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Role of plant-associated bacteria in the remediation of contaminated soils

Cristina Becerra Castro

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Dra. Petra S. Kidd e **Dra. Ángeles Prieto Fernández**, Científicos Titulares do Consejo Superior de Investigaciones Científicas, adscritas ao Instituto de Investigacións Agrobiolóxicas de Galicia e **Prof. Dr. Jaco Vangronsveld**, Profesor e Director do Centrum voor Milieukunde da Universidade de Hasselt (Bélxica)

INFORMAN:

Que a presente memoria titulada "***Role of plant-associated in the remediation of contaminated soils***" presentada por **Dna. Cristina Becerra Castro** para optar ao Grao de Doutora en Bioloxía, foi realizada baixo a nosa dirección.

E considerando que representa traballo de Tese de Doutoramento, autorizamos a súa presentación ante o Tribunal correspondente.

E para que así conste, asinamos o presente en Santiago de Compostela a 8 de Outubro de 2012.

Asdo.:	Asdo.:	Asdo.:
Dra. Petra S. Kidd	Dra. Ángeles Prieto Fernández	Prof. Jaco Vangronsveld

Dra. Mª del Carmen Monterroso Martínez, Profesora titular do Departamento de Edafoloxía e Química Agrícola da Universidade de Santiago de Compostela

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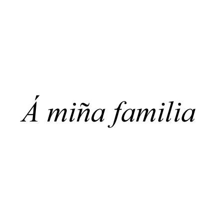
Asdo.: Dra. Mª del Carmen Monterroso Martínez



Á miña familia

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Resumo

Introdución

O solo considérase un recurso non renovable que actúa como plataforma da maioría das actividades humanas e que intervén en moitas funcións como son: a produción de biomasa e alimento; o almacenamento, filtrado e transformación de nutrientes e auga; o aloxamento da reserva da biodiversidade; o aporte de materias primas; a acumulación dunha reserva de carbono; e o almacenamento do patrimonio arqueolóxico e xeolóxico. A súa contaminación debido á presenza (en elevadas concentracións) dunha ou máis substancias químicas (normalmente de orixe antrópica) altera a súa funcionalidade e calidade, e incide con forza noutros aspectos medioambientais como son a calidade da auga, a seguridade alimentaria, a saúde humana e a biodiversidade. Os contaminantes edáficos divídense xeralmente entre contaminantes orgánicos (ex. pesticidas, PAHs, etc.) e inorgánicos (ex. metais pesados ou elementos traza).

O tratamento de solos contaminados pode clasificarse en técnicas de illamento ou en técnicas de descontaminación. As técnicas de illamento teñen como obxectivo a inmobilización do contaminante, mentres que as técnicas de descontaminación están enfocadas cara a recuperación ou a eliminación do mesmo. Estas técnicas poden basearse en procesos físicos (tratamentos térmicos, adsorción con carbono activo, construción de celas de illamento, bombeo de augas contaminadas, etc.), procesos químicos (degradación oxidativa ou redutiva, etc.) e procesos biolóxicos (como a biorremediación ou a fitocorrección). Sen embargo, non se pode falar dunha técnica de corrección universal. En cada caso é necesario un estudo de viabilidade tendo en conta o volume de solo a tratar, a profundidade da contaminación, as condicións ambientais, os obxectivos de recuperación, así como os custos (Mulligan *et al.*, 2001; Kaifer *et al.*, 2004).

En moitos casos as técnicas "convencionais" de recuperación son costosas (especialmente cando é preciso que o tratamento se faga *ex situ*), limítanse a áreas relativamente pequenas e resultan moi agresivas para o solo (destrución da estrutura, eliminación da coberta vexetal, aumento da erosión, etc.) (Cunningham *et al.*, 1995; Barceló e Poschenrieder, 2003). Debido a estas limitacións, nas

últimas dúas décadas os investigadores centráronse no desenvolvemento de "tecnoloxías verdes" de descontaminación que representasen alternativas viables fronte ás técnicas tradicionais máis centradas en métodos de enxeñaría. Un exemplo destas alternativas son aquelas técnicas englobadas dentro das denominadas tecnoloxías de fitocorrección, as cales aproveitan a capacidade natural que teñen as plantas para atenuar a contaminación dun solo (Chaney *et al.*, 1997; Salt *et al.*, 1998; Pilon-Smits, 2004; Kidd *et al.*, 2009). A fitocorrección de solos usa plantas (e os seus microorganismos asociados) para a eliminar, establizar ou detoxificar contaminantes. Dentro destas técnicas pódense distinguir a *fitoestabilización* (uso de plantas para reducir a mobilidade e/ou a toxicidade do contaminante no ambiente); a *fitoextracción* (uso de plantas que extraen os contaminantes do solo, concentrándoos na súa parte aérea); a *fitovolatilización* (uso de plantas para volatilizar contaminantes) e a *fitodegradación* (uso de plantas para degradar contaminantes orgánicos).

No últimos anos, demostrouse que a fitocorrección pode constituír unha alternativa viable para o tratamento de solo contaminados. Con todo, é necesario esclarecer os procesos involucrados, e tamén desenvolver estratexias para mellorar e optimizar estes procesos antes de que se poidan aplicar a gran escala. Existe un número crecente de estudos que indican que as asociacións planta-microorganismos xogan un importante papel nestas técnicas, e que polo tanto o seu aproveitamento podería conducir a un aumento da eficiencia do proceso de limpeza (Wenzel, 2009; Weyens *et al.*, 2009a). Os solos naturalmente ricos en metais, así como as áreas afectadas por contaminación de orixe antrópica considéranse ambientes idóneos para a busca e selección de novas e beneficiosas asociacións de plantas e microorganismos. Este tipo de zonas permite tanto a selección de especies e/ou ecotipos vexetais (Diez *et al.*, 2006; Abhilash *et al.*, 2008), como o illamento de cepas microbianas asociadas a plantas con características adecuadas para a súa aplicación en procesos de fitocorrección (Schippers *et al.*, 1995; Batty, 2005; Ryan *et al.*, 2008; Wenzel, 2009; Mengoni *et al.*, 2010). Nesta tese expoñerase unicamente traballo relativo ás interaccións beneficiosas entre plantas e bacterias, aínda que non hai que esquecer que tamén existen interaccións semellantes entre plantas e fungos que poden aplicarse en fitocorrección (Meharg e Cairney, 2000; Göhre e Paszkowski, 2006; Schulz, 2006; Lebeau *et al.*, 2008).

Sábese que as bacterias poden ser hóspedes comúns das plantas, tanto da súa superficie como do seu interior. De feito, na literatura as bacterias asociadas a

plantas adoitan clasificarse en función do hábitat que ocupan como bacterias endófitas, da filosfera ou da rizosfera (ou do rizoplano), según habiten o interior dos tecidos, a superficie das partes aéreas das plantas ou a interface solo:raíz, respectivamente (Gnanamanickam, 2007). No campo da fitocorrección o aproveitamento das relacións entre plantas e microorganismos pode enfocarse de dúas maneiras:

Por unha parte, as bacterias poden actúar sobre o estado xeral da planta, favorecendo o seu asentamento e crecemento en solos contaminados. Este tipo de bacterias denomínanse bacterias promotoras de crecemento vexetal, ou polas súas siglas en inglés, PGPB (*plant growth-promoting bacteria*) e poden afectar ao crecemento das plantas mediante distintos mecanismos. Así, as PGPB poden aumentar a dispoñibilidade de nutrientes vexetais esenciais como por exemplo mediante a fixación de nitróxeno atmosférico, a solubilización de compostos de fósforo non dispoñibles; ou a síntese de compostos quelantes de ferro (sideróforos) que poden facilitar a absorción de ferro por parte das plantas. Outros mecanismos que inflúen sobre o crecemento e estado fisiolóxico das plantas inclúen a produción de fitohormonoas (por exemplo, o ácido indoleacético, AIA), a redución dos niveis de estrés da planta mediante a produción do enzima ACC deaminasa e o seu papel como axentes de biocontrol (inhibindo ou reducindo os efectos de patóxenos vexetais) (Compant *et al.*, 2005; Lemanceau *et al.*, 2007; Podile e Kishore, 2007).

Por outra parte, pódese recorrer ao uso de cepas que actúan directamente sobre o contaminante diana. O exemplo máis evidente é o uso de cepas bacterianas capaces de degradar os contaminantes (no caso de compostos orgánicos). Pero as bacterias tamén poden modificar a biodispoñibilidade dos contaminantes, afectando deste xeito á súa descomposición, á súa absorción por parte da planta e/ou á súa toxicidade. Por exemplo, no caso dos contaminantes orgánicos a produción de biosurfactantes ou a formación de biofilmes asócianse cun aumento da dispoñibilidade dos mesmos. No caso dos elementos traza, a produción de ácidos orgánicos, de sideróforos ou de biosurfactantes relaciónanse frecuentemente cun aumento de dispoñibilidade, mentres que mecanismos como a biosorción ou a produción de compostos quelantes asócianse cunha inmobilización deste tipo de contaminantes (Weyens *et al.*, 2009a; 2009b). Estas características poden ser utilizadas en técnicas como a fitoextracción de metais e a fitodegradación de compostos orgánicos (no caso de aumento de dispoñibilidade) ou a fitoestabilización de metais (no caso de inmobilización).

Obxectivo e tarefas desenvolvidas

Dentro deste marco, o obxectivo global deste traballo de tese foi o establecemento dunha colección de cepas bacterianas asociadas con especies vexetais tolerantes que se atopan crecendo de forma espontánea en diferentes lugares contaminados, así como a avaliación dunha selección deses illados na súa aplicación en diferentes estratexias de fitocorrección. Co fin de acadar ese obxectivo, realizáronse unha serie de estudos en tres escenarios de contaminación diferentes.

A. Solos ultramáficos

Leváronse a cabo varios estudos con poboacións de diferentes subespecies de *Alyssum serpyllifolium* da Península Ibérica. Dúas destas subespecies (ssp. *lusitanicum* e ssp. *malacitanum*) son hiperacumuladoras de Ni e endémicas en solos ultramáficos, naturalmente ricos en metais pesados. Debido a súa extraordinaria capacidade de acumulación de metais, este tipo de plantas son de especial interese en técnicas de fitoextracción (Baker e Brooks, 1989; Chaney *et al.*, 1997).

Como primeira aproximación, levouse a cabo un estudo (capítulo 1) da comunidade microbiana cultivable do solo da rizosfera de 3 poboacións da subespecie *A. serpyllifolium* ssp. *lusitanicum*. Analizouse a resistencia a Co, Cr e Ni de bacterias heterótrofas, así como a abundancia de diversos grupos microbianos dos ciclos do C e N. A efectos de comparación tamén se analizaron mostras de solo non vexetado, así como mostras de solo rizosférico dunha especie exclusora de metais (*Dactylis glomerata*) tomadas nos mesmos solos ultramáficos. A continuación (capítulo 2), procedeuse ao illamento e caracterización de bacterias procedentes da rizosfera de dúas subespecies de *A. serpyllifolium* hiperacumuladoras de Ni, ssp. *lusitanicum* e ssp. *malacitanum*, e dunha subespecie non acumuladora de metais, ssp. *serpyllifolium*. Os illados caracterizáronse molecularmente mediante BOX-PCR (técnica de tipificación bacteriana que se usou para a análise da diversidade dos illados xa que permite discriminar diferentes cepas bacterianas) e secuenciación do ADNr 16S (que permitiu a súa identificación mediante o cotexo con secuencias depositadas en bases de datos públicas). Ademais, as cepas tamén se caracterizaron fenotípicamente analizando a súa resistencia a Ni, así como a presenza de características promotoras de crecemento e/ou que poidan modificar a dispoñibilidade de metais tales como a produción de sideróforos, de ácido indolacético (AIA), de biosurfactantes ou a

capacidade de solubilización P. Un número reducido desta colección de rizobacterias utilizouse para avaliar a súa capacidade de mobilizar Ni a partir dun solo ultramáfico. Para iso, as cepas seleccionadas crecéronse nun medio de cultivo líquido. Seguidamente, este medio utilizouse para realizar unha extracción de Ni dun solo ultramáfico e mediuse a concentración de Ni no extracto obtido.

Noutro estudo en maior profundidade sobre a mobilización bacteriana de metais, escolléronse dúas cepas (*Arthrobacter nitroguajacolicus* LA44 e *Arthrobacter oxydans* SBA82) da colección anterior (capítulo 3). Neste caso os obxectivos foron a avaliación da capacidade das cepas para mobilizar Ni (e outros elementos) dende unha rocha ultramáfica, así como a súa influencia sobre a dispoñibilidade e fraccionamento de metais no solo, en relación á absorción de Ni por parte de *A. serpyllifolium* ssp. *malacitanum*. Para iso, ambas cepas fixéronse crecer en contacto con rocha ultramáfica moída durante dúas semanas, período no que se tomaron alícuotas para medir a variación co tempo das concentracións de metais e ácidos orgánicos. Ao final do experimento tamén se realizou unha análise do fraccionamento de metais na rocha. Co fin de investigar a influencia destas rizobacterias sobre a absorción de Ni por parte de *A. serpyllifolium* ssp. *malacitanum*, realizouse un experimento no que se inocularon estas mesmas cepas en plantas de *A. serpyllifolium* ssp. *malacitanum* establecidas en solo ultramáfico. Ao final deste ensaio, mediuse a biomasa producida, así como o Ni absorbido por estas plantas. Así mesmo, determinouse a dispoñibilidade e o fraccionamento de metais no solo rizosférico das plantas.

B. Solo dunha mina de Pb/Zn

As poboacións vexetais, e os seus microorganismos asociados, que colonizan de forma natural solos derivados da actividade mineira, son boas candidatas para súa aplicación na rehabilitación ou na fitocorrección de solos contaminados con metais (Whiting *et al.*, 2004; Batty, 2005).

Neste caso levouse a cabo unha caracterización do sistema solo-planta-microorganismo de especies vexetais dominantes en solos dunha mina abandonada caracterizados por un elevado contido en Cd, Pb e Zn (Diez Lázaro, 2008). Os obxectivos deste estudo (capítulo 4) foron determinar a acumulación e/ou exclusión de Cd, Pb e Zn de tres especies pseudometalófitas (*Festuca rubra*, *Cytisus scoparius* e *Betula pendula*) que medran de forma natural nunha na mencionada mina e analizar a influencia desas plantas sobre a dispoñibilidade e fraccionmento de metais no solo da rizosfera. Outro obxectivo paralelo foi o

illamento e caracterización de rizobacterias metalo-tolerantes asociadas con estas especies, así como a avaliación da influencia dunha selección destas cepas sobre o crecemento de dúas especies vexetais (*Festuca pratensis* e *Salix viminalis*) comúnmente utilizadas en técnicas de fitocorrección. Para iso, recolléronse entre 5 e 7 individuos de cada especie vexetal, incluíndo en cada caso o solo do terrón. Determinouse a concentración de Cd, Pb e Zn nos tecidos vexetais, así como a súa dispoñibilidade e fraccionamento no solo rizosférico de cada especie. Ademais, as mostras de solo rizosférico utilizáronse para illar rizobacterias que se caracterizaron xenotípica e fenotípicamente de forma semellante á descrita previamente para o capítulo 2. Unha selección destas rizobacterias utilizouse para realizar un ensaio de promoción de crecemento de dúas especies vexetais (*Festuca pratensis* e *Salix viminalis*). Estas plantas creceron nunha mestura de area:perlita e inoculáronse coas diferentes rizobacterias. Ao final do experimento, determinouse a biomasa das plantas, así como o seu contido en nutrientes e metais e compararónse os resultados cos obtidos con plantas non inoculadas.

C. Solo contaminado con isómeros de hexaclorociclohexano (HCH)

Leváronse a cabo varios estudos coa especie *Cytisus striatus* que se atopa crecendo de forma espontánea nunha área contaminada con residuos da produción do pesticida organoclorado lindano (γ-HCH). Esta poboación de *C. striatus* foi proposta en estudos anteriores para a súa aplicación na fitocorrección de solos contaminados con HCH (Calvelo-Pereira *et al.*, 2006; Calvelo Pereira, 2008; Kidd *et al.*, 2008).

Ao igual que nos casos anteriores o primeiro obxectivo (capítulo 5) consistiu no illamento e caracterización de bacterias asociadas a plantas de *C. striatus* que crecen de forma natural nesa área contaminada. Neste caso illáronse bacterias endófitas e do rizoplano. Os illados bacterianos caracterizáronse tal e como se explicou anteriormente. Ademais tamén se buscou a presenza dos xenes bacterianos *linA* e/ou *linB*, que codifican enzimas envoltos na ruta de degradación aerobia de HCH.

Posteriormente ao establecemento e caracterización desta colección bacteriana, realizáronse dous experimentos de inoculación en maceta. O obxectivo destes ensaios foi avaliar os efectos da inoculación de *C. striatus* con cepas bacterianas sobre a degradación de HCH, así como sobre o crecemento e supervivencia das plantas en presenza de HCH. Nestes ensaios utilizáronse unha cepa endófita (*Rhodococcus erythropolis* ET54b, produtora de sideróforos e

biosurfactantes) e unha cepa degradadora de HCH (*Sphingomonas* sp. D4). No primeiro ensaio (capítulo 6) utilizouse un substrato de area:perlita contaminado de forma artificial con tres niveis de HCH (0, 10 e 35 mg kg^{-1}). As plantas inoculáronse coas dúas cepas bacterianas por separado (ET ou D4) e en combinación (ETD4). Como control utilizáronse plantas sen inocular. A continuación, realizouse un segundo ensaio (capítulo 7) co mesmo esquema de inoculación que no caso anterior, pero neste caso as plantas creceron en dous tipos de solos con diferente contido de materia orgánica e contaminados artificialmente con dous niveis de HCH (0 e 65 mg kg^{-1}). En ambos casos, ao final do experimento, determinouse a biomasa vexetal, a actividade de enzimas implicados na regulación do estrés na planta e as concentracións de HCH en planta e substrato.

Principais resultados e conclusións

Durante a realización deste traballo de tese, illáronse máis de 500 cepas bacterianas que se encontran almacenadas en glicerol a -70ºC. A continuación expoñeránse os resultados obtidos máis relevantes de acordo cos 3 escenarios de contaminación anteriormente descritos.

A. Solos ultramáficos

Os resultados obtidos cos experimentos levados a cabo con *A. serpyllifolium* revelaron que a subespecie hiperacumuladora de Ni *A. serpyllifolium* ssp. *lusitanicum* presenta unha maior dispoñibilidade de Ni na súa rizosfera, así como unha maior densidade de microorganismos metalo-tolerantes que a especie non acumuladora (*D. glomerata*).

Estableceuse unha colección de 346 rizobacterias das diferentes subespecies de *A. serpyllifolium*. Como datos máis relevantes sobre a súa caracterización pódese destacar que a produción de ácido indolacético (AIA) foi unha característica común entre todos os illados, mentres que a porcentaxe de illados capaces de producir sideróforos ou de solubilizar fósforo foi menor. A maioría dos illados capaces de modificar a extractabilidade de Ni foron identificados como membros do xénero *Arthrobacter*. Trece destes illados foron capaces de producir metabolitos que aumentaron a mobilización de Ni, sendo esta característica de especial relevancia para a súa aplicación en técnicas de fitoextracción. Por outra parte, 29 das cepas reduciron significativamente a cantidade de Ni extraído, o cal podería ser beneficioso en técnicas como a fitoestabilización cuxo obxectivo é a inmobilización do metal.

No segundo experimento de mobilización de metais observouse que as dúas cepas ensaiadas (LA44 e SBA82) foron capaces de liberar Ni (e outros elementos) a partir da rocha, aínda que os resultados parecen mostrar que o mecanismo co que actúan é diferente. Isto resulta especialmente interesante, tendo en conta que ambas cepas pertencen ao xénero *Arthrobacter*. A co-liberación de Al, Fe e Si observada no caso da cepa SBA82 suxire que se produce unha meteorización de silicatos ferromagnesianos e parece estar relacionada coa produción de sideróforos por parte desta cepa. Pola contra, no caso de LA44 non ocorre unha liberación deses elementos, pero obsérvase unha mobilización de Mn, Ni e Co, ademais dunha redución de elementos asociados á fracción de óxidos de Mn, o que parece indicar que LA44 está actuando sobre esta fracción. Ademais a análise de ácidos orgánicos levada a cabo indica que a produción de oxalato por parte desta cepa parece tomar parte no ataque aos óxidos de Mn. A inoculación de *A. serpyllifolium* ssp. *malacitanum* con estas mesmas cepas tivo un efecto positivo na cantidade de Ni extraído por parte desta especie, que foi ademais significativo no caso da cepa LA44.

B. Solo dunha mina de Pb/Zn

As tres especies pseudometalófitas estudadas, *Festuca rubra*, *Cytisus scoparius* e *Betula pendula* exclúen Cd, Pb e Zn da súa parte aérea, concentrando estes elementos nas súas raíces. Con todo, a especie *B. pendula* mostrou unha importante translocación de Zn das raíces cara a parte aérea (relación concentración de Zn en parte aérea / concentración de Zn en raíces de 0.8). No outro extremo, a especie *F. rubra* resultou ser unha especie cun gran potencial de exclusión de metal da parte aérea (relación concentración de metal en parte aérea / concentración de metal en raíces menor de 0.2 para os tres metais estudados). Obtívose unha colección de 74 illados rizobacterianos metalo-tolerantes, dos que se pode destacar a súa baixa diversidade, con só 6 xéneros representados (*Streptomyces*, *Tsukamurella*, *Pseudomonas*, *Massilia* e *Rhodococcus*). Ademais, a maioría das cepas mostraron moi poucas características promotoras do crecemento, sendo a produción de AIA a menos común (presente en só dúas cepas).

De entre estas cepas bacterianas, escolléronse 14 para avaliar a súa influencia sobre o crecemento das especies vexetais *Festuca pratensis* e *Salix viminalis*. No caso de *F. pratensis*, tódolos illados bacterianos salvo un (P54) tiveron un efecto positivo sobre a biomasa en comparación co control non

inoculado, e na maioría dos casos este efecto foi estatisticamente significativo. Pola contra, a maioría dos illados tiveron un efecto negativo sobre a biomasa de *S. viminalis*. Só 5 dos 14 illados ensaiados aumentaron a súa biomasa, aínda que este efecto só foi significativo no caso da cepa P87 (produtora de AIA). Polo tanto o efecto da inoculación bacteriana sobre o crecemento vexetal parece ser dependente da especie vexetal, ademais de que non sempre se pode relacionar coa presenza de características promotoras de crecemento na cepa.

C. Solo contaminado con isómeros de hexaclorociclohexano (HCH)

Na etapa de illamento e caracterización de cepas bacterianas asociadas con *C. striatus* obtívose unha colección de 146 illados con potencial en fitocorrección de solos contaminados con HCH. Nesta primeira etapa determinouse que a densidade e diversidade bacteriana foi mayor nas raíces (interior e rizoplano) que na parte aérea (follas e talos). Ademais, varios xéneros bacterianos estiveron representados tanto no interior das raíces como no rizoplano, mentras que na parte aérea detectáronse cepas de xéneros bacterianos que non tiveron representantes nas raíces e que sen encontran frecuentemente na filosfera de plantas, como por exemplo o xénero *Methylobacterium*. Isto apoia a existencia de dúas vías de entrada das bacterias ao interior das plantas, unha dende a rizosfera e o rizoplano, e outra a través da filosfera. A gran maioría dos illados bacterianos mostraron algunha característica promotora de crecemento vexetal, sendo as máis comúns a produción de AIA e de sideróforos. A produción de surfactantes, de especial interés dado o seu posible uso como modificador da dispoñibilidade de HCH, foi sen embargo a característica menos frecuente.

No primeiro ensaio de inoculación utilizando un substrato de perlita:area observouse que as plantas inoculadas coas cepas bacterianas (o endófito *R. erythropolis* ET54b ou o degradador *Sphingomonas* sp. D4) por separado sufriron unha redución en biomasa, tanto aérea como radicular, en tódolos niveis de HCH ensaiados (0, 10 e 35 mg kg^{-1}). Pola contra, cando ambas cepas se inocularon en conxunto (ETD4), a biomasa de *C. striatus* aumentou de forma significativa tanto en ausencia como en presenza de HCH. Sen embargo, ao final do experimento, non se observaron diferenzas entre as concentracións de HCH nos substrato dos diferentes tratamentos de inoculación.

No segundo ensaio de inoculación, realizado con solo contaminado, observouse que tanto o efecto do HCH como o efecto dos inóculos foi dependente do tipo de solo. Así as plantas que creceron sobre o solo B (con menor contido en

materia orgánica) mostraron de forma xeral un menor crecemento e un peor estado nutritivo, o cal levou a que o efecto fitotóxico do HCH resultase máis severo neste solo que no solo A (con maior riqueza en materia orgánica). Nestes dous solos, non se observou un aumento de biomasa cando se inocularon as dúas cepas bacterianas de forma conxunta (ETD4). Sen embargo, neste caso apreciouse un efecto da inoculación sobre a concentración de HCH no solo. Ao final do experimento, nas macetas con plantas inoculadas coa combinación das dúas cepas (ETD4) observouse unha maior diminución do HCH no solo que nas macetas non inoculadas ou inoculadas coas cepas por separado. No solo A este tratamento conseguiu reducir ata un 53 % o HCH presente no solo, e ademais esta redución foi significativamente maior que as reducións observadas nos outros tratamentos. Unha posible explicación pola cal o tratamento de inoculación con ambas cepas aumenta a disipación de HCH no solo podería estar relacionada coas características fenotípicas de ambas cepas. Ao igual que ocorre para outros contaminante orgánicos, a dispoñibilidade de HCH considérase un factor crítico que afecta a súa degradación no solo. A capacidade da cepa ET54b de producir biosurfactantes podería ter levado a un aumento da dispoñibilidade do HCH, e en consecuencia, facelo máis dispoñible para a súa degradación por parte da cepa degradadora D4. No solo B, o tratamento ETD4 tamén provocou unha importante redución de HCH (ata un 43%), aínda que tamén se observou un descenso semellante (37 %) no caso da inoculación coa cepa D4 en solitario. A degradación de HCH neste solo por parte da cepa D4 probablemente se encontrase menos limitada pola dispoñibilidade do HCH debido ao seu baixo contido en materia orgánica (e menor retención), o cal explicaría o feito de que en ambos tratamentos (ETD4 e D4) se observaron reducións de HCH en solo semellantes. Ambos ensaios (perlita e solo) permiten concluír que a inoculación da especie *C. striatus* coa combinación das dúas cepas parece ser unha aplicación moi prometedora no campo da fitocorrección de solos contaminados con HCH.

Finalmente, á vista dos resultados obtidos neste traballo de tese pódese concluír de forma xeral que:

- O illamento e caracterización de bacterias asociadas con plantas tolerantes que crecen de forma espontánea en áreas contaminadas é un procedemento necesario e válido para a identificación de cepas bacterianas, así como de asociacións planta-bacteria beneficiosas e de posible aplicación en técnicas de fitocorrección.

- As cepas rizobacterianas son capaces de producir metabolitos que influencian a extractabilidade de metales do solo, que modifican o fraccionamento metálico e /ou aumentan a meteorización de rochas ultramáficas.
- A inoculación bacteriana de plantas pode influír:
  - na supervivencia e crecemento das plantas, aínda que os efectos parecen ser dependentes da especie vexetal e non sempre se poden relacionar coa presenza de características promotoras de crecemento na cepa bacteriana.
  - na absorción e acumulación de metais por parte das plantas, e como consecuencia na eficiencia das técnicas de fitoextracción.
  - na disipación dos isómeros de HCH, e como consecuencia na fitocorrección de solos contaminados con HCH.

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Introduction

Soil contamination

Soil is considered as a non-renewable resource, performing vital functions in the biosphere and acting as a platform for the majority of human activities (biomass and food production, storage and cycling of nutrients, water regulation, sustaining biodiversity, C sequestration and pools, archiving archaeological and geological heritage, etc.). Soil contamination due to the presence (in elevated concentrations) of chemical substance(s) (normally of anthropogenic origin) alters soil quality and functions, and can negatively affect water quality, biodiversity, food security or human health. The European Union (EU), in its Thematic Strategy for Soil Protection, considers soil contamination as one of the principal causes of soil degradation (Commission of the European Communities, 2006). The most important sources of this contamination are industrial and commercial activities and the treatment and disposal of waste. Soil contaminants are generally divided into inorganic and organic substances, and the most frequent types are trace elements (37 %), mineral oil (34 %) and polycyclic aromatic hydrocarbons (13 %) (Figure 1)[a]. The European Environment Agency (EEA) estimates that the number of potentially contaminated sites in EU countries is close to 3 million, and 250,000 of these are in need of urgent clean-up (Figure 1). On a Spanish level, 15,126 sites have been identified as contaminated, and the total number of potentially contaminated sites is estimated in 33,595[b]. Current Spanish policy and legislation regulating contaminated soils have been developed within the *II Plan Nacional de Suelos Contaminados* (2007-2015) and under the Law 22/2011 of the 28th of July (BOE no. 181, 29 July 2011) and the Royal Decree 9/2005 of the 14th of January (BOE no. 15, 18 January 2005). The Royal Decree 9/2005 presents a regulatory framework to establish those industrial activities which may result in soil contamination, as well as the criteria to be adopted when declaring a soil as contaminated. It establishes the Generic Values of Reference (NGRs according to the Spanish spelling) of contaminants which are defined as the maximum con-

[a] EEA; http://www.eea.europa.eu/data-and-maps/figures/overview-of-contaminants-affecting-soil-and-groundwater-in-europe

[b] EEA; http://www.eea.europa.eu/data-and-maps/data/soil-contamination-1

centration of a soil contaminant which does not present a risk higher than the accepted level for human health or ecosystems. The regulation includes NGRs for sixty priority pollutants and establishes the methodology for setting these triggers. If the NGRs are exceeded, a site-specific risk assessment is required, and on the basis of these results the soil can be declared as contaminated and the required measures to be taken stipulated. The Royal Decree includes the NGRs for organic substances, but does not cover levels for trace elements. This responsibility is transferred to the regional governments, and NGR levels for metals are decided at a more local level. The reason being that metals are found naturally in soils and their background levels are strongly dependent on the geological substrate (whereas organic contaminants are principally introduced into the soil as a result of human activities). In Galicia, these levels are defined in the Soil Contamination Decree 60/2009 of the 6th of February (DOG no. 57, 24 March 2009). Although the regulation of organic substances in soil is, as explained above, established at a national level, it is worth noting that there is a Galician Decree specifically regulating soils affected by residues of hexachlorocyclohexane (Decree 263/1999 of the 30th of September; DOG no. 196, 8 October 1999).

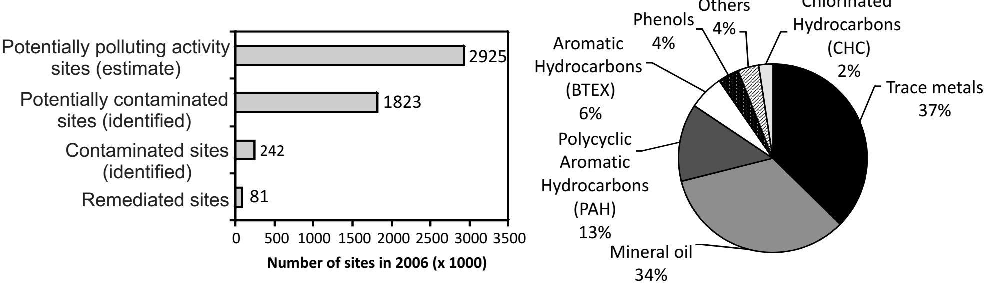

Figure 1. Contaminated sites in Europe and overview of the most frequent soil contaminants, 2006

Within the **inorganic contaminants** are the so-called heavy metals, trace elements or trace metals, terms which are frequently used synonymously in the literature (Adriano, 2001). Heavy metals have a density of > 5 g cm^{-3}, and trace elements are present in the lithosphere in concentrations of < 0.1 %. Metals occur in soils as a result of natural processes or as a result of human activities. Natural concentrations or background levels are strongly dependent on the soil parent material and acting weathering processes. For example, soils derived from ultramafic (serpentine) rocks are characteristically rich in Cr, Co and Ni (Calvo de Anta *et al.*, 1987a; Proctor and Nagy, 1992; Proctor, 1999; Box A). The main anthropogenic

sources of metal contamination include application of metal-based pesticides and metal-enriched sewage sludges in agriculture, combustion of fossil fuel, metallurgical industries and electronics, military training, etc. (Ross, 1994; Adriano, 2001) (Box A). Although some trace elements are required by organisms at low concentrations (essential metals as Cu, Fe, Mn, Zn and Ni), problems of pollution or toxicity are encountered when they are present in concentrations exceeding those normally found in nature (Adriano, 2001). Phytotoxicity of trace metals is due to the formation of free radicals, which can lead to oxidative stress or the substitution of other metals or binding to functional groups in enzymes, pigments or structural proteins, leading to metabolic disruptions (Sharma and Dietz, 2009; Cuypers *et al.*, 2010). Trace metals are also known to affect soil microbial communities, and can lead to reductions in microbial density and biomass, and shifts in community structure, which in turn, affects C and N mineralization, soil enzyme activities and litter decomposition (Chander and Brookes, 1991; Brookes, 1995). Unlike organic pollutants, metals do not biodegrade and their residence time in the soil is prolonged (thousands of years) (Adriano, 2001; Vassilev *et al.*, 2004). Thus, their incorporation into the food chain can cause serious problems to both animals and humans.

