata y ambientalmente sostenible con respecto a las técnicas físico-químicas tradicionales.

Para un correcto desarrollo de la fitorremediación es necesario un conocimiento profundo de las interacciones entre el contaminante y la especie de planta que se vaya a utilizar en la revegetación. La información disponible acerca de las interacciones Cr-planta es escasa y existen pocos estudios disponibles que evalúen el potencial de especies vegetales terrestres para ser aplicadas en suelos contaminados con Cr.

Silene vulgaris es una metalofita facultativa que ha sido propuesta como una especie de interés para la recuperación y revegetación de suelos contaminados. En

nuestro grupo de investigación se cuenta con un banco de material vegetal caracterizado de esta especie y con una amplia experiencia en el estudio de la misma. Dado que no existe en la literatura ninguna información disponible acerca de la respuesta de *S. vulgaris* a Cr, el objetivo general de esta tesis fue estudiar la tolerancia a Cr de esta especie y su acumulación, a fin de evaluar su posible aplicación en emplazamientos contaminados con este metal. Para ello se realizaron una serie de estudios que abarcaron desde los aspectos básicos relacionados con la tolerancia y el desarrollo de la planta en presencia de Cr, como aspectos más aplicados acerca de la influencia de la planta, principalmente vía exudación, sobre las características del suelo y la dinámica del Cr en el mismo. Una de las características de *S. vulgaris* es su elevada variabilidad genética tanto inter como intra poblacional por lo que en la mayor parte de los ensayos se utilizaron como material vegetal esquejes que constituyen clones de genotipos homogéneos. Sin embargo, debido a las limitaciones temporales y técnicas que presenta el procesos de esquejado, también se emplearon plántulas desarrolladas a partir de semillas.

Como se ha podido comprobar a partir de los resultados obtenidos, el Cr presenta distinta disponibilidad y toxicidad dependiendo de la especie química en la que se encuentre. En los estudios en hidroponía, *S. vulgaris* acumuló más Cr en presencia de Cr(VI) que en presencia de Cr(III), lo cual se apoya en la literatura disponible en base a los distintos mecanismos de entrada en la raíz que presenta cada especie química, metabólicamente activos para Cr(VI) y pasivos para Cr(III). Además el Cr(VI), por su mayor poder oxidante, resultó más tóxico que el Cr(III).

En todos los casos, el Cr fue acumulado principalmente en la raíz lo que se identifica como un primer mecanismo de defensa de la planta para proteger la función fotosíntetica.

Los rangos de tolerancia a Cr se establecieron con respecto a Cr(VI) mediante test basados en la longitud de la raíz en los que se expuso a las plantas a concentraciones crecientes de metal. El estudio de la longitud de la raíz es el ensayo más adecuado para estudiar la tolerancia a Cr en *S. vulgaris* porque es el parámetro más

sensible a la exposición a este metal. El contacto directo de las raíces con el metal y el elevado poder oxidante del Cr(VI) producen un efecto inhibitorio inmediato sobre la división celular y, por lo tanto, sobre la elongación de la raíz. Además este tipo de test se ha usado de forma efectiva para estudiar la tolerancia de *S. vulgaris* a otros metales. Los test mostraron un rango de tolerancia a Cr(VI) entre 200 y 1000 µM para los genotipos más sensibles y entre 200 y 1200 µM para los más tolerantes. En el caso de las plántulas desarrolladas a partir de semillas, la tolerancia fue menor, entre 30 y 100 µM. Esta diferencia de tolerancia entre esquejes y plántulas desarrolladas a partir de semillas puede ser atribuida al distinto nivel de desarrollo de cada tipo de planta. Los esquejes constituyen plantas adultas que se recogen del campo y que se enraízan posteriormente por lo que sus estructuras, principalmente los tallos, deben estar mejor formadas y ser más resistentes a la acción del Cr que las plantas jóvenes desarrolladas a partir de semillas, aunque ambos tipos de plántulas tengan el mismo tamaño.

