INSTITUTO DE INVESTIGACIÓNS AGROBIOLÓXICAS DE GALICIA
Consejo Superior de Investigaciones Científicas

IIAG INSTITUTO DE INVESTIGACIÓNS AGROBIOLÓXICAS DE GALICIA

CSIC CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

DEPARTAMENTO DE EDAFOLOXÍA E QUÍMICA AGRÍCOLA
Universidade de Santiago de Compostela

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Nickel hyperaccumulating plants: strategies to improve phytoextraction and a characterisation of *Alyssum* endemic to the Iberian Peninsula

María Isabel Cabello Conejo
Tesis de doctorado, 23 de enero de 2015

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Dra. Petra S. Kidd y **Dra. Ángeles Prieto Fernández**, Científicos Titulares del Consejo Superior de Investigaciones Científicas, adscritas al Instituto de Investigaciones Agrobiológicas de Galicia y **Dra. Mª del Carmen Monterroso Martínez**, Profesor Titular del Departamento de Edafología y Química Agrícola de la Universidad de Santiago

INFORMAN:

Que la presente memoria titulada "**Nickel hyperaccumulating plants: strategies to improve phytoextraction and a characterisation of *Alyssum* endemic to the Iberian Peinsula"** presentada por **Dña. María Isabel Cabello Conejo** para optar al Grado de Doctora en Ciencias Ambientales, fue realizada bajo nuestra dirección.

Y considerando que representa trabajo de Tesis de Doctorado, autorizamos su presentación ante el Tribunal correspondiente.

Y para que así conste, firmamos el presente informe en Santiago de Compostela a 21 de Enero de 2015.

Fdo.: Dra. Petra S. Kid

Fdo.: Dra. Ángeles Prieto Fernández

Fdo.: Dra. Mª del Carmen Monterroso Martínez

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A mis padres

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INDEX

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

RESUMEN

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCCIÓN

Los elementos traza se caracterizan por estar presentes a bajas concentraciones en la litosfera (<1000 mg kg^{-1}) y en los organismos vivos (<100 mg kg^{-1}) (Adriano 2001), y entre ellos se encuentra el grupo de los metales pesados, que tienen una densidad mayor de 5 g cm^{-3} (Bothe 2011). Algunos de estos elementos como Ni, Cu, Fe o Zn, son micronutrientes esenciales para los seres vivos, mientras que otros (Cd, Hg, Pb As) no participan en ninguna función biológica conocida (Adriano 2001). En todo caso todos ellos, si se encuentran a elevadas concentraciones, puede ser tóxicos para los organismos y causar graves daños ambientales (McGrath and Zhao 2013).

En los suelos, sin interferencia humana, la concentración de elementos traza depende sobre todo del material de partida y de los procesos de meteorización que intervienen en su formación. En algunas zonas del planeta, existen afloramientos rocosos con una composición química que se caracteriza por una mayor abundancia de estos elementos. Entre estos afloramientos se encuentran las rocas ultrabásicas o ultramáficas, rocas ígneas o metamórficas con un alto contenido de minerales ferromagnesianos (>70 %) y bajo contenido de sílice (SiO_2 <45 %), en las que la abundancia de Ni varía entre 1400 y 2000 mg kg^{-1} (Kabata-Pendias 2011) y que también presentan contenidos elevados de Co y Cr. Las rocas ultrabásicas se distribuyen de forma dispersa en el planeta ocupando alrededor del 1% de la superficie terrestre. Los suelos serpentiníticos desarrollados sobre estas rocas, aunque muestran una variabilidad considerable en sus propiedades fisicoquímicas, tienen una serie de características que los diferencian de otros suelos. Así, presentan elevadas concentraciones de Mg, Fe y elementos traza potencialmente fitotóxicos como Ni, Co y Cr, bajo contenido en materia orgánica y en macronutrientes esenciales como N, P y K, baja disponibilidad de Ca en relación al Mg y baja capacidad de retención de agua (Brooks 1987; Whittaker 1954). Este conjunto de características limitan el crecimiento de las plantas y se conocen como "síndrome serpentinítico". Por otra parte, los suelos pueden contener elevadas concentraciones de elementos traza debido a causas antropogénicas. En el caso del Ni, las principales fuentes de contaminación del suelo son actividades relacionadas con la industria de fundición y refinación de Ni, y con la aplicación de lodos residuales y fertilizantes fosfatados en agricultura (Li *et al.* 2003).

A pesar de que la elevada concentración de metales traza en el suelo, como es el caso de las zonas serpentiníticas, supone una limitación importante para el crecimiento vegetal, existen plantas que han desarrollado estrategias muy especializadas para crecer en estos ambientes. Baker (1981) clasifica las plantas,

atendiendo a su respuesta a altas concentraciones de metales potencialmente fitotóxicos en el suelo, en exclusoras, que limitan la translocación de metales a sus tejidos; indicadoras, que regulan la absorción de metal de modo que la concentración en sus tejidos refleja el contenido de metal en el suelo; y acumuladoras, que concentran activamente el metal, lo que implica una fisiología altamente especializada. En el grupo de plantas acumuladoras están las metalófitas denominadas hiperacumuladoras, que presentan concentraciones extremadamente altas de metales en sus tejidos aéreos cuando crecen en ambientes enriquecidos en metales. Este término se aplicó por primera vez para describir plantas que contienen >1000 mg kg^{-1} Ni en peso seco (Brooks *et al.* 1977) y posteriormente se aplicó también a especies que acumulan otros elementos traza (Cd, Co, Zn, etc). Para que una determinada especie vegetal se considere hiperacumuladora se han establecido unas concentraciones mínimas que se han de alcanzar en los tejidos aéreos y que dependen del elemento de que se trate. La importancia ecológica y evolutiva del fenómeno de hiperacumulación es objeto de gran debate. Los estudios más recientes indican que este rasgo probablemente apareció como mecanismo de defensa de las plantas frente al ataque de herbívoros y microorganismos (Hörger *et al.* 2013). Hasta la fecha se han identificado aproximadamente unos 500 taxones que hiperacumulan uno o más metales o metaloides y más del 90 % son hiperacumuladores de Ni (Pollard *et al.* 2014). El género con mayor número de hiperacumuladoras de Ni es *Alyssum* (Brassicaceae) (Baker and Brooks 1989). En la Península Ibérica existen dos subespecies hiperacumuladoras de este elemento *Alyssum serpyllifolium* ssp. *lusitanicum* y *Alyssum serpyllifolium* ssp. *malacitanum* (conocidas también como *A. pintodasilvae* y *A. malacitanum*). Ambas son endémicas y se localizan en las principales áreas serpentiníticas de la Península Ibérica: en la región de Trás-os-Montes (NE Portugal), en el norte y centro de Galicia (NW España) y en las Cordilleras Béticas de Málaga (SE España) (Asensi *et al.* 2004; Brooks *et al.* 1981; Menezes de Sequeira and Pinto da Silva 1991).

La mayoría de las hiperacumuladoras de Ni son especies endémicas de zonas serpentiníticas y en ellas el Ni supone 1-3 % del peso seco de los tejidos aéreos (Chaney *et al.* 2010) mientras que en otras plantas de cultivo la cantidad de Ni varía entre >10 mg kg^{-1} de peso seco en especies sensibles y >50 mg kg^{-1} de peso seco en especies moderadamente tolerantes (Asher 1991). Los mecanismos de absorción y translocación de Ni desde la raíz a los tejidos aéreos no se conocen totalmente. Las hiperacumuladoras se caracterizan por una tasa de carga de Ni en el xilema muy alta y a nivel de la hoja se detectan un flujo de entrada de Ni a través de la membrana plasmática y un secuestro en vacuolas elevados (Broadhurst *et al.* 2004; Milner and Kochian 2008). Se asume que en el interior de

la planta la mayor parte del metal está ligado a ácidos orgánicos, aminoácidos, péptidos y proteínas que actúan como mecanismos de detoxificación (Callahan *et al.* 2006; Sharma and Dietz 2006; Verbruggen *et al.* 2009).

Aunque las plantas hiperacumuladoras disponen de una extraordinaria capacidad de acumular Ni, este proceso depende de varios factores como la disponibilidad de Ni en el suelo para la planta, su reposición a través de las formas menos biodisponibles, así como de la capacidad de la planta para absorber el Ni y transportarlo a su parte aérea (Ernst 2000; Wenzel *et al.* 2003). La biodisponibilidad del metal en el suelo se puede definir como la fracción de metal que puede interactuar con organismos vivos. En el caso de las plantas la disponibilidad de metal está gobernada más que por el contenido total de metal en el suelo por un pseudoequilibrio entre la fase líquida, en la que hay iones libres o complejados con aniones y macromoléculas orgánicas y con coloides inorgánicos; y la fase sólida, en la que se encuentra la fracción cambiable, metal complejado con materia orgánica, sorbido y ocluido por óxidos y minerales arcillosos, coprecipitado en minerales secundarios o formando parte de la estructura cristalina de minerales primarios. En plantas hiperacumuladoras la gran cantidad de metal absorbido raramente se puede explicar por la reducción de las fracciones metálicas más lábiles del suelo, por esto algunos autores han propuesto que estas plantas acceden a fracciones no disponibles para otras especies vegetales (Knight *et al.* 1997; McGrath *et al.* 1997); sin embargo, varios estudios han demostrado que las plantas hiperacumuladoras y no hiperacumuladoras acceden a las mismas fracciones metálicas (Echevarria *et al.* 1998; Hammer *et al.* 2006; Hutchinson *et al.* 2000). En general, las plantas ejercen una gran influencia sobre el suelo que está en contacto directo con las raíces, conocido como rizosfera o suelo rizosférico, y pueden modificar sus propiedades a través de procesos como la producción de exudados radiculares o la alteración del pH y del potencial redox (Hinsinger 2001; Jones and Darrah 1994; Marschner 2007). La producción de exudados radiculares puede influir directamente sobre la disponibilidad de nutrientes y metales, liberando las formas menos disponibles del suelo, o indirectamente, influyendo sobre la actividad microbiana del suelo (Adriano 2001; Hinsinger *et al.* 2005; Puschenreiter *et al.* 2003; Uren and Reisenauer 1988). En el caso de las plantas hiperacumuladoras resulta de gran interés estudiar los procesos fisicoquímicos y biológicos que tienen lugar en la rizosfera y su relación con el proceso de hiperacumulación.

Las plantas, como las hiperacumuladoras, que son capaces de prosperar en suelos ricos en metales se consideran de gran interés por su potencial aplicación en tecnologías de descontaminación de suelos (Chaney *et al.* 2010; Dickinson *et al.* 2009; Kidd *et al.* 2009; Mench *et al.* 2009; Vangronsveld *et al.* 2009).

La aplicación de plantas y sus microorganismos asociados en la recuperación de ambientes contaminados se conoce como "*fitocorrección*" e incluye diferentes técnicas (Chaney *et al.* 1997; Salt *et al.* 1995). Las técnicas de fitocorrección se consideran una alternativa eficiente para la corrección de suelos contaminados, menos agresiva que las técnicas convencionales de ingeniería y capaz de restaurar las funciones y estructura del suelo (Mench *et al.* 2010; Moreno-Jiménez *et al.* 2012; Vangronsveld *et al.* 2009). Entre estas técnicas se encuentra la fitoextracción, que se aplica sobre todo a suelos contaminados con metales, y consiste en el uso de plantas (hiper)acumuladoras capaces de absorber, transportar y acumular elevadas concentraciones de metales traza en su biomasa aérea, por tanto, reduciendo la concentración de metal en el suelo (Chaney 1983; Chaney *et al.* 1997; Vassilev *et al.* 2004). La fitoextracción incluye tres categorías: 1) uso de plantas de cultivo y de agentes químicos o biológicos para la movilización y acumulación de elementos traza 2) cultivo de árboles de crecimiento rápido con fenotipos acumuladores para la producción de biomasa para energía 3) cultivo de plantas hiperacumuladoras (Bani *et al.* 2007; French *et al.* 2006; Munn *et al.* 2008). La selección de la opción más adecuada para cada caso dependerá de numerosos factores (Mench *et al.* 2010).

El uso de plantas hiperacumuladoras en técnicas de fitoextracción fue propuesto por Chaney *et al.* (1983) debido a su extraordinaria capacidad para absorber y acumular en su biomasa aérea metales o metaloides. Sin embargo, existen importantes factores limitantes para su aplicación práctica como su reducida biomasa, la ausencia de semillas o plántulas disponibles en el mercado, la sensibilidad de estas plantas a otros contaminantes, el desconocimiento sobre los requerimientos de su cultivo, necesidades climáticas, etc. A pesar de ello, las plantas hiperacumuladoras son una buena alternativa para su aplicación en técnicas de fitoextración, como por ejemplo para la fitominería de Ni en suelos serpentiníticos. La fitominería es un proceso de fitoextracción en el que el metal acumulado tiene valor comercial y puede ser recuperado de la biomasa cosechada. La fitominería de Ni se basa en el cultivo y cosecha de especies hiperacumuladoras y en la posterior incineración de la biomasa cosechada para la obtención de un residuo (ceniza) con elevado contenido en Ni conocido como "bio-mena". La fitominería presenta diferentes ventajas respecto a la minería convencional: permite explotar metales que no serían rentables a través de técnicas de minería convencional, puede mejorar la calidad del suelo, el impacto ambiental es mínimo, y se obtiene un bio-mineral menos tóxico y cuyo procesado requiere menos coste (Anderson *et al.* 1999; Brooks *et al.* 1998). A pesar de estas ventajas, el proceso de fitominería también puede presentar importantes limitaciones como puede ser el hecho de que la mayoría de las hiperacumuladoras son especies de

baja biomasa, crecimiento lento y sistemas radiculares superficiales; además, el proceso de extracción estará influenciado por factores climáticos, estacionales y limitado por factores biogeoquímicos y por la solubilidad y disponibilidad de metales en el suelo (Ghosh and Singh 2005). En general, se acepta que en al proceso de fitominería se incorporen prácticas agronómicas para la optimización de la extracción de Ni, el incremento de la producción de biomasa, la mejora del estado nutricional de la planta, de la calidad del suelo, manejo de plagas, etc. El éxito global del proceso de fitominería dependerá claramente de la concentración del metal de interés en la biomasa cosechable y de la cantidad de biomasa obtenida. Por tanto, la especie vegetal seleccionada debe presentar una alta tolerancia al metal, acumular grandes concentraciones de elementos traza en su biomasa aérea y tener una producción de biomasa suficiente para que la extracción de metal del suelo sea rentable (Glick 2010; Li *et al.* 2003; Vangronsveld *et al.* 2009). Por estas razones, se puede actuar a distintos niveles para mejorar el proceso de extracción de Ni empleando hiperacumuladoras de este metal.

Selección de plantas más adecuadas

Las plantas hiperacumuladoras generalmente muestran una gran variabilidad en cuanto a producción de biomasa y acumulación de metal tanto entre diferentes poblaciones de la misma especie, como dentro de una misma población. Diferentes estudios llevados a cabo con especies del género *Alyssum* han demostrado una elevada variabilidad inter-poblacional en la acumulación de Ni en los tejidos de la planta (Adamidis *et al.* 2014; Kazakou *et al.* 2010). Por otra parte, la selección de especies nativas para su uso en fitoextracción o fitominería en un determinado lugar presenta varias ventajas no sólo debido a cuestiones prácticas, sino también por motivos como la conservación de la biodiversidad de ambientes serpentiníticos, evitándose así la introducción de especies exóticas. A través de técnicas tradicionales de cruzamiento selectivo se pueden explotar la diversidad genética disponible de una determinada especie para combinar los rasgos más adecuados para su uso en procesos de fitoextracción (y fitominería).

Aplicación de prácticas agronómicas

La eficiencia de las técnicas de fitoextracción no sólo depende de la capacidad de la planta para absorber, transportar y acumular el metal en sus tejidos, la aplicación de prácticas agronómicas adecuadas (como fertilización, encalado, aplicación de herbicidas, etc.) puede ser una herramienta eficiente para mejorar el crecimiento y la producción de cultivos de plantas hiperacumuladoras (Li *et al.* 2000). Además de estas técnicas agronómicas tradicionales, se ha visto que la aplicación de fitoreguladores (fitohormonas) como auxinas, citoquininas o

giberelinas puede favorecer el crecimiento de las plantas (El-Saeid *et al.* 2010; Emongor *et al.* 2004; Hussain *et al.* 2011). Algunos estudios recientes han observado que la aplicación de fitoreguladores en especies hiperacumuladoras del género *Alyssum* pueden producir un aumento de biomasa o una mayor translocación y acumulación de Ni (Cassina *et al.* 2011; Qiu *et al.* 2009).

Bioaumento con rizobacterias

Varios autores han propuesto la incorporación de microorganismos asociados a las plantas (rizosféricos, endofíticos y micorrizas) al proceso de fitoextracción (Abou-Shanab *et al.* 2006a; Kidd *et al.* 2009; Ma *et al.* 2009; Rajkumar and Freitas 2008; Sessitsch *et al.* 2013). Las bacterias promotoras de crecimiento (PGP) son capaces de producir sustancias que aumentan el crecimiento y la tolerancia de las plantas en condiciones de estrés causadas por ejemplo por la presencia de elevadas concentraciones de metales en el suelo. Muchas bacterias PGP facilitan el crecimiento de la planta a través de la producción de fitohormonas (citoquininas, giberelinas), de la liberación de nutrientes esenciales (como los microorganismos fijadores de N_2, solubilizadores de fosfatos o productores de sideróforos) o mediante la inducción de mecanismos de defensa de la planta (Glick 2003; Glick *et al.* 1998; Weyens *et al.* 2009). Por otra parte, se ha visto que determinadas cepas bacterianas tolerantes a metales asociadas con plantas hiperacumuladoras son capaces de movilizar metales del suelo y, por tanto, aumentar la cantidad de metal fitodisponible y la absorción de metal por la planta. Las bacterias pueden modificar la disponibilidad de metal mediante distintos mecanismos como la liberación de agentes quelantes, acidificación o cambios redox en la rizosfera (Becerra-Castro *et al.* 2013; Gadd 2004; Glick 2003; Khan 2005; Sessitsch *et al.* 2013).

Algunos estudios han observado que la inoculación con rizobacterias promotoras de crecimiento puede aumentar la disponibilidad de Ni en el suelo, la producción de biomasa y/o la acumulación de Ni en los tejidos de la planta (Abou-Shanab *et al.* 2006b; Abou-Shanab *et al.* 2003; Ma *et al.* 2011).

OBJETIVOS Y PRINCIPALES TAREAS REALIZADAS

Los objetivos de esta Tesis se pueden resumir en los siguientes:

1. El estudio de la variabilidad inter- e intrapoblacional en la tolerancia y acumulación de Ni de las subespecies hiperacumuladoras del género *Alyssum* endémicas de la Península Ibérica: *A. serpyllifolium* ssp. *lusitanicum* del NW de España (Melide) y NE de Portugal (Morais and Samil), y *A. serpyllifolium* ssp. *malacitanum* del S de España (Sierra Aguas y Sierra Bermeja), conocidas también

como *A. pintodasilvae* y *A. malacitanum,* respectivamente. El estudio considera principalmente la variabilidad observada en la biomasa de la planta, acumulación de Ni y/o la capacidad de la planta para movilizar el Ni del suelo, y está dirigido a obtener información que pudiera ser útil en futuros experimentos para la obtención de plantas con una mayor capacidad de extracción de Ni.

Para conseguir este objetivo se evaluaron la tolerancia y acumulación de Ni de cinco poblaciones de plantas (Melide, Morais, Samil, Sierra Aguas y Sierra Bermeja) crecidas en tres condiciones diferentes: en su hábitat natural, en cultivo hidropónico rico en Ni y en maceta con suelo serpentinítico. Además se evaluaron las propiedades fisicoquímicas y la disponibilidad de Ni tanto en suelo no vegetado como en la rizosfera de estas hiperacumuladoras de Ni.

2. La aplicación de dos estrategias diferentes para aumentar la producción de biomasa y/o la concentración de Ni en la parte aérea de diferentes especies hiperacumuladoras de Ni.

a) Aplicación de diferentes reguladores de crecimiento vegetal (fitohormonas) para mejorar la producción de biomasa y la capacidad de fitoextracción de Ni de varias especies hiperacumuladoras de este elemento de los géneros *Alyssum* (*A. corsicum*, *A. malacitanum*, *A. murale*, *A. pintodasilvae*) y *Noccaea goesingense* cultivadas en suelo serpentinítico.

Para lograr este objetivo se realizó un estudio en dos partes: un experimento inicial (Parte I) en el que se aplicaron dos productos comerciales a base de citoquininas y/o giberelinas a dos concentraciones diferentes cada uno, y un segundo experimento (Parte II), en el que se aplicaron cuatro productos comerciales a base de ácido indolacético, citoquininas y/o giberelinas a tres concentraciones diferentes. Se evaluaron los efectos de estas fitohormonas sobre el crecimiento de las plantas, la producción de biomasa, el estado nutricional y la eficiencia en la fitoextracción de Ni.

b) La inoculación con cepas de rizobacterias promotoras de crecimiento vegetal para aumentar la producción de biomasa y la fitoextracción de Ni en la especie hiperacumuladora *A. pintodasilvae.*

Para alcanzar este objetivo se seleccionaron quince aislados bacterianos teniendo en cuenta sus caracteristicas promotoras de crecimiento y se inocularon plantas *A. pintodasilvae* cultivadas en un medio de perlita y arena. En función de los resultados obtenidos se seleccionaron cinco cepas de las quince ensayadas y se inocularon plantas de *A. pintodasilvae* cultivadas en dos suelos con elevadas concentraciones de metal, un suelo serpentinítico rico en Ni y un suelo agrícola con elevadas concentraciones de Ni y Cd como consecuencia de la aplicación de

lodos residuales. Se determinó el efecto del inóculo sobre la disponibilidad del metal en el suelo, el crecimiento de la planta, su estado nutricional y su capacidad de acumulación y fitoextracción de Ni.

RESULTADOS Y CONCLUSIONES

Los resultados de este estudio ponen de manifiesto que existe una gran variabilidad en cuanto al crecimiento de la planta, la tolerancia y acumulación de Ni entre y dentro de las diferentes poblaciones de las subespecies hiperacumuladoras de Ni de *A. serpyllifolium.* Se han observado importantes diferencias inter- e intra-poblacionales en el contenido de nutrientes y en la acumulación de Ni tanto en las plantas de campo como en sus descendientes cultivados en condiciones controladas (cultivo hidropónico y maceta).

En las plantas recogidas en campo la variabilidad en la acumulación de Ni entre poblaciones fue más pronunciada que en plantas cultivadas en condiciones controladas: las dos poblaciones que presentaron una mayor acumulación de Ni en hoja fueron la población de *A. pintodasilvae* de Melide (L) y la de *A. malacitanum* de Sierra Bermeja (SB). Estas diferencias entre poblaciones en la concentración de Ni en hoja no mostraron correlación con la concentración total de Ni en el suelo ni con la fracción de Ni disponible para la planta.

Los experimentos llevados a cabo mostraron que la acumulación de Ni de las plantas de campo no estaba significativamente correlacionada con la de sus descendientes cultivados en condiciones controladas.

Las plantas de campo mostraron una mayor concentración de Ni en hoja que en las plantas cultivadas en suelo serpentinítico en maceta, lo que podría ser debido a diferencias en la edad de la planta, en el desarrollo radicular y a las propiedades edáficas y climáticas de cada población. En los datos de concentración de Ni en hoja obtenidos en cultivo hidropónico y en maceta se observó que la mayor proporción de la variabilidad total estaba relacionada con variabilidad dentro de las poblaciones de *A. serpyllifolium*. La baja variabilidad inter-poblacional observada en general puede ser debida a factores ambientales o como resultado de la historia evolutiva de las poblaciones serpentiníticas de *A. sepyllifolium*. Por otro lado, este estudio demuestra diferencias significativas en la producción de biomasa y en la transferencia de Ni de la raíz a la parte aérea cuando las plantas crecen en condiciones controladas, lo que podría suponer un importante campo de estudio para aumentar la capacidad de extracción de Ni de estas subespecies hiperacumuladoras. Es importante destacar que la producción de biomasa de las subespecies de *A. serpyllifolium* es significativamente inferior que en otras especies de *Alyssum*, como *Alyssum corsicum* y *Alyssum murale*, lo que probablemente puede limitar su aplicación práctica en técnicas de fitominería.

A pesar de sus limitaciones, estas subespecies, bajo determinadas condiciones, pueden ser consideradas como potenciales candidatos en procesos de fitominería. Por ejemplo, la aplicación de fitominería en zonas serpentiníticas de donde estas subespecies son endémicas podría suponer una alternativa a la agricultura tradicional, contribuyendo al desarrollo de áreas rurales. La utilización de estas especies vegetales nativas podría favorecer la conservación de la biodiversidad en zonas serpentiníticas y evitar la introducción de especies exóticas que frecuentemente invaden nuevas áreas perjudicando a las especies locales. Sería necesario la realización de estudios para evaluar la utilización de estas hiperacumuladoras de Ni a escala de campo, evaluando sus patrones de crecimiento y su potencial idoneidad para ser cosechadas mecánicamente. Además, sería necesario aplicar técnicas agronómicas adecuadas para maximizar la producción de biomasa y la extracción de Ni de estas hiperacumuladoras.

El análisis fisicoquímico del suelo indicó que existe un aumento de la biodisponibilidad de Ni en el suelo rizosférico de las hiperacumuladoras estudiadas respecto al suelo no vegetado, aunque este efecto no se observó en las cinco poblaciones de *A. pintodasilvae* y *A. malacitanum*. En algunos casos la concentración de Ni extraíble con $Sr(NO_3)_2$ en suelo rizosférico fue significativamente más elevada en comparación con la extraíble en el suelo no vegetado. Además en algunas poblaciones se observaron cambios inducidos por la planta en el fraccionamiento del Ni del suelo, aumentando la concentración de las fracciones de Ni más solubles a expensas de la fracción residual menos disponible o ligada a silicatos. Por otro lado en el suelo rizosférico de algunas poblaciones se apreció un aumento de pH, del contenido total de C y N, de la capacidad de intercambio catiónico (CIC) y/o de la relación Ca/Mg respecto al suelo no vegetado. La actividad de las raíces de estas plantas hiperacumuladoras de Ni puede aumentar la meteorización de minerales ricos en Ni, lo que provocaría un aumento de las fracciones solubles de Ni. Sin embargo, es fundamental llevar a cabo más estudios de los complejos procesos fisicoquímicos y biológicos que tienen lugar en la rizosfera de plantas hiperacumuladoras. La realización de futuros estudios centrados en los procesos cinéticos involucrados en el aporte de Ni de la fase sólida del suelo podría constituir un pilar clave para un mayor conocimiento de los procesos de hiperacumulación de metales.

La aplicación de reguladores de crecimiento (PGR) o fitohormonas se considera una estrategia interesante para aumentar la producción de biomasa de especies hiperacumuladoras de Ni de los géneros *Alyssum* y *Noccaea* y, en consecuencia, para aumentar su capacidad de fitoextración. En la primera parte de nuestro estudio la aplicación de fitohormonas (Cytokin y Promalin, a base de citoquininas y giberelinas) no tuvo un efecto significativo sobre la producción de

biomasa, la acumulación de Ni o la capacidad de fitoextracción en *A. corsicum*, *A. malacitanum*, *A. murale* y *A. pintodasilvae*. En la segunda parte del experimento se aplicaron cuatro productos (Berelex, Cytoplant, Kelpak y Promalin, a base de citoquininas, giberelinas y ácido indolacético (IAA)) a tres concentraciones diferentes. En esta segunda parte la aplicación de fitohormonas aumentó significativamente el crecimiento en las cuatro especies en cuanto al número de brotes/hojas, tamaño de la hoja y longitud del tallo. Los productos Kelpak y Promalin (IAA y mezcla de citoquininas y giberelinas) aumentaron significativamente la producción de biomasa de las cuatro hiperacumuladoras. Aunque, en general, la aplicación de fitohormonas redujo la acumulación de Ni en hoja en las cuatro especies estudiadas, el tratamiento con Kelpak incrementó la capacidad de fitoextracción de las cuatro especies debido al aumento en el crecimiento y producción de biomasa. Sería recomendable realizar estudios más a largo plazo utilizando diferentes reguladores de crecimiento que contengan IAA con el fin de optimizar los efectos beneficiosos que puede tener este tipo de fitohormonas sobre la capacidad de fitoextracción de Ni en especies hiperacumuladoras. Además, sería necesario evaluar el uso de estas fitohormonas en experimentos de campo antes de ser incorporados en las técnicas de fitominería.

La inoculación con rizobacterias promotoras de crecimiento (PGPR) aumentó la producción de biomasa y/o la acumulación de Ni en *A. pintodasilvae*. Sin embargo, el efecto de los inóculos varió en función del tipo de suelo, lo que indica que la eficiencia de este tipo de inóculos no sólo depende de la especie vegetal, sino que también influyen las condiciones fisiológicas de la planta y las características del suelo. Se seleccionaron cuatro cepas bacterianas por su elevada tolerancia al Ni, sus propiedades promotoras de crecimiento y su capacidad de solubilizar Ni (LA44, SA5b, SA17 y SA40) y se incluyo, además, una cepa bacteriana que no causó efecto alguno sobre la planta en experimentos previos (SBA50). Las cepas LA44, SA5b, SA17 y SA40 aumentaron las cantidades de Ni fitoextraído en plantas cultivadas en suelo serpentinítico. Sin embargo, las propiedades de estas cepas bacterianas observadas *in vitro* no siempre se correspondían con los efectos observados en el complejo planta-microorganismo-suelo, indicando que existen mecanismos adicionales involucrados en el proceso. Es necesario llevar a cabo estudios adicionales para optimizar el método de inoculación, establecer la densidad más adecuada de inóculo bacteriano, edad de la planta (ej.: inoculación en semilla o planta), momento de la inoculación (fase de crecimiento bacteriano) o necesidad de re-inoculación, así como la persistencia y la capacidad competitiva de las cepas inoculadas. Además, en este trabajo las cepas bacterianas fueron inoculadas individualmente, pero hay que tener en cuenta que la inoculación combinando varias cepas bacterianas con diferentes

características promotoras de crecimiento y/o de solubilización de metales podría producir efectos beneficiosos adicionales. Los avances en estos aspectos llevarían a la obtención de efectos más pronunciados de estas bacterias sobre las plantas y, por tanto, a importantes mejoras en la eficiencia en técnicas de fitoextracción y fitominería. En este estudio la cepa SA40 (*Arthrobacter nicotinovorans* SA40) fue capaz de promover el crecimiento vegetal y la cantidad de Ni fitoextraído en dos tipos de suelo: suelo serpentinítico y suelo agrícola contaminado con Ni y Cd, por lo que puede ser considerado como un buen candidato para la realización de futuros ensayos de bioaumento.

Las principales conclusiones de esta Tesis son:

1. Los estudios en los que se evaluó la variabilidad inter- e intra-poblacional en biomasa y acumulación de Ni de tejidos cosechables de *A. pintodasilvae* y *A. malacitanum* indicaron que:

- En el campo se detectaron diferencias significativas en la acumulación de Ni entre poblaciones; sin embargo, la capacidad de acumulación de Ni de las plantas de campo no se transmitió a sus descendientes cultivados en condiciones controladas ya fuese en cultivo hidropónico o en suelo. A pesar de ello, en condiciones controladas se detectaron variaciones en los individuos en producción de biomasa, acumulación de Ni y translocación de Ni de la raíz a la parte aérea que pueden ser exploradas en futuros estudios enfocados al aumento de la capacidad de extracción de estas subespecies hiperacumuladoras.

- La acumulación de Ni observada en las plantas de campo no se relacionó con la cantidad total de Ni ni con la concentración disponible de Ni en el suelo. Sin embargo, en general se observaron cambios en la disponibilidad y fraccionamiento de Ni, y lo que es más importante, en condiciones controladas se vio que un incremento de la concentración de Ni biodisponible en el sustrato de crecimiento provocó un aumento en la acumulación de Ni en la planta. Esto sugiere que un incremento en la biodisponibilidad de Ni en el suelo (hasta concentraciones no fitotóxicas) puede producir un aumento de la cantidad de Ni extraído por estas plantas.

2. Las estrategias empleadas para aumentar la extracción de Ni por especies vegetales hiperacumuladoras de este metal (aplicación de fihohormonas o inoculación de cepas bacterianas asociadas a la planta) permitieron, en algunos casos, conseguir un aumento de biomasa y/o cantidad de Ni fitoextraído.

- El efecto obtenido tras la aplicación de reguladores de crecimiento vegetal (PGRs) o fitohormonas dependió, además de la composición química del producto, de la dosis utilizada y de la especie en la que fue aplicado.

- En el caso de las rizobacterias, los beneficios obtenidos tras la inoculación dependieron no sólo de las propiedades fenotípicas de las cepas bacterianas, sino también de las condiciones fisiológicas de la planta y de las propiedades fisico-químicas del suelo.

Entre los tratamientos empleados para aumentar la producción de biomasa y/o la fitoextracción de Ni los mejores resultados se obtuvieron tras la aplicación de fitohormonas a base de IAA o con la inoculación con una cepa de *Arthrobacter nicotinovorans*.

REFERENCIAS

Abou-Shanab R, Angle J and Chaney R (2006a). Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils. *Soil Biol Biochem* 38: 2882-2889.

Abou-Shanab RA, Angle JS, Delorme TA, Chaney RL, van Berkum P, Moawad H, Ghanem K and Ghozlan HA (2003). Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. *New Phytol* 158: 219-224.

Abou-Shanab RA, Angle JS and Chaney RL (2006b). Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils. *Soil Biol Biochem* 38: 2882-2889.

Adamidis G, Aloupi M, Kazakou E and Dimitrakopoulos P (2014). Intra-specific variation in Ni tolerance, accumulation and translocation patterns in the Ni-hyperaccumulator *Alyssum lesbiacum*. *Chemosphere* 95: 496-502.

Adriano DC (2001). Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals. Springer, New York, NY.

Anderson C, Brooks R, Chiarucci A, LaCoste C, Leblanc M, Robinson B, Simcock R and Stewart R (1999). Phytomining for nickel, thallium and gold. *J Geochem Explor* 67: 407-415.

Asensi A, Rodríguez N, Díez-Garretas B, Amils R, Boyd R, Baker A and Proctor J (2004). Nickel hyperaccumulation of some subspecies of *Alyssum serpyllifolium* (Brassicaceae) from ultramafic soils on the Iberian Peninsula. Ultramafic rocks: Their soils, vegetation and fauna, Proceedings of the fourth International Conference on Serpentine Ecology. Science Reviews, St. Albans, UK. 263-265.

Asher C (1991). Beneficial elements, functional nutrients and possible new essential elements. In: JJ Mortvedt et al. (eds) Micronutrients in agriculture. 2nd edn. Soil Science Society of America, Madison, WI. p. 703-723.

Baker AJM (1981). Accumulators and excluders-strategies in the response of plants to heavy-metals. *J Plant Nutr* 3: 643-654.

Baker AJM and Brooks RR (1989). Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81-126.

Bani A, Echevarria G, Sulçe S, Morel J and Mullai A (2007). In-situ phytoextraction of Ni by a native population of *Alyssum murale* on an ultramafic site (Albania). *Plant Soil* 293: 79-89.

Bani A, Echevarria G, Sulçe S and Morel JL (2013). Improving the agronomy of *Alyssum murale* for extensive phytomining: A five-year field study. *Int J Phytoremediat*.

Becerra-Castro C, Kidd PS, Kuffner M, Prieto-Fernandez A, Hann S, Monterroso C, Sessitsch A, Wenzel W and Puschenreiter M (2013). Bacterially induced weathering of ultramafic rock and its implications for phytoextraction. *Appl Environ Microbiol* 79: 5094-5103.

Bothe H (2011). Plants in heavy metal soils. In: I Sherameti and A Varma (eds) Detoxification of heavy metals, Soil Biology. Springer-Verlag, Berlin. Vol. 30. p. 35-57.

Broadhurst CL, Chaney RL, Angle JS, Maugel TK, Erbe EF and Murphy CA (2004). Simultaneous hyperaccumulation of nickel, manganese, and calcium in *Alyssum* leaf trichomes. *Environ Sci Technol* 38: 5797-5802.

Brooks RR, Lee J, Reeves RD and Jaffre T (1977). Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *J Geochem Explor* 7: 49-57.

Brooks RR, Shaw S and Marfil AA (1981). Some observations on the ecology, metal uptake and nickel tolerance of *Alyssum serpyllifolium* subspecies from the Iberian Peninsula. *Plant Ecol* 45: 183-188.

Brooks RR (1987). Serpentine and its vegetation: A multidisciplinary approach. Dioscorides Press, Portland, OR.

Brooks RR, Chambers MF, Nicks LJ and Robinson BH (1998). Phytomining. *Trends Plant Sci* 3: 359-362.

Callahan DL, Baker AJ, Kolev SD and Wedd AG (2006). Metal ion ligands in hyperaccumulating plants. *J Biol Inorg Chem* 11: 2-12.

Cassina L, Tassi E, Morelli E, Giorgetti L, Remorini D, Chaney RL and Barbafieri M (2011). Exogenous cytokinin treatments of an Ni hyper-accumulator, *Alyssum murale*, grown in a serpentine soil: Implications for phytoextraction. *Int J Phytoremediat* 13: 90-101.

Chaney RL (1983). Plant uptake of inorganic waste constituents. In: JF Parr et al. (eds) Land treatment of hazardous wastes. Noyes Data Corporation, Park Ridge, NJ. p. 50-76.

Chaney RL, Malik M, Li YM, Brown SL, Brewer EP, Angle JS and Baker AJM (1997). Phytoremediation of soil metals. *Curr Opin Biotechnol* 8: 279-284.

Chaney RL, Angle JS, Baker AJM and Li YM (1998). Method for phytomining of nickel, cobalt and other metals from soil. US Patent N° 5.711.784.

Chaney RL, Broadhurst CL and Centofanti T (2010). Phytoremediation of soil trace elements. Blackwell Publishers, Oxford, UK.

Dickinson NM, Baker AJM, Doronila A, Laidlaw S and Reeves RD (2009). Phytoremediation of inorganics: Realism and synergies. *Int J Phytoremediat* 11: 97-114.

Echevarria G, Morel J, Fardeau J and Leclerc-Cessac E (1998). Assessment of phytoavailability of nickel in soils. *J Environ Qual* 27: 1064-1070.

El-Saeid H, Abou-Hussein S and El-Tohamy W (2010). Growth characters, yield and endogenous hormones of cowpea plants in response to IAA application. *Res J Agric & Biol Sci* 6: 27-31.

Emongor VE, Pule-Meulenberg F and Phole O (2004). Effect of Promalin on growth and development of kale (*Brassica oleracea* L. var. *acephala* DC). *J Agron* 3: 208-214.

Ernst WH (2000). Evolution of metal hyperaccumulation and phytoremediation hype. *New Phytol* 146: 357-358.

French CJ, Dickinson NM and Putwain PD (2006). Woody biomass phytoremediation of contaminated brownfield land. *Environ Pollut* 141: 387-395.

Gadd GM (2004). Microbial influence on metal mobility and application for bioremediation. *Geoderma* 122: 109-119.

Ghosh M and Singh S (2005). A review on phytoremediation of heavy metals and utilization of it's by products. *Asian J Energy Environ* 6: 18.

Glick BR, Penrose DM and Li J (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J Theor Biol* 190: 63-68.

Glick BR (2003). Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnol Adv* 21: 383-393.

Glick BR (2010). Using soil bacteria to facilitate phytoremediation. *Biotechnol Adv* 28: 367-374.

Hammer D, Keller C, McLaughlin MJ and Hamon RE (2006). Fixation of metals in soil constituents and potential remobilization by hyperaccumulating and non-hyperaccumulating plants: Results from an isotopic dilution study. *Environ Pollut* 143: 407-415.

Hinsinger P (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant Soil* 237: 173-195.

Hinsinger P, Gobran G, R. , Gregory P, J. and Wenzel W, W. (2005). Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. *New Phytol* 168: 293-303.

Hörger AC, Fones HN and Preston GM (2013). The current status of the elemental defense hypothesis in relation to pathogens. *Front Plant Sci* 4: 395.

Hussain K, Hussain M, Nawaz K, Majeed A and Bhatti KH (2011). Morphochemical response of chaksu (*Cassia absus* L.) to different concentrations of Indole Acetic Acid (IAA). *Pak J Bot* 43: 1491-1493.

Hutchinson JJ, Young SD, McGrath SP, West HM, Black CR and Baker AJ (2000). Determining uptake of 'non-labile'soil cadmium by *Thlaspi caerulescens* using isotopic dilution techniques. *New Phytol* 146: 453-460.

Jones DL and Darrah PR (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant Soil* 166: 247-257.

Kabata-Pendias A (2011). Trace elements in soils and plants. CRC Press, Boca Raton, FL.

Kazakou E, Adamidis GC, Baker AJ, Reeves RD, Godino M and Dimitrakopoulos PG (2010). Species adaptation in serpentine soils in Lesbos Island (Greece): metal hyperaccumulation and tolerance. *Plant Soil* 332: 369-385.

Khan AG (2005). Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. *J Trace Elem Med Biol* 18: 355-364.

Kidd PS, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R and Monterroso C (2009). Trace element behaviour at the root-soil interface: Implications in phytoremediation. *Environ Exp Bot* 67: 243-259.

Knight B, Zhao FJ, McGrath SP and Shen ZG (1997). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. *Plant Soil* 197: 71-78.

Li YM, Chaney RL, Angle JS and Baker AJM (2000). Phytoremediaiton of heavy metal contaminated soils. In: KL Wise et al. (eds) Bioremediation of contaminated soils. Marcel Dekker, New York, NY. p. 837-884.

Li YM, Chaney R, Brewer E, Roseberg R, Angle JS, Baker A, Reeves R and Nelkin J (2003). Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. *Plant Soil* 249: 107-115.

Ma Y, Rajkumar M and Freitas H (2009). Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. *J Hazard Mater* 166: 1154-1161.

Ma Y, Prasad MNV, Rajkumar M and Freitas H (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnol Adv* 29: 248-258.

Marschner P (2007). Plant-microbe interactions in the rhizosphere and nutrient cycling. Springer-Verlag, Heidelberg, Germany.

McGrath S, Shen Z and Zhao F (1997). Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. *Plant Soil* 188: 153-159.

McGrath SP and Zhao FJ (2013). Concentrations of metals and metalloids in soils that have the potential to lead to exceedance of maximum limit concentrations of contaminants in food and feed. *Soil Use Manage*.

Mench M, Schwitzguebel JP, Schroeder P, Bert V, Gawronski S and Gupta S (2009). Assessment of successful experiments and limitations of phytotechnologies: contaminant uptake, detoxification and sequestration, and consequences for food safety. *Environ Sci Pollut Res Int* 16: 876-900.

Mench M, Lepp N, Bert V, Schwitzguébel J-P, Gawronski S, Schröder P and Vangronsveld J (2010). Successes and limitations of phytotechnologies at field scale: outcomes, assessment and outlook from COST Action 859. *J Soils Sed* 10: 1039-1070.

Menezes de Sequeira E and Pinto da Silva AR (1991). Ecology of serpentinized areas of north-east Portugal. In: BA Roberts and J Proctor (eds) The ecology of areas with serpentinized rocks: A world view. Kluwer Academic Publishers, Dordrecht, Netherlands. p. 169-197.

Milner MJ and Kochian LV (2008). Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Ann Bot* 102: 3-13.

Moreno-Jiménez E, Esteban E, Carpena-Ruiz RO, Lobo MC and Penalosa JM (2012). Phytostabilisation with Mediterranean shrubs and liming improved soil quality in a pot experiment with a pyrite mine soil. *J Hazard Mater* 201: 52-59.

Munn J, January M and Cutright TJ (2008). Greenhouse evaluation of EDTA effectiveness at enhancing Cd, Cr, and Ni uptake in *Helianthus annuus* and *Thlaspi caerulescens*. *J Soils Sed* 8: 116-122.

Pollard AJ, Reeves RD and Baker AJM (2014). Facultative hyperaccumulation of heavy metals and metalloids. *Plant Sci* 217: 8-17.

Puschenreiter M, Wieczorek S, Horak O and Wenzel WW (2003). Chemical changes in the rhizosphere of metal hyperaccumulator and excluder *Thlaspi* species. *J Plant Nutr Soil Sci* 166: 579-584.

Qiu R, Liu W, Zeng X, Tang Y, Brewer E and Fang X (2009). Effects of exogenous citric acid and malic acid addition on nickel uptake and translocation in leaf mustard (*Brassica juncea* var. *foliosa* Bailey) and *Alyssum corsicum*. *Int J Environ Pollut* 38: 15-25.

Rajkumar M and Freitas H (2008). Effects of inoculation of plant-growth promoting bacteria on Ni uptake by Indian mustard. *Bioresour Technol* 99: 3491-3498.

Salt DE, Blaylock M, Kumar N, Dushenkov V, Ensley BD, Chet I and Raskin I (1995). Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. *Biotechnol* 13: 468-474.

Sessitsch A, Kuffner M, Kidd PS, Vangronsveld J, Wenzel WW, Fallmann K and Puschenreiter M (2013). The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biol Biochem* 60: 182-194.

Sharma SS and Dietz KJ (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot* 57: 711-726.

Uren NC and Reisenauer HM (1988). The role of root exudates in nutrient acquisition. In: B Tinker and A Lachli (eds) Advances in plant nutrition. Praeger, New York, NY. p. 79-114.

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D and Mench M (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res Int* 16: 765-794.

Vassilev A, Schwitzguébel JP, Thewys T, van der Lelie P and Vangronsveld J (2004). The use of plants for remediation of metal-contaminated soils. *Sci World J* 16: 9-34.

Verbruggen N, Hermans C and Schat H (2009). Molecular mechanisms of metal hyperaccumulation in plants. *New Phytol* 181: 759-776.

Wenzel W, Bunkowski M, Puschenreiter M and Horak O (2003). Rhizosphere characteristics of indigenously growing nickel hyperaccumulator and excluder plants on serpentine soil. *Environ Pollut* 123: 131-138.

Weyens N, van der Lelie D, Taghavi S, Newman L and Vangronsveld J (2009). Exploiting plant-microbe partnerships to improve biomass production and remediation. *Trends Biotechnol* 27: 591-598.

Whittaker RH (1954). The ecology of serpentine soils. *Ecology* 35: 258-288.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1

INTRODUCTION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1.1. Nickel-rich soils: soils developed over ultramafic rocks and anthropogenic-contaminated soils

Soil naturally enriched in nickel

Free of human interference the soil trace element (TE) content is largely dependent on that of the soil parent material and acting weathering processes. The terms "trace element", "trace metal" or "heavy metal" are frequently used synonymously in the literature. Trace elements, metals or metalloids, are present in the lithosphere in concentrations below 1000 mg kg^{-1} dry weight (DW), and are commonly detected in living organisms at concentrations below 100 mg kg^{-1} DW (Adriano 2001). On the other hand, heavy metals refer to those metals with a density of more than 5 g cm^{-3} (Bothe 2011). Several trace metals are essential micronutrients to plants and/or animals (such as Cu, Fe, Mn, Ni and Zn), and deficiencies can occur when either the concentrations in soil are reduced or when the soil conditions limit their bioavailability. In contrast, other elements are considered non-essential and lack any known biological function (for example, elements such as As, Cd, Hg and Pb). However, in all cases, at high concentrations trace metals can have strong toxic effects and pose environmental threats (McGrath and Zhao 2013).

Nickel is considered to be essential for several biological processes in microorganisms, plants and animals (Adriano 2001). In plants, Ni is involved in N metabolism as a metal component of the enzyme urease, being essential for the structure and catalytic function of this enzyme (Hänsch and Mendel 2009). The adequate range for Ni in plants is between 0.01 mg kg^{-1} DW and >10 mg kg^{-1} DW, which is a wide range as compared to other elements (Brown *et al.* 1987; Gerendás *et al.* 1999). The Ni concentration in plants grown on uncontaminated soil ranges from 0.05 to 5.0 mg kg^{-1} DW (Brooks 1980; Welch 1981).

In the Earth's crust, the mean Ni abundance has been estimated at around 20 mg kg^{-1}, whereas in ultramafic rocks Ni ranges from 1400 to 2000 mg kg^{-1} (Kabata-Pendias 2011). Ni concentrations decrease with increasing acidity of rocks, down to the range of 5-20 mg kg^{-1} in granite rocks (Kabata-Pendias 2011). There is a general similarity between the distribution of Ni, Co and Fe in the earth's crust. The term "ultramafic" refers to igneous or metamorphic rocks that contain high quantities of ferromagnesian minerals (>70 %) and low concentrations of silica (SiO_2 <45 %). Ultramafic igneous rocks are composed essentially of ferro-magnesium minerals (more than 90 %), particularly within the olivine and pyro-xene groups (Brooks 1987). The majority have undergone distinct metamorphic processes, of which serpentinization (in varying degrees) is the most frequent. Serpentinization is a hydrothermal process in which primary

minerals, such as olivine and pyroxene, are transformed into serpentine minerals, such as lizardite, antigorite or chrysotile.

Ultramafic rocks are patchily distributed throughout the world (occupying approximately 1 % of the earth's surface area (Proctor 1999)). The soils developed on ultramafic rocks are generically referred to as "ultramafic soils" or "serpentine soils". In the Iberian Peninsula the main serpentinitic areas are located in the Trás-os-Montes region (NE Portugal), in the Northern (Ortigueira) and Central (Melide) areas in the region of Galicia (NW Spain) and in the western Betic Cordillera of Málaga (SE Spain) (Asensi *et al.* 2004; Brooks *et al.* 1981; Carballeira *et al.* 1983; Menezes de Sequeira and Pinto da Silva 1991).

Although ultramafic soils show considerable variability in their physico-chemical properties, they typically present a series of common characteristics that differentiate them from other soils. For example, they are characterised by elevated concentrations of Mg, Fe and potentially phytotoxic trace metals such as Ni, Co and Cr, low organic matter content, deficiency in essential plant macronutrients such as N, P and K, low availability of Ca relative to Mg, low cation exchange capacity (CEC) and a low water-holding capacity (Brooks 1987; Whittaker 1954). A wide range in soil Ni concentrations in serpentine soils have been observed (from approximately 1000 mg kg^{-1} up to 8000 mg kg^{-1}) (Table 1.1). Serpentine soils can therefore be stressful environments for plant growth and these limiting factors are often referred to as the "serpentine syndrome".

Nickel-contaminated soils

In addition to natural sources, elevated concentrations of trace metals may be present in the soil due to anthropogenic activities. In fact, trace metals are one of the most frequent soil contaminants (35 %) present at polluted sites across Europe (EC 2014). Anthropogenic contamination sources include mining, ore processing, agricultural recycling of sewage and municipal wastes, application of agrochemicals, and release of municipal and industrial emissions (Mench *et al.* 2009). Since Ni and other trace metals (such as Cd, Cu, Pb, Hg and Zn) are persistent in the environment and not biodegradable they are considered to be amongst the most dangerous of soil contaminants and have been included on the list of priority pollutants of the US Environmental Protection Agency's (EPA) (Cameron 1992). In the case of Ni, the content in the soil covers a wide range, depending on the site and the Ni source (Table 1.1). The main sources of soil contamination include metal processing plants (Ni refineries and smelters emissions) as well as some sewage sludges and phosphate fertilizers applied to agricultural soils (Li *et al.* 2003a). Emissions from refineries and smelters are clear sources of Ni (and Co) contamination in surrounding soils (Table 1.1). Downwind from a Ni refinery in Port Colborne, Ontario (Canada), the concentration of Ni in

the topsoil (0-5 cm layer) ranged from 800 to over 6000 mg kg^{-1} (Frank *et al.* 1982). An example of extreme contamination by Ni was reported for topsoils near a Ni-Cu smelter at Sudbury (Canada), where concentrations of up to 26000 mg Ni kg^{-1} were recorded (Cox and Hutchinson 1981).

Table 1.1. Mean nickel concentrations or range in natural Ni-enriched soil (serpentine soil) or soils contaminated through human activities.

Site and Pollution Source	Mean/Range (mg kg^{-1})	Country	Reference
Serpentine soil	1700-5000	New Zealand	Lyon *et al.* 1970
	2000-3000	Scotland	Proctor 1969
	1037-4254	Spain	Paz-González *et al.* 2001 Rufo *et al.* 2005
	2962	Portugal	Peterson *et al.* 2003
Metal-processing industry	206-26000	Canada	Cox and Hutchinson 1981 Freedman and Hutchinson 1980 Temple and Bisessar 1981
	304-9288	Russia	Barcan and Kovnatsky 1998
	1243	Albania	Shtiza *et al.* 2005
Sludge-amended soils	50-84	Germany	Diez and Rosopulo 1976
	up to 385	United Kingdom	McGrath and Smith 1990
	up to 245	France	Mench *et al.* 1994

(based on Kabata-Pendias 2011)

The excessive application of sewage sludge amendments containing high concentrations of trace elements may lead to the accumulation of such elements in the soil, and in turn may cause adverse effects on the growth and development of plants and ultimately, may enter the food chain, affecting animal and human health (Kabata-Pendias and Pendias 1984). Ni contamination has been shown in many agricultural soils where sewage sludge has been used as a soil amendment (Table 1.1). For example, Ni concentrations as high as 385 mg kg^{-1} were measured in UK agricultural soils receiving sewage sludge (McGrath and Smith 1990). The European Community has set 75 mg kg^{-1} as the maximum allowable Ni concentration in pasture soils (McGrath *et al.* 1995). Mench *et al.* (1994) carried out a field experiment in a soil contaminated by the application of Cd/Ni-enriched sewage sludge from a treatment plant (Louis Fargue Station) located in Bordeaux, France. In this study the authors evaluated the metal availability and the effect on maize plants eight years after the termination of the sewage sludge application

(sludges were applied during four years). They concluded that part of the sludge-borne Cd and Ni can remain bioavailable in the soil for a long period of time and Cd and Ni uptake by maize were correlated with extractable-metal concentrations in the soil.

In Spain there is a regulation for the agricultural use of sewage sludge, Royal Decree 1310/1990 (Ministry of Agriculture 1990), which is derived from the EU Directive 86/278/CEE (CEC 1986). The permitted limits of trace elements which are established in this legislation are several times higher than the limits of trace elements allowed for compost in the Spanish Law on fertilisers, Royal Decree 824/2005 (Ministry of Agriculture 2005) (Table 1.2). It has been shown that total concentrations of potentially toxic elements are clearly insufficient for risk assessment given that trace elements toxicity depend on their speciation (Greenway and Song 2002; Smith 2009). In consequence, legislation should not only regulate the maximum total concentration of trace elements in these organic amendments but should also contemplate parameters such as mobility, bio-availabilty and ecotoxicity of these elements (Legret 1993; Pérez-Cid *et al.* 1999).

Table 1.2. Maximum permitted concentrations of trace elements (mg kg^{-1}) in compost and sludge destined for agricultural use according to Spanish legislation.

	Compost (a)			***Sludge(b)***	
	Class A	*Class B*	*Class C*	*Soils pH<7*	*Soils pH>7*
Cu	70	300	400	1000	1750
Pb	45	150	200	750	1200
Zn	200	500	1000	2500	4000
Cd	0.7	2	3	20	40
Cr	70	250	300	1000	1500
Cr(VI)	0	0	0	-	-
Ni	25	90	100	300	400
Hg	0.4	1.5	2.5	16	25

(based on Barral and Paradelo 2011; (a) Ministry of Agriculture 2005; (b) Ministry of Agriculture 1990)

1.2. Metallophytes and nickel hyperaccumulating plants

To survive in serpentine environments plants have developed a wide range of mechanisms of adaptation (Brady *et al.* 2005; Kazakou *et al.* 2008). The deficiency in plant macronutrients of the serpentinitic soils (due to low amounts of

organic material and the lack of P and K in parent rocks) is a major limitation in serpentine environments. Many plants have developed root systems to facilitate uptake of nutrients and water (Brooks 1987). The low Ca/Mg ratio observed in serpentinitic soils is an important restriction to the plant growth due to the fact that Mg may act as an antagonist in the absorption of Ca by plants, causing a deficiency in this nutrient (Brooks 1987). The challenge of a low Ca/Mg ratio has led to numerous adaptive plant responses based on ion exclusion at the root/shoot interface, selective translocation of Ca from root to shoot, sequestration of Mg in the vacuole or internal mechanisms of tolerance (O'Dell *et al.* 2006; Walker *et al.* 1955).

Potentially phytotoxic levels of trace metals in serpentine soils, such as Fe, Ni, Co, Cr and Mn, can negatively affect plant growth, causing stunting and chlorosis or antagonism with other nutrients (Antonovics *et al.* 1971; Clemens 2006). Some plants (metallophytes) have developed highly specialized biological mechanisms permitting them to resist, tolerate or thrive in the presence of elevated metal concentrations (Brooks 1987; Menezes de Sequeira and Pinto da Silva 1991; Reeves *et al.* 1996).

Baker (1981) classified plants into three groups according to their response to metals:

- "Excluder" plants: where the translocation of trace metals is limited and the plant maintains a low levels of metals in their aerial tissues over a wide range of soil metal concentrations.
- "Indicator" plants: take up metals over a wider range than 'normal' plants and the concentrations in plant leaves reflect that of the soil, until phytotoxicity prevents further growth and causes death of the plant.
- "Accumulator" plants: where metals are actively concentrated within plant tissues over the full range of soil concentrations, implying a highly specialised physiology.

Within the group of accumulator plants are those metallophytes denominated as "hyperaccumulators", which accumulate extreme concentrations of metals in their shoots when growing in metal-enriched habitats. The term "hyperaccumulator" was first used by Brooks *et al.* (1977) to describe plants containing >1000 mg kg^{-1} Ni in dry material. Later on the use of the term was widened to include those plants that are able to concentrate at least 100 mg kg^{-1} (0.01 % DW) of Cd or As, 1000 mg kg^{-1} (0.1 % DW) of Co, Cu, Cr, Ni and Pb, and 10000 mg kg^{-1} (1 % DW) of Zn or Mn in their aboveground tissues when growing in their natural habitat (Reeves and Brooks 1983). Recently these criteria have been considered somewhat conservative and a lower threshold has been proposed for elements such as Co, Cu and Cr (300 mg kg^{-1}; 0.03 % DW), or for Zn

(3000 mg kg^{-1}; 0.3 % DW) (Kramer 2010; Van der Ent *et al.* 2013). Examples of metal hyperaccumulating plants are given in Figure 1.1. To date, approximately 500 taxa are known to hyperaccumulate one or more metals or metalloids and over 90 % of known hyperaccumulators (>450 taxa) accumulate Ni (Pollard *et al.* 2014). The genus with the greatest number of Ni-hyperaccumulators is *Alyssum* (Brassicaceae) (Baker and Brooks 1989). Figure 1.2 shows the distribution of Ni-hyperaccumulating species from the genus *Alyssum* in the Mediterranean region. The Iberian Peninsula hosts two subspecies of *Alyssum serpyllifolium* Desf. which are both serpentine-endemic and hyperaccumulators of Ni: *Alyssum serpyllifolium* ssp. *lusitanicum* from Galicia (NW Spain) and Trás-os-Montes (NE Portugal) (frequently referred to as *A. pintodasilvae*), and *Alyssum serpyllifolium* ssp. *malacitanum* from Andalusia (S Spain) (also known as *A. malacitanum*).

The ecological and evolutionary significance of the hyperaccumulating trait is an area of much debate. This trait has evolved multiple times independently in the plant kingdom. Recent studies seem to support the idea that this trait has evolved as a means of defence against attack by pathogenic microorganisms and herbivores. A recent review by Hörger *et al.* (2013) summarises the evidence that metal hyperaccumulation acts as a defensive trait in plants. These authors proposed a possible scenario for the evolution of metal hyperaccumulation, in which selective pressure for resistance to pathogens or herbivores, combined with gene flow from non-metallicolous plant populations, increases the likelihood that the metal hyperaccumulating trait becomes established in plant populations.

Ni-hyperaccumulator plants grow naturally on serpentine soils and the vast majority of these (85-90 %) appear to be serpentine-endemic species (Reeves and Adigüzel 2008) and are characterised by 1-3 % DW Ni accumulation in their shoots (Chaney *et al.* 2010). In comparison, critical toxicity levels of Ni in crop species are in the range of >10 mg kg^{-1} DW in sensitive to >50 mg kg^{-1} DW in moderately tolerant species (Asher 1991). Mechanisms of Ni uptake, root-to-shoot translocation and sequestration in hyperaccumulator plants are not fully understood. Ni-hyperaccumulator plants are characterized by a strongly enhanced rate of loading of Ni into the xylem for transport to the shoot and stimulated metal influx across the leaf cell plasma membrane and sequestration in the leaf vacuoles (Broadhurst *et al.* 2004; Milner and Kochian 2008). It is assumed that most of the metals inside plants are bound to organic acids, amino acids, peptides and proteins, functioning as the main mechanism of metal detoxification (Callahan *et al.* 2006; Sharma and Dietz 2006; Verbruggen *et al.* 2009). In the case of Ni, the nature of Ni-ligands in the plants is still controversial. Some authors have suggested that histidine could be involved in the transport and storage of Ni in *Alyssum* species (Kerkeb and Kramer 2003; Kramer *et al.* 1996), whereas other studies have shown that Ni transport in the xylem sap in *Alyssum* species occurs

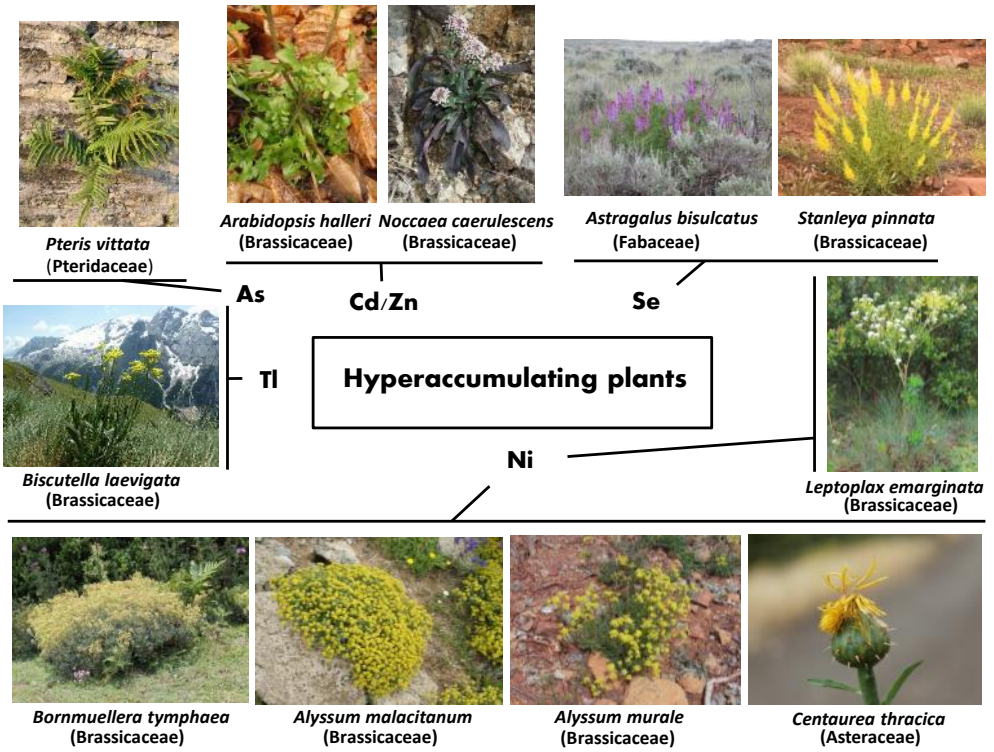


Figure 1.1. Examples of hyperaccumulating plants of the trace metals, Cd, Ni and Zn, and the metalloids, As and Se.
Photos courtesy of Aida Bani, Rufus L. Chaney, Guillaume Echevarria, Elizabeth Pilon-Smits, Aldo De Bastiani, Gianluca Nicolella, Roberto Bottinelli and Acta Plantarum Forum.

mainly as a free hydrated cation and is complexed with carboxylic acids (mainly citric acid) (Alves *et al.* 2011; Centofanti *et al.* 2013).

Although Ni-hyperaccumulator plants have an extraordinary capacity for Ni accumulation**,** this process depends on several factors including Ni bioavailability in the soil, the supply from less plant-available fractions and the ability of the plant to intercept, take up and accumulate trace elements in shoots (Ernst 2000; McGrath *et al.* 1997; Wenzel *et al.* 2003). Metal bioavailability can be defined as the fraction of the total metal content of the soil that can interact with a biological target (Geebelen *et al.* 2003). In the soil solution elements are present as free uncomplexed ions, ion pairs, ions complexed with organic anions, and ions complexed with organic macromolecules and inorganic colloids. The most important metal pools in the solid phase include the exchange complex, metals complexed by organic matter, sorbed onto or occluded within oxides and clay minerals, co-precipitated with secondary pedogenic minerals (e.g. Al, Fe, Mn oxides, carbonates and phosphates, sulphides) or as part of the crystal lattices of

Figure 1.2. Distribution of Ni-hyperaccumulating plants from the genus *Alyssum* in ultramafic outcrops in the Mediterranean region (Geologic map based on Jähne 2014).
1 *Alyssum serpyllifolium* sp *lusitanicum*,**2** *Alyssum serpyllifolium* sp *malacitanum*,**3** *Alyssum argenteum*,**4** *Alyssum bertolonii*,**5** *Alyssum robertianum*,**6** *Alyssum corsicum*,**7** *Alyssum murale*,**8** *Alyssum markgrafii*,**9** *Alyssum sibiricum*,**10** *Alyssum heldreichii*,**11** *Alyssum smolikanum*,**12** *Alyssum fallacinum*,**13** *Alyssum euboeum*,**14** *Alyssum tenium*,**15** *Alyssum lesbiacum*,**16** *Alyssum pinifolium*,**17** *Alyssum floribundum*,**18** *Alyssum davisianum*,**19** *Alyssum virgatum*,**20** *Alyssum dudley*,**21** *Alyssum eriophyllum*,**22** *Alyssum masmenaeum*,**23** *Alyssum peltarioides*,**24** *Alyssum caricum*,**25** *Alyssum huber-morathii*,**26** *Alyssum cypricum*,**27** *Alyssum discolor*,**28** *Alyssum pterocarpum*,**29** *Alyssum trapeziforme*,**30** *Alyssum callichroum*,**31** *Alyssum cassium*,**32** *Alyssum troodii*,**33** *Alyssum akamasicum*,**34** *Alyssum bertolonii* sp *scutarinum*.

primary minerals (Adriano 2001). Availability to plants is governed by the pseudo -equilibrium between aqueous and solid soil phases, rather than by the total metal content.

The reduction in the concentration of labile soil metal pools rarely explains the observed metal uptake by these plants, a phenomenon which led several authors to believe that hyperaccumulators were able to increase their metal uptake by accessing metal fractions which were not available to non-accumulator plants (Knight *et al.* 1997; McGrath *et al.* 1997). However, numerous studies have demonstrated that both hyperaccumulator and non-hyperaccumulator plants access the same soil metal pools (Echevarria *et al.* 1998; Hammer *et al.* 2006; Hutchinson *et al.* 2000). Shallari *et al.* (2001) reported that *A. murale* takes up Ni from the same labile pool of Ni in soils as red clover (non-accumulating plant), suggesting that Ni accumulation by this species is not due to the solubilisation of less-available soil Ni forms. Likewise, Massoura *et al.* (2005) studied an excluder plant species (*Triticum aestivum*), an indicator species (*Trifolium pratense*) and three populations of the Ni-hyperaccumulator *A. murale* to determine whether or not the available Ni pool in the soil varied for the different species. Results showed that, for a given soil, the available pools were similar for all three plant species and that they all accessed the same Ni exchangeable pool regardless of their Ni uptake capacity. Metal uptake is consistently greater in metal hyperaccumulating than in non-accumulating plants; however, the changes observed in labile and non-labile fractions may not necessarily indicate active mobilisation of metals by the plant, but merely the buffering capacity of the soil and replenishment of the soil labile pool (Kidd *et al.* 2009).