The **organic contaminants** are carbon-containing compounds which can be potentially harmful to the environment, animal or human health and/or resistant to degradation and in many cases include compounds synthesised by man (xenobiotic compounds). Today more than 100,000 different organic chemicals are distributed on the European market[c]. The main classes of organic contaminants are polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs), volatile organic contaminants (VOCs) and petroleum hydrocarbons (Swartjes, 2011), although there is some overlap between these categories. *Polycyclic aromatic hydrocarbons* are compounds characterised by two or more fused aromatic rings. Common PAHs in soils included naphthalene, phenanthrene, anthracene, fluoranthene, benzo(a)anthracene, benzo(k)fluoranthene, indeno(1,2,3-cd)pyrene, benzo(g,h,i) perylene and benzo(a)pyrene. The majority of PAHs released into the environment are due to anthropogenic activities such as the processing or burning of fossil fuels and wood treatment, although there are also some natural sources such as forest fires and volcanic eruptions (Swartjes, 2011). The category of *persistent organic pollutants* includes organic contaminants that are resistant to degradation, and present both a long persistence time in the environment and high toxicity to both hu-

[c] European Chemical Substances Information System (ESIS); http://ecb.jrc.it

BOX A. Metal-rich environments

Naturally metal-rich soils and their associated flora: serpentine soils

Serpentine soils, or soils derived from ultramafic rocks, are considered as environments naturally rich in metals. The term ultramafic refers to igneous or metamorphic rocks containing more than 70 % of ferromagnesium minerals and a low content in silicon (< 45 % SiO_2) (Brooks 1987). Ultramafic rocks are widely distributed throughout the world, representing around 13 % of geological substrates (Menezes de Sequeira and Pinto da Silva, 1992) and occupying approximately 1 % of the earth's surface area (Proctor, 1999). The soils developing on these rocks can present contrasting characteristics, but generally show some common traits which are particular to this soil group and are often cited as the cause of a limited vegetation cover: (a) elevated concentrations of Mg and Fe, and a low availability of Ca relative to Mg (unfavourable for Ca absorption); (b) deficiency in essential nutrients such as N, P and K; and (c) high concentrations of potentially phytotoxic trace metals such as Ni, Co and Cr. In addition, these soils are often skeletal, with a low organic matter content and water holding capacity (Brooks, 1987; Roberts and Proctor, 1992; Macías and Calvo de Anta, 2001). Serpentine soils can be stressful environments for plant growth and these limiting factors are often referred to as the *serpentine syndrome* or *serpentine factor*. As a result the plant communities in these areas often present a high number of endemic species, and have evolved both morphological and physiological adaptations differentiating them from the flora of adjacent geological substrates (Whittaker, 1954; Proctor, 1971; Brady *et al.*, 2005). Nickel is believed to play a major role in determining the flora and vegetation in many serpentine areas because of its relatively high availability in serpentine soils and the discovery of a high number of taxa that accumulate Ni in their tissues (Brooks *et al.*, 1977; 1987; Proctor and Nagy, 1992; Diez *et al.*, 2006). Plant species such as these are known as hyperaccumulators and are able to accumulate extraordinarily high concentrations of metals in their aerial biomass. The distribution of Ni hyperaccumulators (especially from the genus *Alyssum* L.) is closely linked to the distribution of ultramafic substrates (Brooks and Radford, 1978; Brooks et al., 1979; Baker and Brooks, 1989).

There are three important areas of serpentine soils in the Iberian Peninsula (Figure A.1): (1), Trás-os-Montes in NE Portugal; (2), Melide in NW Spain (A Coruña); and (3) Sierra Bermeja in S Spain (Málaga). The serpentine outcrop of Trás-os-Montes (near Bragança and Vinhais) (Ferreira, 1965; Menezes de Sequeira, 1969; Alves *et al.*, 2011) hosts a Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf. (Brassicaceae), *A. serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (often re-

ferred to as *A. pintodasilvae*) (Pinto da Silva, 1970; Freitas *et al.*, 2004; Diez *et al.*, 2006). The ultramafic rocks of NW Spain form part of the Capelada-Serra do Careón geological complex (Guitián Ojea and López, 1980; Calvo de Anta *et al.*, 1987a; 1987b; 1987c; Calvo de Anta and Tovar Caballero, 1987). In the locality of Melide, the

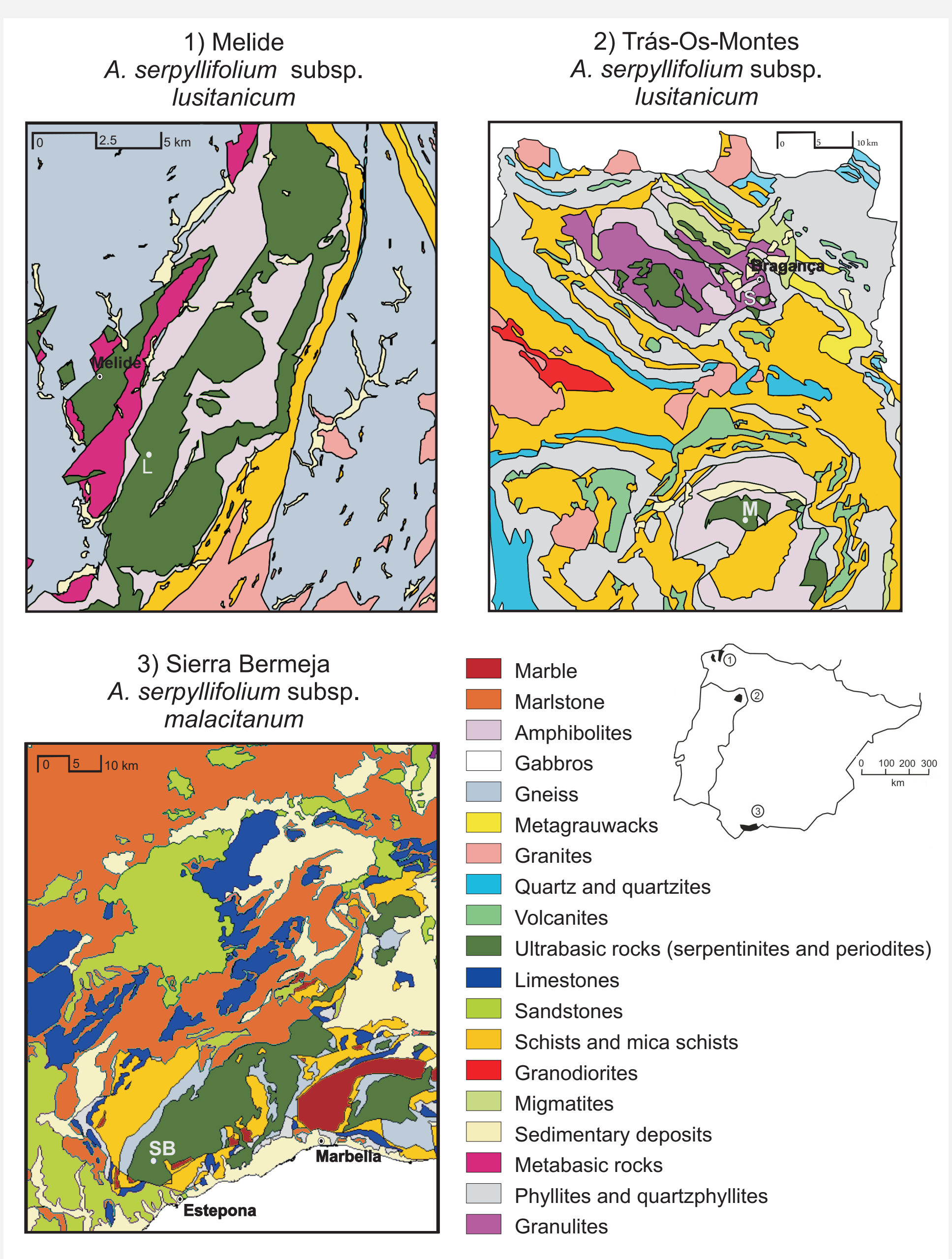

Figure A.1. Map of serpentine outcrops in the Iberian Peninsula showing the geological characteristics of each region and the location of the sampling points.

same Ni hyperaccumulating subspecies as in Portugal can be found, *A. pintodasilvae*. This population 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 *A. guitianae* Brooks (Rodríguez-Oubiña and Ortiz, 1991). The third serpentine area is located in S Spain in Sierra Bermeja (van der Wal and Vissers, 1996; Aguilar Ruiz *et al.*, 1998). In this region, a second Ni-hyperaccumulating subspecies is found, *A. serpyllifolium* subsp. *malacitanum* Rivas Goday (often referred to as *A. malacitanum*) (López González, 1975).

Mine spoils and tailings

Metal mining activities are known to be among the principal causes of soil contamination by heavy metals. Processes involved in the extraction and beneficiation of metals generate large quantities of waste and unwanted byproducts (EPA, 2004). Extraction refers to the removal of metals from concentrated mineral deposits in solid rock, while beneficiation concentrates the metal in the ore by removing unwanted constituents. Mine tailings are the primary component of mine wastes and are considered a major source of pollution (Mendez and Maier, 2008). The average per capita metal consumption has risen from 77 kg in 1950 to 213 kg in 2008 (Haferburg and Kothe, 2010). At 1.4 billion tons, the output of global metal production in 2008 was more than seven times higher than it was in 1950. Worldwide mining operations occupy an expanse of approximately 37,000 km^2 (about 0.2 % of the world's land surface) (Dudka and Adriano, 1997). In addition, approximately 240,000 km^2 is influenced by metals released from waste dumps and open mines (Furrer *et al.*, 2002). Mine tailings and mine spoils are characterized by unfavourable conditions for plant growth, primarily due to an elevated concentration of metals such as As, Cd, Cu, Mn, Pb and Zn (Mendez and Maier, 2008). Additional growth limiting factors include a low nutrient availability, low organic matter content, poor soil structure, absence of topsoil and often high acidity (Monterroso and Macías Vázquez, 1998; Wong, 2003). Soil degradation is one of the most evident impacts of mining activities, but air and water pollution are also of environmental concern. The main air quality issue is the dust produced by the working of open pits and by crushing and grinding operations, and wind erosion from bare or sparsely vegetated sites (UNEP, 2000). Water pollution arises mainly through drainage from surface and underground mines, wastewater from beneficiation, and surface run-off. Of particular concern is acid mine drainage (AMD), acid drainage occurs naturally when mining activities expose sulfide minerals to weathering and they react with water and oxygen. The combination of acids and metals can have severe effects on the ecology of local watercourses. Acid mine water can occur while the mine is operating and even long after its closure, unless specific measures are taken (Calvo de Anta and Otero, 1994; Monterroso and Macías

Vázquez, 1998). Furthermore, in countries with a long mining history, the magnitude of the environmental impacts of abandoned mine sites is often considerable. In Spain, mining activities are mainly dedicated to the production of industrial and non-metallic rocks and minerals (Table A.1). In the early years of this century metal mining suffered a steep decline from a production valued at over 200 million € in 2000 to 130 million € in 2005 and 45 million € in 2008[c]. However, in recent years there has been a renewed interest in metal mining, which reached a value of close to 400 million € in 2010 (Table A.1). This increase is mainly due to the revaluation of metals (such as Cu) in the market, and previously thought uneconomical mines and mining deposits have been reopened.

Table A.1. Performance of mining production (thousand €)[d]

	2005	2006	2007	2008	2009	2010
Energy products	663 704	657 203	657 205	574 603	543 150	521 478
Metallic minerals	128 982	178 052	147 676	45 485	179 517	396 538
Industrial rocks and minerals	2 547 246	2 814 465	2 979 066	2 859 028	2 339 662	2 064 437
Ornamental rocks	632 530	709 644	681 451	635 691	487 251	443 867

Despite the unfavourable conditions for plant growth characteristic to mine tailings and spoils, plant metallophytes have evolved biological mechanisms permitting them to resist and tolerate toxic concentrations of metals, and colonise this type of sub-strate (Whiting et al., 2004; Batty, 2005). Many studies have focused on these plants due to their potential use in the rehabilitation of metal-contaminated land or, more recently, due to their possible application in phytoremediation (Batty, 2005).

mans and wildlife. Many POPs are pesticides which have been banned in many countries, but are still found in soils. Examples of substances classified as POPs according to the Stockholm Convention are 1,2,3,4,5,6-hexachlorocyclohexane (α- and β- isomers as well as γ-isomer, also known as lindane), aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, polychlorinated biphenyls (PCBs), as well as some PAHs. *Volatile organic contaminants* are compounds generally characterised by high enough vapour pressures under normal conditions to significantly vaporize. These contaminants are typically generated from metal degreasing, gasoline, and wood preserving processes. Examples of VOCs include

[d] IGME; http://www.igme.es/internet/PanoramaMinero/PMLin.htm

trichloroethylene, tetrachloroethylene, trichloroethane and BTEX compounds (benzene, toluene, ethylbenzene, and xylenes). *Petroleum hydrocarbons* (often called total petroleum hydrocarbons or TPH) are actually complex mixtures of a whole spectrum of contaminants, mainly hydrocarbons, and additives such as benzene, toluene, xylenes, naphthalene, and fluorene (Swartjes, 2011).

Remediation of contaminated soils. Phytoremediation

At present numerous techniques exist for the remediation of contaminated soils; these can be classified into two groups which are either aimed at contaminant containment or actual decontamination. Containment techniques immobilise contaminants in the substrate, while decontamination techniques aim to eliminate or remove the contaminants from the soil. These treatments can be based on physical processes (thermal treatments, adsorption with active carbon, construction of containment cells, pumping of contaminated waters), chemical processes (oxidative or reductive degradation, chemical binding), or biological processes (such as bioremediation or phytoremediation) (Mulligan *et al.*, 2001a; Kaifer *et al.*, 2004). There is no universal technique for the remediation or recovery of contaminated soils and each case is site-specific. Before implementing any technique, viability studies should be carried out, taking into account the contaminant type and concentration, the volume of soil to be treated, environmental and social factors, the targeted end-point land use and treatment costs (Mulligan *et al.*, 2001a).

In many cases conventional remediation techniques incur high costs (especially when they involve *ex situ* treatment), they are often only applicable to relatively small areas and are frequently aggressive (destroying soil structure and functions, removing plant cover, increasing risks of erosion, etc.) (Cunningham *et al.*, 1995; Barceló and Poschenrieder, 2003). These limitations have led researchers over the last two decades to develop gentle remediation options or "green techniques" which offer viable alternatives to traditional, civil-engineering methods. One alternative are those techniques collectively grouped under the term *phytoremediation* or *phytotechnologies*, and which are based on exploiting the plants natural capacity to attenuate soil contaminants. Phytoremediation of soils use plants and their associated microorganisms to remove, stabilise or detoxify pollutants (Chaney *et al.*, 1997; Salt *et al.*, 1998; Pilon-Smits, 2004; Kidd *et al.*, 2009). This term includes various techniques, such as (Figure 2):

- *Phytostabilisation* uses tolerant plant species to establish a vegetation cover and thus, stabilize and/or reduce the availability of pollutants in soil

(Vangronsveld *et al.*, 1995; Vangronsveld and Cunningham, 1998; Mench *et al.*, 2006; Ruttens *et al.*, 2006; Dary *et al.*, 2010). The use of single or combined amendments can be first incorporated into the soil to decrease the bioavailability and phytotoxicity of the pollutant, and improve plant establishment. This combined use of amendments and plants is sometimes referred as *aided phytostabilisation* (Mench *et al.*, 2005).

- *Phytoextraction* uses plants that take up contaminants from the soil and accumulate them in their aboveground biomass (Kumar *et al.*, 1995; Dickinson and Pulford, 2005; Kidd and Monterroso, 2005; Koopmans *et al.*, 2008). This technique is mainly used for metal contaminants. When phytoextraction generates revenue by recovering marketable trace metals (such as Ni, Au, etc.) from the plant biomass (bio-ores) it is known as *phytomining* (Robinson *et al.*, 1997; Anderson *et al.*, 1999; Chaney *et al.*, 2007; Piccinin *et al.*, 2007). Within phytoextraction is the so-called technique of *induced phytoextraction* (Salt *et al.*, 1998; Lombi *et al.*, 2001) which employs synthetic metal chelators such as EDTA or natural chelators such as citric acid to increase contaminant bioavailability and plant uptake and absorption (Puschenreiter *et al.*, 2001; Evangelou *et al.*, 2004; Meers *et al.*, 2004; Turgut *et al.*, 2004).
- *Phytovolatilisation* exploits the ability of plants to transform pollutants into volatile compounds or to absorb and transport volatile compounds from the soil to the aboveground biomass where they can then be released into the atmosphere (de Souza *et al.*, 1999; McGrath *et al.*, 2002). The term *phytovolatilisation* is generally used when the contaminant is released into the atmosphere after passing through the interior of the plant. When the contaminant is transformed and released directly from the soil surrounding plant roots (rhizosphere), it is usually preferable to use the term *rhizovolatilisation*.
- *Phytodegradation* uses plants (and their associated microorganisms) to degrade organic contaminants (Barac *et al.*, 2004; Calvelo Pereira *et al.*, 2008; Kidd *et al.*, 2008; Weyens *et al.*, 2009a). The term *phytodegradation* or *phytotransformation* is usually used when the degradation of the contaminant occurs *in planta*. In contrast, when the degradation takes place in the rhizosphere of plants, either due to microbial activity or to the release of enzymes from plants, terms such as *phytostimulation* or *rhizodegradation* are more correct (Schwitzguébel *et al.*, 2006; McGuiness and Dowling, 2009).

Phytoremediation is considered cost-effective, incurring costs which are estimated to be from 10 to 1000 times lower than engineering-based remediation

methods such as soil excavation, soil washing or burning, or pump-and-treat systems (Cunningham *et al.*, 1995). Due to the *in situ* nature of these techniques the necessity for soil manipulation is greatly reduced, and they can potentially lead to improvements in soil quality (restoring structure and functions, increasing nutrient content, organic matter, etc.). Moreover, they are generally easily implemented requiring normal agricultural machinery, environmentally-friendly and socially acceptable. Another positive aspect is the possibility of combining these techniques with other methods, such as the use of amendments to aid plant establishment or the excavation of only the most contaminated spots (Salt *et al.*, 1998; Pilon-Smits, 2004; Kidd *et al.*, 2009).

Phytoremediation techniques do of course present a series of limitations, and still require optimisation before they can become fully implemented on a wide -scale. In addition to the inherent problems associated with any agronomical practice (such as the dependence on climate and season, outbreaks of pests or disease, etc.), a major problem associated with these techniques is the length of *time required* for the clean-up process. Time is of particular concern in processes such as phytoextraction, and several authors have proposed that to be realistically viable the necessary clean-up time should preferably not exceed 10 years (Robinson *et al.*, 1998; Blaylock and Huang, 2000). In cases where an immediate clean-up is required phytoremediation may not be the most appropriate strategy. Nonetheless, the bulk of research carried out to date agrees that these are promising techniques for application in large areas with a diffuse or superficial contamination (Wenzel *et al.*, 1999). The *establishment and growth* of plants on contaminated sites is another major obstacle in the application of any phytoremediation strategy (Chaney *et al.*, 1997; Wenzel *et al.*, 1999; Tordoff *et al.*, 2000). Contaminated sites not only present phytotoxic concentrations of pollutants, but are also typically characterised by edaphic conditions which can severely limit plant growth (nutrient deficiency, poor soil structure, low organic matter, etc.). The careful selection of tolerant plant species which are able to establish themselves in this type of environment will therefore be vital (Batty, 2005; Verkleij *et al.*, 2009). The volume of soil that can be treated is limited by the extent of *root proliferation and depth*. The use of deep-rooting plants, mycorrhizal plants or more novel biotechnological methods enhancing plant growth could also improve phytoremediation efficicency (Kidd *et al.*, 2009). Another critical factor is contaminant *bioavailability*, this can be a key element in techniques such as phytoextraction or phytodegradation (Schnoor *et al.*, 1995; Wenzel, 2009). Here bioavailability can be defined as the fraction of the contaminant that can be taken

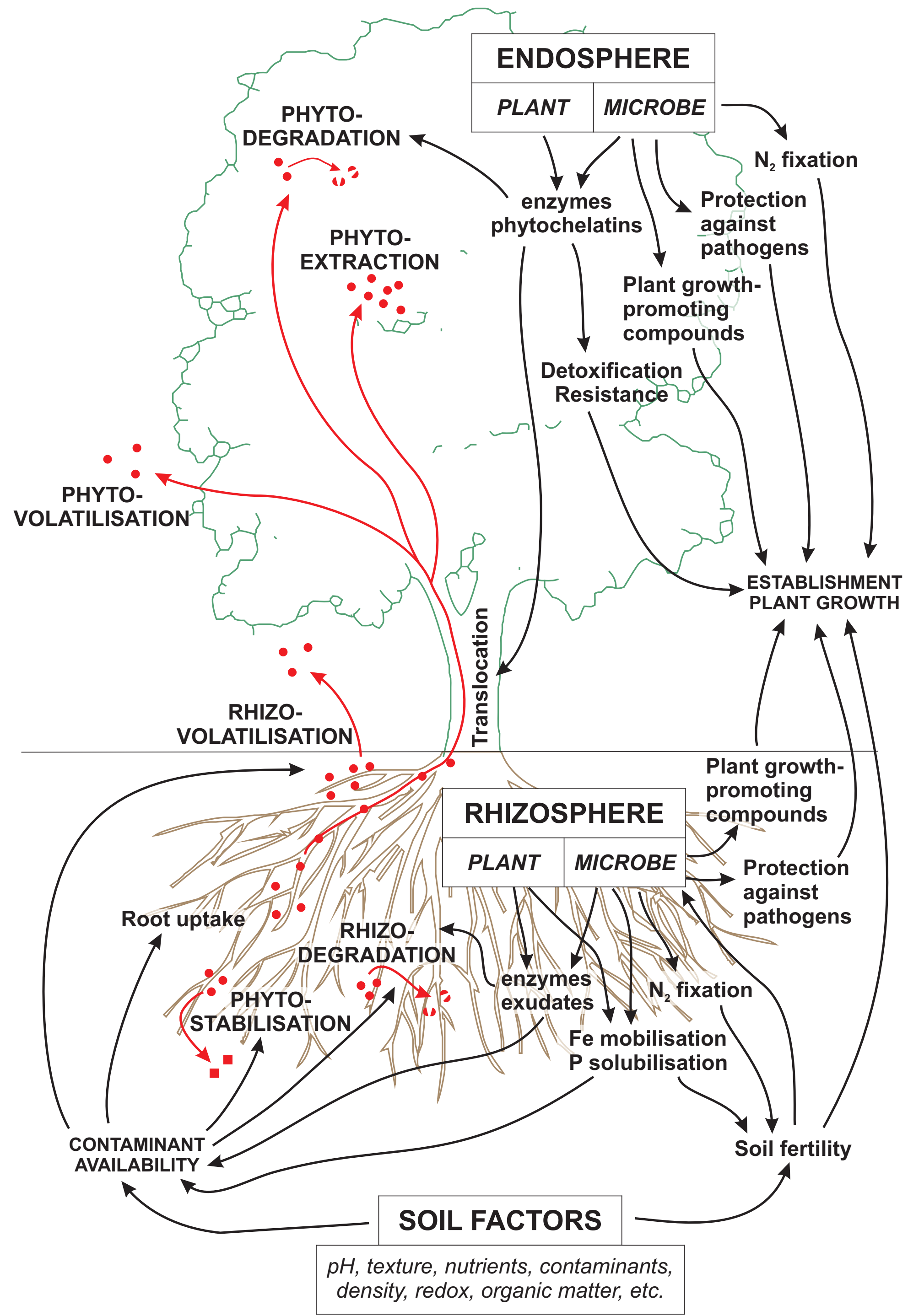

Figure 2. Main phytoremediation processes and simplified overview on plant-microbe-soil interactions involved in phytoremediation (modified from Wenzel *et al.* (1999) and Pilon-Smits (2004)).

up or transformed by living organisms (Semple *et al.*, 2003). Contaminant bioavailability is largely dependant upon the nature of the compound, its physical and chemical properties (solubility, charge, hydrophobicity, diffusion and mass transport, etc.) but is also affected to a great extent by the dominating edaphic properties (content and type of organic matter and clays, pH, redox potential, cation exchange capacity, etc.) (Kidd *et al.*, 2009; Wenzel, 2009). Furthermore, the sensitivity and characteristics of organisms exposed to the contaminant will also influence their bioavailability (Reid *et al.*, 2000). Therefore, the development of methods for modifying contaminant bioavailability could have a positive impact on phytoremediation efficiency. Increasing contaminant bioavailability would improve processes such as phytoextraction or phytodegradation, whereas the use of amendments which reduce contaminant bioavailability could substantially improve phytostabilisation (Wenzel, 2009).

In any case, phytoremediation processes are governed by the interactions between its three key players: soil, plants and microorganisms (Figure 2). The last few years have seen a growing interest in the influence of microorganisms on plant growth and contaminant bioavailability and degradation, and more and more studies are focusing on the role of plant-associated microorganisms in improving phytoremediation efficiency and success (Wenzel *et al.*, 1999; Kidd *et al.*, 2009; Weyens *et al.*, 2009b).

Selection of useful plant species for phytoremediation processes

As mentioned above, when implementing phytoremediation techniques a careful selection of tolerant plant species is vital. Screenings of plant species in contaminated areas can enable the selection of appropriate candidates for different contaminant scenarios (Diez *et al.*, 2006; Abhilash *et al.*, 2008). Within the same plant species different ecotypes, cultivars, varieties or clones can vary greatly in their response to the presence of contaminants. While tolerance to the contaminant in question is vital in all techniques, at other times the selected plant will depend on the remediation option to be used e.g. metal-accumulating plants (phytoextraction, phytomining) or metal-excluding plants (phytostabilisation).

Metalliferous soils, both natural and from anthropogenic origin (Box A), host metal-tolerant plant species of useful application in phytoremediation (Whiting *et al.*, 2004; Batty, 2005). Baker (1981) classified plants into three groups according to their response to metals (Figure 3). *Indicator* plants present

shoot metal concentrations which reflect those in the soil. *Excluder* species are able to restrict the entry of metals into the root and/or their transport to the shoots. This trait is of special interest for revegetating metal-contaminated soils or phytostabilisation processes. Metallicolous populations of metal-excluding grasses, such as *Agrostis capillaris*, *Agrostis gigantea* and *Festuca rubra*, have shown a good ability to colonize Pb-, Zn- and Cu-contaminated soils and have

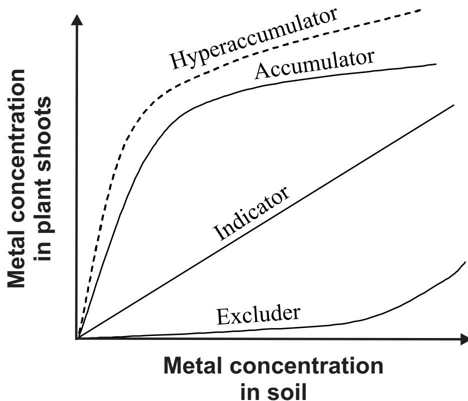

Figure 3. Typical plant responses to increasing soil metal concentrations (Baker, 1981).

been successfully applied in phytostabilisation (Mench *et al.*, 2010). In contrast, *accumulators* actively take up and translocate metals from soils to their shoots. This plant group is of special interest in techniques such as phytoextraction. The use of high-biomass, fast-growing trees such as *Salix* or *Populus* has been recommended for phytoextraction techniques since many clones show reasonable metal accumulation (Pulford and Watson, 2003; Lewandowski *et al.*, 2006). Within the accumulators are those denominated as the *hyperaccumulators* (Brooks *et al.*, 1977), which accumulate extreme amounts of trace metals in their aboveground biomass when growing in metal-enriched habitats. Hyperaccumulators usually present metal concentrations in their shoot biomass between 100 and 1000 times higher than non-accumulating plants (Adriano, 2001; Lasat, 2002). The proposed metal concentration thresholds for hyperaccumulation are 100 mg kg^{-1} (> 0.01 %) for Cd; 1000 mg kg^{-1} (> 0.1 %) for Co, Cu, Cr, Ni and Pb; and 10000 mg kg^{-1} (>1 %) for Mn and Zn (Baker and Brooks, 1989). Approximately 500 plant taxa are known to hyperaccumulate at least one metal according to these criteria, the great majority being hyperaccumulators of Ni from outcrops of serpentine minerals (Box A; Table1). Recently, new thresholds values were proposed for the following elements: 300 mg kg^{-1} (> 0.03 %) for Co; Cr and Cu; and 3000 mg kg^{-1} (> 0.3 %)

Table 1. Hyperaccumulation of trace elements in plants (Krämer, 2010; van der Ent *et al.*, 2012)

Element	Critical deficiency level (mg kg^{-1})	Common levels (mg kg^{-1})	Critical toxicity level (mg kg^{-1})	Hyperaccumlation concentration criterion (mg kg^{-1})		Taxa (No.)
				to date	newly suggested	
Arsenic	n. r.	0.1	<2-80	>1000		15
Cadmium	n. r.	0.05	6-10	>100		5
Cobalt	n. r.	0.2	0.4-several	>1000	> 300	26
Copper	1-5	10	20-30	>1000	> 300	35
Chromium	n. r.	1.5		>1000	> 300	4
Lead	n. r.	1	0.6-28	>1000		14
Manganese	10-20	200	200-3500	>10000		10
Nickel	0.002-0.004	1.5	10-50	>1000		390
Zinc	15-20	50	100-300	>10000	> 3000	15

n. r., no known requirement

for Zn (Krämer, 2010; Van der Ent *et al.*, 2012) (Table1). The application of hyperaccumulating species in phytoremediation (phytoextraction or phytomining) is based on this extraordinary ability to tolerate and accumulate metals in harvestable tissues. Unfortunately, many of these species present a reduced biomass production which can be a serious drawback for phytoremediation (Baker and Brooks, 1989; Chaney *et al.*, 1997; Raskin *et al.*, 1997; Robinson *et al.*, 1997; Anderson *et al.*, 1999; Pilon-Smits, 2004).

Soils contaminated with **organic compounds** can also be a source of tolerant populations of plant species (Madejón *et al.*, 2002; Kaimi *et al.*, 2007; Abhilash *et al.*, 2008). In the case of phytoremediation of organic pollutants, the bioavailability and toxicity of the compound is of extreme importance (Chaudhry *et al.*, 2005; Wenzel, 2009). For instance, hydrophobic compounds are retained by the lipids in the root epidermis and the organic matter surrounding the root, limiting root absorption of the contaminant (Schröder and Collins, 2002). However, several studies have shown that some plants are capable of taking up significant amounts of hydrophobic contaminants, such as dichlorodiphenyldichloroethylene (DDE), and accumulating them in their tissues despite their high hydrophobicity (Chhikara *et al.*, 2010; White, 2010), probably through the exudation of organic acids that increase their availability (White and Kottler, 2002; White *et al.*, 2003). In addition, plants may also release enzymes that degrade organic contaminants (Wenzel *et al.*, 1999) or alternatively, they may stimulate the activity of rhizosphere microorganisms (phytostimulation). Plants often produce

and exude natural chemicals whose structure is close to that of xenobiotic compounds (Siciliano and Germida, 1998). Exudation of phenolic compounds or other secondary metabolites, such as terpenes, by plants has been shown to support or even to stimule the growth of PCB- and PAH-degrading microorganisms in the rhizosphere (Donnelly *et al.*, 1994; Hedge and Fletcher, 1996; Singer *et al.*, 2003).

Plant-associated bacteria: useful bacterial strains for application in phytoremediation

Interactions between plants and their associated bacteria have been studied for many years, and both beneficial and pathogenic effects to their host have been described (Gnanamanickam, 2007). Rhizobia are an example of a highly evolved (and well-studied) mutualistic plant-bacterium interaction (Van Rhijn and Vanderleyden, 1995; Krishnan and Bennett, 2007). Plants offer a wide range of habitats that support microbial growth. In fact, plant-associated bacteria are usually classified on the basis of the habitats they occupy as endophytic, phyllosphere and rhizosphere bacteria. Endophytic bacteria colonize the internal tissues of the plants. Phyllosphere bacteria live on the surface of above-ground parts of the plants. And rhizosphere (or rhizoplane) bacteria are inhabitants of the root:soil interface (Box B). Microbes profit from plants because of the enhanced availability of nutrients, whereas plants can receive benefits from some bacterial associates by growth enhancement or stress reduction (Vessey, 2003; Hardoim *et al.*, 2008; Weyens *et al.*, 2009b; 2009c; Compant *et al.*, 2010). Exploiting the plant-microbial partnerships in phytoremediation is generally based on the capacity of the bacteria to, on one hand, improve establishment, growth and plant survival (plant-growth promotion); and, on the other hand, to act directly on the contaminant (Figure 2). Contaminated sites are not only a source of interesting plant species for application in phytoremediation but also of microorganisms (Schippers *et al.*, 1995; Batty, 2005; Ryan *et al.*, 2008; Mengoni *et al.*, 2010). Numerous studies have focused on the isolation and characterisation of cultivable microorganisms from this type of substrate (Schippers *et al.*, 1995; Willumsen and Karlson, 1997; Siciliano and Germida, 1999; Manonmani *et al.*, 2000; Lodewyckx *et al.*, 2002a; Abou-Shanab *et al.*, 2003b; Hanbo *et al.*, 2004; Idris *et al.*, 2004; Pal *et al.*, 2004; Porteous-Moore *et al.*, 2006; Barzanti *et al.*, 2007; Grandlic *et al.*, 2008; Weyens *et al.*, 2009d).

This introduction will only discuss the beneficial interactions between plants and bacteria and their potential application in phytoremediation, although it

Box B. Plants as a bacterial habitat

Rhizosphere- The *rhizosphere* is defined as the volume of soil surrounding living plant roots that is influenced by root activity (Hiltner, 1904; Darrah, 1993). More precisely, the soil layer surrounding roots is given the term *ectorhizosphere*, whereas the root layer colonised or potentially colonisable by microorganisms is referred to as the *endorhizosphere*. The two areas are separated by the root surface or *rhizoplane*. The term rhizosphere is commonly used when referring to the ectorhizosphere, as it will be used throughout this thesis. In many studies, the rhizosphere is operationally defined as the soil adhering to the roots (Hinsinger, 1998). Its volume can vary both spatially and temporally according to the plant species and the soil physicochemical properties (Hinsinger *et al.*, 2005). The importance of the rhizosphere stems from the unique characteristics differentiating it from the remaining soil (or *bulk soil*). As a result of root growth and activity soil physical, chemical, biochemical and biological properties are modified (Figure B.1). Root growth leads to physical modifications in the rhizosphere soil aggregate stability and structure (Morel *et al.*, 1991). As a result of root activity, absorption, respiration and exudation many chemical properties are altered compared to bulk soil. Root uptake of water and nutrients has been shown to cause gradients in

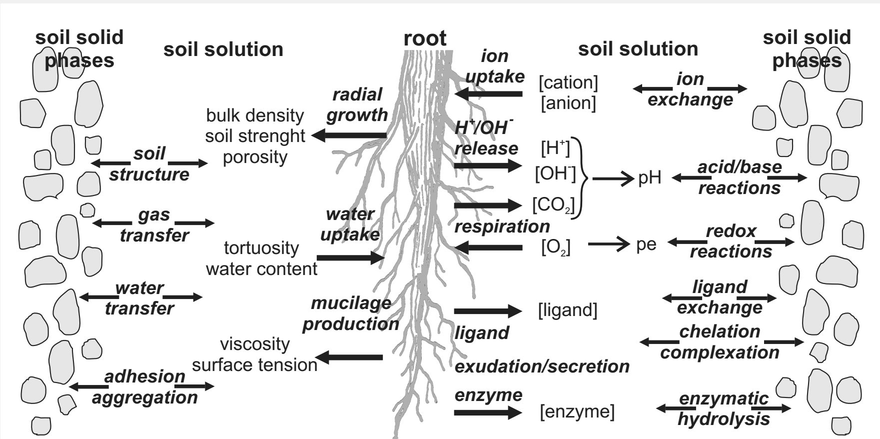

Figure B.1. Schematic representation of root functions involved in changes in physical, biochemical and chemical properties occurring in the rhizosphere (Hinsinger *et al.*, 2005).

nutrient concentrations or depletion zones in the rhizosphere. Both the liberation of protons and respiration can lead to changes in rhizosphere pH of up to 1 to 2 units (Hinsinger *et al.*, 2003). These pH changes can influence mineral dissolution or precipitation, as well as nutrient or trace metal bioavailability (Figure B.1). Between 10 – 50 % of the net photosynthetic carbon is released from plant roots into the rhizosphere (rhizodeposits), principally as root exudates. Root exudates consist of amino

acids, low-molecular-weight carboxylic acids, sugars, fatty acids and sterols, phytohormones, phenolic compounds, flavonoids and enzymes (Curl and Truelove, 1986). Exudates may modify soil physicochemical characteristics and the behaviour of nutrients and trace metals through acidification, chelation, precipitation or redox reactions (Figure B.1; Hinsinger *et al.*, 2005). They also provide a source of labile carbon for soil microorganisms, stimulating microbial density, activity and modifying diversity (Curl and Truelove, 1986; Jones and Darrah, 1994; Bowen and Rovira, 1999; Brimecombe *et al.*, 2001). Microbial densities in the rhizosphere can be up to 1-2 times higher than in bulk soil, reaching densities of 10^7-10^9 culturable rhizosphere bacteria (CFUs) g^{-1} of rhizosphere soil. The composition of rhizosphere microbial communities of different plant species growing in the same soil varied significantly between species and differed from non-vegetated or bulk soil (Garland, 1996; Germida *et al.*, 1998; Steer and Harris, 2000; Smalla *et al.*, 2001; Hartmann *et al.*, 2009). These differences seem to be related to plant species-specific differences in root exudate abundance and composition (or even to different root zones of the same plant species) (Grayston *et al.*, 1998; Pinton *et al.*, 2001; Marschner *et al.*, 2004). Like plant roots, microbial activity in the rhizosphere also modifies physical, chemical and biochemical soil properties, and influences nutrient cycling and availability (Gobran and Clegg, 1996; Andrade, 2004). The rhizosphere is thus a dynamic and complex environment created by the interactions between root, soil and microbial processes (Figure B.2).