La biomasa y el estado de las clorofilas (medido como índice de SPAD) se tomaron como primeros indicadores de la resistencia de los genotipos de *S. vulgaris* al Cr ya que todas las alteraciones metabólicas que ejerce el Cr en la planta se ven traducidas en última estancia en un detrimento en estos dos parámetros. Son especialmente interesantes las curvas dosis-respuesta mostradas para estos dos parámetros en el capítulo 4. Mientras la biomasa de los tallos mostró una disminución aproximadamente lineal con la dosis para todos los genotipos, el peso seco de las raíces mostró distintas tendencias dependiendo del genotipo. A dosis medias de exposición al metal (100 µM), los genotipos experimentaron incrementos en la biomasa de raíz atribuibles al desarrollo de pelos radiculares laterales. Esto confirma el estudio de la longitud de raíz como el mejor método para discriminar el grado de tolerancia entre genotipos sin que los resultados se vean afectados por procesos de hormesis a dosis bajas. Este mismo efecto estimulatorio a dosis bajas de Cr(VI) se observó para el estado de las clorofilas y el contenido en agua a 10 o 10 0µM de Cr(VI) dependiendo del parámetro y el genotipo.

El estudio del balance de nutrientes se llevó a cabo para evaluar la eficiencia de los genotipos y la capacidad de los mismos para mantener unos niveles de nutrientes

adecuados en los procesos fisiológicos vitales. Teniendo en cuenta la visión clásica de eficiencia para plantas herbáceas, los genotipos más eficientes fueron aquellos que mostraron un menor contenido en micronutrientres y una mayor biomasa, como es el caso del genotipo SV21. En exposiciones bajas a Cr, las concentraciones de micronutrientes, especialmente Fe y Mn tendieron a disminuir y las diferencias entre genotipos se incrementaron. El genotipo SV21, identificado como el más eficiente en condiciones normales, también fue el que sufrió un menor descenso en los niveles de micronutrientes cuando fue expuesto a Cr. Al someter a estos mismos genotipos a un rango más amplio de concentraciones de Cr(VI), se observó que tras una disminución inicial de los niveles de nutrientes a dosis bajas de Cr, se produjo un aumento (especialmente en Fe, Ca y Mg) hasta concentraciones iguales o superiores a los niveles mostrados por los controles. Este incremento se relaciona con un mecanismo de defensa de la planta frente a altas concentraciones de Cr, por el que al disminuir su biomasa, es capaz de mantener su homeostasis e invertir estos nutrientes en la síntesis de biomoléculas necesarias para el funcionamiento del metabolismo y/o en sistemas antioxidantes.

En base a estos estudios, el genotipo SV21 se eligió como el más eficiente y tolerante a la exposición a Cr, por ser el que más metal acumuló sufriendo menor efecto sobre su desarrollo. Por contraposición, el genotipo SV38 se consideró el más sensible. Estudios sobre los niveles de estrés oxidativo (medidos como MDA) confirmaron esta diferencia de tolerancia entre ambos genotipos y la mayor toxicidad producida por Cr(VI) que por Cr(III). El estudio comparativo de los niveles de acumulación de Cr con los niveles de exudación para ambos genotipos, reveló que la exudación en *S. vulgaris* es un mecanismo controlado de respuesta a la acumulación de Cr, ya que el genotipo más tolerante fue el que más exudó. A la hora de considerar el uso de una determinada especie en un suelo contaminado es importante caracterizar la exudación radicular, ya que va a ejercer un papel fundamental tanto en la absorción del metal como sobre las características de la rizosfera y la flora bacteriana del suelo.La quercitina y la apiína fueron identificadas como los principales polifenoles presentes en los exudados de *S. vulgaris*. A las dosis aplicadas, las concentraciones de polifenoles totales y quercetina en los exudados experimentaron reajustes relacionados con procesos de hormesis,

atribuibles a su participación en la lignificación de la pared celular para retener el Cr. La apiina sin embargo, que es un antioxidante y quelante de metales muy fuerte, experimentó un aumento significativo de su concentración a la dosis mayor de Cr(VI), por lo que parece estar implicada en mecanismos de protección frente al Cr(VI).