It is well-known that plants influence the surrounding soil (that is the rhizosphere or soil which is in direct contact with the roots) (Hinsinger 2001; Hinsinger and Courchesne 2008; Jones and Darrah 1994; Marschner 2007; Mench and Martin 1991). Important factors influencing soil metal mobility and bioavailability include: 1) root-induced changes in pH of the rhizosphere, 2) increased reducing capacity of the roots, and 3) quantity and composition of root exudates. Changes in pH and redox potential were studied in the rhizosphere of a Ni hyperaccumulator (*Alyssum murale*) and a crop plant (*Raphanus sativus*) growing in metal-contaminated substrates (Bernal *et al.* 1994). Differences in pH and reducing capacity were found between the lateral roots and the main roots of both species, but the acidification and reducing capacity of the roots of *A. murale* were always smaller than those of *R. sativus*. The authors concluded that enhanced metal uptake by the hyperaccumulator plant was not related to metal solubilisation via either a reduction in pH in the rhizosphere, or the release of reductants from roots. These results were confirmed by other studies that, using *Thlaspi*

caerulescens (recently re-classified as *Noccaea caerulescens*), ruled out the role of rhizosphere acidification in metal hyperaccumulation (Knight *et al.* 1997; Luo *et al.* 2000; McGrath *et al.* 1997). Another possibility is that hyperaccumulators release chelating compounds into the rhizosphere (root-soil interface) to mobilise trace metals. Root exudates produced by plants can directly influence soil nutrient and metal availability, through processes such as acidification, chelation, precipitation and redox reactions, releasing the non-labile forms of metals into the soil solution, or indirectly, through their effects on the microbial activity (Adriano 2001; Hinsinger *et al.* 2005; Puschenreiter *et al.* 2003; Tao *et al.* 2004; Uren and Reisenauer 1988). Salt *et al.* (2000) did not find any high-affinity Ni-chelating compounds in the root exudates of *N. goesingense.* Moreover, these authors found that the root exudates of the non-hyperaccumulator *Thlaspi arvense* contained higher levels of known Ni-chelators (histidine and citrate) than the root exudates of *N. goesingense*. However, in this study plants were grown in hydroponic cultures and may therefore not reflect processes operating in soil conditions. For example, in field-collected plants the exudation of organic acids by the Ni hyperaccumulator, *N. goesingense*, was suggested to trigger the replenishment of soluble Ni from sources other than the exchangeable fraction through the dissolution of Ni-rich clay minerals (i.e. non-labile soil solid phase) (Puschenreiter *et al.* 2005; Puschenreiter *et al.* 2003; Wenzel *et al.* 2003).

A more intense weathering of Ni-rich minerals in the rhizosphere of Ni-hyperaccumulators could also lead to the release of labile Ni (be it active mobilisation or not). However, whether this phenomenon is plant- or microbial-induced, or the result of complex plant-microbial interactions, is unknown. Microbial transformation of soil minerals leads to the solubilisation of metals alongside essential nutrients, and to the modification of their form and distribution in the solid phase (Quantin *et al.* 2001; 2002). In the case of hyperaccumulating plant species, their associated microorganisms have already been shown to modify soil Ni mobility (see Section 1.5. *Bioaugmentation with plant-associated microorganisms for increasing nickel phytoextraction*). For example, Becerra-Castro *et al.* (2013) reported that the production of organic acids and siderophores by bacterial strains isolated from the rhizosphere soil of the Ni-hyperaccumulators *A. pintodasilvae* and *A. malacitanum* enhanced the weathering of Ni-rich manganese oxides, iron oxides or serpentine minerals.

1.3. Application of plant metallophytes in phytoextraction and phytomining techniques

Plants which are adapted to thrive on metalliferous soils have received considerable attention due to their potential application in remediation

technologies of trace element-contaminated soils (Chaney *et al.* 2010; Chaney *et al.* 1997; Cunningham *et al.* 1995; Dickinson *et al.* 2009; Kidd *et al.* 2009; Mench *et al.* 2009; Vangronsveld *et al.* 2009). Several techniques have been developed using plants and their associated microorganisms for environmental clean-up, and are collectively known as phytoremediation techniques (Chaney *et al*. 1997; Salt *et al*. 1995). These are considered to be potentially cost-effective options, which are less invasive than conventional civil engineering techniques for soil clean-up (e.g. encapsulation, vitrification, soil washing) and can even restore soil structure and functions (Mench *et al.* 2010; Mench *et al.* 2009; Moreno-Jiménez *et al.* 2012; Vangronsveld *et al*. 2009).

Phytoremediation techniques have been developed to target both organic compounds and trace elements. For trace element-contaminated soils, the objective is to decrease the labile ("bioavailable") pool and/or total contents of metal(loid)s in the soil, or to reduce their entrance in excess into plants (thereby meeting with guideline values for contaminant levels in food or fodder crops) and any related pollutant linkages (e.g. leaching from the root zone, soil erosion and water runoff, etc.).

Phytostabilisation aims to establish a vegetation cover and progressively promote *in situ* inactivation of metal(loid)s by combining the use of trace element-excluding plants and soil amendments (Mench *et al.* 2006; Vangronsveld *et al.* 1996; Vangronsveld *et al.* 2009; Vangronsveld *et al.* 1995). This technology does not lead to a clean-up of the soil, but by altering trace element speciation and mobility it moderates their potential negative environmental impacts and pollutant linkages. Moreover, this technique provides important ecological benefits, such as promoting ecosystem restoration and biodiversity (Schwitzguébel *et al.* 2011). For large contaminated areas, phytostabilisation is probably the most reasonable option for ecosystem restoration (Schwitzguébel 2014).

Phytoextraction uses metal-(hyper)accumulating plant species to transport and accumulate high quantities of trace metals from the soil into the harvestable parts of roots and aboveground shoots, thus removing the metals from the soil (Chaney 1983; Chaney *et al.* 1997; Vassilev *et al.* 2004) (Fig. 1.3). Phytoextraction includes three categories: (1) cultivation of arable crops, with or without additional application of chemical or biological agents to mobilise soil trace elements; (2) cultivation of rapidly growing trees with trace element-accumulating phenotypes, which can additionally produce biomass for energy generation and financial returns; and (3) cultivation of hyperaccumulator plants (Bani *et al.* 2007; French *et al.* 2006; Munn *et al.* 2008). The most appropriate option will be depend on the concentrations of the metal(s), their bioavailability

and risks for relevant pollutant linkages, the remediation objectives and the site management restrictions (Mench *et al.* 2010).

In the early 1980s, Chaney *et al.* (1983) proposed the use of hyperaccumulator plants in phytoextraction techniques due to their ability to accumulate extreme concentrations of metal(loid)s (e.g. Cd, Ni, Zn, Se, and As) in their above-ground biomass. However, a low biomass production can be an important bottleneck limiting the practical application of hyperaccumulators in phytoextraction, as well as the high number of cropping cycles required for clean-up (if the objective is to reduce total trace element concentrations in soils). Additional limiting factors include the absence of commercially available seeds/ seedlings, their sensibility to the presence of contaminants other than the hyperaccumulated trace elements, a lack of knowledge related to their cultivation, climate needs or competition with other trace element-tolerant plants. Over the last two decades high-biomass crops (annuals or perennials) and woody plants have also been recognised as viable plant types for the phytoextraction of trace metals (particularly Cd, Se and Zn) if they show relevant shoot trace metal removals (i.e. moderate-high bioconcentration factor (BCF) and high shoot yield). For example, a large number of *Salix* and *Populus* clones have been screened, and show great variation in biomass production, trace metal tolerance and accumulation patterns in roots and leaves between clones (Gaudet *et al.* 2011; Landberg and Greger 1994; Migeon *et al.* 2009; Pulford *et al.* 2002; Ruttens *et al.* 2011; Van Slycken *et al.* 2013). A field trial implementing a willow short rotation coppice (SRC) system using *Salix*, *Populus* and *Alnus* in brownfields in the UK showed that phytoextraction could potentially reduce Cd and Zn concentrations in hotspots within a 25-30 year life cycle, while reducing the lability of As, Pb, Cu, and Ni (French *et al.* 2006). Fifteen years of willow SRC grown commercially in Swedish fields significantly reduced Cd concentrations in topsoil compared to reference fields with common agricultural crops (Dimitriou *et al.* 2012). Annual crops such as tobacco (*Nicotiana tabacum*) and sunflower (*Helianthus annuus*) have also been shown to accumulate Cd and Zn when growing in soils with Cd and Zn contamination from smelters, mine wastes or historic use of biosolids. Phytoextraction with somaclonal variants of tobacco and sunflower mutant lines (non-GMOs) with enhanced metal uptake and tolerance was shown to lower the labile Zn pool in soil by 45-70 % in a field scale experiment in north-eastern Switzerland, while subplots without phytoextraction treatment maintained labile Zn concentrations (Herzig *et al.* 2014).

In some cases of phytoextraction the accumulated metals can be recovered from the harvested biomass, and when this is the case the process is known as phytomining (Chaney *et al.* 2007a; Li *et al.* 2003a). At present, Ni is the element

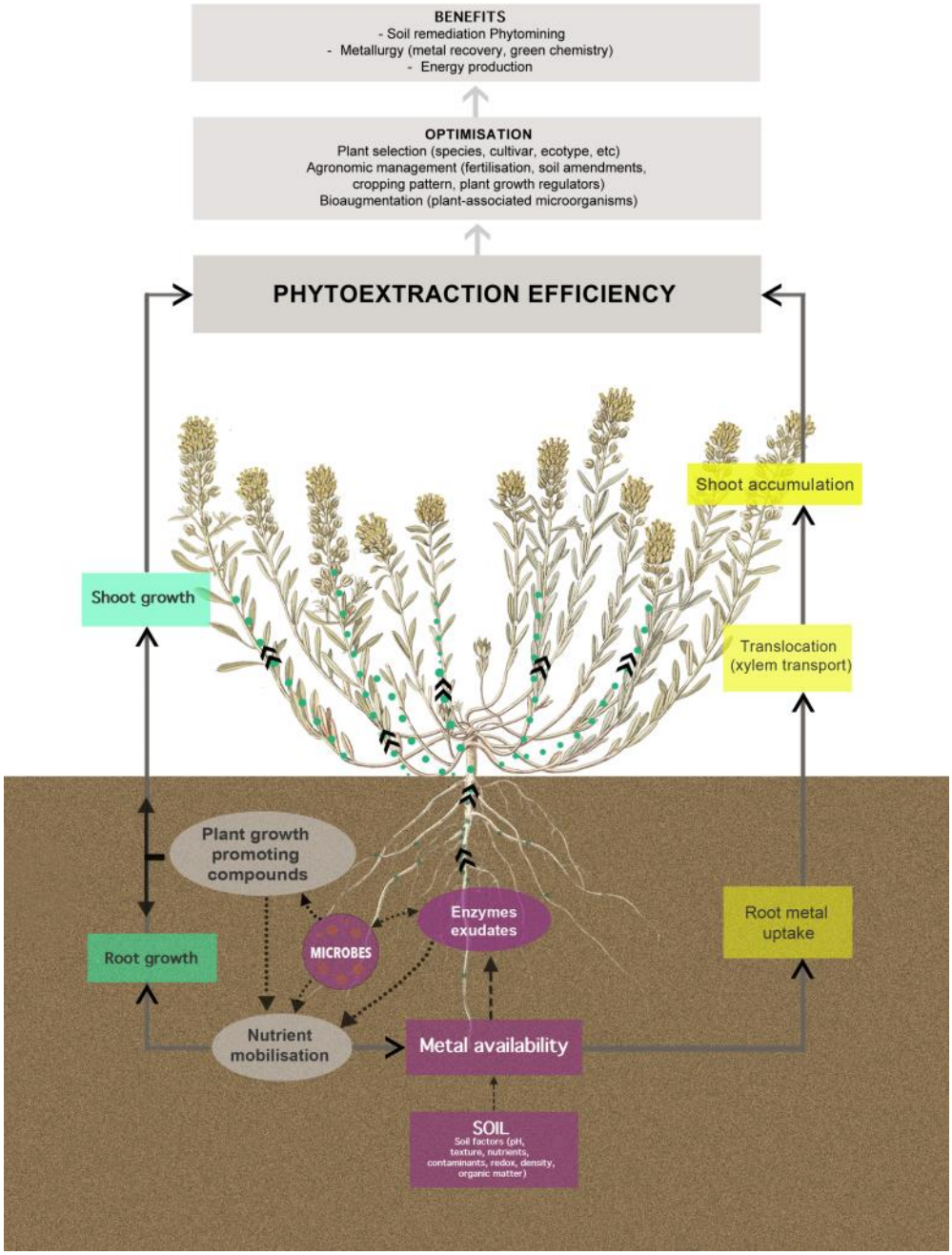


Figure 1.3. Conceptual scheme of processes and factors involved in phytoextraction

for which phytomining appears to be most feasible, and is considered effective for the recovery of this metal from sub-economic ores, such as serpentine soils or Ni-contaminated soils (Chaney *et al.* 2014). A few large-scale field trials demonstrating the potential for Ni phytomining can be found in Albania, Canada and the USA (Figure 1.4 and 1.5). Chaney *et al.* (2014) attributed this to the widespread occurrence and extent of Ni-rich serpentine soils and mine wastes, the wide variety of Ni-accumulating plants (85-90 % of known hyperaccumulators occur on serpentine soil and accumulate Ni), and the ready market for Ni metal, Ni salts and Ni fertilisers (Figure 1.6). In contrast, the price of elements such as Zn in the world market is at present too low to make "Zn-recycling" from trace element contaminated soil economically feasible (Vangronsveld *et al.* 2009).

Currently mined ore materials (associated with ultramafic rocks) typically contain 0.8-2.5 % Ni, while serpentine soils characteristically present a range of 0.05-0.8 % Ni. Ni extraction by conventional mining processes is therefore economically unviable in this type of substrate. However, the Ni concentration of certain hyperaccumulator plants growing on these soils is 1-3 % Ni in DW leaf tissues, and 8-25 % in plant ash, making these plant ashes an ore material with a Ni content which is an order of magnitude higher than mined ores. Furthermore, the plant ash of hyperaccumulators is low in both Fe and Mn oxides and Mg silicates, which are known to complicate Ni recovery from lateritic ores during conventional mining processes. For all these reasons the phytomining of Ni using Ni-hyperaccumulating plants is considered to be economically feasible (Chaney *et al.* 2007a; Chaney *et al.* 2010; Chaney *et al.* 2014).

Already in the early 1990s, a pioneering phytomining trial was carried out using the Ni hyperaccumulator *Streptanthus polygaloides*, an endemic species to serpentine soils in California (USA) (Nicks and Chambers 1994; Nicks and Chambers 1998). Despite a wide variation in Ni concentrations amongst individual plants at the site the authors predicted that, after selective breeding to obtain a high -biomass yielding crop, a 10 t ha^{-1} crop would contain 100 kg of Ni. Incineration of the biomass would then yield approximately 500 kg ha^{-1} of ash containing 20 % Ni. The potential value of the crop of Ni was estimated to be similar to that obtained from a crop of wheat. Robinson *et al.* (1997a) carried out small-scale field trials using the South African Ni-hyperaccumulator *Berkheya coddii* which belongs to the Asteraceae and grows to a height of about 2 m. This high-biomass producing hyperaccumulator can accumulate up to 1.7 % Ni in its dry mass. In the field trials of Robinson *et al.* (1997a) a mean biomass production of 21.4 t ha^{-1} yr^{-1} could be achieved when using appropriate fertilisation. Robinson and colleagues (Robinson *et al.* 1997b) also carried out a small-scale field experiment testing the potential use of the Ni hyperaccumulator *Alyssum bertolonii* in phytomining in

Figure 1.4 Phytomining projects carried out at A) Pojskë, Pogradec (Albania) *Alyssum murale*; B) Port Colborne, Ontario (Canada) and C) Kerby, Oregon (USA) *Alyssum corsicum* and *Alyssum murale.* *Photos courtesy of Aida Bani and Rufus L. Chaney.*

Ni-rich serpentine soils in Tuscany. These authors assessed the biomass productivity of naturally occurring populations of the hyperaccumulator after applying distinct fertiliser treatments. The Ni content of the plants remained fairly constant in the range 0.54-0.77 % for most fertiliser treatments and the maximum annual biomass increase was about 300 %. On a larger scale, Chaney and colleagues successfully demonstrated the phytomining of Ni in field applications with *Alyssum* in Oregon, USA, and in Ontario, Canada (Chaney *et al.* 2007a; Li *et al.* 2003a) (Figure 1.4). They carried out field trials evaluating different genotypes of two Ni-hyperaccumulators, *A. murale* and *A. corsicum*, and obtained shoot Ni concentrations as high as 22 g kg^{-1} and a biomass of up to 20 t ha^{-1} in selected parental lines. The consequent phytoextraction of Ni was shown to be up to 400 kg

Ni ha^{-1}. These authors developed the phytoextraction process on both naturally Ni-enriched soils as well as Ni-contaminated soils (affected by a Ni refinery). On the basis of these studies Chaney *et al.* (1998; 1999; 2007b) obtained US Patents for practical Ni phytomining using *A. murale* and *A. corsicum*.

More recently, in Europe successful field experiments using the Ni hyperaccumulator *A. murale* were carried out by Bani and colleagues in the serpentinitic area of Pojskë-Pogradec in Albania (Bani *et al.* 2007; Bani *et al.* 2013) (Fig. 1.4). A five-year field study was designed to assess the effect of (i) plant phenology and element distribution, (ii) plant nutrition and fertilisation, (iii) plant cover and weed control and (iv), planting technique (natural cover vs. sown crop). The optimal harvest time was found to be the mid-flowering stage when Ni concentration and biomass yield were highest. The application of NPK fertilisers, and especially a split 100 kg ha^{-1} N application, significantly increased the density of *A. murale* compared to all other species, increasing shoot yield but without reducing Ni concentration. The cropping of sown *A. murale* was more efficient than enhancing native stands and resulted in both a higher biomass and phytoextraction yield; biomass yields progressively improved from 0.3 to 9.0 t ha^{-1} and Ni yield increased from 1.7 to 105 kg ha^{-1}.

Several authors have discussed the long-term sustainability of the Ni phytomining process. Chaney *et al.* (2014) suggested that the sustainability of these processes is favourable, with time scales of up to 50 years. This estimation was based on the following calculation: for an area with Ni averaging 2500 mg Ni kg^{-1} to 30 cm rooting depth the total Ni present is approximately 10 t Ni $(ha\text{-}30\ cm)^{-1}$, a single crop of a hyperaccumulator plant with a DW of 10 t ha^{-1} and 2 % Ni will yield 200 kg Ni ha^{-1} (representing 2 % of the whole resource). Sustainability was also dependant on the soil/sub-soil being periodically turned over and that natural soil buffering processes replenish the plant-available Ni fraction within the time scale of the phytoextraction process. These authors also pointed out that for the same site and pH, Ni concentration in phytomining crop shoots will decline over time as the readily phytoavailable pool is depleted, but this process will vary according to the specific site conditions. Nonetheless, the efficiency of the phytomining process requires optimisation, especially on a site-by-site basis and the incorporation of appropriate agronomical practices (planting technology, planting densities, harvest strategies, etc.) and fertilisation regimes.

1.4. Processing of Ni-rich biomass, recovery of Ni bio-ores and production of green Ni products

The technique of phytomining generally involves growing a crop of a metal-hyperaccumulating plant species, harvesting the biomass and burning it to produce

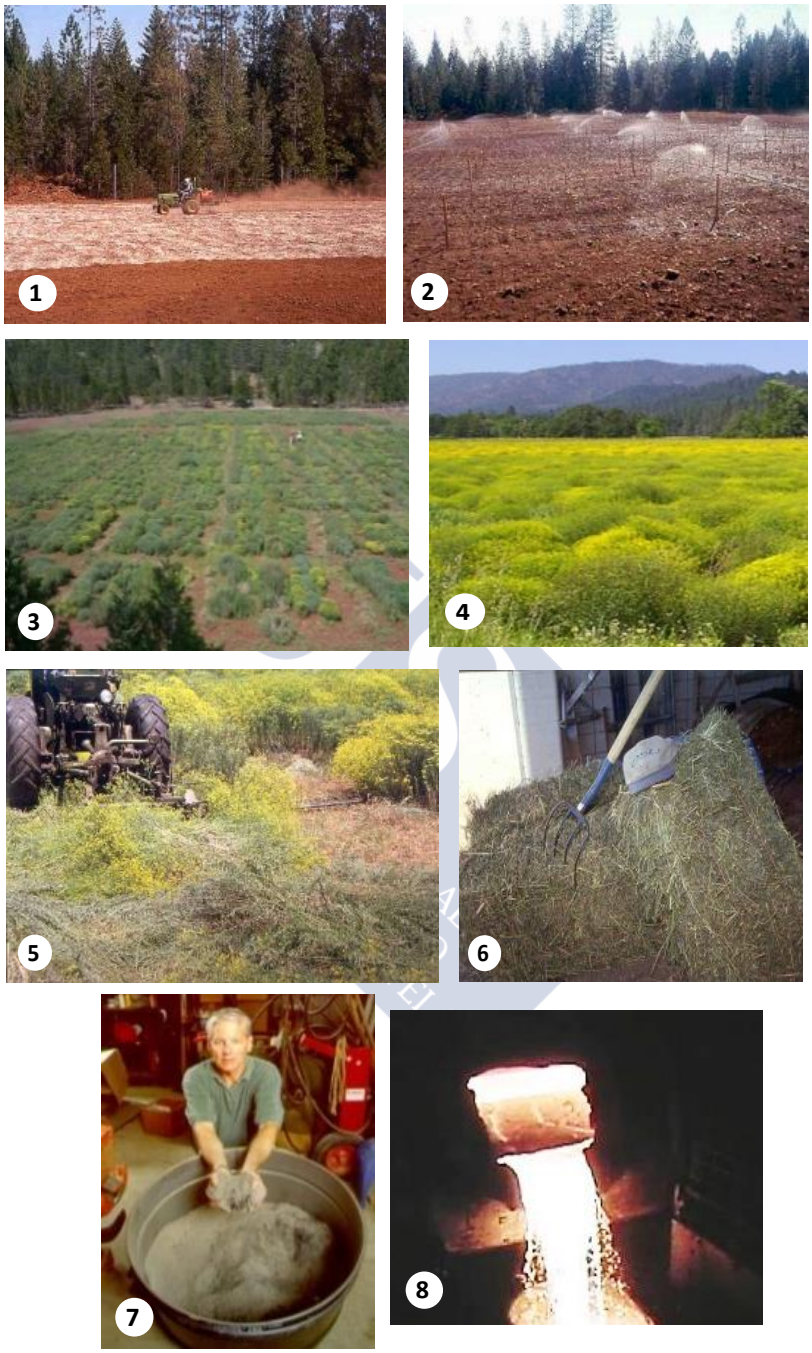


Figure 1.5. The phytomining process: 1) and 2) conventional agronomic practices (ploughing, fertilisation, sowing, irrigation) on a serpentine soil in Oregon (USA); 3) *A. murale* at the start of the flowering stage; 4) *A. murale* before harvesting; 5) Harvesting; 6) Packaging; 7) Combustion to recover biomass energy and Ni-rich ash; 8) Recovery of Ni from ash. *Photos courtesy of Rufus L. Chaney.*

a Ni-rich ash (Figure 1.5). When the plant biomass is ashed there is a possibility for energy recovery from the biomass combustion, thus making the process more cost-effective (to cover the costs of site preparation, seeds, fertilisers, etc.) (Li *et al.* 2003a). The ash obtained, known as a "bio-ore", contains Ni concentrations as high as 30 % and these can be smelted to recover metallic Ni, or alternatively, in periods of low market prices, it can be stored where it does not pose a risk to the environment until the world price improves (Fig. 1.6). Different pyrometallurgical methods have been used when burning the hyperaccumulator biomass and for the recovery of Ni from these bio-ores. To date, the combustion of the plant biomass is the most commonly used method because of the possibility to obtain energy during the process. Boominathan *et al.* (2004) reported that Ni-bearing crystalline residues containing up to 82 % Ni were generated after furnace treatment at 1200 °C of *Alyssum bertolonii* biomass. Results also suggested that reducing the Ca content of the biomass prior to furnace treatment increased the quality of Ni bio-ore produced. Zhang *et al.* (2014) showed that temperature and duration, during the combustion process, were important parameters to ensure a good quality of ashes, and found that the best conditions were a temperature of 550 ºC during 3h. Other authors have proposed the co-incineration of metal-rich plant biomass with municipal solid wastes; obtaining a clean residue (bottom ash) that could be used either as fertiliser or safely disposed of in a landfill, while allowing the recovery of metals from metal concentrates (in this case Zn) (Keller *et al.* 2005). Koppolu and Clements (2003) proposed the use of hyperaccumulator biomass as fuel for pyrolysis with a further step of metal recovery from biochar.

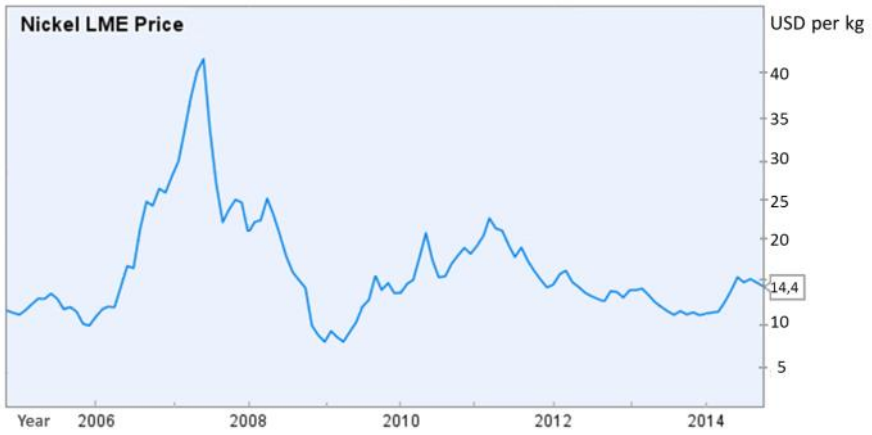


Figure 1.6. Price of cathode nickel on the London Metal Exchange (LME) in the last ten years (based on data from World Bank).

Hydrometallurgical processes can be used to produce Ni-based chemicals in purified forms from Ni-rich ashes (Barbaroux *et al.* 2012; Habashi 2005). Several authors have developed a process to produce a high value Ni salt, Ni ammonium disulphate (containing 13.2 % Ni), from *A. murale* biomass (Barbaroux *et al.* 2011; Barbaroux *et al.* 2012; Mercier *et al.* 2012). The process includes an initial leaching of the ashes with a solution of sulphuric acid at 95 °C, followed by evaporation and precipitation steps and a cold crystallisation of the Ni rich solution. These Ni chemicals have been valued at $20000 per ton in a purified form, thus demonstrating that the combination of phytomining and hydrometallurgy offers a high potential of profitability (Barbaroux *et al.* 2012). Zhang *et al.* (2014) evaluated the potential for Ni recovery from different hyperaccumulating species within the genera *Alyssum*, *Leptoplax* and *Bornmuellera*. The highest Ni concentrations were always recorded in the leaves of the plants, and Ni concentrations in *Leptoplax emarginata* leaves were significantly higher than in the other species. The highest concentration of Ni was also recorded in the ash from the leaves of *L. emarginata*, making this species a particularly interesting candidate for metal recovery. These authors highlighted the need for optimisation so as to enable the upscaling of the hydrometallugical process to produce ammonium sulphate Ni double salt.

Recent studies have considered the use of metal-accumulating plants for the preparation of catalysts that can be used in chemical reactions (Escande *et al.* 2014; Losfeld *et al.* 2012a; Losfeld *et al.* 2012b; Losfeld *et al.* 2012c). Due to the high concentrations of metals in their tissues, hyperaccumulating plants represent an interesting renewable resource for the production of Lewis acid catalysts. Lewis acid catalysis is one of the key technologies for catalysis, green chemistry and asymmetric synthesis and it is used for fine chemistry as well as for large-scale production (Grison and Escarre 2010). Several studies have showed the application of plants containing Zn and Ni nanoparticles as catalysts for the chlorination of alcohols and Friedel-Crafts chemistry (alkylation and acylation reactions) (Losfeld *et al.* 2012a; Losfeld *et al.* 2012c). The application of plants containing metals to environmentally-relevant catalytic reactions could be an innovative outlet for the valorisation of metal-contaminated biomass produced in phytoextraction technologies (Hunt *et al.* 2014).

The biomass of selected metal hyperaccumulators used in soil remediation can be applied for the correction of specific micronutrient deficiencies in crops. Wood *et al.* (2006) demonstrated that the application of aqueous sprays of Ni derived from Ni-containing *Alyssum* biomass are efficacious for correcting Ni deficiency of pecan trees. *Alyssum* biomass appears to be a potential cost-effective

fertiliser and fully compliant with criteria pertaining to organic agriculture certifications.

1.5. Benefits of the phytomining process, possible limitations and strategies for improving phytomining efficiency

Phytomining is receiving increasingly more attention (not only from the scientific community but from all relevant stakeholders) because it can potentially provide a realistic means of meeting with increasing demands on metal resources without causing the environmental damage and contamination associated with conventional mining activities (Chaney *et al.* 2014). These techniques can provide innovative methods to recover metal from low-grade ores which would be economically inviable using conventional mining techniques (Harris *et al.* 2009). Phytomining has several advantages over conventional mining technologies: it offers the possibility of exploiting metals that would not be feasible when using conventional mining methods, it is a potentially low-cost operation, the negative effects on the environment are minimal, and finally, the bio-ores from phytomining are sulphur free (and therefore do not contribute significantly to acid rain) and their smelting requires less energy than sulphide ores (Anderson *et al.* 1999; Brooks *et al.* 1998). In addition to potential metal recovery and energy production, phytomining can restore soil structure, and lead to improvements in soil quality and functions. For example, it has been proposed that the long-term cultivation of *Alyssum*, with use of fertilisers, will result in a permanent increase in soil organic matter levels and sequestration of atmospheric carbon dioxide (Li *et al.* 2003a). Other benefits include the restoration of degraded land after mining activities (Li *et al.* 2003a). Mine-soils are often characterised by a low amount of nutrients and organic matter, often high acidity, phytotoxic concentration of trace metals, and low water retention capacity, properties which convert them in unfavourable environments for plant development and growth. Implementing a phytomining strategy with metal-hypertolerant hyperaccumulating plant species can lead to soil restoration and provision of vital ecosystem services. Phytomining can also provide alternatives to traditional agriculture carried out in serpentine soils that cannot provide a profitable agriculture production, thus supporting the development of rural areas (Bani *et al.* 2007).

Despite these advantages, the phytomining process can also present some important limitations. Most of the natural metal hyperaccumulators are slow growing with a small biomass and shallow root systems (except some Ni-hyperaccumuators such as *Berkheya coddii*). The phytomining process is climate and season dependent, limited by biogeochemical factors (microbial activity, root exudates, temperature, pH, moisture) and the solubility and

availability of the metals in the soil (Ghosh and Singh 2005). The use of solubilising agents to increase metal mobility is generally not recommended due to the associated potential risks of groundwater contamination as a result of leaching of trace elements and may also increase extraction costs (Gramss *et al.* 2004; Greman 2005). Several management techniques have been proposed as strategies for maximising phytomining efficiency and some of these are described in more detail in the following sections. It is generally accepted that the phytomining process requires the successful incorporation of agronomic practices as a means of optimising soil Ni extraction, improving plant biomass production, plant nutritive status, soil quality, pest management, etc. For example, the implementation of agronomic practices such as crop rotations, intercropping, planting density, fertilisation, weed, pest and herbivory management, etc. can significantly improve plant productivity and maximise the metal recovery (Kidd *et al.* 2015). The optimal biomass harvesting schedule (time and conditions) is critical and should be carried out according to the plant metal content versus phenological stage to maximise metal uptake and accumulation from the soils (avoid secondary losses through litter decomposition) and at the same time obtain maximal biomass production of harvestable plant parts. An adequate storage of the harvested biomass is also necessary to prevent the risk of metal transfer to the food chain (Chaney *et al.* 2007a; Ernst 2005).

It is clear that the overall success of the phytomining process will largely depend on the concentrations of the target metals in the harvestable shoot biomass and on biomass yield. Effective hyperaccumulator plants to be applied in phytomining must be highly metal tolerant, able to accumulate large concentrations of trace elements in harvestable shoots, and have a reasonable biomass production so that the annual removal of metal from the site is economic (Glick 2010; Li *et al.* 2003a; Vangronsveld *et al.* 2009). However to be realistic, one of the most important factors in phytomining is also the world price of metals; as mentioned above this technology is currently most viable for Ni, but other valuable metals such as Co and Tl could also be targeted in the future (Anderson *et al.* 1999; Chaney *et al.* 2007a).

Selection of adequate plants for nickel phytomining

Chaney *et al.* (2014) suggested that amongst the Ni hyperaccumulators within the genus *Alyssum*, the most appropriate species for cultivation in areas with a Mediterranean-type climate include *A. murale*, *A. corsicum*, *A. lesbiacum* and *A. pinifolium*, which are short-lived perennials, native to Turkey, Greece, and the Balkan region. The height and growth pattern of these species is suitable for mechanical harvesting. Other genera within the Brassicaceae and endemic to the

Mediterranean region which also show potential as phytomining crops include *Leptoplax emarginata* (Greece) and *Bornmuellera* spp. (Greece and Turkey) (Bani *et al.* 2009; Chardot *et al.* 2005; Reeves and Adigüzel 2008; Shallari *et al.* 1998). Non-hyperaccumulating plants are generally not considered suitable for phytomining purposes (Chaney *et al.* 2010). Several studies have reported an increase in Ni uptake by non-hyperaccumulating plants (such as *Helianthus annuus*) after adding metal-chelating agents, such as EDTA, EDDS and NTA, to the soil (Meers *et al.* 2005). However, such complexing agents can cause unacceptable contaminant leaching and the costs involved are prohibitive (Chaney *et al.* 2007a; Robinson *et al.* 1999).

Selected plants for Ni phytomining purposes should therefore be able to hyperaccumulate this metal and also to tolerate and thrive under the adverse growth conditions which are frequently present in metal-rich environments (Harris *et al.* 2009). There are several advantages to selecting native plant species, due to both practical reasons and also as a means of supporting the conservation of serpentine biodiversity. The ideal choice would be a native plant with high and rapid biomass production, thus avoiding the introduction of exotic plant species that frequently colonise new areas at the expense of native species.

Hyperaccumulating plants often present a large difference in biomass and metal uptake among individual plants of the same population. For this reason, several authors have suggested that traditional plant breeding programmes could use the available genetic diversity within a species to combine the traits needed for successful phytoextraction (and phytomining). Numerous studies have investigated the variation between populations of hyperaccumulating species in their ability to accumulate Zn, Ni and Cd (Assunção *et al.* 2003; Escarré *et al.* 2000; McGrath *et al.* 1993; Pollard and Baker 1996). Nicks and Chambers (1995) observed a large variation in biomass within a *Streptanthus polygaloides* population in a field experiment. They found plants growing less than 20 cm apart to differ in size by a factor of two to three. Brooks and Robinson (1998) reported a very wide range of biomass, ranging from 1 to 64 g, in a *Noccaea caerulescens* population growing over Zn/Pb mine wastes near Montpellier in southern France. Several authors clearly demonstrated a high intraspecific and interspecific variability in metal concentration and metal yield of different populations of hyperaccumulating *Alyssum* species depending on the site of collection (Kazakou *et al.* 2010; Massoura *et al.* 2004; Shallari *et al.* 1998). Populations of *N. caerulescens* are found adapted to calamine (Zn-Cd enriched), serpentine soils, as well as growing on non-metalliferous soils (Molitor *et al.* 2005). All the *N. caerulescens* populations appear to hyperaccumulate Zn, while most of the serpentine populations also hyperaccumulate Ni and finally, only a few of the

calamine populations hyperaccumulate Cd (Lombi *et al.* 2000). However, substantial natural variation between the different populations has been observed, leading to wide differences in metal uptake and tolerance (Assunção *et al.* 2003; Basic *et al.* 2006; Ebbs *et al.* 2009; Lombi *et al.* 2000; Pollard *et al.* 2002; Roosens *et al.* 2003). Pollard *et al.* (2002) studied the within-population variation in metal (Ni/Zn) hyperaccumulation in *N. caerulescens* from five populations representing a variety of soil types in Britain and Spain, including Zn/Pb mine spoil, serpentine soils high in Ni/Co/Cr, and non-metalliferous soils. Significant within-population and between-population variation in their metal hyperaccumulation was found when grown in a uniform hydroponic solution (enriched with either 10 mg L^{-1} Zn or 0.5 mg L^{-1} Ni), and there was no positive association between soil metal concentrations at the site of origin and the mean ability of each population to hyperaccumulate that metal. Macnair (2002) also found genetic variation between and within three populations of *Arabidopsis halleri* growing in the field. However, the results of this author showed that variation in Zn accumulation between plants was heritable and that a small but significant correlation could be found between the field Zn concentration of a maternal plant and the accumulating phenotype of her progeny under standard conditions.

Natural populations of *A. bertolonii* were characterised by widely differing Ni concentrations in their shoots (from 3758 to 21154 mg kg^{-1}), and these were positively correlated with the soil Ni concentration (Galardi *et al.* 2007). Large genetic variations for biomass and Ni shoot concentration were found in *Alyssum murale* and *Alyssum corsicum* grown on an Oregon serpentine soil (Li *et al.* 2003a). In this trial 125 *A. murale* and 45 *A. corsicum* accessions were evaluated. The mean shoot Ni concentrations among *A. murale* and *A. corsicum* ranged from 4200 to 20400 mg kg^{-1}. The genetic diversity within and among nine populations of *A. bertolonii* was assessed using chloroplast microsatellites (cpSSR) in relation to their biogeography and a high level of genetic diversity was found within each of the populations sampled (Mengoni *et al.* 2003). The results demonstrated that each population form a distinct genetic entity and populations within the same serpentine region are more related than populations from different regions.

Knowledge regarding the molecular mechanisms and genetics of metal hyperaccumulation by plants has advanced considerably during the last decade (Kramer 2010; Pollard *et al.* 2014; Verbruggen *et al.* 2009) but the phenomenon is not entirely understood. Hyperaccumulation appears to be a complex multigenic trait which involves many component processes, including an enhanced metal uptake at the root (as result of root foraging, rhizosphere interactions, membrane transport), increased xylem loading and increased detoxification and sequestration in the shoot (mostly inside the vacuoles of leaf cells). Tolerance and hyperaccumulation ability have also been shown to be at least partly under

independent genetic control, and both can vary significantly among and within populations. In the case of the Ni-hyperaccumulators, several authors have suggested that this natural variation in biomass yield and Ni accumulation within and between plant populations can be the basis for the breeding of improved cultivars of Ni hyperaccumulators for application in phytomining (Chaney *et al.* 2010; Pollard *et al.* 2002). Finally, biotechnology offers the possibility to manipulate a plant's capacity to tolerate, accumulate, and/or metabolise pollutants (Maestri and Marmiroli 2011; Pilon-Smits and Pilon 2002). Many genes are reported to be involved in metal uptake, translocation, sequestration, chemical modification, and tolerance (Korenkov *et al.* 2007; Na and Salt 2011). The introduction and overexpression of the hyperaccumulating genes into a non-hyperaccumulator plant could be a possible way to enhance metal uptake, accumulation, tolerance and detoxification process (Clemens *et al.* 2002). A direct method for enhancing the effectiveness of phytoextraction is to overexpress in transgenic plants the genes involved in metabolism, uptake, or transport of specific pollutants. The introduction of these genes has been successfully achieved using *Agrobacterium tumefaciens*-mediated plant transformation. The overexpression of glutamylcysteine synthetase has been accomplished by genetic engineering in *Populus angustifolia*, *Nicotiana tabacum* and *Silene cucubalus* which enhances heavy metal accumulation as compared to the wild type plants (Fulekar *et al.* 2009). It should be noted that the use of transgenics in nature for phytoextraction should be preceded by a thorough risk assessment study and weighing the benefits and risks as compared to alternative technologies (Bhargava *et al.* 2012).

Soil management and agronomic practices to improve nickel phytomining

Effectiveness of phytomining techniques not only depends on the plant's ability to take up and translocate the metal to the shoots but also on the implemented agronomic management practices (such as fertilisation, liming or herbicide regimes) which are required as a means of maximising the performance and yields of hyperaccumulator crops (Li *et al.* 2000). Many studies have shown that modifying soil fertility or soil pH can affect metal bioavailability in the soil and influences the phytoextraction of trace metals such as Ni, Zn, Co and Cd (Kukier *et al.* 2004; Li *et al.* 2003b).

Generally, metal solubility increases with a reduction in soil pH thus making metals more available to plants for uptake (Adriano 2001; Hornburg and Brümmer 1993). Numerous studies have demonstrated that an increase in soil pH causes a decrease in the Ni concentrations of many non-hyperaccumulating plant species growing in Ni-rich soils (Kukier *et al.* 2004; L'Huillier and Edighoffer 1996). However, studies evaluating the influence of pH on Ni uptake and

accumulation in Ni-hyperaccumulating plants show contrasting results. Robinson *et al.* (1999) observed that a decrease in soil pH after the addition of elemental S caused an increase in Ni and Co uptake by *Berkheya coddii*; while, when $MgCO_3$ was added to the soil the pH increased and caused a reduction in the plant uptake of both metals. Conversely, several studies reported that *Alyssum* plants accumulated higher concentrations of Ni in their shoots with increasing soil pH. In a field study, Li *et al.* (2003a) found that limestone treatment significantly increased the Ni concentration in *Alyssum* shoots in most soils. However, for especially Fe-oxide-rich serpentine soils (~20 % Fe), liming above about pH 6.3 was found to reduce Ni accumulation (Kukier *et al.* 2004).

Serpentine soils are typically characterised by edaphic properties which can severely limit plant growth (nutrient deficiency, poor soil structure, low organic matter, etc.). Fertilisation management is therefore an essential factor for commercial phytomining (Li *et al.* 2003a), and regimes can be designed with the aim of improving plant growth and establishment or increasing plant uptake of Ni. Several studies have shown the effect in biomass production and metal accumulation in Ni-hyperaccumulator plants. Numerous authors reported that the application of N, P and K **(**pure chemicals generally added in the forms NH_4NO_3, $Ca(H_2PO_4)_2$/NaH_2PO_4 and KCl/KSO_4) in field trials with Ni hyperaccumulating plant species increased shoot biomass yield without reducing shoot Ni/metal concentration (Chaney *et al.* 1998). After the addition of N, P, K as NH_4NO_3, NaH_2PO_4 and KCl each at 10 g m^{-2}, *Alyssum bertolonii* growing in ultramafic soils showed a three-fold higher biomass than control plants (Robinson *et al.* 1997b). Similarly, after the application of N, P, K fertilisers, Bani *et al.* (2013) observed an increase in the density of *A. murale*. In this field study the fertilisers were applied as NH_4NO_3, $Ca(H_2PO_4)_2$ and K_2SO_4 at 100 kg ha^{-1} of P and K, 65 kg ha^{-1} of Ca and a split 100 kg ha^{-1} N application (a second N fertilisation, 50 kg ha^{-1} N, was performed two weeks later). The application of fertilisers significantly increased shoot yield in *A. murale* without reducing Ni concentration. Serpentine soils characteristically present a low Ca concentration and Ni hyperaccumulators take up large amounts of Ca from them, hence the repeated harvest and removal of the biomass during phytomining processes requires the inclusion of Ca fertilisation to maintain Ca fertility at appropriate levels (Chaney *et al.* 2007a; Kukier and Chaney 2004).

Most plant species selected as appropriate candidates for phytoextraction have been studied as monocultures. However, cropping patterns can be designed so as to improve plant biomass and nutrition, and/or enhance trace element availability and uptake and accumulation, as well as improving soil quality and functions. Moreover, they can be of high importance in terms of enhancing

biodiversity, or even in pest control. Monocultures can lead to a decline in biomass yield due to the depletion of nutrients, occurrence of diseases, pests, and weeds, and have a negative effect on soil fertility (Facknath and Lalljee 2000; Lasat 2000; Mench *et al.* 2010). Any reduction in yield can induce a consequent drop in the plants' phytoextraction capacity. Bañuelos (2000) recommended rotating the trace metal-accumulating plant species with agronomic crops, thereby improving the economic balance of the process and maintaining adequate growth and yield of the phytoextracting species.

Intercropping systems were proposed by Tang *et al.* (2012) for incorporation into the phytoextraction (phytomining) system, since they can promote positive below-ground, plant-soil and plant-plant interactions resulting in improved nutrient availability or increased crop yield. Nutrient deficiency is a common characteristic of serpentine soils, but intercropping with leguminous plants can supply nutrients (through N_2 fixation, transfer of fixed N and mobilisation of P due to rhizosphere acidification) to phytoextracting crops. Intercropped species can access different nutrient pools and they may also alter soil trace metal bioavailability thereby altering their accumulation by phytoextracting crops. Beneficial plants, such as leguminous species, can lead to a reduced reliance on inorganic fertilizers. Legumes include economically important grain crops, oilseed crops, forage crops, and agroforestry species. They are not only a rich source of quality protein for humans and animals but are known soil improvers, due to their ability to establish symbiotic interactions with N_2-fixing bacteria. In summary, intercropping systems can be designed with the aim of (1) "phytoprotecting" the non-accumulating plant crop, (2) enhancing metal accumulation by the phytoextracting crop(s), or (3) improving plant biomass production (and nutrient status) and hence plant performance in phytoextraction (Kidd *et al.* 2015). Until now, few studies have reported the effect of the combination of metal hyperaccumulating plants with other species or in combination with other hyperaccumulating plants (Epelde *et al.* 2012; Whiting *et al.* 2001). A recent study with three Ni hyperaccumulating plants, *Leptoplax emarginata*, *Noccaea tymphaea* and *Alyssum murale*, carried out by Lucisine *et al.* (2014) demonstrated the potential of co-cropping as a strategy for improving phytoremediation and/or phytomining technologies. In this study, each plant species produced more biomass (up to 80 % in *N. tymphaea*) cultivated in combination (multispecies cover) than when cultivated separately (monospecies cover) without significant differences in Ni accumulation between covers (Lucisine *et al.* 2014).

A more recent strategy which was proposed to increase the efficiency of the phytoextraction process is the use of phytohormones or Plant Growth Regulators

(PGRs) (Cassina *et al.* 2011; López *et al.* 2005). Plant growth regulators are a group of naturally occurring organic compounds that regulate physiological processes of a plant at low concentrations (Davies 2010). They can be easily applied and are both cost effective and environmentally friendly (Cassina *et al.* 2011). Plant growth regulators have been commercially developed and are presently used in agriculture for a wide range of purposes, such as increasing plant growth, induction of rooting, lateral branching, promotion of abscission, fruit and nut ripening, and vegetative growth control of cereals and grasses (Carey 2008; Gianfagna 1995). In cotton plantations, defoliants such as DEF (S,S,S-tributlyphosphorotrithioate) are used to remove green leaves that can then fall free of the lint from the open cotton boll. Triiodobenzoic acid (TIBA) was found to increase yield in soybean. This compound reduced plant height and petiole length, and stimulated branching and fruit set. Dinoseb (6-sec-butil-2,4-dinitrofenol) has been found to increase grain yield in corn by 10-15 %. Gibberellic acid is used to reduce the incidence of physiological rind disorders in citrus, and daminozide (N,N,dimethylaminosuccinamic acid) application to apple stimulates color development of the fruit, thus increase the value of the crop (Gianfagna 1995). Other applications of PGRs are confined to high-value horticultural crops. PGRs are used by ornamental plant growers to assist with plant propagation by improving seed germination, improving the rooting of cut stems, and triggering the growth of plant tissues cultures. They are also used to reduce or increase the growth rate of plants, induce buds to break dormancy, break apical dominance, and to delay senescence of buds, flowers, or leaves (Carey 2008).

There are several types of PGRs and these can be classified in seven different groups: auxins, cytokinins, gibberellins, abscisic acid, brassinosteroids, salicylic acid and jasmonates (Kefeli and Kalevitch 2003). Each compound is involved in different processes and affects the plant in a specific way; for example, auxins (such as indole-3-acetic acid, IAA) are known to stimulate cell elongation, growth of roots and shoots and supply from the apical bud represses the growth of lateral buds (this is known as apical dominance), cytokinins (CKs) are known for their ability to induce plant cell division, and gibberellins (GAs) regulate stem growth and elongation, induction of seed germination, and fruit setting and growth (Jones 1973; Taiz and Zeiger 2006). The effect of PGRs on plants depends on a series of factors such as the concentration applied, the physiological plant status and environmental factors conditioning the PGR's absorption (Carey 2008).

In terms of their application in phytoextraction techniques, some studies have used PGRs to increase plant resistance to metal toxicity or have applied PGRs in combination with chelators as part of the induced phytoextraction of Pb/Zn/Cd (Fässler *et al.* 2010; Hadi *et al.* 2010; Israr and Sahi 2008; Liphadzi

Table 1.3. Examples of application of the main types of Plant Growth Regulators (PGRs).

PGR/Experimental conditions	*Plant species*	*Main effects*	*Reference*
AUXINS			
IAA 50-150 ppm (285-855 µM) Foliar spray (pot experiment)	*Cassia absus*	IAA 50-100 ppm increased plant growth and yield	Hussain *et al.* (2011)
IAA 25-100 ppm (140-570 µM) Foliar spray (pot experiment)	*Vigna sinensis*	IAA 25 and 50 ppm increased shoot dry weight. Maximum enhancement 25 ppm	El-Saeid *et al.* (2010)
IAA 1.8 10^{-7}-1.8 10^{-4} ppm (10^{-6}-10^{-3} µM) Hydroponic culture (+Zn, Pb, EDDS)	*Helianthus annuus*	IAA can mitigate the negative effect of Zn/Pb in plant growth and combined with EDDS can increase phytoextraction	Fässler *et al.* (2010)
NAA 1.9 10^{-4}-1.9 10^{-2} ppm IAA 1.8 10^{-4}-1.8 10^{-2} ppm IBA 2 10^{-4} -2 10^{-2} ppm 2, 4-D 2.2 10^{-4} -2.2 10^{-2} ppm (10^{-3}-10^{-1} µM) MS medium culture (+Cd)	*Oryza sativa*	Promoted root elongation (mainly 10^{-2} µM) but inhibition when auxin>0.1 µM. NAA improved shoot growth under Cd stress conditions	Mingming (2010)
IAA 17.5 ppm (100 µM) NAA 18.6 ppm (100 µM) Hydroponic culture (+Pb, EDTA)	*Sesbania drummondii*	IAA and NAA increased Pb accumulation in shoots. No effect in biomass or decreased in presence of EDTA	Israr and Sahi (2008)
IAA 3-6 ppm (17.0-28.5 µM)+EDTA Foliar spray and soil	*Helianthus annuus*	IAA increased Pb and Cd leaves concentration and root growth	Liphadzi *et al.* (2006)
IBA 1000 ppm (5000 µM) NAA 1000 ppm (5370 µM) Foliar spray (pot experiment)	*Zea mays*	IBA increased Pb uptake NAA increased Pb and Zn uptake High mortality rate (>45 %), decreased biomass	Fuentes *et al.* (2000)
IAA 0.175-175 ppm (1-10^{3} µM)	*Pisum sativum*	IAA promoted the stem growth, more effective 175 ppm	Yang *et al.* (1993)
CYTOKININS (CKs)			
KN 15 ppm (70 µM) in shoots KN 3 ppm (14 µM) in soil Foliar spray 3 applications/6 days intervals (pot experiment)	*Alyssum murale*	KN increased biomass and transpiration rate. No effect in Ni concentration	Cassina *et al.* (2011)
KN 20-110 ppm (100-500 µM) Added to soil (pot experiment)	*Parkinsonia aculeata*	KN increased Cr concentration in root, stem and leaf. KN increased root-to-shoot translocation. No effect in root elongation	Zhao *et al.* (2010)
BAP 50-200 ppm (220-880 µM) Foliar spray (plug tray experiment)	*Lactuca sativa* *Apium graveolens*	BAP increased yield production (if applied in pre-transplanting stages)	Araki *et al.* (2007)
KN 1-100 ppm (4.6-460 µM) Foliar spray (experimental beds)	*Cajanus cajan*	KN slightly increased branching and crop yield	Mukherjee and Kumar (2007)

KN 5-20 ppm (20-90 µM) Foliar spray (outdoor pot experiment)	*Carthamus tinctorius*	KN decreased Pb toxicity and increased biomass and chlorophyll content	Sayed (1999)
BA 50-200 ppm (220-880 µM) Foliar spray	*Asparagus officinalis*	BA 100 to 200 ppm stimulated spear sprouting only after the application, not in the following seasons	Uesugi *et al*. (1994)
GIBBERELLINS (GAs)			
GA_3 3.5 ppm (10 µM) Foliar spray (pot experiment)	*Brassica juncea*	GA_3 ameliorated the adverse effects of salt stress and rescued the plant productivity	Shah (2007)
GA_3 0.35 ppm (1 µM) Foliar spray (pot experiment)	*Brassica juncea*	GA_3 increased tolerance to salt treatment, dry weight and seed yields	Afroz *et al*. (2005)
GA_3 0-7.5 ppm (0-21.7 µM) Foliar spray (field experiment)	*Glycine max*	GA_3 7.5 ppm increased leng, leaf area and root and shoot dry weight	Ngatia *et al.* (2004)
GA_3 0-10^{-2} ppm (0-0.03 µM) Foliar spray (field experiment) (+Cd)	*Glycine max*	GA_3 10^{-2} ppm increased root and shoot growth, leaf area, length of stem and reduced Cd toxicity	Ghorbanli *et al*. (2000)
BRASSINOLIDES			
EBR 4.8 10^{-6} -4.8 10^{-2} ppm (10^{-5} -10^{-1} µM) Pre-germination treatment (outdoor pot experiment) (+Cu)	*Brassica juncea*	EBR improved shoot emergence and plant biomass production. Blocked Cu uptake and accumulation	Sharma and Bhardwaj (2007)
EBR 0.2, 0.9 ppm HBR 0.5, 2.5 ppm (1, 5 µM) Foliar spray (pot experiment) (+water stress)	*Phaseolus vulgarus*	Increased root nodulation, ameliorated stress induced, increased pod yield (5 µM). EBR relatively more effective	Upreti and Murti (2004)
COMBINATIONS			
GA_3 50-100 ppm (145-290 µM) IAA 25-75 ppm (145-430 µM) Foliar spray 2 applications 30 and 40 DAS (pot experiment)	*Phaseolus vulgarus*	GA_3 100 ppm improved vegetative growth (plant height, number of leaves and branches, pod yield)	Fawzy *et al*. (2011)
GA 0.4 ppm (1 µM) IAA 0.2 ppm (1 µM) Foliar spray and seed soaking (pot experiment) (+Pb, EDTA)	*Zea mays*	IAA and GA mitigated the negative effect of EDTA in plant growth, increased dry biomass and Pb accumulation. Maximum Pb accumulation in GA+EDTA treatment	Hadi *et al*. (2010)
IAA 1.8 10^{-4} -1.8 10^{-1} ppm GA 3.5 10^{-4} -3.5 10^{-1} ppm ZT 2.2 10^{-4} - 2.2 10^{-1} ppm ET 1.4 10^{-4} -1.4 10^{-1} ppm (10^{-3} -1 µM) Petri plates (plant growth chamber) (+inoculants)	*Triticum aestivum*	GA, IAA, ZT, ET alleviated salinity-induced dormancy in seeds and promoted germination	Egamberdieva (2009)

(Continued)

PGR/Experimental conditions	*Plant species*	*Main effects*	*Reference*
COMBINATIONS			
GA_3 173-346 ppm (500-1000 µM) KN 108-215 ppm (500-1000 µM) SA 69-138 ppm (500-1000 µM) ET 73-145 ppm (500-1000 µM) Foliar spray (pot experiment)	*Taraxacum officinale*	GA_3, KN and SA increased plant growth, ET reduced it	Kim *et al*. (2009)
B. juncea: CA 96-960 ppm MA 67-670 ppm (500-5000 µM) *A. corsicum*: CA 19-96 ppm MA 13-67 ppm (100-500 µM) Hydroponic culture (+Ni 3/300 µM)	*Brassica juncea,* *Alyssum corsicum*	*B. juncea*: CA and MA decreased Ni uptake. CA estimulated translocation. *A. corsicum*: CA reduced Ni concentration in roots. MA 1000 µM enhanced Ni translocation and shoot Ni concentration	Qiu *et al*. (2009)
JA 2.6 ppm (12.5 µM) ABA 2.6 ppm (10 µM) GA 17.3 ppm (50 µM) SA 7 ppm (50 µM) Hydroponic culture (+Cd 50-100 µM)	*Brassica napus*	JA, ABA, GA, SA alleviated Cd toxicity symptoms and increased fresh weight. ABA (+Cd 100 µM) decreased Cd accumulation	Meng *et al.* (2008)
IAA 0.2-18 ppm (1-100 µM) GA 0.4-35 ppm (1-100 µM) KN 0.2-22 ppm (1-100 µM) IAA+KN 18 ppm (100 µM)+22 ppm (100 µM) Hydroponic culture (+Pb, EDTA)	*Medicago sativa*	KN increased Pb concentration in leaves. IAA-KN enhanced the Pb concentration in dry weight compared to plants treated with Pb and/or EDTA without phytohormones	López *et al*. (2007)
GA_3 35 ppm (100 µM) IAA 18 ppm (100 µM) Hydroponic culture (+Cu 80 µM)	*Helianthus annuus.*	IAA decreased toxic effects in root. GA_3 ameliorated the toxic effect mainly to the shoot. IAA and GA_3 improved pigment preservation and water use efficiency	Ouzounidou and Ilias (2005)
BA+GA_{4-7} (Promalin) 25-75 ppm (BA 110-330 µM; GA 70-220 µM) Foliar spray (pot experiment)	*Brassica oleracea*	Promalin increased yield by increasing leaf size, leaf number and plant dry weight. More effective 50 ppm	Emongor *et al*. (2004)
BA 30 ppm (130 µM) GA_3 100 ppm (290 µM) Foliar spray 2 applications/15 days interval (pot experiment)	*Soybean cultivar IAC 17*	GA_3 increased plant height, first node height, stem diameter, leaf area and dry weight. BA was not effective. Joint application inhibited GA_3 effect	Leite *et al*. (2003)

ABA: Abscisic acid, BA: benzyladenine (sintetic = BAP: benzylaminopurine), CA: Citric acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid, DAE: days after emergence, DAS: days after seeding/sowing, EBR: 24-epibrassinolide, ET: Ethephon, GA_3: Gibberellic acid, HBR: homobrassinolide, IAA: Indole-3-acetic acid, IBA: Indolebutyric acid, JA: Jasmonic acid, KN: Kinetin, MA: Malic acid, NAA: Naphthylacetic acid, SA: Salicylic acid.

et al. 2006). Numerous studies have reported the positive effect on plant growth and development after the application of PGRs such as IAA, GB or CK (El-Saeid *et al.* 2010; Emongor *et al.* 2004; Hussain *et al.* 2011). Table 1.3 summarises studies based on the application of different types of PGRs and their effects on plant growth and/or metal tolerance.

Recent studies have demonstrated that the use of PGRs may be considered as a strategy to increased Ni phytoextraction in *Alyssum* species and hence, supporting the Ni phytomining process. Cassina *et al.* (2011) have reported that the foliar and soil application of cytokinins to *A. murale* grown in serpentine soil (pot experiment) increased biomass production up to 75 % in comparison with control plants, without affecting the Ni shoot concentration. More studies are required to optimise the positive effect of PGRs in hyperaccumulator plants so that these can be efficiently incorporated into Ni phytoextraction processes.

Bioaugmentation with plant-associated microorganisms for increasing nickel phytoextraction

Bioaugmentation has been successfully developed for application in soils contaminated with organic pollutants (Weyens *et al.* 2009a; Weyens *et al.* 2009b). However, over the last decade a growing number of studies have indicated the potential use of these techniques to improve the plant's capacity to phytoextract metals from soils. Numerous review articles have been published on the use of these plant-associated microorganisms as a means of accelerating phytoremediation processes (Cherian *et al.* 2012; Glick 2010; Kidd *et al.* 2009; Lebeau *et al.* 2008; Ma *et al.* 2011; Rajkumar *et al.* 2012; Sessitsch *et al.* 2013; Weyens *et al.* 2013). These have focused on different phytoremediation techniques, targeting both trace metal- and organic pollutant-contaminated soils, as well as different microbial groups (e.g. rhizobacteria, endophytes, mycorrihizae) and contrasting host plant species. This introduction will only discuss the beneficial interactions between plants and bacteria and their potential application in the phytoextraction of trace metals, although it should not be forgotten that similar beneficial interactions exist between plants and their associated fungi (see reviews by Göhre and Paszkowski 2006; Lebeau *et al.* 2008; Meharg and Cairney 2000).

In trace metal-rich soils, plant-associated bacteria can enhance plant growth, reduce stress and/or modify soil metal bioavailability (Becerra-Castro *et al.* 2013; Glick 2010; Glick 2014; Kidd *et al.* 2009; Lebeau *et al.* 2008; Sessitsch *et al.* 2013). Plant-associated bacteria include several groups: endophytic bacteria, which colonise the internal tissues of plants without causing negative effects on

their host; phyllospheric bacteria, which inhabit the external surfaces of plant parts (leaves, stems, blossoms and fruits); and rhizospheric bacteria, which are present in the rhizospheric soil in direct contact with the plant roots (Sessitsch and Puschenreiter 2008; Weyens *et al.* 2009b). Many of these bacteria have a beneficial effect on plant growth, a trait which coined the name of plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978).

Trace metal-contaminated soils or natural metal-enriched soils can be a potential source of metal-tolerant bacteria, including plant growth promoting strains. For example, Schlegel *et al.* (1991) found bacterial strains isolated from serpentine soils tolerated up to 10-20 mM Ni (in the culture medium), while strains from other soil types tolerated only 1 mM Ni. Turgay *et al.* (2012) found bacterial strains, isolated from Turkish serpentine soils, could tolerate up to 34 mM Ni in the growth medium. Furthermore, the rhizosphere bacterial communities associated with Ni-hyperaccumulating plants have been shown to differ from those of non-accumulating plants growing at the same site or of non-vegetated soil, and are also characterised by a higher number of Ni-tolerant bacteria (Abou-Shanab *et al.* 2003b; Becerra-Castro *et al.* 2009; Idris *et al.* 2004; Mengoni *et al.* 2001; Schlegel *et al.* 1991). Schlegel *et al.* (1991) reported a higher occurrence of Ni-resistant bacteria in soil samples collected within increasing proximity to the Ni-hyperaccumulating tree *Sebertia acuminata*. Mengoni *et al.* (2001) also found a higher proportion of Ni-resistant colony forming units (CFUs) in proximity to the Ni-hyperaccumulator *Alyssum bertolonii* than in non-vegetated soil. These authors observed simultaneous resistance to a set of metals and highest resistance from isolates of the rhizosphere. Becerra-Castro *et al.* (2009) found higher proportions of Ni-tolerant bacteria in the rhizosphere of *A. serpyllifolium* ssp. *lusitanicum*. These authors observed significant variations among different populations of the Ni-hyperaccumulator in this selective enrichment of Ni-resistant bacteria. In addition, this selective enrichment in Ni-tolerant bacteria in the rhizosphere was correlated with an increase in soil Ni availability (Becerra-Castro *et al.* 2009). Many of these studies have isolated and characterised such rhizosphere bacterial strains as a means of identifying interesting strains for phytoextraction purposes.

For phytoextraction, the aim of these bioaugmentation trials is to enhance the efficiency of the process by increasing the total amount of metal removed from the soil. The use of plant-associated bacteria is generally based on the capacity of the bacteria to, on one hand, improve establishment, growth and plant survival (plant growth promotion (PGP)) and, on the other hand, to modify the mobility and bioavailability of the trace metals in the soil. Plant growth promotion plays a

major role in the extraction and removal of trace elements since a simple improvement in biomass results in an increase in the overall trace element yield (phytoextracted trace elements).

Bacteria can enhance plant growth and resistance to biotic and abiotic stresses by various mechanisms (Gadd 2010; Glick *et al.* 2007; Lebeau *et al.* 2008). Many PGP bacteria directly influence plant growth and physiology through the production of plant-growth promoting compounds or phytohormones, such as indoleacetic acid (IAA). Bacterial IAA stimulates root hair formation while increasing the number and length of lateral and primary roots when it is within an ideal concentration range (Duca *et al.* 2014). Some bacteria can also reduce plant stress levels by producing the enzyme 1-aminocyclopropane-1- carboxylate (ACC) deaminase which can suppress the production of stress ethylene in plants. ACC is the immediate precursor of ethylene which is a phytohormone that plays an important role in root initiation and elongation, nodulation, senescence, abscission and ripening as well as in stress signalling. During situations of stress, plants produce high levels of "stress ethylene", which can inhibit root elongation. Hydrolysis of plant-exuded ACC via the bacterial enzyme ACC deaminase leads the plant to exude more ACC in an attempt to maintain equilibrium between the internal and external ACC levels, and reduce the synthesis of ethylene inside the plant cell. ACC deaminase-producing bacteria can benefit by using ACC as a N source and the plants show a better root elongation as its internal level of ethylene decreases (Dell'Amico *et al.* 2005; Glick 2003; Glick *et al.* 1998). PGPB can increase the availability of essential plant nutrients, such as nitrogen, phosphorus or iron, thus improving plant nutrition in nutrient deficient soils. For example, diazotrophs are capable of fixing atmospheric nitrogen into ammonia using the enzyme nitrogenase, and this biological fixation of N_2 is of paramount importance for plant nutrition. Many bacteria are also capable of converting insoluble phosphates into forms which are accessible to the plant, through the production of organic acids and/or phosphatases (Richardson and Simpson 2011). Inoculation of plants with P-solubilising microorganisms in controlled experiments resulted in improved growth and P nutrition (Leyval and Berthelin 1989; Richardson *et al.* 2001; Richardson and Simpson 2011). Iron is mostly present in soils as Fe(III) in insoluble forms. Microorganisms produce and release Fe(III)-specific chelating agents called siderophores, in response to low concentrations of iron in the environment. These compounds are small molecules (generally less than 1000 Daltons) and show an extremely high affinity for iron (Crowley 2006; Schwyn and Neilands 1987). They can directly mobilise iron from the solid phase minerals or also remove iron from organic complexes (Crowley 2006).

Some authors have isolated metal-tolerant bacterial strains associated with (hyper)accumulating plants and shown that they are able to mobilise metals in soils, and consequently increase the phytoavailable metal fraction in the soil and plant uptake and accumulation. Bacteria can modify trace metal mobility and bioavailability through several mechanisms: the release of chelating agents (such as organic acids and siderophores), acidification or redox changes in the rhizosphere (Gadd 2004; Glick 2003; Khan 2005). Sessitsch *et al.* (2013) reviewed the potential mechanisms for microbial effects on trace element bioavailability in the rhizosphere environment. Sorbed, precipitated and occluded trace elements can be solubilised by acidification, chelation and ligand-induced dissolution. To date, two groups of bacterially produced natural chelators are known: organic acids and siderophores. Bacteria producing trace element-chelating organic acids, such as citric, oxalic or acetic acid have been shown to mobilise various trace elements in soil (Becerra-Castro *et al.* 2013; Li *et al.* 2009). As mentioned above, siderophores form high affinity complexes with Fe(III), but they can also form complexes of lower stability with other trace metals thus affecting their bioavailability (Dimkpa *et al.* 2009; Sessitsch *et al.* 2013).

Examples of bacterial-induced plant growth promotion and metal accumulation in a phytoextraction context can be found in a wide array of plant species, including crop plants, hyperaccumulators and woody tree species. A summary of bioaugmentation studies and the observed effects on plant metal tolerance and plant growth is given in Table 1.4. These studies demonstrate that bacterial inoculants tend to be more successful in promoting plant growth and biomass production (hence increasing the metal yield and metal removal from the soil), rather than increasing the metal concentration in shoots. This was also indicated in a meta-analysis of phytoremediation-orientated inoculation studies carried out by Sessitsch *et al.* (2013). This analysis was based on the results of more than 70 publications and analysed 738 individual cases or treatments, to identify the most frequent effects of plant inoculation on shoot biomass production, trace metal concentration and yield in shoots. In 30 % of the cases studied an increase in shoot biomass was observed (while the shoot metal concentration was unchanged), in contrast only 11 % of treatments were found to increase shoot metal concentration, and 19 % of treatments increased both shoot metal concentration and shoot biomass production.

Several authors have reported an increase in biomass production and Ni accumulation by plants after inoculation with growth-promoting bacterial strains. Ma *et al.* (2009a,b) found that the inoculation of PGPR enhanced Ni solubilisation in soil, increasing the Ni accumulation in *Brassica juncea* and *B. oxyrrhina* and improving the growth of both species (Table 1.4). Similarly, Rajkumar and Freitas

Table 1.4. Effects of plant-associated bacteria isolated for phytoextraction purposes.