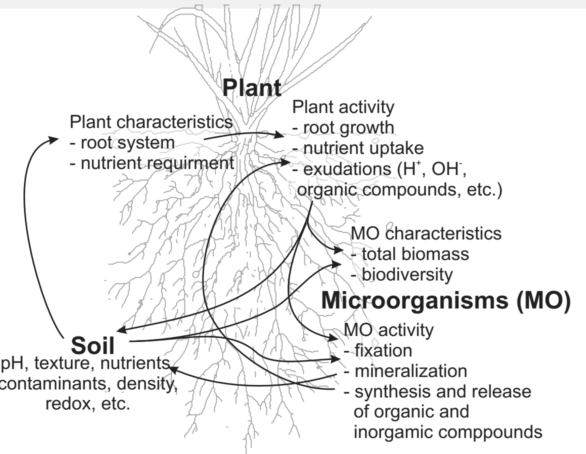

Figure B.2. Plant–soil–microbial interactions in the rhizosphere (Lombi *et al.*, 2001).

Phyllosphere- The *phyllosphere* encompasses the surface area of plant aerial parts (leaves are usually the dominant tissue), and its associated bacteria are normally referred to as *epiphytic* bacteria. The phyllosphere habitat is characterised by pronounced limitations in water and nutrient supply, high variations in temperature and humidity and an important exposition to UV radiation (Lindow and Brandl, 2003). However, this habitat can successfully be colonized and can harbour a diverse microbial community of up to 10^6–10^7 cells per cm^2 leaf surface. Phyllospheric communities vary greatly between, and even within, the same plant species (Kinkel, 1997; Lindow

and Brandl, 2003). Due to these characteristics epiphytic communities from the phyllosphere differ considerably from those of the rhizosphere, and usually present characteristics reflecting their adaptation to this harsh environment. For example, the production of extracellular polysaccharides is related to the formation of bacterial aggregates which is thought to protect bacteria from UV radiation and water stress (Lindow and Brandl, 2003). Production of indole-3-acetic acid (IAA) (common among bacterial epiphytes; Glickmann *et al.*, 1998; Brandl *et al.*, 2001) and biosurfactants has been associated with the ability of epiphytes to modify their local environment and enhance water and nutrient availability in the phyllosphere (Bunster *et al.*, 1989; Schreiber *et al.*, 2005). Another characteristic of these communities is the often high percentage of pigmented bacteria (such as members from the genus *Methylobacterium*, *Xanthomonas*, etc.), presumably because the pigments may serve as a protective screen against solar radiation (Sundin and Jacobs, 1999; Lindow and Brandl, 2003; Jacobs *et al.*, 2004).

Endosphere **-** The *endosphere* can be defined as the microenvironment inside the plant that is colonised by microorganisms. Bacteria benefit from inhabiting the plant's interior because they are protected from biotic an abiotic stresses compared with surface-dwelling bacteria (Hardoim *et al.*, 2008). Bacteria that colonise this habitat without causing negative effects on their host are referred to as endophytic bacteria (Schulz and Boyle, 2006). Intercellular spaces and xylem vessels are the most commonly reported colonised habitats by endophytic bacteria (Sessitsch *et al.*, 2002; Rosenblueth and Martínez-Romero, 2006; Bacon and Hinton, 2007; Weyens *et al.*, 2011). A great diversity of bacterial genera have been isolated from the internal tissues of plants, of both monocotyledons and dicotyledons (see reviews by Sturz *et al.*, 2000; Lodewyckx *et al.*, 2002b; Rosenblueth and Martínez-Romero, 2006). Rhizosphere bacteria seem to be the major source of endophytes (Germaine *et al.*, 2004; Hardoim *et al.*, 2008; Compant *et al.*, 2010), and densities of bacterial endophytes progressively decrease from the roots to the leaves (Lamb *et al.*, 1996; Porteous-Moore *et al.*, 2006; Weyens *et al.*, 2009d). However, it is often possible to isolate strains in aerial plant tissues belonging to genera which are not found to be associated with root tissues, suggesting that the phyllosphere is also a probable route of entry for endophtyes (Hardoim *et al.*, 2008; Compant *et al.*, 2010). Entry points to host plants include tissue wounds, stomata, lenticels, and emerging radicles. Bacteria may also invade intact plants by penetrating root hair cells or by producing cell wall-degrading enzymes (Sessitsch and Puschenreiter, 2008). It has also been shown that certain strains can pass through plant generations via seeds (Van Oevelen *et al.*, 2003; Mastretta *et al.*, 2009).

should not be forgotten that similar beneficial interactions exist between plants and their associated fungi (see reviews by Meharg and Cairney, 2000; Göhre and Paszkowski, 2006; Lebeau *et al.*, 2008).

A. Bacterial-induced improvement of plant growth and establishment

The use of bacterial inoculants to promote plant growth is common practice in agriculture. These types of bacteria are known as plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR). Commercially available inoculants can be found which improve N_2 fixation, stimulate crop growth (PGPR inocula), or act as biocontrol agents for disease prevention (Fravel, 2005). In the following section bacterial mechanisms used by PGPR are discussed in relation to the application of this type of bacteria in phytoremediation processes.

Direct mechanisms

Soil microorganisms influence global soil fertility, as a result of the key roles they play in the decomposition and mineralization of organic material and nutrient cycling (Hayat *et al.*, 2010). Their influence on plant nutrition can also be more localised, especially at the level of the rhizosphere. Microorganisms can increase the **availability of essential plant nutrients**, such as nitrogen, phosphorus or iron .

- *Nitrogen.* Some prokaryotes, known as diazotrophs, are capable of fixing atmospheric nitrogen into ammonia using the enzyme nitrogenase. This biological fixation of N_2 is of paramount importance for plant nutrition. N_2-fixing organisms are usually classified into three principal groups: symbiotic, associative and free-living (Beattie, 2007):

• Symbiotic bacteria: Symbiotic N_2 fixing bacteria share an intimate, highly specific co-existence with their host organism. In this case, N_2 fixation occurs within nodules, which are specialized outgrowths of plant tissue formed specifically in response to the bacterial symbionts. This group includes Gram-negative bacteria such as the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium* which have established symbiotic associations with leguminous plants. These associations are frequently denominated as *Rhizobium*-legume symbioses. Within this bacterial group are also members of the genus *Frankia* which form nodules with species of woody, dicotyledonous trees and shrubs, such as *Alnus* and *Casuarina* sp. A third group of symbiotic diazotrophs include the

cyanobacterias (principally within the genus *Nostoc*) which form symbiotic associations with liverworts, cycads and with the fern *Azolla*.

- Associative and free-living bacteria: there is often confusion when distinguishing between these two groups. In fact, a free-living diazotroph under the direct influence of a host can be considered an associative nitrogen fixer (e.g. rhizosphere bacteria influenced by root exudates or even residing in the plant interior but without nodule formation). Bacterial genera included within this group are *Azospirillum*, *Burkholderia*, *Enterobacter*, *Gluconoacetobacter*, *Herbaspirillum*, *Klebsiella*, etc.

Several rhizospheric and endophytic diazotrophs have been shown to promote plant growth (Vessey, 2003). For instance, endophytic strains from *Burkholderia* sp. and *Gluconacetobacter diazotrophicus* enhance growth of rice and sugarcane (Baldani *et al.*, 2001; Boddey *et al.*, 2001). Similar results were found in the case of wheat after inoculating with N_2-fixing rhizobacterial strains of *Azospirillum* sp., *Azotobacter* sp., and *Bacillus polymyxa* (Boddey *et al.*, 1986; Omar *et al.*, 1996; Mrkovacki and Milic, 2001).

- *Phosphorus*. Phosphorus is the second most important macronutrient for plant growth and is considered as a limited nutrient in many soils since an important fraction of this element is often present in unavailable forms (insoluble phosphates and organic P compounds). Plants display a wide range of root morphological and physiological changes in response to phosphorus deficiency (Hinsinger, 1998; 2001). 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 (Podile and Kishore, 2007; 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). Plants of *Fagus silvatica* inoculated with a P-solubilising rhizobacterium showed an improvement in biomass and uptake of P and other nutrients (Leyval and Berthelin, 1989). Similar results were found by Richardson *et al.* (2001) who showed that P nutrition of several grass species was improved after inoculation with a P-solubilising strain of *Pseudomonas* sp. The production of organic acids frequently associated to the bacterial P solubilisation ability can also induce important changes in contaminant mobility (especially in the case of trace elements), as will be discussed below (see section B.).

- *Iron.* Iron is an essential nutrient for plants and microorganisms since it acts as a cofactor of various enzymes implicated in essential metabolic processes, such as DNA or RNA synthesis. However, in soils iron is mostly present as Fe(III) in insoluble forms. As a result, both plants and microorganisms have evolved specific mechanisms for adquiring Fe from soil. 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 (Schwyn and Neilands, 1987; Crowley, 2006). They can directly mobilize iron from the solid phase minerals or also remove iron from organic complexes (Crowley, 2006). There are now approximately 500 known siderophore structures, which differ in the nature, number and stereochemical arrangement of their metal binding groups (Boukhalfa and Crumbliss, 2002). Siderophores incorporate hydroxamate, catecholate and/or α-hydoxycarboxylic acid binding subunits arranged in different architectures (linear, tripodal, endocyclic or exocyclic). Siderophores produced by rhizosphere bacteria may enhance plant growth by increasing the availability of Fe near the root (Crowley, 2006 and references therein) or by inhibiting colonization by phytopathogens (biocontrol agent). Crowley *et al.* (1988) showed that oat (*Avena sativa*) is able to assimilate Fe from microbial siderophores. Plants of *Vigna radiata* showed a reduction of chlorotic symptoms and enhanced chlorophyll level when inoculated with the siderophore-producing strain *Pseudomonas* GRP3 (Sharma *et al.*, 2003). Although the production of siderophores is primarily induced in Fe-limiting conditions, their production can also be stimulated in the presence of bivalent metals. The formation of complexes between siderophores and metals other than Fe can affect the bioavailability of the latter (Hu and Boyer, 1996; van der Lelie *et al.*, 2000; Dimkpa *et al.*, 2009). This phenomenon can be of special interest in metal phytoextraction, and will be discussed further below (see section B.). Siderophore production can be considered a general phenotype among endophytic bacteria permitting them to growth and prolifer inside plants where they have to cope with extremely low levels of free iron (Idris *et al.*, 2004; Sessitsch *et al.*, 2004). Although siderophore production in the plant interior (or phyllosphere) can be a topic of some controversy. On one hand, a beneficial effect of siderophore production inside plants is the formation of siderophore-metal complexes, in this form the phytotoxicity of metals is reduced (Idris *et al.*, 2004). On the other hand, the same trait can conversely be considered as a virulence factor since the microorganism effectively acquires iron needed by the host plant itself (Taguchi *et al.*, 2010).

Bacteria can also directly influence plant growth and physiology through the production of **plant-growth promoting compounds** such as phytohormones or by **reducing plant stress levels**.

- *Modulation of plant ethylene levels*. Glick *et al.* (1998) developed a model to explain how bacteria capable of producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase 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 (Figure 4). 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 (Glick *et al.*, 1998; Persello-Cartieaux *et al.*, 2003). Later studies showed a positive effect of the activity of this bacterial enzyme on the plant, and this was also shown in contaminated soils (Arshad *et al.*, 2007). For instance, inoculation of *Brassica napus* and *B. campestris* with bacterial strains with ACC deaminase activity enhanced plant growth and protected the plants from metal toxicity (Burd *et al.*, 1998; Belimov *et al.*, 2001).

- *Phytohormones*. Plant hormones are involved in several stages of plant growth and development such as cell elongation, cell division, tissue differentiation, and apical dominance. They also play a key role in plant responses to biotic and abiotic stresses. Many plant-associated microorganisms synthesize phytohormones (such as auxins, cytokinins and gibberellins) and can therefore induce changes in plant morphology and physiology (Persello-Cartieaux *et al.*, 2003). Bacterial synthesis of the auxin, indole-3-acetic acid (IAA) is probably the most studied, its production is well known to enhance plant growth (see review by Vessey, 2003). Studies carried out by Patten and Glick (2002) showed that bacterial production of IAA stimulated root development. However, the impact of bacterial produced IAA on plants can also be negative. For example, inoculation of canola with IAA-producing *Pseudomonas putida* GR12-2 stimulated root elongation, whereas inoculation of the same plant with an IAA overproducer mutant had an inhibitive effect on elongation (Xie *et al.*, 1996). These results indicate that the concentration of IAA available to the plant is an important factor

determining its overall effect. The negative effect of IAA appears to be related to the production of ethylene (Persello-Cartieaux *et al.*, 2003). IAA can stimulate ACC synthase activity, which is implicated in plant ethylene production (Figure 4). This pathogenic effect appears to be related to the IAA biosynthesis pathway. Several bacterial pathways of IAA biosynthesis exist, non-phytopathogenic bacteria principally synthesise IAA via indole-3-pyruvic acid (IPyA pathway) (Spaepen *et al.*, 2007). This is also thought to be a major pathway for IAA biosynthesis in plants. In contrast, phytopathogenic bacteria principally synthesise IAA via indole-acetamide (IAM pathway), a pathway known to be specific to bacteria (Spaepen *et al.*, 2007). Plants do not possess the corresponding regulatory system, and as a result are unable to maintain IAA at non-toxic levels in their tissues (Persello-Cartieaux *et al.*, 2003). Bacterial production of other phytohormones, such as cytokinins and gibberellins, has also been described but to a lesser extent than IAA (Persello-Cartieaux *et al.*, 2003; Vessey, 2003). For instance, growth promotion of *Alnus glutinosa* due to the inoculation of rhizobacteria, *Bacillus pumilus* and *B. licheniformis*, is related to the bacterial synthesis of gibberellins (Guitiérrez-Mañero *et al.*, 2001).

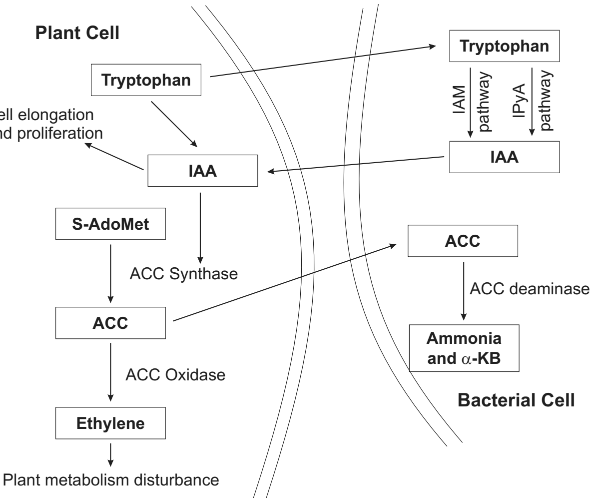

Figure 4. Schematic representation of how bacteria containing ACC deaminase activity lower the ethylene concentration and thereby prevent ethylene-caused inhibition of root elongation (Glick *et al.,* 1998). Abbreviations: IAA, indole acetic acid; ACC, aminocyclopropane carboxylic acid; S-AdoMet, S-adenosyl-methionine; IAM, indoleacetamide; IPyA, indole-3-pyruvic acid; α-KB, α-ketobutyrate.

- *Volatile compounds*. It has recently been suggested that the production of volatile compounds by bacteria can be beneficial for plants (Ryu *et al.*, 2003). The compounds 2,3-butanediol and acetoin liberated by strains of *Bacillus subtilis* and *B. amyloliquefaciens* promoted the growth of *Arabidopsis thaliana* seedlings. Similarly, Blom *et al.* (2011) studied the effects of volatile compounds from 42 bacterial strains on *Arabidopsis thaliana*. They found more than 130 different bacterial volatile compounds, which produced both positive and negative effects

on plant growth. Compounds such as indole, 1-hexanol and pentadecane strongly promoted plant growth.

Indirect mechanisms

Bacteria that inhibit or reduce plant diseases are often referred to as **biocontrol agents**. The principal mechanisms by which they protect plants is by competing for nutrients and space (niche exclusion), producing antimicrobial compounds or through the induction of plant defence mechanisms (Compant *et al.*, 2005; Lemanceau *et al.*, 2007; Podile and Kishore, 2007).

- *Competition for resources.* Beneficial and pathogenic bacteria compete amongst themselves for both nutrient and space. One of the most illustrated cases is that of iron. It has been postulated that the production of iron-chelating compounds (siderophores) by PGPB in the rhizosphere deprive pathogens of this essential element (Kloepper *et al.*, 1980; Compant *et al.*, 2005). For example, *Pseudomonas putida* strain B10 and several strains from *P. fluorescens* have been shown to suppress *Fusarium* sp. by producing siderophores (Kloepper *et al.*, 1980; Kurek and Jaroszuk-Scisel, 2003). Competition for space is intense in the phyllosphere, where sites harboring sufficient nutrients for growth may be low. Colonization of the phyllosphere by *Pseudomonas syringae* mutants (deficient in the production of the ice-nucleation protein) decreased the subsequent colonization of these plants by the wild-type *P. syringae*, thus reducing the incidence of plant frost injury (Lindow, 1987).

- *Antibiosis*. Antibiosis is an important mode of action of many biocontrol agents. Compounds with biocontrol capacity include antimicrobial compounds (antibiotics), biosurfactants and chitinolytic enzymes (Lemanceau *et al.*, 2007). Several bacterial strains from different genera can produce a wide range of antimicrobial compounds (most of them with a broad-spectrum activity). Common examples of antibiotics produced by bacteria are 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), kanosamine, phenazines, oomycin A, pyrrolnitrin, viscosinamide, pyoluteorin, butyrolactones, pantocin A and B, xanthobaccins and zwittermycin A (Whipps, 2001; Raaijmakers *et al.*, 2002). Antimicrobial compounds act, mainly, on four different targets: cell wall synthesis, protein synthesis, nucleic acid replication or cellular membranes. Many of these antibiotic-producing strains have been isolated from soils and plants (see review by Raaijmakers *et al.*, 2002). For instance, bacterial endophytes isolated from potato plants showed antagonistic activity against fungal and bacterial pathogens

(Sessitsch *et al.*, 2004). Antimicrobial activity of several biosurfactants (biological compounds that exhibit high surface-active properties; Georgiou *et al.*, 1992) has also been reported in the literature (Maier, 2003; Cameotra and Makkar, 2004). Biosurfactants produced by *Bacillus subtilis* RB14, have been identified as playing a role in the suppression of damping-off disease of tomato seedlings caused by *Rhizoctonia solani* (Asaka and Shoda, 1996). Another means of biocontrol by PGPB is the capacity to produce cell wall hydrolases, such as quitinases, glucanases, etc. which attack the cell wall of phytopathogenic fungi (Compant *et al.*, 2005; Podile and Kishore, 2007).

- *Degradation of pathogenicity factors*. Another important means of biocontrol is through the detoxification of virulence factors produced by pathogens. For example, the detoxification of fusaric acid produced by *Fusarium* sp. has been shown for several strains of *Pseudomonas* sp. (Toyoda *et al.*, 1988; Thangavelu *et al.*, 2001).

- *Induced systemic resistance.* Some bacterial strains can activate/stimulate plant defence mechanisms without causing visible symptoms of stress on the host plant. This phenomenon is referred as induced systemic resistance (ISR; van Loon *et al.*, 1998). Bacterial determinants of ISR include lipopolysaccharides (LPS), siderophores and salicylic acid (SA) (van Loon *et al.*, 1998). However, the possible involvement of other macromolecules (Ongena *et al.*, 2002), such as volatile compounds (Ryu *et al.*, 2004) or even antibiotics (Iavicoli *et al.*, 2003) can not be discarded. An example of ISR is the treatment of peanut seeds with *Bacillus subtilis* AF1, that conferred protection against crown rot caused by *Aspergillus niger* (Sailaja and Podile, 1998).

B. Bacterial-induced modifications in trace metal bioavailability

The mobility and bioavailability of trace metals in the soil is a critical factor affecting the efficiency of phytoremediation processes. Trace elements in the soil solution can be 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 soil 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 primary minerals (Adriano, 2001). Metal availability is governed by the pseudo-equilibrium between aqueous and solid soil phases (Figure 5),

which is conditioned by numerous processes (acid-base reactions, complexation, precipitation-dissolution, oxidation-reduction and ionic exchange) (Lindsay, 1983). Contaminant bioavailability can be substantially modified in the rhizo-

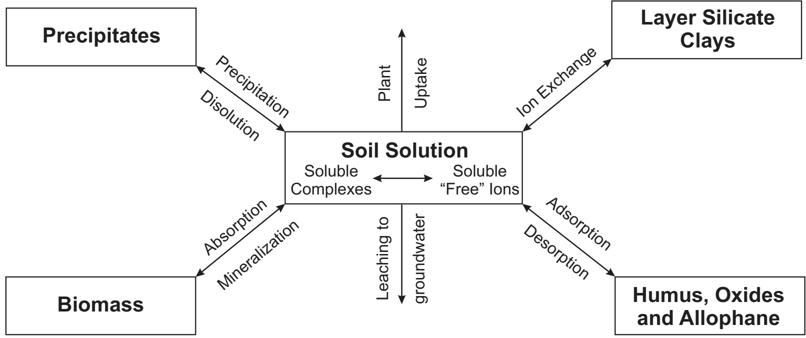

Figure 5. Schematic of key interactive processes in the soil system affecting the partitioning of trace metals between aqueous and solid phases (Adriano, 2001).

sphere as a result of edaphic processes, root exudates or microbial activity (Kidd *et al.*, 2009) (Figure 2). Metabolic processes of microorganisms can lead to either an increase or decrease in contaminant availability, and these processes can be exploited in phytoremediation techniques (Kidd *et al.*, 2009; Wenzel, 2009). Processes such as chemical transformation, chelation and protonation will lead to mobilisation of trace elements, whereas precipitation or sorption decreases trace element availability.

Microbial mechanisms involved in trace element mobilisation

An increase in the phytoavailable pool of metal(s) can facilitate their phytoextraction (or phytomining) from contaminated soils. The use of synthetic chelators such as EDTA, DTPA have been proposed as a means of increasing contaminant bioavailability. However, serious restrictions apply to the use of these complexing agents since they are poorly photo-, chemo- and bio-degradable. Biodegradable chelates are available and has been shown to increase metal availability but they are often expensive, rendering their use economically inviable. Alternative strategies are therefore necessary and the use of plant-microbial partnerships could provide economical means of manipulating contaminant availability at contaminated sites (Lebeau *et al.*, 2008; Kidd *et al.*, 2009; Wenzel, 2009; Weyens *et al.*, 2009c). Metal mobilisation by bacteria can be achieved by acidolysis, chelation or complexation, chemical transformation and/or redox reactions.

- *Acidification.* Various biological products of bacterial metabolism can acidify their environment, these include organic acids such as carbonic acid resulting from CO_2 respiration, and other more complex molecules. This local acidification can lead to metal solubilisation from the solid phase, but can also affect metal speciation in the soil solution (Gadd, 2010). *Pseudomonas* sp. RJ10 and *Bacillus* sp. RJ16 strains were able to solubilize $CdCO_3$ in liquid medium by decreasing pH. Furthermore, *Brassica napus* growing in Cd-contaminated soil and inoculated with the same strains showed an increase in Cd uptake and a simultaneous decrease in rhizosphere pH (Sheng and Xia, 2006).

- *Complexation.* Chelating molecules (such as organic acid anions, siderophores or biosurfactants) can form complexes with free metals in solution, thus promoting metal desorption from the soil matrix. Additionally, they can cause metal desorption through direct contact with the adsorbed metal (Miller, 1995). An increase in metal availability due to the release of bacterial metabolites (and not simply associated with a pH decrease) has been demonstrated by various authors (Whiting *et al.*, 2001; Kuffner *et al.*, 2008; 2010). In many of these studies, it is unclear which metabolites are acting. In fact, depending on soil nutrient availability bacteria can produce distinct metabolites with varying mobilising potential (Bennett *et al.*, 2001; Whiting *et al.*, 2001). To date the bacterial metabolites, siderophores, biosurfactants, and carboxylic acids are among the most studied.

• Siderophores: Siderophore production is widespread among bacteria and siderophores are perhaps the most common bacterial secondary metabolites. As mentioned above, they form high affinity complexes with Fe(III), but can also form complexes of lower stability with other trace metals thus affecting their bioavailability (Hu and Boyer, 1996; van der Lelie *et al.*, 2000; Dimkpa *et al.*, 2009). Furthermore, chelation of Fe from the soil solid phase (from sorption sites and mineral lattices) can lead to the liberation of other metals forming part of these minerals. Diels *et al.* (1999) found they could treat sandy soil contaminated with heavy metals using *Alcaligenes eutrophus* strain CH34 due to its ability to solubilise metals through the production of siderophores. Bacterial culture filtrates containing hydroxamate siderophores secreted by *Streptomyces tendae* F4 significantly promoted plant growth and enhanced plant uptake of Cd and Fe, relative to the control (Dimkpa *et al.*, 2009). In contrast, studies carried out using the siderophore desferrioxamine B (DFOB) showed that the metal-DFOB complex can be resorbed at mineral surfaces (depending on the charge of the siderophore

and the surface charge of soil minerals) thus reducing metal solubility (Neubauer *et al.*, 2000; 2002). A great variety of siderophores with varying structures have been described, and their effects and behaviour will vary greatly. For example, siderophores forming negatively charged metal complexes could be more useful for metal solubilisation than those with similar structures to DFOB (Neubauer *et al.*, 2000).

• Biosurfactants: Biosurfactants are biological compounds that exhibit high surface-active properties, containing at least one hydrophilic and one hydrophobic group. They can be grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds. Most of these compounds are either anionic or neutral (Mulligan *et al.*, 2001b). Several studies have successfully used biosurfactants to solubilise and removed metals in soil-washing applications (also referred as to bioleaching; Gadd, 2010; Herman *et al.*, 1995; Mulligan *et al.*, 1999). However, few studies exist in which microbes capable of producing biosurfactants have been applied in phytoremediation of metal-contaminated soils. Studies carried out by Gunawardana *et al.* (2009; 2011) and Johnson *et al.* (2009) found that rhamnolipids had no effect on the uptake of metals by plant in hydroponic cultures. Contrasting results have been observed in experiments carried out on contaminated soils. The application of rhamnolipids in a Cu-contaminated soil led *Zea mays* to extract up to 3 times more Cu than in control soils. However, the same treatment had the opposite effect in *Atriplex nummuleria,* suggesting that there is a species-specific response to these metabolites (Maier *et al.*, 2001). Other authors found no effect on metal extraction in soil experiments carried out with rhamnolipids (Jordan *et al.*, 2002; Wen *et al.*, 2010). The lack of effect was attributed to the retention of the chelant (or metal-rhamnolipid complex) on soil surfaces (Jordan *et al.*, 2002), or to the exclusion of the metal-rhamnolipid complex at the root (Wen *et al.*, 2010).

- *Organic acids.* Organic acids provide both a source of protons for metal mobilisation (by altering pH) and metal chelating anions to complex metal cations (Devêvre *et al.*, 1996). In fact, organic acids are present as negatively charged anions under a wide range of soil conditions, allowing them to react strongly with metal ions in both soil aqueous and solid phases (Jones and Darrah, 1994; Ryan *et al.*, 2001). Bacteria producing organic acids, such as citric, oxalic or acetic acid have been shown to mobilize various trace elements in soil (Li *et al.*, 2010). An increased trace element uptake in various plants after inoculation with acid-

producing bacteria has been observed (Abou-Shanab *et al.*, 2003a; Ma *et al.*, 2011).

- *Redox transformations.* Microorganisms can reduce metals such as Fe(III) and Mn(IV) thus increasing their solubility. Furthermore, reduction of these metals can indirectly release other metals which are strongly bound to Fe and Mn oxides (Lovley and Coates, 1997; Gadd, 2010). For instance, bacterial-mediated reduction of Fe(III) from goethite resulted in the release of Mn and Co (Bousserrhine *et al.*, 1999).

- *Biomethylation.* Methylation of metal(loid)s by bacteria transforms metals into a gaseous state via the addition of methyl groups (Hietala and Roane, 2009). This phenomenon occurs with trace elements such as As, Hg, Se, Sn, Te and Pb. Methylated metal(loid)s are generally more toxic (Hietala and Roane, 2009), but they are often lost from the soil or plant through volatilization (phytovolatilisation).

Microbial mechanisms involved in trace element immobilisation

Metal immobilisation can be achieved by sorption to cell components or exopolymers, transport and intracellular sequestration, release of metal binding compounds or precipitation as insoluble organic or inorganic molecules (Gadd, 2004).

- *Biosorption.* Biosorption can be defined as the microbial uptake of organic and inorganic metal species, both soluble and insoluble, by physicochemical mechanisms, such as adsorption (Gadd, 2004). Due to the reduced volume (1.5–2.5 μm^3) and the negative net charge of the cell, bacteria have a high surface to volume ratio and can accumulate metal cations from the environment (Haferburg and Kothe, 2007). Peptidoglycan carboxyl and phosphate groups are the main binding sites in Gram-positive and Gram-negative bacterial cells, respectively (Gadd, 2004). Nickel sorption capacity of *Bacillus subtilis* SJ-101 has been shown to alleviate Ni-toxicity in *Brassica juncea* (Zaidi *et al.*, 2006). Biosorption can also provide nucleation sites on the cell envelope for precipitation of minerals. In fact, precipitation on the cell wall and biosorption are sometimes overlapping phenomena and it can be difficult to assign the corresponding contribution of each to metal immobilisation (Haferburg and Kothe, 2007).

- *Intracellular accumulation.* Some cationic species can be accumulated within cells via membrane transport systems. Once inside cells, metals species can be bound, precipitated or translocated to intracellular structures or organelles (Gadd, 2004).

- *Production of metal-binding compounds.* A range of metal-binding compounds can be produced by microorganisms, from simple organic acids and alcohols to macromolecules, such as polysaccharides, humic and fulvic acids. For instance, the strain *Paenibacillus jamilae* CECT 5266 was able to complex up to 230 mg Pb per g of exopolysaccharide produced (Morillo *et al.*, 2006). Specific metal-binding proteins, such as metallothioneins and phytochelatins, can also be produced in response to toxic metals (Gadd, 2004).

- *Redox transformation.* Reduction of metals can lead to a reduced metal mobility and toxicity. Bacteria can, for example, reduce Cr(VI) to Cr(III) and U (VI) to U(IV). Anaerobic sulfate-reducing and metal-reducing bacteria can produce less soluble metal species, such as metal sulfides and phosphates, elemental (Hg, Se), and reduced forms of different metals (Gadd, 2004).

C. Bacterial-induced modifications in organic contaminant bioavailability

The behaviour of organic compounds in the soil is principally governed by their hydrophobicity (lipophilicity) (Alexander, 2000; Reid *et al.*, 2000; Semple *et al.*, 2003). Hydrophobicity is habitually indicated by the compound solubility and the log octanol/water partition coefficient, log K_{OW}. High values of log K_{OW} (log K_{OW} > 3.5) indicate a higher hydrophobicity, a higher resistance to degradation or transformation, a higher persistence, thus tending to bioaccumulate in the food chain. A part from the physicochemical properties of the contaminant, soil properties, such as organic matter content, clay minerals and oxides, soil pH, moisture and cation exchange capacity are also directly implicated in the degree to which these compounds are retained (sorbed) within the soil. It is generally accepted that the main critical factors determining these soil-compound interactions are the amount and nature of the soil organic matter. Although in those soils with low organic matter content, the clay minerals and Fe oxides play an important role. With time, availability of organic contaminants in soils progressively decreases, a phenomenon which is known as "ageing" (Semple *et al.*, 2003). During this process contaminants can be sequestered in the organic matter, or sorbed to surfaces within nano- and micropores in soils containing little organic matter.

Bioavailability of organic pollutants in soils seems to be an important and restrictive factor for effective phytoremediation (Schnoor *et al.*, 1995; Alexander, 2000; Chaudhry *et al.*, 2005). This is particularly the case for contaminants with a low water solubility and high hydrophobicity which impedes microbial degradation or their uptake and absorption within plants. Bacteria can be useful for pro-

moting the bioavailability of organic contaminants, and thus facilitating their degradation and/or uptake (Pandey *et al.*, 2009; Wenzel, 2009). The main mechanisms operating in this process are described below.

- *Biosurfactants*. Biosurfactants can increase the solubility of organic contaminants in water through the formation of micelles, or alternatively, they can modify the hydrophobicity of the bacterial cells membranes and facilitate the attachment of hydrophobic compounds (Maier, 2000; Hickey *et al.*, 2007). For instance, the addition of rhamnolipids increased the degradation of phenanthrene by *Sphingomonas* sp. GF2B, while using the synthetic surfactant Tween 80 had the opposite effect (Pei *et al.*, 2010). Hickey *et al.* (2007) found that biosurfactants significantly increased desorption of PAHs from soil and enhanced their biodegradation by *Pseudomonas alcaligenes* PA-10. The addition of biosurfactants also enhanced the phytoremediation of PAH-contaminated soils inoculated with a PAH-degrading bacterial strain (Liu *et al.*, 2010; Zhang *et al.*, 2010).