Los ácidos orgánicos juegan un papel fundamental en la detoxificación de metales en la planta, como quelantes y transportadores de los mismos a los lugares de almacenamiento. En el caso de *S. vulgaris*, los ácidos cítrico y málico fueron los que experimentaron una mayor respuesta a la exposición a Cr. Ambos disminuyeron su concentración en el exudado y la aumentaron en la planta en proporción directa a la concentración de Cr en los tejidos, especialmente en las raíces.

Como muestran las curvas de dosis-respuesta de biomasa, SPAD, contenido en agua y clorofilas, cada parámetro estudiado puede mostrar respuestas estimulatorias o inhibitorias a distintas dosis (hormesis). Sería interesante estudiar la composición de los exudados y el balance de ácidos orgánicos en un rango de concentraciones de Cr mayor.

Los estudios de espectroscopía de rayos X con fuente sincrotrón revelaron la capacidad de *S. vulgaris* para reducir Cr(VI) a la especie menos tóxica Cr(III) lo que supone el mecanismo fundamental de tolerancia de esta especie a Cr. Los estudios de especiación confirmaron que todo el Cr en planta se encuentra como Cr(III) a pesar de tratarse de plantas expuestas a Cr(VI), y principalmente se encuentra unido a ácidos orgánicos. Esto confirma el papel fundamental de los ácidos orgánicos en el almacenamiento y transporte de Cr en *S. vulgaris*. El estudio de la distribución de Cr en planta confirmó que el Cr es retenido principalmente en las paredes celulares del cortex de la raíz para evitar su presencia en el citosol. A mayores dosis de Cr, éste es transportado por los ácidos orgánicos a las hojas donde se acumula principalmente en la pared de las células que forman los márgenes de las hojas, donde la interferencia con la fotosíntesis es menor. Estudios de FTIR confirmaron el papel de la pared celular en la retención del Cr.

A la luz de los resultados, la disminución de biomasa parece ser un mecanismo por el que las plantas son capaces de mantener los niveles de nutrientes adecuados e

invertir una mayor energía en hacer frente al exceso de Cr sin comprometer la función principal para la supervivencia de la planta, que es la fotosíntesis. Sin embargo, este desajuste entre consumo y asimilación de carbono da lugar a la concentración excesiva de depósitos de almidón en las células de las hojas de plantas tratadas con Cr(VI), como mostraron las imágenes obtenidas por SEM y los resultados del análisis de FTIR. Los espectros de FTIR también revelaron la ausencia de daños sobre las membranas celulares y la síntesis de compuestos aromáticos que podrían estar relacionados con la prevención del daño oxidativo.

El ensayo realizado con suelos contaminados puso de manifiesto la relevancia de las características del suelo y de la especiación química del metal a la hora de determinar la peligrosidad de un vertido de Cr. El suelo con bajos contenidos en materia orgánica y pH alcalino fue más vulnerable, por lo que el vertido de Cr(VI) resultó más peligroso que el de Cr(III). También se puso de manifiesto la importancia desde un punto de vista ambiental de determinar la fracción disponible de metal respecto al contenido total ya que *S. vulgaris* no fue capaz de crecer en los suelos básicos con bajo contenido en MO en los que gran cantidad de Cr(VI) se encontraba disponible. De los resultados de este ensayo podemos concluir que *S. vulgaris* fue capaz de crecer en suelos con concentraciones moderadas de Cr a los que aportó C a través de su mecanismo de exudación. Este aporte de C estimuló la actividad de los microorganismos del suelo contribuyendo a la recuperación del mismo. El efecto positivo de *S. vulgaris* fue mayor en el suelo pobre en MO. Además, la capacidad de *S. vulgaris* para crecer en suelos contaminados con Cr aportaría todos los beneficios propios de una cubierta vegetal: disminución de la escorrentía superficial, prevención de la erosión, aumento de la capacidad de retención de agua y estimulación del proceso de regeneración natural. *S. vulgaris* es una especie con alta capacidad de adaptación que puede desarrollarse en un amplio espectro de condiciones climatológicas y edáficas y con bajos requerimientos nutricionales por lo que sería interesante estudiar su aplicación en combinación otras tecnologías o enmiendas que reduzcan la fracción disponible de Cr.