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
Alyssum murale	*Microbacterium oxydans AY509223*	Acid; Siderophore	Ni	↑ leaf [Ni]	Abou-Shanab *et al.* (2006)
A. murale	*Sphingomonas macrogoltabidus, Microbacterium liquefaciens, Microbacterium arabinogalactanolyticum*	Acid (*M. l, M. a*); PO_4 (*M. l, M. a*); Siderophore (*M. l*)	Ni	↑ soil $[Ni]_{Sr(NO3)2}$ (*M. a*); ↑ shoot [Ni]	Abou-Shanab *et al*. (2003a)
A. serpyllifolium ssp. *malacitanum*	*Arthrobacter* LA44, *Arthrobacter* SBA82	IAA; Siderophore; PO_4	Ni	↑ biomass; ↑ shoot [Ni]; ↑ Ni yield (LA44)	Becerra-Castro *et al.* (2013)
Brassica juncea	*Enterobacter aerogenes* NBRI K24, *Rahnella aquatilis* NBRI K3	IAA; Siderophore; ACCD; PO_4	Cr, Ni	↑ plant growth/biomass; ↑ [Cr, Ni] uptake	Kumar *et al.* (2009)
B. juncea	*Achromobacter xylosoxidans* Ax10	IAA; ACCD; PO_4	Cu	↑ plant growth/biomass; ↑ [Cu]root/shoot	Ma *et al.* (2009b)
B. juncea	*Bacillus biosubtyl, Bacillus licheniformis, Bacillus thuringiensis*	nd	Cd, Cr, Se	↑ Cd accumulation (*B.l*)	Hussein (2008)
B. juncea	*Enterobacter* sp. NBRI K28	IAA; Siderophore; ACCD; PO_4	Cr, Ni	↑ plant biomass; ↑ [Cr, Ni, Zn] uptake	Kumar *et al*. (2008)
B. juncea	*Pseudomonas* sp. Ps29C, *Bacillus megaterium* Bm4C	IAA; Siderophore; ACCD; PO_4	Ni	↑ plant biomass plant protection from Ni toxicity	Rajkumar and Freitas (2008b)
B. juncea	*Pseudomonas* sp. PsA4, *Bacillus* sp. Ba32	IAA; ACCD; PO_4	Cr	↑ plant growth protection of Cr toxicity	Rajkumar *et al*. (2006)
B. juncea	*Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *Bacillus mucilaginosus* HKK-1	N-fixing (*A. c*); PO_4 (*B. me*); K solubilisation (*B. mu*)	Cu, Pb, Zn	↑ removal of Cu, Pb and Zn	Wu *et al*. (2006)
B. juncea	*Bacillus subtilis* strain SJ-101	IAA; PO_4	Ni	↑ plant growth/biomass; ↑ [Ni] shoot	Zaidi *et al*. (2006)
B. juncea	11 bacteria strains, *included: Variovorax paradoxus, Rhodococcus* sp., *Flavobacterium* sp.	Cd-tolerance; IAA; ACCD; Siderophore	Cd	↑ Co, Cu, Ni, Zn tolerance ↑ root elongation	Belimov *et al*. (2005)
B. juncea	Se-tolerant rhizobacteria (strains BJ1, BJ2, BJ2, BJ4)	Produce heat-labile bioactive compound	Se	↑ [Se] shoot	De Souza *et al*. (1999)
B. juncea, Brassica oxyrrhina	*Bacillus* sp. (strains SN3,SN9,SRS5, SRS15, SRI4, SRI11, SRI14), *Pseudomonas* sp. SRI2, *Psychrobacter* sp. SRS8	Ni-solubilisation; IAA; Siderophore (except SRS5); ACCD (SN9, SRI2, SRI4, SRI11,SRI14); PO_4	Ni	↑ plant biomass (mainly SRI2, SRS8, SN9) ↑ [Ni] shoot and root (SN9)	Ma *et al.* (2009a)
B. juncea, B. oxyrrhina	*Psychrobacter* sp. (strains SRA1, SRA2), *Bacillus cereus* sp. (strains SRA10, SRP4), *Bacillus weihenstephanensis* SRP12	IAA; Siderophore (SRA1, SRA10, SRP4, SRP12); ACCD; PO_4	Ni	↑ plant biomass (SRA2) ↑ Ni solubilisation (SRA1, SRA10) ↑ [Ni] shoot and root (SRA1, SRA10)	Ma *et al*. (2009c)

(Continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
B. juncea, Lycopersicon esculentum, Zea mays L. var. Denhai-11	*Burkholderia* sp. J62	Metal-resistance; Antibiotic resistance; IAA; Siderophore; ACCD; PO_4	Cd, Pb	↑ plant biomass (*L. esculentum, Z. mays*); ↑ Cd, Pb uptake (*L. esculentum, Z. mays*);	Jiang *et al*. (2008)
B. juncea, Brassica napus, L. esculentum	*Kluyvera ascorbata* SUD165, *Kluyvera ascorbata* SUD165/26	Siderophore (*K. a. SUD165/26)*	Cu, Ni, Zn	↓ growth inhibition caused by metals	Burd *et al*. (2000)
Brassica napus	*Arthrobacter* sp. MT16, *Microbacterium* sp. JYC17, *Pseudomonas chlororaphis* SZY6, *Azotobacter vinelandii* GZC24, *Microbacterium lactium* YJ7	IAA; Siderophore; ACCD; PO_4	Cu	↑ root length	He *et al.* (2010b)
B. napus	*Firmicutes sp., Actinobacteria sp., Proteobacteria sp.*	IAA; Siderophore; ACCD; arginine decarboxylase production	Cu	↑ plant biomass ↑ [Cu] shoot	Sun *et al*. (2010)
B. napus	*Pseudomonas tolaasii* ACC23, *Pseudomonas fluorescens* ACC9, *Alcaligenes* sp. ZN4, *Mycobacterium* sp. ACC14	Cd-resistance; IAA; ACCD; Siderophore	Cd	↑ root elongation ↑ shoot and root growth	Dell'Amico *et al*. (2008)
B. napus	*Pseudomonas fluorescens* G10, *Microbacterium* sp. G16	IAA; Siderophore; ACCD; PO_4	Pb	↑ plant biomass (root elongation) ↑ Pb uptake (shoot)	Sheng *et al*. (2008b)
B. napus	*Pseudomonas putida* UW4	ACCD	Ni	↑shoot biomass ↑ Ni tolerance	Farwell *et al*. (2007)
B. napus	*Pseudomonas putida* UW4, *Pseudomonas putida* HS2	ACCD High Ni-tolerance (*P. p* HS2)	Ni	↑ plant growth ↑ Ni yield	Farwell *et al*. (2006)
B. napus	Not determined	Cd-resistant	Cd	↑ [Cd] shoot	Sheng and Xia (2006)
B. napus, L. esculentum, Z. mays, Sorghum sudanense	*Bacillus* sp. J119	Metal-resistance; Antibiotic resistance; Biosurfactant; IAA; Siderophore	Cd	↑ shoot/root biomass of *L. esculentum*; ↑ [Cd] shoot (*B. napus*, *L. esculentum*); ↑ [Cd] root (*B. napus*, *L. esculentum*, *Z.mays*)	Sheng *et al*. (2008a)
Heliathnus annuus	*Bacillus weihenstephanensis* SM3	IAA; PO_4; Cu, Ni, Zn mobilisation	Cu, Ni, Zn	↑ plant biomass ↑ Cu, Zn uptake	Rajkumar *et al*. (2008)
H. annuus	*Pseudomonas fluorescens*	IAA, Siderophore	As	↑ shoot biomass; ↑ [As] shoot; ↑ phloem fluxes	Shilev *et al*. (2006)
Lycopersicon esculentum	*Pseudomonas* sp. RJ10, *Bacillus* sp. RJ16	IAA; Siderophore; ACCD; Cd, Pb mobilisation	Cd, Pb	↑ plant biomass and root length ↑ Cd, Pb uptake	He *et al*. (2009)

Medicago sativa	*Pseudomonas fluorescens* (strains Avm, U), *Rhizobium leguminosarum* bv *phaseoli* (strains CPMex44, CPMex46), *Azospirillum lipopherum* (strains UAP40, UAP154)	Siderophore Growth promotion	Cu	↑ Cu, Fe root-shoot translocation (*CPMex46*, *Avm*)	Carrillo-Castañeda *et al.* (2002)
Nicotiana tabacum	*Sanguibacter* sp., *Enterobacter* sp., *Pseudomonas* sp.	nd	Cd	↑ plant biomass (*Sanguibacter* sp S-d2 and consortia) ↑ Cd translocation ↑ Cd, Fe uptake	Mastretta *et al.* (2009)
Noccaea caerulescens	Mixed inoculum (*Microbacterium saperdae*, *Pseudomonas monteilii*, *Enterobacter cancerogenes*)	-	Zn	↑ $[Zn]_{water\text{-}soluble}$; ↑ [Zn] shoot	Whiting *et al.* (2001)
Orychophragmus violaceus	*Bacillus subtilis*, *B. cereus*, *Flavobacterium* sp., *Pseudomonas aeruginosa*	Zn-tolerance	Zn	↑ plant biomass and root length ↑ Zn solubilisation and uptake	He *et al.* (2010a)
Pteris vittata	*Rhodococcus* sp. TS1, *Delftia* sp. TS33, *Comamonas* sp. TS37, *Delftia* sp. TS41, *Streptomyces lividans* sp. PSQ22	As-reducing	As	↑ plant biomass ↑ As uptake ↑ As solubilisation ↓ As leaching	Yang *et al.* (2012)
Populus deltoides	*Agrobacterium radiobacter*	As-tolerance IAA; Siderophore	As	↑ plant biomass ↑ chlorophyll, enzymatic activity ↑ [As] root, stem, leaf ↑ As uptake and translocation	Wang *et al.* (2011)
Ricinus communis	*Pseudomonas* sp. PsM6, *P. jessenii* PjM15	IAA; Siderophore; ACCD; Cu, Ni, Zn mobilisation	Cu, Ni, Zn	↑ plant biomass ↑ Zn translocation and uptake	Rajkumar and Freitas (2008a)
Salix caprea	*Agromyces* sp. AR33, *Streptomyces* sp. AR17	-	Cd, Zn	↑ soil extractable-Cd/Zn; ↑ plant growth ↑ Cd/Zn uptake	Kuffner *et al.* (2008)
Sedum alfredii	*Burkholderia* sp. D54	IAA; Siderophore; ACCD; PO_4	Cd, Pb, Zn	↑ plant biomass; ↑ [Cd] shoot and root; ↑Cd, Pb, Zn uptake	Guo *et al.* (2011)
S. alfredii	5 bacterial strains (unidentified)	nd	Cd, Cu, Pb, Zn	↑ plant biomass ↑chlorophyll and nutrient content ↓ Cd, Cu, Pb, Zn toxicity ↑ Cd, Cu, Pb, Zn uptake from contaminated water	Xiong *et al.* (2008)
S. alfredii	*Burkholderia cepacia*	-	Cd, Zn	↑ plant biomass; ↑ [Cd/Zn] shoot; ↑ metal tolerance; ↑ $[Cd/Zn]_{leaf}$:$[Cd/Zn]_{root}$ ratio	Li *et al.* (2007)

(Continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
Sedum plumbizincicola	*Phyllobacterium myrsinacearum* RC6b	Metal-resistance; Metal mobilization; IAA; Siderophore; ACCD; PO_4	Cd, Pb, Zn	↑ plant biomass ↑ [Cd, Zn] root and shoot	Ma *et al*. (2013)
Sorghum bicolor, *Z. mays*	*Bacillus subtilis*, *B. pumilus*, *Pseudomonas pseudoalcaligenes*, *Brevibacterium halotolerans*	-	Cu, Cr, Pb, Zn	↑ plant biomass (with inoculum mixture); ↑ shoot biomass (*Br. h*, *P. p*); ↑ solubility Cr, Cu (*B. s*, *B. p*); ↑ [Cu, Cr, Pb, Zn] shoot on Cu-rich soil; ↑ [Cr]shoot on Cr-rich soil	Abou-Shanab *et al*. (2008)
S. bicolor	*Pseudomonas monteillii*	nd	Cd	↑ plant biomass ↑ Cd uptake	Duponnois *et al*. (2006)
Trifolium repens Linn.	Bacterial strain mix *(Bacillus cereus)*	Metal-tolerance; IAA	Cd	↑ plant biomass and phytoextraction	Azcón *et al*. (2009)
Trifolium hybridum, *Alopecurus pratensis*, *Poa pratensis*, *Hordeum violaceum*, *Ranunculus kotschyi*, *Cerastium sp.*	*Bacillus megaterium* var. *phosphaticum*	Nutrient-solubilisation; Pathogens control	B, Ni, Mn, Pb, Zn	↑ Ni, Pb, Fe, Zn, Na, B desorption from the soil ↑ Pb, Ni, B, Mn, Zn uptake	Gullap *et al*. (2014)
Zea mays	*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Ralstonia metalidurans*	Siderophore	Cr, Pb	↑ Cr, Pb exchangeable fraction in the soil (*P. aeruginosa*) ↑ [Cr, Pb] shoot	Braud *et al*. (2009)

Bacteria characteristics: ACCD, ACC deaminase activity; Acid, acid-producers; Biosurfactant, biosurfactant-producers; Ethylene, ethylene-producer; IAA, indoleacetic acid-producers; Siderophore, siderophore-producers; PO_4, phosphate solubilisers; nd: not detected.

(2008b) observed that inoculation with PGPR in *B. juncea* caused an increase in aboveground biomass, mainly due to IAA production and phosphate solubilisation, and consequently enhanced the phytoextraction efficiency (Table 1.4). Results obtained by Zaidi *et al.* (2006) demonstrated that inoculation with PGPR not only stimulated the growth and Ni accumulation in *B. juncea*, but also protected the plant from Ni toxicity. Various authors also obtained increases in Ni uptake by *B. juncea* and other non-hyperaccumulating plant species (*B. napus*, *Ricinus communis, Poa pratensis*, etc.) after bacterial inoculation (Farwell *et al.* 2006; Gullap *et al.* 2014; Kumar *et al.* 2008; Kumar *et al.* 2009; Rajkumar and Freitas 2008a) (Table 1.4). With regards to Ni hyperaccumulating species, Abou-Shanab *et al.* (2003a) reported that bacteria isolated from the rhizosphere of *Alyssum murale* increased the availability of Ni in the soil and enhanced the Ni accumulation by *A. murale*. In agreement with these results, a posterior study with *A. murale* grown in Ni-contaminated soils demonstrated that inoculation with selected rhizobacteria strains increased the Ni extraction from the soil and Ni uptake by *A. murale* (Abou-Shanab *et al*. 2006). These authors considered the presence of such rhizobacteria to be an important factor influencing metal hyperaccumulation. Becerra-Castro *et al.* (2013) used two strains of *Arthrobacter* harbouring several plant growth promoting characteristics and able to mobilise Ni from serpentine rock, as an inoculum for *Alyssum serpyllifolium* ssp. *malacitanum* grown in ultramafic soil and observed an increase in plant biomass and shoot Ni concentrations.

1.6 *REFERENCES*

Abou-Shanab RA, Angle JS, Delorme TA, Chaney RL, van Berkum P, Moawad H, Ghanem K and Ghozlan HA (2003a). Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. *New Phytol* 158: 219-224.

Abou-Shanab RA, Delorme TA, Angle JS, Chaney RL, Ghanem K, Moawad H and Ghozlan HA (2003b). Phenotypic characterization of microbes in the rhizosphere of *Alyssum murale*. *Int J Phytoremediat* 5: 367-379.

Abou-Shanab RA, Angle JS and Chaney RL (2006). Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils. *Soil Biol Biochem* 38: 2882-2889.

Abou-Shanab RA, Ghanem K, Ghanem N and Al-Kolaibe A (2008). The role of bacteria on heavy-metal extraction and uptake by plants growing on multi-metal-contaminated soils. *World J Microb Biot* 24: 253-262.

Adriano DC (2001). Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals. Springer, New York, NY.

Afroz S, Mohammad F, Hayat S and Siddiqui MH (2005). Exogenous application of gibberellic acid counteracts the ill effect of sodium chloride in mustard. *Turk J Biol* 29: 233-236.

Alves S, Nabais C, Simoes Goncalves Mde L and Correia Dos Santos MM (2011). Nickel speciation in the xylem sap of the hyperaccumulator *Alyssum serpyllifolium* ssp. *lusitanicum* growing on serpentine soils of northeast Portugal. *J Plant Physiol* 168: 1715-1722.

Anderson C, Brooks R, Chiarucci A, LaCoste C, Leblanc M, Robinson B, Simcock R and Stewart R (1999). Phytomining for nickel, thallium and gold. *J Geochem Explor* 67: 407-415.

Antonovics J, Bradshaw AD and Turner R (1971). Heavy metal tolerance in plants. *Adv Ecol Res* 7: 1-85.

Araki A, Rattin J, Di Benedetto A and Miravé P (2007). Temperature and cytokinin relationships on lettuce (*Lactuca sativa* L.) and celery (*Apium graveolens* L.) nursery growth and yield. *Int J Agric Res* 2: 725-730.

Asensi A, Rodríguez N, Díez-Garretas B, Amils R, Boyd R, Baker A and Proctor J (2004). Nickel hyperaccumulation of some subspecies of *Alyssum serpyllifolium* (Brassicaceae) from ultramafic soils on the Iberian Peninsula. Ultramafic rocks: Their soils, vegetation and fauna, Proceedings of the fourth International Conference on Serpentine Ecology. Science Reviews, St. Albans, UK. 263-265.

Asher C (1991). Beneficial elements, functional nutrients and possible new essential elements. In: JJ Mortvedt et al. (eds) Micronutrients in agriculture. 2nd edn. Soil Science Society of America, Madison, WI. p. 703-723.

Assunção AG, Bookum WM, Nelissen HJ, Vooijs R, Schat H and Ernst WH (2003). Differential metal -specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytol* 159: 411-419.

Azcón R, Medina A, Roldán A, Biró B and Vivas A (2009). Significance of treated agrowaste residue and autochthonous inoculates (arbuscular mycorrhizal fungi and *Bacillus cereus*) on bacterial community structure and phytoextraction to remediate soils contaminated with heavy metals. *Chemosphere* 75: 327-334.

Baker AJM (1981). Accumulators and excluders-strategies in the response of plants to heavy-metals. *J Plant Nutr* 3: 643-654.

Baker AJM and Brooks RR (1989). Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81-126.

Bani A, Echevarria G, Sulçe S, Morel J and Mullai A (2007). *In-situ* phytoextraction of Ni by a native population of *Alyssum murale* on an ultramafic site (Albania). *Plant Soil* 293: 79-89.

Bani A, Echevarria G, Mullaj A, Reeves R, Louis Morel J and Sulçe S (2009). Nickel hyperaccumulation by Brassicaceae in serpentine soils of Albania and Northwestern Greece. *Northeast Nat* 16: 385-404.

Bani A, Echevarria G, Sulçe S and Morel JL (2013). Improving the agronomy of *Alyssum murale* for extensive phytomining: A five-year field study. *Int J Phytoremediat*.

Bañuelos GS (2000). Factors influencing field phytoremediation of selenium-laden soils. CRC Press, Boca Raton, FL.

Barbaroux R, Mercier G, Blais J, Morel J and Simonnot M (2011). A new method for obtaining nickel metal from the hyperaccumulator plant *Alyssum murale*. *Sep Purif Technol* 83: 57-65.

Barbaroux R, Plasari E, Mercier G, Simonnot M, Morel J and Blais J (2012). A new process for nickel ammonium disulfate production from ash of the hyperaccumulating plant *Alyssum murale*. *Sci Total Environ* 423: 111-119.

Barcan V and Kovnatsky E (1998). Soil surface geochemical anomaly around the copper-nickel metallurgical smelter. *Water Air Soil Poll* 103: 197-218.

Barral MT and Paradelo R (2011). Trace elements in compost regulation: The case of Spain. *Waste Manage* 31: 407-410.

Basic N, Salamin N, Keller C, Galland N and Besnard G (2006). Cadmium hyperaccumulation and genetic differentiation of *Thlaspi caerulescens* populations. *Biochem Syst Ecol* 34: 667-677.

Becerra-Castro C, Monterroso C, Garcia-Leston M, Prieto-Fernández A, Acea MJ and Kidd PS (2009). Rhizosphere microbial densities and trace metal tolerance of the nickel hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum*. *Int J Phytoremediat* 11: 525-541.

Becerra-Castro C, Kidd PS, Kuffner M, Prieto-Fernández A, Hann S, Monterroso C, Sessitsch A, Wenzel W and Puschenreiter M (2013). Bacterially induced weathering of ultramafic rock and its implications for phytoextraction. *Appl Environ Microbiol* 79: 5094-5103.

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S and Glick BR (2005). Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biol Biochem* 37: 241-250.

Bernal M, McGrath S, Miller A and Baker A (1994). Comparison of the chemical changes in the rhizosphere of the nickel hyperaccumulator *Alyssum murale* with the non-accumulator *Raphanus sativus*. *Plant Soil* 164: 251-259.

Bhargava A, Carmona FF, Bhargava M and Srivastava S (2012). Approaches for enhanced phytoextraction of heavy metals. *J Environ Manage* 105: 103-120.

Boominathan R, Saha-Chaudhury N, Sahajwalla V and Doran PM (2004). Production of nickel bio-ore from hyperaccumulator plant biomass: Applications in phytomining. *Biotechnol Bioeng* 86: 243-250.

Bothe H (2011). Plants in heavy metal soils. In: I Sherameti and A Varma (eds) Detoxification of heavy metals, Soil Biology. Springer-Verlag, Berlin. Vol. 30. p. 35-57.

Brady KU, Kruckeberg AR and Bradshaw Jr H (2005). Evolutionary ecology of plant adaptation to serpentine soils. *Annu Rev Ecol Evol Syst* 36: 243-266.

Braud A, Jézéquel K, Bazot S and Lebeau T (2009). Enhanced phytoextraction of an agricultural Cr- and Pb-contaminated soil by bioaugmentation with siderophore-producing bacteria. *Chemosphere* 74: 280-286.

Broadhurst CL, Chaney RL, Angle JS, Maugel TK, Erbe EF and Murphy CA (2004). Simultaneous hyperaccumulation of nickel, manganese, and calcium in *Alyssum* leaf trichomes. *Environ Sci Technol* 38: 5797-5802.

Brooks RR, Lee J, Reeves RD and Jaffre T (1977). Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *J Geochem Explor* 7: 49-57.

Brooks RR (1980). Indicator plants for mineral prospecting-A critique. *J Geochem Explor* 12: 67-78.

Brooks RR, Shaw S and Marfil AA (1981). Some observations on the ecology, metal uptake and nickel tolerance of *Alyssum serpyllifolium* subspecies from the Iberian peninsula. *Plant Ecol* 45: 183-188.

Brooks RR (1987). Serpentine and its vegetation: A multidisciplinary approach. Dioscorides Press, Portland, OR.

Brooks RR, Chambers MF, Nicks LJ and Robinson BH (1998). Phytomining. *Trends Plant Sci* 3: 359-362.

Brooks RR and Robinson BH (1998). The potential use of hyperaccumulators and other plants for phytomining. In: RR Brooks (ed) Plants that hyperaccumulate heavy metals-their role in phytoremediation, microbiology, archeology, mineral exploration, and phytomining. CAB International, Cambridge, England. p. 327-356.

Brown PH, Welch RM and Cary EE (1987). Nickel: A micronutrient essential for higher plants *Plant Physiol* 85: 801-803.

Burd GI, Dixon DG and Glick BR (2000). Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Can J Microbiol* 46: 237-245.

Cabello-Conejo MI, Becerra-Castro C, Prieto-Fernández A, Monterroso C, Saavedra-Ferro A, Mench M and Kidd PS (2014). Rhizobacterial inoculants can improve nickel phytoextraction by the hyperaccumulator *Alyssum pintodasilvae*. *Plant Soil*: 1-16.

Callahan DL, Baker AJ, Kolev SD and Wedd AG (2006). Metal ion ligands in hyperaccumulating plants. *J Biol Inorg Chem* 11: 2-12.

Cameron R (1992). Guide to site and soil description for hazardous waste site characterization: Vol. 1. Metals. US Environmental Protection Agency, Washington, DC.

Carballeira A, Devesa C, Retuerto R, Santillán E and Ucieda F (1983). Bioclimatología de Galicia. Fundación Barrié de la Maza, A Coruña, Spain.

Carey DJ (2008). The effects of benzyladenine on ornamental crops. Thesis for Master of Science, Department of Horticultural Science. North Carolina State University, Raleigh, NC.

Carrillo-Castañeda G, Juárez Muños J, Peralta-Videa J, Gomez E, Tiemannb K, Duarte-Gardea M and Gardea-Torresdey J (2002). Alfalfa growth promotion by bacteria grown under iron limiting conditions. *Adv Environ Res* 6: 391-399.

Cassina L, Tassi E, Morelli E, Giorgetti L, Remorini D, Chaney RL and Barbafieri M (2011). Exogenous cytokinin treatments of an Ni hyper-accumulator, *Alyssum murale*, grown in a serpentine soil: Implications for phytoextraction. *Int J Phytoremediat* 13: 90-101.

Centofanti T, Sayers Z, Cabello-Conejo MI, Kidd P, Nishizawa NK, Kakei Y, Davis AP, Sicher RC and Chaney RL (2013). Xylem exudate composition and root-to-shoot nickel translocation in *Alyssum* species. *Plant Soil* 373: 59-75.

Chaney RL (1983). Plant uptake of inorganic waste constituents. In: JF Parr et al. (eds) Land treatment of hazardous wastes. Noyes Data Corporation, Park Ridge, NJ. p. 50-76.

Chaney RL, Malik M, Li YM, Brown SL, Brewer EP, Angle JS and Baker AJM (1997). Phyto-remediation of soil metals. *Curr Opin Biotechnol* 8: 279-284.

Chaney RL, Angle JS, Baker AJM and Li YM (1998). Method for phytomining of nickel, cobalt and other metals from soil. US Patent N° 5.711.784.

Chaney RL, Angle JS, Baker AJM and Li YM (1999). Method for phytomining of nickel, cobalt and other metals from soil. US Patent N° 5.944.872.

Chaney RL, Angle JS, Broadhurst CL, Peters CA, Tappero RV and Sparks DL (2007a). Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. *J Environ Qual* 36: 1429-1443.

Chaney RL, Angle JS, Li YM and Baker AJM (2007b). Recovering metals from soil. US Patent N° 7.268.273 (Continuation-in-Part of US Patent N° 5711784).

Chaney RL, Broadhurst CL and Centofanti T (2010). Phytoremediation of soil trace elements. Blackwell Publishers, Oxford, UK.

Chaney RL, Reeves RD, Baklanov IA, Centofanti T, Broadhurst L, Baker AJM, Van der Ent A and Roseberg RJ (2014). Phytoremediation and Phytomining: Using plants to remediate contaminated or mineralized environments (Chapter 15). In: N Rajakaruna et al. (eds) Plant ecology and evolution in harsh environments. Nova Science Publishers, New York, NY. p. 365-391.

Chardot V, Massoura ST, Echevarria G, Reeves RD and Morel J-L (2005). Phytoextraction potential of the nickel hyperaccumulators *Leptoplax emarginata* and *Bornmuellera tymphaea*. *Int J Phytoremediat* 7: 323-335.

Cherian S, Weyens N, Lindberg S and Vangronsveld J (2012). Phytoremediation of trace element: Contaminated environments and the potential of endophytic bacteria for improving this process. *Crit Rev Environ Sci Technol* 42: 2215-2260.

Clemens S, Palmgren MG and Krämer U (2002). A long way ahead: understanding and engineering plant metal accumulation. *Trends Plant Sci* 7: 309-315.

Clemens S (2006). Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* 88: 1707-1719.

Commission of the European Community CEC (1986). Council directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Off J Eur Communities L181 (86/278/EEC).

Cox R and Hutchinson T (1981). Environmental factors influencing the rate of spread of the grass *Deschampsia cespitosa* invading areas around the Sudbury nickel-copper smelters. *Water Air Soil Poll* 16: 83-106.

Crowley DE (2006). Microbial siderophores in the plant rhizosphere. In: J Barton and J Abadía (eds) Iron nutrition in plants and rhizospheric microorganisms. Springer, Netherlands. p. 169-198.

Cunningham SD, Berti WR and Huang JW (1995). Phytoremediation of contaminated soils. *Tibtech* 13: 393-397.

Davies PJ (2010). The plant hormones: Their nature, occurrence, and functions. In: PJ Davies (ed) Plant hormones: biosynthesis, signal transduction, action! 3rd edn. Springer, Dordrecht, Netherlands. p. 1-15.

De Souza M, Huang C, Chee N and Terry N (1999). Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants. *Planta* 209: 259-263.

Dell'Amico E, Cavalca L and Andreoni V (2005). Analysis of rhizobacterial communities in perennial Graminaceae from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiol Ecol* 52: 153-162.

Dell'Amico E, Cavalca L and Andreoni V (2008). Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. *Soil Biol Biochem* 40: 74-84.

Dickinson NM, Baker AJM, Doronila A, Laidlaw S and Reeves RD (2009). Phytoremediation of inorganics: Realism and synergies. *Int J Phytoremediat* 11: 97-114.

Diez T and Rosopulo A (1976). Schwermetallgehalte in Böden and Pflanzen nach extrem hohen Klärschlammgaben. *Sonderdruck Landw Forsch* 33: 236-248.

Dimitriou I, Mola-Yudego B, Aronsson P and Eriksson J (2012). Changes in organic carbon and trace elements in the soil of willow short-rotation coppice plantations. *Bioenergy Res* 5: 563-572.

Dimkpa CO, Merten D, Svatoš A, Büchel G and Kothe E (2009). Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. *Soil Biol Biochem* 41: 154-162.

Duca D, Lorv J, Patten CL, Rose D and Glick BR (2014). Indole-3-acetic acid in plant-microbe interactions. *A Van Leeuw J Microb*: 1-41.

Duponnois R, Kisa M, Assigbetse K, Prin Y, Thioulouse J, Issartel M, Moulin P and Lepage M (2006). Fluorescent pseudomonads occuring in *Macrotermes subhyalinus* mound structures decrease Cd toxicity and improve its accumulation in sorghum plants. *Sci Total Environ* 370: 391-400.

Ebbs SD, Zambrano MC, Spiller SM and Newville M (2009). Cadmium sorption, influx, and efflux at the mesophyll layer of leaves from ecotypes of the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *New Phytol* 181: 626-636.

Echevarria G, Morel J, Fardeau J and Leclerc-Cessac E (1998). Assessment of phytoavailability of nickel in soils. *J Environ Qual* 27: 1064-1070.

Egamberdieva D (2009). Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. *Acta Physiol Plant* 31: 861-864.

El-Saeid H, Abou-Hussein S and El-Tohamy W (2010). Growth characters, yield and endogenous hormones of cowpea plants in response to IAA application. *Res J Agric & Biol Sci* 6: 27-31.

Emongor VE, Pule-Meulenberg F and Phole O (2004). Effect of Promalin on growth and development of kale (*Brassica oleracea* L. var. acephala DC). *J Agron* 3: 208-214.

Epelde L, Becerril J, Blanco F, Kowalchuk G and Garbisu C (2012). Links between pseudo-metallophytes and rhizosphere microbial communities in a metalliferous soil. *Pedobiologia* 55: 219-225.

Ernst WH (2000). Evolution of metal hyperaccumulation and phytoremediation hype. *New Phytol* 146: 357-358.

Ernst WH (2005). Phytoextraction of mine wastes-options and impossibilities. *Chem Erde-Geochem* 65: 29-42.

Escande V, Olszewski TK, Petit E and Grison C (2014). Biosourced polymetallic catalysts: An efficient means to synthesize underexploited platform molecules from carbohydrates. *ChemSusChem* 7: 1915-1923.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y and 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.

European Commission (2014). Progress in the management of contaminated sites in Europe. Joint Research Centre, Institute for Environment and Sustainability. Publications Office of the European Union, Luxembourg.

Facknath S and Lalljee B (2000). Allelopathic strategies for eco-friendly crop protection. In: SS Narwal et al. (eds) Allelopathy in ecological agriculture and forestry. Kluwer Academic Publishers, London, England. p. 33-46, 267.

Farwell AJ, Vesely S, Nero V, Rodriguez H, Shah S, Dixon DG and Glick BR (2006). The use of transgenic canola (*Brassica napus*) and plant growth-promoting bacteria to enhance plant biomass at a nickel-contaminated field site. *Plant Soil* 288: 309-318.

Farwell AJ, Vesely S, Nero V, Rodriguez H, McCormack K, Shah S, Dixon DG and Glick BR (2007). Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal-contaminated field site. *Environ Pollut* 147: 540-545.

Fässler E, Evangelou MW, Robinson BH and Schulin R (2010). Effects of indole-3-acetic acid (IAA) on sunflower growth and heavy metal uptake in combination with ethylene diamine disuccinic acid (EDDS). *Chemosphere* 80: 901-907.

Fawzy ZF, El-Bassiony AM and El-Nemr MA (2011). Improvement growth, yield and quality of two snap bean (*Phaseolus vulgarus*, L) varieties using some growth regulators. *J Appl Sci Res* 7: 2047-2055.

Frank R, Stonefield K, Suda P and Potter J (1982). Impact of nickel contamination on the production of vegetables on an organic soil, Ontario, Canada, 1980-1981. *Sci Total Environ* 26: 41-65.

Freedman B and Hutchinson T (1980). Pollutant inputs from the atmosphere and accumulations in soils and vegetation near a nickel-copper smelter at Sudbury, Ontario, Canada. *Can J Bot* 58: 108-132.

French CJ, Dickinson NM and Putwain PD (2006). Woody biomass phytoremediation of contaminated brownfield land. *Environ Pollut* 141: 387-395.

Fuentes H, Khoo C, Pe T, Muir S and Khan A (2000). Phytoremediation of a contaminated mine site using plant growth regulators to increase heavy metal uptake. Proceedings of the 5th International Conference on Clean Technologies for the Mining Industry. University of Concepción Press, Concepción, Chile. 427-435.

Fulekar M, Singh A and Bhaduri AM (2009). Genetic engineering strategies for enhancing phytoremediation of heavy metals. *Afr J Biotechnol* 8: 529-535.

Gadd GM (2004). Microbial influence on metal mobility and application for bioremediation. *Geoderma* 122: 109-119.

Gadd GM (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156: 609-643.

Galardi F, Corrales I, Mengoni A, Pucci S, Barletti L, Barzanti R, Arnetoli M, Gabbrielli R and Gonnelli C (2007). Intra-specific differences in nickel tolerance and accumulation in the Ni-hyperaccumulator *Alyssum bertolonii*. *Environ Exp Bot* 60: 377-384.

Gaudet M, Pietrini F, Beritognolo I, Iori V, Zacchini M, Massacci A, Mugnozza GS and Sabatti M (2011). Intraspecific variation of physiological and molecular response to cadmium stress in *Populus nigra* L. *Tree Physiol* 31: 1309-1318.

Geebelen W, Adriano D, van der Lelie D, Mench M, Carleer R, Clijsters H and Vangronsveld J (2003). Selected bioavailability assays to test the efficacy of amendment-induced immobilization of lead in soils. *Plant Soil* 249: 217-228.

Gerendás J, Polacco JC, Freyermuth SK and Sattelmacher B (1999). Significance of nickel for plant growth and metabolism. *J Plant Nutr Soil Sci* 162: 241-256.

Ghorbanli M, Kaveh SH and Sepehr MF (2000). Effects of cadmium and gibberellin on growth and photosynthesis of *Glycine max*. *Photosynthetica* 37: 627-631.

Ghosh M and Singh S (2005). A review on phytoremediation of heavy metals and utilization of it's by products. *Asian J Energy Environ* 6: 18.

Gianfagna T (1995). Natural and synthetic growth regulators and their use in horticultural and agronomic crops. In: PJ Davies (ed) Plant hormones: Physiology, biochemistry and molecular biology. Kluwer Academic Publishers, Dordrecht, Netherlands. p. 751-773.

Glick BR, Penrose DM and Li J (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J Theor Biol* 190: 63-68.

Glick BR (2003). Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnol Adv* 21: 383-393.

Glick BR, Cheng Z, Czarny J and Duan J (2007). Promotion of plant growth by ACC deaminase-producing soil bacteria. *Eur J Plant Pathol* 119: 329-339.

Glick BR (2010). Using soil bacteria to facilitate phytoremediation. *Biotechnol Adv* 28: 367-374.

Glick BR (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol Res* 169: 30-39.

Göhre V and Paszkowski U (2006). Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. *Planta* 223: 1115-1122.

Gramss G, Voigt KD and Bergmann H (2004). Plant availability and leaching of (heavy) metals from ammonium-, calcium-, carbohydrate-, and citric acid-treated uranium-mine-dump soil. *J Plant Nutr Soil Sci* 167: 417-427.

Greenway GM and Song QJ (2002). Heavy metal speciation in the composting process. *J Environ Monit* 4: 300-305.

Greman H (2005). Phytoextraction of heavy metals from contaminated soil: expectations and limitations. *Geophys Res Abstr* 7: 01117.

Grison C and Escarré J (2011). Use of metal-accumulating plants for the preparation of catalysts that can be used in chemical reactions. US Patent N° 0.316.340.

Gullap MK, Dasci M, Erkovan Hİ, Koc A and Turan M (2014). Plant Growth-Promoting Rhizobacteria (PGPR) and phosphorus fertilizer-assisted phytoextraction of toxic heavy metals from contaminated soils. *Commun Soil Sci Plant Anal* 45: 2593-2606.

Guo J, Tang S, Ju X, Ding Y, Liao S and Song N (2011). Effects of inoculation of a plant growth promoting rhizobacterium *Burkholderia* sp. D54 on plant growth and metal uptake by a hyperaccumulator *Sedum alfredii* Hance grown on multiple metal contaminated soil. *World J Microb Biot* 27: 2835-2844.

Habashi F (2005). A short history of hydrometallurgy. *Hydrometallurgy* 79: 15-22.

Hadi F, Bano A and Fuller MP (2010). The improved phytoextraction of lead (Pb) and the growth of maize (*Zea mays* L.): the role of plant growth regulators (GA_3 and IAA) and EDTA alone and in combinations. *Chemosphere* 80: 457-462.

Hammer D, Keller C, McLaughlin MJ and Hamon RE (2006). Fixation of metals in soil constituents and potential remobilization by hyperaccumulating and non-hyperaccumulating plants: Results from an isotopic dilution study. *Environ Pollut* 143: 407-415.

Hänsch R and Mendel RR (2009). Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Curr Opin Plant Biol* 12: 259-266.

Harris A, Naidoo K, Nokes J, Walker T and Orton F (2009). Indicative assessment of the feasibility of Ni and Au phytomining in Australia. *J Clean Prod* 17: 194-200.

He CQ, Tan G, Liang X, Du W, Chen Y, Zhi G and Zhu Y (2010a). Effect of Zn-tolerant bacterial strains on growth and Zn accumulation in *Orychophragmus violaceus*. *Appl Soil Ecol* 44: 1-5.

He LY, Chen ZJ, Ren G-D, Zhang Y-F, Qian M and Sheng X-F (2009). Increased cadmium and lead uptake of a cadmium hyperaccumulator tomato by cadmium-resistant bacteria. *Ecotoxicol Environ Saf* 72: 1343-1348.

He LY, Zhang YF, Ma HY, Su LN, Chen ZJ, Wang QY, Qian M and Sheng XF (2010b). Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants. *Appl Soil Ecol* 44: 49-55.

Herzig R, Nehnevajova E, Pfistner C, Schwitzguebel J-P, Ricci A and Keller C (2014). Feasibility of labile Zn phytoextraction using enhanced tobacco and sunflower: Results of five-and one-year field-scale experiments in Switzerland. *Int J Phytoremediat* 16: 735-754.

Hinsinger P (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant Soil* 237: 173-195.

Hinsinger P, Gobran G, R. , Gregory P, J. and Wenzel W, W. (2005). Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. *New Phytol* 168: 293-303.

Hinsinger P and Courchesne F (2008). Biogeochemistry of metals and metalloids at the soil-root interface. In: A Violante et al. (eds) Biophysico-chemical processes of heavy metals and metalloids in soil environments. John Wiley & Sons, Chichester, England. p. 267-311.

Hörger AC, Fones HN and Preston GM (2013). The current status of the elemental defense hypothesis in relation to pathogens. *Front Plant Sci* 4: 395.

Hornburg V and Brümmer G (1993). Behaviour of heavy metals in soils. 1. Heavy metal mobility. *Z Pflanz Bodenkunde* 156: 467-477.

Hunt AJ, Anderson CW, Bruce N, García AM, Graedel TE, Hodson M, Meech JA, Nassar NT, Parker HL and Rylott EL (2014). Phytoextraction as a tool for green chemistry. *Green Process Synth* 3: 3-22.

Hussain K, Hussain M, Nawaz K, Majeed A and Bhatti KH (2011). Morphochemical response of chaksu (*Cassia absus* L.) to different concentrations of Indole Acetic Acid (IAA). *Pak J Bot* 43: 1491-1493.

Hussein H (2008). Optimization of plant-bacteria complex for phytoremediation of contaminated soils. *Int J Bot* 4: 437-443.

Hutchinson JJ, Young SD, McGrath SP, West HM, Black CR and Baker AJ (2000). Determining uptake of 'non-labile'soil cadmium by *Thlaspi caerulescens* using isotopic dilution techniques. *New Phytol* 146: 453-460.

Idris R, Trifonova R, Puschenreiter M, Wenzel WW and Sessitsch A (2004). Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl Environ Microbiol* 70: 2667-2677.

Israr M and Sahi SV (2008). Promising role of plant hormones in translocation of lead in *Sesbania drummondii* shoots. *Environ Pollut* 153: 29-36.

Jähne F (2014). Geology of Europe. In: C Reimann et al. (eds) Chemistry of Europe's Agricultural Soils Part B: General background information and further analysis of the GEMAS dataset, Geologisches Jahrbuch (Reihe B 103). Schweizerbart Science Publishers, Stuttgart, Germany. p. 47-70.

Jiang CY, Sheng XF, Qian M and Wang QY (2008). Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. *Chemosphere* 72: 157-164.

Jones DL and Darrah PR (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant Soil* 166: 247-257.

Jones R (1973). Gibberellins: their physiological role. *Annu Rev Plant Phys* 24: 571-598.

Kabata-Pendias A and Pendias H (1984). Trace elements in soils and plants. CRC Press, Boca Raton, FL.

Kabata-Pendias A (2011). Trace elements in soils and plants. CRC Press, Boca Raton, FL.

Kazakou E, Dimitrakopoulos P, Baker A, Reeves R and Troumbis A (2008). Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. *Biol Rev* 83: 495-508.

Kazakou E, Adamidis GC, Baker AJM, Reeves RD, Godino M and Dimitrakopoulos PG (2010). Species adaptation in serpentine soils in Lesbos Island (Greece): metal hyperaccumulation and tolerance. *Plant Soil* 332: 369-385.

Kefeli V and Kalevitch MV (2003). Natural growth inhibitors and phytohormones in plants and environment. Kluwer Academic Publishers, Dorderecht, Netherlands.

Keller C, Ludwig C, Davoli F and Wochele J (2005). Thermal treatment of metal-enriched biomass produced from heavy metal phytoextraction. *Environ Sci Technol* 39: 3359-3367.

Kerkeb L and Kramer U (2003). The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiol* 131: 716-724.

Khan AG (2005). Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. *J Trace Elem Med Biol* 18: 355-364.

Kidd PS, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R and Monterroso C (2009). Trace element behaviour at the root-soil interface: Implications in phytoremediation. *Environ Exp Bot* 67: 243-259.

Kidd PS, Mench M, Álvarez-López V, Bert V, Dimitriou I, Friesl-Hanl W, Herzig R, Janssen JO, Kolbas A, Müller I, Neu S, Renella G, Ruttens A, Vangronsveld J and Puschenreiter M (2015). Agronomic practices for improving gentle remediation of trace element-contaminated soils. *Int J Phytoremediat (in press)*.

Kim YH, Hamayun M, Khan AL, Na CI, Kang SM, Han HH and Lee I (2009). Exogenous application of plant growth regulators increased the total flavonoid content in *Taraxacum officinale* Wigg. *Afr J Biotechnol* 8.

Kloepper J and Schroth M (1978). Plant growth-promoting rhizobacteria on radishes. Proceedings of the 4th international conference on plant pathogenic bacteria. Gilbert-Clarey, Tours, France. 879-882.

Knight B, Zhao FJ, McGrath SP and Shen ZG (1997). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. *Plant Soil* 197: 71-78.

Koppolu L and Clements LD (2003). Pyrolysis as a technique for separating heavy metals from hyperaccumulators. Part I: Preparation of synthetic hyperaccumulator biomass. *Biomass Bioenerg* 24: 69-79.

Korenkov V, Hirschi K, Crutchfield JD and Wagner GJ (2007). Enhancing tonoplast Cd/H antiport activity increases Cd, Zn, and Mn tolerance, and impacts root/shoot Cd partitioning in *Nicotiana tabacum* L. *Planta* 226: 1379-1387.

Kramer U, Cotter-Howells JD, Charnock JM, Baker AJM and Smith JAC (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379: 635-638.

Kramer U (2010). Metal hyperaccumulation in plants. *Annu Rev Plant Biol* 61: 517-534.

Kuffner M, Puschenreiter M, Wieshammer G, Gorfer M and Sessitsch A (2008). Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows. *Plant Soil* 304: 35-44.

Kukier U and Chaney RL (2004). In situ remediation of nickel phytotoxicity for different plant species. *J Plant Nutr* 27: 465-495.

Kukier U, Peters CA, Chaney RL, Angle JS and Roseberg RJ (2004). The effect of pH on metal accumulation in two species. *J Environ Qual* 33: 2090-2102.

Kumar KV, Singh N, Behl H and Srivastava S (2008). Influence of plant growth promoting bacteria and its mutant on heavy metal toxicity in *Brassica juncea* grown in fly ash amended soil. *Chemosphere* 72: 678-683.

Kumar KV, Srivastava S, Singh N and Behl HM (2009). Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea*. *J Hazard Mater* 170: 51-57.

L'Huillier L and Edighoffer S (1996). Extractability of nickel and its concentration in cultivated plants in Ni rich ultramafic soils of New Caledonia. *Plant Soil* 186: 255-264.

Landberg T and Greger M (1994). Can heavy metal tolerant clones of *Salix* be used as vegetation filters on heavy metal contaminated land? In: P Aronsson and K Perttu (eds) Willow vegetation filters for municipal wastewaters and sludges: A biological purification system. Swedish University of Agricultural Sciences, Uppsala, Sweden. p. 133-144.

Lasat MM (2000). The use of plants for the removal of toxic metals from contaminated soil. American Association for the Advancement of Science. Environmental Science and Engineering Fellow.

Lebeau T, Braud A and Jézéquel K (2008). Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. *Environ Pollut* 153: 497-522.

Legret M (1993). Speciation of heavy metals in sewage sludge and sludge-amended soil. *Int J Environ Anal Chem* 51: 161-165.

Leite VM, Rosolem CA and Rodrigues JD (2003). Gibberellin and cytokinin effects on soybean growth. *Scientia Agricola* 60: 537-541.

Leyval C and Berthelin J (1989). Interactions between *Laccaria laccata*, *Agrobacterium radiobacter* and beech roots: Influence on P, K, Mg, and Fe mobilization from minerals and plant growth. *Plant Soil* 117: 103-110.

Li WC, Ye Z and Wong M (2007). Effects of bacteria on enhanced metal uptake of the Cd/Zn-hyperaccumulating plant, *Sedum alfredii*. *J Exp Bot* 58: 4173-4182.

Li YM, Chaney RL, Angle JS and Baker AJM (2000). Phytoremediaiton of heavy metal contaminated soils. In: KL Wise et al. (eds) Bioremediation of contaminated soils. Marcel Dekker, New York, NY. p. 837-884.

Li YM, Chaney R, Brewer E, Roseberg R, Angle JS, Baker A, Reeves R and Nelkin J (2003a). Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. *Plant Soil* 249: 107-115.

Li YM, Chaney RL, Brewer EP, Angle JS and Nelkin J (2003b). Phytoextraction of nickel and cobalt by hyperaccumulator *Alyssum* species grown on nickel-contaminated soils. *Environ Sci Technol* 37: 1463-1468.

Li YT, Rouland C, Benedetti M, Li F-b, Pando A, Lavelle P and Dai J (2009). Microbial biomass, enzyme and mineralization activity in relation to soil organic C, N and P turnover influenced by acid metal stress. *Soil Biol Biochem* 41: 969-977.

Liphadzi M, Kirkham M and Paulsen G (2006). Auxin-enhanced root growth for phytoremediation of sewage-sludge amended soil. *Environ Technol* 27: 695-704.

Lombi E, Zhao FJ, Dunham SJ and McGrath SP (2000). Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytol* 145: 11-20.

López ML, Peralta-Videa JR, Benitez T and Gardea-Torresdey JL (2005). Enhancement of lead uptake by alfalfa (*Medicago sativa*) using EDTA and a plant growth promoter. *Chemosphere* 61: 595-598.

López ML, Peralta-Videa JR, Parsons JG, Benitez T and Gardea-Torresdey JL (2007). Gibberellic acid, kinetin, and the mixture indole-3-acetic acid-kinetin assisted with EDTA-induced lead hyperaccumulation in alfalfa plants. *Environ Sci Technol* 41: 8165-8170.

Losfeld G, de La Blache PV, Escande V and Grison C (2012a). Zinc hyperaccumulating plants as renewable resources for the chlorination process of alcohols. *Green Chem Lett Rev* 5: 451-456.

Losfeld G, Escande V, Jaffré T, L'Huillier L and Grison C (2012b). The chemical exploitation of nickel phytoextraction: An environmental, ecologic and economic opportunity for New Caledonia. *Chemosphere* 89: 907-910.

Losfeld G, Escande V, Vidal de La Blache P, L'Huillier L and Grison C (2012c). Design and performance of supported Lewis acid catalysts derived from metal contaminated biomass for Friedel-Crafts alkylation and acylation. *Catal Today* 189: 111-116.

Lucisine P, Echevarria G, Sterckeman T, Vallance J, Rey P and Benizri E (2014). Effect of hyperaccumulating plant cover composition and rhizosphere-associated bacteria on the efficiency of nickel extraction from soil. *Appl Soil Ecol* 81: 30-36.

Luo YM, Christie P and Baker A (2000). Soil solution Zn and pH dynamics in non-rhizosphere soil and in the rhizosphere of *Thlaspi caerulescens* grown in a Zn/Cd-contaminated soil. *Chemosphere* 41: 161-164.

Lyon G, Brooks R and Peterson P (1970). Some trace elements in plants from serpentine soils. *New Zeal J Sci* 13: 133-139.

Ma Y, Rajkumar M and Freitas H (2009a). Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. *J Hazard Mater* 166: 1154-1161.

Ma Y, Rajkumar M and Freitas H (2009b). Isolation and characterization of Ni mobilizing PGPB from serpentine soils and their potential in promoting plant growth and Ni accumulation by *Brassica*. *Chemosphere* 75: 719-725.

Ma Y, Rajkumar M and Freitas H (2009c). Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *J Environ Manage* 90: 831-837.

Ma Y, Prasad MNV, Rajkumar M and Freitas H (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnol Adv* 29: 248-258.

Ma Y, Rajkumar M, Luo Y and Freitas H (2013). Phytoextraction of heavy metal polluted soils using *Sedum plumbizincicola* inoculated with metal mobilizing *Phyllobacterium myrsinacearum* RC6b. *Chemosphere* 93: 1386-1392.

Macnair MR (2002). Within and between population genetic variation for zinc accumulation in *Arabidopsis halleri*. *New Phytol* 155: 59-66.

Maestri E and Marmiroli N (2011). Transgenic plants for phytoremediation. *Int J Phytoremediat* 13: 264-279.

Marschner P (2007). Plant-microbe interactions in the rhizosphere and nutrient cycling. Springer-Verlag, Heidelberg, Germany.

Massoura ST, Echevarria G, Leclerc-Cessac E and Morel JL (2004). Response of excluder, indicator, and hyperaccumulator plants to nickel availability in soils. *Aust J Soil Res* 42: 933-938.

Massoura ST, Echevarria G, Leclerc-Cessac E and Morel JL (2005). Response of excluder, indicator, and hyperaccumulator plants to nickel availability in soils. *Soil Research* 42: 933-938.

Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Gonnelli C, Barac T, Boulet J, Weyens N and Vangronsveld J (2009). Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. *Int J Phytoremediat* 11: 251-267.

McGrath SP and Smith SR (1990). Chromium and nickel. In: BJ Alloway (ed) Heavy metals in soils. John Wiley & Sons, New York, NY. p. 125-147.

McGrath SP, Sidoli C, Baker A and Reeves R (1993). The potential for the use of metal-accumulating plants for the in situ decontamination of metal-polluted soils. In: H Eijsackers and

T Hamers (eds) Integrated soil and sediment research: A basis for proper protection. Kluwer Academic Publishers, Dordrecht, Netherlands. p. 673-677.

McGrath SP, Chaudri A and Giller K (1995). Long-term effects of metals in sewage sludge on soils, microorganisms and plants. *J Ind Microbiol* 14: 94-104.

McGrath SP, Shen Z and Zhao F (1997). Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. *Plant Soil* 188: 153-159.

McGrath SP and Zhao FJ (2013). Concentrations of metals and metalloids in soils that have the potential to lead to exceedance of maximum limit concentrations of contaminants in food and feed. *Soil Use Manage*.

Meers E, Ruttens A, Hopgood M, Samson D and Tack F (2005). Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. *Chemosphere* 58: 1011-1022.

Meharg AA and Cairney JW (2000). Ectomycorrhizas-extending the capabilities of rhizosphere remediation? *Soil Biol Biochem* 32: 1475-1484.

Mench M and Martin E (1991). Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L. and *Nicotiana rustica* L. *Plant Soil* 132: 187-196.

Mench M, Martin E and Solda P (1994). After effects of metals derived from a highly metal-polluted sludge on maize (*Zea mays* L.). *Water Air Soil Poll* 75: 277-291.

Mench M, Vangronsveld J, Bleeker P, Ruttens A, Geebelen W and Lepp N (2006). Phytostabilisation of metal-contaminated sites. In: G Echevarria et al. (eds) Phytoremediation of metal-contaminated soils. Springer, Dordrecht, Netherlands. p. 109-190.

Mench M, Schwitzguebel JP, Schroeder P, Bert V, Gawronski S and Gupta S (2009). Assessment of successful experiments and limitations of phytotechnologies: contaminant uptake, detoxification and sequestration, and consequences for food safety. *Environ Sci Pollut Res Int* 16: 876-900.

Mench M, Lepp N, Bert V, Schwitzguébel J-P, Gawronski S, Schröder P and Vangronsveld J (2010). Successes and limitations of phytotechnologies at field scale: outcomes, assessment and outlook from COST Action 859. *J Soils Sed* 10: 1039-1070.

Menezes de Sequeira E and Pinto da Silva AR (1991). Ecology of serpentinized areas of north-east Portugal. In: BA Roberts and J Proctor (eds) The ecology of areas with serpentinized rocks, a world view. Kluwer Academic Publishers, Netherlands. p. 169-197.

Meng H, Hua S, Shamsi IH, Jilani G, Li Y and Jiang L (2008). Cadmium-induced stress on the seed germination and seedling growth of *Brassica napus* L., and its alleviation through exogenous plant growth regulators. *Plant Growth Regul* 58: 47-59.

Mengoni A, Barzanti R, Gonnelli C, Gabbrielli R and Bazzicalupo M (2001). Characterization of nickel-resistant bacteria isolated from serpentine soil. *Environ Microbiol* 3: 691-698.

Mengoni A, Gonnelli C, Brocchini E, Galardi F, Pucci S, Gabbrielli R and Bazzicalupo M (2003). Chloroplast genetic diversity and biogeography in the serpentine endemic Ni-hyperaccumulator *Alyssum bertolonii*. *New Phytol* 157: 349-356.

Mercier G, Barbaroux R, Plasari E, Blais J-F, Simonnot M-O and Morel J-L (2012). Procédé de production d'un sel de sulfate double de nickel et d'ammonium à partir de plantes hyperaccumulatrices. Canada Patent N° 2.731.457.

Migeon A, Richaud P, Guinet F, Chalot M and Blaudez D (2009). Metal accumulation by woody species on contaminated sites in the North of France. *Water Air Soil Poll* 204: 89-101.

Milner MJ and Kochian LV (2008). Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Ann Bot* 102: 3-13.

MingMing H, Fan H, Kai W and FengYun Z (2010). Effects of different kinds of exogenous auxin on the growth of rice roots under cadmium stress. *J Agr Sci Tech* 11: 45-48.

Ministry of Agriculture (1990). Real Decreto 1310/1990, de 29 de octubre, por el que se regula la utilización de los lodos de depuración en el sector agrario (Royal Decree on the agricultural use of sewage sludge). Spanish Official Bull. 262, 32339-32340.

Ministry of Agriculture (2005). Real Decreto 824/2005, de 18 de junio, sobre fertilizantes (Royal Decree on fertilizers). Spanish Official Bull. 171, 25592-25669.

Molitor M, Dechamps C, Gruber W and Meerts P (2005). *Thlaspi caerulescens* on nonmetalliferous soil in Luxembourg: ecological niche and genetic variation in mineral element composition. *New Phytol* 165: 503-512.

Moreno-Jiménez E, Esteban E, Carpena-Ruiz RO, Lobo MC and Penalosa JM (2012). Phytostabilisation with Mediterranean shrubs and liming improved soil quality in a pot experiment with a pyrite mine soil. *J Hazard Mater* 201: 52-59.

Mukherjee D and Kumar R (2007). Kinetin regulates plant growth and biochemical changes during maturation and senescence of leaves, flowers, and pods of *Cajanus cajan* L. *Biol Plant* 51: 80-85.

Munn J, January M and Cutright TJ (2008). Greenhouse evaluation of EDTA effectiveness at enhancing Cd, Cr, and Ni uptake in *Helianthus annuus* and *Thlaspi caerulescens*. *J Soils Sed* 8: 116-122.

Na G and Salt DE (2011). Differential regulation of serine acetyltransferase is involved in nickel hyperaccumulation in *Thlaspi goesingense*. *J Biol Chem* 286: 40423-40432.

Ngatia T, Shibairo S, Emongor V and Obukosia S (2004). Effect of levels and timing of application of gibberellic acid on growth and yield components of common beans. *Afr Crop Sci J* 12: 123-131.

Nicks L and Chambers M (1994). Nickel farming. *Discover Mag* 19: 22-23.

Nicks L and Chambers M (1995). Farming for metals. *Min Environ Manage* 11: 15-18.

Nicks L and Chambers M (1998). A pioneering study of the potential of phytomining for nickel. In: RR Brooks (ed) Plants that hyperaccumulate heavy metals. CAB International. Wallingford, UK. p. 313-326.

O'Dell RE, James JJ and Richards JH (2006). Congeneric serpentine and nonserpentine shrubs differ more in leaf Ca: Mg than in tolerance of low N, low P, or heavy metals. *Plant Soil* 280: 49-64.

Ouzounidou G and Ilias I (2005). Hormone-induced protection of sunflower photosynthetic apparatus against copper toxicity. *Biol Plant* 49: 223-228.

Paz-González A, Taboada Castro MT and Vieira S (2001). Geostatistical analysis of heavy metals in a one-hectare plot under natural vegetation in a serpentine area. *Can J Soil Sci* 81: 469-479.

Pérez-Cid B, Lavilla I and Bendicho C (1999). Comparison between conventional and ultrasound accelerated Tessier sequential extraction schemes for metal fractionation in sewage sludge. *Fresenius J Anal Chem* 363: 667-672.

Peterson LR, Trivett V, Baker AJ, Aguiar C and Pollard AJ (2003). Spread of metals through an invertebrate food chain as influenced by a plant that hyperaccumulates nickel. *Chemoecology* 13: 103-108.

Pilon-Smits E and Pilon M (2002). Phytoremediation of metals using transgenic plants. *Crit Rev Plant Sci* 21: 439-456.

Pollard AJ and Baker AJM (1996). Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytol* 132: 113-118.

Pollard AJ, Powell KD, Harper FA and Smith JAC (2002). The genetic basis of metal hyperaccumulation in plants. *Crit Rev Plant Sci* 21: 539-566.

Pollard AJ, Reeves RD and Baker AJ (2014). Facultative hyperaccumulation of heavy metals and metalloids. *Plant Sci* 217: 8-17.

Proctor J (1969). Studies in serpentine plant ecology. Thesis. University of Oxford, Oxford, UK.

Proctor J (1999). Toxins, nutrient shortages and droughts: the serpentine challenge. *Trends Ecol Evol* 14: 334-335.

Pulford I, Riddell-Black D and Stewart C (2002). Heavy metal uptake by willow clones from sewage sludge-treated soil: the potential for phytoremediation. *Int J Phytoremediat* 4: 59-72.

Puschenreiter M, Wieczorek S, Horak O and Wenzel WW (2003). Chemical changes in the rhizosphere of metal hyperaccumulator and excluder *Thlaspi* species. *J Plant Nutr Soil Sci* 166: 579-584.

Puschenreiter M, Schnepf A, Millan IM, Fitz WJ, Horak O, Klepp J, Schrefl T, Lombi E and Wenzel WW (2005). Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. *Plant Soil* 271: 205-218.

Qiu R, Liu W, Zeng X, Tang Y, Brewer E and Fang X (2009). Effects of exogenous citric acid and malic acid addition on nickel uptake and translocation in leaf mustard (*Brassica juncea* var. *foliosa* Bailey) and *Alyssum corsicum*. *Int J Environ Pollut* 38: 15-25.

Quantin C, Becquer T, Rouiller J and Berthelin J (2001). Oxide weathering and trace metal release by bacterial reduction in a New Caledonia Ferralsol. *Biogeochemistry* 53: 323-340.

Quantin C, Becquer T, Rouiller J and Berthelin J (2002). Redistribution of metals in a New Caledonia Ferralsol after microbial weathering. *Soil Sci Soc Am J* 66: 1797-1804.

Rajkumar M, Nagendran R, Lee KJ, Lee WH and Kim SZ (2006). Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard. *Chemosphere* 62: 741-748.

Rajkumar M and Freitas H (2008a). Effects of inoculation of plant-growth promoting bacteria on Ni uptake by Indian mustard. *Bioresour Technol* 99: 3491-3498.

Rajkumar M and Freitas H (2008b). Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. *Chemosphere* 71: 834-842.

Rajkumar M, Ma Y and Freitas H (2008). Characterization of metal-resistant plant-growth promoting *Bacillus weihenstephanensis* isolated from serpentine soil in Portugal. *J Basic Microbiol* 48: 500-508.

Rajkumar M, Sandhya S, Prasad M and Freitas H (2012). Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnol Adv* 30: 1562-1574.

Reeves RD and Brooks RR (1983). Hyperaccumulation of lead and zinc by two metallophytes from mining areas of Central Europe. *Environ Pollut* 31: 277-285.

Reeves RD, Baker AJM, Borhidi A and Berazain R (1996). Nickel-accumulating plants from the ancient serpentine soils of Cuba. *New Phytol* 133: 217-224.

Reeves RD and Adigüzel N (2008). The nickel hyperaccumulating plants of the serpentines of Turkey and adjacent areas: a review with new data. *Turk J Biol* 32: 143-153.

Richardson AE, Hadobas PA, Hayes JE, O'hara C and Simpson R (2001). Utilization of phosphorus by pasture plants supplied with myo-inositol hexaphosphate is enhanced by the presence of soil micro-organisms. *Plant Soil* 229: 47-56.

Richardson AE and Simpson RJ (2011). Soil microorganisms mediating phosphorus availability update on microbial phosphorus. *Plant Physiol* 156: 989-996.

Robinson BH, Brooks RR, Howes AW, Kirkman JH and Gregg PEH (1997a). The potential of the high-biomass nickel hyperaccumulator *Berkheya coddii* for phytoremediation and phytomining. *J Geochem Explor* 60: 115-126.

Robinson BH, Chiarucci A, Brooks RR, Petit D, Kirkman JH, Gregg PEH and De Dominicis V (1997b). The nickel hyperaccumulator plant *Alyssum bertolonii* as a potential agent for phytoremediation and phytomining of nickel. *J Geochem Explor* 59: 75-86.

Robinson BH, Brooks RR and Clothier BE (1999). Soil amendments affecting nickel and cobalt uptake by *Berkheya coddii*: potential use for phytomining and phytoremediation. *Ann Bot* 84: 689-694.

Roosens N, Verbruggen N, Meerts P, XIMÉNEZ-EMBÚN P and Smith J (2003). Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from western Europe. *Plant, Cell Environ* 26: 1657-1672.

Rufo L, Rodríguez N and de la Fuente V (2005). Análisis comparado de metales en suelos y plantas de la Sierra Bermeja. II Simposio Nacional de control de la degradación de suelos. Universidad Autónoma de Madrid, Madrid, Spain. 197-201.

Ruttens A, Boulet J, Weyens N, Smeets K, Adriaensen K, Meers E, Van Slycken S, Tack F, Meiresonne L and Thewys T (2011). Short rotation coppice culture of willows and poplars as energy crops on metal contaminated agricultural soils. *Int J Phytoremediat* 13: 194-207.

Salt DE, Blaylock M, Kumar N, Dushenkov V, Ensley BD, Chet I and Raskin I (1995). Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. *Biotechnol* 13: 468-474.

Salt DE, Kato N, Krämer U, Smith R and Raskin I (2000). The role of root exudates in nickel hyperaccumulation and tolerance in accumulator and nonaccumulator species of *Thlaspi*. In: N Terry and G Bañuelos (eds) Phytoremediation of contaminated soil and water. CRC Press Boca Raton, FL. p. 189-200.

Sayed SA (1999). Effects of lead and kinetin on the growth, and some physiological components of safflower. *Plant Growth Regul* 29: 167-174.

Schlegel H, Cosson JP and Baker A (1991). Nickel-hyperaccumulating plants provide a niche for nickel-resistant bacteria. *Bot Acta* 104: 18-25.

Schwitzguébel JP, Page V, Martins-Dias S, Davies LC, Vasilyeva G and Strijakova E (2011). Using plants to remove foreign compounds from contaminated water and soil. In: P Schröder and CD
Collins (eds) Organic xenobiotics and plants. Springer, Netherlands. p. 149-189.

Schwitzguébel JP (2014). Phytoremediation of polluted soils: hype, hope and facts. Invited lecture presented at the International Congress on Phytoremediation of Polluted Soils. École Polytecnique Fédérale de Lausanne, Switzerland.

Schwyn B and Neilands J (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160: 47-56.

Sessitsch A and Puschenreiter M (2008). Endophytes and rhizosphere bacteria of plants growing in heavy metal-containing soils. In: P Dion and CS Nautiyal (eds) Microbiology of extreme soils. Springer, Heidelberg, Germany. p. 317-332.

Sessitsch A, Kuffner M, Kidd PS, Vangronsveld J, Wenzel WW, Fallmann K and Puschenreiter M (2013). The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biol Biochem* 60: 182-194.

Shah S (2007). Effects of salt stress on mustard as affected by gibberellic acid application. *Gen Appl Plant Physiol* 33: 97-106.

Shallari S, Schwartz C, Hasko A and Morel J (1998). Heavy metals in soils and plants of serpentine and industrial sites of Albania. *Sci Total Environ* 209: 133-142.

Shallari S, Echevarria G, Schwartz C and Morel J (2001). Availability of nickel in soils for the hyperaccumulator *Alyssum murale* Waldst. & Kit. *S Afr J Sci* 97: 568-570.

Sharma P and Bhardwaj R (2007). Effects of 24-epibrassinolide on growth and metal uptake in *Brassica juncea L*. under copper metal stress. *Acta Physiol Plant* 29: 259-263.

Sharma SS and Dietz KJ (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot* 57: 711-726.

Sheng XF and Xia JJ (2006). Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere* 64: 1036-1042.

Sheng XF, He L, Wang Q, Ye H and Jiang C (2008a). Effects of inoculation of biosurfactant-producing *Bacillus* sp. J119 on plant growth and cadmium uptake in a cadmium-amended soil. *J Hazard Mater* 155: 17-22.

Sheng XF, Xia J-J, Jiang C-Y, He L-Y and Qian M (2008b). Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environ Pollut* 156: 1164-1170.

Shilev S, Fernández A, Benlloch M and Sancho E (2006). Sunflower growth and tolerance to arsenic is increased by the rhizospheric bacteria *Pseudomonas fluorescens*. In: JL Morel (ed) Phytoremediation of metal contaminated soils. Springer, Netherlands. p. 315-326.

Shtiza A, Swennen R and Tashko A (2005). Chromium and nickel distribution in soils, active river, overbank sediments and dust around the Burrel chromium smelter (Albania). *J Geochem Explor* 87: 92-108.

Smith SR (2009). A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. *Environ Int* 35: 142-156.

Sun LN, Zhang Y-F, He L-Y, Chen Z-J, Wang Q-Y, Qian M and Sheng X-F (2010). Genetic diversity and characterization of heavy metal-resistant-endophytic bacteria from two copper-tolerant plant species on copper mine wasteland. *Bioresour Technol* 101: 501-509.

Taiz L and Zeiger E (2006). Plant physiology. Sinauer Associates Publishers, Sunderland, MA.

Tang YT, DENG T-H-B, WU Q-H, WANG S-Z, QIU R-L, WEI Z-B, GUO X-F, WU Q-T, LEI M and CHEN T-B (2012). Designing cropping systems for metal-contaminated sites: A review. *Pedosphere* 22: 470-488.

Tao S, Liu W, Chen Y, Xu F, Dawson R, Li B, Cao J, Wang X, Hu J and Fang J (2004). Evaluation of factors influencing root-induced changes of copper fractionation in rhizosphere of a calcareous soil. *Environ Pollut* 129: 5-12.