- *Biofilms*. Biofilms are assemblages of single or multiple microbial populations that are attached to abiotic or biotic surfaces through a self-produced matrix of extracellular polymeric substances (Singh *et al.*, 2006). Degrading bacteria can access target compounds if they are present in solution or by direct contact with the adsorbed compound. These processes can be facilitated through the formation of biofilms (Pandey *et al.*, 2009). Biofilm formation has been shown to be a specific response of *Mycobacterium* sp. LB501T to optimize anthracene bioavailability (Wick *et al.*, 2002).

- *Organic acids*. Although studies evaluating the effects of organic acids of bacterial origin on organic contaminant mobility and bioavailability are rare, there are studies which assess the influence of organic acids and other metabolites of non-bacterial origin. White *et al.* (2002; 2003) have shown that organic acids can increase the plant uptake of 2,2-bis(p-chlorophenyl)- 1,1-dichlorethylene (DDE). Similarly, exudates of *Cytisus striatus* have been shown to improve bioavailability of hexachlorocyclohexane (HCH) isomers (Rodríguez Garrido, 2009).

- *Degradation.* Although it is not a direct mechanism, the degradation of organic compounds has been shown to enhance desorption rates of contaminants from sorbed surfaces to the aqueous phases (Bosma *et al.*, 1997).

- *Chemotaxis.* Certain microorganisms are capable of actively moving towards a contaminant via chemotaxis (Andreu and Picó, 2004; Golubev *et al.*, 2008; Strobel *et al.*, 2011). Chemotaxis can play an important role in the formation of biofilms on hydrophobic contaminants (Pandey and Jain, 2002; Harms and Wick,

2006; Singh *et al.*, 2006). Although this phenomenon is not considered a direct mechanism, it is considered a means of overcoming the limited bioavailability of a contaminant.

D. Microbial degradation of organic contaminants

Biodegradation is the transformation of organic contaminants that occurs due to microbial activity. As such, the contaminants can be considered as a microbial substrate, acting as a source of C and energy, or nutrients (for example N in the case of the degradation of TNT) or as an electron acceptor. When the transformation is complete and the organic compound is converted into inorganic substances, the process is known as *mineralisation*. Some compounds can be degraded through *co-metabolism*, where there is a transformation of the contaminant but the co-metabolising organism receives no energy to support its growth (Skladany and Metting, 1993; Maier, 2000).

The high adaptability and metabolic versatility of microorganisms, and ability to rapidly develop numerous enzymatic mechanisms for transforming organic compounds, make them of especial importance in the clean-up of this type of contaminants (Maier, 2000; Lal *et al.*, 2010). Microbial communities in contaminated soils are considered as an important source of degrading strains or consortia. The presence of contaminants leads to the selective-enrichment of these resistant or degrading microorganisms and contaminated soils are generally noted for their biodegradation potential (Urbance, 2003). Several studies have also demonstrated the contaminant tolerance and biodegradative capacity of bacterial strains associated to plants growing in contaminated sites. The majority of isolated bacteria from oak and ash trees, growing on a TCE-contaminated site, showed tolerance to TCE and toluene, and TCE degradation capacity was observed in some rhizosphere strains (Weyens *et al.*, 2009d). Siciliano *et al.* (2001) showed that plants growing in soils contaminated with petroleum hydrocarbons and nitroaromatics naturally recruited root endophytes with the necessary contaminant-degrading genes. This natural capacity to degrade organic compounds presented by many microorganisms is a characteristic that can be exploited in phytoremediation methods.

The complementary action of plants and their associated microorganisms on contaminant degradation has been studied in detail in the rhizosphere zone (rhizodegradation or phytostimulation). Numerous studies demonstrate a significantly enhanced dissipation and/or mineralisation of organic pollutants in the rhizosphere (Schwitzguébel *et al.*, 2006; Azaizeh *et al.*, 2011). This plant-induced

effect is generally attributed to a stimulation in microbial density, diversity and/or activity in the rhizosphere, or the selective enrichment of degrading microorganisms (Anderson *et al.*, 1993; Wenzel *et al.*, 1999; Kuiper *et al.*, 2004; Chaudhry *et al.*, 2005; Schwitzguébel *et al.*, 2006; Dzantor, 2007). Due to the release of plant rhizodeposits, bacterial metabolic biodiversity in the rhizosphere can be greater than in non-vegetated soil and can additionally stimulate contaminant degradation through co-metabolism. *Medicago sativa* enhanced densities of PAH-degrading bacteria in the rhizosphere (Muratova *et al.*, 2003). Siciliano *et al.* (2003) observed a greater level of hydrocarbon-related catabolic genes (*ndoB*, *alkB* and *xylE*) in the rhizosphere soils of *Festuca arundinacea* relative to bulk soil. Kidd *et al.* (2008) observed a higher dissipation of HCH in soils planted with *Cytisus striatus* and *Holcus lanatus* than in non-planted soils. These authors attributed this result to the phytostimulation of bacterial activity in the rhizosphere.

More recently, the degradative capacity of endophytic bacterial communities has been shown to play an important role in contaminant dissipation, especially in the case of volatile compounds. Plant-endophyte associations have successfully enhanced phytoremediation of organic contaminants such as toluene (Barac *et al.*, 2004). Inoculation of *Lupinus luteus* with the bacterial endophyte *Burkholderia cepacia* L.S.2.4 harbouring the pTOM toluene-degradation plasmid protected the plants from toluene phytotoxicity. *In planta* toluene degradation was enhanced and reduced evapotranspiration of toluene by 50-70 % (Barac *et al.*, 2004). The same strategy was applied in the field by Weyens *et al.* (2009a). *In situ* inoculation of poplar trees, growing on a TCE-contaminated site, with the TCE-degrading strain *Pseudomonas putida* W619-TCE reduced evapotranspiration of the contaminant by 90 %. Furthermore, inoculation with degrading strains can lead to the transfer of the degradation pathway among the endogenous members of the community, via horizontal gene transfer (Taghavi *et al.,* 2005; Weyens *et al.*, 2009a).

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Objectives

In recent years, it has been shown that phytoremediation could provide a viable alternative for the remediation of contaminated soils. Nonetheless, there is a need for further research to fully elucidate the processes that are involved and also towards developing strategies that improve and optimise these processes before they can be fully deployed on a large-scale. A growing number of studies indicates an important role of plant-microbial associations in these techniques, and their exploitation could lead to an enhanced efficiency of the clean-up process. Natural metalliferous soils and sites with anthropogenic-induced pollution are considered ideal environments for the screening of new and beneficial plant-microbial associations. This type of area allows for the selection of useful plant species and/or ecotypes, as well as the isolation of plant-associated microbial strains, with suitable characteristics for application in phytoremediation processes.

On this basis, the global objective of this thesis was to establish a collection of plant-associated bacteria, obtained from tolerant plants growing spontaneously at different contaminated areas (geogenic or anthropogenic) and to evaluate the potential application of a selected group in diverse phytoremediation strategies. To achieve this, a series of studies were carried out targeting three contaminant scenarios.

Scenario 1: Serpentine soils (naturally metal-rich soils)
The objective of the first study (Chapter 1) was to investigate differences in the rhizosphere microbial community of three populations of the Ni-hyperaccumulator, *Alyssum serpyllifolium* subsp. *lusitanicum*, in comparison with the Ni-excluder, *Dactylis glomerata* or non-vegetated soil from the same site. The main focus of this work was to determine the resistance of heterotrophic bacteria to Co, Cr and Ni, as well as the abundance of several microbial groups involved in C and N cycling, in relation to their plant host.
The following study (Chapter 2) had two main objectives, firstly, the isolation and characterisation (metal resistance and plant-growth promoting traits) of rhizobacterial strains associated with three subspecies of *A. serpyllifolium*, two Ni hyperaccumulating subsp. (subsp. *lusitanicum* and subsp. *malacitanum*) and a closely related non-hyperaccumulator (subsp. *serpyllifolium*), and secondly, to evaluate the capacity of these bacterial isolates to modify soil Ni extractability.

Finally, two bacterial strains isolated from the rhizosphere of Ni-hyperaccumulating subspecies of *A. serpyllifolium* were selected for a more in-depth study (Chapter 3). Here the objectives were to evaluate their weathering capacity and ability to mobilise Ni from ultramafic rocks, as well as their influence on soil metal availability and soil metal pools in relation to Ni uptake and accumulation by *A. serpyllifolium* subsp. *malacitanum*.

Scenario 2: Mine tailings from a Pb/Zn-mining area

In this study (Chapter 4) the plant-microorganism-soil system of the dominant plant species found in an old Pb/Zn mining area was characterised. Within this context the objectives were firstly, to determine Cd, Pb and Zn accumulation and/or exclusion by three pseudometallophytes colonizing the mine-spoils, and the plant-induced modifications in metal "bioavailability" and fractionation in the rhizosphere. A second objective was to isolate and characterise (metal resistance and plant-growth promoting characteristics) metal-tolerant rhizobacterial strains associated with these plant species, and to assess the ability of selected rhizobacteria on the growth of two plant species (*Festuca pratensis* and *Salix viminalis*) commonly used in phytoremediation strategies.

Scenario 3: Urban soils contaminated with isomers of hexachlorocyclohexane (HCHs) as a result of the fabrication of the organcochlorine pesticide lindane (γ-HCH)

In this contaminant scenario a study (Chapter 5) was carried out with two initial objectives. Firstly, to assess the diversity of the culturable endophytic and rhizoplane bacterial communities associated with *Cytisus striatus* growing spontaneously in an HCH-contaminated site; and secondly, to characterise the isolated strains by focusing on their plant growth-promoting traits and their ability to produce biosurfactants or degrade HCHs.

Following on from this, two further studies were carried out with the objective of evaluating the effects of the inoculation of *C. striatus* with selected bacterial strains on the dissipation and/or degradation of HCH isomers, as well as on the growth and survival of these plants when grown in HCH spiked artificially composed growth substrates (Chapter 6) or HCH spiked natural soils (Chapter 7).

Chapter 1

Rhizosphere microbial densities and trace metal tolerance of the nickel hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum*

ABSTRACT

In this study we determine culturable microbial densities (total heterotrophs, ammonifiers, amylolytics and cellulolytics) and bacterial resistance to Co, Cr and Ni in bulk and rhizosphere soils of three populations of the Ni-hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum* and the excluder *Dactylis glomerata* from ultramafic sites (two populations in Northeast (NE) Portugal (Samil (S), Morais (M)) and one population in Northwest (NW) Spain (Melide (L)). The relationship between water-extractable metal concentrations and microbial densities were analysed. Significant differences in microbial densities and metal-resistance were observed between the two species and their three populations. The hyperaccumulator showed higher microbial densities (except cellulolytics) and a greater rhizosphere effect, but this was only observed in S and M populations. These populations of *A. serpyllifolium* also showed selective enrichment of Ni-tolerant bacteria at the rhizosphere where Ni solubility was enhanced (densities of Ni-resistant bacteria were positively correlated with H_2O-soluble Ni). These rhizobacteria could solubilise Ni in the soil and potentially improve phytoextraction strategies.

This study formed part of the following publication:

Becerra-Castro C, Monterroso C, García-Lestón M, Prieto-Fernández A, Acea MJ, Kidd PS 2009. Rhizosphere microbial densities and trace metal tolerance of the nickel hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum*. *International Journal of Phytoremediation* 11:525-541.

1.1 Introduction

Plants known as hyperaccumulators can accumulate extreme amounts of trace metals in their aboveground biomass when growing in metal-enriched habitats, such as ultramafic soils (in mg kg^{-1}; > 10000 (Mn or Zn), > 1000 (Cu, Co, Cr, Ni, Pb) or > 100 (Cd); Baker *et al.*, 2000). Phytoextraction strategies often exploit this type of plant for the clean-up of trace metal-contaminated soils.

Physiological mechanisms involved in the translocation, sequestration and detoxification of metals within tissues of hyperaccumulator plants have been studied in detail (Brooks, 1998; Salt and Kramer, 2000; Lasat, 2002). In contrast, processes operating at the root-soil interface (rhizosphere), and mechanisms by which these plants are able to access non-labile metals in soils, continue to be debated. Trace metal bioavailability is typically higher in the rhizosphere of hyperaccumulators than non-hyperaccumulating plants. Recent evidence suggests that hyperaccumulator plants may induce mineral weathering and metal liberation through root secretions or exudates (Wenzel *et al.*, 2003; Puschenreiter *et al.*, 2005). Fewer studies have considered the importance of rhizosphere bacteria in the tolerance, mobilization and hyperaccumulation of trace metals (Khan, 2005). Hyperaccumulating plants, such as *Alyssum bertolonii*, *Sebertia acuminata* or *T. caerulescens* subsp. *calaminaria*, have been shown to host higher proportions of Cd-, Ni- or Zn-resistant bacteria in the rhizosphere compared to non-hyperaccumulating plants or non-vegetated soil (Schlegel *et al.*, 1991; Delorme *et al.*, 2001; Mengoni *et al.*, 2001; Lodewyckx *et al.*, 2002; Abou-Shanab *et al.*, 2003).

Soil microorganisms can potentially enhance phytoextraction in different ways: they can improve the solubility, availability and transport of trace elements (such as Fe) and nutrients by reducing soil pH, secretion of chelators and siderophores or redox changes; they can enhance plant biomass production, root growth and root hair development increasing the functional surface area for metal uptake (plant growth-promoting rhizobacteria (PGPR)); and they can increase heavy metal tolerance of plants, for example, through the production of 1-aminocyclopropane-1 -carboxylic acid (ACC) deaminase which modulates plant ethylene levels (Khan, 2005; Abou-Shanab *et al.*, 2006). Plant-microbe associations or developed inoculums could be exploited to improve metal uptake and accumulation in phytoextraction systems.

The objectives of this study were to investigate bulk and rhizospheric microbial groups associated with three populations of the Ni-hyperaccumulator, *Alyssum*

serpyllifolium subsp. *lusitanicum* and the resistance of heterotrophic bacteria to Co, Cr and Ni. The Ni-excluder, *Dactylis glomerata*, was also studied for comparative purposes. Considerable natural variation in metal uptake and accumulation has been observed among different populations of hyperaccumulators but few studies have evaluated such population-specific differences in microbial groups and metal tolerance. Furthermore, there is a lack of information about physiological groups of bulk and rhizosphere soil microorganisms associated with hyperaccumulating plants.

1.2 Materials and methods

Study site and collection of plant and soil samples

This study was carried out using samples collected from two serpentine outcrops of the NW Iberian Peninsula (Figure A.1): (1), Trás-os-Montes in NE Portugal; and (2), Melide in NW Spain. Trás-os-Montes has a Mediterranean climate, with a mean annual temperature of 12.4 ºC and annual precipitation of 720 mm (Carballeira *et al.*, 1983; Menezes de Sequeira and Pinto da Silva, 1992). Melide has a European humid-temperate climate with a mean annual temperature of 12.9 ºC and mean annual precipitation of 1381 mm (Carballeira *et al.*, 1983). Two sites were sampled in the Trás-os-Montes region (Samil (S) and Morais (M)) and one site in Melide (L) (Figure A.1).

Two plant species were studied from each site: the Ni-hyperaccumulator, *Alyssum serpyllifolium* Desf. ssp. *lusitanicum* Dudley and P. Silva (Brassicaceae) and the Ni-excluder, *Dactylis glomerata* ssp. *hispanica* (Roth) Nyman (Gramineae). *Alyssum serpyllifolium* ssp. *lusitanicum*, commonly referred to as *Alyssum pintodasilvae* Dudley, is the only Ni hyperaccumulator in NE Portugal and was first described as a Ni hyperaccumulator in 1969 (Menezes de Sequeira, 1969). This endemic species occurs in all typical serpentinic associations, but especially in *Taeniathero-Alyssetum pintodasilvae* (Menezes de Sequeira and Pinto da Silva, 1992). Plants growing on ultramafic soils of this region contained Ni concentrations as high as 38105 mg kg^{-1} in their shoot tissues (Freitas *et al.*, 2004; Diez *et al.*, 2006). In this chapter all three populations (S, M and L) are referred to as *Alyssum serpyllifolium*.

The whole plant and root system (including root ball) of 5-10 individuals of each species were collected from all three populations. Operationally defined rhizosphere soil was separated by gently crushing the root ball and shaking the root

system. Tightly held soil (< 3 mm from the root surface) was considered rhizosphere soil. The remaining loosely held soil was considered bulk soil. To the extent possible, root debris included in the collected rhizosphere soil were removed using tweezers or by sieving. In addition, surface soil samples (0-10 cm) were collected at each site from bare patches where no plants were found growing (non-vegetated soil). Fresh soil samples were used for microbial analyses and air-dried soil samples for physico-chemical analyses.

Soil analyses

Soil analyses were carried out on the air-dried, < 2 mm fraction of samples. Soil pH was measured in H_2O and 0.1 M KCl using a 1:2.5 soil:solution ratio. Organic C and total N were analysed by combustion with a LECO analyser (Model SC-144 DR, LECO Corp., St Joseph, MI). Exchangeable cations were extracted with 1 M NH_4Cl. Sodium and K in the extracts were determined by emission spectrophotometry; Ca and Mg by atomic absorption spectroscopy (AAS) (Perkin-Elmer 2380, Norwalk, CT). Soils (0.5 g) were digested in a 2:1 mixture of concentrated HNO_3:HCl and total concentrations of metals were analysed by atomic absorption as above (Co_T, Cr_T and Ni_T). The plant-available metal fraction (Co_{EDTA}, Cr_{EDTA}, Ni_{EDTA}) was estimated in bulk soils using 0.5 M NH_4OAc + 0.5 M HOAc + 0.02 M EDTA (1:5 soil:extractant ratio, pH 4.65, 30 min shaking). Water-soluble Co, Cr and Ni concentrations were analysed by AAS with graphite furnace (Perkin-Elmer 4110 ZL) in both bulk and rhizosphere soil extracts after 30 min shaking using a 1:2.5 soil:H_20 ratio (Cr_{H2O}, Co_{H2O}, Ni_{H2O}).

Microbial analyses

Culturable populations

Culturable heterotrophic bacteria (HP), the ammonifying (AM), amylolytic (SM) and cellulolytic (CE) populations were determined in soils by the most probable number (MPN) technique, as follows. Five grams of soil were suspended in 45 ml sodium hexametaphosphate solution (1 %) and shaken for 30 min in an end-over-end shaker. These soil suspensions were diluted in 10-fold series and 50 µl aliquots were used to inoculate microtiter plates containing selective liquid media (150 µl/well) (HP, AM, SM), or 1 ml aliquots were used to inoculate tubes containing 9 ml selective liquid media (CE). Five wells were inoculated per suspension-dilution. Heterotrophic population was estimated in yeast-extract medium (1.0 g yeast extract, 1.0 g glucose, 0.5 g KNO_3, 0.2 g $MgSO_4.7H_2O$, 0.5 g

K_2HPO_4, 0.1 g $CaCl_2$, 0.1 g NaCl, 0.01 g $FeCl_3$, in 1 l deionised water) plus oligo-elements (1.5 mg $FeSO_4.7H_2O$, 0.3 mg H_3BO_4, 0.19 mg $CoCl_2.H_2O$, 0.1 mg $MnCl_2.4H_2O$, 0.08 mg $ZnSO_4.7H_2O$, 0.02 mg $CuSO_4.5H_2O$, 0.036 mg $Na_2MoO_4.2H_2O$, 0.024 mg $NiCl_2.6H_2O$). Amylase-producers were cultured in Winogradsky`s saline medium (containing 5 g $K_2HPO_4$, 5.1 g $MgSO_4.7H_2O$, 1.0 g $NH_4NO_3$, 2.5 g NaCl, 0.04 g $Fe_2(SO_4)_3.7H_2O$, 0.05 g $MnSO_4.H_2O$ in 1 l deionised water) plus soluble starch (1.5 g $l^{-1}$) and oligoelements (as above). Ammonifiers were evaluated in Winogradsky`s saline solution and L-asparagine (0.2 g l^{-1}) as the only N and C source plus oligoelements (as above). The redox dye, tetrazolium violet (2,5-diphenyl-3-(α-naphthyl)tetrazolium chloride (TV), 15 mM) was used to indicate growth (dehydrogenase activity), and was added to media using the ratio 1:100 (v/v, TV:media) (Kennedy, 1994). Cellulase-producing microbes were counted in tubes with 10 ml of medium containing 0.3 g NH_4NO_3 l^{-1}, Winogradsky`s saline medium and oligoelements (as above). One strip (10 x 1 cm^2) of cellulose filter paper was placed in each tube; the breakdown of the paper was positive evidence of growth of cellulolytic microorganisms.

All microbial groups were cultured at pH 6.8 and counts were performed after 1 week of incubation at 25 ºC. Each MPN was determined from the appropriate table, corrected for the initial dilution and inoculant volume, and expressed as log_{10}MPN g^{-1} dry soil. The rhizosphere effect was calculated for each physiological group as the ratio of the number (MPN g^{-1} soil) of microorganisms in the rhizosphere over the number (MPN g^{-1} soil) of microorganisms in bulk soil (R/B), and as the ratio of the number (MPN g^{-1} soil) of microorganisms in the rhizosphere over the number (MPN g^{-1} soil) of microorganisms in non-vegetated soil (R/NV).

Carbon substrate utilisation

The ability of microbial communities to utilise different C substrates was determined (community level physiological profiles (CLPP) analysis). Carbon substrate microtiter (MT) plates were made up by selecting 28 carbon sources (Figure 1.3) from those included in Ecoplate^{TM} from Biolog Inc. or recommended by Kennedy (1994). Individual carbon source stock solutions were prepared at a concentration of 10 %, filter-sterilised and added to saline medium (1.75 g K_2HPO_4, 0.5 g KH_2PO_4, 0,582 g NH_4Cl and 0.25 g $MgSO_4.7H_2O$ in 1 l deionised water, adjusted to pH 7.0) using the ratio 1:100 (v/v, C source:saline medium). The indicator TV was added as above (1:100 TV:media (v/v)). Each well of microtiter plates was filled by dispensing 150 µl of C substrate:saline medium-TV, and each set of carbon sources was replicated three times in a single 96 well MT plate. Wells contain-

ing saline medium-TV without C substrates were also prepared as controls. A ten-fold dilution of the first soil suspension (10^{-2}) was used to inoculate the MT plates (bulk and rhizosphere soils). The soil suspension was allowed to sediment for 2 hours and 50 µl of the supernatant were inoculated into each well of the MT plate. Substrate utilisation was indicated by colour development of the tetrazolium violet redox dye, and recorded after 1 week incubation at 25 ºC. The total number of C sources utilised, and the number of C sources utilised within each group of substrates (amines, amino acids, carbohydrates, carboxylic acids, glucose 1-phosphate, polymers), was determined.

Trace metal resistance of heterotrophic bacteria

Resistance of heterotrophic bacteria to Co, Cr or Ni was determined in bulk and rhizosphere soils by the MPN technique. Microtiter plates containing yeast-extract medium were supplemented with 1000 µM of either Ni (as $NiSO_4.6H_2O$), Co (as $CoCl_2.6H_2O$) or Cr (as CrO_4K_2) and inoculated as described above for culturable populations. A concentration of 1000 µM has been used to indicate metal resistance in previous studies (Nieto *et al.*, 1987).

Statistical analyses

Differences in soil physicochemical parameters and microbial densities between plant species and populations were determined using analyses of variance (ANOVA). Comparison of means was achieved by the Students *t* test for independent means (comparison of species) and related means (comparison of bulk/ rhizosphere soils). A multiple comparison of means was determined by the "post-hoc" Least Significance Difference test for unequal sample sizes. Data were log transformed where necessary to achieve homogeneity of variance. Regressions were carried out between densities of metal-tolerant microorganisms and soil water-extractable metal concentrations (Co_{H2O}, Cr_{H2O}, Ni_{H2O}).

1.3 Results

Bulk soil physicochemical characteristics

Physicochemical characteristics of the three study sites were typical of ultramafic soils (Table 1.1). All three soils presented pH values close to neutrality (pH_{H2O} 6.9-7.2) and were characterized by a predominance of Mg in the exchange complex. Both exchangeable Ca and Mg were significantly greater at the Portuguese sites compared to the Spanish site ($p < 0.05$) but Ca/Mg quotients were < 1 at all three

sites (S (0.57); M (0.33); L (0.29)). Soil organic C and N were generally low but significantly greater in S and M (3.0 %C and 0.3 %N) compared to L (1.3 %C and 0.1 %N). While the C:N ratio tended to be lower in the Portuguese soils.

Concentrations of Ni_T were significantly greater in the Portuguese sites (2724-3028 mg kg^{-1}) compared to L (2199 mg kg^{-1}). Total Co concentrations (Co_T) were similar at all three sites (148-176 mg kg^{-1}), while Cr_T was significantly greater at M compared to both S and L (up to 3-fold greater). EDTA-extractable concentrations of Ni and Co were 1.7- and 1.5-fold greater at S and M compared to L,

Table 1.1. Physicochemical characteristics of the soils of the three study sites: S and M (NE Portugal) and L (NW Spain). Within each row different letters indicate significant differences at $p < 0.05$.

	S	M	L
pH_{H2O}	6.9 ± 0.1 *a*	6.9 ± 0.1 *a*	7.2 ± 0.1 *a*
pH_{KCl}	6.3 ± 0.1 *a*	6.2 ± 0.1 *a*	6.0 ± 0.1 *a*
Organic C (%)	3.05 ± 0.24 *a*	3.00 ± 0.27 *a*	1.28 ± 0.11 *b*
Total N (%)	0.28 ± 0.02 *a*	0.26 ± 0.02 *a*	0.10 ± 0.01 *b*
C/N	10.8 ± 0.4 *a*	11.5 ± 0.3 *ab*	12.7 ± 0.6 *b*
Exchangeable cations ($cmol_c$ kg^{-1})			
Ca^{2+}	8.0 ± 0.7 *a*	5.4 ± 0.4 *b*	2.4 ± 0.3 *c*
Mg^{2+}	14.6 ± 0.8 *a*	16.5 ± 0.5 *a*	9.0 ± 0.6 *b*
Na^{+}	0.1 ± 0.0 *a*	0.2 ± 0.0 *a*	0.2 ± 0.0 *a*
K^{+}	0.5 ± 0.0 *a*	0.4 ± 0.0 *a*	0.2 ± 0.0 *b*
CEC	23.2 ± 1.1 *a*	22.4 ± 0.5 *a*	11.8 ± 0.3 *b*
Ca/Mg	0.6 ± 0.1 *a*	0.3 ± 0.0 *b*	0.3 ± 0.0 *b*
Total metal concentration (mg kg^{-1})			
Ni_T	3028 ± 121 *a*	2724 ± 40 *a*	2199 ± 185 *b*
Co_T	148 ± 3 *a*	176 ± 7 *b*	149 ± 12 *ab*
Cr_T	909 ± 66 *a*	2997 ± 279 *b*	1066 ± 60 *a*
EDTA-extractable metal concentration (mg kg^{-1})			
Ni_{EDTA}	189 ± 19 *a*	185 ± 7 *a*	109 ± 20 *b*
Co_{EDTA}	27 ± 1 *a*	29 ± 1 *a*	19 ± 2 *b*
Cr_{EDTA}	0.4 ± 0.0 *ab*	0.3 ± 0.0 *a*	0.4 ± 0.0 *b*
H_2O-soluble metal concentration (µg kg^{-1})			
Ni_{H2O}	401 ± 51 *a*	375 ± 49 *a*	90 ± 26 *b*
Co_{H2O}	5 ± 1 *ab*	6 ± 0 *a*	6 ± 1 *b*
Cr_{H2O}	93 ± 6 *a*	108 ± 9 *a*	203 ± 24 *b*

respectively. Mean concentrations of Ni_{EDTA}, Co_{EDTA} and Cr_{EDTA} represented 5-7 %, 13-19 % and < 0.05 % of Ni_T, Co_T and Cr_T, respectively.

In agreement with Ni_T and Ni_{EDTA}, water-soluble concentrations of Ni (Ni_{H2O}) were significantly greater in Portuguese soils (4 to 5-fold) than in the Spanish site (90.1 µg kg^{-1}). Co_{H2O} ranged between 5.3 and 6.1 µg kg^{-1}, and no differences were observed between the three sites. In contrast, Cr_{H2O} concentrations (93–203 µg kg^{-1}) were significantly greater (2-fold) in L compared to S and M (Table 1.1).

Metal extractability in the rhizosphere

A significant increase in concentrations of water-soluble Ni was observed in the rhizosphere of the Ni hyperaccumulator, *A. serpyllifolium*, but only in the Portuguese populations (S, $p < 0.01$; M, $p < 0.001$) (Figure 1.1). No differences were observed in bulk and rhizosphere soils of the L population of this species, and differences in bulk and rhizosphere concentrations were less important in all three populations of *D. glomerata*. Rhizosphere concentrations of water-soluble Co and Cr were either no different or lower, than corresponding bulk soils, and this was irrelevant of the plant species or population.

Microbial populations and C substrate utilisation

The highest densities of heterotrophic bacteria were associated with the two Portuguese populations of *A. serpyllifolium* and the L population of *D. glomerata*. Den-

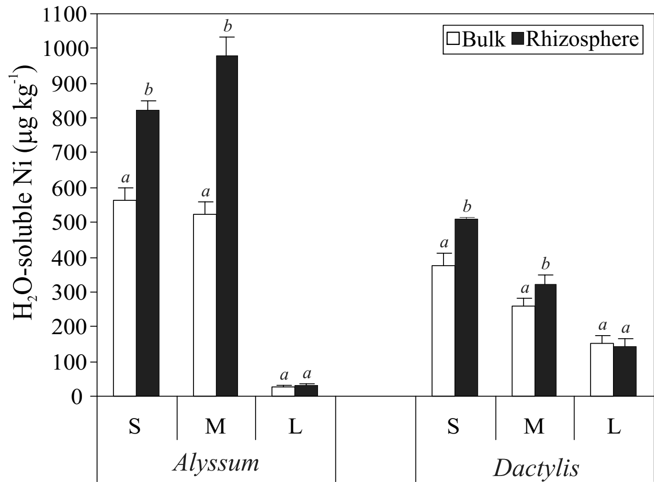

Figure 1.1. Concentrations of Ni in water extracts of bulk and rhizosphere soils of *Alyssum serpyllifolium* and *Dactylis glomerata* from the three ultramafic sites (S, M and L). Different letters indicate significant differences between bulk and rhizosphere soils ($p < 0.05$).

sities in bulk soils of the Portuguese populations of *A. serpyllifolium* were up to 1.5 logarithmic units greater than those of *D. glomerata*, while in L, densities in bulk soils of the hyperaccumulator were almost one logarithmic unit lower than in those of *D. glomerata* (Figure 1.2 and Table 1.2). Numbers of ammonifying and amylolytic microorganisms were generally of a similar magnitude, and species- and population-specific differences followed a similar pattern to that observed in total heterotrophs (Figure 1.2 and Table 1.2). In S and M mean densities of ammonifiers in bulk soils of *A. serpyllifolium* ranged from 5.9 x 10^5 - 1.4 x 10^6 g^{-1} soil and of *D. glomerata* from 1.3 x 10^4 – 4.3 x 10^4 g^{-1} soil, while corresponding densities in L were 1.7 x 10^5 g^{-1} soil (*A. serpyllifolium*) and 4.4 x 10^6 g^{-1} soil (*D. glomerata*). In S and M mean densities of amylolytics in bulk soils of *A. serpyllifolium* ranged from 7.1 x 10^5 – 8.5 x 10^5 g^{-1} soil and of *D. glomerata* from 1.1 x 10^4 – 1.3 x 10^4 g^{-1} soil, while corresponding densities in L were 1.6 x 10^5 g^{-1} soil (*A. serpyllifolium*) and 9.9 x 10^6 g^{-1} soil (*D. glomerata*). On the other hand, densities of cellulolytics were 2-4 logarithmic units lower (1.5 x 10^2 – 2.3 x 10^4 g^{-1} soil) than these groups and no differences were observed between species or populations.

In general, densities of heterotrophs, ammonifiers and amylolytics were significantly greater in the rhizosphere than corresponding bulk or nonvegetated soils (in many cases by more than one logarithmic unit; Figure 1.2). This rhizosphere effect (R/B and R/NV ratio) was generally observed for both species, although it was most apparent in *A. serpyllifolium* and especially in the M population (Table 1.3 and Figure 1.2). In contrast, the number of cellulolytics tended to decrease in the rhizosphere of both species compared to bulk soils: mean densities of cellulolytics in bulk soils of *A. serpyllifolium* ranged from 9.1 x 10^2 – 2.3 x 10^4 g^{-1} soil and of *D. glomerata* from 1.5 x 10^2 – 1.5 x 10^3 g^{-1} soil, while corresponding densities

Table 1.2. Effects of plant species (spp), population (pop) and soil type (bulk or rhizosphere soil) on microbial counts (MPN g^{-1} soil) of total heterotrophs, ammonifiers and amylolytics (results of three separate 3-way ANOVAs).