En este último ensayo en suelo de seis meses de duración, también se emplearon los genotipos SV21 y SV38. Sin embargo las diferencias observadas en los

estudios de hidroponía (2-3 semanas de exposición a Cr) no se manifestaron en este ensayo lo que parece indicar que a largo plazo, incluso los genotipos aparentemente menos tolerantes a Cr son capaces de desarrollarse, por lo que la tolerancia a Cr parece ser una característica "*per se*" de *S. vulgaris.*

Teniendo en cuenta los siguientes resultados obtenidos en esta tesis, *S. vulgaris:* i) es capaz de reducir Cr(VI) a la especie menos tóxica Cr(III) con la consiguiente reducción de riesgo ambiental; ii) acumula el Cr principalmente en la raíz reduciendo el riesgo de que pase a la cadena trófica y, por último, iii) es capaz de desarrollarse en suelos con contaminación moderada de Cr y distintas características edáficas, aportando C al mismo y mejorando su calidad. Por todo ello, *S. vulgaris* puede ser considerada una especie candidata para su uso en revegetación y recuperación de suelos contaminados con Cr.

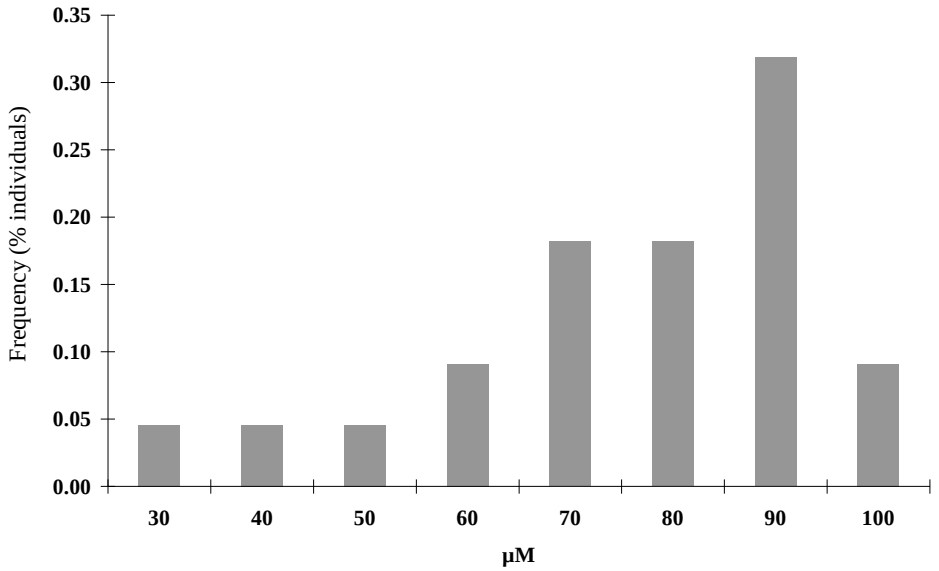
8.2. CONCLUSIONES GENERALES

1. *Silene vulgaris* acumula más Cr en presencia de Cr(VI) que de Cr(III) y el Cr(VI) induce mayor efecto tóxico sobre la planta.
2. *Silene vulgaris* cuenta con diversos mecanismos internos que la hacen tolerante al Cr:
    a. El mecanismo de tolerancia principal es la reducción de Cr(VI) a Cr(III) en la raíz.
    b. La asociación del Cr a los polisacáridos y las proteínas de la pared celular protege la funcionalidad de las células.
    c. Se preserva la función fotosíntetica mediante la retención preferente del Cr en la raíz. El exceso de Cr que no puede ser retenido en la raíz es complejado con ácidos orgánicos y transportado a la pared celular de las células de los márgenes de la hoja. En la hoja se sintetizan compuestos aromáticos destinados a la protección de las membranas celulares
    d. La disminución de biomasa permite a la planta gestionar el exceso de Cr y mantener niveles adecuados de nutrientes sin embargo, genera un desajuste entre el carbono asimilado consumido que da lugar a la acumulación de gránulos de almidón en las hojas.
3. El proceso de exudación es un proceso controlado que también parece estar relacionado con la acumulación y la tolerancia a Cr, principalmente en el caso de los ácidos orgánicos y la apiína. Además ésta liberación de compuestos de C juega un papel fundamental en la rizosfera de *S. vulgaris*:
    a. Incrementa el pH de la rizosfera y del suelo
    b. Aumenta la solubilidad del Cr(III) intensificando su absorción por la planta
    c. Estimula la actividad de la flora microbiana en el suelo