Temple PJ and Bisessar S (1981). Uptake and toxicity of nickel and other metals in crops grown on soil contaminated by a nickel refinery. *J Plant Nutr* 3: 473-482.

Turgay OC, Görmez A and Bilen S (2012). Isolation and characterization of metal resistant-tolerant rhizosphere bacteria from the serpentine soils in Turkey. *Environ Monit Assess* 184: 515-526.

Uesugi T, Koshioka M, Nishijima T and Yamazaki H (1994). Stimulation of asparagus spear sprouting with benzyladenine. *Acta Hort* 394: 241-250.

Upreti KK and Murti GSR (2004). Effects of brassmosteroids on growth, nodulation, phytohormone content and nitrogenase activity in french bean under water stress. *Biol Plant* 48: 407-411.

Uren NC and Reisenauer HM (1988). The role of root exudates in nutrient acquisition. In: B Tinker and A Lachli (eds) Advances in plant nutrition. Praeger, New York, NY. p. 79-114.

Van der Ent A, Baker AJM, Reeves RD, Pollard AJ and Schat H (2013). Hyperaccumulators of metal and metalloid trace elements: facts and fiction. *Plant Soil* 362: 319-334.

Van Slycken S, Witters N, Meiresonne L, Meers E, Ruttens A, Van Peteghem P, Weyens N, Tack FM and Vangronsveld J (2013). Field evaluation of willow under short rotation coppice for phytomanagement of metal-polluted agricultural soils. *Int J Phytoremediat* 15: 677-689.

Vangronsveld J, Van Assche F and Clijsters H (1995). Reclamation of a bare industrial area contaminated by non-ferrous metals: *In situ* metal immobilization and revegetation. *Environ Pollut* 87: 51-59.

Vangronsveld J, Colpaert J, van and Van Tichelen K (1996). Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. *Environ Pollut* 94: 131-140.

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D and Mench M (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res Int* 16: 765-794.

Vassilev A, Schwitzguébel JP, Thewys T, van der Lelie P and Vangronsveld J (2004). The use of plants for remediation of metal-contaminated soils. *Sci World J* 16: 9-34.

Verbruggen N, Hermans C and Schat H (2009). Molecular mechanisms of metal hyperaccumulation in plants. *New Phytol* 181: 759-776.

Walker RB, Walker HM and Ashworth P (1955). Calcium-magnesium nutrition with special reference to serpentine soils. *Plant Physiol* 30: 214.

Wang Q, Xiong D, Zhao P, Yu X, Tu B and Wang G (2011). Effect of applying an arsenic-resistant and plant growth-promoting rhizobacterium to enhance soil arsenic phytoremediation by *Populus deltoides* LH05-17. *J Appl Microbiol* 111: 1065-1074.

Welch RM (1981). The biological significance of nickel. *J Plant Nutr* 3: 345-356.

Wenzel W, Bunkowski M, Puschenreiter M and Horak O (2003). Rhizosphere characteristics of indigenously growing nickel hyperaccumulator and excluder plants on serpentine soil. *Environ Pollut* 123: 131-138.

Weyens N, Van Der Lelie D, Artois T, Smeets K, Taghavi S, Newman L, Carleer R and Vangronsveld J (2009a). Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation. *Environ Sci Technol* 43: 9413-9418.

Weyens N, van der Lelie D, Taghavi S, Newman L and Vangronsveld J (2009b). Exploiting plant-microbe partnerships to improve biomass production and remediation. *Trends Biotechnol* 27: 591-598.

Weyens N, Beckers B, Schellingen K, Ceulemans R, Croes S, Janssen J, Haenen S, Witters N and Vangronsveld J (2013). Plant-associated bacteria and their role in the success or failure of metal phytoextraction projects: first observations of a field-related experiment. *J Microbial Biotech* 6: 288-299.

Whiting SN, Leake JR, McGrath SP and Baker AJ (2001). Assessment of Zn mobilization in the rhizosphere of *Thlaspi caerulescens* by bioassay with non-accumulator plants and soil extraction. *Plant Soil* 237: 147-156.

Whittaker RH (1954). The ecology of serpentine soils. *Ecology* 35: 258-288.

Wood BW, Chaney R and Crawford M (2006). Correcting micronutrient deficiency using metal hyperaccumulators: *Alyssum* biomass as a natural product for nickel deficiency correction. *HortScience* 41: 1231-1234.

Xiong J, He Z, Liu D, Mahmood Q and Yang X (2008). The role of bacteria in the heavy metals removal and growth of *Sedum alfredii* Hance in an aqueous medium. *Chemosphere* 70: 489-494.

Yang Q, Tu S, Wang G, Liao X and Yan X (2012). Effectiveness of applying arsenate reducing bacteria to enhance arsenic removal from polluted soils by *Pteris vittata* L. *Int J Phytoremediat* 14: 89-99.

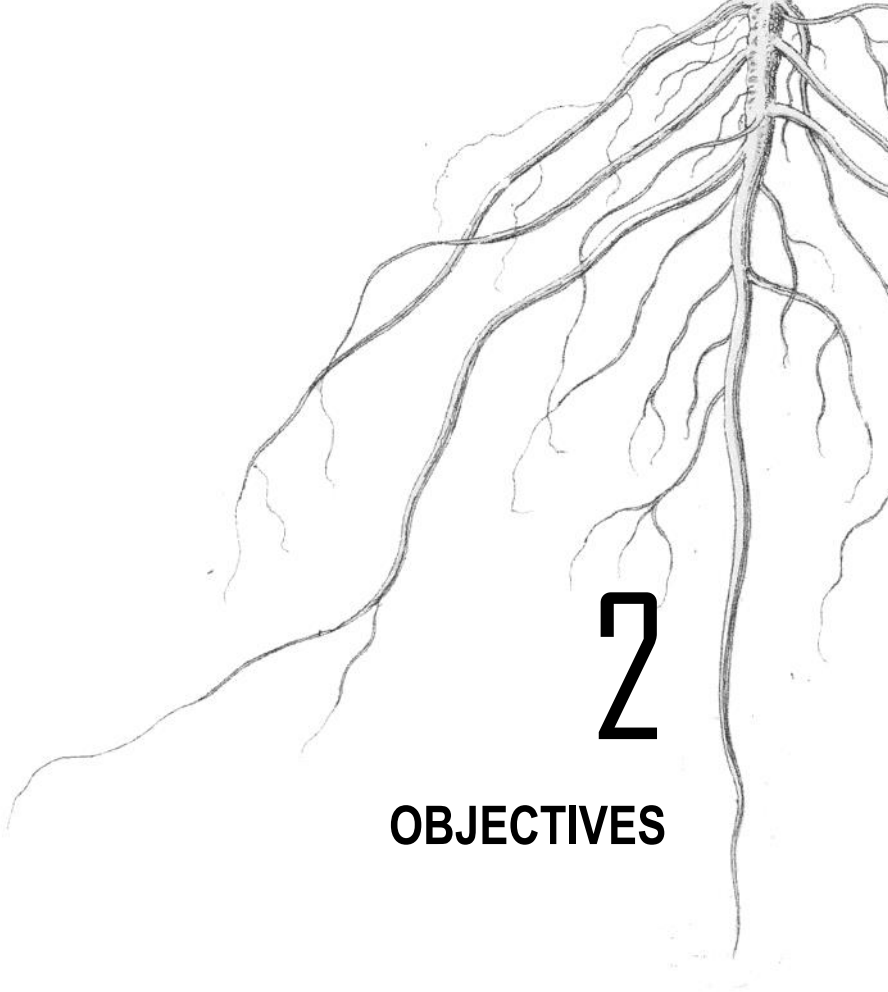
Yang T, Law DM and Davies PJ (1993). Magnitude and kinetics of stem elongation induced by exogenous indole-3-acetic acid in intact light-grown pea seedlings. *Plant Physiol* 102: 717-724.

Zaidi S, Usmani S, Singh BR and Musarrat J (2006). Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. *Chemosphere* 64: 991-997.

Zhang X, Houzelot V, Bani A, Morel JL, Echevarria G and Simonnot M-O (2014). Selection and combustion of Ni-hyperaccumulators for the phytomining process. *Int J Phytoremediat* 16: 1058-1072.

Zhao Y, Peralta-Videa JR, Lopez-Moreno ML, Ren M, Saupe G and Gardea-Torresdey JL (2010). Kinetin increases chromium absorption, modulates its distribution, and changes the activity of catalase and ascorbate peroxidase in Mexican palo verde. *Environ Sci Technol* 45: 1082-1087.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



2

OBJECTIVES

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Phytoextraction cultivates plants that are able to accumulate trace metals from metal-rich soils and transport them to the shoots which can then be harvested, thus removing the metals from the soil. Phytoextraction provides the opportunity to recover highly valuable metals from the plant biomass, a process known as phytomining. Phytomining is receiving increasingly more attention because it can potentially provide a realistic means of meeting with increasing demands on metal resources without causing the environmental damage and contamination associated with conventional mining activities. Until now it has been shown to be feasible for the recovery of Ni from sub-economic ores, such as serpentine soils or Ni-contaminated soils. Plants must be highly metal tolerant, able to accumulate large concentrations of the targeted trace elements in harvestable shoots, and have a reasonable biomass production so that metal removal from the site is economic. To date the Ni-hyperaccumulating *Alyssum* species, *Alyssum murale* and *Alyssum corsicum* (native to Mediterranean serpentine soils), have shown high potential application in the phytomining process. The Iberian Peninsula hosts two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf.: *Alyssum serpyllifolium* ssp. *lusitanicum* and *Alyssum serpyllifolium* ssp. *malacitanum*. Both subspecies are serpentine-endemic and are distributed throughout the main serpentinitic areas of the Peninsula (NE Portugal and NW and S Spain). Several authors have studied these Ni-hyperaccumulating subspecies from an ecological and physiological perspective but few studies have evaluated their Ni bioaccumulation capacity and considered their potential use in phytomining purposes. Moreover, differences in biomass and metal uptake among individual plants of the same population or amongst different populations of these two subspecies have not been assessed. It has been suggested that traditional plant breeding programmes could use the available genetic diversity within hyperaccumulating plant species to combine the traits needed for successful phytoextraction (and phytomining).

Although the phytomining process appears to be viable for Ni recovery it can also present some important limitations. For example, many natural metal hyperaccumulators are slow growing with a small biomass and shallow root systems; the process is climate and season dependent, and can be limited by biogeochemical factors (microbial activity, root exudates, temperature, pH, moisture) and the solubility and availability of the metals in the soil. Incorporating different management techniques could maximise the performance and yields of hyperaccumulator crops and thus increase phytomining efficiency. Conventional agronomic practices such as fertilisation, liming or herbicide regimes have been used to maximise the biomass production of Ni-hyperaccumulators. Plant growth regulators (PGRs) are a group of naturally occurring organic compounds that

regulate physiological processes of a plant at low concentrations and have been commercially developed and are presently used in agriculture for a wide range of purposes. However, few studies have considered the application of PGRs to hyperaccumulating plants as a means of increasing biomass production and/or metal accumulation and, hence, their phytoextraction capacity. Finally, a growing number of studies indicate an important role of plant-microbial associations in the phytoextraction process. It has been shown that the inoculation of phytoextractor crops with plant growth promoting rhizobacteria (PGPR) can increase soil metal availability and accumulation. The use of this type of strategy could lead to an increase in the plant biomass production and Ni uptake and accumulation by Ni-hyperaccumulating plants, thus enhancing their phytoextraction capacity.

On this basis, the objectives of this PhD thesis can be summarised as follows:

1. To study the inter- and intra-population variability in Ni tolerance and accumulation patterns of the Ni-hyperaccumulating species of the genus *Alyssum* endemic to the Iberian Peninsula: *A. serpyllifolium* ssp. *lusitanicum* from NW Spain and NE Portugal and *A. serpyllifolium* ssp. *malacitanum* from S Spain, also known as *A. pintodasilvae* and *A. malacitanum*. The analysis intended to detect significant variability in plant biomass, Ni accumulation and/or an ability to mobilise soil Ni, that could be further explored to increase the Ni yield of these hyperaccumulating *A. serpyllifolium* subspecies in future plant breeding experiments.

To achieve this objective the Ni tolerance and accumulation was evaluated in five populations (Melide, Morais, Samil, Sierra Aguas and Sierra Bermeja) which were grown in three different conditions: *in situ* plants growing in the field, plants cultivated in hydroponic culture solutions enriched with Ni and plants cultivated in a pot experiment using serpentine soil. In addition, the soil physicochemical properties and Ni availability was evaluated in the rhizosphere of these Ni-hyperaccumulators.

2. To evaluate the potential use of two contrasting strategies for increasing biomass production and/or Ni concentration in the harvestable tissues of different Ni-hyperaccumulating species:

a) To assess the use of different plant growth regulators (PGRs) or phytohormones to enhance biomass production and Ni phytoextraction of several Ni-hyperaccumulating species from the genus *Alyssum* (*A. corsicum*, *A. malacitanum*, *A. murale*, *A. pintodasilvae*) and *Noccaea goesingense* grown in serpentine soil.

To achieve this objective a study was carried out in two parts: an initial experiment (Part I) tested two different phytohormones based on cytokinins and/or gibberellins applied at two concentration rates, and a second experiment (Part II) tested four commercial products based on combinations of indoleacetic acid, cytokinins and/or gibberellins and applied at three different concentrations. Effects on plant growth and biomass production, nutrient status and Ni phytoextraction efficiency were determined.

b) To assess the use of plant growth promoting (PGP) rhizobacterial strains for improving the biomass production and Ni phytoextraction of the Ni-hyperaccumulator *A. pintodasilvae*.

To achieve this objective fifteen bacterial isolates were screened for their PGP capacities and used to inoculate *A. pintodasilvae* growing in a simple perlite:sand mixture. On the basis of the results obtained five bacterial strains were selected to inoculate *A. pintodasilvae* growing in two soils, a naturally Ni-rich serpentine soil and a sewage sludge-amended agricultural soil with Ni and Cd as the main contaminants. The effects of the bacterial inoculants on soil metal availability, plant growth and nutrient status, and plant Ni accumulation and phytoextraction were evaluated.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3

NATURAL VARIATION IN PLANT GROWTH, NICKEL TOLERANCE *and accumulation in five populations of* the Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium*

ABSTRACT

The Iberian Peninsula hosts two subspecies of *Alyssum serpyllifolium* which are both serpentine-endemic and hyperaccumulators of Ni: *Alyssum serpyllifolium* ssp. *lusitanicum* from Galicia (NW Spain) and Trás-os-Montes (NE Portugal), and *Alyssum serpyllifolium* ssp. *malacitanum* from Andalusia (S Spain). The aim of this study was to assess the inter- and intra-population variability in Ni tolerance and accumulation patterns of these Ni-hyperaccumulating subspecies. This was evaluated in five populations which were grown in three different conditions: *in situ* plants growing in the field, plants cultivated in hydroponic culture solutions enriched with Ni and plants cultivated in a pot experiment using serpentine soil. In addition, the soil physicochemical properties and Ni availability was evaluated in the rhizosphere of these Ni-hyperaccumulators. Population-specific effects on the physicochemical properties of the rhizosphere soil were observed. However, in general, rhizosphere soils presented a higher pH, organic C and total N content, cation exchange capacity and Ca/Mg ratio. In addition, root activity generally led to an increase in plant-available soil Ni fractions and modifications in the soil Ni fractionation. In the field-collected plants the inter-population variance in Ni accumulation patterns was more pronounced than when the progeny were grown in controlled conditions. In both the hydroponic and pot experiments a high variability in the measured parameters was found within populations rather than amongst populations. Nonetheless, the significant differences revealed under controlled conditions in aspects such as biomass production and root-shoot Ni transfer could be further explored as a means of increasing the Ni yield of *Alyssum serpyllifolium*.

This study forms part of the following publications:

Cabello-Conejo MI, Monterroso C, Prieto-Fernández A, Becerra-Castro C, Ginzo-Villamayor MJ and Kidd PS (2015). A characterisation of the Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium*. Part I: Populational variation in Ni accumulation and plant-induced effects on soil rhizosphere properties. *Environ Exper Bot* (submitted).

Cabello-Conejo MI, Monterroso C, Prieto-Fernández A, Ginzo-Villamayor MJ and Kidd PS (2015). A characterisation of the Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium*. Part II: Populational variation in growth and Ni accumulation of offspring cultivated under controlled conditions. *Environ Exper Bot* (submitted).

3.1 *INTRODUCTION*

Serpentine soils are derived from weathered ultramafic rocks, where the term ultramafic refers to igneous or metamorphic rocks containing more than 70 % of ferromagnesian minerals and a low content in silicon (<45 % SiO_2) (Brooks 1987). They are characterised by a deficiency in essential nutrients (such as N, P, K and Ca), a low Ca:Mg ratio (unfavourable for Ca absorption) and elevated concentrations of Mg and Fe, as well as potentially phytotoxic trace metals (such as Ni, Co and Cr) (Brooks 1987). In addition, these soils are often skeletal, with a low organic matter content and water holding capacity (Brooks 1987; Kruckeberg 1984; Proctor and Roberts 1992). These extreme edaphic properties (which are generally referred to as the "serpentine syndrome") make these soils inhospitable to most plant species. As a result, the plant communities which develop in these areas are floristically distinct with a high proportion of endemic or disjunctly distributed species and adaptive morphologies (such as a reduced leaf size and high degree of sclerophylly) (Proctor 1999). Serpentine soils are typically recognized across landscapes as patchily distributed rocky outcrops with stunted vegetation. The potentially toxic Mg level, Ca/Mg imbalance, phytotoxic Ni level and P deficiency have been implicated as the primary reasons for serpentine soil infertility which in turn causes evolution of serpentine flora (Proctor and Baker 1994; Reeves 1992).

One of the most remarkable plant adaptations to serpentine soils is the hyperaccumulation of trace metals and especially Ni (Brady *et al.* 2005; Kazakou *et al.* 2010; Kazakou *et al.* 2008). The term *hyperaccumulator* was first used to describe plants that were able to accumulate more than 1000 mg kg^{-1} DW of Ni in their above-ground tissue when growing in their natural habitat (Brooks *et al.* 1977). Since then hyperaccumulators have been recorded and experimentally confirmed for elements such as Ni, Zn, Cd, Mn, As and Se (Van der Ent *et al.* 2013). Nonetheless, the Ni hyperaccumulators represent over 90 % of known hyperaccumulators; and the genus with the greatest number of Ni hyperaccumulators is *Alyssum* (Brassicaceae) (Baker and Brooks 1989). Most hyperaccumulating species of the *Alyssum* genus are serpentine endemics restricted to ultramafic soils enriched in Ni (Pollard *et al.* 2002). Pollard *et al.* (2014) differentiated between obligate metallophytes, species that are restricted to metalliferous soils, and facultative hyperaccumulators that hyperaccumulate trace metals when occurring on metalliferous soils, yet also occur commonly on normal, non-metalliferous soils.

Tolerance to trace metals and hyperaccumulation ability are at least partly under independent genetic control (Assunção *et al.* 2003b; Macnair *et al.* 1999),

and numerous studies have shown both parameters to vary significantly among and within plant populations (Bert *et al.* 2000; Dechamps *et al.* 2005; Escarré *et al.* 2000; Meerts and Van Isacker 1997; Meyer *et al.* 2010). Some authors have found this intraspecific variation in metal hyperaccumulation to be correlated with the metal concentration in the soil of origin; however, the results are somewhat controversial (Escarré *et al.* 2000; Meerts and Van Isacker 1997). Hyperaccumulating plant species were thought to be able to increase their metal uptake by accessing metal fractions which were not available to non-accumulating plants (Knight *et al.* 1997; McGrath *et al.* 1997). However, most studies have demonstrated that both plant groups access the same metal pools (Echevarria *et al.* 1998; Massoura *et al.* 2004; Shallari *et al.* 2001), although it has been suggested that the rate of replenishment of labile Ni pools may be faster in the rhizosphere of hyperaccumulating plants (Kidd *et al.* 2009).

The hyperaccumulators, *Noccaea caerulescens* and *Arabidopsis halleri*, are considered model plants for studying trace metal hyperaccumulation and tolerance. Variation in Cd/Zn accumulation has frequently been documented between different ecotypes (Assunção *et al.* 2003a; Frérot *et al.* 2003; Meerts and Van Isacker 1997; Roosens *et al.* 2003) and between populations within ecotypes (Escarré *et al.* 2000; Lombi *et al.* 2000; Meerts and Van Isacker 1997; Pollard and Baker 1996). Significant variation within populations has also been reported (Pollard and Baker 1996; Meerts and Van Isacker 1997; Escarré *et al.* 2000). In *N. caerulescens* the properties of Zn tolerance and hyperaccumulation occur species-wide (albeit with some degree of variability) (Pollard *et al.* 2014). Tolerance in different populations of *N. caerulescens* was frequently found to be positively correlated with soil Zn concentration in their natural habitat, but was inversely related to the capacity for Zn hyperaccumulation (Assunção *et al.* 2001; Escarré *et al.* 2000; Meerts and Van Isacker 1997). Roosens *et al.* (2003) investigated Cd tolerance and metal accumulation for seven contrasting populations of *N. caerulescens* grown under controlled conditions in solution culture: the Ganges populations (South France) was highlighted for its ability to combine a high level of Cd accumulation with an exceptional degree of tolerance. The large differences in Cd accumulation capacity between the Ganges population of *N. caerulescens* and a lower-accumulating population Prayon (Belgium) has been partly explained by the heavy metal ATPase 3 (HMA3) tonoplast transporter (Ueno *et al.* 2011). HMA3 expression in the Ganges population was 7-fold higher than in Prayon, and this difference was shown to be partly due to gene copy number expansion (Ueno *et al.* 2011).

Fewer studies have focused on intra- and inter-population variation in Ni hyperaccumulation. Nonetheless, a high variability in both, Ni concentration and

Ni yield, have been observed between different populations of *Alyssum* species, and this variation often depended on the soil Ni concentrations (Kazakou *et al.* 2010; Massoura *et al.* 2004; Shallari *et al.* 1998). Kazakou *et al.* (2010) studied the Ni-hyperaccumulation capacity of different populations of *Alyssum lesbiacum*, a serpentine endemic of Lesbos Island (Greece). Population differences in Ni hyperaccumulation varied according to soil Ni availability. Similar results were obtained by Shallari *et al.* (1998) who found that different populations of *A. murale* in Albania varied in their shoot Ni concentration according to the soil Ni concentration at the origin. Massoura *et al.* (2004) evaluated Ni accumulation in three populations of *A. murale* (seed were collected from Albania) when grown in a serpentine soil. Dry weight (DW) yield, shoot Ni concentration and soil Ni removal varied amongst the three populations by up to 2.5- (DW yield), 2.7- (shoot Ni concentration) or 3.5-fold (soil Ni removal). These populational differences in Ni accumulation could not be attributed to the plant's capacity to modify the pool of available soil Ni, and rather they were attributed to contrasting capacities of the root membrane to take up Ni.

The most important serpentinitic outcrops in the Iberian Peninsula are found in the Trás-os-Montes region (NE Portugal), the Melide complex in the region of Galicia (NW Spain) and in the western Betic Cordillera of Málaga (SE Spain) (Asensi *et al.* 2004; Brooks *et al.* 1981; Menezes de Sequeira and Pinto da Silva 1991). The Iberian Peninsula hosts two subspecies of *Alyssum serpyllifolium* Desf. which are both serpentine-endemic and hyperaccumulators of Ni: *Alyssum serpyllifolium* ssp. *lusitanicum* from Galicia (NW Spain) and Trás-os-Montes (NE Portugal), and *Alyssum serpyllifolium* ssp. *malacitanum* from Andalusia (S Spain). The objectives of this study were to evaluate differences in Ni accumulation and plant growth of five populations of the Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* from the main serpentinitic areas in the Iberian Peninsula. Intra- and inter-specific variability in Ni accumulation was assessed in plants sampled in the field, and in plants grown under controlled conditions in different substrates (hydroponic solutions with increasing Ni concentration and in serpentine soil). In addition, the effects of these plants on the physicochemical properties and Ni bioavailability in the rhizosphere soil were determined.

3.2 *MATERIALS AND METHODS*

Geographical description of the study areas and sampling of soils and plant material

The study areas included in this work represent the main serpentinitic areas of the Iberian Peninsula: Melide (L) (NW Spain), Morais (M) and Samil (S) (NE

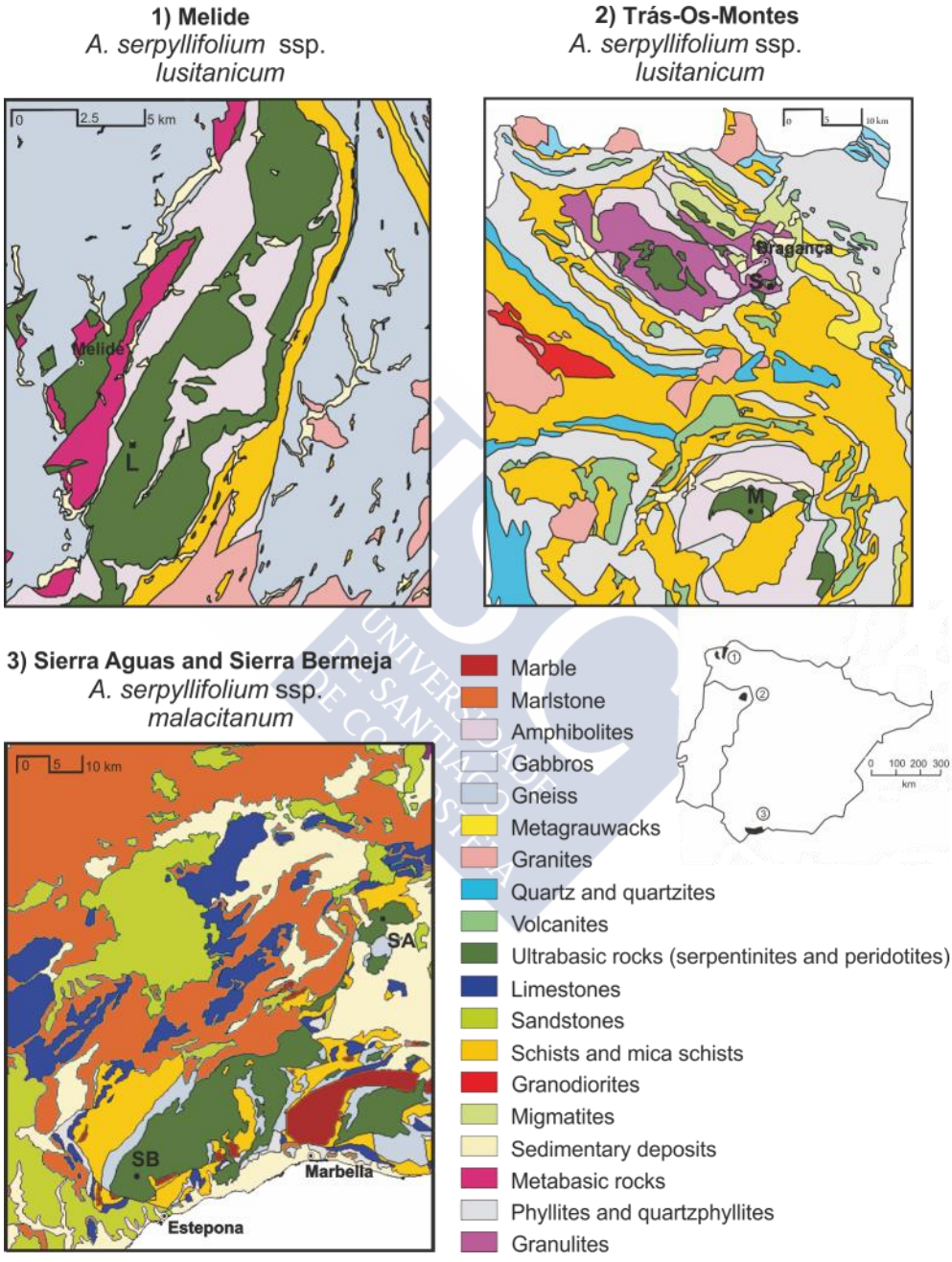
Portugal), Sierra Aguas (SA) and Sierra Bermeja (SB) (S Spain). The Melide ultramafic complex is included in the Serra do Careón, covering an area of approximately 65 km^2 it is considered a Special Area of Conservation (SAC) and included within the Natura 2000 Network. Melide has an European humid-temperate climate with a mean annual temperature of 12.9 ° C and mean annual precipitation of 1381 mm (Carballeira *et al.* 1983). Serpentinitic outcrops of the Trás-os-Montes region, in the Vinhais-Bragança (Samil) and Morais Massifs (Morais), are the largest and the richest in endemic species within Portugal, covering an area of about 80 km^2. This region has a Mediterranean climate, with a mean annual temperature of 12.4 ° C and mean annual precipitation of 720 mm (Carballeira *et al.* 1983; Menezes de Sequeira and Pinto da Silva 1991). Sierra Aguas and Sierra Bermeja are situated in the western Betic Cordillera of Málaga, they are located in one of the largest areas (more than 430 km^2) of ultramafic rocks in the Iberian Peninsula (Asensi *et al.* 2004). The area has a Mediterranean climate, with a mean annual temperature of 16 ° C and mean annual precipitation of 600 mm in Sierra Aguas and a mean annual temperature of 15 ° C and mean annual precipitation of 1200 mm in Sierra Bermeja (Consejería de Medio Ambiente 2009; Gómez-Zotano *et al.* 2014). A map of the serpentine outcrops in the Iberian Peninsula showing the geological characteristics of each region, the location of each sampling point and some photos of the sites can be found in Fig. 3.1, and the UTM coordinates of each sampling site are shown in Table 3.1.

Two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf. (*Brassicaceae*) are found in these study areas: *A. serpyllifolium* ssp. *lusitanicum* Dudley and P. Silva (hereafter referred to as *A. pintodasilvae*) and *A. serpyllifolium* ssp. *malacitanum* Rivas Goday (hereafter referred to as *A. malacitanum*). *A. pintodasilvae* was sampled from Melide (L) and Trás-os-Montes (Morais (M) and Samil (S)) and *A. malacitanum* was sampled from Sierra

Table 3.1. Geographical (UTM) coordinates and altitude of each of the five study sites.

Sampling site	UTM Coordinates	Altitude (m)
1) Melide (L)	29 T 580001 4744900	319
2) Morais (M)	29 T 681706 4599052	651
Samil (S)	29 T 687286 4627805	715
3) Sierra Aguas (SA)	30 S 341390 4080295	842
Sierra Bermeja (SB)	30 S 0302892 4039289	1178

(a)


1) Melide
A. serpyllifolium ssp.
lusitanicum
0 2.5 5 km
Melide
L
2) Trás-Os-Montes
A. serpyllifolium ssp.
lusitanicum
0 5 10 km
Bragança
M
3) Sierra Aguas and Sierra Bermeja
A. serpyllifolium ssp.
malacitanum
0 5 10 km
SA
SB
Marbella
Estepona
Marble
Marlstone
Amphibolites
Gabbros
Gneiss
Metagrauwacks
Granites
Quartz and quartzites
Volcanites
Ultrabasic rocks (serpentinites and peridotites)
Limestones
Sandstones
Schists and mica schists
Granodiorites
Migmatites
Sedimentary deposits
Metabasic rocks
Phyllites and quartzphyllites
Granulites
0 100 200 300
km

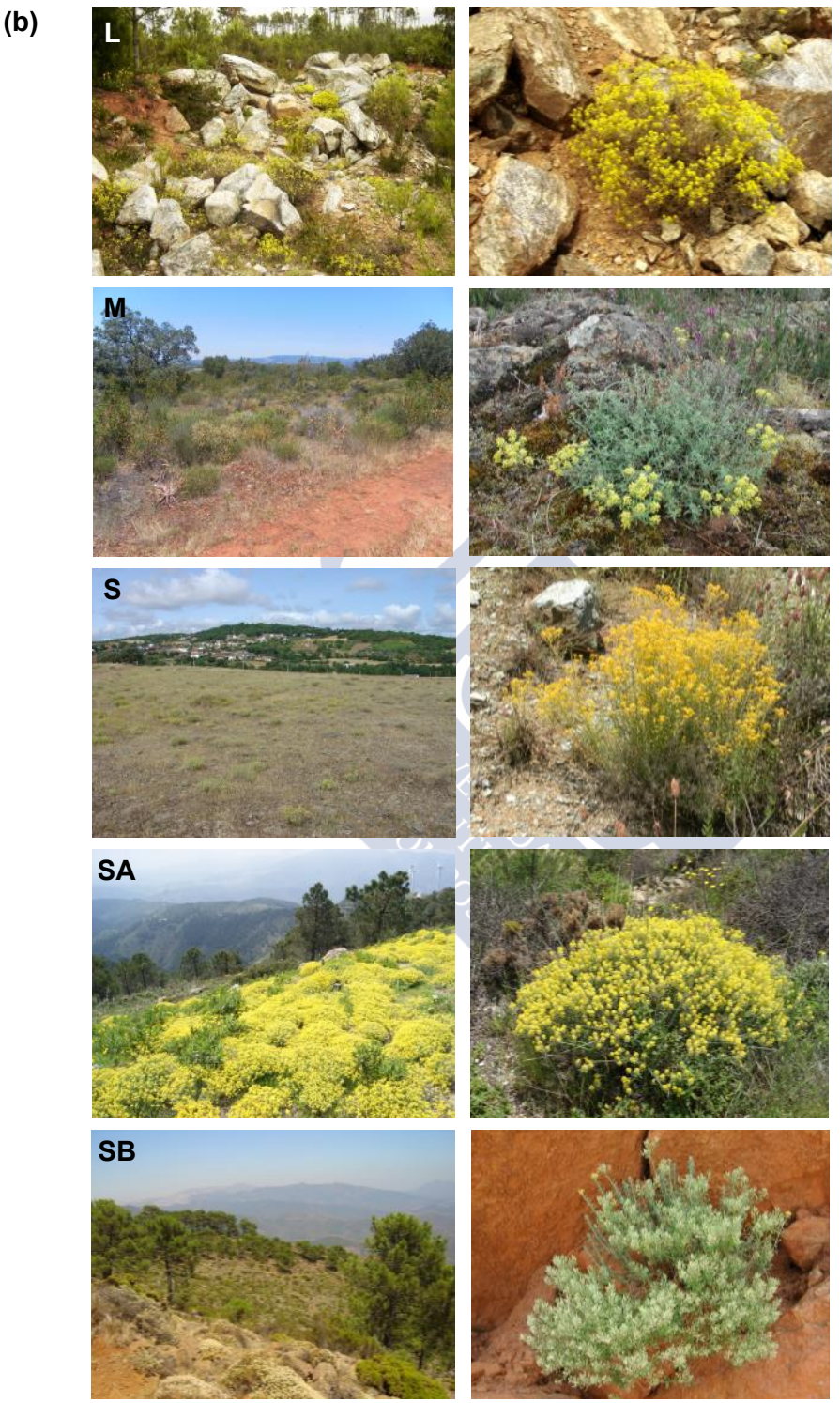


Figure 3.1. Geological maps of the main serpentine outcrops in the Iberian Peninsula (a) and photos of the sampling sites (b) Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB).

Aguas (SA) and Sierra Bermeja (SB). *A. pintodasilvae* is the only Ni hyperaccumulator in NE Portugal and was first described as a Ni hyperaccumulator in 1969 (Menezes de Sequeira 1969). The Spanish population of this subspecies (L) has been suggested to differ sufficiently from the Portuguese populations so as to merit classification of a new species, and is sometimes referred to as *Alyssum guitianae* Brooks (Rodriguez-Oubiña and Ortiz 1991). However, recent studies observed that differentiation within all Iberian populations of *A. serpyllifolium* was weak and no phylogenetic divergence was found between them, supporting their conspecific status (Cecchi *et al.* 2013).

A non-destructive sampling technique was carried out in which aerial biomass (stems plus leaves) and seeds from fifteen random individual (mother) plants were collected from each of the three populations of *A. pintodasilvae* (L, M and S) and two populations of *A. malacitanum* (SA (only shoot biomass) and SB). Seeds were stored in glassine seed envelopes at 4 ºC.

In addition, the whole plant and root system (including the root ball) of five to seven individuals were collected at each site in order to obtain the rhizosphere soil. The rhizosphere soil was operationally defined as the soil attached to roots after gentle crushing of the root ball and shaking the root system. Tightly held soil (<3 mm from the root surface) was considered rhizosphere soil. To the extent possible, root debris included in the collected rhizosphere soil were removed using tweezers or by sieving. Finally, five surface soil samples (0-15 cm, coinciding with the depth where most *Alyssum* roots were present) were collected at each site from bare patches where no plants were found growing (non-vegetated soil).

Elemental analysis of field-collected soils and plant material

Soil samples (non-vegetated and rhizosphere soil) were air-dried and sieved through a 2-mm stainless steel sieve. Soil pH was measured in H_2O and KCl using a 1:2.5 soil:solution ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Exchangeable cations (Ca, Mg, Al, Na and K) were extracted with 1 M NH_4Cl and determined by inductively coupled plasma optical emission spectrometry (ICP-OES, model Vista-PRO, Varian). Soils were digested in a 3:1 mixture of concentrated HNO_3:HCl and the total concentrations of Co, Cr y Ni were analysed by ICP-OES. Soil metal availability was evaluated after extraction with 1 M NH_4Cl (16 h shaking) and after extraction with 10 mM $Sr(NO_3)_2$ according to the method described by Everhart *et al.* (2006). A metal fractionation scheme was carried out following a modified BCR protocol (Rauret *et al.* 1999). This targets the following metal fractions: water-soluble, exchangeable and carbonate-bound metal fraction (exchangeable; 0.11 M CH_3COOH, 16h shaking); iron and manganese

oxide-bound forms (reducible; 0.1 M $NH_2OH.HCl$ adjusted to pH 2.0 with high purity HNO_3, 16 h shaking); organically bound and sulphide metals (oxidisable; the residue is digested with 30 % H_2O_2, taken to dryness on a water bath heated to 85 ºC, and shaken with 1M NH_4OAc adjusted to pH 5.0 for 16 h) and finally, residual fraction (silicate-bound metals; acid digestion as above). The concentrations of Ni, Co and Cr were analysed in the filtered supernatants of each extraction by ICP-OES. All metal concentrations were expressed in mg kg^{-1} dry weight (DW) soil.

Plant material collected in the field was separated in leaves, stems and roots, and washed with pressurised tap water followed by deionised water, oven-dried at 45 ° C, weighed and ground. Plant tissues (approximately 0.1 g) were digested in a 2:1 concentrated HNO_3:HCl mixture on a hot plate at 160 ºC, and the concentration of Ca, Co, Cr, K, Mg, Ni and P were measured by ICP-OES and expressed in mg kg^{-1} DW plant material. The Bioconcentration Factor (BCF) was calculated as the ratio of the shoot Ni concentration and the pseudo-total Ni concentration in the soil.

Seed germination and plant growth in controlled conditions: serpentine-like hydroponic nutrient solution and serpentine soils

Plant cultivation in hydroponic nutrient solutions

Seeds were obtained from fifteen individual (mother) plants at each site, with the exception of Sierra Aguas where they were not mature at the time of sampling. Seeds were allowed to germinate on plastic flats filled with a 2:1 perlite:quartz sand mixture (2:1 v/v) in a growth chamber under controlled conditions (temperature 22-25 ºC, PPFD of 190 mmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle). Seeds were watered daily with deionised water until germination and thereafter with a serpentine-like nutrient solution which consisted of 2 mM $MgSO_4$, 0.8 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 0.1 mM K_2HPO_4, 75 µM KCl, 20 µM FeEDDHA chelate, 10 µM H_3BO_3, 2 µM $MnCl_2$, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.2 µM Na_2MoO_4 and 10 µM $NiSO_4$ in deionised water (based on Chaney *et al.* 2009). Seedlings were maintained in these flats for four weeks (for the hydroponic solution experiment) and for 8 weeks (for the soil experiment) after germination.

For each of the four plant populations (L, S, M and SB), 30-45 seedlings (progeny) from each mother plant (10-15 mothers) were transplanted into various 2.5 L plastic trays containing the nutrient solution described above. The nutrient solution also contained 2 mM 2-(N-morpholino)ethanesulfonic acid (MES) to buffer the solution pH at 7.0. Seedlings were suspended from 1 cm thick

polystyrene trays (floating on the solution surface) and remained in this nutrient solution without Ni treatment for 30 days as an adaptation period to allow the root system to recover from transplanting. The nutrient solution was continuously aerated. After this time seedlings were treated with three different Ni concentrations in the nutrient solution: 32 µM (Control), 320 µM (Low-Ni) and 1000 µM (High-Ni), added as $NiSO_4.6H_20$. The Ni concentration range was in line with that of other hydroponic cultures based on *Alyssum* species (Centofanti *et al.* 2013; Chaney *et al.* 2008). For each plant population, ten to fifteen progeny from each mother were grown in each Ni treatment. Nutrient solutions were renewed every 3 days; this was the optimum period for ensuring a constant Ni concentration of each treatment. The hydroponic experiment was carried out in a growth chamber under controlled conditions (temperature 22-25 ºC, PPFD of 190 mmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle). Plants were harvested after four weeks of treatment and shoots and roots were separated, washed, dried at 45 °C and weighed to determine DW yield.

Plant cultivation in serpentine soils

For each of the four plant populations (L, S, M, and SB), six seedlings (progeny) from at least 10 mother plants (6 in the case of SB) were transplanted into 500 mL plastic pots filled with serpentine soil (in total 60 pots were used). Seedlings were selected for a similar size (2-3 cm tall) and plants were watered every other day with deionised water. The experiment was carried out in a growth chamber under controlled conditions (temperature 22-25 ºC, PPFD of 190 mmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle). Plants were harvested 4 months after transplanting: shoots and roots were separated, washed in pressurised tap water to remove any adhering soil particles and rinsed in deionised water, dried at 45 °C and weighed to determine DW yield.

The soil used in this experiment was collected from the A horizon of an Eutric leptosol (Magnesic) profile in the serpentinitic region of Melide (L). Soil was air-dried, sieved through an 8-mm stainless steel sieve and mixed for pot preparation and soil analysis. Perlite was added to the soil in the ratio of 10:1 (v/v) to improve aeration and drainage.

Analysis of plant material obtained from experiments in controlled conditions (hydroponic nutrient solution and in serpentine soil)

Plant tissues were washed and acid-digested as described above (Section Elemental analysis of field-collected soils and plant material) and the concentrations of Ca, Co, Cu, Fe, K, Mg, Mn, Ni and P were measured by

ICP-OES. Results were expressed in mg kg^{-1} DW plant material/soil. Shoot:root Ni concentration ratio was determined as the Ni concentration in shoots divided by the Ni concentration in roots. The Ni phytoextracted (soil Ni removal) was calculated as the product of the shoot DW and the Ni concentration in shoots. For the hydroponic experiment, the Tolerance Index (TI) for shoot and roots was calculated as the mean DW value in either the Low/High-Ni treatment divided by the mean DW value in the Control treatment. For the pot experiment, the Bioconcentration Factor (BCF) was calculated as the ratio of the shoot Ni concentration and the pseudo-total Ni concentration in the soil.

Statistical methods

Differences in soil physicochemical parameters between rhizosphere and non-vegetated soils were determined using analyses of variance (ANOVA). A multiple comparison of means was determined by the "post-hoc" Least Significance Difference (LSD) test.

ANOVAs were performed to examine the main effects of plant populations and Ni treatment (in the case of the hydroponic experiment) on the DW yields, Ni accumulation, shoot to root Ni transport and nutrient status of plants. To confirm the requisites for ANOVA applications Shapiro Wilk or Lilliefors and Levene tests were applied. If requisites were not satisfied a non-parametric test Kruskal-Wallis was used. Following ANOVA or Kruskal-Wallis test, the Tukey's test was used for pairwise comparisons. To analyse the inter- and intra-population variability of the studied variables a mixed model was used. The population was considered as fixed effect and mother plants from the field were considered as random effect. In general, the proportion of explained variance by the random effects was very small, less than the 1 %. The model was also applied without considering the random effect. The relative quality of both models were compared using the Akaike Information Criterion (AIC) and the application of a simple model without random effects was the more adequate option. In order to evaluate any potential relationship between the studied variables in sampled soils and plant tissues the Pearson correlation coefficient was calculated. In addition the Pearson coefficient was used to analyse a potential correlation between the characteristics of mother plants from the field and their progeny grown in controlled conditions. The Pearson correlation was considered significant when $r \geq |0.7|$.

The statistical software used was R, version R-3.1.1, and the packages were nortest (Gross and Ligges 2012), nlme (Pinheiro *et al.* 2007) and lme4 (Bates *et al.* 2012) for the fixed model.

3.3 *RESULTS*

Soil physicochemical properties, metal content and fractionation, and plant metal accumulation

Physicochemical properties of soils from the five study areas

The physicochemical characteristics of the non-vegetated topsoils of each of the five sites were typical of serpentine soils (Table 3.2). All soil samples presented pH values close to neutrality, ranging from 6.9 to 7.3, with the exception of Sierra Aguas (SA) where the soil pH was alkaline and significantly higher

Table 3.2. Physicochemical characteristics of soils from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB). Different letters denote significant differences between populations (P <0.05).

Soil properties	L	M	S	SA	SB
pH_{H2O}	7.3 ±0.1a	6.9 ±0.0b	7.0 ±0.1b	8.4 ±0.1c	7.2 ±0.0a
% C	1.15 ±0.19a	3.17 ±0.12c	2.10 ±0.26bc	2.44 ±0.41c	1.58 ±0.03ab
% N	0.10 ±0.01b	0.28 ±0.01d	0.22 ±0.02c	0.03 ±0.01a	0.11 ±0.01b
C/N	11.5 ±0.7ab	11.2 ±0.2ab	9.6 ±0.4a	42.9 ±4.9c	14.3 ±1.5b
Exchangeable cations ($cmol_c\ kg^{-1}$)					
Ca^{2+}	0.9 ±0.0a	3.2 ±0.1b	3.3 ±0.1b	11.4 ±1.0c	2.6 ±0.1ab
Mg^{2+}	12.3 ±0.2ab	14.7 ±0.2ab	16.7 ±0.2b	32.5 ±3.0c	10.8 ±0.3a
Na^{+}	0.07 ±0.00a	0.04 ±0.00a	0.06 ±0.02a	0.06 ±0.01a	0.06 ±0.00a
K^{+}	0.07 ±0.00a	0.31 ±0.01c	0.18 ±0.01b	0.19 ±0.06b	0.18 ±0.00ab
CEC	13.2 ±0.2a	18.3 ±0.3ab	20.2 ±0.3b	44.1 ±3.3c	13.7 ±0.3a
Ca/Mg	0.1 ±0.0a	0.2 ±0.0b	0.2 ±0.0b	0.4 ±0.0c	0.2 ±0.0b
Pseudo-total metal concentration ($mg\ kg^{-1}$)					
Ni	2553 ±130c	2745 ±39c	2713 ±15c	1811 ±97a	2293 ±34b
Co	170 ±11d	182 ±4d	141 ±2c	68 ±5a	105 ±2b
Cr	958 ±44a	3560 ±64c	847 ±82a	827 ±27a	1408 ±62b
$Sr(NO_3)_2$-extractable metal concentration ($mg\ kg^{-1}$)					
Ni	2.87 ±0.00e	1.49 ±0.01d	1.27 ±0.06c	0.25 ±0.02a	1.01 ±0.03b
Co	0.023 ±0.002c	0.000 ±0.002a	0.010 ±0.006ab	0.004 ±0.003a	0.020 ±0.004bc

compared to the other populations (pH 8.4; P <0.05). Soil total C and N were generally low, with values ranging from 1.15 to 3.17 % and from 0.03 to 0.28 %, respectively. The highest contents of total C and N were generally found in Portuguese serpentine soils (S and M), although for total C a similar value was observed in soil from SA (Table 3.2). Soil from Melide (L) presented the lowest total C (1.15 %), and total N was lowest in L, Sierra Bermeja (SB) and SA (dropping to 0.03 % in SA). As a result of the low % N in SA soil this site presented a significantly higher C/N ratio (42.9) compared to the other sites (C/N ranged from 9.6 to 14.3) (P <0.05; Table 3.2). Cation exchange capacity (CEC) varied from 13.2 to 44.1 $cmol_c$ kg^{-1} and was significantly higher in SA (P <0.05) compared to the other sites. Exchangeable Ca and Mg, as well as the Ca/Mg quotient were significantly greater in SA compared to the other sites (P <0.05). The lowest Ca/Mg ratio was observed in L (with values as low as 0.07). However, all the soils were characterised by a predominance of Mg in the exchange complex, showing a Ca/Mg quotient <1 in all five sites (Table 3.2).

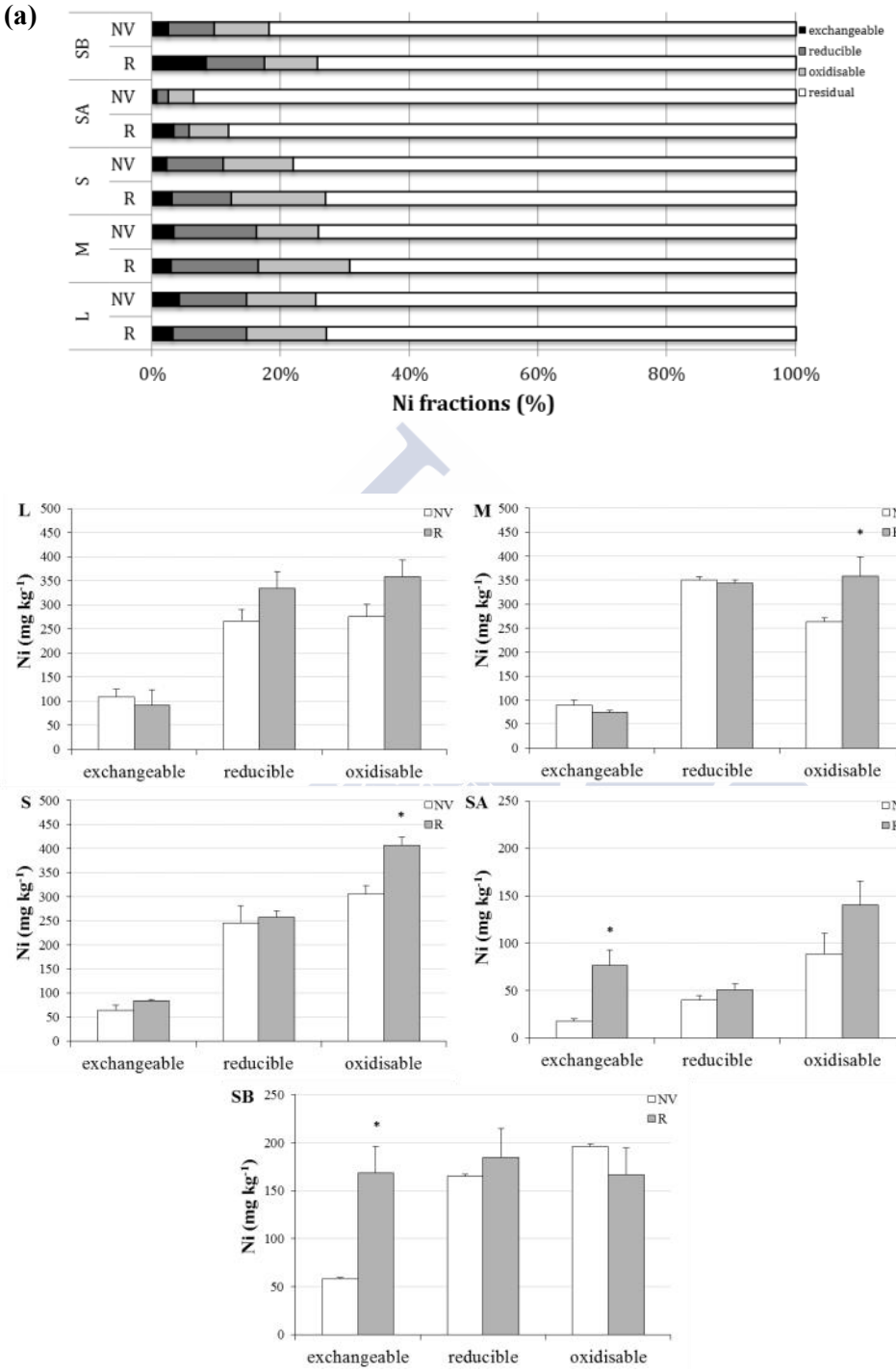
The mean concentration of total Ni in the soils varied from 1811 to 2745 mg kg^{-1} (Table 3.2). The lowest soil Ni concentrations were found in the S Spain serpentine sites (SA and SB). In contrast, L, M and S presented total Ni concentrations of a similar magnitude (2553-2745 mg kg^{-1}), and these were significantly higher compared to either SA or SB (P <0.05; Table 3.2). The total Cr concentration in the soils varied from 827 to 3560 mg kg^{-1}, and the M soil showed the highest total Cr concentration (2.5- to 4.2-fold higher than the other soils). Total Co concentrations were significantly lower than both Ni and Cr. Concentrations of this metal were similar in L and M (170 and 182 mg kg^{-1}, respectively), and significantly lower in S, SB and SA (141, 105 and 68 mg kg^{-1}, respectively) (P <0.05; Table 3.2). The M soil consistently presented the highest concentration of all three metals (although in the case of Ni this difference was not statistically significant), whereas the lowest concentrations of Ni, Cr and Co were found in the SA soil. The concentration of plant-available Ni, estimated using the $Sr(NO_3)_2$ extraction, ranged from 0.25 to 2.87 mg kg^{-1}, following the decreasing order: L>M>S>SB>SA. This order more or less coincides with the total soil Ni concentration that is, the sites in NW Spain and NE Portugal (L, M and S) presented a higher total Ni concentration and also present a higher $Sr(NO_3)_2$-extractable concentration, while the lowest concentrations in both total and $Sr(NO_3)_2$-extractable Ni were found in the SA (Table 3.2). This $Sr(NO_3)_2$-extractable fraction represented at most 0.1 % (in L soil) of the total Ni concentration in the soil. The $Sr(NO_3)_2$-extractable Co concentrations were at least two orders of magnitude lower (ranging from below the detection limit to 0.023

mg kg^{-1}). SA was the only site where detectable concentrations of available Cr were found (0.047 mg kg^{-1}).

The sum of the Ni, Co and Cr fractions (exchangeable, reducible, oxidisable and residual) corresponded well with the total concentrations of each metal. The majority of Ni in all the soils was found in the residual fraction (representing 74-94 % of total Ni) (Fig. 3.2a). Of the non-residual fractions, Ni was primarily found to be associated with organic matter (4-11 %) and Fe and Mn oxides (2-13 %). A minor percentage of the total concentration was found in the exchangeable pool (1-4 %). L, M and S presented a significantly higher Ni concentration in exchangeable, reducible and oxidisable fractions compared to SA and SB (P <0.05; Fig. 3.2a). Like Ni, the residual fraction was also dominant for Cr (representing more than 95 % of the total Cr concentration). After the residual fraction, Cr was found mainly in association with organic matter (this pool represented from 0.5 % (in M) to 4.3 % (in L and SB). In contrast, the residual pool was not always dominant in the case of Co, where the majority of this metal was often associated with Fe and Mn oxides. That is, the reducible fraction of Co represented 51-66 % of the total Co, with the exception of the SA soil where only 15 % of Co was bound to Fe and Mn oxides (Fig. 3.2b). The reducible fraction of Co found in SA (12.3 mg kg^{-1}) was significantly lower compared to the other sites, whereas the highest concentrations were observed in M (114.0 mg kg^{-1}) (P <0.05). The percentage of the exchangeable Co pool varied from 1 to 5 % and the percentage bound to organic matter (oxidisable fraction) from 5 to 18 %. The M and SB showed significantly higher exchangeable Co concentrations compared to the other sites (5.8 and 5.6 mg kg^{-1}, respectively), whereas the highest concentrations in Co oxidisable fraction were observed in M and SA, 13.3 and 15.3 mg kg^{-1}, respectively (P <0.05). The residual fraction was dominant in SA (representing 66 % of the total concentration compared to 21-42 % in the remaining soils) (Fig. 3.2b).

Influence of plant root activity on soil physicochemical properties and metal bioavailability

The influence of plant root activity on the physicochemical characteristics of the soil differed according to the plant population and site. Significant differences were observed between the non-vegetated (NV) soil pH and the rhizosphere (R) soil pH in M, S and SA. The R soil pH was significantly higher in M and S compared to NV soil (P <0.05; Fig. 3.3): in the case of M the pH increased from 6.9 to 7.3, and in S from 7.0 to 7.4. In contrast, in SA, pH values were slightly lower in the R soil than the NV soil (the mean pH value decreased from 8.4 to 8.2 (P <0.05). In L and SB, no significant differences were observed in soil pH (Fig. 3.3).


(a)
SB
SA
S
M
L
NV
R
exchangeable
reducible
oxidisable
residual
0%
20%
40%
60%
80%
100%
Ni fractions (%)
L
M
S
SA
SB
Ni (mg kg-1)
exchangeable
reducible
oxidisable
*

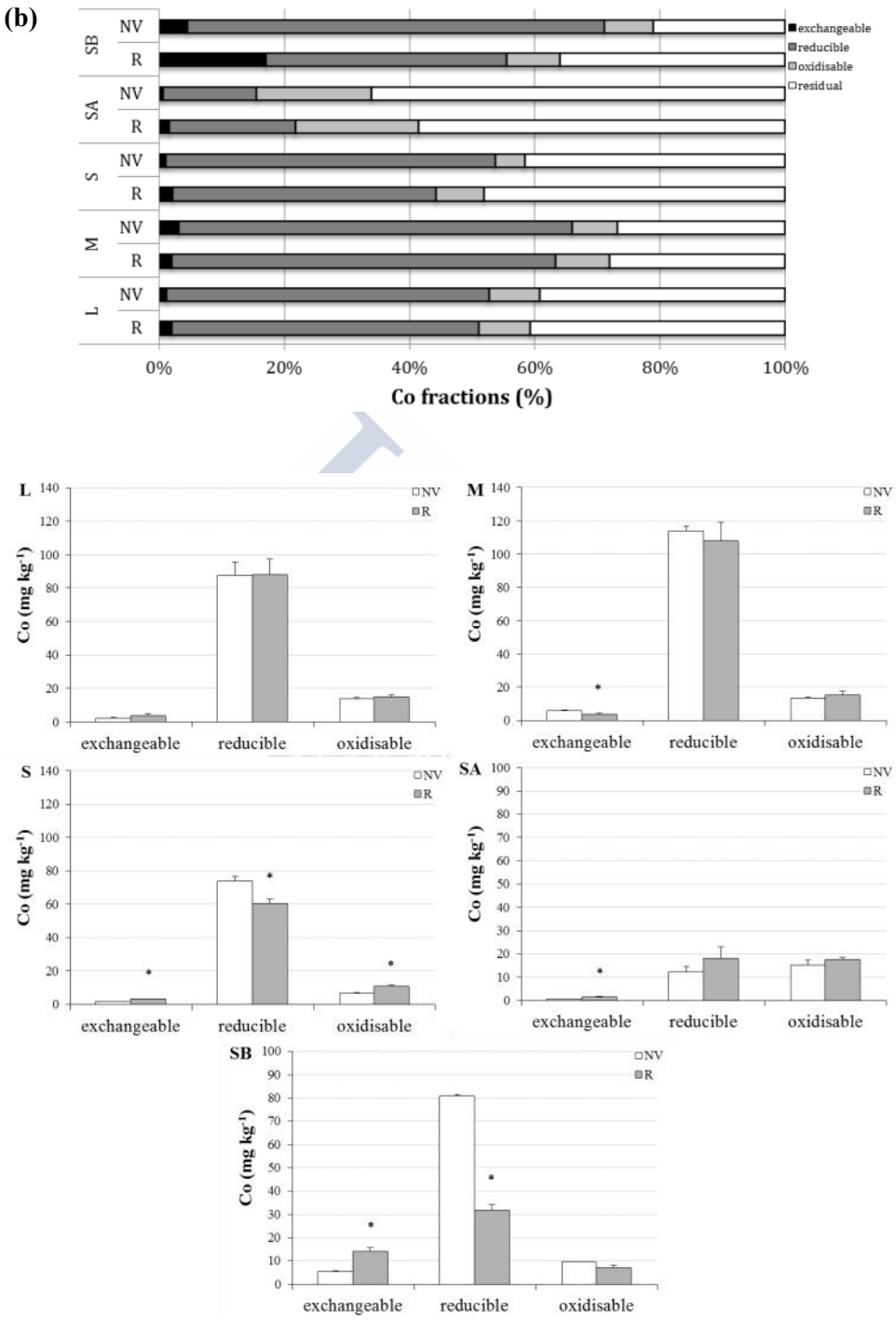


Figure 3.2. Ni (a) and Co (b) fractionation (%, mg kg^{-1}) in the non-vegetated (NV) and rhizospheric (R) soil from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB). Asterisks denote significant differences between NV and R soil for each metal fraction (P <0.05).

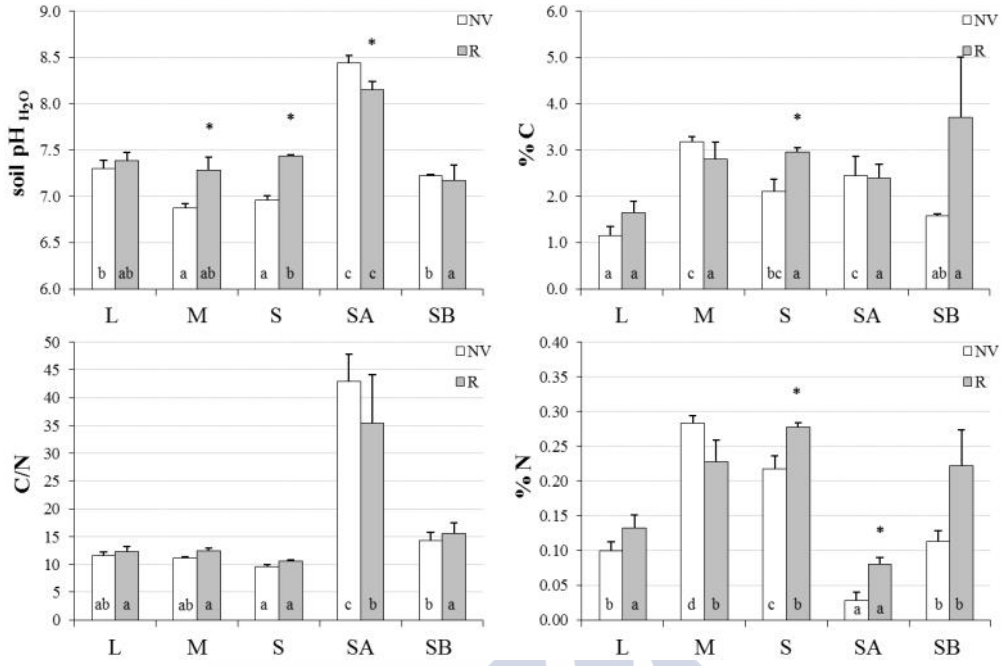


Figure 3.3. Physicochemical properties (pH, % C, % N and C/N ratio) of non-vegetated (NV) and rhizosphere (R) soil from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB). Significant differences between NV and R soil for each population are shown by asterisks (P <0.05). Different letters denote significant differences in either NV or R soils between the five populations (P <0.05).

Rhizosphere soils generally presented a higher total C and N content compared to non-vegetated soils (albeit not always statistically significant). This was observed in L, S, SA (only total N) and SB (but was only statistically significant in S and SA (only total N)) (Fig. 3.3). In the case of S, total C and N content increased from 2.1 % and 0.2 % in non-vegetated soil, respectively, to 2.95 % and 0.28 %, in rhizosphere soil, respectively (P <0.05). Likewise, in the case of SA, the total N content increased from 0.03 to 0.08 % (P <0.05). No significant differences in the C/N ratio between rhizosphere soil and non-vegetated soil were observed, and this was the case for all five sites.

As observed in non-vegetated soils, the CEC of rhizosphere soils was dominated by Mg. Rhizosphere soil tended to present a higher CEC than non-vegetated soil (except for L and SB), although this was only significant in the case of M where CEC increased from 18.3 $cmol_c\ kg^{-1}$ in non-vegetated soil to 25.0 $cmol_c\ kg^{-1}$ in rhizosphere soil (P <0.05; Fig. 3.4). In contrast, a small but significant decrease in CEC was observed in the rhizosphere soil of SB compared to non-vegetated soil (11.4 and 13.7 $cmol_c\ kg^{-1}$ in R and NV, respectively). An

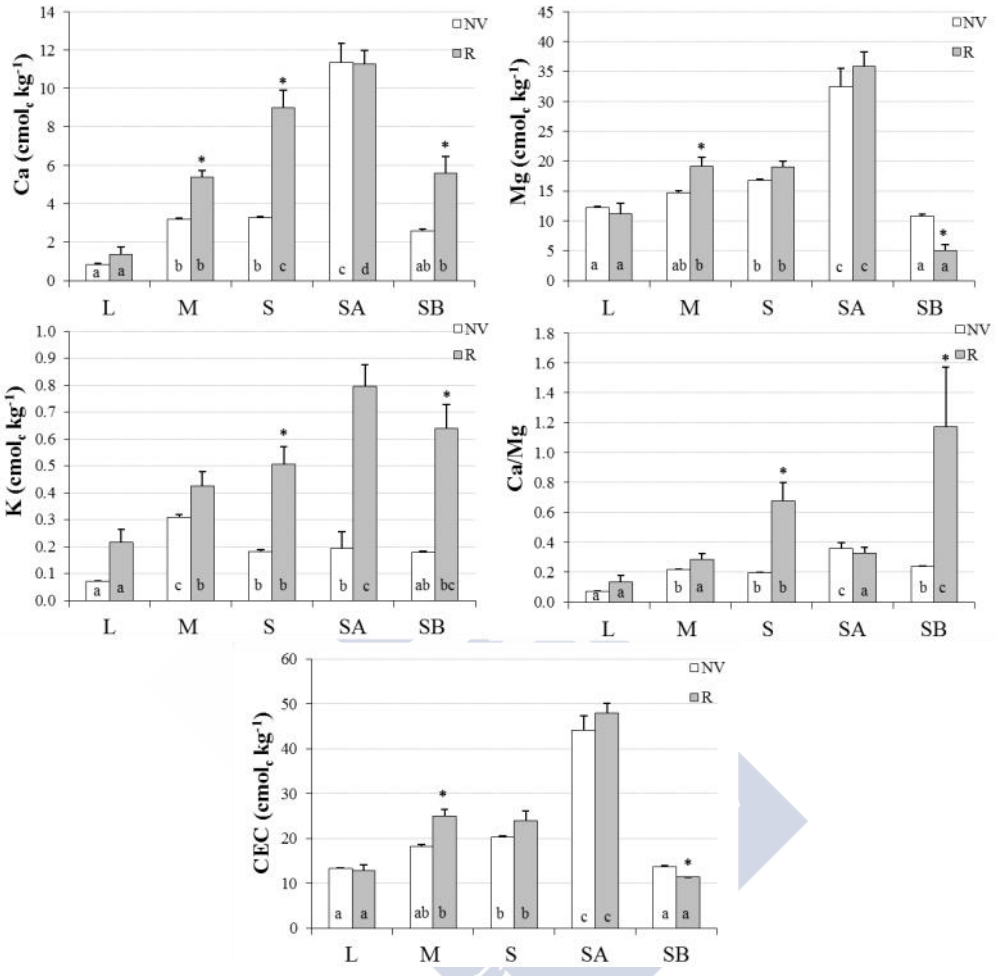


Figure 3.4. Exchangeable cations (Ca^{2+}, Mg^{2+}, K^{+}), Cation Exchange Capacity (CEC) and the Ca/Mg ratio of non-vegetated (NV) and rhizosphere (R) soils from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB). Significant differences in rhizospheric soil compared to the non-vegetated soil from each population are shown by asterisks (P <0.05). Different letters denote significant differences between populations (P <0.05).

increase in exchangeable Ca, K and Mg (except in SB where exchange Mg was significantly lower in the R soil) was generally observed in rhizosphere soils (albeit not always significantly) (Fig. 3.4). In all populations (except SA) a higher Ca/Mg ratio was found in the rhizosphere soil compared to the non-vegetated soil (Fig. 3.4). In the case of S and SB this Ca/Mg ratio increased significantly from 0.2 to 0.7 and from 0.2 to 1.2 in NV and R soil, respectively (P <0.05).