		Heterotrophs		Ammonifiers		Amylolytics	
	df	MS	F	MS	F	MS	F
spp	1	3.6	31.9^{***}	5.9	94.0^{***}	8.0	120.2^{***}
pop	2	1.5	13.0^{***}	2.6	41.5^{***}	3.6	53.8^{***}
soil type	1	3.9	34.2^{***}	3.8	61.5^{***}	3.1	47.1^{***}
spp* pop	2	5.8	50.9^{***}	11.2	179.2^{***}	15.5	233.8^{***}
spp*soil type	1	0.01	0.1	0.4	6.6^{*}	0.9	14.2^{**}
pop* soil type	2	0.4	3.2	0.1	1.3	0.1	1.7
spp*pop*soil type	2	0.1	0.9	0.1	0.9	0.5	7.4^{**}

$^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$

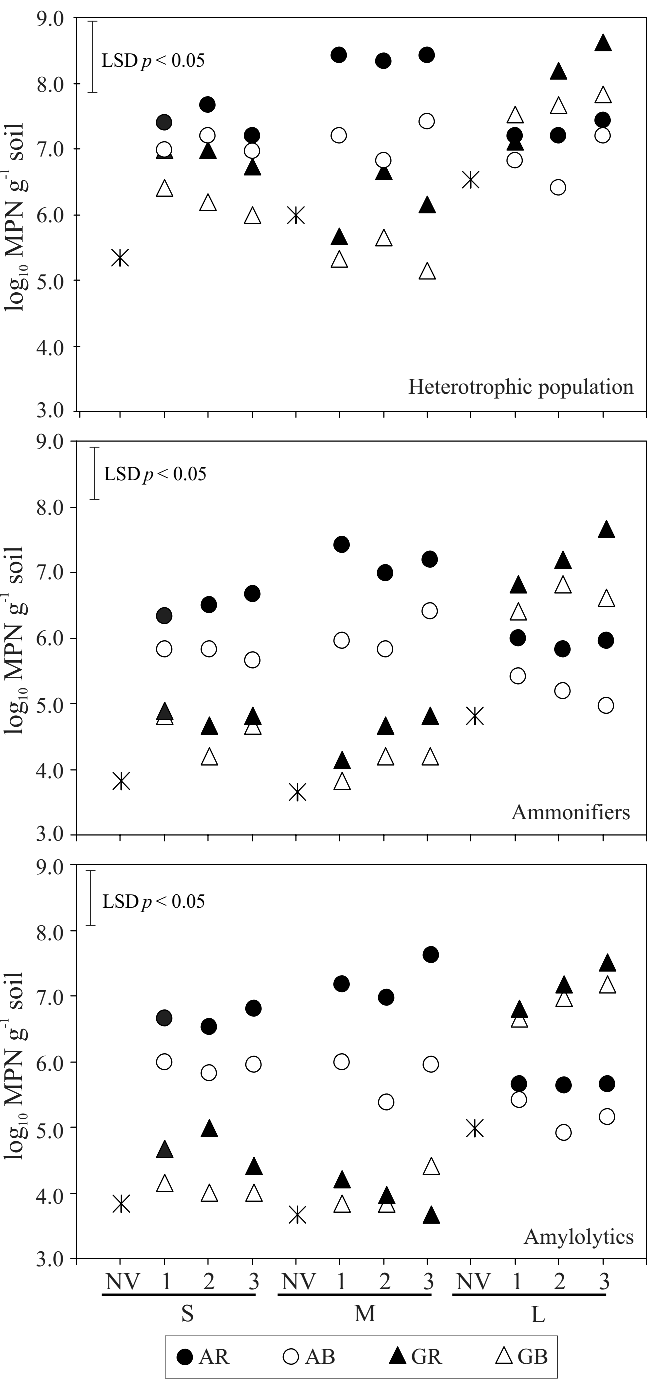

Figure 1.2. Microbial densities (MPN g^{-1} soil) of total heterotrophs, ammonifiers and amylolytics observed in nonvegetated soil (NV), and bulk (B) or rhizosphere (R) soils of three individuals of *Alyssum serpyllifolium* (A) and *Dactylis glomerata* (G) from the three ultramafic sites (S, M and L). Results are presented for 3 plant individuals from each population.

Table 1.3. The rhizosphere effect (R/B and R/NV ratio; number (NMP g^{-1} soil) of microorganisms in rhizosphere soil/number (NMP g^{-1} soil) of microorganisms in bulk or non vegetated soil) for each physiological group and plant species. For each site, different letters indicate significant differences between the two plant species ($p < 0.05$).

	S		M		L	
	Alyssum	*Dactylis*	*Alyssum*	*Dactylis*	*Alyssum*	*Dactylis*
Heterotrophic population						
R/B	2_a	5_b	20_a	8_a	3_a	3_a
R/NV	135_a	39_b	252_a	2_b	6_a	25_a
Ammonifiers						
R/B	6_a	2_a	16_a	3_a	6_a	5_a
R/NV	498_a	10_b	3717_a	9_b	13_a	342_a
Amylolytics						
R/B	6_a	5_a	35_a	1_b	3_a	2_a
R/NV	739_a	9_b	5025_a	2_b	5_a	191_a

in the rhizosphere were 1.5 x 10^2 – 1.5 x 10^3 g^{-1} soil and 7.0 x 10^1 – 1.5 x 10^3 g^{-1} soil.

After one week of incubation, microorganisms of bulk and rhizosphere soils were able to degrade almost all of the C substrates tested. Differences in degradation between species, populations and soil type (bulk or rhizosphere) were mostly observed for polymers and amino acids (Figure 1.3). In general, microorganisms in rhizosphere soils were able to degrade more C substrates than those in bulk soils. This rhizosphere effect was particularly evident in the hyperaccumulator *A. serpyllifolium* (Figure 1.3).

Metal tolerance of heterotrophic bacteria

Microbial densities of heterotrophs were higher in the presence of Ni, followed by Co and Cr, and this was irrelevant of soil type (non-vegetated, bulk or rhizosphere soil), plant species or population. In non-vegetated soils, Ni-, Co- and Cr-tolerant microorganisms represented up to 21 %, 10 % and 5 % of the total heterotrophic population, respectively. This percentage was increased by the presence of plants but only in the case of Ni-tolerant microorganisms: in some individuals there was no difference in densities between total heterotrophs and Ni-tolerant bacteria. In S and M, *A. serpyllifolium* consistently host a higher density of Ni-tolerant bacteria, and frequently a higher density of Co- and Cr-tolerant (only M) bacteria than *D. glomerata* (Table 1.4 and Figure 1.4). On the other hand, in L Ni- and Co-tolerant bacterial densities were greater in *D. glomerata* than *A. serpyllifolium* (plant spe-

cies x plant population interaction significant at $p < 0.01$; Table 1.4 and Figure 1.4).

Densities of metal-tolerant bacteria were significantly greater in the rhizosphere than in corresponding bulk or non-vegetated soils (Table 1.4 and Figure 1.4). This rhizosphere effect was generally more pronounced in Ni-tolerant bacteria associated with the hyperaccumulator *A. serpyllifolium*. Ratios of R/NV for Ni-tolerant bacteria of S, M and L populations of the hyperaccumulator were 207, 186 and 35, respectively (Table 1.5). In the Portuguese populations these R/NV ratios are 6- (S) and 69-fold (M) greater than corresponding ratios for *D. glomerata*, while in L they do not differ significantly between the two species. The highest R/

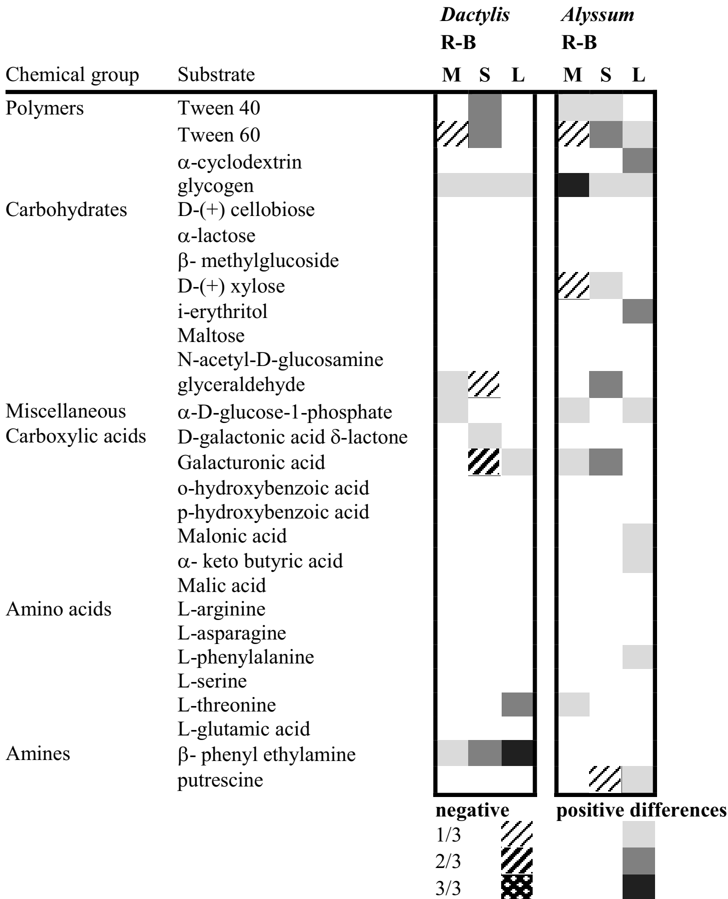

Figure 1.3. Differences in C substrate utilisation between bulk (B) and rhizosphere (R) soils in *Dactylis glomerata* and *Alyssum serpyllifolium* from the three study sites (S, M and L). Positive or negative differences are indicated by broken or full squares (trends observed for 1, 2 or 3 individuals out of a total of 3).

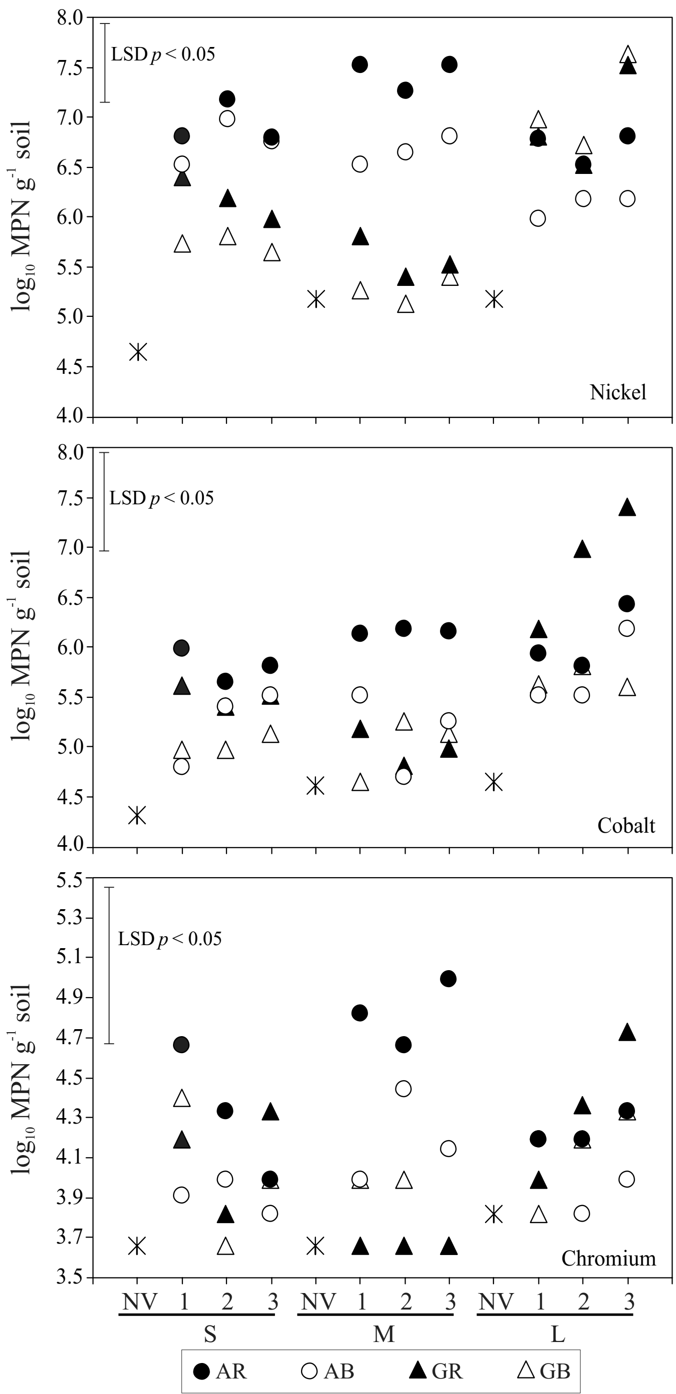

Figure 1.4. Microbial densities (MPN g^{-1} soil) of Co-, Cr- or Ni-tolerant heterotrophs observed in nonvegetated soil (NV), and bulk (B) or rhizosphere (R) soils of three individuals of *Alyssum serpyllifolium* (A) and *Dactylis glomerata* (G) from the three ultramafic sites (S, M and L). Results are presented for 3 plant individuals from each population.

Table 1.4. Effects of plant species (spp), population (pop) and soil type (bulk or rhizosphere soil) on Ni-tolerant, Co-tolerant and Cr-tolerant microorganisms (MPN g^{-1} soil) (results of three separate 3-way ANOVAs).

		Ni-tolerant		Co-tolerant		Cr-tolerant	
	df	MS	F	MS	F	MS	F
spp	1	3.6	54.9^{***}	0.3	3.1	0.4	7.8^{*}
pop	2	0.7	11.3^{***}	2.1	21.6^{***}	0.02	0.4
soil type	1	1.2	17.9^{***}	3.1	32.3^{***}	0.5	8.7^{**}
spp*pop	2	3.9	60.7^{***}	0.8	8.3^{**}	0.6	10.7^{***}
spp*soil type	1	0.2	3.3	0.02	0.2	0.3	5.9^{*}
pop*soil type	2	0.1	1.3	0.1	0.6	0.002	0.04
spp*pop*soil type	2	0.2	3.3	0.7	6.8^{*}	0.1	2.5

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

NV ratios of Co- and Cr-tolerant heterotrophs were also consistently found for the M population of *A. serpyllifolium* (R/NV ratios of 33-34 (Co) and 6-15 (Cr)).

1.4 Discussion

Soil physicochemical properties were similar at the two Portuguese sites: M showed somewhat lower values of exchangeable Ca and greater concentrations of Co_T and Cr_T compared to S. However, the plant-available concentrations of metals (EDTA-extractable and H_2O-soluble) did not differ significantly between these sites. Concentrations of Cr and Ni have been shown to vary considerably in the

Table 1.5. The rhizosphere effect (R/B and R/NV ratio; number (NMP g^{-1} soil) of microorganisms in rhizosphere soil/number (NMP g^{-1} soil) of microorganisms in bulk or non vegetated soil) for Co-, Cr– and Ni-tolerant heterotrophs for plant species. For each site, different letters indicate significant differences between the two plant species ($p < 0.05$).

	S		M		L	
	Alyssum	*Dactylis*	*Alyssum*	*Dactylis*	*Alyssum*	*Dactylis*
Ni-tolerant						
R/B	2_a	3_a	6_a	2_a	4_a	1_b
R/NV	207_a	37_b	186_a	3_b	35_a	32_a
Co-tolerant						
R/B	6_a	3_a	14_a	2_a	2_a	27_a
R/NV	33_a	16_a	34_a	2_b	31_a	270_a
Cr-tolerant						
R/B	3_a	1_a	5_a	1_b	2_a	2_a
R/NV	6_a	3_a	15_a	1_b	3_a	4_a

Trás-os-Montes region but these results fall within previously published values (Menezes de Sequeira and Pinto da Silva, 1992; Freitas *et al.*, 2004; Diez *et al.*, 2006). On the other hand, soil at the Spanish site (L) showed a significantly lower % of organic C and N, CEC, Ni_T, Ni_{EDTA}, Co_{EDTA} and Ni_{H2O} than both S and M soils, and significantly greater concentration of Cr_{H2O}. Soil properties for L were also in accordance with previous results for this region (López López and Guitián Ojea, 1981; Calvo de Anta *et al.*, 1987).

A higher density of heterotrophic bacteria would be expected in the rhizosphere of *D. glomerata* considering its more vigorous root system, which presumably provides a larger root surface area for microbial colonization and probably greater rhizodeposition. In fact mean concentrations of total dissolved organic C (1:2.5 soil:H_2O extraction) are generally greater in the rhizosphere of *D. glomerata* (117.6 ± 34.0 mg kg^{-1}) than *A. serpyllifolium* (59.1 ± 17.1 mg kg^{-1}) (García-Lestón *et al.*, 2007). Surprisingly, total bacterial densities were significantly greater in both bulk and rhizosphere soils of the hyperaccumulator, *A. serpyllifolium*. However, this was only observed in the two Portuguese populations of this species, and was particularly evident in M. These species- and population-specific differences were even more pronounced in ammonifying and amylolytic bacterial populations.

As expected, the rhizosphere hosted higher microbial densities than bulk soil (or non-vegetated soil), presumably in response to the presence of readily available carbon sources and growth factors present in root exudates and sloughed root cells. Densities of heterotrophic bacteria in the rhizosphere were up to 20- or 250-fold greater than corresponding bulk or non-vegetated soil. Carbon source utilisation also indicated a change in the CLPP in the rhizosphere compared to bulk/non-vegetated soil. The most pronounced rhizosphere effect and change in CLPP was observed in the Portuguese populations of the hyperaccumulator, and in particular M. Previous studies have also shown an increase in numbers of culturable bacteria at the rhizosphere of the Ni hyperaccumulator, *A. murale*, but not population-specific differences (Abou-Shanab *et al.*, 2003). This rhizosphere effect was also observed in amylolytics and ammonifiers, but not in cellulolytics. The lack of a rhizosphere effect in the cellulolytic population could be due to the fact that cellulose is not easily degradable and the majority of cellulase-producers are fungi. Easily labile C present in the rhizosphere could lead to preferential proliferation of bacteria over these cellulase-producers.

In this study, soil microorganisms showed a high tolerance to Ni, and low tolerance to Co and Cr. The soils of all three sites are characterised by low concen-

trations of water-soluble Co (less than 10 μg kg^{-1}) and it is therefore not surprising that the microbial community of these soils do not show a high degree of tolerance to toxic concentrations of available Co. The lack of tolerance to Cr is also expected given that the reduction of toxic Cr(VI) to less toxic Cr(III) is favoured under these soil conditions (Fendorf, 1995). It should be noted that in the culture medium, Cr was added as CrO_4K_2 (Cr(VI)), which will not be the dominating form under natural soil conditions. No significant correlation was observed between concentrations of Co or Cr in soil water extracts and corresponding microbial densities.

Only in the case of the Portuguese populations did we observe a significant positive relationship between densities of Ni-tolerant bacteria and concentrations of water-soluble Ni in soils (Figure 1.5). Species-specific differences in densities of Ni-resistant bacteria were also only found in S and M. In these populations, R/NV ratios of Ni-tolerant bacteria were up to two orders of magnitude greater for *A. serpyllifolium* than *D. glomerata*, indicating a selective enrichment of Ni-resistant bacteria in the rhizosphere of the hyperaccumulator. This has been shown for other hyperaccumulators such as *A. murale* (Abou-Shanab *et al.*, 2003). Recent studies on the hyperaccumulator *Thlaspi goesingense*, suggest that this plant is able to accumulate extreme concentrations of Ni due to the enhanced solubility of this metal in the rhizosphere. Moreover, this enhanced solubility is proposed to be the

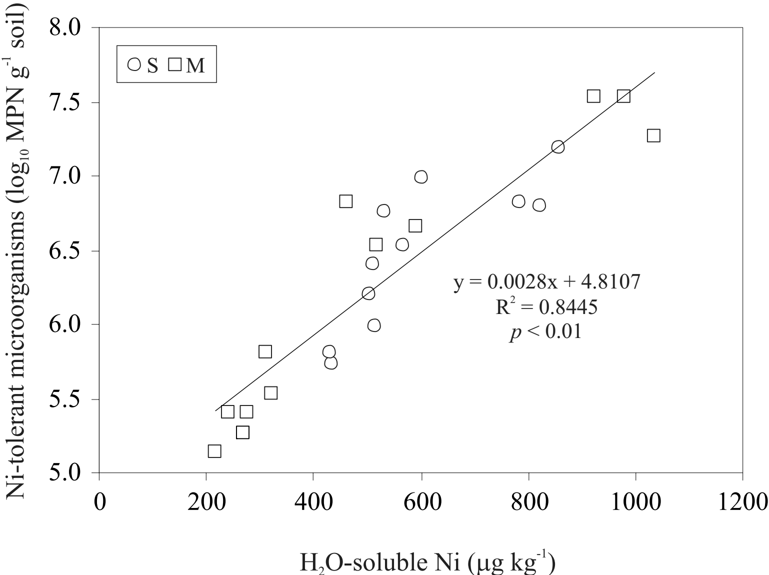

Figure 1.5. Linear regression between soil water-soluble Ni and densities of Ni-tolerant heterotrophs in Portuguese populations of *Alyssum serpyllifolium* and *Dactylis glomerata* (S and M).

result of ligand-induced dissolution of Ni-bearing ferromagnesium minerals (Wenzel *et al.*, 2003; Puschenreiter *et al.*, 2005). The release of organic ligands in *A. serpyllifolium* root exudates could also favour such metal dissolution, leading to an enhanced Ni solubility in the rhizosphere. The lower Ni concentrations observed in water extracts of soils from L could represent differences in exudates composition between Spanish and Portuguese populations, or differences in the potential weathering of the Ni-rich ferromagnesium minerals present at these sites. Alternatively, higher densities of Ni-resistant rhizobacteria could also induce mineral weathering and dissolution of Ni, explaining the significant increase in Ni_{H2O} concentrations in the rhizosphere of the two Portuguese populations of *A. serpyllifolium*. Bacteria have frequently been shown to increase the solubility of Mn, Mg and Fe (Berthelin *et al.*, 1995; Amir and Pineau, 2003). Enhanced release of Co and Ni has also been associated with the activity of specialized organotrophic microorganisms in ultramafic soils of New Caledonia (Amir and Pineau, 2003). The inoculation with *Microbacterium oxydans* AY509223, a rhizobacteria of *A. murale*, increased soil $Sr(NO_3)_2$-extractable Ni and foliar Ni concentrations in the hyperaccumulator by up to 72 % (Abou-Shanab *et al.*, 2006).

As expected the rhizosphere of both plant species hosted higher microbial densities compared to bulk soils, presumably in response to the presence of readily available carbon sources and growth factors present in root exudates and sloughed root cells. Significant differences in the microbial community (physiological groups and CLPP) were observed between the Ni-hyperaccumulator and non-accumulator. In addition, higher densities of Ni-resistant bacteria were observed in the rhizosphere of the hyperaccumulator which may play a role in Ni solubilisation. Moreover, this study shows important natural variation in this selective enrichment of Ni-resistant bacteria among different populations of the Ni-hyperaccumulator. Further studies of the rhizospheric community composition and structure, and the isolation of Ni-resistant bacterial strains from selected populations may lead to the production of inoculates for increasing the efficiency of phytoextraction strategies.

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Chapter 2

Nickel solubilising capacity and characterisation of rhizobacteria isolated from hyperaccumulating and non-hyperaccumulating subspecies of *Alyssum serpyllifolium*

ABSTRACT

Bacterial strains were isolated from the rhizosphere of three populations of the Ni-hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum* (*A. pintodasilvae*; M, S and L), one population of Ni-hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* (*A. malacitanum*; SB), and one population of the non-hyperaccumulator *A. serpyllifolium* subsp. *serpyllifolium* (*A. serpyllifolium*; SN). Isolates were characterized genotypically by BOX-PCR genomic DNA fingerprinting and comparative sequence analysis of partial 16S rRNA gene, and phenotypically by their Ni tolerance (0-10 mM), presence of plant growth-promoting traits (indoleacetic acid (IAA)-, siderophore-, or organic acid-production, and phosphate solubilisation) or capacity to produce biosurfactants. Among the collection of rhizobacteria, 84 strains were selected (according to their BOX-PCR profiles and phenotypic characteristics) to assess their ability to modify Ni extractability from Ni-rich (serpentine) soils. Metabolites produced by 13 of the isolates mobilised soil Ni (originating from the rhizosphere of both Ni-hyperaccumulators and non-hyperaccumulator). In contrast, Ni extraction using culture medium filtrates which had supported the growth of 29 strains was significantly reduced. The remaining strains had no effect on Ni mobility. Bacterial induced Ni mobilisation was not related to Ni resistance or the phenotypic traits tested.

This study formed part of the following publication:

Becerra-Castro C, Prieto-Fernández A, Álvarez-López V, Monterroso C, Cabello-Conejo M, Acea MJ, Kidd PS 2011. Nickel solubilizing capacity and characterization of rhizobacteria isolated from hyperaccumulating and non-hyperaccumulating subspecies of *Alyssum serpyllifolium*. *International Journal of Phytoremediation* 13 (supl. 1):229-244.

2.1 Introduction

The last two decades have seen the emergence of plant-based, gentle soil remediation techniques (phytoremediation). Recent reviews indicate a promising future for the use of these techniques in the remediation of contaminated soils (Vangronsveld *et al.*, 2009; Kidd *et al.*, 2009). Phytoextraction aims to remove trace elements from the soil through their uptake and accumulation by plants. Metal hyperaccumulating plants are the perfect candidates for the phytoextraction strategy given their extraordinary capacity for metal uptake (Baker *et al.*, 2000). Optimizing the plant-soil system can lead to a significant improvement in the overall efficiency of the process. Metal hyperaccumulators typically show a wide range of genetic variation in shoot metal concentration, and plant selection can be used to breed improved hyperaccumulator cultivars (Chaney *et al.*, 2007). Agronomic management practices (pH adjustment, fertilization etc.) can also further increase plant performance and biomass production (Li *et al.*, 2003). More recently, researchers have proposed the use of microbial inoculants to improve plant performance and Ni extraction (Abou-Shanab *et al.*, 2003; 2006).

Plant-associated microorganisms can improve the efficiency of phytoextraction techniques by (a), simply improving plant biomass production and hence metal extraction (total metal yield) (using plant growth-promoting rhizobacteria, PGPR); or (b), altering trace metal mobility and availability to the plant. A prominent mechanism used by many PGPR to facilitate plant growth is through the production of plant growth regulators and hormones (such as indoleacetic acid (IAA) cytokinins, gibberellins) or suppression of stress ethylene production through synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick *et al.*, 1998). Other known mechanisms include enhancing nutrient uptake (e.g. N_2 fixers, P solubilizers, siderophore-producers), production of antibiotics or cell wall lytic enzymes such as gluconases or chitinases to inhibit pathogens, and the induction of plant defense mechanisms (Lin *et al.*, 1983; Kapulnik *et al.*, 1996). Various mechanisms have been postulated by which microorganisms potentially alter trace metal bioavailability: production of organic acids; production of iron chelators and siderophores which may mobilize trace metals other than Fe; production of biosurfactants; metal dissolution by bacterial production of strong acids such as H_2SO_4 (e.g. *Thiobacillus*); production of ammonia or organic bases resulting in metal hydroxide precipitates; extracellular metal precipitation (e.g. with sulphate reducing bacteria); production of extracellular polysaccharides that can chelate metals; bio-

transformation via biomethylation, volatilization, oxidation or reduction (Vecchio *et al.*, 1998; Chen and Cutright, 2003). Inoculating *B*rassica *juncea* with the Ni-mobilizing rhizobacteria, *Psychrobacter* sp. SRA1 and *Bacillus cereus* SRA10, led to a significant increase in root and shoot Ni accumulation (Ma *et al.*, 2009). Nickel accumulation and yield was improved after inoculating the Ni-hyperaccumulator *Alyssum murale* with the Ni-resistant rhizobacteria *Microbacterium arabinogalactanolyticum* AY509224 (Abou-Shanab *et al.*, 2006). The authors suggested that production of siderophores and organic acids, and phosphate solubilisation, by the bacterial isolates facilitated soil Ni solubility.

The Ni-hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum* hosts higher proportions of Ni-resistant bacteria in the rhizosphere compared to non-hyperaccumulating plants or non-vegetated soil. Furthermore, this selective enrichment of Ni-resistant bacteria differs significantly between different populations of the hyperaccumulator (see Chapter 1). In this study, rhizobacteria were isolated from different populations of three subspecies of *A. serpyllifolium* (two of which are Ni-hyperaccumulators). Isolates were characterized genotypically (BOX -PCR, 16s rDNA) and phenotypically (Ni resistance, plant growth promotion, biosurfactant production), and evaluated for their ability to modify Ni extractability from Ni-rich soils. The global objective of the work was to identify candidate rhizobacterial isolates with potential to improve Ni extraction by hyperaccumulating subspecies of *A. serpyllifolium*.

2.2 Materials and methods

Study species

Two Ni-hyperaccumulating subspecies of *Alyssum serpyllifolium* Desf. (Brassicaceae) were studied: three populations of *A. serpyllifolium* subsp. *lusitanicum* Dudley and P. Silva (*A. pintodasilvae*) and one population of *A. serpyllifolium* subsp. *malacitanum* Rivas Goday (*A. malacitanum*). *Alyssum pintodasilvae* populations were sampled from three sites located in two serpentinitic areas in NW Iberian Peninsula (L, Melide (NW Spain); S, Samil (Trás-os-Montes, NE Portugal); and M, Morais (Trás-os-Montes, NE Portugal)). *Alyssum pintodasilvae* is the only Ni hyperaccumulator in NE Portugal and was first described as a Ni hyperaccumulator in 1969 (Menezes de Sequeira, 1969). The population of *A. malacitanum* (SB) was sampled from the serpentinitic area in Sierra Bermeja (Málaga, S Spain). Finally, one population of the non-hyperaccumulator *A. serpyllifolium* subsp. *ser-*

pyllifolium Desfontaines (*A. serpyllifolium*; SN) was sampled from the calcareous dolomitic area of Sierra Nevada (Granada, S Spain).

Soil physicochemical analysis and plant Ni accumulation

At each site (L, S, M, SB and SN) 5 replicate soil samples (0-10 cm) were taken, and analyses were carried out on the air-dried, < 2 mm fraction. Soil pH (in H_2O and KCl), organic C, total N and exchangeable cations were determined as described in Chapter 1. Total Ni concentration in soils were determined after digestion as described in Chapter 1. A metal fractionation scheme was carried out following the BCR protocol (Rauret *et al.*, 1999). First, soils were shaken at room temperature with 0.11 M CH_3COOH for 16 h. This extracts the water-soluble, exchangeable and carbonate-bound metal fraction (acid-extractable). Second, the resulting residue was shaken for 16 h at room temperature with 0.10 M $NH_2OH.HCl$ adjusted to pH 2.0 with high purity HNO_3. This step extracts mainly iron and manganese oxide bound forms (reducible). Third, the residue was digested with 30 % H_2O_2, taken to dryness on a water bath heated to 85 ºC, and shaken with 1 M NH_4OAc adjusted to pH 5.0 with HOAc for 16 h. This step extracts primarily organically bound and sulphide metals (“oxidisable” fraction). The residual fraction (silicate-bound metals) was digested as above, and the concentration of Ni was analysed in the filtered supernatants of each extraction by atomic absorption spectroscopy (AAS) (Perkin-Elmer 2380, Norwalk, CT).

Plant aerial biomass was collected from 15 random individuals of each plant population (L, S, M, SB, SN). Leaves and stems were separated, washed in deionized water, oven-dried at 45 ºC, weighed and ground. Plant tissues (0.1g) were digested in a 2:1 HNO_3:HCl mixture on a hot plate at 160ºC and the concentration of Ni measured as above.

Isolation of rhizobacteria associated with different subspecies of *A. serpyllifolium*

The whole plant and root system (including root ball) of 5-10 individuals of *A. serpyllifolium* were collected from each population. Plants of a similar size were sampled, and all of them were in the flowering stage. Operationally defined rhizosphere soil was separated by gently crushing the root ball and shaking the root system. Tightly held soil (< 3 mm from the root surface) was considered rhizosphere soil. Five grams of fresh rhizosphere soil were suspended in 45 ml sterile sodium hexametaphosphate solution (1 %) and shaken for 30 min in an end-over-

end shaker. Soil suspensions were diluted in 10-fold series and plated in duplicate onto 284 agar medium (Schlegel *et al.*, 1961) supplemented with 100 mg ml^{-1} of the fungicide cycloheximide. The 284 medium contains (per liter medium): 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.04 g $Na_2HPO_4.2H_2O$, 10 ml Fe(III)NH_4 citrate solution (containing 48 mg/100ml) plus oligoelements (1.5 mg $FeSO_4.7H_2O$, 0.3 mg H_3BO_4, 0.19 mg $CoCl_2.H_2O$, 0.1 mg $MnCl_2.4H_2O$, 0.08 mg $ZnSO_4.7H_2O$, 0.02 mg $CuSO_4.5H_2O$, 0.036 mg $Na_2MoO_4.2H_2O$) adjusted to pH 7. The medium was supplemented with a mixture of different carbon sources: lactate (0.7 g l^{-1}), glucose (0.5 g l^{-1}), gluconate (0.7 g l^{-1}), fructose (0.5 g l^{-1}) and succinate (0.8 g l^{-1}). After 7 days incubation at 28 °C distinct morphotypes (1-5 colonies) associated with each of the five populations of *A. serpyllifolium* (L, S, M, SB, SN) were sub-cultured at least three times to ensure purity and cryopreserved at -70 °C in culture medium supplemented with 15 % (v/v) glycerol.

Phenotypic characterization

Rhizobacterial strains were screened for Ni resistance, the ability to produce biosurfactants, and for various plant growth promoting characteristics: phosphate solubilisation capacity, siderophore production, organic acid production and indoleacetic acid (IAA) production.

Nickel resistance of the strains was tested using 284 agar medium (see above) supplemented with an increasing concentration of Ni (0 mM, 1.0 mM, 2.5 mM, 5.0 mM, 10.0 mM; added as $NiSO_4.6H_2O$) and incubated at 28 °C for 7 d. The Maximal Tolerable Concentration (MTC) of Ni was recorded for each isolate, as the highest Ni concentration tested where the isolate was able to grow. The ability to solubilise inorganic phosphate was assessed in a modified NBRIP agar medium (1.8 %) supplied with 5 g l^{-1} of hydroxyapatite and incubated at 28 °C for 5 d (10.0 g glucose, 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 0.1 g yeast extract in 1 l deionized water adjusted to pH 7.0; modified from Nautiyal, 1999). A clear halo around the bacterial colony indicated solubilisation of mineral phosphate. Yeast extract was added since some strains were unable to grow in yeast-free NBRIP medium. Siderophore production was detected in a modified 284 liquid medium (without Fe) using the Chrome Azurol S (CAS) method described by Schwyn and Neilands (1987). All glassware used in this assay was previously cleaned with 30 % HNO_3 followed by washing in distilled water (Cox, 1994). Each isolate was screened for acid production. Single colonies were plated

on agar medium containing 0.002 % bromocresol purple (per liter medium): 10.0 g glucose, 1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.03 g $CaCl_2.2H_2O$. Colonies forming a yellow halo after 1 d growth at 28 ºC indicated a pH change in the medium and were considered acid producers. The ability of isolated strains to produce IAA were evaluated in liquid medium (5.0 g glucose, 1.0 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 0.5 g $CaCO_3$, 0.5 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.1 g yeast extract adjusted to pH 7; modified from Sheng *et al.*, 2008) supplemented with 0.5 mg ml^{-1} of tryptophan. After 5 d incubation at 28 ºC, cultures were centrifuged and the presence of IAA was evaluated after incubating 1 ml of the supernatant with 2 ml of Salkowski's reagent (50 ml 35 % $HClO_4$ + 1 ml 0.5 M $FeCl_3$) for 25 min. The production of IAA was recognized by the presence of red coloring and evaluated spectrophotometrically (535 nm) using pure IAA as a standard calibration curve. Strains were screened for potential biosurfactant production using the qualitative method of Chen *et al.* (2007). The strains were inoculated in 284 liquid medium and cultured overnight at 28 ºC, at 150 rpm on a rotary shaker. A 100 µl sample was taken from the supernatant of each strain and added to a microwell of a 96-microwell plate. The plate was then viewed using a backing sheet of paper with a black and white grid. The optical distortion of the grid provided a qualitative assay for the presence of surfactants.