4. *S. vulgaris* puede desarrollarse en suelos con contaminación moderada de Cr aportando a los mismos los beneficios propios de una cobertura vegetal
5. Todo ello hace que *S. vulgaris* pueda ser considerada una buena candidata para ser utilizada en la recuperación de suelos contaminados con Cr.

8.3. CONCLUSIONS

1. *Silene vulgaris* accumulates more Cr in presence of Cr(VI) than Cr(III) and Cr(VI) is more toxic than the trivalent form.
2. *Silene vulgaris* shows different mechanisms that make it tolerant to Cr:
    a. Main tolerance mechanism is Cr(VI) reduction to Cr(III) in roots.
    b. Cell funcionality is protected by the association of Cr with polyssacharides and proteins in cell wall.
    c. Phosynthetic function is preserved by Cr retention in roots. Cr excess that is not retained in roots is complexed by organic acids and transported to the cell wall in the leaf margins. Aromatics compounds are synthesized in leaves to protect membrane integrity.
    d. Plant manages the excess of Cr and keeps its nutrient balance through biomass reduction. The impartment between C assimilated and C consumed leads to starch accumulation in leaves.
3. Exudation is a controled mechanism that is related to Cr accumulation and tolerance, mainly in the case of realeasing organic acids and apiin. The realease of organic compounds plays an important role in the rhizosphere of *S. vulgaris* as it:
    a. Increasse soil and rhizosphere pH.
    b. Increases Cr(III) solubility to enhance Cr uptake.
    c. Estimulates microbial activity in soil.
4. *S. vulgaris* can growth in soils with moderate Cr pollution providing the benefits of vegetal cover.
5. Therefore *S. vulgaris* can be considered a good candidate to be used in the recovery of Cr polluted soils.

Tolerance test of *S. vulgaris* populations



Supplementary Figure 1: EC_{100} frequency distribution values for the effect of Cr(VI) on root growth in a population of *S. vulgaris* (Rozas del Puerto REal, Madrid, Spain) as established in a sequential exposure test using concentration steps of 10 µM Cr(VI).

Anexo 2/Annex 2

Currículum Vitae

Ana Elena Pradas del Real
Date of birth: 25/07/1984

Current position held

- **Position / Title:** PhD candidate
- **Advisors:** Dr Araceli Pérez-Sanz and Dr MªCarmen Lobo Bedmar
- **Institution:** Department of Agroenvironmental Research, **Madrid Institute for Research and Rural Development, Agriculture and Food (IMIDRA**), Finca "El Encín" A-II Km 38,200- E-28800 Alcalá de Henares, SPAIN
- **Telephone:** 0034 626 29 03 40
- **Email:** ana.elena.pradas@madrid.org/anaepra@gmail.com

Education

- MSc. **Environmental Science and Technology - Rey Juan Carlos University** (Madrid, Spain) (2008).Thesis: *Nitric oxide protection against oxidative stress during rehydration of Ramalina farinacea*.