In general, the total metal (Ni, Cr and Co) concentrations did not differ significantly between the NV and R soils. However, in some cases significant

differences were found. For example, in L, the rhizosphere soil of *A. pintodasilvae* presented a significantly higher Ni concentration than the non-vegetated soil: the total Ni concentration in non-vegetated soil was 2553 ±130 mg kg^{-1} while in rhizosphere soil it was 3157 ±47 mg kg^{-1} (Fig. 3.5). In contrast, the total concentration of both Ni and Cr were significantly lower in the rhizosphere soil in M: the Ni concentration decreased from 2745 ±39 to 2513 ±56 mg kg^{-1}, and the Cr concentration from 3560 ±64 to 2040 ±145 mg kg^{-1} (P <0.05). Similarly, a significantly lower concentration of total Ni, Cr and Co was found in the rhizosphere soil of *A. malacitanum* compared to non-vegetated soil in SB: total metal concentrations were 2293 ±34 (Ni), 1408 ±62 (Cr) and 105 ±2 (Co) mg kg^{-1} in NV soil and 1814 ±66 (Ni), 653 ±78 (Cr) and 69 ±4 (Co) mg kg^{-1} in R soil.

Concentrations of $Sr(NO_3)_2$- or NH_4Cl-extractable metals did not follow the same order as total concentrations. For example, soils from M, which presented the highest total concentrations, showed relatively low concentrations of $Sr(NO_3)_2$- and NH_4Cl- extractable Ni (Fig. 3.5). Concentrations of $Sr(NO_3)_2$-extractable Ni were significantly higher in the rhizosphere of L, S, SA and SB compared to corresponding non-vegetated soils (P <0.05; Fig. 3.5). This was most pronounced in the S Spain populations, where Ni concentrations increased by 6.2- to 11.7-fold (SA and SB, respectively). In L and S populations the Ni concentration increased by 1.5- to 4.2-fold (L and S, respectively). The highest $Sr(NO_3)_2$- and NH_4Cl- extractable Ni were observed in L populations (only in NV soil in the case of $Sr(NO_3)_2$-extractable Ni) (Fig. 3.5).

A similar pattern was found for Co, where a significantly higher $Sr(NO_3)_2$-extractable concentration was observed in the rhizosphere of L, S and SB (0.09 ±0.00, 0.11 ±0.03 and 0.11 ±0.02 mg kg^{-1}, respectively) compared to the corresponding non-vegetated soils: concentrations were between 4.1- and 11.5-fold higher (P <0.05). In this case the increase was most pronounced in S. With the exception of SA, $Sr(NO_3)_2$-extractable Cr concentrations were <0.01 mg kg^{-1}. In SA, a significant increase in $Sr(NO_3)_2$-extractable Cr, from 0.05 ±0.01 to 0.15 ±0.03 mg kg^{-1}, was observed in the R soil compared to NV soil (P <0.05).

The effect of plant root activity on the soil metal fractionation varied according to the metal fraction in question and the plant population. As observed in NV soils, the residual Ni fraction was the dominant pool for this metal in R soils (Fig. 3.2a). However, the residual Ni fraction in the rhizosphere of all the plant populations was lower than in their corresponding non-vegetated soils: when expressed as a % of the total Ni, this fraction decreased from 74.2-93.6 % in NV soil to 69.4-88.1 % in R soil (P <0.05; only significant in the case of M and SB). This effect was most pronounced in SB. Apart from the general decrease in the residual fraction the most important plant-induced change in Ni fractionation was an increase in the Ni concentration associated with organic matter (Fig. 3.2a). This

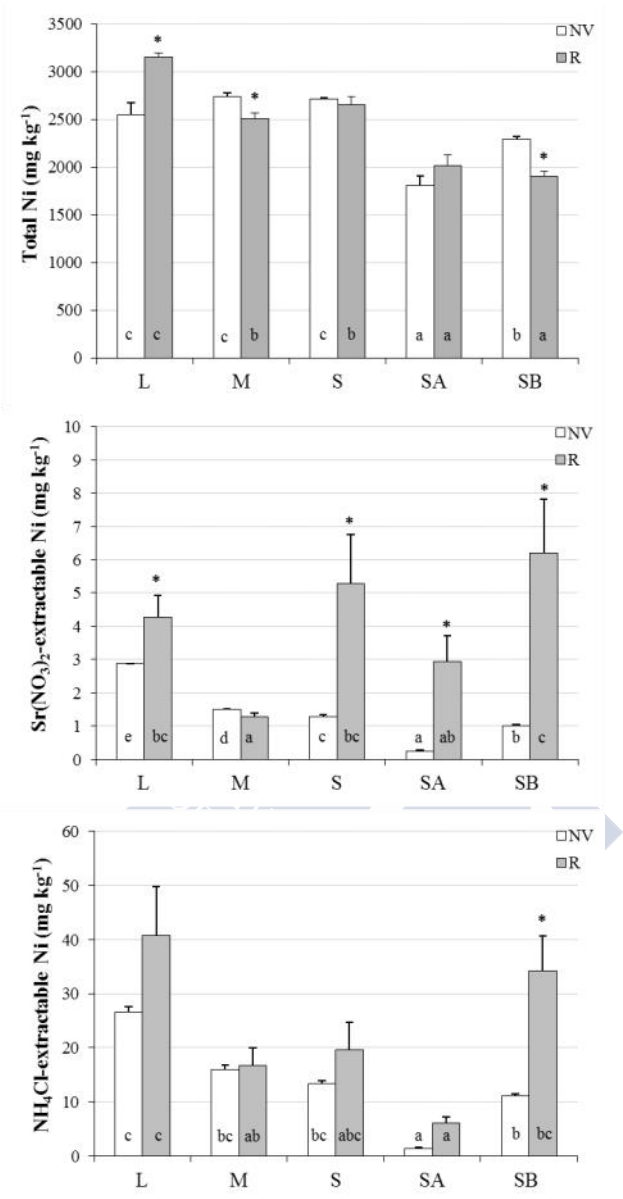


Figure 3.5. $Sr(NO_3)_2$- and NH_4Cl- extractable Ni concentrations (mg kg^{-1}) in the non-vegetated (NV) and rhizospheric (R) soil from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB). Significant differences in rhizospheric soil compared to the non-vegetated soil from each population are shown by asterisks (P <0.05). Different letters denote significant differences between populations (P <0.05).

effect was observed in all populations, but it was statistically significant only in M and S populations (from 263 and 306 mg kg^{-1} in non-vegetated soil to 359 and 408 mg kg^{-1} in rhizosphere soil, respectively) (P <0.05; Fig. 3.2a). A slight increase in the reducible Ni fraction was found in the rhizosphere, but this was not statistically

significant. In the populations from the South of Spain, SA and SB, a strong increase in the exchangeable pool was observed in the R soil compared to NV soil; this increase ranged from 0.8 % to 3.4 % in SA and from 2.5 to 8.3 % in SB (P <0.05; Fig. 3.2a). In the case of Cr, the residual fraction represented up to 99 % of the total Cr in the R soils. A slight increase in Cr associated with organic matter (oxidisable fraction) was observed in the R soils of all the populations (in S and SB this was statistically significant, and increased from 1.7 to 2.4 % and from 4.3 to 5.8 %, respectively) (P <0.05; Fig. 3.5). Significant changes in the R soil were also observed in Co fractionation (P <0.05; Fig. 3.2b). The most pronounced changes in all Co fractions were observed in the rhizosphere of *A. pintodasilvae* of S and *A. malacitanum* of SB. A lower % of total Co tended to be associated with Fe and Mn oxides in the rhizosphere: this reducible fraction decreased from 52.5 to 42.1 % in S and from 66.5 to 38.3 % in SB. On the other hand, a slight increase in Co associated with organic matter was observed in the rhizosphere: in S this fraction represented 4.7 % in NV soil and increased to 7.6 % in R soil. A small but significant increase in the exchangeable pool of Co was also observed in the rhizosphere of L (not significant), S, SA and SB populations (P <0.05; Fig. 3.2b). This was particularly pronounced in SB, where exchangeable Co increased from 4.6 to 17.1 % (from 5.6 to 14.2 mg kg^{-1}), representing an increase of 3.7-fold. In contrast, in the rhizosphere soil of M this exchangeable fraction showed a significant decrease from 3.2 to 2.1 % (from 5.8 to 3.7 mg kg^{-1}) (Fig. 3.2b).

In general, in both NV and R soils the concentrations of Ni and Co were significantly correlated, and this was observed in all the populations (r=0.80, P <0.05). This positive correlation between these two elements was found for most of the metal fractions. In addition, a significant negative correlation between the soil Ca/Mg ratio and the different Ni fractions was observed in both soils, with the exception of SA for non-vegetated soil and L for rhizosphere soil. In the rhizosphere soil of SB, the Ca/Mg ratio and all Co and Cr fractions were also negatively correlated (P <0.05).

Plant ionome and nickel accumulation

Table 3.3 presents the mean concentration of macronutrients and trace elements in the stem and leaf tissues for each plant population, while Fig. 3.6 presents the plant Ni concentrations. A wide variability was observed in the plant ionome amongst the different populations: mean concentrations of Ca, K, Mg and P varied by up to 7.8-, 2.9-, 6.9- and 3.6-fold, respectively. In the leaves, the larger part of the total variance of both nutrient and trace element (Ni, Co and Cr) concentrations was found to be amongst the populations (inter-population) rather than within populations (intra-population) (Table 3.4 and Fig. 3.6). In stem tissues the larger part of the total variance of Ca, Co and Cr concentrations was explained

Table 3.3. Macro- and micro-nutrient concentrations in *Alyssum pintodasilvae* and *Alyssum malacitanum* (mean concentration ± standard error) from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB). Different letters denote differences between populations (P <0.05).

Plant tissue	Population	Ca	Mg	P	K	Co	Cr	Ca/Mg
		g kg^{-1}				mg kg^{-1}		
Leaf	**L**	24.0 ±2.0ab	15.8 ±1.5c	1.18 ±0.16cd	7.0 ±0.8b	16.8 ±1.7a	15.7 ±3.0b	1.8 ±0.3a
	M	18.7 ±1.0a	8.9 ±0.6b	0.57 ±0.05a	4.5 ±0.4a	8.7 ±1.0a	15.9 ±1.8b	2.2 ±0.1a
	S	34.4±1.3c	12.0 ±0.8c	1.36±0.05d	5.4 ±0.5ab	9.7 ±0.8a	19.9 ±1.6b	3.0 ±0.2b
	SA	30.1±2.0b	5.7 ±0.4a	0.95 ±0.09bc	10.7 ±0.7c	8.7 ±0.6a	1.1 ±0.3a	5.5 ±0.4c
	SB	26.7 ±1.0bc	7.8 ±0.6b	0.78 ±0.06b	4.5 ±0.5a	32.6 ±4.3b	18.0 ±1.4b	3.8 ±0.3b
Stem	**L**	4.4 ±0.4a	3.0 ±0.2a	0.38 ±0.04a	3.7 ±0.5a	2.4 ±0.3b	33.2 ±4.7c	1.5 ±0.2a
	M	6.5 ±0.5ab	2.3 ±0.2a	0.44 ±0.07a	4.3 ±0.3a	2.2 ±0.3b	12.9 ±2.6b	3.0 ±0.2bc
	S	6.9±0.3bc	2.5 ±0.2a	1.07 ±0.13b	4.4 ±0.2a	2.3 ±0.3b	9.2 ±1.1b	2.9 ±0.2b
	SA	23.3 ±1.3d	2.8 ±0.2a	0.65 ±0.09a	8.3 ±0.7b	0.3 ±0.1a	0.7 ±0.2a	8.9 ±0.8d
	SB	9.5 ±0.7c	2.8 ±0.2a	0.53 ±0.04a	4.8 ±0.4a	4.1 ±0.5b	13.1 ±1.6b	3.6 ±0.2c

by the population factor, while for Mg, K, P and Ni the variance was mainly explained by the intra-population variability (Table 3.4). In accordance, significant differences in concentrations were found between populations (P <0.05), with the

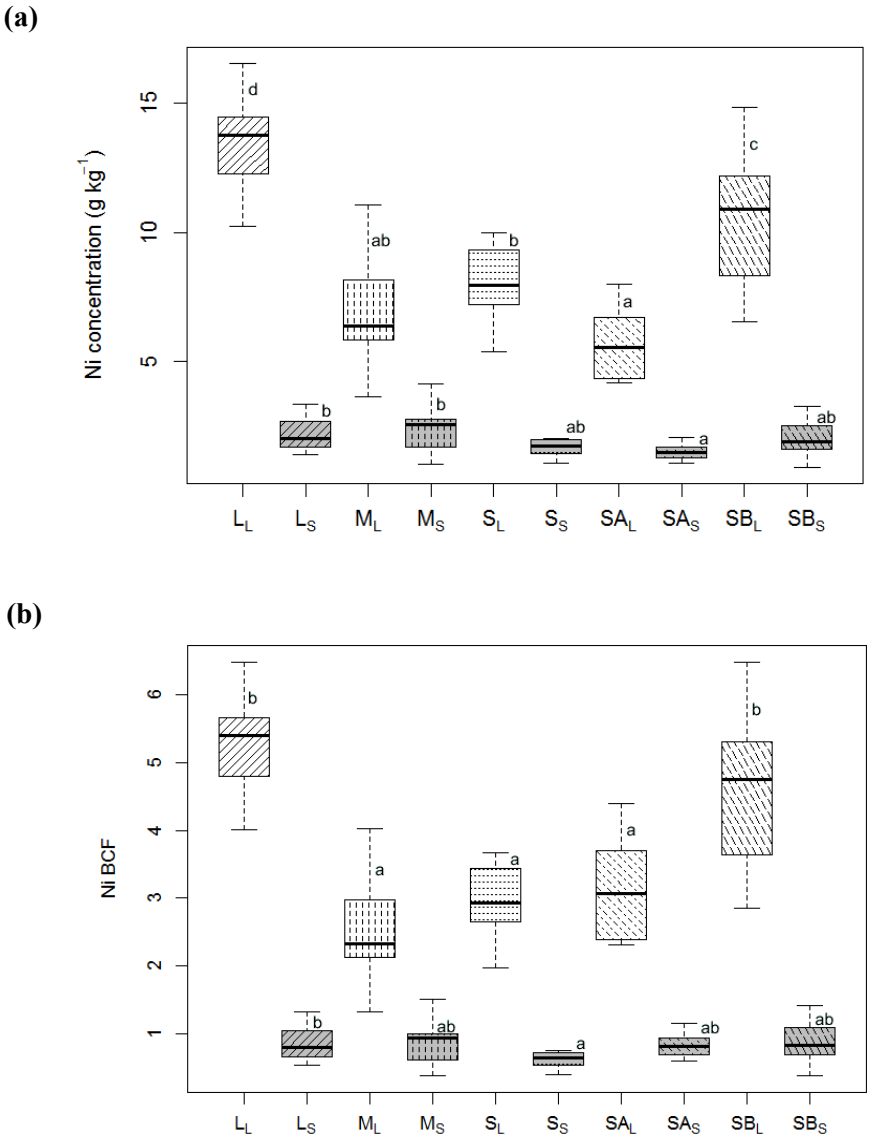


Figure 3.6. Ni concentration (a) and Bioconcentration Factor (BCF) (b) in leaves and stems of *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S), Sierra Aguas (SA) and Sierra Bermeja (SB) grown in the field. Subscripts L and S denote leaves and stems, respectively. Different letters denote significant differences between populations for either leaves of stems (P <0.05).

exception of the stem Mg concentration for which no significant differences were observed (P=0.09).

In general, all macronutrient concentrations in the leaves were significantly higher than in stems (Table 3.3; P <0.05). The highest leaf Ca concentrations were observed in S and SA populations (34.4 and 30.1 g kg^{-1}, respectively), whereas L and M populations showed the lowest leaf Ca concentrations (24.0 and 18.7 g kg^{-1}, respectively). Stem Ca concentrations followed a similar pattern (Table 3.3). Leaf Mg concentrations were significantly higher in L and S populations (15.8 and 12.0 g kg^{-1}, respectively) compared to the other populations (P <0.05). The stem Mg concentration was similar in all the populations and ranged from 2.3 to 3.0 g kg^{-1}. The Ca/Mg ratio in both leaves and stems was consistently >1, and varied from 1.8 to 5.5 and from 1.5 to 8.9, respectively (Table 3.3). It was also consistently highest in SA and lowest in L (P <0.05). Leaf P concentrations were highest in the L and S populations (1.18 and 1.36 g kg^{-1}, respectively) and lowest in M and SB populations (0.57 and 0.78 g kg^{-1}, respectively). Leaf K concentrations were highest in the L and SA (leaf and stem) populations (7.0 and 10.7 g kg^{-1}, respectively) and did not differ significantly in the remaining three populations (Table 3.3).

Significant differences were found in trace metal accumulation between populations (P <0.05; Table 3.3 and Fig. 3.6). As observed for macronutrients the stem concentrations of metals were generally lower than corresponding leaf concentrations (in the case of Ni stem concentrations were up to one order of magnitude lower), with the exception of Cr which showed similar concentrations in both plant parts. The leaf Ni concentrations followed the decreasing order: L>SB>S≈M>SA, whereas the stem Ni concentration decreased as follows: L≈M>SB>S>SA (Fig. 3.6a). Differences in Ni concentrations between populations were more pronounced in the leaves (up to 2.4-fold) than in the stems (up to 1.6-fold). No significant relation between leaf/stem Ni concentration and either the total soil Ni or available Ni concentration in the site of origin were found. The highest Ni concentrations were generally observed in the L and SB populations, with mean values of 13.6 and 10.5 g kg^{-1} in leaves, respectively, and 2.4 and 2.1 g kg^{-1} in stem tissues, respectively (Fig. 3.6a). In contrast, the lowest Ni concentrations were always found in the SA population (5.6 and 1.5 g kg^{-1} in leaf and stem tissues, respectively). Figure 3.6 shows the dispersion in leaf and stem Ni concentrations within each population (29.4 % and 78.6 % of variance in leaf and stem concentrations was attributed to intra-population variability, respectively; Table 3.4). Dispersion was greatest within the SB population where minimum and maximum values of 6.5 and 14.8 g kg^{-1} in leaf Ni concentrations were found. Plants from SB showed significantly higher leaf Co concentrations

Table 3.4. Inter- and intra-population variability (%) in macro- and micro-nutrient concentrations and Ni and Co Bioconcentration Factors (BCF) in *Alyssum pintodasilvae* and *Alyssum malacitanum* collected in the field.

	Variability (%)	Ca	Co	Cr	K	Mg	Na	Ni	P	Ca/Mg	Co BCF	Ni BCF
Leaf	**Inter-population**	56.1	55.6	51.5	58.4	61.6	78.7	70.6	52.6	62.7	52.0	66.7
	Intra-population	43.9	44.4	48.5	41.6	38.4	21.3	29.4	47.4	37.3	48.0	33.3
Stem	**Inter-population**	85.7	57.6	68.5	44.8	6.7	77.8	21.5	38.1	74.3	48.5	11.1
	Intra-population	14.3	42.4	31.5	55.2	93.3	22.2	78.5	61.9	25.7	51.5	88.9

(with a mean leaf Co concentration of 32.6 mg kg^{-1}), whereas no significant differences were observed in the remaining populations (Table 3.3). Leaf Cr concentrations were of a similar magnitude to Co concentrations, and few differences between populations were observed. A significantly lower leaf and stem Cr concentration were determined in the SA population (Table 3.3). Significant positive correlations between concentrations of macronutrients and trace metals in plant tissues were mainly observed in stem tissues. In stems of plants from L, M, S and SB, Ni concentrations were correlated with Ca and Mg concentrations (only Mg in the case of L and S) (r=0.7-0.9; P <0.05). Similarly, in stems of plants from SB, Co concentrations were correlated with Na and Cr contents (r=0.7, P <0.05). In stems of plants from S a positive correlation was observed between Cr, Ca and Mg (r=0.8, P <0.05) and in stems of plants from M a correlation was found between Cr and Mg (r=0.7, P <0.05), P and Ni (r=0.8, P <0.05). In leaves, a significant positive correlation was found between Ni and Mg in the population of M (r=0.8, P <0.05).

As observed in the plant tissue Ni concentrations, the variance in Ni BCF was principally explained by the population factor for leaf tissues (67 % of the total variance) and by inter-population variability for stem tissues (Table 3.4). Likewise, Ni BCF values were significantly higher for leaves compared to stems (P <0.05; Fig. 3.6b). In agreement with leaf Ni concentrations, the leaf Ni BCF were significantly higher in the L and SB populations, with mean values of 5.4 and 4.6, respectively, whereas the populations of M, S and SA showed a lower and similar Ni BCF (ranging from 2.6 to 3.1). Again in a similar manner to stem Ni concentrations, the stem Ni BCF presented similar values for the different populations, ranging from 0.8 to 0.9, with the exception of S where a significantly lower stem Ni BCF was observed (0.6) (Fig. 3.6b). The Co BCF was also calculated and as expected showed values significantly lower than the corresponding Ni BCF (consistently <0.6 in the leaves and <0.1 in stems). The highest leaf Co BCF value was found in the SB population (mean BCF of 0.3) (P <0.05).

Plant growth, Ni tolerance and bioaccumulation in populations of *Alyssum serpyllifolium* subspecies grown in controlled conditions

Experiments carried out in hydroponic nutrient solutions

Plant growth and nickel tolerance

In general plants grown in hydroponic solutions showed a healthy appearance, and only at the highest Ni solution concentration (1000 μM) did some plants show signs of chlorosis. In all the Ni treatments, both shoot and root DW

was found to vary widely between different plant individuals (progeny): the larger part of the total variability was found within populations (from 63 to 88 % for shoot DW and from 77 to 98 % for root DW, depending on the treatment (Control, Low-Ni, High-Ni), rather than amongst populations (from 12 to 34 % for shoot DW and from 2 to 22 % in root DW) (Table 3.5). When considering all four plant populations together, shoot DW yields varied between individual plants (progeny) by a factor of 181, 344 and 103 in Control, Low-Ni and High-Ni treatments, respectively. Likewise, root DW yields varied by a factor of 256, 435 and 300 in Control, Low-Ni and High-Ni treatments, respectively. Nevertheless, some significant differences in both shoot and root DW yields between populations were found (P <0.05), with the exception of the root DW yield of plants in the Control treatment. In addition, significant differences in root and shoot DW yields were observed between the three Ni treatments in all four populations (P <0.05).

Shoot DW yields followed the decreasing order: S>L~M>SB (Fig. 3.7a). The S population showed the highest shoot DW when grown in all three Ni concentrations: presenting mean values of 0.05 ±0.01 g plant^{-1}, 0.05 ±0.00 g plant^{-1} and 0.04 ±0.00 g plant^{-1} in Control, Low-Ni and High-Ni treatments, respectively (Fig. 3.7a). Mean shoot DW yields of this population were significantly higher than those of SB in all three Ni treatments. On the other hand, shoot DW yields of L and M were only significantly higher than SB in the Low-Ni and High-Ni treatments. The High-Ni treatment led to a decrease in shoot DW yields in all four populations; however this decrease was only significant in the case of L, M and SB. Shoot DW of the L and M populations in the High-Ni treatment was 0.6-fold (M and SB) that of the control (P <0.05; Fig. 3.7a). More pronounced was the effect of Ni treatments on the growth of the SB population: in this case both the Low-Ni and High-Ni treatments caused a significant reduction in the shoot DW compared to the control, and mean values were 0.8- and 0.6-fold of those in the control (P <0.05; Fig. 3.7a).

Root DW yields followed a similar trend to that observed for shoot DW, with the decreasing order: S>L~M>SB (Fig. 3.7b). However, in the case of root DW, differences were less pronounced and did not always reach statistical significance. The S population showed the highest mean root DW yield when grown in all three Ni concentrations: presenting mean values ≤0.01 ±0.00 g plant^{-1} in all the treatments (Fig. 3.7b). In the M population root DW yields tended to be higher in Low-Ni compared to control but this was not significant (Fig. 3.7b). The High-Ni treatment led to a reduction in root DW in all four populations, however, this was only significant in L and SB, which showed root DW yields with values as low as 0.5-fold of controls (P <0.05; Fig. 3.7b).

The Tolerance Index (TI) based on shoot and root growth was determined

Table 3.5. Inter- and intra-population variability (%) in dry weight (DW) yield, macro- and micro-nutrient concentrations, Ca/Mg ratio, Ni phytoextracted (Ni Phyto) and Ni shoot:root (S:R) ratio in *Alyssum pintodasilvae* and *Alyssum malacitanum* grown in hydroponic solutions with different Ni concentrations (Control, Low-Ni and High-Ni treatment).

Treatment	Plant tissue	Variability (%)	DW	Ca	Cu	Fe	K	Mg	Mn	Ni	P	Zn	Ca/Mg	Ni Phyto	Ni S:R
CONTROL	*Shoot*	Inter-population	11.5	44.2	5.8	2.3	17.6	32.8	13.5	45.5	55.6	48.1	52.3	0.0	23.7
		Intra-population	88.5	55.8	94.2	97.7	82.4	67.2	86.5	54.5	44.4	51.9	47.7	100.0	76.3
	Root	Inter-population	2.4	4.3	33.4	9.2	27.2	36.3	33.5	37.3	51.8	44.5	31.7	-	-
		Intra-population	97.6	95.7	66.6	90.8	72.8	63.7	66.5	62.7	48.2	55.5	68.3	-	-
LOW-Ni	*Shoot*	Inter-population	37.0	7.0	29.0	21.2	49.1	56.9	42.3	33.2	44.7	56.7	70.0	25.9	1.4
		Intra-population	63.0	93.0	71.0	78.8	50.9	43.1	57.7	66.8	55.3	43.3	30.0	74.1	98.6
	Root	Inter-population	14.1	2.3	27.3	15.3	26.4	21.9	34.9	6.3	32.1	25.9	16.8	-	-
		Intra-population	85.9	97.7	72.7	84.7	73.6	78.1	65.1	93.7	67.9	74.1	83.2	-	-
HIGH-Ni	*Shoot*	Inter-population	33.7	5.3	11.6	12.4	25.4	57.0	64.5	16.2	60.0	62.3	49.9	22.2	11.6
		Intra-population	66.3	94.7	88.4	87.7	74.6	43.0	35.5	83.8	40.0	37.7	50.1	77.8	88.4
	Root	Inter-population	22.5	0.0	41.5	31.8	18.6	11.8	43.0	8.1	36.3	2.1	7.1	-	-
		Intra-population	77.5	100.0	58.5	68.2	81.4	88.2	57.0	91.9	63.7	97.9	92.9	-	-

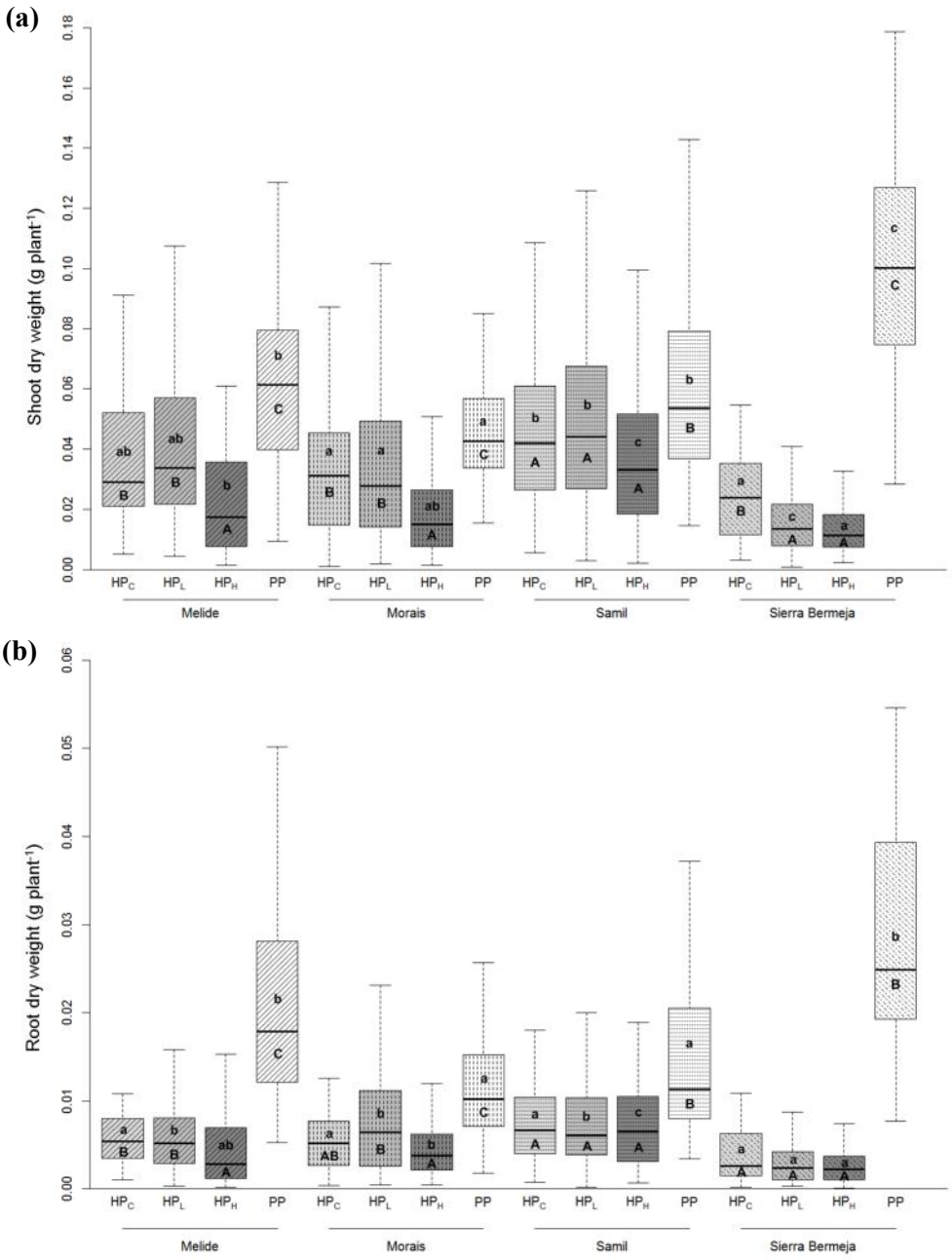


Figure 3.7. Shoot (a) and root (b) dry weight (DW, g plant^{-1}) of *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) grown in hydroponic culture at different Ni concentrations (HP_C: Hydroponic Progeny in Control treatment, HP_L: Hydroponic Progeny in Low-Ni treatment, HP_H: Hydroponic Progeny in High-Ni treatment) and in pots filled with serpentine soil (PP: Pot Progeny). Different capital letters denote significant differences between each growth medium within each population and different lower case letters denote significant differences between plants from the different populations within each growth medium (P <0.05).

for each population and treatment (Low-Ni and High-Ni). Shoot TI ranged from 1.07 to 0.78 in the Low-Ni treatment and from 0.76 to 0.54 in the High-Ni treatment. Plants grown in Low-Ni treatment showed a TI for shoots close to or slightly higher than 1 (with the exception of SB), indicating that plants grown in this treatment produced a similar shoot biomass to that of control plants (Table 3.6). However, plants grown in the High-Ni treatment showed a shoot TI lower than 1 in all four populations (only 0.54 in L), in concordance with the significant reduction observed in shoot biomass in plants from the four populations. In accordance, the TI in roots varied from 1.14 to 0.75 in the Low-Ni treatment and from 0.82 to 0.61 in the High-Ni treatment. In general, the TI for roots was lower than 1, only the Portuguese populations (M and S) showed a TI higher than 1 in the Low-Ni treatment (Table 3.6).

Nutrient concentration and metal accumulation in plant tissues

Table 3.7 shows the shoot and root macro- and micro-nutrient concentrations for each population after treatment in the hydroponic solutions with different Ni concentrations (Control, Low-Ni, High-Ni). The plant ionome showed a large intra-and inter-population variability (Table 3.5). In the Control treatment, a higher proportion of the variance in shoot concentrations of elements such as Ca, Cu, Fe, K and Mn was explained by the intra-population factor rather than through differences between populations (inter-population) (Table 3.5). However, the variance in concentrations of other nutrients, such as, Mg, P or Zn, was explained

Table 3.6. Shoot and root tolerance index (TI) of *Alyssum pintodasilvae* and *Alyssum malacitanum* from the different populations (Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB)) grown in hydroponic solutions with Ni.

Population	Treatment	Tolerance Index (TI)	
		Shoot	Root
L	***Low-Ni***	1.07	0.88
	High-Ni	0.54	0.61
M	***Low-Ni***	1.06	1.14
	High-Ni	0.57	0.77
S	***Low-Ni***	0.98	1.08
	High-Ni	0.76	0.82
SB	***Low-Ni***	0.78	0.75
	High-Ni	0.60	0.61

Table 3.7. Macro- and micro-nutrients concentrations in *Alyssum pintodasilvae* and *Alyssum malacitanum* (mean concentration ± standard error) from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) grown in hydroponic solutions with Ni. Asterisks denote significant differences between Low- or High-Ni treatment and control treatment ($P < 0.05$). Different letters indicate significant differences between populations within the same treatment ($P < 0.05$).

Plant tissue	Population	Treatment	Ca	K	Mg	Ni	P	Cu	Fe	Mn	Zn
			g kg^{-1}					mg kg^{-1}			
Shoot	**L**	**Control**	47.7 ±0.8b	32.5 ±0.8a	8.4 ±0.2b	5.4 ±0.2a	7.5 ±0.4b	8 ±1a	220 ±38a	226 ±9b	935 ±74c
		Low-Ni	45.6 ±0.7b	33.8 ±0.7a	9.6 ±0.2b*	11.1 ±0.3ab*	8.3 ±0.4b	13 ±1b*	148 ±17b	146 ±4c*	648 ±30c*
		High-Ni	36.5 ±0.7a*	37.0 ±0.1a*	9.6 ±0.2b*	19.1 ±0.4b*	13.3 ±0.8b*	17 ±1a*	270 ±38b	151 ±6c*	521 ±22b*
	M	**Control**	54.1 ±1.2c	33.4 ±1.1a	6.8 ±0.2a	7.2 ±0.2b	10.0 ±0.1b	16 ±3b	296 ±55ab	187 ±7a	344 ±26a
		Low-Ni	49.7 ±0.7c*	31.9 ±0.8a	6.2 ±0.1a*	12.5 ±0.2b*	9.9 ±0.5b	27 ±5b	204 ±27b	106 ±3a*	380 ±46b
		High-Ni	39.7 ±0.8b*	34.4 ±1.1a	6.2 ±0.2a*	17.4 ±0.4b*	16.8 ±0.8c*	21 ±3a	143 ±16a*	95 ±4a*	192 ±11a*
	S	**Control**	46.1 ±1.4b	34.5 ±0.9a	6.2 ±0.2a	4.5 ±0.2a	4.0 ±0.1a	7 ±1a	143 ±19a	183 ±7a	392 ±21b
		Low-Ni	48.8 ±0.6c	39.1 ±0.7b*	6.4 ±0.1a	10.5 ±0.2a*	4.7 ±0.1a*	9 ±1a	92 ±4a*	112 ±2b*	304 ±12a*
		High-Ni	43.8 ±0.8c*	42.8 ±0.9b*	6.5 ±0.1a	15.0 ±0.3a*	6.4 ±0.1a*	14 ±2a*	105 ±10a*	102 ±3b*	247 ±11a*
	SB	**Control**	35.3 ±1.0a	40.0 ±1.5b	8.5 ±0.3b	8.0 ±0.4b	15.2 ±1.1c	15 ±4b	406 ±115b	183 ±9a	414 ±34bc
		Low-Ni	41.2 ±0.9a	48.7 ±1.1c*	9.6 ±0.2b*	14.5 ±0.3c*	22.4 ±1.2c	18 ±3b	359 ±67c	202 ±8d	351 ±17ab
		High-Ni	39.6 ±1.0 b	47.9 ±1.4c*	8.7 ±0.2b	18.1 ±0.4b*	23.1 ±1.2d*	16 ±2a	217 ±21b	206 ±9d	271 ±21a*
Root	**L**	**Control**	9.5 ±0.5b	45.5 ±2.8bc	4.6 ±0.4c	2.6 ±0.2b	11.1 ±0.7b	51 ±4b	1230 ±143b	85 ±7b	1397 ±129a
		Low-Ni	9.1 ±0.4a	39.2 ±1.7b	4.9 ±0.3b	5.4 ±0.3ab*	11.0 ±0.5b	66 ±4b	862 ±76a*	37 ±2c*	807 ±84a*
		High-Ni	8.8 ±0.4b	28.2 ±2.1a*	3.6 ±0.3a*	7.6 ±0.5b*	11.1 ±0.6b	150 ±13b*	1864 ±176c*	64 ±5c*	834 ±84a*
	M	**Control**	11.1 ±1.1b	23.9 ±1.6a	2.3 ±0.1a	1.9 ±0.2a	10.0 ±0.6b	104 ±8c	882 ±90ab	46 ±3a	1906 ±110a
		Low-Ni	8.3 ±0.4a	23.0 ±1.1a	3.2 ±0.2a*	6.2 ±0.4b*	10.8 ±0.6b	142 ±16c	1257 ±153a	35 ±4b	1442 ±121c
		High-Ni	6.8 ±0.3a*	28.1 ±1.0a	2.7 ±0.1a*	6.1 ±0.4a*	14.9 ±0.6c*	138 ±5b	650 ±35a	20 ±1a*	1032 ±62a*
	S	**Control**	9.8 ±0.7b	39.4 ±2.7b	3.0 ±0.2b	2.2 ±0.2ab	3.7 ±0.2a	30 ±3a	721 ±67a	61 ±5b	1672 ±91a
		Low-Ni	8.8 ±0.4a	40.6 ±2.1b	2.9 ±0.2a	4.6 ±0.2a*	4.1 ±0.1a	33 ±2a	763 ±34a	22 ±1a*	1348 ±70b
		High-Ni	7.7 ±0.3b*	43.4 ±1.7b	2.7 ±0.1a	7.4 ±0.3b*	5.1 ±0.3a*	54 ±3a*	855 ±36b	30 ±2b*	925 ±39a*
	SB	**Control**	6.9 ±0.5a	48.6 ±2.8c	4.9 ±0.4c	4.6 ±0.5c	16.6 ±1.5c	84 ±10c	1197 ±203b	145 ±20c	3929 ±436b
		Low-Ni	8.6±0.4a	28.7 ±1.9a*	4.3 ±0.2b	7.5 ±0.4c*	12.6 ±0.7c*	169 ±9d*	2162 ±192b*	98 ±7d*	2094 ±156d*
		High-Ni	9.0 ±0.7b	34.5 ±2.5a*	4.2 ±0.2b	10.2 ±0.5c*	19.6 ±1.9c	208 ±15c*	2113 ±167c*	121 ±12d	1353 ±202b*

in almost equal parts by both factors (intra- and inter-population) (Table 3.5). Similar patterns were observed in the Low-Ni and High-Ni treatments, but the total variance explained by differences between populations was higher for elements such as Cu, Fe, K, Mg and Mn, whereas a higher % of variance in shoot Ni concentrations could be explained by differences within populations (Table 3.5). Likewise, the variance in root concentrations of nutrients was largely explained by the within population factor, rather than by variability between populations (Table 3.5).

Nonetheless, some significant differences in shoot and root nutrient concentrations were found between populations, and this was the case for all Ni treatments (P <0.05; Table 3.7). The highest shoot Ca concentrations were generally observed in M and S populations, and the lowest concentrations in L and SB populations (Table 3.7). On the other hand, the SB population showed the highest shoot and root concentrations of Fe and P. Both the L and SB populations presented the highest Mg concentrations in shoots and roots, and differences were significantly higher than the other populations (P <0.05). Shoot Ca/Mg ratios were significantly higher in plants grown in the hydroponic solutions than in plants collected in the field (up to 2.4-fold higher), but values were consistently >1 in both field and hydroponic-grown plants (Fig. 3.8). In general, the shoot Ca/Mg ratio decreased when the Ni concentration in the hydroponic solution increased; this was most pronounced in L (decreasing from 5.8 in control to 3.9 in High-Ni), and only in the SB population were no significant differences in the Ca/Mg ratio observed between treatments. The highest shoot Ca/Mg ratios were observed in the M and S populations (mean Ca/Mg ratio of 8.4 and 7.8 in controls, respectively) (Fig. 3.8).

Significant positive correlations (r ≥0.7) between some nutrients were found for some populations and Ni treatments. In M and S a positive significant correlation between shoot Ni and Mg concentrations was found in the High-Ni treatment (r=0.8). Likewise a positive significant correlation between Ni and Mg concentrations was observed in the root tissues, and this was seen in all four populations and all treatments (r=0.8).

Shoot Ni accumulation by the four populations when grown in hydroponic solutions was compared to the corresponding accumulation when grown in their natural habitats (leaf Ni accumulation in the case of field collected plants) (Fig. 3.9). The Ni concentration in leaves of the mother plants collected from the different serpentinitic areas was not correlated with the shoot Ni concentrations in their progeny when grown in hydroponic culture solutions which simulated serpentine conditions (r <0.7); the Pearson coefficient (r) ranged from -0.14 in the Control treatment to 0.10 in the High-Ni treatment. Shoot Ni concentrations of

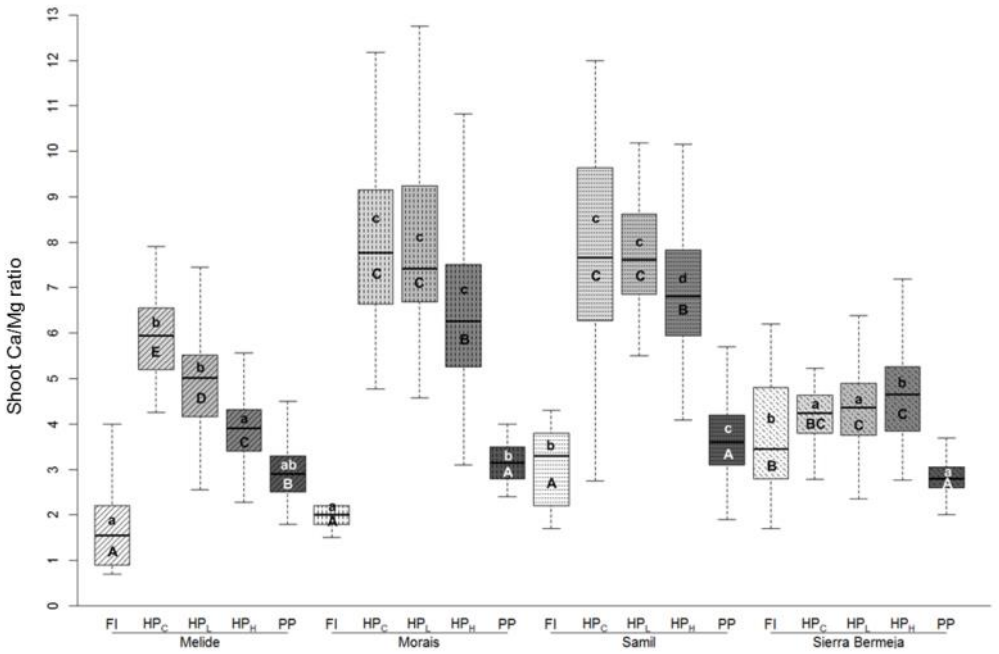


Figure 3.8. Shoot Ca/Mg ratio in *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) grown in the field (IF: Individual Field), in hydroponic culture at different Ni concentrations (HP$_C$: Hydroponic Progeny in Control treatment, HP$_L$: Hydroponic Progeny in Low-Ni treatment, HP$_H$: Hydroponic Progeny in High -Ni treatment) and in pots filled with serpentinitic soil (PP: Pot Progeny). Different capital letters denote significant differences between each growth medium within each population and different lower case letters denote significant differences between plants from the different populations within each growth medium (P <0.05).

plants collected in the field were significantly higher than the concentrations observed in their progeny when grown in the control solution (except for M where no significant differences were found between mother plants and their progeny) (P <0.05). The difference in shoot Ni concentrations between field and hydroponic -grown plants was most pronounced in L, the mean shoot Ni concentration in field plants was 13.6 ±0.6 g kg^{-1} and in the control treatment was 5.4 ±0.2 g kg^{-1}. In contrast, in the M population the mean shoot Ni concentration in field plants was 7.1 ±0.5 g kg^{-1} and in the control treatment it was 7.2 ±0.2 g kg^{-1}. With the exception of L (where mother plants presented a significantly higher shoot Ni concentrations), plants grown in the Low-Ni treatment showed significantly higher Ni concentrations in their shoots compared to corresponding mother plants from the field (P <0.05). Finally, the shoot Ni concentrations of plants grown in the

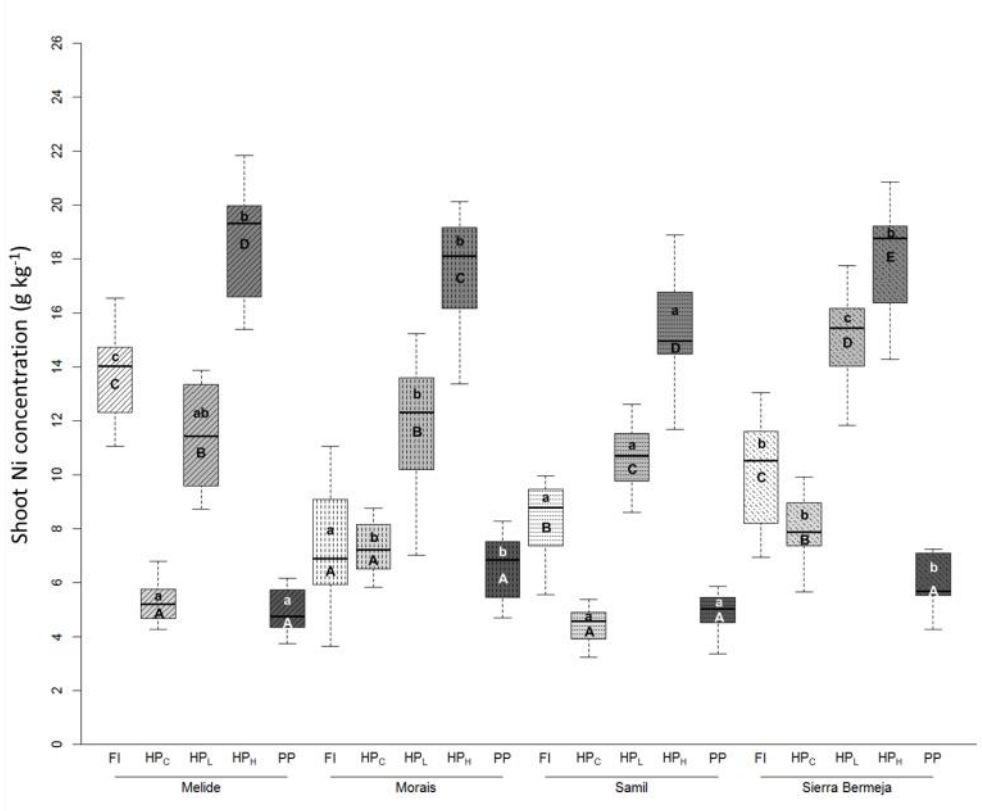


Figure 3.9. Shoot(*) Ni concentrations in *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) growing in the field (IF: Individual Field), cultivated in hydroponic cultures with different Ni concentrations (HP_C: Hydroponic Progeny in Control treatment, HP_L: Hydroponic Progeny in Low-Ni treatment, HP_H: Hydroponic Progeny in High-Ni treatment) or in pots filled with serpentine soil (PP: Pot Progeny). (*)Leaf Ni concentration in the case of individual (mother) plants from the field (IF). Different capital letters denote significant differences between each growth medium within each population and different lower case letters denote significant differences between plants from the different populations within each growth medium (P <0.05).

High-Ni treatment were significantly higher than corresponding concentrations observed in the mother plants (shoot Ni concentrations in High-Ni were up to 3.5-, 2.4-, 3.3- and 2.3-fold higher than corresponding concentrations in the field for the populations L, M, S and SB, respectively), and this was the case for all four populations (P <0.05) (Fig. 3.9).

The variance in shoot Ni concentrations of hydroponically-grown plants was mainly explained by the intra-population factor and this was most pronounced in the High-Ni treatment. Shoot Ni concentrations increased with an increase in solution Ni concentration (i.e. from Control to High-Ni; Fig. 3.9), presenting mean values of 6.1 ±0.2 g kg^{-1}, 12.1 ±0.1 g kg^{-1} and 17.3 ±0.2 g kg^{-1} in Control, Low-Ni and High-Ni treatments, respectively. The most pronounced differences between

populations were observed in the Control and Low-Ni treatments: the mean shoot Ni concentration was 5.4 ±0.2 g kg^{-1}, 7.2 ±0.2 g kg^{-1}, 4.5 ±0.2 g kg^{-1} , 8.0 ±0.4 g kg^{-1} in the Control treatment, and 11.1 ±0.3 g kg^{-1}, 12.5 ±0.2 g kg^{-1}, 10.5 ±0.2 g kg^{-1}, 14.5 ±0.3 g kg^{-1} in the Low-Ni in L, M, S and SB, respectively. Shoot Ni concentration in control plants varied significantly between populations following the decreasing order: SB~M>L~S (P <0.05; Table 3.7 and Fig. 3.9). Significant differences were also found in Low-Ni and High-Ni treatments and followed the decreasing order of SB>M~L~S in Low-Ni and of L~SB~M>S in High-Ni (P <0.05). The highest shoot Ni concentration was observed in the L population in the High-Ni treatment (with a mean value of 19.1 ±0.4 g kg^{-1}), while the lowest concentration was found in S (15.0 ±0.3 g kg^{-1}). In the low-Ni treatment the SB population presented the highest mean shoot Ni concentration (14.5 ±0.3 g kg^{-1}), and the lowest concentration was found in the S population (10.5 ±0.2 g kg^{-1}) (Fig. 3.9). Similar to shoot Ni concentrations, the root Ni concentrations gradually increased with an increase in the concentration of Ni in the solution (Table 3.7). SB showed the highest root Ni concentration in Control, Low-Ni and High-Ni treatments: 4.6 ±0.7 g kg^{-1}, 7.6 ±0.6 g kg^{-1} and 10.8 ±1.1 g kg^{-1}, respectively, while S and M (depending on the treatment) showed the lowest root Ni concentrations (Table 3.7).

The shoot:root Ni concentration ratios of hydroponic-grown plants (considering all three treatments) ranged from 0.6 to 9.2 in individuals of L, from 0.5 to 16.7 in M, from 0.4 to 13.2 in S and finally, from 0.6 to 10.5 in SB. The variance in the Ni S:R ratios was again related to variability within the different populations rather than between populations; however, some significant differences were observed between the populations in all Ni treatments (P <0.05; Table 3.5 and Fig. 3.10). The highest Ni S:R ratio was generally observed in M, presenting mean values of 6.1 ±0.6, 3.5 ±0.3 and 3.6 ±0.2 in Control, Low-Ni and High-Ni treatments, respectively, whereas the SB population showed the lowest Ni S:R ratio in all three treatments, presenting mean values of 2.7 ±0.4, 2.6 ±0.1 and 2.1 ±0.1 (Fig. 3.10). In the M and S populations significant differences in Ni S:R ratio were found between the different Ni treatments: the Ni S:R ratios were significantly reduced in Low-Ni and High-Ni treatments (only High-Ni treatment in S) compared to the Control treatment (P <0.05; Fig. 3.10). L and SB populations did not show any significant differences between the different treatments (Fig. 3.10). The Ni S:R ratio and shoot/root Ni concentrations were not significantly correlated (r <0.4), and this was the case for all the different treatments and populations.

The phytoextracted Ni was calculated as the product of the DW yield produced and the shoot Ni concentration. The amount of Ni extracted by plants was both treatment- and population-dependent and it generally increased with an

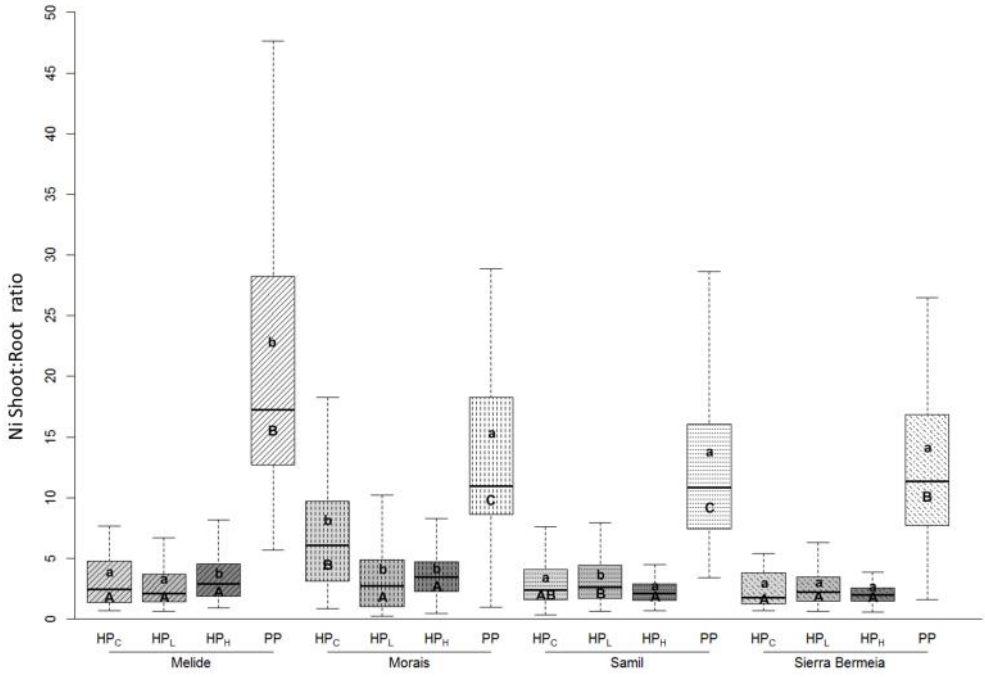


Figure 3.10. Shoot:root (S:R) Ni concentration ratios in *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) grown in hydroponic culture at different Ni concentrations (HP_C: Hydroponic Progeny in Control treatment, HP_L: Hydroponic Progeny in Low-Ni treatment, HP_H: Hydroponic Progeny in High-Ni treatment) and in pots filled with serpentinitic soil (PP: Pot Progeny). Different capital letters denote significant differences between each growth medium within each population and different lower case letters denote significant differences between plants from the different populations within each growth medium (P <0.05).

increase in solution Ni concentration. The larger proportion of the total variance in this Ni yield was related to intra-population variability (Table 3.5); however, some significant differences in the amount of Ni phytoextracted between the different populations in Low-Ni and High-Ni treatments were found (P <0.05; Fig. 3.11). In the Control treatment there were no differences between populations in the Ni phytoextracted, which generally followed the decreasing order: S>L≈M>SB. For all three treatments the highest values of mean phytoextracted Ni were observed in the S population, which was also the population that showed the highest shoot DW yields. In contrast, phytoextracted Ni was lowest in the SB population which showed the lowest DW yields. In the Control treatment the Ni phytoextracted ranged from 0.19 ±0.03 to 0.24 ±0.08 mg (Fig. 3.11), whereas in the Low-Ni treatment this varied from 0.25 ±0.04 to 0.54 ±0.07 mg and in the High-Ni treatment from 0.25 ±0.04 to 0.56 ±0.05 mg. In L, M and S populations the Ni phytoextracted in the low-Ni and High-Ni treatments were significantly higher than in the Control treatment (P <0.05; Fig. 3.11).

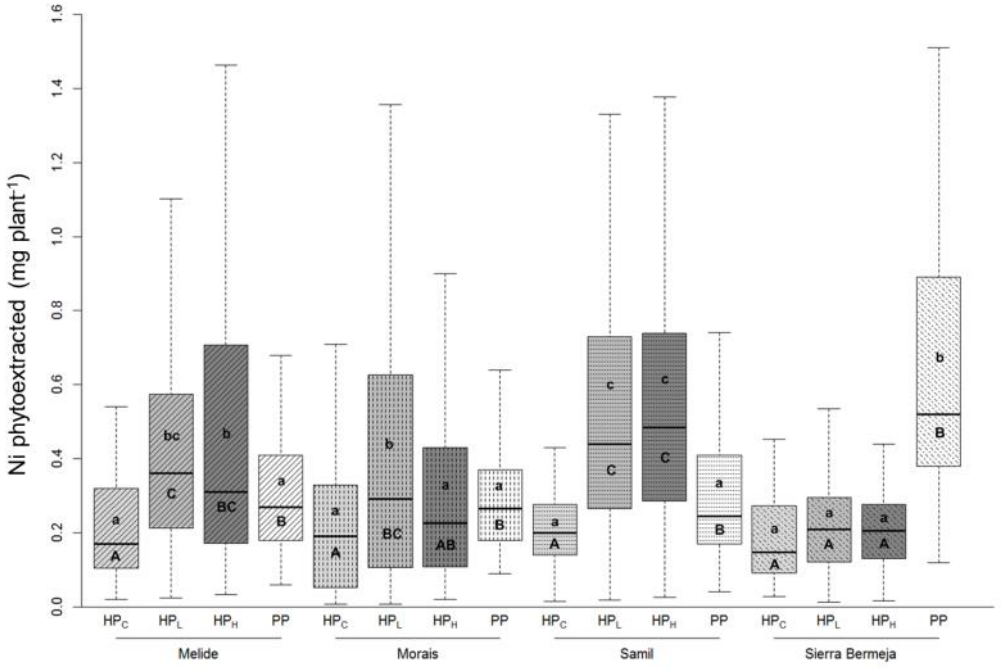


Figure 3.11. Ni phytoextracted (mg plant^{-1}) by *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) grown in hydroponic culture at different Ni concentrations (HP_C: Hydroponic Progeny in Control treatment, HP_L: Hydroponic Progeny in Low-Ni treatment, HP_H: Hydroponic Progeny in High-Ni treatment) and in pots filled with serpentinitic soil (PP: Pot Progeny). Different capital letters denote significant differences between each growth medium within each population and different lower case letters denote significant differences between plants from the different populations within each growth medium (P <0.05).

Experiments carried out in serpentine soil

Plant growth and biomass production

After 16 weeks growth in serpentine soil all plants showed a healthy appearance and both shoot and root DW yields were significantly greater than in plants grown in hydroponic conditions (P <0.05; Fig. 3.7). As observed in the hydroponic study, the larger part of the total variance in shoot and root DW was explained by the intra-population factor (64 and 72 %) rather than through differences between populations (36 and 28 %) (Table 3.8). However, some significant differences in shoot and root DW yields were observed between the different populations (P <0.05; Fig. 3.7). When considering all four plant populations together the shoot DW yield ranged from 0.01 to 0.29 g plant^{-1} whereas the root DW varied from 0.01 to 0.13 g plant^{-1}. The shoot DW yields of the different populations followed the decreasing order: SB>L≈S>M (Fig. 3.7a). In contrast to what was observed in hydroponic solutions, the SB population

showed a significantly higher shoot DW than the other populations (P <0.05), with a mean value of 0.11 ±0.02 g plant^{-1} which was almost 2-fold the values observed in either L or S (both these populations showed a mean shoot DW yield of 0.06 ±0.01 g plant^{-1}). In this experiment, M was the population with the lowest shoot DW (mean value of 0.05 ±0.01 g plant^{-1}) which was significantly lower than in the other populations (P <0.05; Fig. 3.7a). Root DW yields followed a similar behaviour to that found for shoots and decreased as follows: SB>L>S≈M (Fig. 3.7b). The highest root DW yield was observed in SB, with a mean value of 0.03 ±0.01 g plant^{-1} which was significantly higher than the other populations (P <0.05). Once again, the M population showed the lowest root DW yield (0.01 ±0.00 g plant^{-1}).

Nutrient concentration and nickel accumulation in plant tissues

The concentrations of Fe, Mg and Mn in the plant tissues were of a similar magnitude to concentrations observed in plants grown in hydroponic conditions, whereas the concentrations of Ca, K, Ni and P were significantly lower in the pot experiment than the hydroponic experiment (Table 3.9). As observed in the hydroponic experiment, the larger part of the total variance in plant ionome was due to intra-population variability (from 57 to 96 % in shoot and from 79 to 100 % in root) rather than through differences between populations (from 4 to 46 % in shoot and from 0 to 21 % in root). Nonetheless, there were some significant differences in nutrient concentrations (with the exception of Fe and Mg concentration), in the shoot Ca/Mg ratio, Ni concentration, Ni S:R ratio, Ni BCF and Ni phytoextracted, between the different populations (P <0.05).

Differences in the plant ionome between populations did not follow the same pattern as observed for plants grown in the hydroponic solutions. In general, the SB population showed significant differences in nutrient concentrations compared to the other populations (P <0.05; Table 3.9): SB presented a significantly lower shoot concentration of Ca, Mg, K, P and Cu than the other populations (P <0.05; Table 3.7). In contrast, L presented significantly higher concentrations of Mg and P in shoots compared to the other populations. In the case of roots, plants from SB showed significantly higher concentrations of K, P and Cu compared to the other populations, whereas plants from L showed the lowest concentrations of Ca, Mg and Mn in roots (P <0.05; Table 3.9). Similar values in Mg and Mn root concentrations were found in plants from M, S and SB, showing values significantly lower than the other populations (P <0.05). In general, the shoot Ca/Mg ratio observed in plants grown in serpentine soil were lower than those observed in plants grown in hydroponic conditions but higher than the Ca/Mg ratio found in plants from the field. In the pot experiment the

Table 3.8. Inter- and intra-population variability (%) in dry weight (DW), macro- and micro-nutrient concentrations, Ca/Mg ratio, Co and Ni BCF, Ni phytoextracted (Ni Phyto) and Ni shoot:root (S:R) ratio in *Alyssum pintodasilvae* and *Alyssum malacitanum* grown in serpentine soil.

Variability (%)	DW	Ca	Co	Cu	Fe	K	Mg	Mn	Ni	P	Ca/Mg	Co	Ni	Ni	Ni S:R
Inter-population	36.3	26.8	28.3	5.6	3.7	4.2	23.5	43.2	19.9	20.1	21.7	28.9	19.6	34.3	11.2
Intra-population	63.7	73.2	71.7	94.4	96.3	95.8	76.5	56.8	80.1	79.9	78.3	71.1	80.4	65.7	88.8
Inter-population	28.4	20.6	n.d.	15.5	0.0	9.7	0.0	10.7	15.1	2.7	4.8	-	-	-	-
Intra-population	71.6	79.4	n.d.	84.5	100.0	90.3	100.0	89.3	84.9	97.3	95.2	-	-	-	-

Table 3.9. Macro- and micro-nutrient concentrations in the shoots and roots of *Alyssum pintodasilvae* and *Alyssum malacitanum* grown in serpentine soils (mean value ±standard error). Different letters denote differences between populations (P <0.05).

Plant tissue	Population	Ca	K	Mg	Ni	Co	Cu	Fe	Mn	P
		g kg^{-1}				mg kg^{-1}				
Shoot	***L***	32.5 ±0.6b	17.9 ±0.4b	11.0 ±0.2b	4.9 ±0.2a	13 ±0a	9 ±1b	318 ±23ab	169 ±4a	954 ±54c
	M	32.0 ±0.6b	17.4 ±0.4ab	10.2 ±0.2b	6.5 ±0.2b	22 ±1c	9 ±0b	259 ±18a	282 ±9c	730 ±25b
	S	31.5 ±0.6b	17.5 ±0.3ab	8.9 ±0.2a	4.9 ±0.2a	16 ±1b	9 ±1b	377 ±36b	258 ±7c	663 ±20ab
	SB	25.9 ±0.9a	15.8 ±0.5a	9.2 ±0.2a	5.9 ±0.4b	18 ±1b	6 ±1a	361 ±52ab	203 ±10b	618 ±41a
Root	***L***	2.9 ±0.1a	8.7 ±0.4a	2.2 ±0.2a	0.3 ±0.0a	n.d.	125 ±13a	1772 ±258a	60 ±5a	741 ±67b
	M	3.5 ±0.1b	9.4 ±0.5a	2.6 ±0.1a	0.8 ±0.2ab	n.d.	173 ±13b	1842 ±151a	104 ±6b	620 ±47a
	S	4.0 ±0.1c	9.0 ±0.3a	2.6 ±0.1a	0.5 ±0.0ab	n.d.	139 ±18a	1610 ±151a	93 ±6b	625 ±19ab
	SB	3.5 ±0.1b	11.4 ±0.4b	2.7 ±0.2a	1.4 ±0.8b	n.d.	259 ±31b	1725 ±244a	86 ±6ab	815 ±93b

highest shoot Ca/Mg ratio was observed in the S population (3.7 ±0.1); it was significantly higher than the values observed in plants from the other populations (P <0.05; Fig. 3.8). The same population also showed the highest root Ca/Mg ratios (data not shown). Significant positive correlations were observed between the concentrations of some nutrients in plant tissues (r ≥0.7). Plants from S showed a significant positive correlation between Ni and Ca concentrations in shoots (r ≥0.7). In roots a significant correlation was found between Mg, Fe and Mn concentrations in the different populations (with the exception of S). In the M population, Mg and Ca shoot concentrations were also positively correlated. The shoot Ni accumulation in plants grown in serpentine soil was compared to the Ni accumulation of the mother plants growing in their natural habitats (Fig. 3.9). There was no correlation in the shoot Ni concentrations between the mother plants (collected in the field) and their progeny when grown in the serpentine soil under controlled conditions (r =-0.26). Shoot Ni concentrations in plants from the pot experiment were generally lower than field plants, with the exception of plants from M, which showed similar shoot Ni concentrations. In general, plants grown in the serpentine soil (pot experiment) showed a similar shoot Ni concentration to that of plants grown in the Control treatment of the hydroponic experiment. Plants from both M and SB showed significantly higher Ni concentrations in shoots compared to L and S populations (P <0.05; Fig. 3.9). The highest shoot Ni concentration was observed in plants from M, showing a mean value of 6.5 ±0.4 g kg^{-1}, and plants from the SB population accumulated a mean Ni concentration of 5.9 ±0.4 g kg^{-1}. Whereas the populations of L and S showed mean shoot Ni concentrations of 4.9 ±0.3 g kg^{-1} and 4.9 ±0.2 g kg^{-1} respectively. Root Ni concentrations were significantly lower than shoot Ni concentrations (P <0.05; Table 3.9). As observed for shoots, both M and SB showed higher mean root Ni concentrations compared to L and S; in this case the differences were only significant between SB and L (1.4 ±0.8 and 0.3 ±0.0 g Ni kg^{-1}, respectively). The shoot:root Ni ratio was significantly higher in plants from L compared to the other populations (P <0.05; Fig. 3.10). The mean shoot:root Ni ratio observed in L was 22.5 ±2.7,while in M, S and SB the shoot:root Ni ratios ranged from 13.0 ±1.8 to 13.7 ±2.0. The shoot Co concentration observed in plants from M (22 ±1 mg kg^{-1}) was significantly higher than that of the other populations (P <0.05). S and SB showed similar Co concentrations (16 ±1 and 18 ±1 mg kg^{-1}, respectively) and L showed the lowest concentrations (13 ±0 mg kg^{-1}) compared to the other populations (P <0.05; Table 3.9). Root Co concentrations were under the detection limit. The BCF for Ni and Co is presented in Table 3.10. Plants from M showed the highest Ni and Co BCF (4.04 ±0.24 and 0.17 ±0.01, respectively); and these were significantly higher than those observed in both L and S populations

Table 3.10. Mean bioconcentration factor (BCF) for Ni and Co in the shoots of *Alyssum pintodasilvae* and *Alyssum malacitanum* from Melide (L), Morais (M), Samil (S) and Sierra Bermeja (SB) grown in serpentine soil (concentrations ± standard error). Different letters denote differences between populations (P <0.05).

Population	Ni BCF	Co BCF
L	3.04 ±0.16a	0.10 ±0.00a
M	4.04 ±0.24b	0.17 ±0.01b
S	3.01 ±0.14a	0.13 ±0.01a
SB	3.67 ±0.28ab	0.14 ±0.01ab

(P <0.05). The highest amount of Ni phytoextracted was observed in plants from SB which was also the population with the highest shoot DW. Mean phytoextracted Ni in SB was 2-fold higher than in L, M or S. Plants from SB showed a mean value of phytoextracted Ni of 0.62 ±0.11 mg, whereas L, M and S showed mean values of 0.31 ±0.03 mg (Fig. 3.11).

3.4 *DISCUSSION*

To the best of our knowledge this is the first time that the Ni tolerance and accumulation characteristics of different populations of the Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* (*Alyssum pintodasilvae* and *Alyssum malacitanum*) from the main serpentine outcrops of the Iberian Peninsula have been simultaneously assessed under three different growth conditions: *in situ* plants growing in the field, plants cultivated in hydroponic culture solutions enriched with Ni and plants cultivated in a pot experiment using serpentine soil. Moreover, it is the first time that the soil physicochemical properties and Ni availability are evaluated in the rhizosphere of more than one population of both of these Ni-hyperaccumulating subspecies.