Genotypic characterization

BOX-PCR genomic DNA profiling

BOX-PCR fingerprinting was used to genotype and group bacterial strains within each isolate collection (L, S, M, SB, SN). Crude cell lysates (colonies suspended in 100 µl and heated to 100 ºC for 5 min) were used as DNA templates for BOX-PCR reactions. BOX reactions were performed in a total volume of 20 µl containing: 1x Taq buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 U Taq polymerase (Invitrogen), 2 µM of BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic *et al.*, 1994), and 2 µl of cell lysate. Thermocycling conditions were: 1 cycle of 94 °C for 5 min; 35 cycles of 1 min at 94 ºC; 1.5 min at 50 ºC and 8 min at 68°C; and 1 cycle of 8 min at 68 ºC. The obtained PCR products were separated by gel electrophoresis in 1.8 % agarose run for 3 h at 3.3 V cm^{-1} gel. Gel images were analyzed, using the Pearson correlation coefficient and UPGMA clustering algorithm of the Gel Compar Bionumerics program (Bionumerics Version 5.1, Applied Maths, Belgium).

DNA extraction and 16S rRNA gene amplification

Only those rhizobacterial isolates found to influence Ni extraction (see below) were identified using 16S rDNA sequencing. For DNA extraction, purified strains were grown in 1/10 strength 869 liquid medium (1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.1 g glucose, 0.035 g $CaCl_2.2H_2O$ in 1 l deionized water) (Mergeay *et al.*, 1985) and genomic DNA was extracted from bacterial cell pellets. Briefly, the method consists of alkaline cell lysis followed by phenol/chloroform/isopropanol alcohol purification. DNA quality was checked by gel electrophoresis on a 0.8 % agarose gel. PCR amplification targeting the 16S rRNA gene was carried out using the primers 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-TACGGYTACCTTGTTA CGACTT-3') (Lane, 1991). PCR reactions were performed in a total volume of 50 µl containing: 1x Taq buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1.75U Taq polymerase (Invitrogen), 0.4 µM of each primer, and 1 µl of extracted DNA. Thermocycling conditions were: 2 min at 94 °C; 30 cycles of 1 min at 94 °C; 1 min at 55 °C and 2 min at 72 °C; and 1 cycle of 10 min at 72 °C. PCR products were partially sequenced (approximately 750 bases) using the primer 16S-27F (Lane, 1991). Sequence data were checked using the Chromas v. 1.45 software (Technelysium Pty. Ltd., Australia) and assessed for similarity with sequences of the Ribosomal Database Project (RDP; (Cole *et al.*, 2009). Since several isolates were closely related to the *Arthrobacter* genus, their sequences and those of the nearest neighbors found in public databases were aligned using Clustal W (Thompson *et al.*, 1994) and phylogenetics analyses were performed using MEGA version 4 (Tamura *et al.*, 2007). The neighbor-joining tree was constructed based on the Maximum Composite Likelihood method (Tamura *et al.*, 2004). Bootstrap values were calculated from 2000 replicated analyses. The sequences used for identification of the cultivable strains are available in the EMBL database (www.ebi.ac.uk) under accession numbers FN908759 through to FN908797.

Soil Ni extraction using bacterial metabolites

The potential capacity of the bacterial isolates to mobilise Ni from a Ni-rich serpentine soil (M, Table 2.1) was evaluated. A reduced number of isolates were used for this experiment. This selection was made according to BOX-PCR profiles, MTCs and phenotypic traits. Within each collection, isolates were selected so as to represent the main BOX-PCR profiles and where possible the entire range of MTCs. For each MTC, isolates showing different PGP traits were included when-

ever possible. Bacterial strains were grown to early stationary phase in 1/10 strength 869 medium (see above). Five milliliters of this culture was used to inoculate 50 ml of 1/10 strength 869 medium in 100 ml Erlenmeyer flasks (10^7-10^8 CFUs/ml). Additional flasks of medium were prepared as sterile (axenic) controls. The bacterial cultures and control flasks were incubated at 28 °C on an orbital shaker at 180 rpm until early stationary phase. Thereafter, culture media were centrifuged at 6000 g for 10 min; the supernatant decanted and vacuum filtered (0.22 µm pore size). The pH and Eh of the filtrate were determined prior to soil extraction. The ability of the filtrate to extract Ni was determined by shaking three replicate 0.1 g samples of soil with 10 ml of each of the bacterial filtrates or axenic filtrate for 2 h in an end-over-end shaker. Soil suspensions were centrifuged at 10000 g for 10 min and filtered (0.45 µm pore size), and the concentration of Ni, Fe, Mg and K in the HNO_3 acidified filtrate were determined by atomic absorption spectroscopy (Perkin-Elmer 2380, Norwalk, CT), and P by colorimetry using the Murphy-Riley method (Murphy and Riley, 1962). For each strain two early stationary cultures were prepared and from each culture three 10 ml aliquots were used for soil extractions.

Statistical analyses

Differences between Ni extracted with the filtrates of bacterial cultures and Ni extracted with sterile controls were determined by the Students *t* test for independent means.

2.3 Results

The four ultramafic soils (L, S, M, SB) presented pH values close to neutrality, a predominance of Mg in the exchange complex, and Ca/Mg quotients of < 1. In contrast, SN soils presented alkaline pH values and Ca dominated the cation exchange complex. Total Ni concentrations of up to 3028 mg kg^{-1} were determined; values were similar in the Portuguese sites (S and M) and significantly lower in the L and SB soils. As expected, Ni concentrations were minimal in the SN soil. Nickel fractionation indicates the accumulation of Ni in the residual fraction, and somewhat lower concentrations of labile Ni in the M and SB soils (Table 2.1).

A total of 75, 51 and 75 rhizobacteria were isolated from the L, M and S populations of *A. pintodasilvae*; 95 rhizobacteria were isolated from the SB population of *A. malacitanum*; and finally, 50 rhizobacteria from the SN population of the non-hyperaccumulator *A. serpyllifolium*. Eighty four isolates (10-20 from each

Table 2.1. Leaf Ni accumulation and physicochemical characteristics (mean ± SE) of the soils where the different populations of *Alyssum serpyllifolium* were growing: S and M (Samil and Morais, NE Portugal), L (Melide, NW Spain), SB and SN (Sierra Bermeja and Sierra Nevada, S Spain).

	S	M	L	SB	SN
Leaf Ni concentration (mg kg^{-1})					
	11022 ±1169	10879 ±744	15464 ±719	10391 ±774	n.d.
Soil physicochemical properties					
pH_{H2O}	6.9 ±0.1	6.9 ±0.1	7.2 ±0.1	7.4 ±0.0	8.0 ±0.0
pH_{KCl}	6.3 ±0.1	6.2 ±0.1	6.0 ±0.1	5.6 ±0.0	7.2 ±0.0
Total C (%)	3.05 ±0.24	3.00 ±0.27	1.28 ±0.11	2.42 ±0.10	9.73 ±0.05
Total N (%)	0.28 ±0.02	0.26 ±0.02	0.10 ±0.01	0.21 ±0.00	0.46 ±0.01
C/N	10.8 ±0.4	11.5 ±0.3	12.7 ±0.6	11.5 ±0.4	21.2 ±0.2
CEC ($cmol_c$ kg^{-1})	23.2 ±1.1	22.4 ±0.5	11.8 ±0.3	25.9 ±0.5	23.9 ±0.5
Ca/Mg	0.6 ±0.1	0.3 ±0.0	0.3 ±0.0	0.5 ±0.0	2.9 ±0.0
Total Ni (mg kg^{-1})	3028 ±121	2724 ±40	2199 ±185	2098 ±26	32 ±3
BCR Ni fractionation (mg kg^{-1})					
Acid extractable	72 ±6	59 ±5	71 ±16	42 ±7	0.3 ±0.1
Reducible	272 ±23	214 ±12	228 ±22	215 ±6	0.5 ±0.3
Oxidisable	322 ±7	191 ±18	234 ±23	332 ±11	2.6 ±0.0
Residual	2370 ±90	2260 ±37	1656 ±142	1567 ±78	28.7 ±3.3

n.d., not detectable

collection) were assessed for their ability to mobilise Ni, their BOX-PCR profiles and phenotypic characteristics are presented in Table 2.2. None of the tested isolates were able to produce biosurfactants, and the most common traits observed were the secretion of organic acids and production of IAA (37-39 % of isolates), followed by siderophore production (24 % of isolates). Only 3 isolates were able to solubilize P (SA40, SBA68, SBA70) (Table 2.2).

Sterile 869 medium (1/10 strength) had a pH of 6.5 and Eh of 293 mV, and extracted 59 mg Ni kg^{-1} (representing the acid-extractable, or labile, Ni pool of the M soil; Table 2.1). Bacterial growth always led to an increase in the pH of the culture medium, and in general, to a reduction in redox potential. The pH and Eh values of bacterial cultures at the early stationary phase ranged from 6.8-8.1 and 201-294 mV. However, there was no correlation between Ni extraction and medium pH or Eh (Figure 2.1). The cell-free bacterial cultures of 13 of the tested strains led to Ni mobilisation (Figure 2.2). In contrast, Ni extraction was reduced using filtered media which had supported the growth of 29 of the rhizobacterial strains tested (Figure 2.2). The remaining 42 strains did not significantly affect Ni extraction.

Table 2.2. Rhizobacterial isolates used for the Ni mobilisation study, their BOX-PCR profiles and phenotypic characteristics (Ni resistance and plant growth-promoting traits). MTC, Maximal Tolerable Concentration mM; P, phosphate solubilisation; Sd, siderophore production; OA, organic acid production; IAA, indoleacetic production in mg l^{-1}.

Isolate	Box profile	Ni MTC	P	Sd	OA	IAA
Morais						
MA105		0	-	-	-	-
MA66		0	-	-	-	9.6
MA67		0	-	-	-	5.4
MA106		0	-	-	+	-
MA73		1	-	-	-	-
MA87		2.5	-	-	-	-
MA61		2.5	-	-	-	4.6
MA62		2.5	-	-	-	6.2
MA63		2.5	-	-	-	4.6
MA64		2.5	-	-	-	7.6
MA100		2.5	-	-	-	5.8
MA102		2.5	-	-	+	-
MA89		2.5	-	-	+	11.9
MA92		5	-	-	-	-
MA69		5	-	-	+	6.9
MA79		10	-	-	-	-
MA82		10	-	-	-	-
MA77		10	-	-	-	4.8
MA78		10	-	-	-	5.2
Samil						
SA43		0	-	-	-	4.7
SA56		1	-	-	-	-
SA62		1	-	-	-	-
SA7		1	-	-	-	4.9
SA38		2.5	-	-	-	-
SA11		2.5	-	-	-	4.1
SA34		2.5	-	-	-	4.9
SA36		2.5	-	-	-	5.2
SA5		2.5	-	-	+	-
SA33		2.5	-	-	+	6.0
SA57		2.5	-	+	-	-
SA73		2.5	-	+	-	4.2
SA17		2.5	-	+	+	-
SA19		2.5	-	+	+	-
SA20		2.5	-	+	+	-
SA22		2.5	-	+	+	-
SA26		2.5	-	+	+	-
SA40		2.5	+	+	-	7.6
SA28		5	-	-	+	-
SA77		10	-	+	-	-
SA18		10	-	+	+	6.6

Isolate	Box profile	Ni MTC	P	Sd	OA	IAA
Melide						
LA18		0	-	-	-	9.6
LA23		0	-	-	-	7.5
LA41		0	-	-	-	7.2
LA11		1	-	-	-	-
LA51		1	-	-	-	6.4
LA27		1	-	-	+	9.6
LA75		1	-	-	+	-
LA85		2.5	-	-	-	-
LA10		2.5	-	-	+	16.9
LA45		2.5	-	-	+	-
LA31		5	-	-	-	-
LA56		5	-	-	-	-
LA60		5	-	+	-	-
LA1		5	-	+	-	8.8
LA2		5	-	-	+	-
LA4		5	-	-	+	-
LA7		5	-	-	-	-
LA66		5	-	-	-	-
LA44		10	-	-	+	81.7
LA80		10	-	-	-	89.6
Sierra Bermeja						
SBA44		0	-	+	+	-
SBA29		1	-	-	+	15.2
SBA7		2.5	-	+	-	-
SBA3		2.5	-	+	+	-
SBA86		2.5	-	+	+	-
SBA52		5	-	-	+	-
SBA68		5	+	-	-	-
SBA5		5	-	-	-	39.5
SBA50		10	-	-	-	-
SBA55		10	-	-	-	-
SBA103		10	-	-	-	-
SBA107		10	-	-	-	-
SBA70		10	+	-	-	-
Sierra Nevada						
SNA69		0	-	-	-	-
SNA111		0	-	-	-	-
SNA116		0	-	-	-	-
SNA81		0	-	-	-	4.6
SNA89		0	-	+	-	-
SNA93		0	-	-	+	-
SNA94		0	-	-	+	-
SNA87		0	-	-	+	-
SNA72		0	-	+	+	-
SNA77		0	-	+	+	4.8
SNA92		0	-	+	+	-

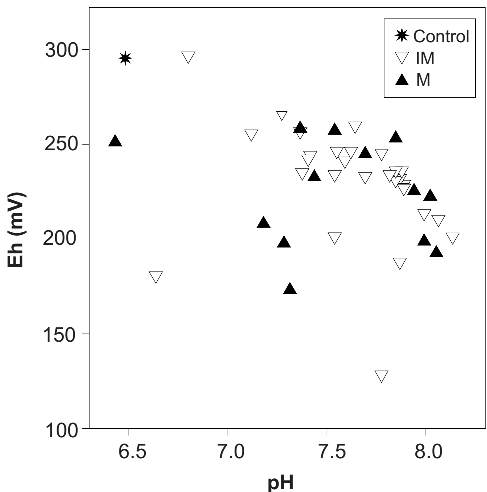

Figure 2.1. The pH and redox potential (Eh, mV) of cell-free bacterial cultures which significantly altered soil Ni extraction. IM, Ni-immobilisers; M, Ni-mobilisers.

The phylogenetic affiliation (according to comparative analysis of partial 16S DNA sequence) of those strains shown to alter Ni bioavailability (either increase or decrease Ni extractability) is given in Table 2.3.

Ni-mobilising bacterial strains increased Ni extraction to values 1.1- to 1.5-fold greater than those obtained using sterile culture medium (Figure 2.2), and the amount of Ni extracted was higher than the labile (acid-extractable) fraction found in the tested soil (Table 2.1). Several of the Ni-mobilising strains were identified as *Streptomyces* sp. (strains LA66, SA38, SBA50), while others included members of the genera *Arthrobacter*, *Curtobacterium*, *Microbacterium* and *Pseudomonas* (Table 2.3). The majority of these Ni-mobilizing isolates originated from the rhizosphere of the S and L populations of *A. pintodasilvae* (SA5, SA26, SA28, SA38, SA62, LA7, LA66, LA75 and LA85). No strains associated with the M population of *A. pintodasilvae* were found to solubilise Ni. Two strains isolated from rhizosphere soil of *A. malacitanum* increased Ni extraction from 59.0 mg kg^{-1} to 67.3 and 69.2 mg kg^{-1} (SBA44 and SBA50). Surprisingly, two strains isolated from the rhizosphere soil of the non-hyperaccumulator (*A. serpyllifolium*) also solubilised soil Ni (extracted Ni increased from 59.0 mg kg^{-1} to 64.3 mg kg^{-1} (SNA92) and 72.3 mg kg^{-1} (SNA116)) (Figure 2.2). These two strains originate from calcareous soils and, as expected, showed no resistance to Ni (Tables 2.1 and 2.2). Nickel resistance of the remaining strains ranged from 1 to 10 mM (the highest Ni concentration tested); however, there was no significant correlation between the level of Ni resistance and Ni-solubilising capacity. *Streptomyces* SBA50 showed the high-

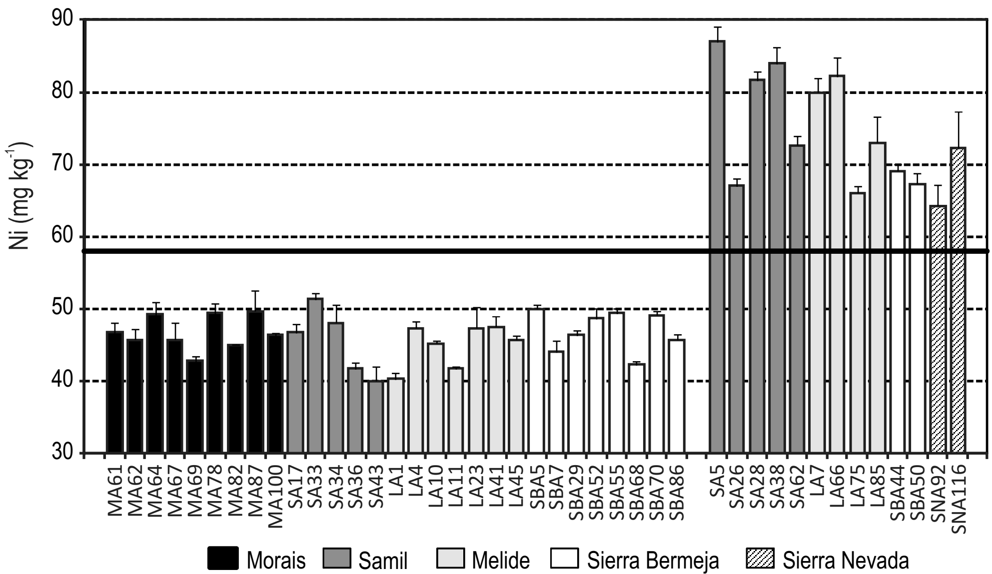

Figure 2.2. Concentrations of Ni (mg kg^{-1}) extracted from ultramafic soil using cell-free rhizobacterial cultures (mean ± SE). Soil was shaken with filtrates of bacterial cultures (early stationary phase in 1/10 strength 869 medium). Only those strains significantly altering Ni extraction compared to controls are presented ($p < 0.05$).

est Ni resistance (MTC of 10 mM) but one of the lower Ni solubilising capacities, while the highest Ni extraction was obtained using filtrates of *Arthrobacter oxydans* SA5 (99.9 % similarity) with an MTC of 2.5 mM Ni (Tables 2.2 and 2.3). Strains SA5, SA26, SA28, SBA44 and SNA92 were able to produce organic acids, and strains SA26, SBA44 and SNA92 also secreted siderophores. The production of the phytohormone IAA was not observed in any of the strains capable of mobilising soil Ni, while this trait was found in almost all remaining strains (Table 2.2).

The majority of the potentially Ni-immobilising strains belonged to the genus *Arthrobacter*, 17 of these strains were members of this genus (Table 2.3). However, most of them were clearly separated phylogenetically from Ni-mobilising strains. Moreover, strain SA5 (identified as *Arthrobacter oxydans*; 98.9 % similarity) which mobilised the most soil Ni (87 mg kg^{-1}) was distant from all of the Ni-immobilising strains (Figure 2.3). Other isolates within this group were affiliated with the genera *Bacillus*, *Curtobacterium*, *Microbacterium*, or *Phyllobacterium* (Table 2.3). These isolates originated from the rhizosphere of both the Ni hyperaccumulating *A. serpyllifolium* subspecies (*A. pintodasilvae* and *A. malacitanum*). In contrast, none of the Ni-immobilising strains were isolated from the rhizosphere soil of the non-hyperaccumulator (*A. serpyllifolium*). As observed for the

Table 2.3. Partial 16S rDNA sequence identification of rhizobacterial isolates significantly influencing soil Ni extraction. Act., actinobacteria; Bact., bacteroidetes; Prot., proteobacteria; Firm., firmicutes.Aminimum fragment size of 750 bp were used for strain identification

Isolate	Phylum	Most similar type strain (% identity)	Most similar strain (% identity)
MA61	Act.	*Arthrobacter globiformis* X80736 (99.7)	*A. globiformis* AY167856 (99.8)
MA62	Act.	*Arthrobacter nicotinovorans* X80743 (99.8)	*A. nicotinovorans* GQ284330 (99.9)
MA64	Act.	*Arthrobacter globiformis* X80736 (99.7)	*A. globiformis* AY167856 (99.8)
MA67	Act.	*Arthrobacter nitroguajacolicus* AJ512504 (99.9)	*A. aurescens* DQ016989 (99.9)
MA69	Firm.	*Bacillus megaterium* D16273 (99.4)	*Bacillus sp.* FN423794 (100)
MA78	Act.	*Arthrobacter nitroguajacolicus* AJ512504 (99.8)	*A. boritolerans* AB288059 (100)
MA82	Act.	*Arthrobacter nitroguajacolicus* AJ512504 (100)	*Arthrobacter sp.* FN293207 (100)
MA100	Act.	*Arthrobacter globiformis* X80736 (99.7)	*A. globiformis* AY167856 (99.8)
SA5	Act.	*Arthrobacter scleromae* AF330692 (99.8)	*A. oxydans* EU086811 (99.9)
SA17	Act.	*Microbacterium hydrocarbonoxydans* AJ698726 (99.7)	*M. hydrocarbonoxydans* EU373354 (99.9)
SA26	Act.	*Curtobacterium flaccumfaciens* AJ312209 (98.4)	*C. flaccumfaciens* AJ310414 (98.4)
SA28	Act.	*Curtobacterium flaccumfaciens* AJ312209 (100)	*C. flaccumfaciens* AJ310414 (100)
SA33	Firm.	*Bacillus megaterium* D16273 (99.6)	*Bacillus sp.* DQ323079 (100)
SA34	Act.	*Arthrobacter nitroguajacolicus* AJ512504 (99.7)	*Arthrobacter sp.* FN293207 (100)
SA36	Act.	*Arthrobacter nicotinovorans* X80743 (99.8)	*Arthrobacter sp.*AJ785759 (100)
SA38	Act.	*Streptomyces olivochromogenes* AY094370 (99.8)	*S. lincolnensis* X79854 (99.5)
SA43	Act.	*Curtobacterium flaccumfaciens* AJ312209 (99.1)	*Curtobacterium sp.*DQ238838 (99.3)
SA62	Act.	*Microbacterium oxydans* Y17227 (99.0)	*Microbacterium sp.* AY864634 (99.2)
LA1	Act.	*Arthrobacter nicotinovorans* X80743 (99.0)	*Arthrobacter sp.* AY5767707 (100)
LA4	Act.	*Arthrobacter defluvii* AM409361 (100)	*Arthrobacter sp.* AY581444 (100)
LA7	Bact.	*Chitinophaga ginsengisegetis* AB264798 (98.4)	*Ch. arvensicola* AM237312 (98.4)
LA10	Act.	*Arthrobacter defluvii* AM409361 (99.7)	*Arthrobacter sp.* DQ519082 (99.8)
LA11	Prot.	*Phyllobacterium myrsinacearum* AY785315 (99.0)	*P. myrsinacearum* EU169173 (99.7)
LA23	Act.	*Amycolatopsis coloradensis* AJ293753 (99.6)	*Amycolatopsis sp.* FJ581021 (99.8)
LA41	Prot.	*Devosia neptuniae* AF469072 (98.7)	*D. yakushimanensis* AB361068 (97.0)
LA45	Act.	*Arthrobacter defluvii* AM409361 (97.4)	*Arthrobacter sp.* AB242641 (99.4)
LA66	Act.	*Streptomyces prunicolor* DQ026659 (99.6)	*Streptomyces sp.* EU098034 (99.7)
LA75	Act.	*Arthrobacter defluvii* AM409361 (99.6)	*Arthrobacter sp.* AY581444 (100)
LA85	Act.	*Arthrobacter nitroguajacolicus* AJ512504 (100)	*Arthrobacter sp.* FN293207 (99.9)
SBA5	Act.	*Curtobacterium flaccumfaciens* AJ312209 (99.6)	*C. flaccumfaciens* AJ310414 (99.6)
SBA7	Act.	*Arthrobacter globiformis* X80736 (99.6)	*Arthrobacter sp.* AY581444 (100)
SBA29	Act.	*Arthrobacter globiformis* X80736 (99.8)	*Arthrobacter sp.* EF599994 (100)
SBA44	Act.	*Arthrobacter defluvii* AM409361 (99.9)	*Arthrobacter sp.* AY581444 (100)
SBA50	Act.	*Streptomyces lincolnensis* AB184279 (99.6)	*S. lincolnensis* X79854 (99.6)
SBA68	Act.	*Rhodococcus globerulus* X80619 (99.5)	*R. globerulus* X81931 (99.6)
SBA70	Act.	*Rhodococcus erythropolis* X79289 (100)	*Rhodococcus sp.* AJ786781 (100)
SBA86	Act.	*Arthrobacter nitroguajacolicus* AJ512504 (100)	*A. aurescens* DQ016989 (100)
SNA92	Firm.	*Bacillus simplex* AJ439078 (100)	*Bacillus simplex* AJ6287746 (100)
SNA116	Prot.	*Pseudomonas lutea* AY364537 (98.8)	*Ps. lutea* EU118771 (98.8)

Ni-mobilising strains, there was no correlation between the reduction in Ni extraction and isolate resistance to Ni. Nine of the strains were able to produce organic acids (MA69, SA33, SA17, LA10, LA45, LA4, SBA29, SBA86, SBA53), three strains secreted siderophores (SA17, LA1, SBA86), and two strains (both from the SB population) were able to solubilise inorganic phosphate (SBA68 and SBA70). Soil Ni extraction was reduced by 10-30 % when using the cell-free cultures of this group of isolates (Figure 2.2).

2.4 Discussion

Half of the isolates tested in this study modified Ni extractability from the Ni-rich, ultramafic soil (M, Table 2.1). In the case of Ni-mobilizing strains, these isolates seem to be able to mobilize Ni from non-labile soil fractions (associated with organic matter or bound to Fe/Mn oxides). Our results are therefore in agreement with other studies demonstrating a role of bacterial isolates in modifying soil metal availability (Whiting *et al.*, 2001; Abou-Shanab *et al.*, 2003; 2006; Kuffner *et al.*, 2008; Ma *et al.*, 2009). Strains isolated from the rhizosphere of *A. pintodasilvae* and *A. malacitanum* included members of genera, which include strains that have been shown to mobilize soil metals (*Bacillus* spp., *Streptomyces* spp., *Microbacterium* spp.). *Streptomyces* AR17 (isolated from rhizosphere soil in a Pb mining area in Austria) increased the uptake of Zn and Cd in *Salix caprea* (Kuffner *et al.*, 2008). Inoculating a high-Ni soil with *Microbacterium arabinogalactanolyticum* AY509224 increased Ni extraction (Abou-Shanab *et al.*, 2003). Most of these studies tested metal-tolerant bacterial strains which were isolated from contaminated soil or from the rhizosphere of (hyper)accumulating plants growing in metal-enriched soils. The ability of some of these isolates to increase metal availability was suggested, together with plant root activity, to explain the frequently observed increase in metal bioavailability in the rhizosphere of hyperaccumulators (see review by Kidd *et al.*, 2009). In this study, 13 isolates were able to increase Ni extractability but this phenomenon was not found to be restricted to metal-tolerant bacteria. In fact, two of the Ni-mobilising strains were isolated from the non-hyperaccumulator *A. serpyllifolium*, with MTCs of <1 mM Ni. Moreover, the presence of Ni-mobilising bacteria does not seem to be correlated to plant Ni hyperaccumulation. For instance, M, S and SB populations accumulate a similar level of Ni in leaves but no mobilising strains were found in the M collection, and only one isolate was found in the SB collection. On the other hand, Ni bioaccumulation was

highest in the L population, but we did not identify a greater number of Ni-mobilising strains associated with this population.

Our results suggest that the release of labile Ni could merely be an indirect result of microbial-induced mechanisms to promote nutrient availability, and not due to active Ni mobilisation. Microbial weathering of soil minerals is known to release major nutrients (such as K, P, Fe, Ca, Mg) and trace elements which are essential for microbial and plant growth (see review by Uroz *et al.*, 2009). In ultramafic soils, solubilisation of Ni may occur indirectly as a result of the weathering of their characteristic Ni-rich, ferromagnesium minerals (such as serpentine, chlorite). Concentrations of K, Mg and Fe were analyzed in the soil extracts since their presence could indicate such mineral dissolution. No clear trends were observed in the concentration of Mg or Fe, however, K concentrations tended to increase or decrease in the same manner as Ni (data not presented).

Microbial mechanisms reported to be involved in soil mineral weathering include acidolysis, chelation and oxidoreduction processes (Uroz *et al.*, 2009). In

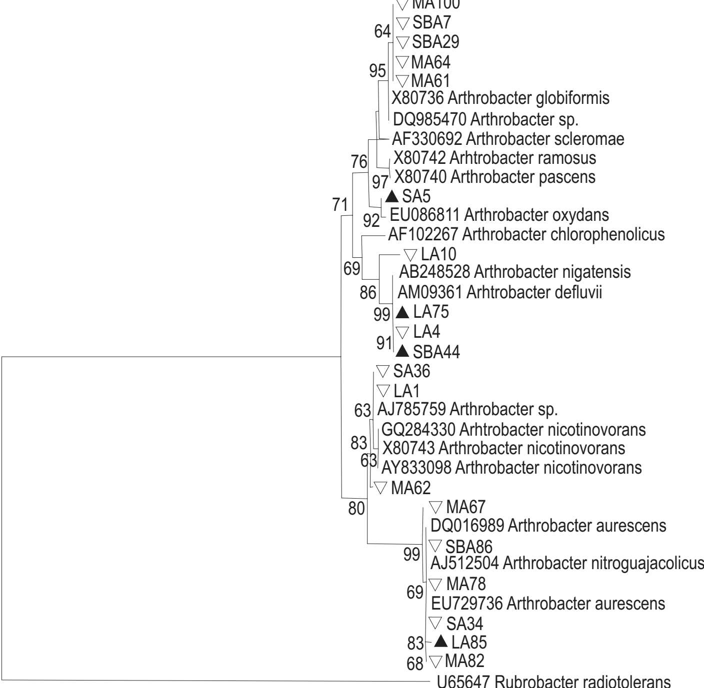

Figure 2.3. Phylogenetic relationships of strains related to the genus *Arthrobacter* based on 16S rDNA sequences obtained from the rhizobacteria associated with *Alyssum serpyllifolium* and closely related sequences (neighbor-joining algorithm using Maximum Composite Likelihood method; 2000 bootstrap replicates performed; fragments of 850 bp were used for tree construction). The strain *Rubrobacter radiotolerans* was used as an outgroup. Filled triangles, Ni-mobilising strains; open triangles, Ni-immobilising strains.

the present study we did not detect acidification: bacterial growth always led to an increase in medium pH, probably due to the release of ammonia from the decomposition of proteins. On the other hand, Ni solubilisation may have been due to the release of Ni chelating compounds (such as low molecular weight organic anions or siderophores). Differences in pH and redox potential of the cell-free cultures may reflect differences in the quantity and composition of bacterial metabolites. Production of gluconic acid is the most commonly cited mechanism of phosphate (apatite) solubilisation. Bacterial secretion of siderophores (specific Fe(III) chelating agents) are known to mobilise extremely insoluble Fe(III)(hydr)oxides in the soil, and have been proposed to mobilise other trace metals at the same time. However, based on the phenotypic characterisation carried out in this study Ni mobilisation cannot be directly associated with the release of this type of compound. It is likely that chelating molecules other than those tested here (organic acids, siderophores, P solubilising compounds) are involved in the Ni-mobilisation process. For example, catechol derivatives produced by *Azotobacter* sp. were shown to augment dissolution of olivine (Page and Huyer, 1984). Nonetheless, since the bacteria were grown in a nutrient-rich culture medium mechanisms inducing siderophore production, or solubilisation of P, may not have been activated. The extraction of Ni using filtrates of the same bacterial isolates grown in minimal culture medium may be very different to that obtained with the 1/10 strength 869 medium used in this study. This aspect will be studied in the future.

The reduction in extractable-Ni observed in some cases could be indicative of a Ni-immobilising effect due to the type of chelating compounds produced by these strains, or could be due to a change in the medium composition during microbial growth leading to a reduction in its Ni extraction potential. Decomposition of tryptone during microbial growth could diminish the capacity of the 1/10 strength 869 medium to extract Ni, since tryptone acts as a complexing agent. As a result, extracted Ni concentrations may reflect the exchangeable Ni pool of this soil due to displacement of Ni from exchange sites by cations in the culture medium. In fact, exchangeable Ni (estimated using 1M NH_4Cl) in the M soil was 41 mg kg^{-1} (Cabello-Conejo, 2010), and Ni extracted by immobilising strains ranged from 40 to 51 mg kg^{-1}. Metal immobilisation by microbial inoculants is particularly interesting for phytostabilisation techniques where the aim is to reduce metal bioavailability. Chen and Cutright (2003) demonstrated that a rhizosphere microbial consortium, able to resist high levels of trace metals, significantly reduced the bioavailability of metals by altering the pH of its surroundings from 4.5 to near neu-

tral. The microbial activity and change in pH significantly reduced water-soluble concentrations of Cd, Cr and Ni. Pishchik *et al.* (2002) suggested that Cd-tolerant PGPR (*Anthrobacter mysorens* 7, *Flavobacterium* sp. L30, *Klebsiella mobilis* CIAM 880) migrated from the rhizoplane to the rhizosphere where they bound soluble free Cd ions in biologically unavailable complex forms. The increase in pH of the 1/10 strength 869 medium after bacterial growth and formation of insoluble Ni hydroxides could explain the reduction in Ni extraction observed in this study. However, this increase in pH after bacterial growth was also observed in Ni -mobilising isolates. As observed in the Ni-mobilising strains, there was no correlation between Ni immobilisation or isolate phenotypic characteristics. Repeating these soil extractions using minimal mineral medium (with simple C sources) could confirm whether or not these bacterial strains are truly able to immobilise Ni. Further research is also necessary to confirm the mechanisms operating during the immobilisation process.

Overall, the results indicate that the protocol used in this study was suitable for screening a relatively large number of strains for their Ni-solubilising potential, and permitted the selection of potentially useful strains for phytoextraction (or phytostabilisation) processes. Furthermore, PGP traits found in some of the isolates could be exploited for improving plant growth and performance in metal-contaminated soils. Five of the Ni-mobilising strains were able to produce organic acids (SA5, SA26, SA28, SBA44, SNA92), and three of these acid-producers also secreted siderophores (SA26, SBA44, SNA92). Amongst the potential Ni-immobilising isolates, strains SBA86 and SA17 produce siderophores and organic acids, and strains LA10, SBA5 and SBA29 produce significant quantities of IAA (> 15 mg/l). However, the behavior of selected strains and effects on plant Ni accumulation will need to be studied in the plant-soil system.