- BSc. **Environmental Sciences - Rey Juan Carlos University** (Madrid, Spain) (2007).

Current research interest: *Silene vulgaris*; genotypes, Chromium speciation, soil-plant interactions, nutrient balance in plants, oxidative stress, rhizosphere process.

Science disciplines: Environmental sciences, soil science, heavy metals, phytomanagement, ecotoxicology.

Experience :

- **PhD student**. Departement of Agroenvironmental Research, (IMIDRA). Madrid (Spain). 11/2008 – 11/2012. (4 years).

- **Visiting Scholar:** Department of Plant and Soil Sciences. University of Kentucky (Lexington, KY, USA). 10/2011-04/2012 (6 months).

- **Research assistant**. PROGEPY (Centre de Promotion du Génie des Procédés dans l'industrie) Nancy (France). 1/10/2008 – 15/11/2008 (6 weeks).

- **Graduate student**. Department of Biology and Geology, Rey Juan Carlos University (Madrid, Spain). 09/2007-09/2008 (1 year).

Languages

- Languages (R = regular, G = good, F = fluent)

Language	**speaking**	**reading**	**writing**
Spanish (mother tongue)	F	F	F
English	G	F	G
French	G	F	G

Grants

- **PhD Grant** in Madrid Institute for Research and Rural Development, Agriculture and Food (IMIDRA) .20 November 2008 to 20 November 2012.

- **Mobility ARGO GRANT** (European Programme Leonardo da Vinci). Oxidation treatments in PAHs contaminated soils. PROGEPY (Centre de Promotion du Génie des Procédés dans l'industrie) Nancy (France). 1 September – 15 November 2008.

Participation in Research projects

- **Environmental Impact Assessment and recovery of the natural environment in contaminated sites.** (2010-2013). EIADES PROGRAM S2009/AMB-1478 Consejería de Educación, Comunidad de Madrid**,**

- **Use of wild grasses to recover degraded soil. Evaluation of plant tolerance to heavy metal as function of soil characteristics**. Spanish Minister of Science and Innovation-INIA (RTA2009-00150-00-00, December, 2009-2012).

Publications:

- **Pradas del Real, A.E.,** García-Gonzalo, P., Alarcón, R., González,A., Lobo, M.C., Pérez-Sanz, A. *Effect of Genotype, Cr(VI) and Cr III) on Plant Growth and Micronutrient Status in Silene vulgaris (Moench).* **Spanish Journal of Agricultural Research**. (Accepted).

- Catalá, M., Gasulla, F., **Pradas del Real, A.E**., García-Breijo., F, Reig-Armiñana, J., Barreno, E. *The organic air pollutant cumene hydroperoxide interferes with NO antioxidant role in rehydrating lichen*. **Environmental Pollution** 179,277-284.

- Montalbán, B., **Pradas del Real, A E**., García, P., Lobo, M.C., Pérez-Sanz, A. 2012. *Germination and Early Development of Brassica napus and Brachypodium distachyon Growth with Zn, Cr(VI), As(V) or Cd*: Preliminary Results. **Acta Phytopathologica et Entomologica Hungarica**.47 (2), 363-371.