Influence of plant root activity on soil Ni availability and physicochemical properties

As expected, the soil physico-chemical properties of each study site were characteristic of serpentine soils: low in essential plant nutrients and organic matter, a dominance of Mg in the exchange complex, Ca/Mg ratios of <1, and high concentrations of the trace metals, Ni, Cr and Co. The most marked differences

observed between the different serpentine outcrops were found in SA: this site had an alkaline soil pH and corresponding higher concentrations of total C. As a result part of the total C in this site is likely to be associated with carbonates; which is in concordance with the low total N content and the corresponding high C/N ratio, as well as the high exchangeable Ca and Mg concentrations observed in this soil. It was also noted as the site with the lowest concentrations of Ni, Co and Cr. On the other hand, M differed from the other Northern serpentine outcrops (S and L) in its exceptionally high concentration of total Cr (more than 4-fold higher), while L presented a notably higher concentration of available ($Sr(NO_3)_2$-extractable) Ni.

It is well known that plants induce changes in the physicochemical characteristics of the rhizosphere soil, leading to gradients in general soil properties, nutrient and trace metal availability (Hinsinger *et al.* 2001, 2005). Similarly, plant-induced changes in rhizosphere soil properties have also been observed in metal hyperaccumulating plant species (Lombi *et al.* 2001; Marschner *et al.* 1987; Puschenreiter *et al.* 2005). In this study significant differences were observed between the non-vegetated and rhizosphere soils associated with populations of both Ni-hyperaccumlators, *A. pintodasilvae* and *A. malacitanum*. However, there were no generalised effects which could be consistently attributed to the activity of the hyperaccumulators, and the plant-induced changes observed in the soil properties were population-dependent.

Several authors have reported a higher pH in the rhizosphere soil of contrasting metal(loid)-hyperaccumulating plant species compared to bulk or non-vegetated soil. Silva Gonzaga *et al.* (2006) observed an increase in the rhizosphere soil pH of the As-hyperaccumulator, *Pteris vittata*, when grown in a sandy calcareous soil. Both Hammer and Keller (2002) and Knight *et al.* (1997) observed increases in rhizosphere soil pH after cultivating *N. caerulescens* in a range of Cd/Zn-contaminated soils. These studies were carried out in greenhouse experiments, while Wenzel *et al.* (2003) found an increase of 0.4 pH units in the rhizosphere of the Ni-hyperaccumulator *Noccaea goesingense* in samples collected from plants growing in the field (Redlschlag serpentine site, E Austria). In agreement with these authors, our results showed a higher pH in the rhizosphere soils of *A. pintodasilvae* from both the M and S populations compared to the non-vegetated soil. Moreover, both these studies were carried out in serpentine soils. Kidd and Monterroso (2005) found that one of the same populations of *A. pintodasilvae* which was used in this study (S population) also led to an increase in the rhizosphere soil pH when grown in metal-contaminated lignite mine tailings. However, a similar increase in pH was not observed in either the L population of *A. pintodasilvae* or the two populations of *A. malacitanum*. In fact, the SA population of *A. malacitanum* presented a significantly lower pH in the

rhizosphere soil compared to non-vegetated soil. Other authors have also reported a decrease in rhizosphere soil pH after cultivating hyperaccumulators (Delorme *et al.* 2001; Li *et al.* 2011). For example, McGrath *et al.* (1997) found a lower pH and a higher concentration of mobile Zn in the rhizosphere of *N. caerulescens* than in the bulk soil when grown in Cd/Zn-contaminated agricultural soils (with either a sandy loam or loam texture). On the other hand, several studies exist in the literature in which no change in pH was recorded between non-vegetated and rhizosphere soil (Al-Najar *et al.* 2003; Puschenreiter *et al.* 2003), which was the case for the L population of *A. pintodasilvae* and the SB population of *A. malacitanum* in this study. The differences in plant-induced changes in rhizosphere soil pH observed amongst these different studies could be attributed to differences in soil buffering capacity in addition to specific plant activity.

Some common effects of both hyperaccumulators could be seen on soil properties: such as an increase in soil total C and N, and an increase in the cation exchange capacity in the rhizosphere compared to non-vegetated soil. Serpentine soils are characterised by an unfavourable Ca/Mg ratio (<1) but it appears that these hyperaccumulators are able to improve this Ca:Mg ratio in their rhizosphere soil. This was most pronounced in the SB population of *A. malacitanum* where in some cases this ratio was even increased to >1. A similar improvement in the Ca/Mg ratio in the rhizosphere soil of non-hyperaccumulating plants (*Dactylis glomerata* and *Holcus lanatus*) growing at the same serpentine sites was not observed (unpublished data), suggesting that this trait is particular to the hyperaccumulating species. The increase in organic C in the rhizosphere is to be expected since plants are known to release high amounts of nutrients and organic compounds into the rhizosphere in the form of root exudates and rhizodeposits. Root exudation of organic compounds (especially organic acid anions) has also been suggested to increase metal mobility and availability in the rhizosphere of certain hyperaccumulating plant species (Kidd and Monterroso 2005; Knight *et al.* 1997; Puschenreiter *et al.* 2003; Uren 2000; Wenzel *et al.* 2003). Moreover, an increase in metal availability in the rhizosphere of hyperaccumulating plant species has often been suggested to explain the enhanced metal uptake and accumulation observed in these plants. There is still no general consensus regarding the ability of this type of plant to access less available soil metal fractions, however, most recent studies indicate that both hyperaccumulating and non-hyperaccumulating species access the same soil metal fractions (Echevarria *et al.* 1998; Hammer *et al.* 2006; Hutchinson *et al.* 2000; Shallari *et al.* 2001). Numerous studies can be found in the literature showing an increase, a decrease, or even no change in plant-available metal fractions in the rhizosphere of metal-hyperaccumulating plant species (reviewed by Kidd *et al.* (2009)). Many of

these studies were however carried out in controlled conditions (greenhouse or hydroponics) and may not reflect the processes occurring *in situ*. Wenzel *et al.* (2003) observed an increase in water-soluble concentrations of Ni in the rhizosphere soil of *N. goesingense* plants which were collected *in situ* in serpentine soils. In agreement, in this study the $Sr(NO_3)_2$-extractable Ni concentrations were generally higher in the plant rhizosphere of both *A.pintodasilvae* and *A. malacitanum* (albeit more or less pronounced across the different plant populations). Moreover, plant-induced shifts in the soil Ni fractionation were observed, whereby more plant-available fractions (e.g. exchangeable or oxidisable) were increased at the expense of less plant-available or silicate-bound (residual) fractions. Such changes in the soil Ni fractionation were generally not observed in non-hyperaccumulating plants growing in the same serpentine sites (unpublished data). The root activities of these Ni-hyperaccumulator plants could enhance the weathering of Ni-rich clay minerals (i.e. non-labile and less plant-available solid fractions) which in turn would lead to the replenishment of soluble or labile Ni pools. This release of labile Ni does not necessarily indicate an active mobilisation of Ni by the hyperaccumulator but since these plant species have a high capacity for Ni absorption the replenishment process is likely to be enhanced in the rhizosphere of hyperaccumulators rather than non-hyperaccumulating plant species which avoid Ni absorption and bioaccumulation. Moreover, mineralogical studies (based on selective extractions and X-ray diffraction (XRD)) of the clay fraction (<2 μm) of rhizosphere soils from *A. pintodasilvae* (M population) supported the idea of a more intense weathering of Ni-rich ferromagnesium minerals in the rhizosphere of this hyperaccumulator compared to the non-accumulator, *Dactylis glomerata*, growing in the same site (Kidd *et al.* 2009). This was reflected through an increase in poorly crystallised Fe oxyhydroxides and greater concentration of Ni associated with this mineral phase in the rhizosphere of the Ni hyperaccumulator. Moreover, chlorite and serpentine were identified as the dominant clay minerals in these soils but the presence of smectite (a weathering product of serpentine) was only identified in the rhizosphere soil of the hyperaccumulator. Without doubt further research is necessary to further understand the complexity of the physicochemical and biological processes occurring in the soil-rhizosphere-hyperaccumulator plant system. Studies investigating the kinetics of soil Ni replenishment (and rate of supply from the soil solid phase) in the rhizosphere of Ni-hyperaccumulating plant species may shed further light on our understanding of the metal hyperaccumulation process.

Interactions between plant macro- and micro-nutrient tissue concentrations

Leaf concentrations of plants collected in the different field sites presented similar concentrations of macro-nutrients (such as Ca, Mg) and Ni to those previously reported for a range of Ni-hyperaccumulating *Alyssum* species (Bani *et al.* 2010; Galardi *et al.* 2007b; Zhang *et al.* 2014). In the field collected plants there was a significant positive correlation between shoot Ca and Mg concentrations and the shoot Ni concentration (r >0.8-1), and this was a general trend in all populations except SA. Moreover, the same correlation between these elements was also observed in plants grown in controlled conditions, especially in the roots of plants grown in hydroponic culture solutions. A similar relationship between Ca and Ni was also reported by Chaney *et al.* (2008) in two different *Alyssum* species (*A. murale* and *A. pintodasilvae*) when grown in hydroponic cultures (this effect was only linear up to a solution Ca concentration of 2 mM). Furthermore, the same authors showed that the solution Ca concentration also influenced shoot yield and Ni translocation from roots to shoots. Similarly, Brooks *et al.*(1981) in pot trials found a significant Ca-Ni association in populations of the same Ni-hyperaccumulating subspecies of *A. serpyllifolium* from the Iberian Peninsula that were used in this study (Trás-os-Montes region, Sierra Aguas and Sierra Bermeja). Gabbrielli and Pandolfini (1984) suggested that Ca had a detoxifying effect towards Mg and Ni toxicity in the root development of *Alyssum bertolonii*. Proctor and McGowan (1976) demonstrated that Mg could ameliorate Ni toxicity, possibly by a similar mechanism to that of Ca. In contrast to the soils, the Ca/Mg ratios in plants were consistently >1, and up to 5.5 in SA (where soil exchangeable Ca was also higher). It has been suggested that the ability to maintain a high leaf Ca/Mg ratio (by selective translocation of Ca and/or inhibited transport of Mg from roots) is a key evolutionary change which was needed by plants for survival on serpentine soils (O'Dell *et al.* 2006). In this study the *Alyssum* subspecies in the field, or those cultivated in hydroponic culture or serpentine soil in the pot experiment all had the capacity to improve the Ca/Mg ratio in their tissues, thus inverting the low Ca/Mg ratio observed in the serpentine soils of their origin. A better understanding of these complex biogeochemical interactions between Ni and other relevant ions should be necessary to clarify the main processes involved in tolerance and adaptation capacity of hyperaccumulating plants from serpentinitic environments.

Nickel tolerance and accumulation in different growth substrates

Ni concentrations in the stems of the field-collected plants ranged from 0.9 to 4.1 g kg^{-1}, and they were always lower than in leaf tissues (ranging from 3.6 to 16.6 g kg^{-1} across the five populations). A higher Ni concentration in the leaves

rather than in the stems has been previously found for several Ni-hyperaccumulating species, including other *Alyssum* species, *Bornmuellera thymphaea* or *Leptoplax emarginata* (Bani *et al.* 2009; Zhang *et al.* 2014). Nickel has been shown to be concentrated in epidermal cell vacuoles (Broadhurst *et al.* 2004). The leaf Ni concentrations found for the five populations of the Ni-hyperaccumulating subspecies were within the same range as that observed for different populations of *A. murale*, *A. bertolonii* and *Alyssum markgrafii* collected in Greece and Albania (Bani *et al.* 2007; Zhang *et al.* 2014). Even higher shoot Ni concentrations were found in field-grown *A. murale* and *Alyssum corsicum* plants (ranging from 4.2 to 20.4 g kg^{-1}) (Li *et al.* 2003a).

Several studies have associated the variability in metal tolerance or metal accumulation of hyperaccumulating plants to the concentration of these metals in the soil. Some studies have shown that plants with the highest levels of tolerance or with the strongest ability to concentrate metals are those found growing in soils with the lowest metal concentration (Dechamps *et al.* 2005; Escarré *et al.* 2000; Meerts and Van Isacker 1997; Shallari *et al.* 1998; Wu *et al.* 2009). In contrast, other studies have reported that those plants growing in higher soil metal concentrations also showed the highest metal accumulation in their tissues, especially for Cd (Lombi *et al.* 2000; Reeves *et al.* 2001; Roosens *et al.* 2003). In the present study the total soil Ni concentration did not reflect the tissue Ni concentrations which were found in the different populations. Although plants from L growing in the field showed the highest nickel concentration in leaves and the highest concentration of $Sr(NO_3)_2$-extractable Ni in their rhizosphere soil. Similarly, Kazakou *et al.* (2010) observed that Ni hyperaccumulation in different populations of *A. lesbiacum* varied according to the soil Ni availability. These results support the general consensus that available soil metal fractions are more closely related to plant uptake than the total soil metal concentration (Adriano 2001). However, in the present study we did not find any significant correlation between soil Ni concentrations and accumulation in the different populations of *A. serpyllifolium* growing in the field.

Many studies evaluating the tolerance of hyperaccumulating plant species (including *Noccaea* and *Alyssum* spp.) in hydroponic conditions observed a reduction in plant biomass production with an increase in the metal concentration in the hydroponic solution (Escarré *et al.* 2013; Ghasemi and Ghaderian 2009; Keller *et al.* 2006; Roosens *et al.* 2003). Similarly, in our study the general reduction in plant growth observed in the High-Ni treatment (1000 µM $NiSO_4$) suggested that this Ni concentration was phytotoxic for both *A. pintodasilvae* and *A. malacitanum*. In the hydroponic study, shoot and root growth were strongly correlated, and this has been observed in numerous studies (e.g. Assunçao *et al.*

2003). Moreover, several studies (Galardi *et al.* 2007a; Meyer *et al.* 2010) have reported a high variability in metal tolerance index between and within populations in hyperaccumulators such as *A. halleri* or *A. bertolonii* when grown in hydroponic culture solutions.

Plant growth in the pot experiment was significantly higher compared to that obtained in the hydroponic solutions, which could be attributed to the longer growth time in the pot experiment and also that these plants are adapted to growing in serpentine soils and that hydroponic solutions do not reflect the physicochemical characteristics of soils. Differences in plant growth between experiments carried out in hydroponic cultures and in soils are well known (Escarré *et al.* 2000; Li *et al.* 2003b; Lombi *et al.* 2000). This difference between growth substrates was most pronounced in the case of the SB population, which presented the lowest DW yields in hydroponic solutions but when cultivated in the soils this was the highest yield-producing population.

The highest shoot Ni concentrations were observed in plants grown in hydroponic conditions, presumably due to the higher Ni availability in the solutions than in the soil (either field or pot experiment). In fact, the water-soluble Ni concentrations in the soil used for the pot experiment were close to the Ni concentrations in the Control treatment of the hydroponic solutions (data not shown). In accordance, the shoot Ni concentrations were also similar between the Control treatment in the hydroponic solutions and that observed in the pot experiment. However, the field plants presented a significantly higher shoot/leaf Ni concentration than that obtained in plants grown in the pot experiment. This can be explained by the differences in plant age, root proliferation (plants have a restricted space for root proliferation in pots), and differences in the edaphic properties of each site. In hydroponic cultures the shoot and root Ni concentration of plants from the different populations was directly influenced by the concentration of Ni in the solution, and increased with an increase in solution concentration. As mentioned above the High-Ni treatment was phytotoxic and the variability in shoot Ni concentrations was reduced compared to the Low-Ni treatment where a higher proportion of variance in Ni accumulation was attributed to inter-population differences. In this treatment, the SB population showed a higher capacity for Ni accumulation but this population was also the least efficient biomass producer. Whereas the S population produced a higher biomass this resulted in a higher Ni yield (or phytoextracted Ni). Pollard *et al.* (2014) suggested that in order to evaluate the plant hyperaccumulation capacity under controlled laboratory conditions it is essential that none of the applied treatments exceed the limits of tolerance of the population under study. In the hydroponic study the most appropriate Ni concentration for evaluating the hyperaccumulation capacity of

these five populations of *A. serpyllifolium* was therefore that of the Low-Ni treatment.

In the hydroponic study, the Portuguese populations (S and M) showed a lower Ni S:R ratio when the solution Ni concentration increased, which would coincide with the fact that the higher concentration of Ni was phytotoxic. Similarly, Visioli *et al.* (2014) observed a reduction in the Ni S:R ratio in two metallicolous *N. caerulescens* populations when grown in hydroponic conditions (these authors used a lower concentration than those used in this study). In contrast, studies carried out by Galardi *et al.* (2007a) and Adamidis *et al.* (2014) did not observe any tendency towards a reduction in the Ni S:R ratio of either *A. bertolonii* or *A. lesbiacum* as the Ni treatment increased. This was also the case for both the L and SB populations in this study. It is also noteworthy that the L population in the pot experiment tended to present higher Ni S:R ratios which is an important trait for phytoextraction applications. The Ni S:R ratio was not significantly correlated to either shoot or root Ni concentration in any of the *A. serpyllifolium* populations when grown in either hydroponic culture or the pot experiment. A similar result was found by Richau and Schat (2009) who suggested that Ni/Zn accumulation by *N. caerulescens* when grown in nutrient solutions was related to the plant's capacity for metal uptake rather than to the root to shoot translocation capacity.

Comparison of Ni accumulation between mother plants (growing in their natural habitats) and their progeny when grown in controlled conditions

Natural variation in metal accumulation is an important issue to be considered in phytoextraction or phytomining processes due to the possibility for improving related hyperaccumulation traits in plants through selective breeding (Pollard *et al.* 2002). Several authors have investigated the relationship in tolerance and hyperaccumulation capacity between individual plants and their progeny as a means of estimating genetic variation and the potential heritability of these traits. These studies have concentrated on two model hyperaccumulating species: *N. caerulescens* and *A. halleri*, and have been carried out in a range of growth conditions (Escarré *et al.* 2000; Frérot *et al.* 2010; Frérot *et al.* 2003; Pollard and Baker 1996; Richau and Schat 2009).

In our study, we have documented important inter-population differences in Ni accumulation in plant populations growing in the field. In contrast, Ni accumulation of mother plants was not significantly correlated with the shoot Ni concentration of their descendants when these were grown in either hydroponic culture solutions or in serpentine soil under controlled conditions. This result is in accordance to Galardi *et al.* (2007a), who observed a positive correlation between

Ni tolerance and accumulation in serpentine *Alyssum bertolonii* populations when grown in hydroponic culture solutions, but they did not observe any correlation between the data obtained in hydroponics with either, the metal concentrations determined in plants collected in the field, or the variation observed in genetic diversity.

The lack of any clear relationship between mother plants and their progeny could be due to differences in the growth conditions (i.e. field grown plants versus hydroponics or pot experiments), and the different environmental conditions that affect each population in the field. Along these lines, Galardi *et al.* (2007b) concluded that the observed variation in Ni accumulation amongst different populations of *A. bertolonii* in the field was mainly due to the influence of the soil properties. This could in part also explain the results observed in this study with the two subspecies of *A. serpyllifolium*; e.g. the L population which presented the highest Ni accumulation in the field was also the population with the highest concentration of $Sr(NO_3)_2$-extractable Ni in rhizosphere soils. However, the same is not true of the SB population which also accumulated significantly higher concentrations of Ni in the field than either S or M populations.

In addition, in controlled conditions (i.e. the hydroponic solutions and pot experiment) most of the variance observed in the different variables considered (biomass, tolerance index, macro- and micronutrients, metal bioaccumulation factor) occurred at the intra-population level, which is clearly contradictory to what was observed in the field-collected plant material. Moreover, the mixed model analysis seems to reveal that the variability in the Ni accumulation observed in the different populations of the hyperaccumulating *A. serpyllifolium* subspecies was not related to maternal effects. Similarly, Gonneau *et al.* (2014) found that most of the variability in biomass production and elemental composition amongst *N. caerulescens* plants was attributed to within population differences (these authors included both serpentine and calamine populations in their study, and also evaluated Zn accumulation in calamine populations).

As discussed above, the differences observed between populations collected in the field can be greatly affected by the differences in environmental conditions at each site. At the same time, the low inter-population effects in the variance in Ni hyperaccumulation may be the result of the evolutionary history of serpentine populations of *A. serpyllifolium*. Genetic analyses seem to indicate that the Ni-hyperaccumulating serpentine populations of *A. serpyllifolium* sub-species are the result of local evolutionary events (i.e. they have evolved from non-hyperaccumulating populations growing on limestone or other calcareous soils) (Celestino Quintela-Sabarís, personal communication). The microevolution event that allowed the adaptation to the harsh environmental conditions on

serpentine outcrops (nutrient deficiency, metal toxicity, drought stress, higher insolation…) and the colonisation of these areas, may have acted as a genetic filter (only the plants with serpentine-adapted genotypes survive) that contributes to reducing the inter-population genetic differences.

Escarré *et al.* (2000), analysed several metallicolous and non-metallicolous populations of *N. caerulescens*, and suggested that a sufficient degree of genetic variation in both biomass production and metal bioaccumulation exists in this species, making it possible to breed hyperaccumulator plant cultivars with increased extraction capacity. The results of this study, which are based on the analysis of Ni-hyperaccumulating populations only (i.e. metalliferous), revealed a lower variability than that observed in the study of Escarré *et al.* (2000). However, under controlled conditions the present study revealed significant differences in biomass production and root-shoot Ni transfer that could be explored to increase the Ni yield of *A. serpyllifolium*.

A recent study by (Maestri *et al.* 2013) suggests that phenotypic variability in *N. caerulescens* is not supported by genotypic variability in selected genes which are involved in metal homeostasis, and therefore different genetic factors may be involved in the observed variability in phenotype. It has been shown that the hyperaccumulation trait is not dependent on a single mechanism, but a result of multiple processes which may include metal uptake, chelation and transport, metal sequestration into specific organelles or tissues, and rhizosphere interactions, and is likely to be controlled by multiple genetic factors (Fasani 2012; Kramer 2010). Therefore, numerous genetic factors can be responsible for the significant variability amongst and within populations of hyperaccumulating plant species (Galardi *et al.* 2007b; Macnair 2002; Maestri *et al.* 2013). Further studies related to the genetic basis of the Ni tolerance and hyperaccumulation in these Ni-hyperaccumulating *A. serpyllifolium* subspecies are therefore needed.

3.5 *REFERENCES*

Adamidis G, Aloupi M, Kazakou E and Dimitrakopoulos P (2014). Intra-specific variation in Ni tolerance, accumulation and translocation patterns in the Ni-hyperaccumulator *Alyssum lesbiacum*. *Chemosphere* 95: 496-502.

Adriano DC (2001). Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals. Springer, New York, NY.

Al-Najar H, Schulz R and Römheld V (2003). Plant availability of thallium in the rhizosphere of hyperaccumulator plants: a key factor for assessment of phytoextraction. *Plant Soil* 249: 97-105.

Asensi A, Rodríguez N, Díez-Garretas B, Amils R, Boyd R, Baker A and Proctor J (2004). Nickel hyperaccumulation of some subspecies of *Alyssum serpyllifolium* (Brassicaceae) from ultramafic soils on the Iberian Peninsula. Ultramafic rocks: Their soils, vegetation and fauna, Proceedings

of the fourth International Conference on Serpentine Ecology. Science Reviews, St. Albans, UK. 263-265.

Assunção AGL, Bookum WM, Nelissen HJ, Vooijs R, Schat H and Ernst WH (2003). A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytol* 159: 383-390.

Baker AJM and Brooks RR (1989). Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81-126.

Bani A, Echevarria G, Sulçe S, Morel J and Mullai A (2007). In-situ phytoextraction of Ni by a native population of *Alyssum murale* on an ultramafic site (Albania). *Plant Soil* 293: 79-89.

Bani A, Echevarria G, Mullaj A, Reeves R, Louis Morel J and Sulçe S (2009). Nickel hyperaccumulation by Brassicaceae in serpentine soils of Albania and Northwestern Greece. *Northeast Nat* 16: 385-404.

Bani A, Pavlova D, Echevarria G, Mullai A, Reeves RD, Morel JL and Sulçe S (2010). Nickel hyperaccumulation by the species of *Alyssum* and *Thlaspi* (Brassicaceae) from the ultramafic soils of the Balkans. *Botanica Serbica* 34: 3-14.

Bates D, Maechler M and Bolker B (2012). lme4: Linear mixed-effects models using S4 classes. R package version 0.999999-0.

Brady KU, Kruckeberg AR and Bradshaw H (2005). Evolutionary ecology of plant adaptation to serpentine soils. *Annu Rev Ecol Evol Syst* 36: 243-266.

Broadhurst CL, Chaney RL, Angle JS, Maugel TK, Erbe EF and Murphy CA (2004). Simultaneous hyperaccumulation of nickel, manganese, and calcium in *Alyssum* leaf trichomes. *Environ Sci Technol* 38: 5797-5802.

Brooks RR, Lee J, Reeves RD and Jaffre T (1977). Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *J Geochem Explor* 7: 49-57.

Brooks RR, Shaw S and Marfil AA (1981). Some observations on the ecology, metal uptake and nickel tolerance of *Alyssum serpyllifolium* subspecies from the Iberian Peninsula. *Plant Ecol* 45: 183-188.

Brooks RR (1987). Serpentine and its vegetation: A multidisciplinary approach. Dioscorides Press, Portland, OR.

Carballeira A, Devesa C, Retuerto R, Santillán E and Ucieda F (1983). Bioclimatología de Galicia. Fundación Pedro Barríe de la Maza, Conde de Fenosa, A Coruña.

Cecchi L, Colzi I, Coppi A, Gonnelli C and Selvi F (2013). Diversity and biogeography of Ni-hyperaccumulators of *Alyssum* section *Odontarrhena* (Brassicaceae) in the central western Mediterranean: evidence from karyology, morphology and DNA sequence data. *Bot J Linn Soc* 173: 269-289.

Centofanti T, Sayers Z, Cabello-Conejo MI, Kidd P, Nishizawa NK, Kakei Y, Davis AP, Sicher RC and Chaney RL (2013). Xylem exudate composition and root-to-shoot nickel translocation in *Alyssum* species. *Plant Soil* 373: 59-75.

Chaney RL, Chen KY, Li YM, Angle JS and Baker AJM (2008). Effects of calcium on nickel tolerance and accumulation in *Alyssum* species and cabbage grown in nutrient solution. *Plant Soil* 311: 131-140.

Chaney RL, Fellet G, Torres R, Centofanti T, Green CE and Marchiol L (2009). Using chelator-buffered nutrient solutions to limit Ni phytoavailability to the Ni-hyperaccumulator *Alyssum murale*. *Northeast Nat* 16: 215-222.

Consejería de Medio Ambiente (2009). REDIAM, Red de Información Ambiental de Andalucía. Consejería de Medio Ambiente. Junta de Andalucía, Sevilla, Spain.

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

Delorme TA, Gagliardi JV, Angle JS and Chaney RL (2001). Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. and the non metal accumulator *Trifolium pratense* L. on soil microbial populations. *Can J Microbiol* 47: 773-776.

Echevarria G, Morel J, Fardeau J and Leclerc-Cessac E (1998). Assessment of phytoavailability of nickel in soils. *J Environ Qual* 27: 1064-1070.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y and 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.

Escarré J, Lefebvre C, Frérot H, Mahieu S and Noret N (2013). Metal concentration and metal mass of metallicolous, non metallicolous and serpentine *Noccaea caerulescens* populations, cultivated in different growth media. *Plant Soil* 370: 197-221.

Everhart JL, McNear D, Jr., Peltier E, van der Lelie D, Chaney RL and Sparks DL (2006). Assessing nickel bioavailability in smelter-contaminated soils. *Sci Total Environ* 367: 732-744.

Fasani E (2012). Plants that hyperaccumulate heavy metals. In: A Furini (ed) Plants and Heavy Metals. Springer, Netherlands. p. 55-74.

Frérot H, Petit C, Lefèbvre C, Gruber W, Collin C and Escarré J (2003). Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* (Brassicaceae). *New Phytol* 157: 643-648.

Frérot H, Faucon MP, Willems G, Godé C, Courseaux A, Darracq A, Verbruggen N and Saumitou-Laprade P (2010). Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL× environment interactions. *New Phytol* 187: 355-367.

Gabbrielli R and Pandolfini T (1984). Effect of Mg^{2+} and Ca^{2+} on the response to nickel toxicity in a serpentine endemic and nickel-accumulating species. *Physiol Plant* 62: 540-544.

Galardi F, Corrales I, Mengoni A, Pucci S, Barletti L, Barzanti R, Arnetoli M, Gabbrielli R and Gonnelli C (2007a). Intra-specific differences in nickel tolerance and accumulation in the Ni-hyperaccumulator *Alyssum bertolonii*. *Environ Exp Bot* 60: 377-384.

Galardi F, Mengoni A, Pucci S, Barletti L, Massi L, Barzanti R, Gabbrielli R and Gonnelli C (2007b). Intra-specific differences in mineral element composition in the Ni-hyperaccumulator *Alyssum bertolonii*: A survey of populations in nature. *Environ Exp Bot* 60: 50-56.

Ghasemi R and Ghaderian SM (2009). Responses of two populations of an Iranian nickel-hyperaccumulating serpentine plant, *Alyssum inflatum* Nyar., to substrate Ca/Mg quotient and nickel. *Environ Exp Bot* 67: 260-268.

Gómez-Zotano J, Román-Requena F, Hidalgo-Triana N and Pérez-Latorre AV (2014). Biodiversidad y valores de conservación de los ecosistemas serpentínicos en España: Sierra Bermeja (Provincia de Málaga). *B Asoc Geogr Esp* 65: 187-206.

Gonneau C, Genevois N, Frérot H, Sirguey C and Sterckeman T (2014). Variation of trace metal accumulation, major nutrient uptake and growth parameters and their correlations in 22 populations of *Noccaea caerulescens*. *Plant Soil* 384: 271-287.

Gross J and Ligges U (2012). Nortest: Tests for Normality. R package version: 1.0-1.

Hammer D and Keller C (2002). Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractants. *J Environ Qual* 31: 1561-1569.

Hammer D, Keller C, McLaughlin MJ and Hamon RE (2006). Fixation of metals in soil constituents and potential remobilization by hyperaccumulating and non-hyperaccumulating plants: Results from an isotopic dilution study. *Environ Pollut* 143: 407-415.

Hinsinger P (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant Soil* 237: 173-195.

Hinsinger P, Gobran GR, Gregory PJ and Wenzel WW (2005). Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. *New Phytol* 168: 293-303.

Hutchinson JJ, Young SD, McGrath SP, West HM, Black CR and Baker AJ (2000). Determining uptake of 'non-labile'soil cadmium by *Thlaspi caerulescens* using isotopic dilution techniques. *New Phytol* 146: 453-460.

Kazakou E, Dimitrakopoulos PG, Baker AJM, Reeves RD and Troumbis AY (2008). Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. *Biol Rev* 83: 495-508.

Kazakou E, Adamidis GC, Baker AJM, Reeves RD, Godino M and Dimitrakopoulos PG (2010). Species adaptation in serpentine soils in Lesbos Island (Greece): metal hyperaccumulation and tolerance. *Plant Soil* 332: 369-385.

Keller C, Diallo S, Cosio C, Basic N and Galland N (2006). Cadmium tolerance and hyperaccumulation by *Thlaspi caerulescens* populations grown in hydroponics are related to plant uptake characteristics in the field. *Funct Plant Biol* 33: 673-684.

Kidd PS and Monterroso C (2005). Metal extraction by *Alyssum serpyllifolium* ssp. *lusitanicum* on mine-spoil soils from Spain. *Sci Total Environ* 336: 1-11.

Kidd PS, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R and Monterroso C (2009). Trace element behaviour at the root-soil interface: Implications in phytoremediation. *Environ Exp Bot* 67: 243-259.

Knight B, Zhao FJ, McGrath SP and Shen ZG (1997). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. *Plant Soil* 197: 71-78.

Kramer U (2010). Metal hyperaccumulation in plants. *Annu Rev Plant Biol* 61: 517-534.

Kruckeberg AR (1984). California serpentines: Flora, vegetation, geology, soils, and management problems. University of California Press, Berkeley, CA.

Li T, Di Z, Islam E, Jiang H and Yang X (2011). Rhizosphere characteristics of zinc hyperaccumulator *Sedum alfredii* involved in zinc accumulation. *J Hazard Mater* 185: 818-823.

Li YM, Chaney R, Brewer E, Roseberg R, Angle JS, Baker A, Reeves R and Nelkin J (2003a). Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. *Plant Soil* 249: 107-115.

Li YM, Chaney RL, Brewer EP, Angle JS and Nelkin J (2003b). Phytoextraction of nickel and cobalt by hyperaccumulator *Alyssum* species grown on nickel-contaminated soils. *Environ Sci Technol* 37: 1463-1468.

Lombi E, Zhao FJ, Dunham SJ and McGrath SP (2000). Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytol* 145: 11-20.

Lombi E, Wenzel WW, Gobran GR and Adriano DC (2001). Dependency of phytoavailability of metals on indigenous and induced rhizosphere processes: A review. In: GR Gobran et al. (eds) Trace elements in the rhizosphere. Crc Press-Taylor and Francis Group, Boca Raton, FL. p. 3-24.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P and Petit D (1999). Zinc tolerance and hyperaccumulation are genetically independent characters. *Proc R Soc Lond B* 266: 2175-2179.

Macnair MR (2002). Within and between population genetic variation for zinc accumulation in *Arabidopsis halleri*. *New Phytol* 155: 59-66.

Maestri E, Pirondini A, Visioli G and Marmiroli N (2013). Trade-off between genetic variation and ecological adaptation of metallicolous and non-metallicolous *Noccaea* and *Thlaspi* species. *Environ Exp Bot* 96: 1-10.

Marschner H, Römheld V and Cakmak I (1987). Root-induced changes of nutrient availability in the rhizosphere. *J Plant Nutr* 10: 1175-1184.

Massoura ST, Echevarria G, Leclerc-Cessac E and Morel JL (2004). Response of excluder, indicator, and hyperaccumulator plants to nickel availability in soils. *Soil Res* 42: 933-938.

McGrath SP, Shen Z and Zhao F (1997). Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. *Plant Soil* 188: 153-159.

Meerts P and Van Isacker N (1997). Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecol* 133: 221-231.

Menezes de Sequeira E (1969). Toxicity and movement of heavy metals in serpentinic soils (north-eastern Portugal). *Agron Lusit* 30: 115-154.

Menezes de Sequeira E and Pinto da Silva AR (1991). Ecology of serpentinized areas of north-east Portugal. In: BA Roberts and J Proctor (eds) The ecology of areas with serpentinized rocks: A world view. Kluwer Academic Publishers, Dordrecht, Netherlands. p. 169-197.

Meyer CL, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M and Frérot H (2010). Variability of zinc tolerance among and within populations of the pseudometallophyte species *Arabidopsis halleri* and possible role of directional selection. *New Phytol* 185: 130-142.

O'Dell RE, James JJ and Richards JH (2006). Congeneric serpentine and nonserpentine shrubs differ more in leaf Ca: Mg than in tolerance of low N, low P, or heavy metals. *Plant Soil* 280: 49-64.

Pinheiro J, Bates D, DebRoy S and Sarkar D (2007). Linear and nonlinear mixed effects models. R package version 3.1-97.

Pollard AJ and Baker AJM (1996). Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytol* 132: 113-118.

Pollard AJ, Powell KD, Harper FA and Smith JAC (2002). The genetic basis of metal hyperaccumulation in plants. *Crit Rev Plant Sci* 21: 539-566.

Pollard AJ, Reeves RD and Baker AJM (2014). Facultative hyperaccumulation of heavy metals and metalloids. *Plant Sci* 217: 8-17.

Proctor J and McGowan ID (1976). Influence of magnesium on nickel toxicity. *Nature* 260: 134.

Proctor J and Roberts BA (1992). The ecology of areas with serpentinized rocks: A world view. Kluwer Academic Publishers, Dordrecht, Netherlands.

Proctor J and Baker AJM (1994). The importance of nickel for plant growth in ultramafic (serpentine) soils. In: SM Ross (ed) Toxic metals in soil-plant systems. John Wiley and Sons, Chichester, England. p. 417-432.

Proctor J (1999). Toxins, nutrient shortages and droughts: the serpentine challenge. *Trends Ecol Evol* 14: 334-335.

Puschenreiter M, Wieczorek S, Horak O and Wenzel WW (2003). Chemical changes in the rhizosphere of metal hyperaccumulator and excluder *Thlaspi* species. *J Plant Nutr Soil Sci* 166: 579-584.

Puschenreiter M, Schnepf A, Millan IM, Fitz WJ, Horak O, Klepp J, Schrefl T, Lombi E and Wenzel WW (2005). Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. *Plant Soil* 271: 205-218.

Rauret G, Lopez-Sanchez J, Sahuquillo A, Rubio R, Davidson C, Ure A and Quevauviller P (1999). Improvement of the BCR three step sequential extraction procedure prior to the certification of new sediment and soil reference materials. *J Environ Monit* 1: 57-61.

Reeves RD (1992). The hyperaccumulation of nickel by serpentine plants. In: AJM Baker et al. (eds) The vegetation of ultramafic (serpentine) soils. Intercept Ltd, Andover, MA. p. 253-277.

Reeves RD, Schwartz C, Morel JL and Edmondson J (2001). Distribution and metal-accumulating behavior of *Thlaspi caerulescens* and associated metallophytes in France. *Int J Phytoremediat* 3: 145-172.

Richau KH and Schat H (2009). Intraspecific variation of nickel and zinc accumulation and tolerance in the hyperaccumulator *Thlaspi caerulescens*. *Plant Soil* 314: 253-262.

Rodriguez-Oubiña J and Ortiz S (1991). Los pastizales pioneros vivaces de los suelos serpentínicos del NO ibérico. *Lazaroa* 12: 333-344.

Roosens N, Verbruggen N, Meerts P, Ximénez-Embún P and Smith JAC (2003). Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from western Europe. *Plant, Cell Environ* 26: 1657-1672.

Shallari S, Schwartz C, Hasko A and Morel JL (1998). Heavy metals in soils and plants of serpentine and industrial sites of Albania. *Sci Total Environ* 209: 133-142.

Shallari S, Echevarria G, Schwartz C and Morel J (2001). Availability of nickel in soils for the hyperaccumulator *Alyssum murale* Waldst. & Kit. *S Afr J Sci* 97: 568-570.

Silva Gonzaga MI, Santos JA and Ma LQ (2006). Arsenic chemistry in the rhizosphere of *Pteris vittata* L. and *Nephrolepis exaltata* L. *Environ Pollut* 143: 254-260.

Ueno D, Milner MJ, Yamaji N, Yokosho K, Koyama E, Clemencia Zambrano M, Kaskie M, Ebbs S, Kochian LV and Ma JF (2011). Elevated expression of TcHMA3 plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Plant J* 66: 852-862.

Uren NC (2000). Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In: R Pinton et al. (eds) The rhizosphere: Biochemistry and organic substance at the soil-plant interface. Marcel Dekker, New York, NY. p. 1-21.

Van der Ent A, Baker AJM, Reeves RD, Pollard AJ and Schat H (2013). Hyperaccumulators of metal and metalloid trace elements: facts and fiction. *Plant Soil* 362: 319-334.

Visioli G, Gullì M and Marmiroli N (2014). *Noccaea caerulescens* populations adapted to grow in metalliferous and non-metalliferous soils: Ni tolerance, accumulation and expression analysis of genes involved in metal homeostasis. *Environ Exp Bot* 105: 10-17.

Wenzel W, Bunkowski M, Puschenreiter M and Horak O (2003). Rhizosphere characteristics of indigenously growing nickel hyperaccumulator and excluder plants on serpentine soil. *Environ Pollut* 123: 131-138.

Wu F, Leung H, Wu S, Ye Z and Wong M (2009). Variation in arsenic, lead and zinc tolerance and accumulation in six populations of *Pteris vittata* L. from China. *Environ Pollut* 157: 2394-2404.

Zhang X, Houzelot V, Bani A, Morel JL, Echevarria G and Simonnot M-O (2014). Selection and combustion of Ni-hyperaccumulators for the phytomining process. *Int J Phytoremediat* 16: 1058-1072.

4

IMPROVING THE GROWTH AND Ni YIELD OF *hyperaccumulating plants using phytohormones*

ABSTRACT

The application of plant growth regulators (PGRs) or phytohormones could be an interesting option for stimulating biomass production of hyperaccumulating plants and, consequently, their metal phytoextraction capacity. The effect of exogenous applications of phytohormones (PGRs) on the Ni phytoextraction capacity of different Ni hyperaccumulating species was evaluated. This study was carried out in two parts (Part I and Part II). A preliminary study was carried out (Part I) in which two commercial products (Cytokin® and Promalin®), based on cytokinins and/or gibberellins, were applied at two concentration rates on the shoot biomass of four Ni hyperaccumulating *Alyssum* species (*A. corsicum*, *A. malacitanum*, *A. murale*, and *A. pintodasilvae*). Although the application of phytohormones had no clear positive effect, a slightly positive response to Promalin treatment was observed in the biomass production and Ni phytoextraction efficiency of *A. corsicum*. Therefore a wider study was carried out (Part II) to identify the most adequate phytohormone treatments as well as the appropriate concentration. In this study four commercially available phytohormones (Berelex®, Cytoplant®, Kelpak® and Promalin®), based on gibberellins, cytokinins and auxins (indoleacetic acid), were applied to the aerial tissues of four Ni hyperaccumulating species (*Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense*). Each product was applied at three concentrations. The effect on biomass production was dependent on the species, the PGR type and the concentration at which it was applied. Two of the four products (Kelpak® and Promalin®) consistently increased biomass production compared to untreated control plants in all plant species. Moreover, the application of PGRs led to a significant increase in the number of branches (and leaves in the case of *N. goesingense*) of all species compared to control plants. Application of phytohormones led to a reduction in shoot Ni concentration. Nonetheless, in some cases as a consequence of the increase observed in biomass after the application of phytohormones a significant increase in the Ni phytoextraction efficiency was also observed. The results show that PGRs can be successfully used to improve the growth and biomass production of hyperaccumulating species such as *Alyssum* and *Noccaea*. The most effective PGR for increasing Ni removal was the IAA-based product (Kelpak®).

This study formed part of the following publications:

Cabello-Conejo MI, Centofanti T, Kidd PS, Prieto-Fernández A and Chaney RL (2013). Evaluation of plant growth regulators to increase nickel phytoextraction by *Alyssum* species. *Int J Phytoremediat* 15: 365-375.

Cabello-Conejo MI, Prieto-Fernández A and Kidd PS (2014). Exogenous treatments with phytohormones can improve growth and nickel yield of hyperaccumulating plants. *Sci Total Environ* 494-495: 1-8.

4.1 *INTRODUCTION*

Plant Growth Regulators (PGRs) are a group of naturally occurring organic compounds that at low concentrations regulate physiological processes in plants (Pazurkiewicz Kocot 2003). Nowadays, PGRs are used in many areas in agriculture, horticulture and floriculture for a wide range of purposes, such as increasing plant growth, delaying or promoting ripening, induction of rooting, lateral branching, promotion of abscission or weed control (Emongor 1995). Numerous studies have shown positive effects on the growth and development of a wide range of forage, cereal or fruit crops after the application of PGRs (Nickell 1982; Weaver 1972). Kefeli and Kalevitch (2003) have classified PGRs into seven different groups: auxins, cytokinins, gibberellins, abscisic acid, brassinosteroids, salicylic acid and jasmonates. Each of these phytohormones is involved in different processes and affects the plant in a specific way. For example, auxins (such as indoleacetic acid, IAA) are known to stimulate cell elongation, growth of roots and shoots, and suppress the development of lateral buds (apical dominance) (Thimann and Skoog 1934). Cytokinins (CKs; such as kinetin, benzyladenine) are known for their ability to induce plant cell division and have also been shown to play an important role in the regulation of plant response to environmental stress (Ha *et al.* 2012). Gibberellins (GAs; such as GA_3, GA_4, GA_7) have been shown to regulate stem growth and elongation, induction of seed germination and fruit setting and growth (Jones 1973; Salisbury and Ross 1992; Taiz and Zeiger 2006). The PGR effects on the plant vary according to the applied concentration, environmental factors influencing their absorption, and on the physiological status of the plant at the time of application (Carey 2008).

Due to the fact that PGRs may stimulate plant growth or reduce abiotic stress their use has also been considered as a means of enhancing the efficiency of remediation techniques such as phytoextraction (Barbafieri and Tassi 2010; Cassina *et al.* 2011). Phytoextraction is based on the cultivation of plants to accumulate trace metals from contaminated soils and transport them to the shoots which can then be harvested. In those cases where the economic value of the recovered metal is the primary motive the process is known as phytomining (Chaney 1983). The technique of phytomining involves growing a crop of a metal-hyperaccumulating plant species, harvesting the biomass and burning it to produce a bio-ore. Metal-hyperaccumulating plants are ideal candidates due to their extraordinary capacity to absorb and accumulate metals in their harvestable parts (Baker *et al.* 1994). To be used in phytoextraction technologies, hyperaccumulators must be highly metal tolerant, able to accumulate large

concentrations of the targeted trace elements in harvestable shoots, and have a reasonable biomass production so that metal removal from the site is economic (Li *et al.* 2003; Vangronsveld *et al.* 2009). The efficiency of the process can be limited by poor plant survival and growth, metal phytotoxicity or restricted soil metal bioavailability, making the application of PGRs a potential means of overcoming some of these bottlenecks. Three groups of PGRs have been proposed as being useful for phytoextraction purposes: auxins, cytokinins, and gibberellins (Bulak *et al.* 2014). Several studies have demonstrated that the application of auxins (IAA) can increase shoot metal accumulation, resulting in a higher metal removal yield which is the primary objective of phytoextraction. Hadi *et al.* (2010) have observed that applying a foliar spray of IAA at a concentration of 0.175 mg L^{-1} significantly increases the total Pb accumulation in *Zea mays*. Liphadzi *et al.* (2006) have applied IAA on *Helianthus annuus* and observed an increased Pb and Cd accumulation in leaves. Recent studies have tested the effects of PGRs on Ni phytoextraction by hyperaccumulators within the *Alyssum* genus. Cassina *et al.* (2011) have demonstrated that applications of cytokinins (Cytokin®) to *Alyssum murale* grown in serpentine soil improves Ni phytoextraction (mg Ni pot^{-1}) due to a higher biomass production in treated plants compared to control plants. Qiu *et al.* (2009) evaluated the effects of exogenous citric acid and malic acid on the uptake of Ni by the Ni hyperaccumulator *Alyssum corsicum* and the non-accumulator leaf mustard grown in hydroponic culture. These authors observed that citric acid reduced Ni concentration in roots of *A. corsicum*, whereas the application of malic acid enhanced Ni translocation and shoot Ni concentration. These studies highlight the need to study more types of PGRs for application in phytoextraction processes, as well as the optimum application method, concentration and timing of exogenous treatments.

The objective of these studies was to evaluate the effect of different phytohormones on the biomass production and Ni phytoextraction of several Ni -hyperaccumulating species (*A. corsicum*, *A. malacitanum*, *A. murale*, *A. pintodasilvae* and *Noccaea goesingense*) grown in serpentine soil. This was carried out in two stages: a preliminary experiment (Part I) tested two different phytohormones, based on cytokinins and/or gibberellins, and applied at two concentration rates on four Ni-hyperaccumulating *Alyssum* species; and a wider study (Part II) tested the effects of four commercial products (based on combinations of indoleacetic acid, cytokinins and/or gibberellins) when applied at three different concentrations, on Ni-hyperaccumulating species within the *Alyssum* and *Noccaea* genera. Effects on plant growth and biomass production, nutrient status and Ni phytoextraction efficiency were determined.

4.2 *MATERIALS AND METHODS*

Part I. Application of cytokinins and gibberellins at two concentration rates in four Ni-hyperaccumulating *Alyssum* species

The soil was collected from a vineyard area in Josephine County, Oregon, USA. It is a natural Brockman variant gravelly loam serpentine soil (Typic Xerochrepts) (Soil Survey Staff 2010). The collected soil was air-dried, sieved through a 2-mm stainless steel sieve and mixed for pot preparation and soil analysis. Chemical and physical properties of the serpentine soil used were described by Kukier *et al.* (2004). The soil has a slightly acid pH (pH_{H2O} 6.30), organic C content of 3.1 % and total Ni concentration of 4707 mg kg^{-1}. Basal fertilisers were added to the soil and thoroughly mixed to obtain a homogenous mixture. Nitrogen was added at 150 kg ha^{-1} as NH_4NO_3, phosphorus was added at 100 kg ha^{-1} as $Ca(H_2PO_4)_2 \cdot 2H_2O$, potassium was added at 150 kg ha^{-1} as half KCl and half K_2SO_4, calcium was added at 1000 kg ha^{-1} as $CaSO_4 \cdot 2H_2O$ (gypsum), and finally, boron was added at 1 kg ha^{-1} as H_3BO_3. In addition to the fertilisers, 10 % dry weight Pro-mix® potting soil was added to improve drainage. After mixing, approximately 1.5 kg of soil was weighed into each pot (drainage holes of pots were covered with a plastic mesh to retain the soil) and placed in a saucer to prevent loss of nutrients. A total of 60 pots of 15 cm diameter were used.

Four species of *Alyssum* were used: *A. corsicum, A. malacitanum*, *A. murale* and *A. pintodasilvae.* Seed of *A. corsicum* were collected from Turkey (Koycegiz), and *A. murale* 'Kotodesh' from Albania. *A. malacitanum* and *A. pintodasilvae* are endemic to the Iberian Peninsula (Asensi *et al.* 2004; Brooks *et al.* 1981; Menezes de Sequeira 1969). Seed of *A. malacitanum* were collected from Sierra Bermeja, Málaga (S Spain) and *A. pintodasilvae* from Morais, Trás-os-Montes (NE Portugal). Seeds were germinated on a 10:1 Promix®:vermiculite mixture in plastic flats kept in the greenhouse under controlled conditions. Seeds were watered daily with deionised water until germination and then twice per week with a 1:10 mixture of serpentine-like macro -nutrient solution which consisted of 2 mM $MgSO_4$, 0.8 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 0.1 mM K_2HPO_4, 20 μM FeEDDHA, 10 μM H_3BO_3, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.2 μM Na_2MoO_4 and 300 μM $NiSO_4$ (Chaney *et al.* 2008). The growth time prior to transferring into pots was different for every species so as to obtain similar sized plants at the time of transplanting. *A. corsicum* and *A. murale* were grown on the germination substrate for six weeks, *A. malacitanum* for ten weeks and *A. pintodasilvae* for fifteen weeks before transplanting. Seedlings were 2-3 cm tall when they were transferred into pots with serpentine soil. Five healthy plants of uniform size were transplanted

into each pot. After transplantation, the seedlings were grown on soil without phytohormone treatments for 39 days to allow the plants to adjust to the new substrate and recover from any stress caused by transplantation. After this adjustment period, two different commercially available phytohormones were applied: Cytokin® (Miller Chemical & Fertilizer Corporation, Hanover, Pensilvania, USA), a mixture of three naturally occurring cytokinins (kinetin), and Promalin® (Abott Laboratories, North Chicago, USA), a mixture of cytokinins (benzyladenine) and gibberellins in a 1:1 ratio. Treatment concentrations were based on Cassina *et al.* (2011) and Emongor *et al.* (2004). Four different treatments were applied: Cytokin® at a concentration of 15 mg L^{-1} (Low-CK), Cytokin® at a concentration of 60 mg L^{-1} (High-CK), Promalin® at a concentration of 60 mg L^{-1} and a control treatment (no PGRs were applied). Four replicates of each treatment were established, except for *A. pintodasilvae*, this species only received the High-CK treatment and the Promalin treatment due to a lack of plants. The pots were arranged in a randomised complete block design. The treatments were applied as a foliar spray; 20 mL pot^{-1} of each solution was sprayed on the plant shoots three times at 2 week intervals. Plants were watered (approx. 20 mL pot^{-1}) every other day with deionized water from the top of the pot, and were grown on the serpentine soil for a total of 90 days before harvesting. Twenty ml was found to be the optimum volume for spraying the plants without high losses due to dripping from the leaf surfaces. The experiment was carried out in the greenhouse with the following controlled conditions: 26 °C max. and 21 °C min. for both day and night: 16/8 day/night photoperiod. During the growth period, plants were observed to see if the treatments caused any toxicity or change in growth patterns. At harvest, shoots were separated from the root by cutting the stem 1 cm above the soil, and shoot fresh weight was recorded. Shoots were then rinsed in deionised water to remove any adhering soil particles, dried for 24 h at 60 °C and weighed to determine dry biomass; roots were not harvested. The samples were ashed in an oven at 480 °C for 16 h. After cooling, the ash was digested with 2 mL concentrated HNO_3, swirled and taken to dryness. The sample was then dissolved in 10 mL 3N HCl, filtered through Whatman #40 filter paper and brought to volume in a 25 mL volumetric flask using 0.1 N HCl. Ca, Fe, K, Mg, Mn, Ni, P and Zn concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-OES). The Ni phytoextraction efficiency (soil Ni removal) was calculated as the product of the shoot dry weight (DW) and the Ni concentration in shoots in relation to the total Ni content in the soil.

Part II. Application of cytokinins, gibberellins and auxins (IAA) at three concentration rates in four Ni hyperaccumulating plants

The soil used in this experiment was collected from the serpentinitic region of Barazón, located in Galicia (NW Spain). Soil was air-dried, sieved through a 2-mm stainless steel sieve and mixed for pot preparation and soil analysis. The soil had a pH_{H2O} of 6.7 and as expected for a serpentine soil, presented high concentrations of Ni, Co and Cr (2092, 175 and 1346 mg kg^{-1}, respectively), and a Ca/Mg ratio <1. Soil organic C and N were 1.97 % C and 0.15 % N. Basal fertilisers were added to the soil and thoroughly mixed to obtain a homogenous mixture. Nitrogen was added at 100 kg ha^{-1} as NH_4NO_3, phosphorus and potassium were added as K_2HPO_4 at 100 kg ha^{-1} and 125 kg ha^{-1}, respectively. In addition the soil was mixed with perlite in the ratio of 10:1 (v/v) to improve aeration and drainage. After mixing, approximately 700 g of soil was weighed into each pot. A total of 312 pots of 12.5 cm diameter were used.

Four Ni-hyperaccumulating plant species were used: *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense* (previously named *Thlaspi goesingense*). Seeds of *A. corsicum* were collected from Turkey (Koycegiz), and *A. murale* 'Kotodesh' from Albania. Seeds of *Alyssum malacitanum* were collected from Sierra Bermeja, Málaga (S Spain). Seeds of *N. goesingense* were collected from Redlschlag (E Austria). Seeds were germinated on a perlite:quartz sand mixture (2:1 v/v) in a growth chamber under controlled conditions (temperature 22-25 ºC, PPFD of 190 mmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle). Seeds were watered daily with deionised water until germination and then twice per week with a serpentine-like macro-nutrient solution as in Part I. Three-month-old seedlings (2-3 cm tall) were transferred into the pots containing the serpentine soil. Seedlings were grown in pots for one month before applying any treatments so as to allow them to adapt to the new substrate and recover from transplantation.

After this adjustment period, four different commercially available phytohormones were applied: B (Berelex® purchased from Kenogard, Barcelona, Spain), C (Cytoplant® from Daymsa, Zaragoza, Spain), K (Kelpak® from Daymsa, Zaragoza, Spain) and P (Promalin® purchased from Kenogard, Barcelona, Spain). Berelex is based on gibberellic acid (also called GA_3) (16000 mg L^{-1}). Cytoplant is based on natural seaweed extracts and has a cytokinic activity equivalent to 400 mg L^{-1} kinetin. Kelpak is also derived from marine algae extracts and contains 11 mg L^{-1} auxins (IAA, indoleacetic acid). Both Cytoplant and Kelpak may contain small quantities of other plant growth promoting substances, such as polysaccharides, micronutrients and/or vitamins, but are free of heavy metals. Both products are certified treatments for use in Organic

Agriculture (Regulation 2007). Promalin is a mixture of cytokinins (benzyladenine) and gibberellins (GA_4 and GA_7) in a 1:1 ratio. Three different concentrations of each product were applied: Berelex at a concentration of 0.1, 1 and 10 mg L^{-1} (B1, B2 and B3, respectively), Cytoplant at 1, 5 and 10 mg L^{-1} (C1, C2 and C3, respectively), Kelpak at 0.01, 0.05 and 0.1 mg L^{-1} (K1, K2 and K3, respectively) and Promalin at 5, 30 and 50 mg L^{-1} (P1, P2 and P3, respectively). A control treatment was included with no application of PGRs. Six replicates of each treatment were established and the pots were arranged in a randomised complete block design. The treatments were applied as a foliar spray; 20 mL pot^{-1} of each solution was sprayed on the plant shoots three times at 2 week intervals. Plants were watered (approx. 20 mL pot^{-1}) every other day with deionised water from the top of the pot, and were grown on the serpentine soil for a total of 90 days before harvesting. At harvest, shoots were separated from the root by cutting the stem 1 cm above the soil, shoots were then rinsed in deionised water to remove any adhering soil particles and shoot fresh weight was recorded. For those treatments where a significant effect was noted on shoot biomass, roots were also separated, thoroughly washed in deionised water and the fresh weight determined. Samples were dried for 48 h at 60 °C and weighed to determine DW yield. For each plant, the number of branches at the time of harvest (number of leaves in the case of *N. goesingense*) was also recorded. The length of the main stem of each plant in the *Alyssum* species was determined; likewise, in the case of *N. goesingense* the width and length of the largest leaf in each individual plant were recorded. Plant tissues were digested in a 2:1 HNO_3 (65 %):HCl (37 %) mixture and Ca, Co, Fe, K, Mg, Mn, Ni, P and Zn were measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Vista Pro; Varian Inc., Australia). Shoot:root Ni concentration ratio was determined as the Ni concentration in shoots divided by the Ni concentration in roots. The Ni phytoextraction efficiency (soil Ni removal) was calculated as the product of the shoot DW and the Ni concentration in shoots in relation to the total Ni content in the soil.

Statistical Analysis

Data from Part I were analysed using SAS PC version 6.0. Data required log transformation to attain homogeneity; geometric means and standard errors are shown in the tables and figures. The PROC MIXED procedure was utilized for analysis of variance of plant yield and tissue metal concentration for differences of treatments. In Part II the significant effects of phytohormone treatments (type and concentration) on plant growth and biomass production, nutrient and metal content were determined using analyses of variance (ANOVA) followed by the "post-hoc" Minimum Significance Difference test using SPSS (Version 21).

4.3 *RESULTS*

Part I. Influence of exogenous cytokinins and gibberellins in four *Alyssum* species

Aboveground biomass production

A. corsicum and *A. murale* had a shoot biomass of 5-7 g pot $^{-1}$, whereas *A. pintodasilvae* and *A. malacitanum* produced a lower biomass in the range of 1-5 g pot^{-1} (Fig. 4.1). In untreated control plants, shoot biomass was significantly greater in *A. corsicum* and *A. murale* (6.60 and 7.03 g pot^{-1}, respectively), followed by *A. malacitanum* (4.61 g pot^{-1}) and *A. pintodasilvae* (2.28 g pot^{-1}) (P <0.05). Contrary to what was expected, the application of the different PGR treatments generally had a negative effect on biomass production of all four *Alyssum* species (Fig. 4.1). The cytokinin-based treatments caused a decrease in biomass at both concentrations and in all species. In the Low-CK treatment this reduction in biomass was only significant in the case of *A. murale*. The High-CK treatment was the most phytotoxic, and resulted in a significant reduction in the biomass of all four *Alyssum* compared to control plants (shoot biomass in High-CK was reduced by 20-57 % compared to control). In contrast, the Promalin treatment did not have a negative effect on plant growth of *Alyssum* species. In fact, *A. corsicum* showed a higher shoot biomass after Promalin treatment, unfortunately this trend was not statistically significant.

Shoot ionome

Shoot macro- and micronutrient concentrations were adequate for normal growth of *Alyssum* (Table 4.1). Ca concentration was slightly higher in *A. malacitanum* control plants relative to the PGR treated plants. Significant increase in Fe (in *A. malacitanum* and *A. pintodasilvae*), Mg and Mn (in *A. corsicum*), and Zn (in *A. pintodasilvae*) was observed in plants treated with high concentration of cytokinin. However, in the case of Fe the high values are influenced by one outlier.

After 90 days, control plants accumulated up to 4000 mg kg^{-1} Ni in the shoots (Fig. 4.2), confirming their ability to hyperaccumulate this element. There were no significant differences between the four species in their Ni accumulation. However, *A. murale* and *A. pintodasilvae* tended to accumulate more Ni (approx. 3800-4000 mg kg^{-1}) in their shoots than *A. corsicum* and *A. malacitanum* (approx. 3100-3200 mg kg^{-1}) (Fig. 4.2). In general, PGR treatments had no significant effects on Ni bioaccumulation by *Alyssum*. Although Promalin application caused an increase in the biomass production of *A. corsicum* (Fig. 4.1) this did not result in a significant increase in Ni uptake in the shoots: mean shoot Ni concentrations

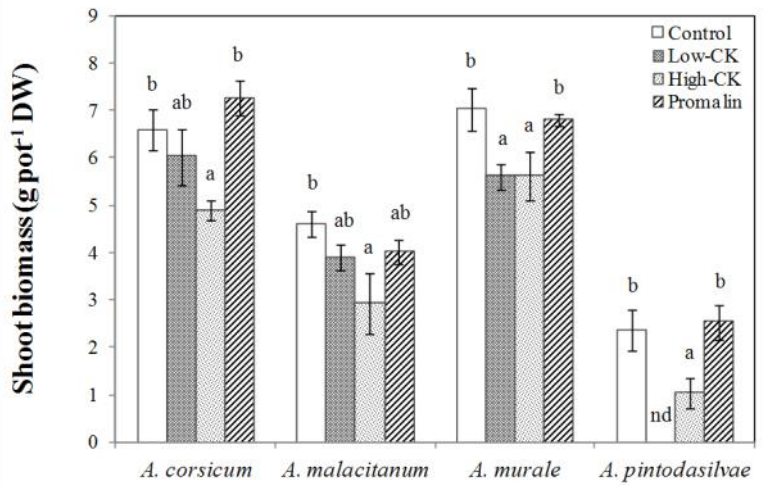


Figure 4.1. Effect of different PGR treatments (Control; Low-CK (15 mg L^{-1} Cytokin); High-CK (60 mg L^{-1} Cytokin); Promalin (60 mg L^{-1})) on the biomass production of *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Alyssum pintodasilvae*. Mean shoot biomass (±standard error) of four replicates are shown. *A. pintodasilvae* was not treated with Low-CK treatment. Differences between treatments within species are shown by a different letter (P <0.05) (nd: no data).

were 3160 mg kg^{-1} in control plants and 2860 mg kg^{-1} in Promalin-treated plants (Fig. 4.2). In *A. malacitanum* and *A. pintodasilvae* Promalin treatment tended to decrease plant Ni uptake relative to controls, although this was only significant in the case of *A. malacitanum* (Fig. 4.2). Finally, in *A. murale* Promalin treatment had no effect on shoot Ni content (Fig. 4.2). The Low-CK treatment (15 mg L^{-1}) did not influence Ni concentration in *A. murale* or *A. corsicum*, and led to a reduction in Ni accumulation in *A. malacitanum* (although not significant).

Ni phytoextraction efficiency

The nickel phytoextraction efficiency was calculated as the product of the dry weight produced and the Ni accumulation in shoots in relation to the total Ni content in the soil. Total Ni phytoextracted ranged from 0.05 to 0.40 %, and generally followed the order *A. murale* > *A. corsicum* > *A. malacitanum* > *A. pintodasilvae*. The effect of PGR treatments on Ni phytoextraction was species dependant. In *A. corsicum* and *A. murale* the Ni phytoextracted was similar to the control for all treatments: ranging from 0.31-0.39 % in *A. murale* and from 0.26-0.31 % in *A. corsicum* (Fig. 4.3). In *A. malacitanum*, phytoextraction of Ni was significantly lower (0.15-0.20 %) than the control after application of cytokin and Promalin due to the decrease in plant biomass and Ni concentration in shoots (Figs. 4.1 and 4.2). Similarly, Ni phytoextraction by *A. pintodasilvae* was lower

Table 4.1. Mean macro- and micro-nutrient concentrations in shoots of *A. corsicum*, *A. malacitanum*, *A. murale*, *A. pintodasilvae*.

	Ca	Fe	K	Mg	Mn	P	Zn
	g kg^{-1}						
A. corsicum							
Control	19.90a	0.08a	29.78a	4.20a	0.21a	2.63a	0.07ab
Low-CK	21.68a	0.10a	36.51b	4.70ab	0.21a	2.63a	0.06ab
High-CK	24.30a	0.10a	44.10c	5.40b	0.27b	2.95a	0.08b
Promalin	20.37a	0.09a	34.31a	4.50ab	0.20a	2.20a	0.05a
A. malacitanum							
Control	44.69b	0.14a	32.51a	5.45a	0.26a	3.03a	0.06a
Low-CK	37.10a	0.15a	33.85a	4.19a	0.26a	3.18b	0.06a
High-CK	38.03a	0.32b	35.13a	4.69a	0.26a	3.81b	0.08a
Promalin	33.27a	0.16a	32.74a	4.27a	0.22a	2.20a	0.05a
A. murale							
Control	28.96a	0.18a	40.37a	3.39a	0.30a	2.57a	0.08a
Low-CK	25.45a	0.22a	40.28a	3.04a	0.31a	2.61a	0.09a
High-CK	25.86a	0.18a	43.22a	3.33a	0.29a	2.40a	0.08a
Promalin	26.44a	0.16a	36.51a	3.02a	0.27a	2.42a	0.07a
A. pintodasilvae							
Control	44.81a	0.31a	33.17ab	4.21a	0.30a	2.67a	0.08a
High-CK	42.68a	0.64b	39.22b	5.07a	0.31a	3.62a	0.12b
Promalin	44.29a	0.29a	28.49a	4.30a	0.25a	2.72a	0.07a

Letters show significant differences (P <0.05) between treatments (Control; Low-CK (15 mg L^{-1} Cytokin); High-CK (60 mg L^{-1} Cytokin); Promalin (60 mg L^{-1})) and within the same species.

(<0.15 %) than the control after the High-CK treatment, but there was no effect of Promalin on Ni phytoextraction in this species.

Part II. Application of cytokinins, gibberellins and auxins (IAA) at three concentration rates in four Ni hyperaccumulating plants

Plant biomass production

All the study species showed normal growth and no visual symptoms of toxicity were observed after application of treatments. In control plants, the shoot biomass of *A. corsicum*, *A. murale* and *N. goesingense* (mean DW yield of 1.07, 1.18 and 0.89 g pot^{-1}, respectively) was more than 2-fold greater than that of *A. malacitanum* (0.42 g pot^{-1}) (P <0.05; Fig. 4.4). The effect of the PGR

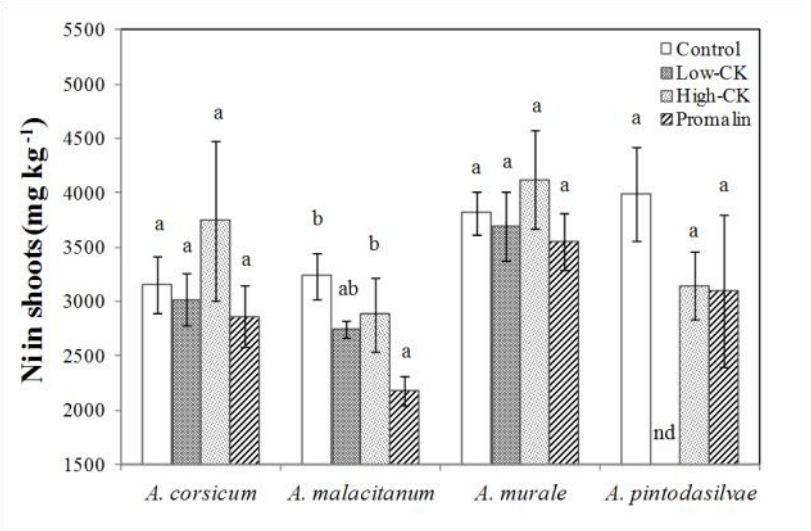


Figure 4.2. Effect of different PGR treatments (Control; Low-CK, 15 mg L^{-1} Cytokin; High-CK, 60 mg L^{-1} Cytokin; Promalin 60 mg L^{-1}) on Ni concentration in shoots of *A. corsicum*, *A. malacitanum*, *A.murale* and *A. pintodasilvae*. Mean Ni concentration (± standard error) of four replicates is shown. *A. pintodasilvae* was not treated with Low-CK treatment (15 mg L^{-1} Cytokin). Differences between treatments within species are shown by a different letter (P <0.05) (nd: no data).

treatments on biomass production varied according to which PGR was applied, the concentration and the plant species: either no effect was observed or they led to a significant increase in biomass production. None of the treatments negatively affected plant growth (Fig. 4.4). The most pronounced effects on biomass production were found after treatment with Kelpak and Promalin, and this increase in biomass was again concentration- and species-dependent (only species-dependent in the case of Promalin). A significant increase in shoot DW yield compared to control plants was obtained after application of Kelpak in all four study species (P <0.05). Moreover, DW yield tended to increase with an increase in the treatment concentration (K1 to K3). The highest increment was obtained in the K3 treatment for all four species: shoot DW yields increased in *A. corsicum* by 1.4-fold, in *A. malacitanum* by 1.8-fold, in *A. murale* by 1.6-fold and in *N. goesingense* by 2.1-fold (Fig. 4.4). The most marked effects of the Kelpak treatment were observed in *N. goesingense*. The increase in shoot DW yield after treatment with Kelpak was accompanied by a significant increase in root DW yields (data not shown; P <0.05). As observed for shoot biomass this effect was most pronounced at the highest concentration (K3) in all four study species. Root DW yields of *A. corsicum* and *A. malacitanum* increased from 0.16 ±0.03 to 0.29 ±0.05 g pot^{-1} and from 0.03 ±0.01 to 0.14 ±0.02 g pot^{-1} in control plants and the K3 treatment, respectively. The same treatment led to a significant rise in root

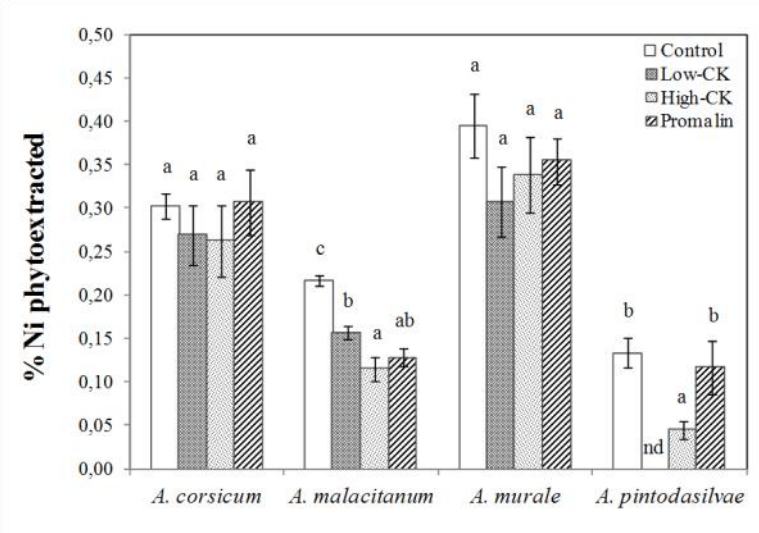


Figure 4.3. Effect of different PGR treatments (Control; Low-CK, 15 mg L^{-1} Cytokin; High-CK, 60 mg L^{-1} Cytokin; Promalin 60 mg L^{-1}) on Ni phytoextraction efficiency by *A. corsicum*, *A. malacitanum*, *A.murale* and *A. pintodasilvae*. Average and standard error (error bars) of four replicates are shown. *A. pintodasilvae* was not treated with Low-CK treatment (15 mg L^{-1} Cytokin). Differences between treatments within species are shown by a different letter (P <0.05) (nd: no data).