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Chapter 3

Bacterial-induced weathering of ultramafic rock: implications in phytoextraction

ABSTRACT

Soil metal bioavailability is often cited as a limiting factor of phytoextraction (or phytomining). Bacterial metabolites, such as organic acids, siderophores, or biosurfactants, have been shown to mobilise metals, and their use to improve metal extraction has been proposed by several authors. In this study, the weathering capacity and Ni mobilisation by bacterial strains was evaluated. Minimal medium containing ground ultramafic rock was inoculated with two strains of *Arthrobacter* spp.: LA44 (IAA-producer) or SBA82 (siderophore-producer, PO_4-solubiliser and IAA-producer). Trace elements and organic compounds were determined in aliquots taken at different time intervals. Trace metal fractionation was carried out on remaining rock at the end of the experiment. Results suggest that the strains act upon different mineral phases. LA44 is a more efficient Ni-mobiliser, apparently solubilising Ni associated with Mn oxides and this appeared to be related to oxalate production. SBA82 also leads to release of Ni and Mn, albeit to a much lower extent. In this case, the concurrent mobilisation of Fe and Si indicates preferential weathering of Fe oxides and serpentine minerals possibly related to siderophore production.

The same bacterial strains were then used to inoculate the Ni-hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* growing in ultramafic soil in a rhizobox system. Biomass production and shoot Ni concentrations tended to be higher in inoculated plants than non-inoculated plants. Ni yield was significantly enhanced in plants inoculated with LA44. These results suggest that Ni-mobilising inoculants could be useful for improving Ni uptake and accumulation during phytoextraction (or phytomining).

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3.1 Introduction

Microorganisms play an essential role in the global biogeochemical cycling of metals and nutrients. Their activity can either increase or reduce the mobility of these elements in soils (Gadd, 2010). Bacteria influencing the availability of plant nutrients (such as N, Fe or P) have been used as “biofertilizers” to enhance plant nutrient uptake and alleviate nutrient deficiencies (Vessey, 2003). Inoculation of pine seedlings with *Burkholderia glathei* PML1(12) significantly improved plant growth and nutrition through the weathering of biotite and release of nutrients such as K and Mg (Calvaruso *et al.*, 2006). Similarly, the use of metal-mobilising bacteria to enhance plant metal uptake has been proposed by several authors as a promising method to improve phytoextraction (or phytomining), since metal bioavailability can be a limiting factor in these processes (Whiting *et al.*, 2001; Chapter 2; Abou-Shanab *et al.*, 2003; Kuffner *et al.*, 2008). Such microbial inoculants offer an alternative to the controversial use of metal chelants which can increase metal bioavailability but at the same time lead to environmental problems due to their limited biodegradability or to an enhanced leaching of metals to groundwater. Several microbial metabolites have been shown to enhance rock weathering through chemical interaction or, oxidation-reduction reactions, leading to mineral dissolution and metal solubilisation. These metabolites include inorganic acids (HNO_3 and H_2SO_4), organic acids (such as citric, oxalic, gluconic acid) and metal-chelating ligands such as iron-complexing siderophores or biosurfactants (Ehrlich, 1998). Microbial activity was associated with an enhanced release of Co and Ni in ultramafic soils of New Caledonia (Amir and Pineau, 2003). Quantin *et al.* (2002) showed that bacterial reduction of oxides led to the solubilisation of Fe, Mn, Ni and Co and modified soil metal distribution. Li *et al.* (2010) related the production of short-chain organic acids by rhizosphere bacteria with the mobilisation of Cd and Zn. Cell-free culture filtrates of rhizobacterial strains have been shown to mobilise soil Ni (Abou-Shanab *et al.*, 2003; Chapter 2), Zn and Cd (Whiting *et al.*, 2001; Kuffner *et al.*, 2010). This bacterial-promoted solubilisation of metals led, in some cases, to an increase in metal uptake by plants. For instance, inoculation with Ni-mobilising rhizobacteria enhanced Ni uptake by the Ni-hyperaccumulator *Alyssum murale* (Abou-Shanab *et al.*, 2003; Abou-Shanab *et al.*, 2006) and by the non-hyperaccumulator *Brassica juncea* (Ma *et al.*, 2009). Similarly, Zn/Cd-mobilising bacteria enhanced metal accumulation in *Salix caprea* (Kuffner *et al.*, 2010) and Zn concentration in *Thlaspi caerulescens* (Whiting *et al.*, 2001). These changes in

metal uptake and accumulation are generally attributed to bacterial induced modifications in metal solubility through the release of metabolites such as those mentioned above. However, in many of these studies no clear connection was found between microbial-induced changes in soil properties and plant response.

In this study, two bacterial strains isolated from the rhizosphere of the Ni-hyperaccumulator *Alyssum serpyllifolium* (see Chapter 2) were evaluated for their weathering capacity and ability to mobilise Ni from ultramafic rock. The influence of these strains on metal availability and plant uptake was further evaluated by growing inoculated plants in ultramafic soil in a rhizobox system.

3.2 Materials and methods

Bacterial strains

Two bacterial isolates identified as members of the *Arthrobacter* genus were selected for this study. These strains were isolated from the rhizosphere soil of two subspecies of the Ni-hyperaccumulator *Alyssum serpyllifolium,* and were previously shown to influence metal solubilisation from ultramafic rock in batch cultures (Saavedra Ferro, 2011). These included the strains (identified by partial sequencing of 16S rDNA) *Arthrobacter nitroguajacolicus* LA44 (an IAA-producer) isolated from the rhizosphere of *Alyssum serpyllifolium* subsp. *lusitanicum* (Melide, NW Spain) and *A. oxydans* SBA82 (a siderophore-producer, PO_4-solubiliser and IAA-producer) isolated from *Alyssum serpyllifolium* subsp. *malacitanum* (Sierra Bermeja, S Spain).

Batch culture experiment

To evaluate the ability of the bacterial strains to mobilise Ni they were cultivated in minimal medium containing sterile ultramafic rock. The ultramafic rock was collected from the serpentinitic area of Morais (NE Portugal) where *A. serpyllifolium* subsp. *lusitanicum* is found growing. The chemical composition of this rock is given in Table 3.1. As is expected for an ultramafic rock, the SiO_2 content is less than 45 %, the Al_2O_3, K_2O and CaO contents are low, whereas the MgO content

Table 3.1. Chemical composition of the rock used in batch culture experiment obtained by Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF)

Major elements (wt.% oxides)								Trace metals (mg kg^{-1})		
SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	Ni	Cr	Co
42.79	1.49	15.73	0.39	34	0.98	0.54	0.05	3530	2660	130

and total Ni, Co and Cr concentrations are elevated. Mineral associations and element distribution were characterised by scanning electron microscope (SEM EVO LS 15) with EDX microprobe analysis (INCA X-act, Oxford Instruments, United Kingdom) (Figure 3.1).

A modified 284 medium (see Chapter 2) was used with no trace of Ni, Mn or Co and a reduced concentration of K and Fe. This modified 284 medium contains (per liter medium): 6.06 g Tris-HCl, 4.68 g NaCl, 0.015 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.20 g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.04 g $Na_2HPO_4.2H_2O$, 1 ml Fe(III)NH_4 citrate solution (containing 48 mg/100ml) plus oligoelements (0.3 mg H_3BO_4, 0.02 mg $CuSO_4.5H_2O$, 0.036 mg $Na_2MoO_4.2H_2O$) adjusted to pH 7. The medium was supplemented with glucose (0.5 g l^{-1}) and fructose (0.5 g l^{-1}). Flasks containing 2 g of ground rock (< 100 µm) were autoclaved three times with a 24h interval between cycles. 100 ml of modified 284 medium was added to each flask and inoculated with 1 ml of each bacterial inoculant, LA44 or SBA82, or 1 ml of 10 mM $MgSO_4$ (control treatment). To prepare the bacterial inoculants, strains were cultivated in 869 medium (see Chapter 2) for 3 days, harvested by centrifugation (4000 g, 15 min, 4 ºC), washed and re-suspended in 10 mM $MgSO_4$ to an optical density of 1.0 at 600 nm (about 10^8 cells per ml). Flasks were incubated in the dark for two weeks at 28 ºC and 150 rpm. Five replicates were prepared for each treatment.

Aliquots (of 3 ml) were taken at different time intervals (1, 2, 4, 7, 10 and 14 days). Samples were centrifuged, the supernatant was decanted and filtered (0.22 µm pore size) and immediately frozen until analysis. Samples were analysed for element concentrations, organic acids and phenolic compounds. At the same time intervals, bacterial densities were determined by plating out serial dilutions of the samples on 1:10 diluted 869 agar medium (see Chapter 2), for three replicates of each treatment. At the end of the experiment (14 days), the medium pH was determined. The sterility of the control flasks was checked by plating on 1:10 diluted 869 agar medium. Finally, the cultivated bacterial strains (LA44 and SBA82) were compared with the original inoculants using BOX-PCR and following the methods described in Chapter 2.

The concentration of Al, Co, Cr, Fe, Mn, Ni and Si in the culture medium was determined by ICP-MS (Elan 9000 DRCe, Perkin Elmer) and K by emission spectrophotometry (AAS; Perkin Elmer 2380, Norwalk, CT).

Carboxylic acids were separated by reversed-phase liquid chromatography on a C_{18} column with 5 µm particle size (GraceSmart, RP18, and 2.1 x 150 mm

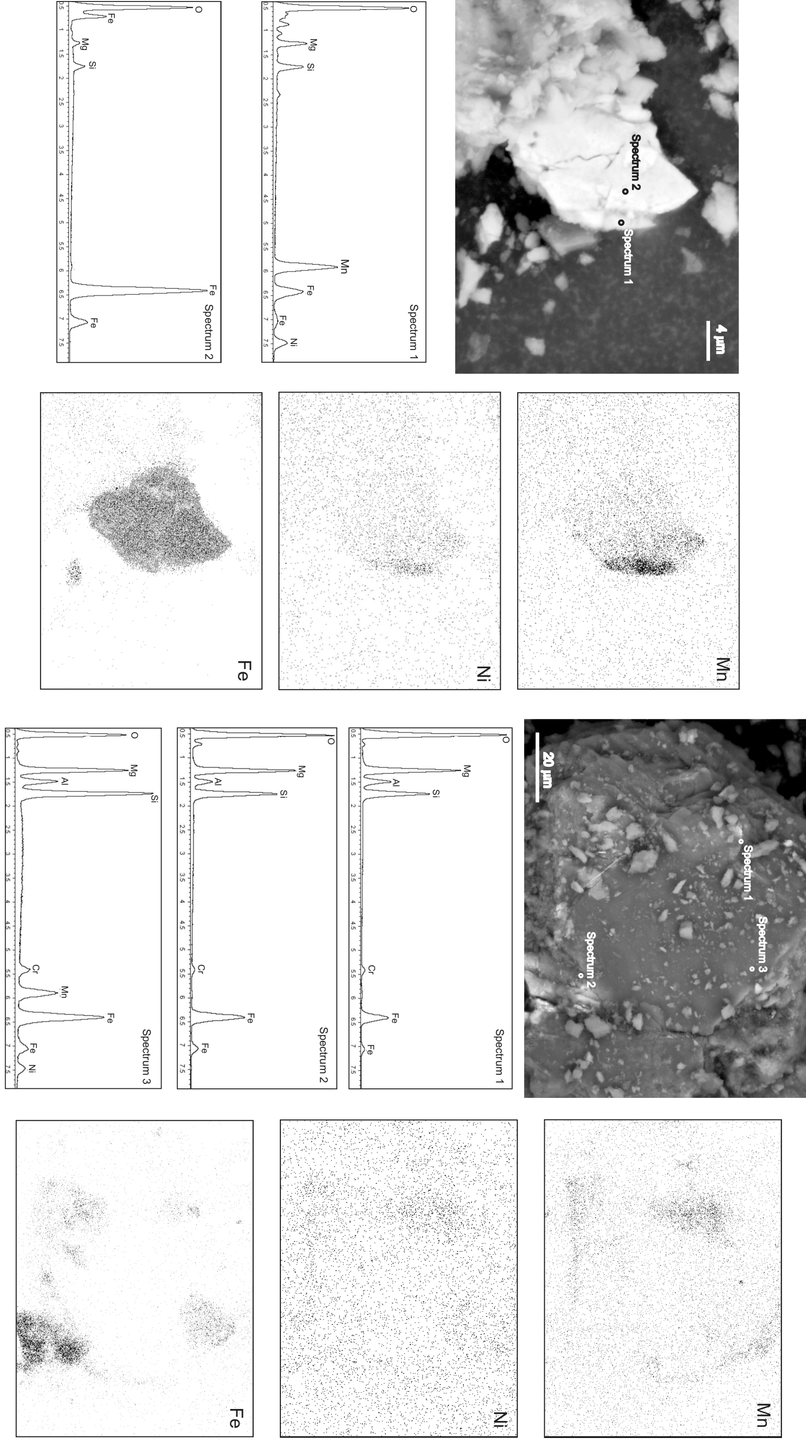

Figure 3.1. SEM photomicrographs of original ground ultramafic rock, element mapping and results of microprobe analysis.

column from Grace Davison Discovery Sciences, Deerfield, Il, United State) and analysed by liquid chromatography -electrospray ionization - time of flight mass spectrometry (LC-ESI-TOFMS, Agilent Technologies, Palo Alto, CA, United States) as described by Jaitz *et al.* (2011). The freeze-dried medium was re-dissolved in 2 ml deionized water. Afterwards 20 µl of the internal standard (2 µmol l^{-1} $^{13}C_2$-oxalic acid) were added and subsequently the solvent was removed using a speed vac system (Eppendorf Concentrator, 5301 Eppendorf AG, Hamburg, Germany). Afterwards, 50 µl of benzylalcohol and 20 µl of clorotrimethyl-silane (TMS-Cl) were added to perform the derivatisation of the carboxylic groups. Samples were placed for 45 min in an ultrasonic bath and for further 45 min at 80 ºC in an oven. The reaction was stopped by adding 20 µl of 0.3 mmol l^{-1} tetramethylammonium fluoride (TMAF) in tetrahydrofuran (THF). $^{13}C_2$-carboxylic acids were added as internal standard to all the samples.

Quantification of selected phenolic compounds (caffeic acid, catechin, p-coumaric acid, 2.5-dihydroxybenzoic acid, epicatechin, ferulic acid, gallic acid, 4-hydroxy-3-methoxycinnamidaldeyhde, resveratrol (3,5,4'-trihydroxy-trans-stilbene), sinapic acid, syringic acid and vanillic acid) was performed via LC–MS/MS in negative ionisation mode on a 6410 triple quadrupole mass spectrometer from Agilent Technologies (Palo Alto, CA, United States) equipped with an electrospray ionisation (ESI) interface (Jaitz *et al.*, 2010). Nitrogen was used as a desolvation- and collision gas. The mass spectrometer was connected to a liquid chromatography system (1200 series, Agilent). C-18 reversed phase separation was performed on a Rapid Resolution HT 2.1 x 50 mm column with 1.8 µm particle diameter (Agilent). Mass Hunter software (version B.01.03) was used for instrument control and data processing. Solvent A was 99 % water, 1 % acetonitrile and 0.1 % formic acid and solvent B consisted of 99 % acetonitrile, 1 % water and 0.1 % formic acid. The flow rate was set to 0.4 ml min^{-1}, column temperature was at 20 ºC and the injection volume was 3 µl. The gradient profile was 0.0–1.5 min 2 % B, from 1.5 to 11.25 min 45 % B, from 11.25 to 12.75 min 70 % and at 12.82 min back to the initial conditions of 2 % B.

The remaining ground rock at the end of the experiment was recovered by centrifugation, washed 4 times with sterile deionized water and air-dried. A metal fractionation scheme was carried out on the residual rock samples according to Zeien and Brümmer (1989) (Table 3.2):

- First, soil samples were shaken with 1 M NH_4NO_3 (unbuffered) for 24 h at room temperature, the solution was separated by centrifugation (15 min, 2500

min^{-1}) and filtered and stabilized with HNO_3 (65 %). This extracts the *water soluble and exchangeable* metals.

- Second, the resulting residue was shaken for 24 h at room temperature with 1 M NH_4OAc (adjusted with 50 % HOAc to pH 6), centrifuged, filtered and stabilized with HNO_3 (65 %). 25 ml NH_4NO_3 was added to the remaining residue and shaken for 10 min. The solution was separated by centrifugation, filtered and combined with the NH_4NOAc extract. This step extracts *easily mobilisable* metals.
- Third, the residue was shaken with 0.1 M NH_2OH-HCl + 1 M NH_4OAc (adjusted to pH 6 with diluted HCl) for 30 min, separated by centrifugation, filtered and stabilized with HCl (37 %). 25 ml 1 M NH_4OAc were added to the residue and shaken for 10 min, centrifuged, filtered (repeated once) and combined with the above solution. This extracts metals bound to *Mn oxides*.
- Fourth, 50 ml 0.2 M NH_4-oxalate (= 0.2 M Di-ammonium oxalate-monohydrate + 0.2 M oxalic acid-dihydrate adjusted to pH 3.25 with diluted NH_4OH) were added to the residue, and after shaking (over-head) for 4 h in the dark, the solution was separated by centrifugation (15 min, 2500 min^{-1}) and filtered. A second volume of 25 ml 0.2 M NH_4-oxalate (pH 3.25) was added to the residue and shaken over-head in the dark for 10 min. The solution was separated by centrifugation (15 min, 2500 min^{-1}), filtered and the two filtrates were combined. This step targets those metals bound to *amorphous Fe oxides*.
- Fifth, 50 ml 0.1 M ascorbic acid + 0.2 M NH_4-oxalate (= 0.1 M L(+)-ascorbic acid + 0.2 M NH_4-oxalate buffer: 0.2 M oxalic acid-dihydrate adjusted to pH 3.25 with diluted NH_4OH) were added, and after shaking horizontally in a water bath for 30 min at 96 ºC ± 3 ºC, the solution was separated by centrifugation (15 min, 2500 min^{-1}) and filtered. The remaining solution was extracted with 25 ml 0.2 M NH_4-oxalate (pH 3.25) by shaking over-head in the dark for 10 min. The solution was separated by centrifugation (15 min, 2500 min^{-1}), filtered and the filtrates were combined. This extracts metals bound to *crystalline Fe oxides*.
- Finally, the remaining residue was digested with *aqua regia* to determine the *residual* (silicate-bound) fraction.

The concentration of Al, Co, Fe, Mn, Ni and Si were analysed in the filtered supernatants of each extraction by ICP-OES (Vista Pro; Varian Inc., Australia). In the case of Si, the residual fraction was obtained by subtracting the sum of all frac-

tions from the total Si (obtained by Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF).

Table 3.2. Metal fractionation scheme according Zeien and Brümmer (1989).

Fraction		Extractant	pH	Condition
F1	Water soluble and exchangeable	NH_4NO_3		24 h
F2	Easily mobilisable	NH_4OAc	6	24 h
F3	Mn oxides	$NH_2OH{\cdot}HCl + NH_4OAc$	6	30 min
F4	Organically-bound metals*	NH_4-EDTA	4.6	90 min
F5	Amorphous Fe oxides	NH_4-Oxalat	3.25	4 h
F6	Crystalline Fe oxides	NH_4-Oxalat + ascorbic acid	3.25	30 min; 96ºC
F7	Residual	aqua regia		digestion

* This fraction was not determined in residual rock (see text)

Rhizobox experiment

Ultramafic soil was collected from a serpentine site in Redschlag, Austria (Wenzel *et al.*, 2003). The soils at this site present a slightly acidic pH (pH_{CaCl2} 6.55) with an organic C content of 13 g kg^{-1}, a predominance of Mg in the exchange complex and high concentration of total Ni (2580 mg kg^{-1}). The soils were air-dried and sieved to < 2 mm before being filled into the rhizoboxes (bulk density, 1.2 g cm^{-3}). The rhizobox used in this study was based on the system of Fitz *et al.* (2003) (Figure 3.2). Root growth was restricted to a central compartment by a 30 µm mesh size nylon net (Labor Becker, Vienna, Austria) to avoid growth of root hairs into the adjacent 2-mm-thick root-free rhizosphere soil compartment. The root-free rhizosphere compartment was separated from bulk soil by the same 30 µm mesh size nylon net. The rhizoboxes were made of Perspex acrylic material, allowing observation of root growth. Rhizoboxes were wrapped with aluminium foil during the experiment to avoid growth of photosynthetic soil organisms and weed germination.

For this experiment, the subspecies *malacitanum* of the Ni-hyperaccumulating *A. serpyllifolium* was selected. Surface-sterilized seeds were germinated on perlite and transplanted into rhizoboxes (2 seedlings per rhizobox). After 5 weeks, 20 ml of LA44 or SBA82 bacterial suspensions (prepared as described previously) were added to the rhizoboxes at the base of the plants. The same amount of 10 mM $MgSO_4$ was added to non-inoculated plants in control rhizoboxes. All treatments were replicated five times. Plants were grown for a further

2 months. At harvest, the shoots and roots of plants were separated, washed with pressurised tap water (and 0.05 M $CaCl_2$ in the case of roots) followed by deionised water, oven-dried at 45 ºC and ground. Shoot tissues (0.1 g) were digested in a 2:1 mixture of concentrated HNO_3:HCl on a hot plate at 160 ºC, and the Ca, Mg, K, P, Fe and Ni concentration was measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed on the basis of dry weight plant material. The full recovery of plant roots was not possible and only shoot dry weight was taken into account when assessing the effects of inoculation on plant biomass production or Ni yield.

Soil analyses were carried out on the < 2 mm fraction of rhizosphere and bulk soil samples. Soil pH was measured in H_2O using a 1:2.5 soil:solution ratio. Exchangeable cations were extracted with 0.1 M $BaCl_2$, and Al, Ca, K, Mg and Na determined by ICP-OES (Vista Pro; Varian Inc., Australia). Water-soluble Ni concentration was analysed by ICP-OES in soil extracts after 30 min shaking using a 1:2.5 soil:H_2O ratio. $Ca(NO_3)_2$- and $Sr(NO_3)_2$-extractable Ni concentrations were determined by ICP-OES in soil extracts after 2 h shaking using a 1:4 soil:extractant ratio for both extractants. A metal fractionation scheme was carried out as explained above (Table 3.2). However, in this case an additional step was incorporated to target metals associated with organic material (*organically-bound metals*). After the extraction of metals associated with Mn oxides, 50 mL 0.025 M NH_4EDTA (adjusted to pH 4.6 with diluted NH_4OH) were added to the residue, and after shaking over-head for 90 min the solution was separated by centrifuga-

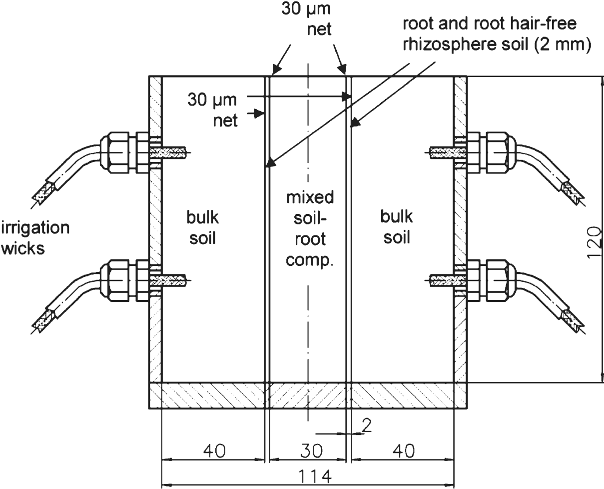

Figure 3.2. Croos section of the rhizobox design used (Fitz *et al.*, 2003).

tion (15 min, 2500 min^{-1}) and filtered. For extraction of the remaining solution, 25 mL 1 M NH_4OAc (adjusted to pH 4.6 by 100 % HOAc) were added. After shaking over-head for 10 min, the solution was separated by centrifugation (15 min, 2500 min^{-1}), filtered and combined with the NH_4EDTA extract. The concentration of Al, Co, Fe, Mn, Ni and Si was analysed in the filtered supernatants of each extraction by ICP-OES (Vista Pro; Varian Inc., Australia). In the case of Si, the residual fraction was obtained by subtracting the sum of all fractions from the total Si (obtained by Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF).

Statistical analysis

Differences in element solubilisation and organic acid production in the batch culture experiment were determined using a repeated measures analysis of variance (ANOVA). A multiple comparison of means was determined by the "post-hoc" Bonferroni. Data were log transformed where necessary to achieve homogeneity of variance. Mann–Whitney U-tests were used to detect significant differences between microbial densities.

Changes in the culture medium composition (element and organic acid anion concentrations) were also analysed by principal component analysis (PCA). Values below detection limits (DL) were recorded as the ½ DL for statistical analysis. A varimax rotation was applied to the PCAs in order to facilitate the interpretation of the extracted principal components.

Differences in plant biomass and plant metal/nutrient concentration in the rhizobox-grown plants were determined using analyses of variance (ANOVA). A multiple comparison of means was determined by the "post-hoc" Least Significance Difference test. Data were log transformed where necessary to achieve homogeneity of variance. Comparison of means between bulk and rhizosphere soil was achieved by the Student's t test for related means.

3.3 Results

Batch culture experiments

Bacterial growth in medium with ground rock

During the experiment, no significant differences were found in the bacterial densities of the two strains (Table 3.3). They presented a similar growth rate, reaching a stable density of 1.1-6.4 x 10^8 CFUs ml^{-1} medium by day 2. The identity of the recovered strains at the end of the experiment was confirmed by BOX-PCR to be

the same as the originally inoculated strains.

Table 3.3. Bacterial densities (mean ± SE) of LA44 and SBA82 (log CFUs g^{-1} soil) in culture medium during incubation of 14 days (n = 3).

	Days					
	1	2	4	7	10	14
LA44	4.3 ± 0.4 x 10^7	6.4 ± 0.4 x 10^8	6.3 ± 0.6 x 10^8	5.8 ± 0.3 x 10^8	6.2 ± 0.7 x 10^8	4.2 ± 0.8 x 10^8
SBA82	2.0 ± 0.3 x 10^7	1.4 ± 0.4 x 10^8	1.2 ± 0.3 x 10^8	1.9 ± 0.1 x 10^8	1.1 ± 0.3 x 10^8	1.5 ± 0.1 x 10^8

Element solubilisation from rock and exudation of organic compounds

Element concentrations barely showed variation over time in the control treatment and were generally lower than in bacterial cultures (Figure 3.3). Iron and K were added in low concentrations to the initial liquid medium (0.09 and 7.8 mg l^{-1}, respectively), while Co, Mn and Ni were absent. Bacterial activity and growth either led to a depletion in some elements compared to the non-inoculated control (such as K), or alternatively, to the release of some elements from the rock (such as Fe, Co, Mn, Ni, or Si). At the end of the experiment there was no difference in the culture medium pH between treatments. The repeated measures ANOVA showed that the time, inoculant treatment, and the time x inoculant interaction factors, significantly affected changes in the medium composition ($p < 0.01$; Table 3.4).

Concentrations of K in controls were similar to those added in the initial medium (7.8 mg l^{-1}) and remained stable throughout incubation, values varied from 8.5 mg l^{-1} on day 2 to 9.8 mg l^{-1} on day 14 (Figure 3.3e). In contrast, bacterial growth led to a significant decrease in the original K concentration of the medium, and concentrations were lower than controls throughout the duration of the experiment. Minimum values of 0.8 mg l^{-1} and 4.5 mg l^{-1} were detected on day 2 in LA44 and SBA82 cultures, respectively. From this time onwards however, there was a steady release of K into the medium in the presence of both strains (although concentrations never reached values of controls). On day 14 the K concentration was 4.1 mg l^{-1} and 8.2 mg l^{-1} in LA44 and SBA82 cultures, respectively.

Concentrations of Ni and Mn in LA44 cultures on day 2 were 8.9- and 7.1-fold higher than in controls, and 3.1- and 2.7-fold higher than in SBA82 cultures (Figure 3.3c and d), and both elements were significantly correlated ($R^2 = 0.79$, $p < 0.001$). Inoculation with LA44 induced a rapid release of both Ni and Mn, and concentrations in the medium peaked on day 2. This bacterial strain also led to a higher dissolution of Co than either SBA82 cultures or the control (Figure 3.3f), although in this case concentrations decreased with time. Concentrations of Fe or

Si in LA44 cultures were similar to those in the non-inoculated control (Figure 3.3a and b).

In contrast to what was observed in the LA44 cultures or controls, the presence of strain SBA82 led to a significant release of Fe and Si into the medium solution. By day 7 concentrations of these two elements were up to 4.1- and 3.8-fold higher than those detected in controls, respectively. Both elements followed a similar pattern with time, a higher rate of solubilisation occurred during the initial days which then stabilised between days 4-7 (Figure 3.3a and b). Concentrations of Fe and Si in SBA82 cultures were significantly correlated ($R^2 = 0.93$; $p < 0.001$). Values for Ni and Mn in SBA82 cultures were also significantly higher than controls (although lower than in LA44 cultures): by day 4 concentrations of these two elements were up to 2.8- and 2.6-fold higher than the control, respectively (Figure 3.3c and d), and were also significantly correlated ($R^2 = 0.95$; $p <$

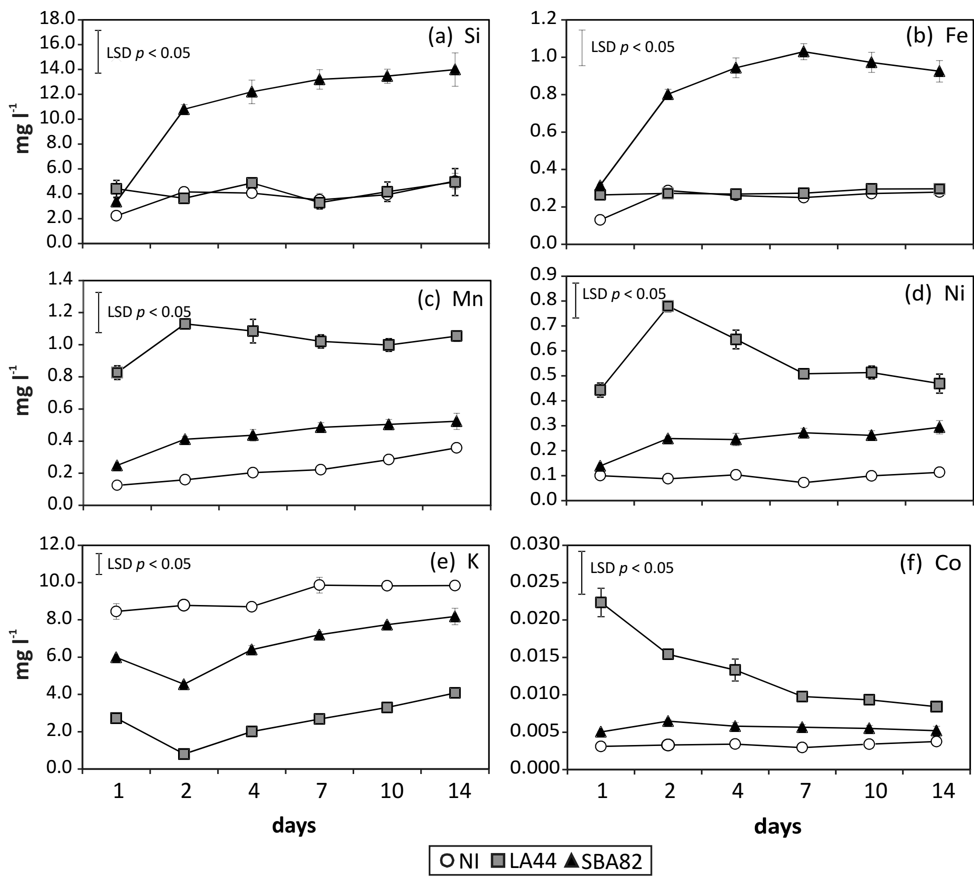

Figure 3.3. Concentrations (mean ± SE) of Si (a), Fe (b), Mn (c), Ni (d), K (e) and Co (f) in medium of LA44, SBA82 and non-inoculated (NI) treatments during incubation of 14 days (n = 5).

Table 3.4. Effects of inoculant treatment (strain) and time (days) on element solubilisation and organic acid production (results of eight separate 2-way repeated measure ANOVAs).

		Si		Fe		Mn		Ni	
	df	MS	F	MS	F	MS	F	MS	F
days	5	39.9	20.7*	0.1	18.5*	0.3	13.7*	0.1	17.2*
strain	2	514.2	329.4*	2.9	298.5*	5.4	283.1*	1.8	336.0*
days x strain	10	23.2	12.1*	0.1	24.3*	0.1	4.6*	0.1	15.7*
		K		Co		Oxalate		Succinate	
	df	MS	F	MS	F	MS	F	MS	F
days	5	17.2	66.0*	1.4×10^{-4}	15.5*	422.4	44.9*	13	27.4*
strain	2	336.8	3710.5*	1.5×10^{-4}	63.1*	7169	585.9*	9.7	12.0*
days x strain	10	1.8	6.8*	8.8×10^{-4}	16.0*	135.5	14.4*	15	32.0*

* $p < 0.01$

0.001). The presence of this strain also induced the release of Al into the medium; concentrations of between 9 ng l^{-1} and 12 ng l^{-1} were detected. In contrast, Al was consistently below the detection limit in both controls and LA44 cultures.

None of the phenolic compounds were detected in the culture media. In contrast, detectable levels of malate, malonate, oxalate and succinate were measured. Aconitate and citrate were below the detection limit. Malate concentrations in control and SBA82 cultures were detected in a similar range, and remained always below 0.70 µM. LA44 cultures showed somewhat higher values of this anion, between 0.20 – 1.15 µM. In control treatments the malonate concentration did not exceed 0.30 µM, and in SBA82 values ranged from 0.20 – 0.55 µM. Again higher values were found in LA44 cultures, which varied from 0.30 – 0.90 µM (data not shown). Significant differences were observed between inoculant treatments for both oxalate and succinate ($p < 0.05$; repeated measures ANOVA; Table 3.4). Succinate was only detected in control cultures at some sampling points (2, 7, 11 and 14 days), and, with the exception of day 1, was generally higher in SBA82 cultures (1.9 – 3.8 µM) than in LA44 cultures (0.6 – 1.9 µM) (Figure 3.4a). The difference in oxalate concentrations was more marked between the two strains (Figure 3.4b): concentrations in SBA82 cultures were similar to control treatments (4.1 – 5.4 µM), while oxalate increased from 18 µM on day 1 to close to 50 µM on day 14 in LA44 cultures.

Element solubilisation and organic acid production were subjected to principal components analysis (PCA). The Kaiser-Meyer-Oklin value was 0.71 and the

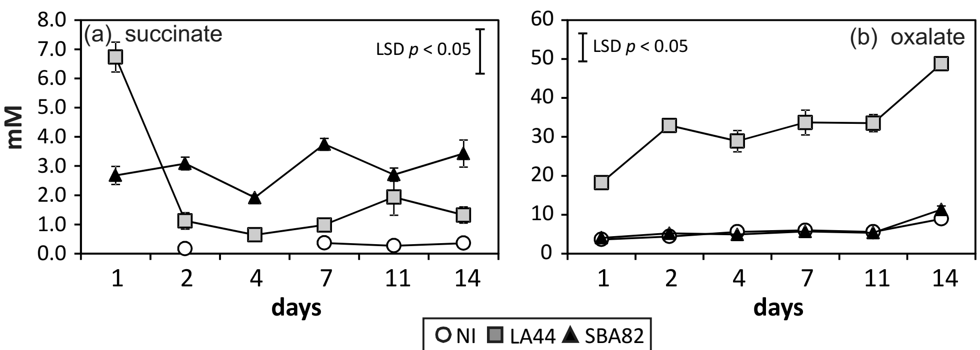

Figure 3.4. Succinate (a) and oxalate (b) concentrations (mean ± SE) in medium of LA44, SBA82 and non-inoculated (NI) treatments during incubation of 14 days (n = 5).