- Catalá, M., Gasulla, F**.**, **Pradas del Real, A**.**E**, García-Breijo, F., Reig-Armiñana, J. & Barreno, E. 2010. *Fungal-associated NO is involved in the regulation of oxidative stress during rehydration in lichen symbiosi*s. **BMC Microbiology** 2010, 10:297

Book Chapters

- **Pradas del Real, A.E.,** Lobo, M.C., Pérez-Sanz. *Study of Cr^{+6} behavior in three agricultural soils*. In **Proceedings of 15th International Conference of Heavy Metals in the Environment** E3S Web of Conferences 1 08008 (2013). DOI:http://dx.doi.org/10.1051/e3sconf/20130108008

- **Pradas del Real, A.E.,** García-Gonzalo, Lobo, M.C., Pérez-Sanz, A. *Influencia del Cr(III) y el Cr(VI) en la absorción de Fe y Mn de seis genotipos de Silene vulgaris*. In "La nutrición Mineral de las Plantas como Base de una Agricultura Sostenible. Procedings of **XIV Simposio Hispano-Luso de Nutrición Mineral de las Plantas. Nutriplanta.** Pp 279-283 ISBN-10:84-695-5571-5. 2012.

- **Pradas del Real, A.E.,** Alarcón, R., García, P., Gil-Díaz, M.M., Lobo, MC, Pérez-Sanz, A. *Cd and Cr uptake by six genotypes of Silene vulgaris (Moench) Garcke*. In **Proceedings of 15th International Conference of Heavy Metals in the Environment**. Pp 563-566 ISBN 978-83-928986-5-8. 2010

- Catalá M, Gasulla F, **Pradas del Real A.E.**, García-Breijo F, Reig-Armiñana J, Barreno E: *Nitric Oxide Is Involved in Oxidative Stress during Rehydration of Ramalina farinacea (L.) Ach. in the Presence of the Oxidative Air Pollutant Cumene Hydroperoxide*. In **Biology of Lichens: Ecology, Environm, Monitoring, Systematics and Cyber Applications**. Edited by Thomas H. Nash III et al.

Stuttgart: E. Schweizerbart Science Publishers 2010, 256pp. [J. Cramer in der Gebrüder Borntraeger Verlagsbuchhandlung (Series Editor): Bibliotheca Lichenologica, vol 105].

Awards:

- **Pradas del Real, A.E.,** Lobo, M.C, Pérez-Sanz. Phytomanagement of Cr polluted sites with *Silene vulgaris*. **Oral presentation. Society of Environmental Toxicology and Chemistry (SETAC) Europe 23nd Annual Meeting.** Glasgow (UK). 12-16 May 2013.

- **Pradas del Real, A.E.,** Lobo, M.C, Pérez-Sanz, A. *Study of Cr(VI) evolution in three agricultural soils*. **Poster**. **16th International Conference on Heavy Metals in the Environment.** Rome (Italia) 23-27 September 2012.

- Pérez-Sanz, A., **Pradas del Real, A.E**. Montalbán, B., García, P., Tardío, J., Lobo, M. C. *Evaluation of different species from Rumex genus to phytotechnologies*. **Oral presentation**. **9th Internacional Conference on Phytothecnologies**. Hasselt (Belgium). 11-14 September 2012.

- Pérez-Sanz, A., **Pradas del Real, A.E**., Montalbán, B., García, P. ,Lobo, M.C. *Especies silvestres en recuperación de suelos*. **Oral presentation**. **XIV Simposio Hispano-Luso de Nutrición Mineral de las Plantas. Nutriplanta.** Madrid (España). 23-26 July 2012

- **Pradas del Real, A.E.,** García, P., Lobo, M.C., Pérez-Sanz, A. *Absorción y efectos de Cr(III) y Cr(VI) sobre Silene vulgaris.* **Oral presentation**. **XIV Simposio Hispano-Luso de Nutrición Mineral de las Plantas. Nutriplanta**. Madrid (España). 23-26 July 2012.

- Domingo, C., Pérez-Sanz, A., **Pradas del Real, A.E**., Lobo, M.C., García P. *Genotipic variation in metal-tolerant Silene vulgaris clones.* **Poster**. **Society of Environmental Toxicology and Chemistry (SETAC) Europe 22nd Annual Meeting.** Berlin (Germany). 20-24 May 2012.