DW yield in *A. murale* (from 0.20 ±0.04 to 0.50 ±0.05 g pot^{-1}) and in *N. goesingense* (from 0.10 ±0.02 to 0.44 ±0.05 g pot^{-1}) (P <0.05). Application of the phytohormone Promalin also significantly increased shoot biomass in all plant species except *A. malacitanum*, but in this case biomass did not tend to increase with an increase in the treatment concentration. The maximum DW yield was obtained at P1 for *A. corsicum* (mean DW yield increased by 1.4-fold), while the maximum yields of *A. murale* and *N. goesingense* were obtained at P2 (DW yield increased by 1.4- and 1.8-fold, respectively (P <0.05)). A further increase in the treatment concentration (P3) had no additional beneficial effect on biomass production. In contrast to what was observed after treatment with Kelpak, applying the phytohormone Promalin had no significant effect on root DW yield. Finally, the phytohormones Berelex and Cytoplant had little effect on shoot DW yields, except in the case of *N. goesingense* where shoot DW yield was increased by 1.5-fold compared to control plants at the lowest concentration of Cytoplant (C1, P <0.05; Fig. 4.4). The same treatment (C1) also significantly increased root DW yield in this species (root DW yield in control plants was 0.10 ±0.02 g pot^{-1} and 0.27 ±0.06 g pot^{-1} in C1). DW yields of *N. goesingense* also tended to increase after treatment with phytohormone Berelex, and almost reached statistical significance at B3 (P=0.06).

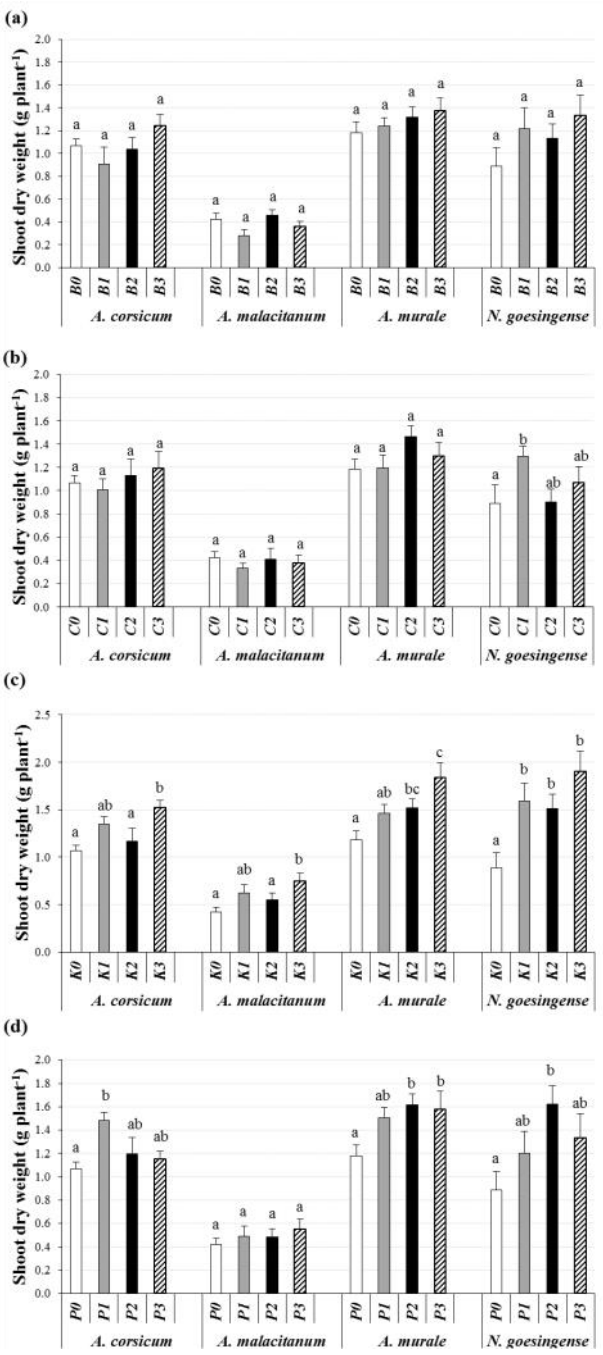


Figure 4.4. Effect of different PGR treatments (Control; B1 (0.1 mg L^{-1}); B2 (1 mg L^{-1}); B3 (10 mg L^{-1}); C1 (1 mg L^{-1}); C2 (5 mg L^{-1}); C3 (10 mg L^{-1}); K1 (0.01 mg L^{-1}); K2 (0.05 mg L^{-1}); K3 (0.1 mg L^{-1}); P1 (5 mg L^{-1}); P2 (30 mg L^{-1}); P3 (50 mg L^{-1})) on shoot dry weight (g) of *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense*. Mean shoot biomass (±standard error) of six replicates are shown. Differences between treatments within species are shown by a different letter (P <0.05).

The application of the different PGR treatments also influenced plant growth in terms of the number of branches/leaves (Fig. 4.5) and leaf size or stem length. Treatment with all four phytohormones led to a significant increase in the number of branches compared to control plants, and this was observed in all three *Alyssum* species (P <0.05; Fig. 4.5). As observed in shoot biomass production, the phytohormones Promalin and Kelpak showed the most marked effect on branching in *Alyssum* species, especially at the highest concentrations. In *A. corsicum*, after application of Promalin, the number of branches increased with increasing treatment concentration, reaching up to 4.8-fold more branches in the P3 treatment compared to control plants. On the other hand, the maximum number of branches for *A. malacitanum* and *A. murale* was obtained after applying the Promalin phytohormone at concentration P2: the number of branches increased by 5.7- and 6.4-fold compared to control plants, respectively (P <0.05; Fig. 4.5). Application of Kelpak led to a significant increase in the number of branches in all three *Alyssum* species compared to control plants, and this was again most pronounced at the highest treatment concentration (K3) (P <0.05). Branching in *A. corsicum, A. malacitanum* and *A. murale* increased by 2.1-, 4.5- and 4.1-fold, in K3 compared to control plants, respectively (P <0.05; Fig. 4.5). Treatment with the Berelex phytohormone caused a significant increase in the number of branches in B2 and B3 treatments in all three *Alyssum* species (only in B2 for *A. corsicum*) compared to untreated plants (P <0.05; Fig. 4.5). In *A. corsicum* and *A. murale* the number of branches increased by 1.4- and 2.4-fold, respectively, in the B2 treatment compared to control. *A. malacitanum* showed an increase in branching of 2.1-fold in B3 compared to control plants (Fig. 4.5). C treatment also led to a significant increase in branching in *A. malacitanum* and *A. murale* (78 % and 108 %, respectively) compared to control plants in C2 treatment (P <0.05). In *A. corsicum* there was no effect on branching after applying C (Fig. 4.5).

Similarly, a significant increase in the number of leaves was found in *N. goesingense* after application of all four phytohormones (P <0.05; Fig. 4.5). The treatment concentration did not have a significant effect on this increase in leaf number, except in the case of the phytohormone Promalin where the two highest concentrations (P2 and P3) led to the highest increment in leaf number (2.7-fold; Fig. 4.5). The treatments Berelex, Cytoplant and Kelpak caused an increase in the number of leaves by up to 1.9-fold compared to control plants (Fig. 4.5).

There was no effect of PGRs on stem length in the *Alyssum* species, with the exception of *A. murale* (data not shown). After the application of Berelex and Promalin, *A. murale* showed a significant increase in stem length compared to control plants (P <0.05), values increased from 23.1 ±2.0 cm in control plants to 33.5 ±1.0 and 37.6 ±0.7 cm at the highest concentrations of Berelex (B3) and

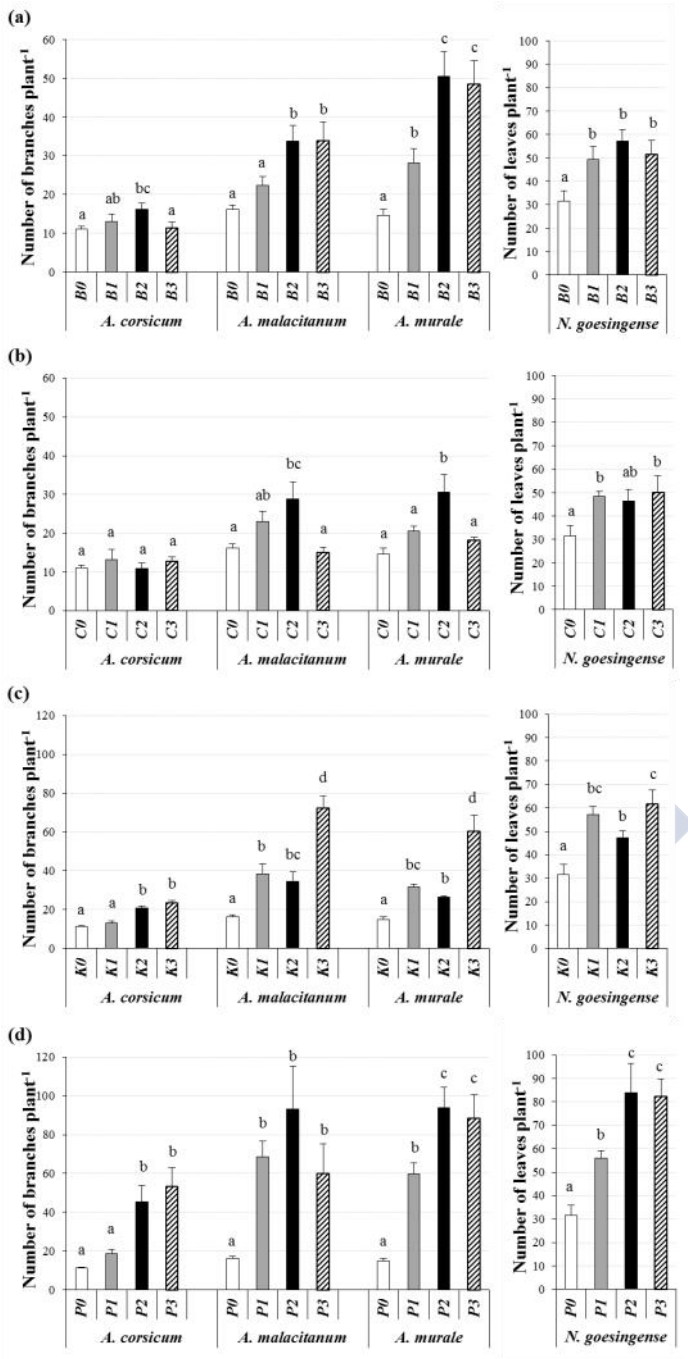


Figure 4.5. Effect of different PGR treatments (Control; B1 (0.1 mg L^{-1}); B2 (1 mg L^{-1}); B3 (10 mg L^{-1}); C1 (1 mg L^{-1}); C2 (5 mg L^{-1}); C3 (10 mg L^{-1}); K1 (0.01 mg L^{-1}); K2 (0.05 mg L^{-1}); K3 (0.1 mg L^{-1}); P1 (5 mg L^{-1}); P2 (30 mg L^{-1}); P3 (50 mg L^{-1})) on the number of branches/leaves of *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense*. Mean shoot biomass (± standard error) of six replicates are shown. Differences between treatments within species are shown by a different letter (P <0.05).

Promalin (P3), respectively. In *N. goesingense* there was an increase in leaf length after the application of the four different treatments compared to control plants: a significant increase was observed after treatments B2, C1, K1, P2 and P3, values increased from 10.3 ±0.5 (control) to 13.0 ±0.6, 11.8 ±0.5, 12.0 ±0.6, 13.1 ±0.3 and 13.0 ±0.7 cm, respectively (P <0.05). No effect of PGRs was found on leaf width.

Shoot and root ionome

In general, the application of PGRs caused a decrease in the concentration of several macro- and micronutrients (Ca, Co, Cu, K, Mg, Mn and P) in shoot tissues compared to control plants, albeit not always significant or at all treatment concentrations (Table 4.2). On the other hand, shoot Fe concentrations were often increased in *A. corsicum* and *A. malacitanum* after treatment. Some exceptions to these generalised trends were observed. In *A. malacitanum*, Cytoplant at the highest concentration caused a significant increase in the shoot Ca concentration (the mean shoot Ca concentration in control plants was 21795 ±1516 and increased to 27746 ±3487 mg Ca kg^{-1} in C3; P <0.05). In the same species, the application of all three concentrations of Cytoplant significantly increased the shoot Mg concentration (the mean shoot Mg concentration in control plants was 16729 ±771 and increased to 18696 ±1713 mg kg^{-1} in C2; P <0.05). Similarly, a significant increase in shoot Fe concentration was observed in *A. corsicum* after application of Kelpak and Promalin treatments at the higher concentrations (P <0.05). The mean shoot Fe concentration in control plants was 172 ±10 mg kg^{-1} and increased to 252 ±31 and 234 ±19 mg kg^{-1} in K2 and P2, respectively. A significant increase in the shoot P concentration in all four species was observed after the application of Kelpak (especially at the highest concentration, K3) relative to control plants (shoot concentrations were up to 2.5-fold higher in *A. malacitanum*; P <0.05). In *A. malacitanum* a significant increase was also observed in the shoot P concentration in the B1 treatment (Table 4.2). The application of PGRs also influenced the concentration of nutrients in the root tissues (Table 4.3). As observed in shoot tissues, nutrient concentrations in roots were generally also lower after the application of phytohormones. However, the application of Kelpak led to a significant increase in Cu concentration in *A. corsicum* (from 13 ±2 to 111 ±14 mg kg^{-1}, K3), *A. malacitanum* (from 23 ±4 to 72 ±14 mg kg^{-1}, K3), *A. murale* (from 17 ±3 to 128 ±25 and 82 ±2 mg kg^{-1}, K2 and K3 respectively) and *N. goesingense* (from 12 ±1 to 386 ±56, 228 ±77 and 166 ±20 mg kg^{-1}, K1, K2 and K3, respectively) compared to control plants (P <0.05). Likewise, root Co concentrations significantly increased in *N. goesingense* after the application of Cytoplant at 1 mg L^{-1} (C1) and Promalin at

Table 4.2. Macro- and micro-nutrients content (mg kg^{-1}) in shoots of *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense* (mean concentrations ±standard error).

Species	Treatment	Ca	Co	Cu	Fe	K	Mg	Mn	Ni	P
						mg kg^{-1}				
A. corsicum	Control	15356±1162	36.1±8.3	13.5±3.3	172±10	28608±1237	9643±1193	251±23	20802±1012	4179±176
	B1	15838±1621	32.7±2.5	12.3±1.6	200±7	27755±911	11033±851	193±18*	17060±1411*	3512±272*
	B2	18427±1843	27.8±3.5	14.0±1.1	190±26	21993±1740*	10777±1379	185±10*	16135±942*	2614±232*
	B3	9790±590*	23.8±2.2	7.4±0.5*	150±10	22333±1358*	9797±796	162±17*	14762±1100*	2546±75*
	C1	13219±1149	35.7±4.2	15.3±4.0	198±18	22905±1494*	10474±734	207±21	17212±1243*	3449±269*
	C2	17978±1756	36.4±2.4	17.8±2.6	221±27	25281±1674	10819±1111	193±6*	19790±1026	3457±350
	C3	12209±946	27.9±2.5	15.2±2.4	119±11	18658±1947*	8840±384	199±15*	15605±763*	2431±307*
	K1	14012±569	30.7±7.6	9.5±0.9	189±24	22844±940*	10128±1160	150±17*	16831±2208	3455±376
	K2	13067±1380	31.2±3.7	9.0±0.7	252±31*	24499±1270*	9477±855	162±16*	16864±1267	4594±290
	K3	14590±1453	30.3±2.6	12.2±1.2	248±16*	28040±1654	9754±672	206±19	16570±619*	6074±243*
	P1	12674±918	27.7±2.8	15.2±1.6	198±21	27094±1691	8494±734	168±11*	15611±663*	3244±274*
	P2	13412±586	20.9±2.3*	12.2±0.8	234±19*	30704±1596	8197±1148	144±15*	11032±1036*	2311±178*
	P3	13494±618	16.8±2.3*	7.5±4.3	232±17*	24305±1363	9035±743	98±16*	9251±1271*	2422±263*
A. malacitanum	Control	21795±1516	16.9±1.0	9.2±1.1	330±10	35926±1995	16729±771	161±9	11405±416	3357±173
	B1	23373±864	21.0±2.1	15.2±2.5	419±65	43588±2293*	19150±988	157±8	11401±686	4510±375*
	B2	19253±1505	15.3±1.9	13.3±2.4	347±19	36165±1899	14995±671	131±11*	9404±1103	3728±416
	B3	14467±202*	20.2±4.4	6.8±2.2	249±28	34578±1782	15226±552	160±26	10023±826	3282±205
	C1	21385±536	20.3±1.2	11.6±1.1	245±23*	34214±3253	17905±837	144±9	11457±457	3637±230
	C2	22191±1522	18.6±2.0	26.8±2.7*	321±36	28770±2011	18696±1713	152±10	11038±596	3445±251
	C3	27746±3487*	21.5±2.9	15.0±2.6*	316±55	33271±1479	18280±615	171±14	13838±1109*	3721±270
	K1	21925±1631	19.3±1.2	7.6±0.5	347±37	36336±1510	16929±657	156±9	11392±363	4279±284*
	K2	22921±725	17.6±0.5	7.7±1.0	361±44	38455±2268	16861±851	153±12	10648±759	5163±271*
	K3	18924±1319	20.1±2.3	10.7±1.0	404±47	37865±3713	16528±1083	143±12	10707±943	8253±397*
	P1	19687±890	16.9±1.2	6.0±1.9	314±18	37434±1386	16961±829	125±9	8478±532*	2774±239*
	P2	14170±795	16.6±1.8	10.5±2.2	593±243	43540±2710*	13710±642	124±9	5552±700*	2865±164*
	P3	20308±804	18.2±2.9	5.9±1.3	399±81	37834±2134	14664±542	98±16*	5643±648*	2916±480*

A. murale	Control	19876±1203	39.3±4.7	11.4±1.9	418±48	33343±2603	8340±591	400±56	18191±609	3868±204
	B1	17297±1728	29.5±2.4	12.4±1.4	335±30	30333±2712	9302±1354	305±14	17651±1035	3883±501
	B2	11961±765*	18.3±2.1*	10.9±1.3	218±14*	26537±1524*	5931±504*	181±16*	12344±763*	3008±142*
	B3	18464±2066*	26.6±2.8*	8.5±0.7	332±21*	30491±1390	10824±1662	242±24*	14149±1051*	2979±206
	C1	18605±1220	35.6±3.9	24.5±9.3	284±18*	22436±1740*	7585±646	304±49	18633±1203	3492±225
	C2	22017±2227	49.0±5.7	13.8±2.0	419±55	26595±1431*	7816±990	437±40	20277±2067	3640±499
	C3	19733±1920	33.8±3.5	17.7±2.9	266±22*	23095±2000*	7218±721	289±28	16548±1152	3018±307
	K1	18581±1709	25.2±3.0*	10.3±1.6	461±108	28969±1686	7542±790	251±17*	16069±1160	3930±293
	K2	16179±2373	24.6±4.3*	8.8±1.0	385±44	25175±1226*	7875±909	207±22*	15167±1694	4305±125
	K3	16887±1568	40.0±4.7	10.2±0.9	565±59	38484±5341	8927±1302	302±44	17193±1872	7572±940*
	P1	11558±680*	24.2±2.3*	14.0±3.1	244±29*	26147±1819*	5970±411*	232±29*	13980±872*	2804±93*
	P2	15323±1142*	24.8±2.8*	7.9±2.7	323±30	23850±1642*	7375±956	209±36*	11122±777*	2631±207*
	P3	16497±802*	21.3±1.5*	11.5±2.4	434±29	35016±1186	6590±447	193±13*	10611±821*	2994±57*
N. goesingense	Control	11818±318	29.7±2.2	14.6±1.0	856±120	41682±3670	12318±844	149±9	11610±679	4334±247
	B1	13186±1760	15.5±2.0*	10.8±0.7	409±40*	36768±2564	12711±1115	111±9*	7760±325*	4004±300
	B2	12118±1567	17.3±2.6*	12.3±2.8	469±86*	31815±2538*	9639±1060	103±9*	7381±516*	3458±163*
	B3	6650±522*	16.3±1.5*	6.2±0.5*	309±57*	32923±1072*	10823±849	130±10	8067±467*	3071±256*
	C1	10345±2072	28.2±2.5	11.0±1.2	453±59*	26705±2243*	7591±832*	130±15	9740±459	3746±213
	C2	11573±1619	32.2±3.2	5.4±1.5*	839±100	25966±3374*	7944±750*	143±20	10826±807	3535±130*
	C3	8826±625	27.1±2.2	9.8±2.2*	592±170	22645±2601*	7338±505*	125±13	10149±1027	3065±283*
	K1	12815±931	20.9±0.5*	8.8±0.6*	536±82*	31667±1891*	10805±1099	119±5	9756±833	4006±248
	K2	10517±1347	28.0±2.1	9.2±0.8*	445±32*	45036±1765	12293±926	157±27	11808±413	5505±365*
	K3	10166±820	29.6±2.2	12.1±1.1	584±44*	37506±3016	10855±1167	120±15	9983±848	5863±370*
	P1	9083±842*	20.4±2.3*	10.0±2.5	389±93*	35037±2170	11655±1239	108±12*	7435±471*	3420±274*
	P2	9560±775*	15.7±1.6*	13.3±3.3	357±44*	32421±2667*	9757±813*	104±12*	3314±385*	2028±106*
	P3	9104±693*	15.3±1.6*	5.7±1.3*	360±42*	27054±1515*	9283±689*	72±5*	3283±380*	1813±98*

Asterisks indicate significant differences between the treated plants and the control plants (P <0.05) within the same species.

30 mg L^{-1} (P2) compared to non-treated plants (P <0.05). In contrast to what was observed in shoot tissues, the application of Kelpak led to a reduction in root P concentration (P <0.05).

Shoot Ni concentrations in control plants differed significantly between the four study species, and followed the order *A. corsicum* (20802 ±1012 mg Ni kg^{-1}) > *A. murale* (18191 ±609 mg Ni kg^{-1}) > *N. goesingense* (11610 ±679 mg Ni kg^{-1}) ~ *A. malacitanum* (11405 ±416 mg Ni kg^{-1}) (P <0.05; Table 4.2). In general, PGR treatments led to a reduction in shoot Ni concentrations of either the *Alyssum* species or *N. goesingense* (albeit not always statistically significant). In the case of *A. corsicum*, both Kelpak and Cytoplant caused a similar and significant decrease in Ni shoot concentration (values were approx. 20 % lower than in control plants, declining from 20800 to a minimal of 15605 mg Ni kg^{-1}) (P <0.05). These two treatments did not lead to significant reductions in shoot Ni concentrations in the remaining study species. On the other hand, both Berelex and Promalin treatments caused a significant decrease in shoot Ni concentration in all four species (except *A. malacitanum* treated with B) compared to control plants (P <0.05). In the case of plants treated with Berelex, shoot Ni concentrations decreased by 18-29 % in *A. corsicum*, 3-32 % in *A. murale* and 30-36 % in *N. goesingense* compared to control plants (P <0.05). In the case of plants treated with Promalin, shoot Ni concentrations were reduced by 25-50 % in *Alyssum* species, and by up to 70 % in some cases in *N. goesingense*. This decrease in Ni concentration was more pronounced with an increase in treatment concentration. In contrast, two treatments led to an increase in shoot Ni concentration: C3 in *A. malacitanum* and C2 in *A. murale*. After phytohormone treatments, root Ni concentrations were significantly lower than control plants in all four species (by up to 60 %) (P <0.05; Table 4.2). The K3 treatment caused a decrease in root Ni concentration from 5231 ±492 mg kg^{-1} to 3718 ±324 mg kg^{-1} in *A. corsicum*, from 932 ±109 mg kg^{-1} to 604 ±64 mg kg^{-1} in *A. malacitanum*, from 4314 ±757 mg kg^{-1} to 3117 ±296 mg kg^{-1} in *A. murale* and from 2186 ±153 mg kg^{-1} to 1002 ±133 mg kg^{-1} in *N. goesingense*. In *A. murale* treatment with Promalin at the highest concentration (P3) caused the most marked reduction in root Ni concentrations, from 4314 ±757 mg kg^{-1} to 1924 ±119 mg kg^{-1} (representing a decrease of 55 %) (Table 4.3). In the case of *N. goesingense*, the lowest concentration of Ni in the roots was found in plants treated with K2 (865 ±32 mg kg^{-1}), representing a reduction of 60 % compared to control plants.

The shoot:root Ni concentration ratio was calculated in those plants where PGR treatments significantly affected both shoot and root DW yields (P <0.05). There was a significant increase in the shoot:root Ni ratio in *A. malacitanum* treated with Kelpak (at concentration K3) and *N. goesingense* (at concentrations K2 and K3) (P <0.05). Shoot:root Ni concentration ratios increased from 10.8 to

18.7 in *A. malacitamum* and from 5.4 to 13.7 and 10.7 in *N. goesingense* (data not shown).

Ni phytoextraction efficiency

The % Ni phytoextracted by control plants varied between the four species (P <0.05) in the following order: *A. corsicum* (1.25 %) > *A. murale* (1.21 %) > *N. goesingense* (0.60 %) > *A. malacitanum* (0.28 %) (Table 4.4). The effect of PGRs on Ni removal varied according to the PGR type and/or concentration applied. Application of Cytoplant to *A. murale* at concentration C2 significantly increased the % Ni phytoextracted from 1.21 % in control to 1.68 % in treated plants (P <0.05). However, a further increase in phytoextracted Ni was not observed at the highest application concentration (C3). Likewise, application of Kelpak increased % Ni phytoextracted by all four species. This increase was significant at the highest concentration (K3 treatment) in *A. malacitanum*, *A. murale* and *N. goesingense* (P <0.05; Table 4.4): Ni phytoextracted increased from 0.28 % to 0.45 %, from 1.21 % to 1.81 % and from 0.60 % to 0.99 % in *A. malacitanum*, *A. murale* and *N. goesingense*, respectively. In the case of *N. goesingense* the intermediate dose of Kelpak (K2) also significantly enhanced the % Ni phytoextracted. In contrast, after application of Berelex, *A. corsicum* showed a significant decrease in phytoextracted Ni in the B2 treatment (equivalent to 1 mg L^{-1}) (P <0.05): Ni phytoextracted was reduced from 1.25 % in control plants to 0.93 %. *A. malacitanum* also showed a lower % Ni phytoextracted in plants treated with this phytohormone; this decrease was significant in the B1 treatment, from 0.28 % in control plants to 0.18 % (P <0.05; Table 4.4). Treatment with Berelex had no effect on Ni removal by *A. murale* and *N. goesingense*. Application of Promalin also tended to cause a decrease in the % Ni phytoextracted compared to control plants (P <0.05), especially at the highest concentration rates. In the P3 treatment, the % Ni phytoextracted was significantly reduced from 1.25 % to 0.57 % in *A. corsicum*, from 0.28 % to 0.18 % in *A. malacitanum* and from 0.60 % to 0.24 % in *N. goesingense* (P <0.05; Table 4.4).

4.4 *DISCUSSION*

Plant biomass production

Shoot biomass production was found to vary significantly between the two experiments (Parts I and II). Although the plants were grown in serpentine soils with similar characteristics in both experiments, a higher shoot biomass production was obtained in Part I. This was probably due to the application of

Table 4.3. Macro- and micro-nutrients content (mg kg^{-1}) in roots of *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense* (mean concentrations ±standard error).

Species	Treatment	Ca	Co	Cu	Fe	K	Mg	Mn	Ni	P
						mg kg^{-1}				
A. corsicum	Control	17055±3268	9.4±1.6	13±2	2198±458	11616±892	3217±328	89±14	5231±492	3781±38
	K3	12139±1366	6.4±0.8	111±14*	1392±201	4794±472*	3130±224	59±6	3718±324*	1706±74
A. malacitanum	Control	40530±10111	6.8±1.0	23±4	3924±920	17560±943	5300±695	102±16	932±109	2620±54
	K3	27611±5508	2.5±0.6*	72±14*	1614±271	1626±501*	2591±447*	36±8*	604±64*	1099±11
A. murale	Control	19374±3563	9.2±2.0	17±3	1948±394	19119±3947	2815±598	91±18	4314±757	4409±70
	K2	13484±1839*	10.9±1.3	128±25*	2935±703	8872±1218*	3543±429	135±20	3362±366*	3240±420
	K3	6132±515*	9.5±1.0	82±2*	2274±332	9152±543*	2987±201	104±9	3117±296*	2905±124
	P2	5432±688*	8.2±0.9	10±1	3175±410	9260±1381*	3351±211	113±12	2209±317*	3018±437
	P3	6245±814*	10.8±0.5	10±1	5071±746*	6664±356*	4223±471	133±14	1924±119*	2501±149
N. goesingense	Control	12872±2130	4.5±0.5	12±1	1627±425	22584±1235	4370±484	89±8	2186±153	6284±40
	C1	6361±802*	7.0±0.3*	14±1	2073±182	13713±1488*	3944±297	92±7	1525±260*	5637±106
	K1	8905±253	4.2±0.7	504±129*	1580±379	9162±1658	3206±340	68±8	1143±127*	4663±541
	K2	9371±272	6.0±0.6	228±77	2393±343	8799±846	3542±251	92±13	865±32*	3464±363
	K3	11519±1163	4.8±0.6	166±20	2146±535	13874±1364	3522±407	75±9	1002±133*	4915±432
	P2	6643±696*	8.6±1.0*	15±3	2919±466	7168±2016*	4289±485	116±16	1377±309*	2310±517

Asterisks indicate significant differences between the treated plants and the control plants (P <0.05) within the same species.

higher amounts of fertilisers (NPK), as well as Ca addition, in Part I compared to Part II. To date the majority of studies evaluating the effects of PGRs have been carried out on agricultural crops. These results indicated that the application of PGRs can also have a positive effect on the vegetative growth and biomass production of hyperaccumulating plant species. Moreover, this positive effect was observed in hyperaccumulating plants belonging to two distinct genera (*Alyssum* or *Noccaea*) with contrasting growth habits and morphology (observed in Part II). In addition, PGRs can also attenuate the stress caused by the presence of high concentrations of metals in the soil, as has been suggested by previous authors (Ouzounidou and Ilias 2005; Sayed 1999).

The treatments based exclusively on either gibberellic acid (Berelex) or cytokinins (Cytoplant) did not affect or reduced the plant biomass production in *Alyssum* species but promoted growth of *N. goesingense*, indicating that plant growth response to this type of compound can be plant-specific. These results did not coincide with Cassina *et al.* (2011) who have found a 1.5-fold increase in shoot biomass of *A. murale* after a foliar treatment of cytokinins at 15 mg L^{-1} (equivalent to Low-CK (Part I) and C3 (Part II) treatments in these experiments). The discrepancy in the results obtained in the present study and that of Cassina *et al.* (2011) may be due to the fact that different application times were used between the two studies. Similarly, Tassi *et al.* (2008) have observed an increase in biomass production of *Helianthus annuus* after applying PGRs based on cytokinins.

In Part I, a low growth of *A. pintodasilvae* was observed compared to the other *Alyssum* species, and in consequence this species was not included in Part II. In Part I, the Promalin treatment which was based on a combination of cytokinins and gibberellins, caused a slight increase in shoot biomass but this was only observed in *A. corsicum*. In contrast, in Part II the same treatment enhanced plant biomass production in all the study species with the exception of *A. malacitanum* (where shoot biomass was unaffected). This may have been due to the fact that in Part I a treatment concentration of 60 mg L^{-1} has been used while in Part II the highest treatment concentration applied was 50 mg L^{-1}. In fact, plant response was found to be concentration-dependent and the maximal promotion in plant growth was obtained at the two lower treatment concentrations (5 and 30 mg L^{-1}). This result reinforces the need to optimize the concentration of PGRs applied to the plants. Indeed, the range between beneficial and toxic effects of phytohormones has been shown to be narrow (Salisbury and Ross 1992).

In Part II, an IAA-based treatment (Kelpak) was included in the study. The Kelpak treatment caused the most marked increase in plant growth in all four species. IAA is known to be involved in cell division processes and plant growth

Table 4.4. Effect of different PGR treatments on Ni phytoextraction efficiency (Ni phytoextracted/yield divided by total Ni in the soil) of *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale* and *Noccaea goesingense* (mean values ± standard error).

Species	Treatment												
	Control	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**	**K1**	**K2**	**K3**	**P1**	**P2**	**P3**
A. corsicum	1.25 ±0.10	0.94 ±0.10	0.93 ±0.08*	1.04 ±0.12	1.00 ±0.13	1.26 ±0.17	1.04 ±0.12	1.28 ±0.18	1.13 ±0.18	1.43 ±0.10	1.22 ±0.13	0.72 ±0.07*	0.57 ±0.07*
A. malacitanum	0.28 ±0.03	0.18 ±0.04*	0.24 ±0.04	0.20 ±0.02	0.21 ±0.03	0.25 ±0.05	0.31 ±0.07	0.40 ±0.05	0.28 ±0.05	0.45 ±0.06*	0.22 ±0.03	0.13 ±0.03*	0.18 ±0.05*
A. murale	1.21 ±0.11	1.23 ±0.10	0.91 ±0.08	1.12 ±0.16	1.26 ±0.15	1.68 ±0.24*	1.20 ±0.12	1.33 ±0.15	1.28 ±0.14	1.81 ±0.30*	1.19 ±0.12	0.99 ±0.08	0.95 ±0.11
N. goesingense	0.60 ±0.11	0.54 ±0.07	0.46 ±0.06	0.59 ±0.08	0.72 ±0.06	0.54 ±0.06	0.64 ±0.13	0.86 ±0.11	1.00 ±0.10*	0.99 ±0.13*	0.48 ±0.06	0.31 ±0.06*	0.24 ±0.01*

Asterisks indicate significant differences between the treated plants and the control plants ($P < 0.05$).

rate, which might explain the observed increases in growth after applying this treatment. Previous studies in which IAA has been applied to plants using a foliar spray also observe significant improvements in plant growth and yield. For example, Hussain *et al.* (2011) have applied IAA as foliar spray at different concentrations (50 mg L^{-1} and 100 mg L^{-1}) to *Cassia absus* and found up to a 2-fold increase in shoot dry weight. Likewise, El-Saeid *et al.* (2010) have reported that IAA treatment at a concentration of 50 mg L^{-1} increases shoot dry weight of *Vigna sinensis* by 0.4-fold compared to control plants. The concentrations used in this study were far lower than those used by these authors (maximum concentration of 0.1 mg L^{-1}) but the observed increase in shoot dry weight was as high as 2-fold. Shoot biomass and plant growth (leaf/branch number and size) increased with an increase in treatment concentration, suggesting that this enhancing effect could be more pronounced. Nickel removal was also highest at K3 (albeit not always significantly) indicating that, for phytoextraction purposes, it would be interesting to test further concentrations of this phytohormone so as to determine at which concentration the maximum Ni removal is obtained. The positive effects on plant growth caused by the application of both Kelpak and Promalin did support the fact that auxins and gibberellins are considered the strongest accelerators of shoot growth amongst the different PGRs (Tanimoto 2005).

Alongside the improvement in shoot biomass, Kelpak also increased root DW yields. An increase in root growth and proliferation has been found in crops after application of IAA-based PGRs. Liphadzi *et al.* (2006) have observed an increase in root growth of *H. annuus* grown in sewage sludge-amended soil when IAA is applied. Similarly, Hadi *et al.* (2010) have reported that the application of IAA and GA_3 increase root length, together with plant height, of *H. annuus* grown on Pb-contaminated soil. On the other hand, treatment with Promalin at 30 and 50 mg L^{-1} did not have any effect on root DW yields, despite the observed promotion in shoot growth. These results did agree with those obtained by Tanimoto (2005), who found that externally applied gibberellins have little effect on root growth in a range of plants. In contrast Emongor *et al.* (2004) have observed a significant increase in root growth of kale plants after the application of Promalin at 25, 50 and 75 mg L^{-1} (concentrations similar to those used in the present study).

As observed in Part II, the positive influence of phytohormones on plant branching, production of leaves and leaf size has been reported in numerous studies. For example, Fawzy *et al.* (2011) have shown that using a foliar spray of GA_3 (at a concentration of 100 mg L^{-1}) improves vegetative growth, expressed as plant height and number of leaves and branches in *Phaseolus vulgaris*. In a pot experiment, Emongor *et al.* (2004) have reported that spraying *Brassica oleracea* with 50 or 75 mg L^{-1} of Promalin causes a significant increase in plant leaf

number. Likewise, Tassi *et al.* (2008) have observed an increase in the leaf number by 30 % in *H. annuus* after applying cytokinins at 100 mg L^{-1}. The increase in stem length after application of Berelex and Promalin in *A. murale* confirmed the involvement of gibberellins in processes such as stem length elongation (Salisbury and Ross, 1992). These results were in agreement with those found by Leite *et al.* (2003), who have reported that the foliar application of GA_3 in soybean plants leads to an increase in plant height, first node height and stem diameter. The application of IAA has also previously been seen to promote stem growth (e.g. in *Pisum sativum* seedlings; Yang *et al.* 1993). However, the Kelpak treatments had no effect on stem length in the three *Alyssum* species.

The enhancing effect of PGRs on plant biomass production was not consistently observed amongst the four products applied in Part II of the experiment; however, all four PGRs stimulated vegetative growth in terms of plant branching or leaf production. It is therefore possible that the growth period was not long enough for this stimulation in growth to be reflected in terms of biomass production. Longer-term studies would be interesting to evaluate the effect of time on plant growth. In addition, the optimal number of PGR applications remains unknown and should be further studied.

Shoot and root ionome

Contrasting results in macro- and micronutrient contents in plant tissues were found. The effect of PGRs in nutrient contents varied depending on the treatment and the plant species. In Part I, a significant increase in Fe (in *A. malacitanum* and *A. pintodasilvae*), K, Mg and Mn (in *A. corsicum*), and Zn (in *A. pintodasilvae*) in plants after treatment with cytokinins at 60 mg L^{-1} was observed. In Part II, a generalised increase in plant nutrient concentrations was not observed. However, the treatments with the cytokinin-based products (Cytoplant and Promalin) as well as the auxin-based product (Kelpak), led to occasional increases in shoot nutrient concentrations e.g. in Fe, Mg and P. This finding was difficult to explain, and to our knowledge such an effect has not been reported in the literature to date. The increase in shoot phosphorus concentration after applying Kelpak could be due to the fact that this product is an algae extract and may contain small amounts of micronutrients and/or vitamins in addition to the active ingredient. However, this increase in P nutrition did not always coincide with an increase in the plant DW yield. Furthermore, the Cytoplant product is also from an algae extract and either had no effect or significantly reduced the phosphorus concentration in shoots. Several authors have shown that plant hormones in general, and auxins in particular, influence the regulation of nutrient uptake and transport within the plant (Arteca 1996; San-Francisco *et al.* 2005; Wang *et al.* 2007).

Despite the occasional increases observed in the contents of some elements, in Part II a general decrease in macro- and micro-nutrient concentrations in shoot and root tissues was observed after application of the PGRs. This decrease did not seem to be related to a dilution effect caused by a parallel increase in biomass production since reductions in the concentration of nutrients was also observed in plants where no promotion in biomass production was observed. Similar responses of Ca and Fe shoot contents were observed in both experiments. In Part I, shoot Ca concentration was slightly lower in *A. malacitanum* plants treated with Promalin and Cytokin relative to control plants. This finding is in line with the reduction in Ca concentrations found in most cases after PGR treatments in Part II of the study, and in this case the decrease was observed in both *Alyssum* sp. and *N. goesingense*. Similarly, in a hydroponic experiment with rice plants treated with Ni, Rubio *et al.* (1994) applied GA_3 at 3.5 mg L^{-1} and also observed a significantly lower total Ca content in shoots than plants treated with Ni alone. Wang *et al.* (2007) have observed that treatment with IAA causes significant decreases in K, Ca, Mn and Cu contents in shoots and roots of *Zea mays*. A general decrease of Ca, Fe and Mg content in roots of *Picris divaricata* due to IAA application has been observed by Du *et al.* (2011). Fässler *et al.* (2010) found a reduction in shoot Pb concentrations of *Helianthus* after treating with IAA; while Liu *et al.* (2007) found that IAA-increased shoot Pb accumulation in *Sedum alfredii*. It should be pointed out however, that the majority of these studies were carried out in hydroponic solutions.

In Part I, lower Ni concentrations in shoots were observed compared to previous studies. Li *et al.* (2003) reported concentrations of up to 11700 mg kg^{-1} Ni in the shoots of *A. murale* grown for 120 days on the same serpentine soil as that used in this experiment, a serpentine Brockman gravelly silt loam (Typic Xerochrepts) collected in Josephine County, Oregon. One possible reason for the discrepancy is that these authors grew the plants in bigger pots (4 L) compared to the pots (1.5 L) used in this study. In this experiment the plant roots at harvest had completely exploited the entire soil volume, so the lower Ni accumulation in this study could be due to the effect of pot size (soil volume) on Ni hyperaccumulation in these *Alyssum* species. In both experiments, PGR treatments had no significant effects on shoot Ni concentrations. Cassina *et al.* (2011) also observed no significant effect of cytokinin treatment on Ni accumulation in *A. murale*. On the other hand, in Part I, the High-CK treatment (60 mg L^{-1}) induced a higher Ni uptake in *A. corsicum* and *A. murale* but this was not statistically significant (the same plants showed a significantly reduced biomass production in this treatment) (Fig. 4.1). In contrast, High-CK tended to reduce Ni concentration in shoots of *A. malacitanum* and *A. pintodasilvae* (again not significantly). In Part II there was a general reduction in tissue Ni concentrations of treated plants (shoots and roots),

and this was not associated with an increase in biomass (dilution effect). Nonetheless, the least affected plant species was *A. malacitanum* which was also the species in which the least effect on biomass production was observed. Only the gibberellic acid-based PGR induced a significant reduction in shoot Ni concentrations of *Alyssum* spp. in Part I. While in Part II, the most pronounced reductions in shoot Ni concentrations were observed after treatment with Promalin and Berelex, PGRs which both contain gibberellic acid. These results contrast with Meng *et al.* (2008) who did not find any effect on the Cd concentration of *Brassica napus* tissues by applying GA treatments. On the other hand, Fässler *et al.* (2010) have found a reduction in shoot Pb concentrations of *H. annuus* after treating with indoleacetic acid. The decrease in root Ni concentrations in *A. malacitanum* and *N. goesingense* after PGR treatments might be explained as a consequence of the increase in the transport of Ni from roots to shoots (increased shoot:root Ni ratio) observed in K2 and K3 treatments. Several authors have suggested that metal translocation from root to shoot in plants may be improved through the application of phytohormones. For example, Hadi *et al.* (2010) have found that the foliar spray of GA_3 and IAA promotes a significant increase in Pb uptake in roots and its translocation into the stem and leaves of *Zea mays* when compared to control plants. Similarly, Zhao *et al.* (2010) have shown the effectiveness of cytokinins (kinetin) in increasing Cr translocation from root to shoots in *Parkinsonia aculeata*.

Ni phytoextraction efficiency

The amount of metal removed from the soil by a plant depends on both the concentration of the metal in the aerial plant biomass and on the amount of that biomass. An increase in plant biomass can therefore enhance Ni removal if the Ni concentration of the shoot tissues is not greatly reduced. In Part I the amount of Ni phytoextracted was of a similar magnitude as observed in *A. murale* by Cassina *et al.* (2011). In general, PGR treated and untreated *A. malacitanum* and *A. pintodasilvae* produced a lower biomass and lower Ni phytoextraction efficiency compared to *A. corsicum* and *A. murale.* It would therefore be necessary to greatly improve the growth of *A. malacitanum* and *A. pintodasilvae* before considering them as good candidates for Ni phytomining.

In Part II, *A.corsicum* and *A. murale* were again the most efficient species in terms of Ni removal from the soil. The increase in the % Ni phytoextracted observed after application of Kelpak (IAA) in all four species (albeit not always significant) was mainly due to the increase in shoot DW yields, since Ni concentrations in shoots were not significantly affected after applying this PGR. However, the increase in % Ni phytoextracted observed in *A. malacitanum* after Kelpak treatment was due to both the increase in plant biomass and shoot Ni

concentration. The significant increase in % Ni phytoextracted by *A. murale* after treatment with Cytoplant (cytokinins) was also due to both an increase in biomass and shoot Ni concentration. Unfortunately the enhanced biomass production after application of phytohormone Promalin (containing cytokinins and gibberellic acid) did not lead to an increase in Ni removal from the soil due to the strong reduction in shoot Ni concentration.

In conclusion, in Part I the application of phytohormones (Promalin and Cytokin) had no clear positive effect on biomass production, Ni accumulation and Ni phytoextraction efficiency in *A. corsicum*, *A. malacitanum*, *A. murale* and *A. pintodasilvae*. The effect of the Cytokin treatment significantly reduced the Ni accumulation and Ni phytoextraction efficiency in *A. malacitanum*. However, a positive response to Promalin treatment was observed in the biomass production and Ni phytoextraction efficiency of *A. corsicum*. Although this PGR-induced effect was not statistically significant it showed the need for further development of these approaches towards increasing Ni phytoextraction efficiency. In Part I, only two types of PGR and two concentrations were tested. Given the wide array of PGRs, and their known importance in all aspects of plant development, there was a clear need for evaluating a fuller range of products and a wider range of concentrations; and this was the reason for carrying out Part II. In Part II, the application of phytohormones significantly enhanced the growth of all four hyperaccumulators studied in terms of their branching, number of leaves, stem length or leaf size. Two PGR products (based on either IAA or a combination of cytokinins and gibberellic acid) significantly stimulated biomass production in the four hyperaccumulators. This effect was most pronounced after application of the Kelpak treatment (IAA). In general, the application of phytohormones reduced the shoot Ni concentration in all four species studied. However, the Kelpak treatment was found to improve Ni phytoextraction capacity of all four study species, and this was mainly due to the increase in plant growth and biomass production. To conclude, it would therefore be recommended to carry out more in-depth and longer-term studies using distinct IAA-based PGRs so as to fully optimize the beneficial effects that they can have on Ni phytoextraction efficiency of hyperaccumulating plant species.

4.5 *REFERENCES*

Arteca RN (1996). Plant growth substances: Principles and applications. Chapman & Hall New York, NY.

Asensi A, Díez-Garretas B and de la Fuente V (2004). Vegetation of ultramafic rocks in the Iberian Peninsula. Ultramafic rocks: their soils, vegetation and fauna, Proceedings of the fourth International Conference on Serpentine Ecology. Science Reviews, St. Albans, UK. 137-143.

Baker AJM, Reeves RD and Hajar ASM (1994). Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. *New Phytol* 127: 61-68.

Barbafieri M and Tassi E (2010). Brassinosteroids for phytoremediation application. In: ES Hayat and A Ahmad (eds) Brassinosteroids: A class of plant hormone. Springer Science, Heidelberg, Germany. p. 403-438.

Brooks RR, Shaw S and Marfil AA (1981). Some observations on the ecology, metal uptake and nickel tolerance of *Alyssum serpyllifolium* subspecies from the Iberian Peninsula. *Plant Ecol* 45: 183-188.

Bulak P, Walkiewicz A and Brzezińska M (2014). Plant growth regulators-assisted phytoextraction. *Biol Plant* 58: 1-8.

Cabello-Conejo MI, Centofanti T, Kidd PS, Prieto-Fernández A and Chaney RL (2013). Evaluation of plant growth regulators to increase nickel phytoextraction by *Alyssum* species. *Int J Phytoremediat* 15: 365-375.

Carey DJ (2008). The effects of benzyladenine on ornamental crops. Thesis for Master of Science, Department of Horticultural Science. North Carolina State University, Raleigh, NC.

Cassina L, Tassi E, Morelli E, Giorgetti L, Remorini D, Chaney RL and Barbafieri M (2011). Exogenous cytokinin treatments of an Ni hyper-accumulator, *Alyssum murale*, grown in a serpentine soil: Implications for phytoextraction. *Int J Phytoremediat* 13: 90-101.

Chaney RL (1983). Plant uptake of inorganic waste constituents. In: JF Parr *et al.* (eds) Land treatment of hazardous wastes. Noyes Data Corporation, Park Ridge, NJ. p. 50-76.

Chaney RL, Chen KY, Li YM, Angle JS and Baker AJM (2008). Effects of calcium on nickel tolerance and accumulation in *Alyssum* species and cabbage grown in nutrient solution. *Plant Soil* 311: 131-140.

Du RJ, He EK, Tang YT, Hu PJ, Ying RR, Morel JL and Qiu RL (2011). How phytohormone IAA and chelator EDTA affect lead uptake by Zn/Cd hyperaccumulator *Picris divaricata*. *Int J Phytoremediat* 13: 1024-1036.

El-Saeid H, Abou-Hussein S and El-Tohamy W (2010). Growth characters, yield and endogenous hormones of cowpea plants in response to IAA application. *Res J Agric & Biol Sci* 6: 27-31.

Emongor VE (1995). Thinning activity of benzyladenine on empire apples application timing and fruit storage. Ph.D. Thesis, University of Guelph, ON.

Emongor VE, Pule-Meulenberg F and Phole O (2004). Effect of Promalin on growth and development of kale (*Brassica oleracea* L. var. *acephala* DC). *J Agron* 3: 208-214.

Fässler E, Evangelou MW, Robinson BH and Schulin R (2010). Effects of indole-3-acetic acid (IAA) on sunflower growth and heavy metal uptake in combination with ethylene diamine disuccinic acid (EDDS). *Chemosphere* 80: 901-907.

Fawzy ZF, El-Bassiony AM and El-Nemr MA (2011). Improvement growth, yield and quality of two snap bean (*Phaseolus vulgarus* L) varieties using some growth regulators. *J Appl Sci Res* 7: 2047-2055.

Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K and Tran L-SP (2012). Cytokinins: Metabolism and function in plant adaptation to environmental stresses. *Trends Plant Sci* 17: 172-179.

Hadi F, Bano A and Fuller MP (2010). The improved phytoextraction of lead (Pb) and the growth of maize (*Zea mays* L.): the role of plant growth regulators (GA_3 and IAA) and EDTA alone and in combinations. *Chemosphere* 80: 457-462.

Hussain K, Hussain M, Nawaz K, Majeed A and Bhatti KH (2011). Morphochemical response of chaksu (*Cassia absus* L.) to different concentrations of Indole Acetic Acid (IAA). *Pak J Bot* 43: 1491-1493.

Jones R (1973). Gibberellins: their physiological role. *Annu Rev Plant Phys* 24: 571-598.

Kefeli V and Kalevitch MV (2003). Natural growth inhibitors and phytohormones in plants and environment. Kluwer Academic Publishers, Dorderecht, Netherlands.

Kukier U and Chaney RL (2004). *In situ* remediation of nickel phytotoxicity for different plant species. *J Plant Nutr* 27: 465-495.

Leite VM, Rosolem CA and Rodrigues JD (2003). Gibberellin and cytokinin effects on soybean growth. *Scientia Agricola* 60: 537-541.

Li YM, Chaney R, Brewer E, Roseberg R, Angle JS, Baker A, Reeves R and Nelkin J (2003). Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. *Plant Soil* 249: 107-115.

Liphadzi M, Kirkham M and Paulsen G (2006). Auxin-enhanced root growth for phytoremediation of sewage-sludge amended soil. *Environ Technol* 27: 695-704.

Liu D, Li T, Yang X, Islam E, Jin X and Mahmood Q (2007). Enhancement of lead uptake by hyperaccumulator plant species *Sedum alfredii* Hance using EDTA and IAA. *Bull Environ Contam Toxicol* 78: 280-283.

Menezes de Sequeira E (1969). Toxicity and movement of heavy metals in serpentinic soils (north-eastern Portugal). *Agron Lusit* 30: 115-154.

Meng H, Hua S, Shamsi IH, Jilani G, Li Y and Jiang L (2008). Cadmium-induced stress on the seed germination and seedling growth of *Brassica napus* L., and its alleviation through exogenous plant growth regulators. *Plant Growth Regul* 58: 47-59.

Nickell LG (1982). Plant growth regulators. Agricultural uses. Springer-Verlag, Berlin, Germany.

Ouzounidou G and Ilias I (2005). Hormone-induced protection of sunflower photosynthetic apparatus against copper toxicity. *Biol Plant* 49: 223-228.

Pazurkiewicz Kocot K (2003). The effect of selenium on the accumulation of some metals in *Zea mays* L. plants treated with indole-3-acetic acid. *Cell Mol Biol Lett* 8: 97-103.

Qiu R, Liu W, Zeng X, Tang Y, Brewer E and Fang X (2009). Effects of exogenous citric acid and malic acid addition on nickel uptake and translocation in leaf mustard (*Brassica juncea* var. *foliosa* Bailey) and *Alyssum corsicum*. *Int J Environ Pollut* 38: 15-25.

Regulation EEC (2007). Council Regulation (EC) N° 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) N° 2092/91. Official Journal of the European Union, Brussels, Belgium.

Rubio MI, Escrig I, Martínez-Cortina C, López-Benet FJ and Sanz A (1994). Cadmium and nickel accumulation in rice plants. Effects on mineral nutrition and possible interactions of abscisic and gibberellic acids. *Plant Growth Regul* 14: 151-157.

Salisbury FB and Ross CW (1992). Plant physiology. Wadsworth Publishing Company, Belmont, CA.

San-Francisco S, Houdusse F, Zamarreño AM, Garnica M, Casanova E and García-Mina JM (2005). Effects of IAA and IAA precursors on the development, mineral nutrition, IAA content and free polyamine content of pepper plants cultivated in hydroponic conditions. *Sci Hort* 106: 38-52.

Sayed SA (1999). Effects of lead and kinetin on the growth, and some physiological components of safflower. *Plant Growth Regul* 29: 167-174.

Soil Survey Staff (2010). Keys to soil taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington, DC.

Taiz L and Zeiger E (2006). Plant physiology. Sinauer Associates Publishers, Sunderland, MA.

Tanimoto E (2005). Regulation of root growth by plant hormones-roles for auxin and gibberellin. *Crit Rev Plant Sci* 24: 249-265.

Tassi E, Pouget J, Petruzzelli G and Barbafieri M (2008). The effects of exogenous plant growth regulators in the phytoextraction of heavy metals. *Chemosphere* 71: 66-73.

Thimann KV and Skoog F (1934). On the inhibition of bud development and other functions of growth substance in *Vicia faba.* Proceedings of the Royal Society of London, Series B. Vol. 114: 317-339.

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D and Mench M (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res Int* 16: 765-794.

Wang HH, Shan Xq, Wen B, Owens G, Fang J and Zhang S-z (2007). Effect of indole-3-acetic acid on lead accumulation in maize (*Zea mays* L.) seedlings and the relevant antioxidant response. *Environ Exp Bot* 61: 246-253.

Weaver RJ (1972). Plant growth substances in agriculture. Freeman & Company, San Francisco, CA.

Yang T, Law DM and Davies PJ (1993). Magnitude and kinetics of stem elongation induced by exogenous indole-3-acetic acid in intact light-grown pea seedlings. *Plant Physiol* 102: 717-724.

Zhao Y, Peralta-Videa JR, Lopez-Moreno ML, Ren M, Saupe G and Gardea-Torresdey JL (2010). Kinetin increases chromium absorption, modulates its distribution, and changes the activity of catalase and ascorbate peroxidase in Mexican palo verde. *Environ Sci Technol* 45: 1082-1087.

5

RHIZOBACTERIAL INOCULANTS CAN IMPROVE NICKEL *phytoextraction by the hyperaccumulator Alyssum pintodasilvae*

ABSTRACT

Rhizobacteria can influence plant growth and metal accumulation. The aim of this study was to evaluate the effect of rhizobacterial inoculants on the Ni phytoextraction efficiency of the Ni-hyperaccumulator *Alyssum pintodasilvae*. In a preliminary screening 15 metal-tolerant bacterial strains were tested for their plant growth promoting (PGP) capacity or effect on Ni bioaccumulation. Strains were selected for their Ni tolerance, plant growth promoting traits and Ni solubilizing capacity. In a re-inoculation experiment five of the previously screened bacterial isolates were used to inoculate *A. pintodasilvae* in two contrasting Ni-rich soils (a serpentine (SP) soil and a sewage sludge-affected agricultural (LF) soil). Plant growth was greater in serpentine soil (where it grows naturally) than in the LF soil, probably due to Cd phytotoxicity. Rhizobacterial inoculants influenced plant growth and Ni uptake and accumulation, but the effect of the strains was dependent upon soil type. The increase in plant biomass and/or Ni accumulation significantly promoted shoot Ni removal. One strain (*Arthrobacter nicotinovorans* SA40) was able to promote plant growth and phytoextraction of Ni in both soil types and could be a useful candidate for future field-based trials.

This study formed part of the following publication:

Cabello-Conejo MI, Becerra-Castro C, Prieto-Fernández A, Monterroso C, Saavedra-Ferro A, Mench M, Kidd PS 2014. Rhizobacterial inoculants can improve nickel phytoextraction by the hyperaccumulator *Alyssum pintodasilvae*. *Plant Soil* 1-16.

5.1 *INTRODUCTION*

Over the last three decades there has been increasing interest in developing plant-based technologies for the remediation of contaminated soils (Chaney *et al.* 1997; Mench *et al.* 2009). For trace metal-contaminated soils, phytoextraction has been proposed as a potentially cost-effective option, which is less invasive than conventional civil engineering techniques for soil clean-up (e.g. encapsulation, vitrification, soil washing) and can even restore soil structure and functions (Moreno-Jiménez *et al.* 2012; Vangronsveld *et al.* 2009). Phytoextraction cultivates plants to accumulate trace metals from contaminated soils and transport them to the shoots which can then be harvested. Metal-hyperaccumulating plants are ideal candidates due to their extraordinary capacity to absorb and accumulate metals in their harvestable parts (Baker *et al.* 1994). The metal accumulation levels of hyperaccumulating plants can be several magnitudes higher than common values for other plants, although they are often only able to accumulate one or two trace elements (Chaney *et al.* 2007; Van der Ent *et al.* 2013). Phytoextraction using hyperaccumulators has been described as a cost-effective method to mine Ni from naturally Ni-rich ultramafic soils (Ni phytomining), or to remediate Ni phytotoxic soils (Bani *et al.* 2007; Chaney *et al.* 2007; He *et al.* 2012). Ash from incineration of *Alyssum murale* biomass contains approximately 20 % Ni and can be used as an ore in electric furnace refining of Ni (Chaney *et al.* 2007).

To be effective phytoextractors, hyperaccumulators must be highly metal tolerant, able to accumulate large concentrations of the targeted trace elements in harvestable shoots, and have a reasonable biomass production so that metal removal from the site is cost-effective (Li *et al.* 2003; Vangronsveld *et al.* 2009). Agronomic management practices (such as fertilisation, liming or herbicide regimes) have been proposed as a means of maximising the performance and yields of hyperaccumulator crops (Kukier *et al.* 2004; Li *et al.* 2003). Biotechnological approaches have also been suggested and several authors have proposed incorporating plant-associated microorganisms (rhizosphere and endophytic bacteria, as well as mycorrhizal fungi) into phytoextraction systems (Abou-Shanab *et al.* 2006; Kidd *et al.* 2009; Ma *et al.* 2009; Rajkumar and Freitas 2008a; Sessitsch *et al.* 2013).

Some microorganisms present plant growth promoting traits. Plant Growth Promoting Rhizobacteria (PGPR) can enhance tolerance, growth and survival under the stress conditions of metal-rich soils (e.g. nutrient deficiency, phytotoxic concentrations of trace metals). Many PGPR facilitate plant growth through the production of plant growth regulators and phytohormones (i.e. indoleacetic acid

(IAA), gibberellins or cytokinins), or via the release of essential nutrients (e.g. N_2-fixers, phosphate-solubilisers, and siderophore-producers), or the induction of plant defence mechanisms (Glick 2003; Glick *et al.* 1998; Weyens *et al.* 2009a). Zaidi *et al.* (2006) reported that an IAA-producing *Bacillus subtilis* strain was able to promote the growth of *Brassica juncea* and thereby increased Ni extraction. Inoculation with the plant growth-promoting bacterium *Psychrobacter* sp. SRS8 stimulated growth and Ni accumulation in *Ricinus communis* and *Helianthus annuus* grown in Ni-contaminated soil (Ma *et al.* 2011). Furthermore, microorganisms can modify trace metal mobility and phytoavailability through the release of chelating agents (organic acids and siderophores), acidification or redox changes (Gadd 2010; Lebeau *et al.* 2008). Rhizobacteria increased soil Ni availability and hyperaccumulation of Ni in *Alyssum murale* (Abou-Shanab *et al.* 2006; Abou-Shanab *et al.* 2003). Cd- and Pb-mobilizing rhizosphere bacterial strains enhanced the uptake of metals in tomato (Jiang *et al.* 2008) and a Zn-mobiliser promoted Zn accumulation in *Ricinus communis* (Rajkumar and Freitas 2008b).

In many cases the effects of these plant-microbial associations have been shown to be plant-species specific (Becerra-Castro *et al.* 2012). However, few studies have evaluated their efficiency in relation to the properties of the growth substrate. It seems likely that their effects may be both plant- and substrate-dependent. This study aimed at evaluating the effect of selected rhizobacterial strains on plant biomass production and Ni phytoextraction by the Ni-hyperaccumulator *Alyssum pintodasilvae* in two contrasting soils. Firstly, fifteen bacterial isolates were screened for their PGP capacities by growing *Alyssum pintodasilvae* in a simple perlite:sand mixture (2:1 v/v). Secondly, *Alyssum pintodasilvae* was grown in two soils, a naturally Ni-rich serpentine soil and a sewage sludge-amended agricultural soil with Ni and Cd as the main contaminants, which were inoculated with five selected bacterial isolates. The effects of bacterial inoculants on soil metal availability, plant growth, nutrient status, Ni accumulation and extraction were evaluated.

5.2 *MATERIAL AND METHODS*

Screening of rhizobacterial isolates for promoting plant growth and Ni accumulation

Bacterial strains were previously isolated by Becerra-Castro *et al.* (2011) from the rhizosphere soil of two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf. (*Brassicaceae*): *A. serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (commonly referred to as *A. pintodasilvae*) and *A. serpyllifolium* subsp. *malacitanum* Rivas Goday (*A. malacitanum*). Both subspecies are endemic

to the Iberian Peninsula (Asensi *et al.* 2004; Brooks *et al.* 1981; Menezes de Sequeira 1969). *Alyssum pintodasilvae* is found in the serpentinitic region of Trás-os-Montes in NE Portugal (Morais (M) and Samil (S)) and in the vicinity of Melide (L) in NW Spain, and *A. malacitanum* grows in the serpentinitic area of Sierra Bermeja (SB), Málaga in S Spain. The isolates were previously screened for Ni resistance, the ability to produce organic acids, and for various plant growth promoting (PGP) characteristics: phosphate solubilisation capacity, siderophore production and indoleacetic acid (IAA) production. In addition, they were characterised genotypically by BOX-PCR fingerprinting and comparative sequence analysis of partial 16S rRNA gene (Becerra-Castro *et al.* 2011). Isolate nomenclature (L, S, M or SB) indicates the serpentine site from which they originate. For this study fifteen rhizobacterial strains were chosen, strains were selected according to their phenotypic traits (Table 5.1).

Seeds of *A. pintodasilvae* were collected from Trás-os-Montes (NE Portugal), surface-sterilised in 10 % sodium hypochlorite solution and then rinsed

Table 5.1. Phenotypic characteristics of the fifteen rhizobacterial strains selected for the screening test. Strains marked in bold were used for the soil pot experiment.

Isolate	Most similar type strain	Plant host	Site	Ni MTC (mM)	PO_4	Sid	OA	IAA (mg L^{-1})
LA1	*Arthrobacter nicotinovirans*	*A. pintodasilvae*	L	5	-	+	-	8.8
LA10	*Arthrobacter defluvii*	*A. pintodasilvae*	L	2.5	-	-	+	16.9
LA44	***Arthrobacter nitroguajacolicus***	***A. pintodasilvae***	**L**	**10**	-	-	+	**81.7**
LA80	*Arthrobacter defluvii*	*A. pintodasilvae*	L	10	-	-	-	89.6
SA5b	***Microbacterium* sp.**	***A. pintodasilvae***	**S**	**2.5**	-	-	+	-
SA17	***Microbacterium hydrocarbonoxydans***	***A. pintodasilvae***	**S**	**2.5**	-	+	+	-
SA26	*Curtobacterium flaccumfaciens*	*A. pintodasilvae*	S	2.5	-	+	+	-
SA37	*Arthrobacter nitroguajacolicus*	*A. pintodasilvae*	S	2.5	+	-	-	6.4
SA40	***Arthrobacter nicotinovorans***	***A. pintodasilvae***	**S**	**2.5**	+	+	-	**7.6**
MA72	*Arthrobacter globiformis*	*A. pintodasilvae*	M	2.5	-	-	-	12.6
SBA5	*Curtobacterium flaccumfaciens*	*A. malacitanum*	SB	5	-	-	-	39.5
SBA29	*Arthrobacter globiformis*	*A. malacitanum*	SB	1	-	+	+	15.2
SBA50	***Streptomyces lincolnensis***	***A. malacitanum***	**SB**	**10**	-	-	-	-
SBA82	*Arthrobacter humicola*	*A. malacitanum*	SB	1	+	+	-	29.8
SBA86	*Arthrobacter nitroguajacolicus*	*A. malacitanum*	SB	2.5	-	+	+	-

MTC, Maximal Tolerable Concentration of Ni; PO_4, Phosphate solubilisation; Sid, Siderophore production; Org acid, Organic acid production; IAA, indoleacetic production (mg L^{-1}) (Becerra-Castro *et al.* 2011).

in sterile deionised water. Seeds were germinated on a 2:1 perlite:quartz sand mixture (2:1 v/v) in a growth chamber under controlled conditions (temperature 22-25 ºC, PPFD of 190 µmol m^{-2} s^{-1}, under a 16/8 h light/dark cycle). Seeds were watered daily with deionised water until germination and thereafter with a Ni-rich serpentine-like macro-nutrient solution which consisted of 2 mM $MgSO_4$, 0.8 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 0.1 mM K_2HPO_4, 20µM FeEDDHA, 10 µM H_3BO_3, 2 µM $MnCl_2$, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.2 µM Na_2MoO_4 and 300 µM $NiSO_4$ (based on Chaney *et al.* 2008). One-month-old *A. pintodasilvae* seedlings were transferred into pots with the same perlite:quartz sand substrate. Three weeks after transferring into pots, seedlings were inoculated with one of the fifteen bacterial strains. Fresh cultures of bacterial strains were grown in 869 medium (Mergeay *et al.* 1985) for three days, harvested by centrifugation (6000 rpm, 15min) and re-suspended in 10 mM $MgSO_4$ to an OD_{660} of 1.0 (about 10^8 cells per mL). Each plant pot was inoculated with 9 mL of each bacterial suspension. The same amount of sterile 10 mM $MgSO_4$ was added to non-inoculated plants. Eight replicates were prepared for each inoculation treatment. After inoculation, plants were watered with the Ni-rich nutrient solution (as above). Seven weeks after inoculation, plants were harvested and rinsed in deionised water to remove any adhering particles. Shoots and roots were separated, dried for 48 h at 40 °C and weighed to determine dry biomass. Plant aerial biomass was digested in a 2:1 HNO_3:HCl mixture and the concentration of K, Ca, Mg, Fe, Cd, Cu, Mn, Ni, P, Pb and Zn were measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed in mg kg^{-1} dry weight (DW) plant material. Shoot Ni removal was calculated as the product of the shoot Ni concentration and shoot DW yield.