- Montalbán, B., Lobo., M.C, **Pradas del Real, A.E**., Pérez-Sanz, A. *Helianthus tuberosus L. grown with multiple pollution in greenhouse conditions*. **Poster**. **Society of Environmental Toxicology and Chemistry (SETAC) Europe 22nd Annual Meeting.** Berlin (Germany). 20-24 May 2012.

- **Pradas del Real, A.E.,** Montalbán, B., García Gonzalo, P., Lobo, M.C Pérez-Sanz, A. *Exploring the phytoremediation potencial of Rumex pulcher L. using agar based medium*. **Oral presentation**. **Sustainable Approaches to Remediation of Contaminated Land in Europe. Contaminated Site Management in Europe,** Gante (Belgium). 24-26 October 2011.

- Montalbán, B., Pérez-Sanz, A., **Pradas del Real, A.E.**, García, P., Plaza, A.,Lobo, M.C. *Germination and early development of Brassica napus L. and Brachypodium distachyon (L.) Beauv. grown with Zn, Cr(VI), As(V) or Cd*. **Poster**. **International Conference on Agri-environmental Chemistry and Toxicology.** Budapest (Hungary). 20-22 September 2011.

- **Pradas del Real, A.E**, Alarcón, R., García. P., Lobo, M.C., Pérez-Sanz, A. *Hexavalent Chromium uptake by six genotypes of Silene vulgaris (Moench).* **11th ICOBTE.** Julio 3-7, 2011, Florencia, Italia

- Gil-Díaz, M., **Pradas del Real, A** , Pérez-Sanz, A., Vicente MA.,Tejerina J.I, Lobo, MC. "*Evaluación de la actividad. biológica de un suelo de olivar tratado con estiércol porcino"*. **Poste**r. **III Congreso de Microbiología Industrial y Biotecnología**. Alcalá de Henares (Spain). 2010.

- **Pradas del Real AE**., Alarcón, R., Gil-Díaz, M., García, P., Lobo, MC., Pérez-Sanz, A. *Cd and Cr up.take by six genotypes of Silene vulgaris (Moench) Garcke*. **Poster**. **15th International Coference of Heavy Metals in the Environment.** Gdânsk, (Poland). 2010.

- **Pradas del Real A.E**, Alarcón, R., Gil-Díaz, M., García, P., Lobo, MC., Pérez-Sanz, A. *Cr(III) and Cr(VI) uptake by different genotypes of Silene vulgaris*. **Poster corner**. **SETAC Europe 20th Annual Meeting**. Sevilla (Spain). 2010.

- Catalá, M, García-Breijo F, Gasulla F, Guéra A, **Pradas del Real A.E**, Reig-Armiñana J, Barreno E. *Nitric oxide protection against oxidative stress during rehydration of Ramalina farinacea (L.)* **Oral presentation**. Ach. **IAL6-ABLS Joint Meeting (Air Pollution Symposium)**. Monterey (EEUU), 2008.

- **Pradas del Real, A.E**; García-Breijo,F; Rodríguez-Gil,J.L; Catalá, M; Barreno, E. *Protective role on NO in Lichens: environmental implications.* **Poster**. **Society of Environmental Toxicology and Chemistry (SETAC) Europe 18th Annual Meeting**. Warsaw (Poland), 2008.

Training courses and technical conferences

- **Pradas del Real, A.E.,** Lobo, M.C, Pérez-Sanz (2010). *Evaluación de la tolerancia al cromo de distintos genotipos de Silene vulgaris"*. **Jornadas Anuales del Programa EIADES.** IMIDRA, (Madid, Spain) 11 de Noviembre de 2010.

- Seminario de Presentaciones Eficaces. **Madrid+d. Fundación Universidad-Empresa** Madrid (Spain) .11 -14 may, 2009 (16 h).

- Curso de "Procesos de Degradación y Recuperación de Suelos", **Unidad de Formación en Energía, Tecnología y Medio Ambiente del Ciemat Madrid (Spain).** 28 septembre to 9 october 2009 (56h).