Effect of selected rhizobacterial inoculants on Ni phytoextraction by *Alyssum pintodasilvae* in two contrasting soils

Soil was collected from the serpentinitic region of Trás-os-Montes (SP) in Portugal (where *A. pintodasilvae* is a native species) and from the Louis Fargue (LF) field experiment in Villenave d'Ornon, Gironde, France (Boisson *et al.* 1998; Mench *et al.* 2006). The LF soil was treated with sewage sludge between 1976-1980 (total sludge input of 300 t DM ha^{-1}) which showed high Ni and Cd concentrations (Mench *et al.* 2006; Weissenhorn *et al.* 1995). Soils were air-dried, sieved through a 2-mm stainless steel sieve and homogenised. Soil pH was measured in H_2O using a 1:2.5 soil:solution ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Exchangeable cations were extracted with 1M NH_4Cl. Calcium and Mg were determined by atomic absorption spectrometry (AAS; Perkin-Elmer 2380, Norwalk, CT). Available P was determined by Olsen's $NaHCO_3$ method (Olsen *et*

al. 1954). Soils were digested in a 2:1 mixture of concentrated HNO_3:HCl and pseudo-total concentrations of metals were analysed by AAS. Soil Ni availability was evaluated after extraction with 10 mM $Sr(NO_3)_2$ (Everhart *et al.* 2006). For pot preparation, the soils were mixed with perlite in the ratio of 10:1 (v/v) to improve aeration and drainage. Plastic pots (500 mL) were filled with either SP or LF soil (36 pots per soil), and one four-week-old seedling of *A. pintodasilvae* (germinated under the same conditions as described above) was transplanted into each pot.

Five bacterial isolates were used for this study, these were selected to represent different phenotypic traits and also according to the results obtained in the preliminary screening described above. The selected strains were identified (by partial sequencing of 16S rDNA) as *Microbacterium hydrocarbonoxydans* SA17, *Arthrobacter nicotinovorans* SA40, *Arthrobacter nitroguajacolicus* LA44, *Microbacterium* sp. SA5b and *Streptomyces lincolnensis* SBA50. Strains SA5b, SA17 and SA40 significantly increased shoot DW yield of *A. pintodasilvae* in the preliminary screening, while root DW yield was highest in plants inoculated with strain LA44. Strain SBA50 was the only strain found to negatively affect plant biomass and was therefore used for comparative purposes. Phenotypic traits such as the production of organic acids or siderophores have been implicated in soil metal mobilisation and can influence metal uptake and bioaccumulation. The strains SA5b, SA17 and LA44 are organic acid-producers, SA17 and SA40 produce siderophores, LA44 and SA40 are IAA-producers, and SA40 is able to solubilise inorganic phosphate (Table 5.1; Becerra-Castro *et al.* 2011). In addition, the metabolites produced by strains SA5b, SA17 and SBA50 can solubilise Ni from serpentine soil (Becerra-Castro *et al.* 2011). Bacterial inoculants were prepared as mentioned above and three weeks after transferring into pots each plant was inoculated with 2 mL of bacterial suspension. The same amount of sterile 10 mM $MgSO_4$ was added to non-inoculated pots. Six replicates were prepared for each inoculation treatment. Plants were grown in an environmentally controlled growth chamber for five months. At harvest, plants were rinsed in deionised water to remove any adhering soil particles. Shoots and roots were separated, dried for 48 h at 40 °C and weighed to determine dry biomass. Plant tissues were digested in a 2:1 HNO_3:HCl mixture and Ca, Cu, Fe, K, Mg, Mn, Ni, P and Zn were measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed in mg kg^{-1} dry weight (DW) plant material. The ability of *A. pintodasilvae* to bioconcentrate Ni in its aboveground biomass from either LF or SP soil (Bioconcentration Factor, BCF) was calculated as the ratio of the shoot Ni concentration and the pseudo-total Ni concentration in the soil. The effect of soil type and/or microbial inoculation on the overall Ni phytoextraction efficiency

was assessed by taking into account plant growth, and was calculated as the product of the shoot DW yield and the shoot Ni concentration in relation to the total soil Ni content.

Statistical analyses

Significant effects of bacterial strains on biomass production, nutrient and metal content in both inoculation experiments were determined using ANOVA followed by the "post-hoc" Dunnett test whenever data were normally distributed, or using the Mann-Whitney test for non-parametric data when homogeneity of variance and normality could not be met.

5.3 *RESULTS*

Influence of rhizobacteria on the growth and shoot ionome of *Alyssum pintodasilvae* grown in a perlite:sand substrate

Plant biomass production

The effects of the bacterial inoculants on the growth of *A. pintodasilvae* depended on the strains. Fig. 5.1 shows the mean plant tissue dry weights (shoots and roots) in non-inoculated and inoculated plants. Five strains (SA5b, SA17, SA40, SBA5 and SBA82) significantly improved shoot biomass production (Fig. 5.1a). Shoot biomass increased by 1.7- to 2.3-fold compared to non-inoculated plants. Root biomass was only significantly increased in the case of SA5b, which increased root DW yield by 1.7-fold. These growth-promoting strains were originally isolated from the rhizosphere soil of two populations of *Alyssum pintodasilvae* (L and S) and one population of *Alyssum malacitanum* (SB). Plants inoculated with the siderophore-producer SA17 (*Microbacterium hydrocarbonoxydans*) showed the highest shoot DW yield, whereas those inoculated with the IAA-producer LA44 (*Arthrobacter nitroguajacolicus*) showed the highest root biomass (Fig. 5.1b). Strain SBA50 (*Streptomyces lincolnensis*) was the only strain which negatively affected the growth of *A. pintodasilvae* (both shoot and root biomass were reduced by approximately 60 % compared to non-inoculated plants; $P < 0.05$; Fig. 5.1).

Shoot ionome and shoot Ni removal

Although there was no clear generalised effect amongst bacterial inoculants and/or specific macro- or micro-nutrients, several strains significantly influenced the plant nutritional status (Fig. 5.2). Shoot concentrations (in mg kg^{-1}) of Ca, Fe, K, Mg, Mn and P in non-inoculated plants were on average 31900, 176, 53000, 7400, 184 and 8400, respectively. Several strains (but not only those strains which

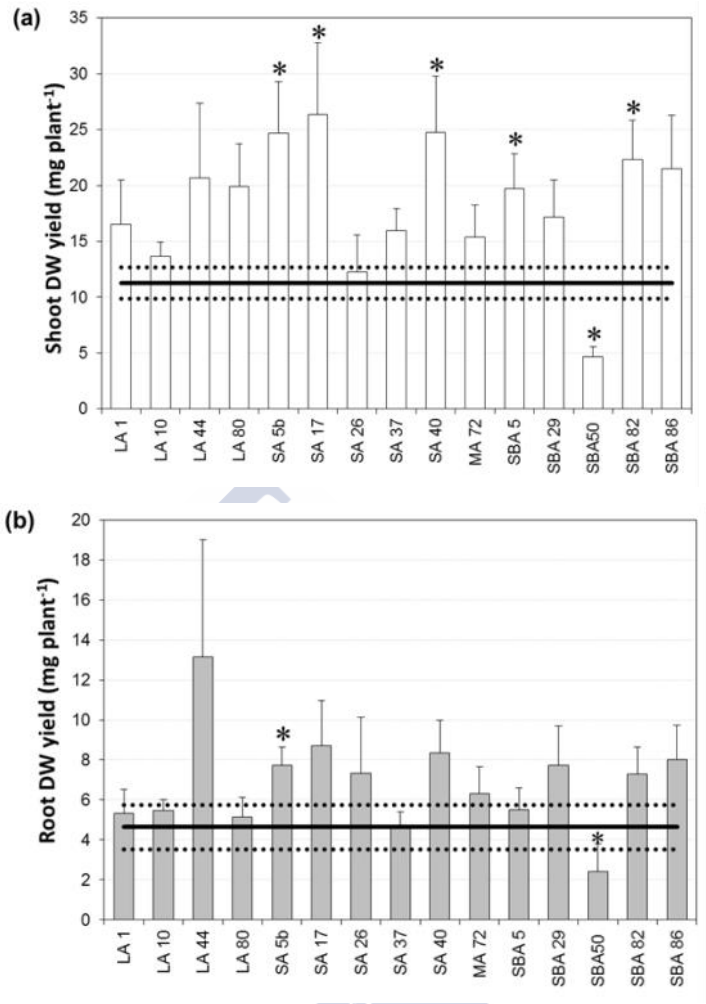


Figure 5.1. Effect of fifteen different rhizobacterial inoculants on the mean shoot (a) and root (b) DW yields (mg plant^{-1}) of *A. pintodasilvae.* Values of non-inoculated controls are indicated by a continuous line (±standard error (broken lines)). Asterisks indicate significant differences from the control (P <0.05).

improved biomass production) led to a significant increase in shoot Ca concentration (LA1, SA17, SA37, SBA82 and SBA86), K (LA80, SA5b and SA26), Mg (LA80), and Mn (LA1, LA10, LA80, SA37, SBA50, SBA82 and SBA86). One strain, identified as *Arthrobacter oxydans* SBA82 and which is able to solubilise inorganic phosphate, also tended to increase shoot P concentration, while the siderophore-producing strain SBA86 (*Arthrobacter nitroguajacolicus*) tended to increase shoot Fe content.

The mean shoot Ni concentration in non-inoculated plants was 6.2 ±1.1 g kg^{-1}, and in general, inoculation of plants did not lead to a significant change in their shoot Ni concentration (values varied from 4.0 ±0.4 to 9.3 ±0.8 g Ni kg^{-1}

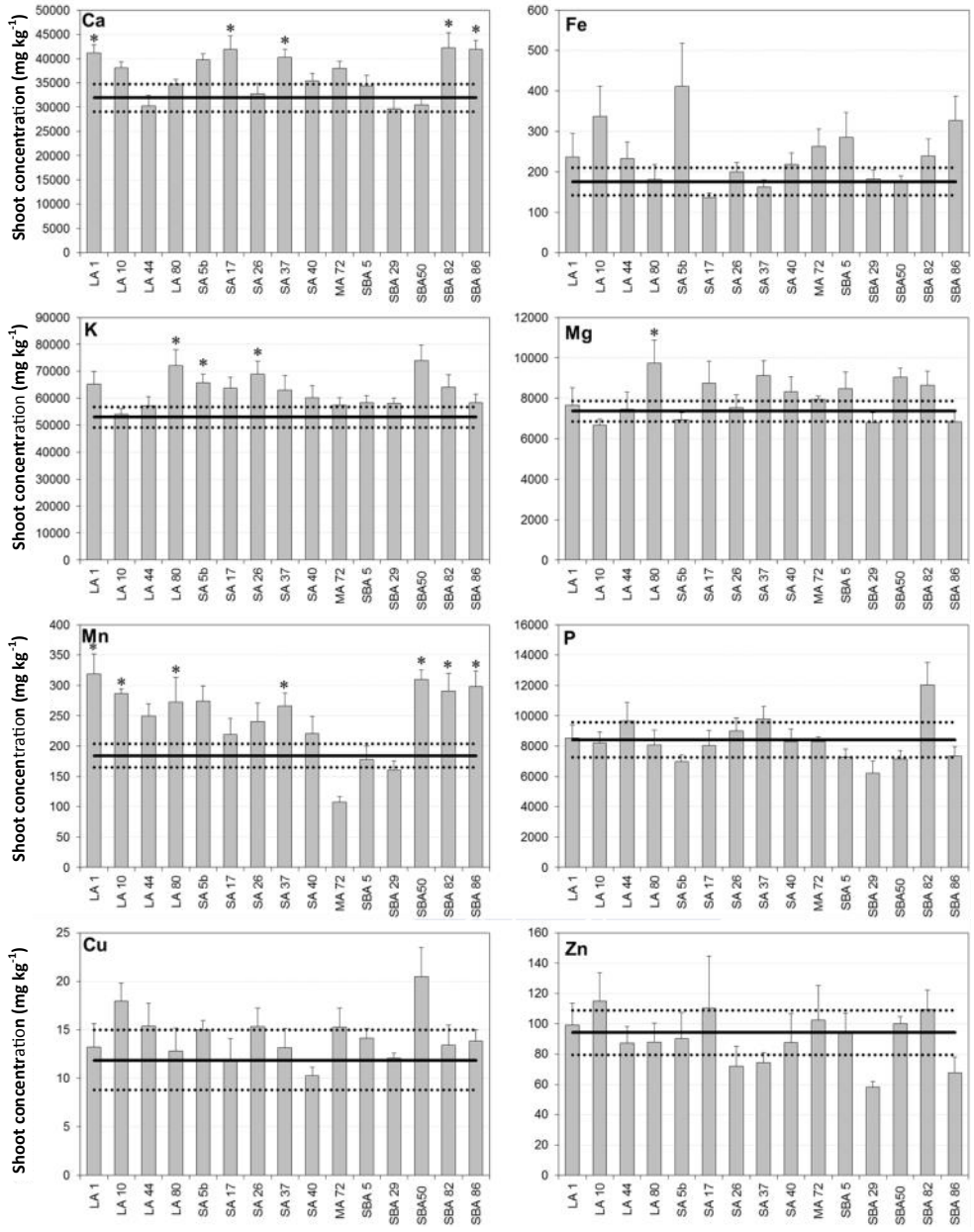


Figure 5.2. Concentrations of macro- and micro-nutrients in the shoots (mean ±SE) of *Alyssum pintodasilvae* grown in sand/perlite mixtures and inoculated with 15 rhizobacterial strains. Values of non-inoculated controls are indicated by a continuous line (±standard error (broken lines)). Asterisks indicate significant differences from the control (P <0.05).

(Fig. 5.3a). However, three inoculants significantly increased shoot Ni concentration, reaching values up to 1.5-fold higher than in controls: LA1 (9.1 ±0.7 g Ni kg^{-1}), MA72 (8.6 ±0.8 g Ni kg^{-1}) and SBA82 (9.3 ±0.8 g Ni kg^{-1}).

For strains SA40 and SBA82 the increase in plant biomass and/or shoot Ni concentration led to a significant increase in shoot Ni removal compared to that obtained with non-inoculated plants (Fig. 5.3b): mean Ni removal of control plants was 78 ±17 µg plant^{-1} compared to 161 ±16 and 157 ±32 µg plant^{-1} in SA40- and SBA82-inoculated plants, respectively (P <0.05). Conversely, the negative effect of SBA50 on both growth and Ni accumulation significantly reduced (by 48 %) shoot Ni removal (P <0.05; Fig. 5.3b).

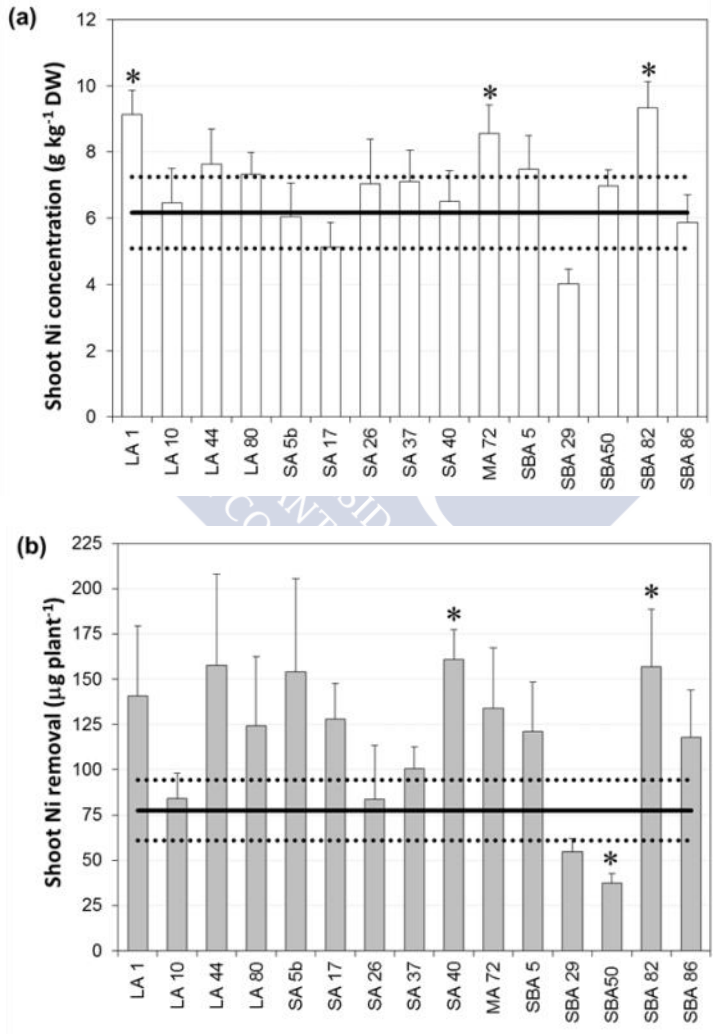


Figure 5.3. Effect of fifteen different rhizobacterial inoculants on shoot Ni concentration of *A. pintodasilvae* (a) and the shoot Ni removal (b). Values of non-inoculated controls are indicated by a continuous line (±standard error (broken lines)). Asterisks indicate significant differences from the control (P < 0.05).

Table 5.2. Physicochemical characteristics of soils used in the re-inoculation experiment.

	Serpentine soil (SP)	Agricultural soil (LF)
pH_{H2O}	7.0 ±0.0	6.9 ±0.0
%C	2.36 ±0.21	1.09 ±0.07
%N	0.29 ±0.01	0.21 ±0.00
CEC (cmol kg^{-1})	18.3 ±0.2	9.7 ±0.3
Ca/Mg	0.1 ±0.0	24.0 ±0.8
Available P (Olsen)	6.2 ±0.0	37.0 ±1.1
Pseudo-total metal concentration (mg kg^{-1})		
Cd	1.4 ±0.1	65.5 ±4.5
Co	1534 ±2	9 ±1
Cr	2587 ±122	14 ±2
Cu	32 ±1	33 ±2
Mn	1641 ±25	64 ±3
Ni	3569 ±189	153 ±10
Zn	50 ±0	105 ±7

Effect of selected rhizobacterial inoculants on the Ni phytoextraction efficiency of *A. pintodasilvae* grown in Ni-rich soils

To test these plant-microbial associations under contrasting soil conditions a reduced number of bacterial isolates were selected (four strains which stimulated plant growth and one which had a negative effect on growth).

Soil physicochemical characteristics and Ni phytoavailability

The serpentine (SP) soil presented a neutral pH (pH_{H2O} 7.0), high concentrations (in mg kg^{-1} soil) of total Ni (3569), Co (154) and Cr (2587), and a predominance of Mg in the exchange complex. The LF agricultural soil had a pH close to neutrality (pH_{H2O} 6.9), a significantly higher concentration of available P and a higher CEC (in this case dominated by Ca) compared to the SP soil (Table 5.2). The problematic trace metals in the LF soil were Ni (153 mg kg^{-1}) and Cd (65 mg kg^{-1}): the concentrations of both metals are higher than the maximum permitted by the EC in soils receiving sewage sludge (75 mg Ni kg^{-1} and 3 mg Cd kg^{-1}) (Ewers 1991).

Table 5.3. Concentrations of $Sr(NO_3)_2$-extractable Ni (mg kg^{-1}) before planting and at harvest. Plants were inoculated with rhizobacterial strains LA44, SA5b, SA17, SA40 or SBA50 or not inoculated (NI).

Treatment	SP soil	LF soil
Before planting	1.49 ± 0.01	2.18 ± 0.05
NI	1.36 ± 0.05	1.33 ± 0.12
LA44	1.21 ± 0.03*	1.13 ± 0.16
SA5b	1.26 ± 0.09	1.09 ± 0.07
SA17	1.40 ± 0.02	1.52 ± 0.06
SA40	1.33 ± 0.10	0.99 ± 0.07*
SBA50	1.02 ± 0.05*	1.53 ± 0.10

An asterisk denotes significant differences between the inoculated and non-inoculated (NI) treatment in the same soil (P <0.05).

Before planting, the Ni phytoavailability assessed by the $Sr(NO_3)_2$-extractable Ni concentration was similar in both soils (1.49 ±0.01 and 2.18 ±0.05 mg kg^{-1} soil in SP and LF, respectively) despite the differences in total Ni concentration (Table 5.3). After plant growth and in non-inoculated treatments, $Sr(NO_3)_2$-extractable Ni concentrations were reduced to 1.36 ±0.05 and 1.33 ±0.12 mg Ni kg^{-1} in SP and LF. Bacterial inoculants led to some numerical changes in Ni phytoavailability compared to non-inoculated treatments, but this depended on the soil type and was generally not statistically significant. After inoculation with strains LA44 and SBA50 a decrease in Ni phytoavailability was observed in SP, while strain SA40 led to significant decrease in LF (P <0.05), compared to the respective non-inoculated samples (Table 5.3).

Plant biomass production

After five months, plants produced a significantly higher biomass when grown in the SP soil compared to the LF soil (up to 9- and 2-fold higher shoot and root biomass, respectively) (P <0.001; Fig. 5.4). Depending on the soil type, the microbial inoculants influenced plant biomass production; however these differences generally did not reach statistical significance (Fig. 5.4). The mean shoot DW yield of non-inoculated plants grown in SP soil was 239 ±60 mg $plant^{-1}$, and this increased to 344 ±49, 439 ±94, 496 ±187 and 390 ±47 mg $plant^{-1}$ in plants

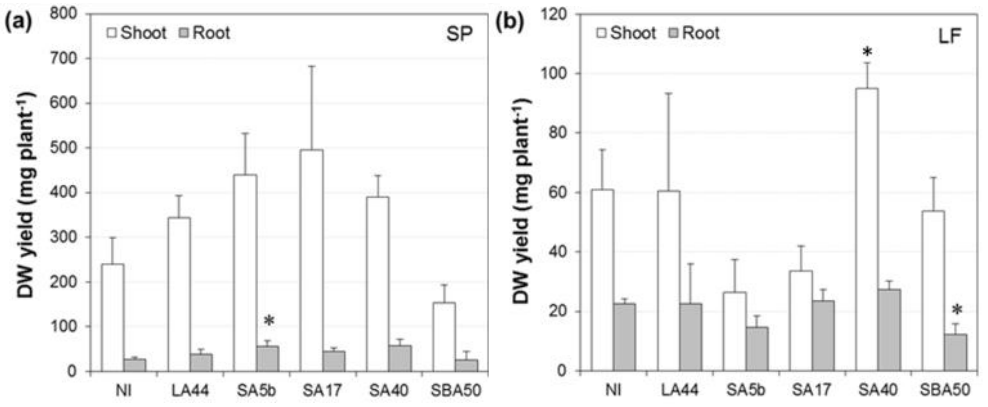


Figure 5.4. Effect of five selected rhizobacteria inoculants (strains LA44, SA5b, SA17, SA40, SBA50) compared to non-inoculated plants (NI) on the shoot and root DW yields (mg plant^{-1}) of *A. pintodasilvae* grown in (a) SP soil and (b) LF soil. Asterisks indicate significant differences from the control (P <0.05).

inoculated with strains LA44, SA5b, SA17 and SA40, respectively. Similarly, root dry weight production in plants inoculated with SA5b was significantly higher than control plants (P <0.05). The effect of the bacterial inoculants on the growth of *Alyssum pintodasilvae* differed in the LF soil, in this case one isolate significantly improved plant growth (strain SA40). The mean shoot DW yield increased from 61 ±13 mg (non-inoculated plants) to 95 ±9 mg DW plant^{-1} after inoculation with this strain (*Arthrobacter nicotinovirans* SA40). Strain SBA50 had no effect on the shoot DW yield of *Alyssum pintodasilvae* in the LF soil but significantly reduced root DW yield (Fig. 5.4b).

Shoot ionome and shoot Ni removal

Plant shoot tissues showed similar concentrations of the nutrients, Ca, Fe and K, when grown in either soil (Table 5.4). Shoot Mg concentrations however were significantly lower in the LF plants than in the SP ones, while shoot P and Zn concentrations were significantly higher in LF compared to SP plants. In general, bacterial inoculants did not significantly influence the shoot ionome. Only a few significant differences were found, and these were mainly in the LF soil (Table 5.4). In this soil, some inoculants significantly increased the shoot content of Ca (SBA50), Mg (SA17) or P (SBA50). Conversely, in the same soil some inoculants led to a significant decrease in nutrient concentrations, such as Ca (SA5b), Fe (SA40), Mg (SA5b and SA40) or Zn (SA5b and SA40). The mean shoot Cd concentration of non-inoculated plants grown in the LF soil was 462 ±25 mg kg^{-1}. All inoculants (except SBA50) led to a significantly lower accumulation of Cd in shoots compared to non-inoculated plants (Table 5.4). In SP soil, the only

significant effect of inoculation on shoot ionome was an increase in the shoot K concentration after inoculation with strain SA40 (Table 5.4).

Nickel accumulation by plants was significantly affected by soil type (P <0.001). This was most pronounced in shoot tissues where Ni concentrations were 8-fold lower in LF plants than SP plants, while root Ni concentrations were only 1.6-fold lower in LF plants than SP plants (Fig. 5.5). In the SP soil, non-inoculated *A. pintodasilvae* had a mean shoot Ni concentration of 6892 ±387 mg kg^{-1} DW, whereas the mean Ni concentration in the shoots of LF plants was 839 ±94 mg kg^{-1} DW. For the SP plants the highest shoot Ni concentrations were found in those plants inoculated with strain LA44: mean concentrations increased from 6892 ±387 mg Ni kg^{-1} to 11282 ±1856 mg Ni kg^{-1} (representing an increase of 64 %) (P <0.05; Fig. 5.5a). Moreover, this increase was accompanied by a reduction in root Ni concentrations, which resulted in a significant increase in the shoot:root Ni concentration ratio (from 6 to 9.7, Fig. 5.5a). For the LF plants there was no clear effect of inoculation on shoot Ni accumulation (Fig. 5.5b).

The LF plants showed a BCF of up to 2.8-fold higher than the SP plants (Table 5.5). For the SP plants, Ni bioaccumulation was significantly higher in plants inoculated with strains LA44 and SA40 (showing BCF values up to 1.6-fold higher; P <0.05). For the LF plants no significant differences in BCF values were observed after inoculation.

Ni phytoextraction efficiency

The percentage of Ni phytoextracted by plants grown in the SP soil was significantly higher than total Ni phytoextracted in LF soil (P <0.05), mean values ranged from 0.17 ±0.04 % to 0.48 ±0.10 % and from 0.06 ±0.02 % to 0.21 ±0.01 %, respectively (Fig. 5.6). In SP soil, the rhizobacterial inoculants LA44, SA5b, SA17 and SA40 significantly increased the phytoextracted Ni (not significant in case of SA17 (P=0.096)), while strain SBA50 did not affect the phytoextracted Ni from the soil. In LF soil, only strain SA40 significantly improved phytoextracted Ni compared to non-inoculated plants (P <0.05; Fig. 5.6b).

5.4 *DISCUSSION*

The effect of bacterial inoculants on plant growth and metal accumulation has previously been shown to be plant species-specific (Becerra-Castro *et al.* 2012). Here, plant-microbial associations were evaluated in relation to the growth substrate. In general, there was a higher variability in the measured parameters (e.g. DW yields, shoot element concentrations) in inoculated plants than non-inoculated plants, and some tendencies regarding plant growth or Ni accumulation after inoculation were not always significant. However, in the initial

Table 5.4. Mean macro- and micro-nutrient concentrations in the shoots of *A. pintodasilvae* grown in either SP or LF soil.

	SP						LF						
	Ca	**Fe**	**K**	**Mg**	**P**	**Zn**	**Ca**	**Cd**	**Fe**	**K**	**Mg**	**P**	**Zn**
	g kg^{-1}						**g kg^{-1}**						
NI	61.2 ±3.2	0.64 ±0.11	22.2 ±3.4	17.2 ±2.0	2.29 ±0.26	0.08 ±0.01	59.0 ±2.7	0.46 ±0.02	1.18 ±0.32	17.2 ±6.4	4.3 ±1.4	6.57 ±0.14	0.92 ±0.12
LA44	68.1 ±7.7	0.69 ±0.12	23.3 ±2.6	16.0 ±3.0	2.88 ±0.53	0.08 ±0.02	69.3 ±2.6	0.29 ±0.05*	0.86 ±0.11	8.6 ±1.9	3.0 ±0.1	7.03 ±0.56	0.70 ±0.18
SA5b	59.4 ±3.0	1.56 ±0.53	25.3 ±3.5	15.2 ±1.6	2.63 ±0.16	0.08 ±0.01	52.0 ±1.4*	0.28 ±0.03*	0.71 ±0.20	19.9 ±2.9	2.0 ±0.1*	5.52 ±0.16	0.57 ±0.03*
SA17	73.0 ±13.9	1.09 ±0.70	21.5 ±3.4	19.5 ±3.8	2.55 ±0.47	0.07 ±0.02	59.3 ±1.4	0.27 ±0.04*	1.57 ±0.47	11.8 ±4.9	7.2 ±1.7*	6.56 ±0.12	0.80 ±0.20
SA40	56.5 ±6.3	0.98 ±0.17	32.1 ±2.8*	16.9 ±1.1	3.03 ±0.50	0.09 ±0.01	53.8 ±2.1	0.28 ±0.05*	0.31 ±0.03*	17.1 ±2.1	1.6 ±0.7*	4.92 ±0.74*	0.48 ±0.08*
SBA50	61.3 ±3.2	1.00 ±0.22	25.1 ±1.7	20.6 ±2.7	2.78 ±0.25	0.10 ±0.02	79.3 ±5.0*	0.41 ±0.02	0.90 ±0.40	23.8 ±4.3	2.6 ±0.3	7.98 ±0.61*	0.59 ±0.06

An asterisk denotes a significant difference (P <0.05) between the inoculated plant (LA44, SA5b, SA17, SA40 or SBA50) and the non-inoculated (NI) control.

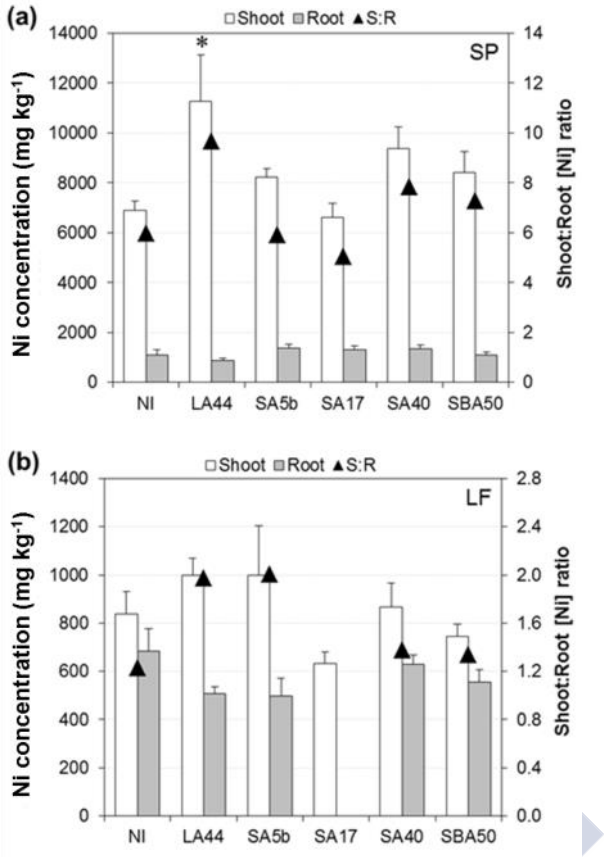


Figure 5.5. Effect of five selected rhizobacteria inoculants (strains LA44, SA5b, SA17, SA40, SBA50) compared to non-inoculated plants (NI) on the shoot and root Ni concentration (mg kg^{-1}), and the shoot:root [Ni] ratio, of *A. pintodasilvae* grown in (a) SP soil and (b) LF soil.

screening experiment, inoculation with five bacterial strains significantly promoted the growth of *A. pintodasilvae*. Moreover, in two cases this enhancement in shoot biomass production led to an increase in phytoextracted Ni. These PGP strains included members of the genus *Arthrobacter* (SA40 and SBA82), *Microbacterium* (SA5b and SA17) or *Curtobacterium* (SBA5). No individual phenotypic trait was consistently found amongst strains which promoted growth. Two strains that produced moderate to high levels of the phytohormone IAA (>25 mg L^{-1}) also significantly increased plant growth (Fig. 5.1; SBA5 and SBA82). Beneficial effects of bacterial inoculants on the growth of metal-exposed plants have often been attributed to the production of this phytohormone (Dell'Amico *et al.* 2008; Shilev *et al.* 2006). However, some of the strains used in the screening which stimulated plant growth (such as SA5b or SA17) did not show the capacity to produce IAA, and there was no clear

Table 5.5. Bioconcentration Factor (BCF, calculated as the ratio of the shoot Ni concentration and the pseudo-total Ni concentration in the soil) of *A. pintodasilvae* grown in either SP or LF soil.

	BCF	
Treatment	**SP**	**LF**
NI	1.9 ±0.1	5.5 ±0.6
LA44	3.2 ±0.5*	6.5 ±0.5
SA5b	2.3 ±0.1	6.5 ±1.3
SA17	1.9 ±0.2	4.2 ±0.3
SA40	2.6 ±0.2*	5.7 ±0.7
SBA50	2.4 ±0.2	4.9 ±0.3

An asterisk denotes a significant difference (P <0.05) between the inoculated plants (LA44, SA5b, SA17, SA40 or SBA50) and the non-inoculated (NI) control plants.

correlation between their PGP traits and the induced growth promotion (these strains were either able to produce organic acids or siderophores). In the initial screening method, nutrient supply (via Hoagland solution) was presumably adequate for plant growth, whereas in a soil system essential nutrients (such as P or Fe) may be limiting. Under nutrient deficiency the PGP traits of the bacterial inoculants are more likely to be activated. Thus, in a plant-microorganism-soil system the bacterial response may differ from that observed when using a simple perlite/sand growth substrate.

Both serpentine soils and anthropogenic-contaminated soils have been suggested as suitable for Ni phytomining (Li *et al.* 2003). Serpentine soils develop from ultramafic parent material and are therefore frequently enriched in trace metals other than Ni, such as Co, Cr, Mn or Fe. In order to use hyperaccumulating plants to extract Ni from these soils they must be tolerant to these co-contaminants (Tappero *et al.* 2007). In the soil experiment, the soil type strongly affected the growth of *A. pintodasilvae*. High total Co and Cr concentrations in the SP soil did not negatively affect its growth or Ni bioaccumulation capacity. This is unsurprising since the SP soil was collected from a serpentine outcrop where this species is found growing naturally, and the soils are characterised by an elevated concentration of Co, Cr and Ni but low labile pools of Co and Cr. *Alyssum pintodasilvae* is adapted to serpentine soils, and to the unfavourable conditions

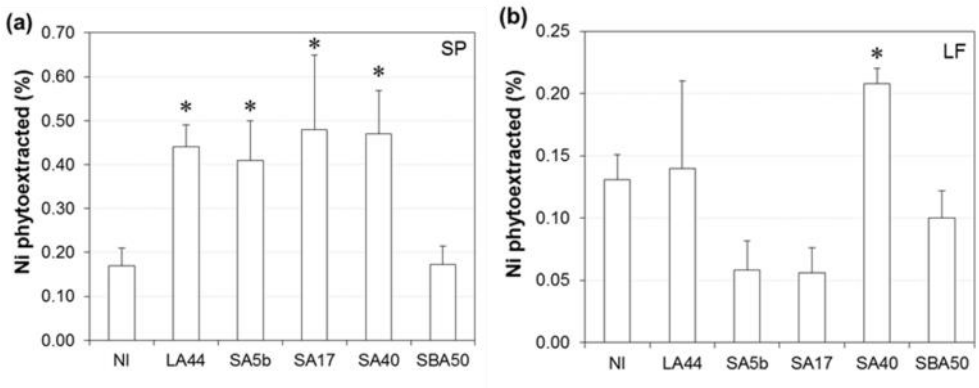


Figure 5.6. Effect of five selected rhizobacteria inoculants compared to non-inoculated plants (NI) on the Ni phytoextracted (shoot Ni removal/total soil Ni, %) of *A. pintodasilvae* grown in (a) SP soil and (b) LF soil. Asterisks indicate significant differences from the control (P <0.05).

that these present for plant growth and development, such as a high Ni phytoavailability, but also to poor fertility (deficiency in N, P and K), a high Mg:Ca quotient and low Fe solubility due to the near-neutral soil pH. In contrast, shoot biomass of *A. pintodasilvae* was up to 8-fold lower in the sewage sludge-amended soil (LF). The LF soil was co-contaminated with both Ni and Cd ($Sr(NO_3)_2$-extractable concentrations of Cd of 0.62 ±0.02 mg kg^{-1} soil was determined) and the poorer growth observed in this soil could have been due to the phytotoxicity of Cd. Cadmium is an element which is rarely found in appreciable concentrations in serpentine soils. Evidence of co-tolerance of hyperaccumulating *Alyssum* species to other metals (other than the hyperaccumulated metal) can be found in the literature. Elevated concentrations of Co or Zn had no effect on the plant's ability to accumulate Ni in hydroponically-grown *A. murale* (Tappero *et al.* 2007). The authors concluded that *A. murale* could therefore be used to recover Ni from most metal-enriched soils containing these metal co-contaminants. Conversely, in hydroponics, the growth of the Ni hyperaccumulator *Alyssum bertolonii* was significantly reduced when the solution Cd concentration increased (0 to 10 µM $CdSO_4$), and Cd was primarily accumulated in the root tissues (Barzanti *et al.* 2011). Cadmium is considered as a non-essential element for metabolic processes, and can negatively affect plant growth and development since it can replace essential elements that play a key role in active sites of enzymes or due to its high affinity for sulfhydryl groups (Vangronsveld and Clijsters 1994). Furthermore, compared to the SP soil, the LF soil presented significantly higher availability of nutrients such as P, and a CEC dominated by Ca (and not Mg). Calcium has been shown to depress both growth and nickel uptake by the Ni hyperaccumulator *Alyssum bertolonii* (Gabbrielli *et al.* 1990).

The shoot Ni concentrations of *A. pintodasilvae* were far above the criteria for Ni hyperaccumulators (>1000 mg Ni kg^{-1}) when grown in the serpentine (SP) soil, and were close to the threshold value when grown in the agricultural (LF) soil (Van der Ent *et al.* 2013). In both soils, shoot:root Ni transport ratios were above 1, confirming their ability to hyperaccumulate this element in the aboveground biomass. Soil Ni bioavailability ($Sr(NO_3)_2$-extractable Ni concentrations) was similar in both soils at the beginning of the experiment (and BCF values were even higher for LF plants). It is worth noting however that $Sr(NO_3)_2$-extractable concentrations of Ni were reduced after growth in the LF soil to a greater extent than in the SP soil, which could have contributed to the lower shoot Ni concentrations in the LF plants. Competitive interactions have also been shown to occur between Cd and Ni during the hyperaccumulation process (Assunção *et al.* 2008). In a hydroponic study, Ni uptake by *Noccaea caerulescens* was strongly suppressed in the presence of both Cd and Zn (Assunção *et al.* 2008). Antagonistic interactions such as these could explain the lower shoot Ni concentrations of *A. pintodasilvae* grown in the LF soil, since the phytoavailable concentration of Ni in the LF soil was not strongly in excess of that of Cd (while the opposite would be the case in SP soils).

Cabello-Conejo *et al.* (2013) found that the Ni phytoextraction efficiency of different Ni-hyperaccumulating *Alyssum* species grown in serpentine soil was, in decreasing order: *A. murale* > *A. corsicum* > *A. malacitanum* > *A. pintodasilvae*. Consequently, for considering *A. pintodasilvae* as a suitable candidate for Ni phytomining of serpentine soils, its biomass production and Ni extraction efficiency would need to be optimised. Similarly, in the case of the agricultural (LF) soil methods would need to be implemented to alleviate the Cd phytotoxicity symptoms as well as improve plant growth and biomass production. Plant growth promotion clearly plays a major role in the extraction and removal of trace elements since a simple improvement in biomass results in an increase in the overall shoot metal(loid) removal. Numerous studies have isolated and characterised rhizosphere or endophytic bacteria associated with trace element-tolerant or trace element-(hyper)accumulating plants as a means of identifying interesting strains for phytoextraction purposes (Rajkumar and Freitas 2008b; Weyens *et al.* 2009b). However, fewer studies have evaluated the application of these strains in contrasting soil types.

Five strains were selected for the bioaugmentation experiment in soils: four of these were selected for their positive influence on growth in the first screening experiment and based on their phenotypic characteristics. This allowed for evaluating the response of these bacterial inoculants under soil conditions, as well as studying the soil-specificity of bacterial-induced modifications in plant

growth/Ni extraction efficiency. The rhizosphere isolate LA44 shows intense IAA-production, is an organic-acid producer and highly Ni-resistant. While SA5b, SA17 and SA40 present intermediate Ni resistance, and either produce organic acids (SA5b, SA17), siderophores (SA17, SA40) or solubilise inorganic phosphates (SA40). Strain SBA50 (highly Ni-resistant, no PGP trait), which had a negative effect on plant growth, was also included for comparative means. Bacterial-induced effects were found to be soil-specific: in the SP soil inoculation generally led to an enhanced plant growth and shoot Ni removal, whereas in the LF soil there was a general lack of a plant-growth promoting effect. The growth-promoting effect demonstrated in the first screening was also seen in inoculated plants grown in the SP soil (with strains SA5b, SA17 and SA40). However, strain SBA50 (*Streptomyces lincolnensis*), which reduced plant growth in the perlite/sand substrate did not significantly reduce biomass production in the SP soil. The two *Microbacterium* spp. (SA5b and SA17) which significantly improved Ni removal in the SP soil had no effect on plants grown in LF soil. However, strain SA40 (*Arthrobacter* sp.) improved shoot DW yields of plants grown in both soils (SP and LF). As mentioned above plant growth was greatly reduced in the LF soil compared to SP soil, possibly due to Cd phytotoxicity. Bacterial inoculants have been shown to reduce plant stress levels, for example, by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which suppresses the production of stress ethylene in plants (Glick *et al.* 1998). The beneficial effect of strain SA40 on plant growth makes it very interesting for bioaugmentation of anthropogenic-contaminated soils. Moreover, the identification of a bacterial strain which has a growth-promoting effect in contrasting soil types is valuable for application in real-life scenarios, where edaphic properties are likely to vary greatly. At least in the case of the SP soil, the congruent results obtained between the initial screening experiment and the soil experiment, suggest that this screening method can be a useful tool for the rapid selection of interesting strains which can then be tested under more realistic conditions. Moreover, this screening method was more helpful in identifying potentially useful strains than the *in vitro* phenotypical characterisation of the strains since the effect of these inoculants cannot always be related to their PGP traits.

For strains SA5b, SA17 and SA40 the increase in shoot Ni removal was largely a consequence of the microbial-induced stimulation in plant biomass. For strain SA40, this was the case in both SP and LF soils. Inoculation with metal-resistant PGP bacteria has previously been shown to increase the biomass of several crops (e.g. *Brassica juncea*, *Ricinus communis*, *Helianthus annuus*) and other hyperaccumulators (e.g. *Sedum alfredii*) growing in metal-contaminated

soils (Dell'Amico *et al.* 2008; Jiang *et al.* 2008; Mastretta *et al.* 2009; Zaidi *et al.* 2006). However, plant-associated microorganisms can also modify soil metal mobility, by acidification, chelation or ligand-induced solubilisation (Abou-Shanab *et al.* 2003; Abou-Shanab *et al.* 2006). The literature generally cites two main groups of bacterially produced natural chelators: organic acids and siderophores. Here, strain LA44 (identified as *Arthrobacter nitroguajacolicus*) significantly enhanced shoot Ni concentrations in *A. pintodasilvae* in SP soil, which could presumably be a result of an enhanced Ni phytoavailability and hence plant uptake. Strain LA44 has been shown to be an efficient mobiliser of Ni from ultramafic rocks under *in vitro* conditions, and principally liberates Ni associated with Mn oxides through the exudation of oxalate (Becerra-Castro *et al.* 2013). Nickel shoot:root transport ratios were also significantly increased, suggesting this bacterial inoculant led to an increase in Ni translocation to aboveground plant parts. It is possible that strain LA44 enhances the replenishment of Ni labile phases in the soil thus increasing plant Ni uptake. The dynamic nature of these solution-solid phase interactions would explain why no corresponding increase in $Sr(NO_3)_2$-extractable Ni concentrations were observed after inoculating with this strain. Inoculating ultramafic soils with the actinobacterial *Microbacterium arabinogalactanolyticum* AY509224 increased soil Ni extractability (Abou-Shanab *et al.* 2003; Abou-Shanab *et al.* 2006). Becerra-Castro *et al.* (2011) showed that culture filtrates of strains SA5b and SBA50 (also used in this study) increased Ni extraction from ultramafic soils. However, no corresponding increase in soil Ni phytoavailability or shoot Ni concentrations were observed in *A. pintodasilvae* inoculated with these two strains. In fact, $Sr(NO_3)_2$-extractable Ni concentrations were reduced after plant growth and no differences were observed between inoculants, although this is likely to be due to root uptake.

In conclusion this study has identified candidate strains which could be useful for future field-based trials. Plant growth-promoting effects by associated bacteria can improve plant performance and also result in higher amounts of phytoextracted Ni. They also seem to be able to mobilise trace metals in soils, thereby increasing the phytoavailable fraction and plant uptake. It has been shown that Ni phytoextraction (or phytomining) can be optimised under field conditions using distinct agronomic practices (e.g. fertilisation regimes; Bani *et al.* 2007) but it remains to be seen whether or not plant-associated microorganisms can further improve the shoot Ni removal on a field scale. Further studies are also required to establish the optimal method of inoculation, regarding inoculum bacterial densities, plant stage and age for inoculation (e.g. inoculating seed or plants), timing of inoculation (bacterial growth phase) or the need for re-inoculation events, as well as the persistence and competition capacity of inoculant strains.

Advances in these aspects could lead to more pronounced effects of these plant-associated bacteria and further improvements in phytoextraction efficiency.

5.5 *REFERENCES*

Abou-Shanab RA, Angle JS, Delorme TA, Chaney RL, van Berkum P, Moawad H, Ghanem K and Ghozlan HA (2003). Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. *New Phytol* 158: 219-224.

Abou-Shanab RA, Angle JS and Chaney RL (2006). Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils. *Soil Biol Biochem* 38: 2882-2889.

Asensi A, Rodríguez N, Díez-Garretas B, Amils R, Boyd R, Baker A and Proctor J (2004). Nickel hyperaccumulation of some subspecies of *Alyssum serpyllifolium* (Brassicaceae) from ultramafic soils on the Iberian Peninsula. Ultramafic rocks: Their soils, vegetation and fauna, Proceedings of the fourth International Conference on Serpentine Ecology. Science Reviews, St. Albans, UK. 263-265.

Assunção AL, Bleeker P, Bookum W, Vooijs R and Schat H (2008). Intraspecific variation of metal preference patterns for hyperaccumulation in *Thlaspi caerulescens*: evidence from binary metal exposures. *Plant Soil* 303: 289-299.

Baker AJM, McGrath SP, Sidoli CMD and Reeves RD (1994). The possibility of *in situ* heavy metal decontamination of polluted soils using crops of metal-accumulating plants. *Resour Conserv Recy* 11: 41-49.

Bani A, Echevarria G, Sulçe S, Morel J and Mullai A (2007). *In-situ* phytoextraction of Ni by a native population of *Alyssum murale* on an ultramafic site (Albania). *Plant Soil* 293: 79-89.

Barzanti R, Colzi I, Arnetoli M, Gallo A, Pignattelli S, Gabbrielli R and Gonnelli C (2011). Cadmium phytoextraction potential of different *Alyssum* species. *J Hazard Mater* 196: 66-72.

Becerra-Castro C, Prieto-Fernández A, Álvarez-Lopez V, Monterroso C, Cabello-Conejo MI, Acea MJ and Kidd PS (2011). Nickel solubilizing capacity and characterization of rhizobacteria isolated from hyperaccumulating and non-hyperaccumulating subspecies of *Alyssum serpyllifolium*. *Int J Phytoremediat* 13: 229-244.

Becerra-Castro C, Monterroso C, Prieto-Fernández A, Rodríguez-Lamas L, Loureiro-Viñas M, Acea MJ and Kidd PS (2012). Pseudometallophytes colonising Pb/Zn mine tailings: A description of the plant-microorganism-rhizosphere soil system and isolation of metal-tolerant bacteria. *J Hazard Mater* 217-218: 350-359.

Becerra-Castro C, Kidd PS, Kuffner M, Prieto-Fernandez A, Hann S, Monterroso C, Sessitsch A, Wenzel W and Puschenreiter M (2013). Bacterially induced weathering of ultramafic rock and its implications for phytoextraction. *Appl Environ Microbiol* 79: 5094-5103.

Boisson J, Mench M, Sappin-Didier V, Solda P and Vangronsveld J (1998). Short-term *in situ* immobilization of Cd and Ni by beringite and steel shots application to long-term sludged plots. *Agronomie* 18: 347-359.

Brooks RR, Shaw S and Marfil AA (1981). Some observations on the ecology, metal uptake and nickel tolerance of *Alyssum serpyllifolium* subspecies from the Iberian Peninsula. *Plant Ecol* 45: 183-188.

Cabello-Conejo MI, Centofanti T, Kidd PS, Prieto-Fernández A and Chaney RL (2013). Evaluation of plant growth regulators to increase nickel phytoextraction by *Alyssum* species. *Int J Phytoremediat* 15: 365-375.

Chaney RL, Malik M, Li YM, Brown SL, Brewer EP, Angle JS and Baker AJM (1997). Phytoremediation of soil metals. *Curr Opin Biotechnol* 8: 279-284.

Chaney RL, Angle JS, Broadhurst CL, Peters CA, Tappero RV and Sparks DL (2007). Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. *J Environ Qual* 36: 1429-1443.

Chaney RL, Chen KY, Li YM, Angle JS and Baker AJM (2008). Effects of calcium on nickel tolerance and accumulation in *Alyssum* species and cabbage grown in nutrient solution. *Plant Soil* 311: 131-140.

Dell'Amico E, Cavalca L and Andreoni V (2008). Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. *Soil Biol Biochem* 40: 74-84.

Everhart JL, McNear D, Jr., Peltier E, van der Lelie D, Chaney RL and Sparks DL (2006). Assessing nickel bioavailability in smelter-contaminated soils. *Sci Total Environ* 367: 732-744.

Ewers U (1991). Standards, guidelines, and legislative regulations concerning metals and their compounds. In: E Merian (ed) Metals and their compounds in the environment: Occurrence, analysis and biological relevance. VCH, Weinheim. p. 687-711.

Gabbrielli P, Pandolfini T, Vergnano O and Palandri MR (1990). Comparison of two serpentine species with different nickel tolerance strategies. *Plant Soil* 122: 271-277.

Gadd GM (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156: 609-643.

Glick BR, Penrose DM and Li J (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J Theor Biol* 190: 63-68.

Glick BR (2003). Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnol Adv* 21: 383-393.

He S, He Z, Yang X and Baligar VC (2012). Chapter three: Mechanisms of nickel uptake and hyperaccumulation by plants and implications for soil remediation. In: LS Donald (ed) Advances in agronomy. Academic Press. Vol. 117. p. 117-189.

Jiang CY, Sheng XF, Qian M and Wang QY (2008). Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. *Chemosphere* 72: 157-164.

Kidd PS, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R and Monterroso C (2009). Trace element behaviour at the root-soil interface: Implications in phytoremediation. *Environ Exp Bot* 67: 243-259.

Kukier U, Peters CA, Chaney RL, Angle JS and Roseberg RJ (2004). The effect of pH on metal accumulation in two species. *J Environ Qual* 33: 2090-2102.

Lebeau T, Braud A and Jézéquel K (2008). Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. *Environ Pollut* 153: 497-522.

Li YM, Chaney R, Brewer E, Roseberg R, Angle JS, Baker A, Reeves R and Nelkin J (2003). Development of a technology for commercial phytoextraction of nickel: economic and technical considerations. *Plant Soil* 249: 107-115.

Ma Y, Rajkumar M and Freitas H (2009). Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. *J Hazard Mater* 166: 1154-1161.

Ma Y, Prasad MNV, Rajkumar M and Freitas H (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnol Adv* 29: 248-258.

Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Gonnelli C, Barac T, Boulet J, Weyens N and Vangronsveld J (2009). Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. *Int J Phytoremediat* 11: 251-267.

Mench M, Renella G, Gelsomino A, Landi L and Nannipieri P (2006). Biochemical parameters and bacterial species richness in soils contaminated by sludge-borne metals and remediated with inorganic soil amendments. *Environ Pollut* 144: 24-31.

Mench M, Schwitzguebel JP, Schroeder P, Bert V, Gawronski S and Gupta S (2009). Assessment of successful experiments and limitations of phytotechnologies: contaminant uptake, detoxification and sequestration, and consequences for food safety. *Environ Sci Pollut Res Int* 16: 876-900.

Menezes de Sequeira E (1969). Toxicity and movement of heavy metals in serpentinic soils (north-eastern Portugal). *Agron Lusit* 30: 115-154.

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P and Van Gijsegem F (1985). *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J Bacteriol* 162: 328-334.

Moreno-Jiménez E, Esteban E, Carpena-Ruiz RO, Lobo MC and Penalosa JM (2012). Phytostabilisation with Mediterranean shrubs and liming improved soil quality in a pot experiment with a pyrite mine soil. *J Hazard Mater* 201: 52-59.

Olsen SR, Cole CV and Watanabe FS (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate. US Dept Agric, Washington. Circular 939.

Rajkumar M and Freitas H (2008a). Effects of inoculation of plant-growth promoting bacteria on Ni uptake by Indian mustard. *Bioresour Technol* 99: 3491-3498.

Rajkumar M and Freitas H (2008b). Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. *Chemosphere* 71: 834-842.

Sessitsch A, Kuffner M, Kidd PS, Vangronsveld J, Wenzel WW, Fallmann K and Puschenreiter M (2013). The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biol Biochem* 60: 182-194.

Shilev S, Fernández A, Benlloch M and Sancho E (2006). Sunflower growth and tolerance to arsenic is increased by the rhizospheric bacteria *Pseudomonas fluorescens*. In: JL Morel (ed) Phytoremediation of metal contaminated soils. Springer, Netherlands. p. 315-326.

Tappero R, Peltier E, Gräfe M, Heidel K, Ginder-Vogel M, Livi KJT, Rivers ML, Marcus MA, Chaney RL and Sparks DL (2007). Hyperaccumulator *Alyssum murale* relies on a different metal storage mechanism for cobalt than for nickel. *New Phytol* 175: 641-654.

Van der Ent A, Baker AJM, Reeves RD, Pollard AJ and Schat H (2013). Hyperaccumulators of metal and metalloid trace elements: facts and fiction. *Plant Soil* 362: 319-334.

Vangronsveld J and Clijsters H (1994). Toxic effects of metals. In: ME Farago (ed) Plants and the chemical elements: biochemistry, uptake, tolerance and toxicity. VCH, Weinheim. p. 149.

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D and Mench M (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res Int* 16: 765-794.

Weissenhorn I, Mench M and Leyval C (1995). Bioavailability of heavy metals and arbuscular mycorrhiza in a sewage-sludge-amended sandy soil. *Soil Biol Biochem* 27: 287-296.

Weyens N, van der Lelie D, Taghavi S, Newman L and Vangronsveld J (2009a). Exploiting plant-microbe partnerships to improve biomass production and remediation. *Trends Biotechnol* 27: 591-598.

Weyens N, van der Lelie D, Taghavi S and Vangronsveld J (2009b). Phytoremediation: plant-endophyte partnerships take the challenge. *Curr Opin Biotechnol* 20: 248-254.

Zaidi S, Usmani S, Singh BR and Musarrat J (2006). Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. *Chemosphere* 64:991-997 991-997.

6

FINAL SYNTHESIS
a n d
c o n c l u s i o n s

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The study assessing the natural variation in plant growth, Ni tolerance and accumulation within and amongst populations of the Ni-hyperaccumulating *A. serpyllifolium* subspecies highlighted important differences in the nutrient status and Ni accumulation between and within the populations when analysing field-grown plants or plants cultivated in either hydroponic conditions or serpentine soil. In the field-collected plants the inter-population variance in Ni accumulation patterns was more pronounced than when the progeny were grown in controlled conditions. In the field the variation in leaf Ni concentrations was mainly explained by differences between populations rather than within populations: two populations were identified as presenting higher leaf Ni concentrations, which were the L population of *A. pintodasilvae* and the SB population of *A. malacitanum*. However, these inter-population differences in leaf Ni concentration were not correlated with either the total soil Ni or plant-available soil Ni fractions at the site of origin. The experiments following on from this indicated that the Ni accumulation of the mother (field-collected) plants was not significantly correlated with the shoot Ni concentration of their progeny when these were grown under controlled conditions. Field plants also presented a significantly higher leaf Ni concentration than that observed in the plants cultivated in the serpentine soil (pot experiment), which could reflect differences in plant age, root proliferation, and the edaphic properties and climate of each site. In both the hydroponic culture and the pot experiment the larger part of the total variability in shoot Ni concentration and yield was related to variance within the populations of *A. serpyllifolium* subspecies rather than between populations. The generally low contribution of the inter-population factor to the variance in Ni hyperaccumulation may be due to environmental factors or the result of the evolutionary history of serpentine populations of *A. serpyllifolium.* Nonetheless, under controlled conditions the present study revealed significant differences in biomass production and root-shoot Ni transfer that could be further explored in the future to increase the Ni yield of these hyperaccumulating *A. serpyllifolium* subspecies. However, it is important to point out that when compared to other *Alyssum* species, such as *Alyssum corsicum* and *Alyssum murale*, the biomass production of the *A. serpyllifolium* subspecies are significantly lower and this is likely to limit their practical application in phytomining strategies. On the other hand, under certain situations, the use of these subspecies may still be promising from a phytomining point of view. For example, in serpentine areas where these subspecies are endemic, phytomining could provide an alternative to traditional agriculture and support the development of rural areas. The use of these native plant species would support the conservation of serpentine biodiversity and avoid the introduction of exotic plant species that frequently colonise new areas at the expense of native species. Future studies

evaluating the use of these Ni-hyperaccumulators on a field-scale would be necessary, particularly those focusing on their growth habit and potential for mechanical harvesting. Moreover, additional agronomic and management measures would be necessary to further maximise biomass production and plant Ni yield.

The physico-chemical analysis of the rhizosphere soils of the five populations of *A. pintodasilvae* and *A. malacitanum* showed that an increase in Ni bioavailability in the rhizosphere was not observed for all populations of the hyperaccumulators. However, in some cases the $Sr(NO_3)_2$-extractable Ni concentration in the rhizosphere of the Ni-hyperaccumulators was significantly higher compared to the non-vegetated soil. Moreover, plant-induced shifts in the soil Ni fractionation were observed in some populations, whereby the more plant-available fractions were increased at the expense of less plant-available or silicate -bound fractions. In addition, some generalised effects were observed in other soil properties, such as an increase in the pH, total C and N content, the cation exchangeable capacity and the Ca:Mg ratio. The root activities of these Ni-hyperaccumulating plants could enhance the weathering of Ni-rich clay minerals which in turn would lead to the replenishment of soluble or labile Ni pools. However, more research is necessary to further understand the complexity of the physico-chemical and biological processes occurring in the soil-rhizosphere-hyperaccumulator plant system. Future studies focusing on the kinetics of soil Ni replenishment (and rate of supply from the soil solid phase) in the rhizosphere of Ni-hyperaccumulating plant species may shed further light on our understanding of the metal hyperaccumulation process.

The application of plant growth regulators (PGRs) was found to be an interesting option for stimulating the biomass production of Ni hyperaccumulating species such as *Alyssum* and *Noccaea* and, consequently, for increasing their metal phytoextraction capacity. In the Part I of our study the application of phytohormones (Cytokin and Promalin, based on cytokinins and gibberellins) had no a clear positive effect on biomass production, Ni accumulation and Ni phytoextraction efficiency in *A. corsicum*, *A. malacitanum*, *A. murale* and *A. pintodasilvae*. However, in Part II where a fuller range of products (Berelex, Cytoplant, Kelpak and Promalin, based on a combination of cytokinins, gibberellins and indoleacetic acid (IAA)) and a wider range of concentrations were evaluated it was shown that the application of PGRs significantly enhanced the growth of four Ni-hyperaccumulators (belonging to two different genera) in terms of their branching, number of leaves, stem length or leaf size. Two PGR products, Kelpak and Promalin (based on either IAA or a combination of cytokinins and gibberellic acid), significantly stimulated biomass production in

all four hyperaccumulators. Although PGRs tended to reduce the shoot Ni concentration, the IAA-based (Kelpak) treatment was found to improve the overall Ni phytoextraction capacity of all four species due to the increase in plant growth and biomass production. Future studies are therefore recommended to carry out more in-depth and longer-term evaluations of distinct IAA-based PGRs so as to fully optimise the beneficial effects that these can have on Ni phytoextraction efficiency of hyperaccumulating plant species. In addition, a field-based evaluation of the use of PGRs will be necessary before they can be successfully incorporated into a phytomining strategy.

The use of the selected plant growth promoting (PGP) rhizobacterial strains was also shown to successfully increase the biomass and/or Ni accumulation in *A. pintodasilvae*. However the effect of the strains was dependent upon the soil type, suggesting that the efficiency of this type of bacterial inoculant will not only be plant species-specific but also influenced by the plant physiological status and soil characteristics. Four strains were selected for their Ni tolerance, plant growth promoting traits and Ni solubilizing capacity (LA44, SA5b, SA17, SA40) and all of these led to an increase in the Ni phytoextracted. However, the *in vitro*-assessed traits of such bacterial strains do not always correspond with the bacterial-induced effects observed in the plant- microbial-soil system, suggesting that different mechanisms other than those generally studied are operating. Further studies are required to establish the optimal method of inoculation, regarding inoculum bacterial densities, plant stage and age for inoculation (e.g. inoculating seed or plants), timing of inoculation (bacterial growth phase) or the need for re-inoculation events, as well as the persistence and competition capacity of inoculant strains. In addition, only single strains were tested in this PhD thesis, but combinations or consortia of bacterial strains which combine different PGP and/or soil metal-solubilising capacities may be more beneficial when inoculated together. Advances in these aspects could lead to more pronounced effects of these plant-associated bacteria and improvements in phytoextraction efficiency, and efforts should be made towards making these methods ready for field applications. Nonetheless, this study identified one strain (*Arthrobacter nicotinovorans* SA40) which was able to promote plant growth and phytoextraction of Ni in contrasting soils: a serpentine soil and a Ni- and Cd-contaminated agricultural soil, and is therefore a useful candidate for future bioaugmentation trials.

The main conclusions of this PhD thesis can be summarised as:

The studies assessing the inter- and intra-population variation in plant biomass and Ni accumulation in harvestable plant tissues revealed that

a) Significant differences in Ni accumulation were found between plant populations in the field, but the Ni accumulation capacity of mother plants was

not transmitted to their descendants when these were grown in either hydroponic solutions or soil. Nonetheless, under these controlled conditions, the variation found in biomass production, Ni accumulation and root-shoot Ni transfer could be further explored in the future to increase the Ni yield of these hyperaccumulating *A. serpyllifolium* subspecies.

b) Plant Ni accumulation of field plants was not related to either the total Ni or plant-available Ni concentration in the soil of their origin. Nonetheless, the hyperaccumulating subspecies of *A. serpyllifolium* were often able to modify soil Ni availability and fractionation and, more importantly, under controlled conditions an increase in bioavailable Ni in the growth substrate led to an increase in Ni accumulation. This suggests that strategies which increase soil Ni bioavailability (to non-phytotoxic levels) could also lead to an increase in the Ni yield of these plants.

The strategies which were implemented as a means of enhancing Ni extraction by plant species that hyperaccumulate this metal (use of plant growth regulators (PGRs) or plant-associated bacterial strains) were, in some cases, found to successfully achieve an improvement in biomass and/or phytoextracted Ni.

a) In the case of the PGRs, the positive results obtained using this strategy were not only dependent on the chemical composition of the regulator applied, but also on the dose used, as well as the plant species to which they were applied.

b) In the case of the rhizobacterial strains, the benefits of these inoculants not only depended on the phenotypical traits of the bacterial strain but also on the physiological status of the host plant species and the soil physico-chemical properties.

Amongst all the treatments tested for improving plant biomass production and/or Ni phytoextraction the best results were obtained with an IAA-based PGR product or with an *Arthrobacter nicotinovorans* inoculum.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Agradecimientos…

Quiero dar las gracias a Petra y a Ángeles, en primer lugar por dirigir este trabajo y orientarme en cada paso que he dado, desde el momento cero de mi experiencia en investigación. En segundo lugar y no menos importante, no han sido sólo directoras, desde que llegué me han tratado como una más y me han hecho sentir, de verdad, como en casa. Soy muy consciente de la suerte que he tenido y que tengo. Gracias también a Carmela, como tutora y por los momentos que hemos podido compartir, sobre todo por sus consejos con las palabras justas y en el momento adecuado.

Tengo que dar las gracias también a Bea, dispuesta siempre a aclarar una duda o a echar una mano. A Lidia, que (aunque nos haya abandonado por el momento) siempre ha estado ahí, dentro y fuera del laboratorio. Gracias también a Marián, por su prudencia y su respeto infinito a los demás y a Mariana, por su eficiencia. Gracias a Tino, por su disposición siempre para todo y por su buen carácter y positivismo. Gracias también al grupo de enzimas: Carmen, Diana, Ana, Elena y Yoli, por estar siempre disponibles para ayudar en lo que haga falta y por compartir tantos cafés y momentos de tertulia. Gracias al grupo vecino, Serafín, Chus, Ángela y Jorge, por poder contar con ellos cuando se les necesita. Y a Mili, por ser una gerente molona y solucionar siempre todos los contratiempos. Gracias a Fina, por sus palabras siempre positivas y llenas de fuerza.

Quiero agradecer a Rufus L. Chaney su ayuda durante mi estancia en el USDA, proponiendo líneas de investigación que han enriquecido este trabajo. Además, agradecerle su apoyo continuo y su disposición en cualquier momento para ayudarme. A Tiziana Centofanti, por pasar tantas horas rodeadas de hidropónico y xilema y, sobre todo, por ser mi principal apoyo durante mi estancia en USA, también fuera del trabajo. Me gustaría agradecer a Lorenzo Cecchi su disponibilidad para echar una mano siempre.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A mis niñas quiero agradecerle muchas cosas, sobre todo, por haber compartido un período tan importante como éste. Cada una aportando sus cosas; gracias a la profe, por su disposición infinita para todo y su falta de maldad, aparte de su increíble capacidad para organizar eventos; a Vanessa por ser clara y directa, por ser la peque no tan peque y por enseñarme lo que es el Butelo claro; a Cristina, que no como grelos desde que se fue, por su gran ayuda y apoyo; a Liberty por ser auténtica y por Antón; a Leganés, la última incorporada, por su cordura. Gracias al Yayo, el hombre del grupo y gallego adoptado como yo, siempre preparado tanto para ayudarte en lo que sea como para sacarte una sonrisa, o dos!

Además quería dar las gracias a todos los que han puesto su granito de arena en este trabajo (Tito Juan, Juanmi) y a los que han hecho de los muestreos una excursión llena de risas (Paíno, Rosi, Resu, Antonio, Fernando).

Gracias cugina mía, gracias por ayudarme a que esta tesis sea mucho más bonita.

Gracias a Alberto, sobre todo, por aguantarme en este último período, en este trabajo también hay un trocito de él.

Y gracias infinitamente a quien va dedicada esta tesis, por dármelo TODO...