Barlett's Test of Sphericity reached statistical significance ($p < 0.05$), supporting the factorability of the correlation matrix. The PCA extracted two principal components that explained 70 % of total variance (Figure 3.5). The first component (PC1; 41 %) was mainly represented by Co, Ni and Mn concentration and by malonate, oxalate and malate concentrations. The second principal component (PC2; 29 %) was related to the concentration of Fe, Al, Si, and to a lesser extent to Cr. In the PCA plot, control samples are grouped together, with negative scores on both axes. SBA82 samples are mainly placed on the positive axis of the second component (PC2) represented by Al, Fe and Si concentrations in the medium, whereas LA44 samples are grouped on the positive axis of the first component (PC1) associated with Co, Mn and Ni concentrations and organic acids (Figure 3.5). The Principal component analysis confirmed the main differences observed between the two inoculants in the release of trace elements and organic acids into the medium.

Metal fractionation in rock samples

Figure 3.6 shows the element fractionation in the recovered rock from the control treatment, as well as the bacterial-induced depletion/increase of each fraction relative to the control treatment. The residual fraction was the most important geochemical phase for Al, Cr and Si (representing more than 85 % of the total content). This phase was also dominant for Fe and Ni (representing more than 60 %) but for these two elements an important fraction was also associated with either amorphous or crystalline Fe oxides (32-35 % in total). Mn was principally associated with the Mn oxide fraction (41 % of total Mn). EDX microprobe analysis confirmed the presence of Ni associated with Mn oxides (Figure 3.1), Co was dis-

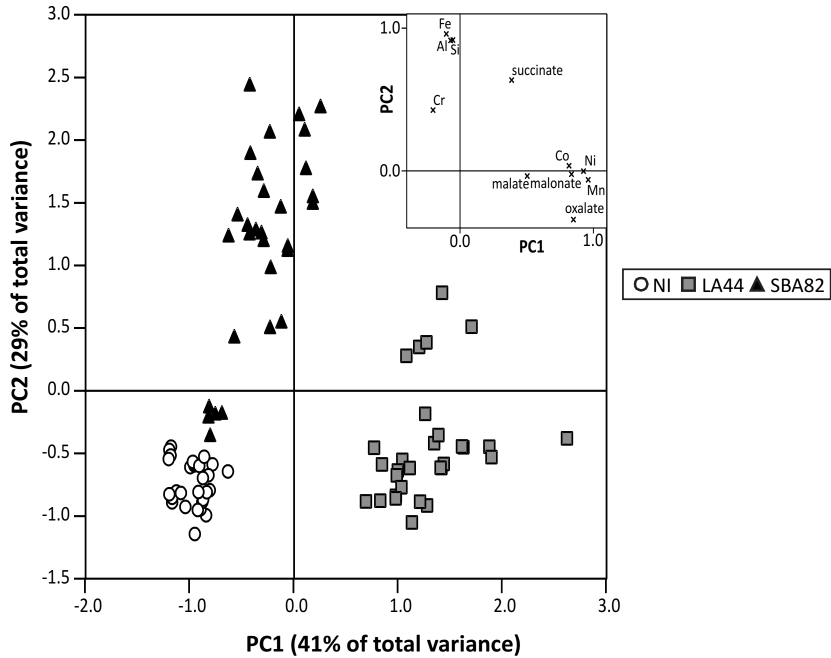

Figure 3.5. Score and loading (upper right-hand corner) plots of principal component analysis (PCA) of elements and organic acids concentrations in medium of LA44, SBA82 and non-inoculated (NI) treatments.

tributed among Mn oxide, amorphous Fe oxide and residual fractions.

Bacterial activity significantly influenced element fractionation in the rock, and these changes were dependent on the bacterial strain (Figure 3.6). After incubation with strain LA44, all elements associated with the Mn oxide fraction were significantly reduced compared to the control. In the case of Mn and Ni, concentrations were reduced from 492 to 393 mg kg^{-1} and from 108 to 87 mg kg^{-1}, respectively (Figure 3.6d and f). These metals were then re-distributed among the more labile fractions (F1 and F2) and amorphous Fe oxides (F5). In F1, Ni concentrations increased from 16.9 to 40.0 mg kg^{-1}, and Mn from 26.6 to 72.8 mg kg^{-1}. Corresponding shifts in F2 were less pronounced: Ni increased from 31.6 to 35.5 mg kg^{-1} and Mn from 21.9 to 37.0 mg kg^{-1} Although not always statistically significant, a similar pattern was observed for Co (Figure 3.6g): an increase in the first two fractions and a decrease in Co associated with the Mn oxide fraction. This effect of bacterial activity on Mn oxides was either not detectable, or far less pronounced, in rock samples which were incubated with strain SBA82 (Figure 3.6).

In the case of SBA82, the presence of this bacterial strain led to a significant

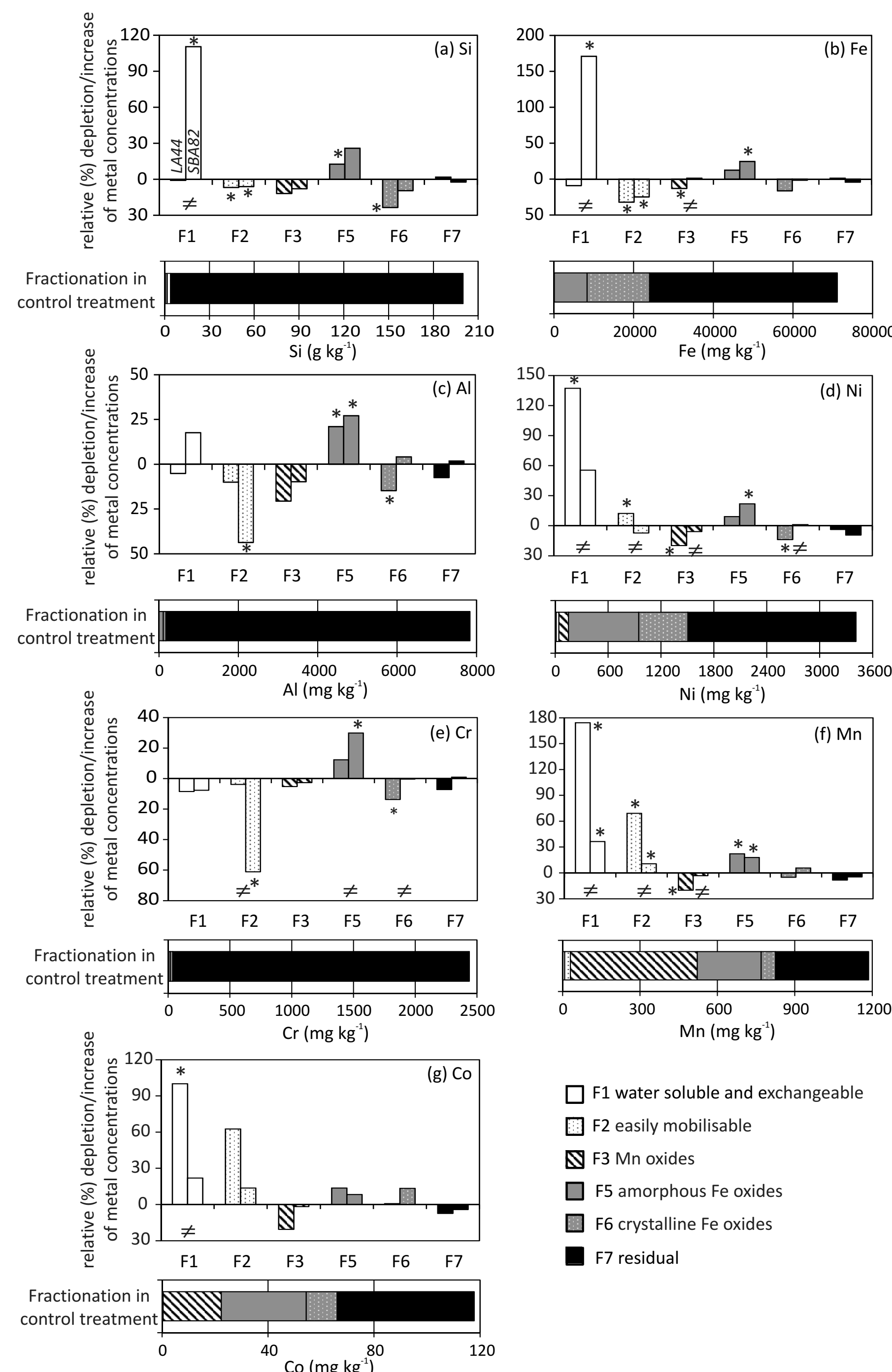

Figure 3.6. Element fractionation in the recovered rock from the control treatment and mean relative (%) bacterial-induced depletion/increase of each fraction relative to the control treatment (n = 5). Depletion/increase was calculated by substraction of the amount of metals in each fraction at bacterial treatment (LA44 or SBA82) from the amount in non-inoculated treatment. A signficant depletion/increase is denoted with an asterisk. Differences between inoculant treatments are denotes with "≠".

increase in soluble and exchangeable concentrations of the major elements Al (from 1.5 to 1.8 mg kg^{-1}), Si (from 399 to 840 mg kg^{-1}) and Fe (from 17.8 to 48.2 mg kg^{-1}) (Figure 3.6a-c). This strain also induced a significant increase in all elements associated with amorphous Fe oxide fractions compared to controls. Concentrations of the major elements (Al, Fe and Si) associated with this phase were increased by 20-27 %, while the trace metals, Ni and Cr, increased from 797 to 971 mg kg^{-1} and from 13.9 to 18.0 mg kg^{-1}, respectively. In parallel, a reduction in the residual Ni fraction was observed (from 2322 to 2115 mg Ni kg^{-1}). These effects were far less pronounced in the LA44 cultures (Figure 3.6).

Rhizobox experiment

Plant biomass production, nutrition and Ni accumulation

After three months growth, inoculated plants presented a higher biomass than non-inoculated plants, although the difference was not significant for either inoculant (Figure 3.7a). Inoculation did not significantly influence nutrient contents in shoot tissues (Table 3.4). However, some tendencies could be observed, for instance, inoculated plants tended to have a higher Fe and Ca shoot content than non-inoculated plants. Furthermore, inoculation with SBA82 also tended to increase shoot P content (Table 3.5).

No differences were observed in root Ni concentrations due to inoculation: values were in the range of 806 to 849 mg Ni kg^{-1} for all three treatments. In contrast, strain LA44 tended to increase Ni accumulation in shoots (reaching up to 11873 mg kg^{-1}) compared to non-inoculated plants (mean Ni concentration of 9700 mg kg^{-1}), although this increase was not significant (Figure 3.7b). Moreover, inoculation of plants with both LA44 and SBA82 increased the total Ni phytoextracted by 1.4- and 1.3-fold, respectively, compared to non-inoculated plants. This

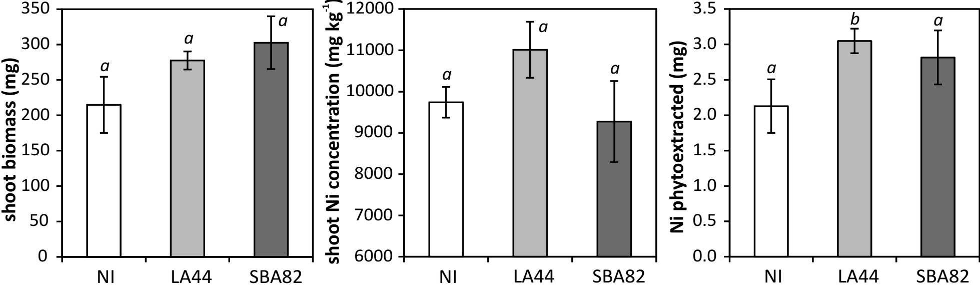

Figure 3.7. Plant biomass, Ni shoot concentration and Ni phytoextracted (mean ± SE) of *Alyssum serpyllifolium* subsp. *Malacitanum* (n = 5). Bars with different letter indicate significant differences ($p < 0.05$).

increase was statistically significant in the case of LA44-inoculated plants ($p < 0.05$; Figure 3.7c).

Plant- and bacterial-induced changes in rhizosphere soil properties

Plant growth induced few changes in physico-chemical soil properties and no sig-

Table 3.5. Shoot nutrients concentrations (mean ± SE) of *Alyssum serpyllifolium* subsp. *malacinatum*. For the same element, different letters indicate significant differences ($p < 0.05$).

	Ca (g kg^{-1})	Mg (g kg^{-1})	K (g kg^{-1})	P (g kg^{-1})	Fe (mg kg^{-1})
NI	24.9 ± 2.1 *a*	10.4 ± 0.7 *a*	16.5 ± 1.3 *a*	1.8 ± 0.2 *a*	69 ± 13 *a*
LA44	27.1 ± 1.7 *a*	12.0 ± 1.0 *a*	16.0 ± 1.6 *a*	1.7 ± 0.3 *a*	75 ± 4 *a*
SBA82	33.0 ± 2.9 *a*	11.8 ± 0.9 *a*	16.0 ± 1.6 *a*	2.5 ± 0.7 *a*	79 ± 4 *a*

nificant effects of bacterial inoculation were observed. In non-inoculated plants, rhizosphere soil pH was slightly higher than bulk soil pH: 8.4 ± 0.1 compared to 8.3 ± 0.1. As expected, the cation exchange complex was dominated by Mg (8.1 – 9.4 cmol$_c$ kg^{-1}). The CEC was 10.3 and 10.9 cmol$_c$ kg^{-1} in bulk and rhizosphere soil, respectively, and Ca/Mg quotients were consistently less than 1 (around 0.2). Water-soluble Ni concentration was significantly higher ($p < 0.01$) in the rhizosphere compared to bulk soil (0.12 and 0.06 mg kg^{-1}, respectively). In contrast, Sr $(NO_3)_2$- or $Ca(NO_3)_2$-extractable Ni concentrations tended to be depleted in the rhizosphere (falling from 0.49 to 0.44 mg kg^{-1} and 6.44 to 5.89 mg kg^{-1}, respectively). Bacterial inoculation did not lead to any significant changes in these general physicochemical properties.

Figure 3.8 shows the element distribution among the seven operationally defined fractions of the bulk soil, and the mean relative (%) depletion/increase in the rhizosphere soil after growth of *A. serpyllifolium* subsp. *malacitanum* (non-inoculated plants). The distribution of metals in the different geochemical phases was similar to those observed in the ultramafic rock used in the batch culture experiment. The residual fraction was the most important phase for Al and Si (78 and 84 %, respectively). Fe and Ni were also mainly associated with the residual fraction (65 and 55 %, respectively), although Fe oxides were also an important phase (35 and 36 %, respectively). As expected, Mn was principally associated with Mn oxides and the residual fraction (35 and 43 %, respectively). Co was distributed among Mn oxides (42 %), organically bound fraction (15 %) and crystalline Fe oxides (15 %). In rhizosphere soils there was a general depletion of elements (in many cases significant) in labile fractions (F1 and F2) and from organic material or Fe-oxide pools (F4, F5 and F6). Concentrations of labile Mn, Ni and

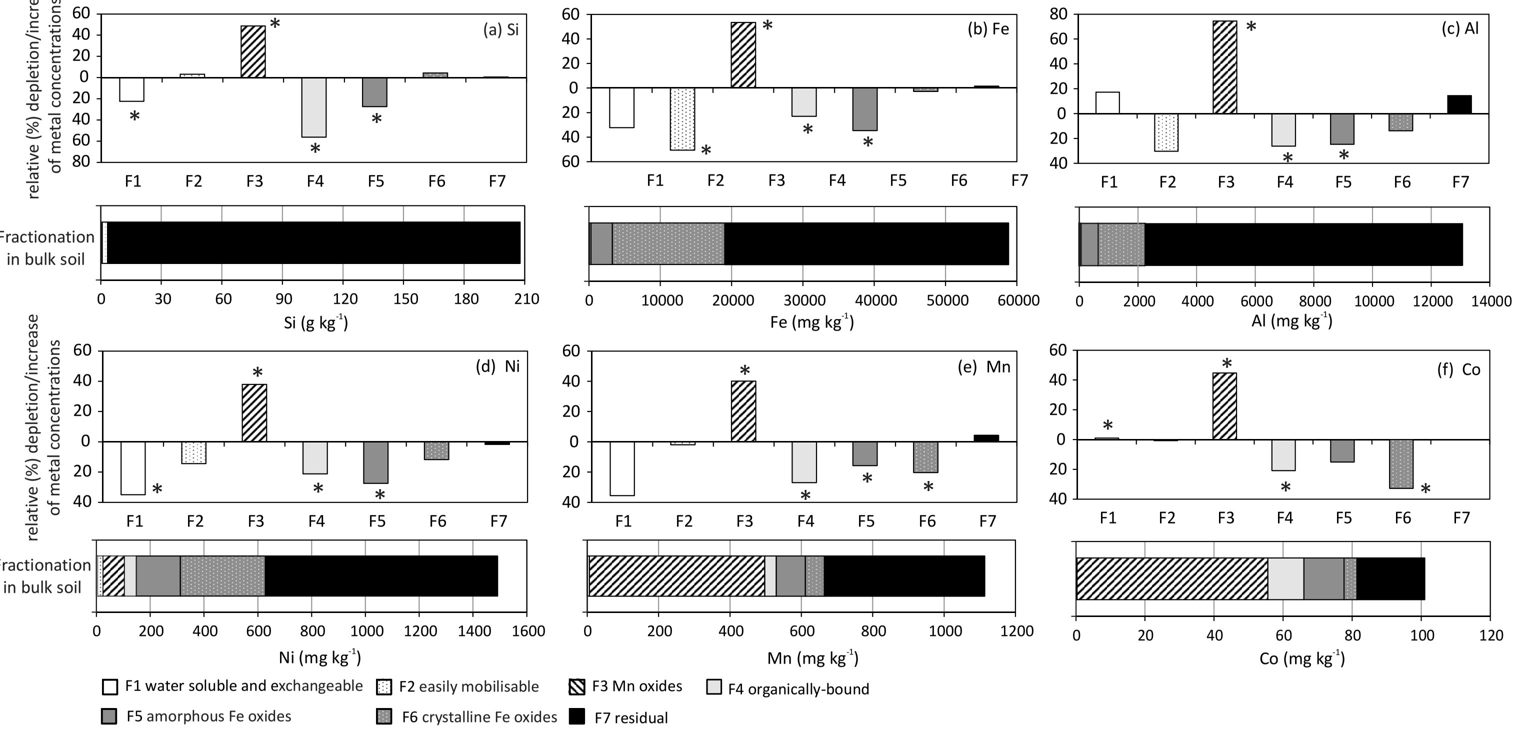

Figure 3.8. Element fractionation in the bulk soil from the non-inoculated plants and mean relative (%) depletion/increase of metal concentration in each fraction from the rhizosphere soil relative to the bulk soil (rhizosphere effect) (n = 5). Depletion/increase was calculated by substraction of the amount of metal in each fraction at rhizosphere soils from the amount of metal in bulk soils. A signficant depletion/increase in metal concentration respect to bulk soil is denoted with an asteris ($p < 0.05$).

Fe were reduced by up to 50 %. On the other hand, plant root activity led to a significant increase in elements associated with Mn oxides ($p < 0.05$), and this was the case for Al, Co, Fe, Mn and Ni. Concentrations of these elements associated with Mn oxides increased from 38 to 74 % in the rhizosphere soil, and this was particularly important for metals such as Co, Mn and Ni where this phase represents a significant proportion of the total metal content. Plant growth had no effect on the residual fraction.

Some differences in the influence of bacterial inoculants on element fractionation in the rhizosphere soil could be seen between the two strains (data not shown). In a similar manner to what occurred in the rock samples incubated with bacterial strains, in rhizosphere soils there was also a general increase in elements associated with amorphous Fe-oxides and organic matter, and this was significant in the case of Al, Fe, Mn and Ni for SBA82 ($p < 0.05$).

3.4 Discussion

The influence of two bacterial strains on the weathering and solubilisation of elements from an ultramafic rock was assayed. Bacterial activity was found to have a significant influence on mineral weathering, as indicated by the release of structural elements (Al, Fe, Si) or adsorbed or interlayer cations (such as Mn, Ni, Co) into the medium solution and their re-distribution among the different geochemical compartments. The release of elements into the medium was most pronounced during the first two days of incubation, coinciding with the period of maximum bacterial growth. Furthermore, based on the differences observed between the two bacterial strains, and on the PCA analysis, our results suggest that the strains preferentially act upon different mineral phases.

In the case of SBA82, the co-release of Al, Fe and Si into the culture medium suggest a preferential weathering of Ni-rich ferromagnesium silicates. Serpentine and chlorite have been identified as the dominant primary minerals in these rocks (unpublished data). Most Fe was included in primary silicate minerals and crystalline Fe oxides, and appears to be rendered amorphous during microbial weathering. Other elements released during weathering were also incorporated into amorphous Fe oxide phases (Al, Si, Co, Cr, Mn, Ni) or released into soluble and exchangeable pools. Amorphous Fe oxides have a high specific surface area and can act as important sorbents of trace metals (Quantin *et al.*, 2008). Our results are in line with previously described weathering sequences of serpentine minerals (Caillaud *et al.*, 2009). Since strain SBA82 is a siderophore-producer the

mechanisms involved in this weathering process are likely to involve siderophore-induced mineral dissolution. Siderophores are iron-chelating secondary metabolites which are known to be produced under Fe-limiting conditions. The reduced Fe concentration of the culture medium used in this study will presumably have induced siderophore production by strain SBA82. Previous studies have demonstrated siderophore-promoted dissolution of goethite and hornblende and consequent release of Fe, Si, and Al (Kalinowski *et al.*, 2000; Liermann *et al.*, 2000b). Although siderophores have a high affinity for Fe(III) they are known to form complexes with other trace elements (Banfield *et al.*, 1999; Schalk *et al.*, 2011). Their presence in the culture medium would therefore maintain elements such as Al, Fe and Si in solution (which was the case in SBA82 cultures). Siderophore mediated Fe solubilisation from Fe-rich minerals could also lead to the solubilisation and re-distribution of their companion trace metals (Cr, Ni). On the contrary, since LA44 is not a siderophore-producer the concentration of major elements (Al, Fe and Si) was no different from controls. However, it cannot be completely ruled out that LA44 is unable to alter ferromagnesian minerals. LA44 could also alter these phases through the secretion of oxalic acid and/or other metabolites. Bacterial secretion of organic acids has been related to the weathering of silicates and Fe (hydr)oxides (Dhungana *et al.*, 2007). Extracellular polymers produced by bacteria have also been shown to affect mineral solubility (Ullman *et al.*, 1996). Nonetheless, the strong mobilisation of Co, Mn and Ni into the culture medium combined with a general reduction of elements associated with Mn oxides suggests that the LA44 strain principally acts on this mineral phase. Although Ni associated with Mn oxides is not the principal Ni fraction in this rock, these oxides seem to be important in determining Ni availability. The analysis of rock samples by SEM confirmed an association between Mn and Ni in this rock. The role of Mn-oxides in determining Ni availability has also been demonstrated by other authors in ultramafic areas. In a study evaluating Ni and Cr extractability in ultramafic soils, Quantin *et al.* (2008) concluded that Ni behaviour appeared to be partly controlled by pH and partly by Mn-oxides. Antić-Mladenović *et al.* (2011) suggested that the dissolution and precipitation of Fe/Mn oxides, organic matter transformations, and adsorption on solids were important processes controlling Ni solubility in ultramafic soils. Similarly, a study carried out in the ultramafic region of Morais, in the same serpentine outcrop where the rock sample used in this study was collected, revealed that the Ni bioavailability in these soils is linked to the Mn oxide fraction (Alves *et al.*, 2011). The strong release of Ni and Mn, and to a lesser degree of Co,

in LA44 cultures seems to be related to the release of organic acids by this bacterial strain. Low molecular weight organic acids, and in particular oxalic acid, are most often cited as the main component in biogeochemical weathering of silicate minerals. These organic ligands can attack minerals directly by complexing with ions at the surface, weakening metal-oxygen bonds, or catalysing dissolution reactions. Indirectly they affect weathering rates by complexing ions in solution, thus lowering the solution saturation rate (Barker *et al.*, 1997). The production of organic acid anions by bacterial strains was related to the weathering of hornblende (Liermann *et al.*, 2000a) and the mobilisation of metals from carbonates and oxides (Li *et al.*, 2010). In the present study, oxalate production seems to have an important role in the solubilisation of Mn and Ni. Oxalate-promoted dissolution of Mn oxides by reduction of Mn(IV) to Mn(II) has been proposed by several authors (Stone, 1987; Pérez-Benito *et al.*, 1996; Banerjee and Nesbitt, 1999). The higher concentrations of Ni released into the culture medium (or the re-distribution of Ni towards labile phases) may be related to the ability of strain LA44 to create reducing conditions. Although no differences in Eh were detected in culture mediums inoculated with either strain, LA44 may generate redox microgradients at the rock surface which were not detected. Solubilisation of Ni in the case of SBA82 mainly came from serpentine group minerals, although a possible influence of this strain on Mn oxides cannot be discarded. Siderophores can also interact with Mn oxides (Duckworth and Sposito, 2007) and could therefore explain, at least partially, the solubilisation of Mn induced by SBA82. In fact, Mn oxides were also reduced after incubation with this strain, although its influence on this phase is far less pronounced than for the LA44 strain.

Metal availability in the soil, or the replenishment of labile metal pools from solid soil phases, is a key element to successful phytoextraction. There has been considerable debate as to whether or not hyperaccumulating plants are able to access metal fractions not available to non-accumulator plants thus increasing metal uptake (Knight *et al.*, 1997; McGrath *et al.*, 1997; Kidd *et al.*, 2009). This study demonstrates the capacity of rhizobacterial strains associated with hyperaccumulating species to mobilize metals such as Ni from rocks. By increasing soil labile metal fractions these bacterial inoculants could also potentially increase metal uptake by metal-(hyper)accumulating plants. In phytoextraction (or phytomining) this would lead to an overall improvement in the efficiency of the process. The effect of these two strains on metal uptake by the hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* was tested in the rhizobox experiment.

In rhizospheric studies of hyperaccumulators some authors show a depletion of labile metal fractions in the rhizosphere (attributed to plant uptake), while others indicate an increase in bioavailable metal fractions in the rhizosphere. In either case, the differences in the concentration of labile metal fractions do not explain the extreme metal uptake by these plants. It therefore continues to be a point of controversy whether or not these plants are able to access metal fractions not available to non-accumulating plants (thus increasing metal uptake), or if their root activity leads to a faster replenishment of soluble metal pools. In this study, water-soluble Ni concentrations increased in the rhizosphere compared to bulk soil of *A. serpyllifolium* subsp. *malacitanum*. The same effect was surprisingly not seen in $Sr(NO_3)_2$- or $Ca(NO_3)_2$-extractable Ni concentrations. However, a similar increase in water-soluble Ni and a decrease in labile Ni in the rhizosphere has been observed in other Ni hyperaccumulators such as *Thlaspi goesingense* (Wenzel *et al.*, 2003; Puschenreiter *et al.*, 2005) and *A. serpyllifolium* subsp. *lusitanicum (A. pintodasilvae*) (Chapter 1). The influence of root activity on metal fractionation in the rhizosphere soil was assessed by selective sequential extraction. A general reduction in the more available metal forms (soluble and exchangeable or F1 and easily mobilisable metals or F2) or fractions associated with organic matter and Fe-oxides (amorphous and crystalline) was observed in the rhizosphere compared to bulk soil. In contrast, plant root activity led to a significant increase in metals associated with Mn oxides. The depletion observed in labile forms of Mn and Ni was most likely due to plant uptake, while reductions in organic matter- and Fe oxide-bound fractions suggest the plant is able to access most soil metal pools, and only the residual fraction was not plant-available. In fact, X-ray diffraction studies of the clay fraction (< 2 µm) of rhizospheric soils of plants growing on serpentinitic soils indicated a more intense weathering of Ni-rich ferromagnesium minerals at the rhizosphere of the hyperaccumulator, *Alyssum serpyllifolium* subsp. *lusitanicum* compared to non-accumulating species such as *Dactylis glomerata* (Kidd *et al.*, 2009). This weathering could lead to the adsorption or co-precipitation of released elements by amorphous Mn-oxides, explaining the observed increase in this fraction in the rhizosphere. The As hyperaccumulator, *Pteris vittata*, also reduced several soil As fractions (including amorphous and crystalline hydrous-oxide bound), and this occurred to a greater extent than the non-hyperaccumulator *Nephrolepis exaltata* (Silva Gonzaga *et al.*, 2006). Wenzel *et al.* (2003) and Puschenreiter *et al.* (2005) also suggested a more intense weathering of Ni-rich minerals in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*, and a

concurrent release of labile Ni.

After 14 weeks growth, plant biomass of inoculated plants tended to be higher than that of non-inoculated plants: shoot biomass was up to 1.3- or 1.4-fold greater in plants inoculated with LA44 or SBA82, respectively (although differences were not statistically significant). This increase in shoot biomass could be related to the ability of both of these strains to produce IAA (Weyens *et al.*, 2009). Nutrient shoot concentrations, such as Ca, Fe, and P also tended to be higher in inoculated plants than in non-inoculated plants, especially in those plants inoculated with strain SBA82. This could be due to bacterial-induced mineral weathering; SBA82 is a phosphate-solubiliser and a siderophore-producer and could lead to an improvement in the plant P and Fe status. Both characteristics have previously been related to an improvement in plant nutrition (De Maria *et al.*, 2011). This improvement in plant nutrition could also be associated with the observed increase in biomass production.

Shoot Ni concentrations were far above the criteria given for Ni hyperaccumulation (> 1000 mg Ni kg^{-1}) (Baker and Brooks, 1989) and were similar to concentrations found in field collected plants of hyperaccumulating subspecies of *Alyssum serpyllifolium* (Diez *et al.*, 2006; Cabello-Conejo, 2010). Shoot Ni concentrations tended to be higher in the LA44-inoculated plants. Although bacterial activity did not significantly influence, either biomass or Ni concentration, the combined effect led to an increase in Ni phytoextracted, and this was significant in the case of LA44-inoculated plants. Similar results were obtained by Cabello-Conejo *et al.* (2011) when they inoculated *A. pintodasilvae* with the same *Arthrobacter* strain LA44 and grew the plants in ultramafic soil from Trás-os-Montes (NE Portugal). The positive influence of bacterial strains on metal uptake by hyperaccumulator plants has been shown by several authors. For instance, inoculation with Ni-mobilising rhizobacteria enhanced Ni uptake by the Ni-hyperaccumulator *Alyssum murale* (Abou-Shanab *et al.*, 2003; Abou-Shanab *et al.*, 2006) and by the non-hyperaccumulator *Brassica juncea* (Ma *et al.*, 2009). The increase in shoot Ni concentration observed in LA44 treatment could be due to the ability of this strain to act on Mn oxides through the production of organic acids, and consequent release of associated Ni. This could effectively help to replenish metals in the more labile fractions and enhance metal uptake by the plant.

In conclusion, the bacterial activity led to the weathering of ultramafic rock in *in vitro* batch cultures. The two bacterial strains studied acted on distinct mineral phases and the mechanisms involved in this process were isolate-specific. Fur-

ther studies should be carried out using pure mineral phases (e.g. olivine, Mn oxides) to study the different mechanisms operating in more detail. Nonetheless, bacterial activity led to an increase in the availability of metals such as Mn, Ni and Co. The inoculation of *A. serpyllifolium* subsp. *malacitanum* with both bacterial strains (applied separately) had a positive, although not significant, effect on plant growth and Ni shoot concentration. Moreover, a significant increase in phytoextracted Ni was observed with the bacterial inoculum which was able to solubilise Ni associated with Mn oxides, a fraction which increased in the rhizosphere of *Alyssum* and which has previously been associated with Ni bioavailability in serpentine soils. In conclusion, rhizobacterial strains could be selected for improving phytoextraction (phytomining) efficiency.

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Chapter 4

Pseudometallophytes colonising Pb/Zn mine tailings: a description of the plant-microorganism-rhizosphere soil system and isolation of metal-tolerant bacteria

ABSTRACT

The plant-microorganism-soil system of three pseudometallophytes (*Betula celtiberica*, *Cytisus scoparius* and *Festuca rubra*) growing in a Pb/Zn mine was characterised. Plant metal accumulation, soil metal fractions (rhizosphere and non-vegetated) and bacterial densities were determined. Total Cd, Pb and Zn in non-vegetated soils was up to 50, 3000 and 20000 mg kg^{-1} dry weight, respectively. The residual fraction dominated non-vegetated soils, whereas plant-available fractions became important in rhizosphere soils. All plant species effectively excluded metals from the shoot. *Festuca rubra* presented a shoot:root transport factor of $\leq$0.2 and this population could be useful in future phytostabilisation trials. Culturable bacterial densities and diversity were low (predominantly *Actinobacteria*). Rhizosphere soils hosted higher total and metal-tolerant bacterial densities. Seventy-four metal-tolerant rhizobacteria were isolated, and characterised genotypically (BOX-PCR, 16S rDNA) and phenotypically (Cd/Zn tolerance, biosurfactant production and plant growth-promoting (PGP) traits). Several isolates resisted high concentrations of Cd and Zn, and only a few presented PGP traits. Fourteen isolates were evaluated for promoting plant growth of two species (*Salix viminalis* and *Festuca pratensis*). Thirteen inoculants enhanced growth of *F. pratensis*, while only three enhanced growth of *S. viminalis*. Growth enhancement could not always be related to isolate PGP traits. In conclusion, some isolates show potential application in phytostabilisation or phytoextraction techniques.

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4.1 Introduction

Mining activities are known to be among the principal causes of soil contamination. The mine-spoils and tailings generated by this industry generally present hostile environments for plant growth, due to low nutrient availability, low organic matter content, high acidity and often elevated trace metal content (Batty, 2005; Barrutia *et al.*, 2011). Despite these unfavourable conditions, plant metallophytes have evolved biological mechanisms permitting them to resist and tolerate toxic concentrations of metals, and colonise this type of substrate (Whiting *et al.*, 2004). Many studies have focused on these plants due to their potential use in the rehabilitation of metal-contaminated land or, more recently, due to their possible application in phytoremediation. Phytoextraction aims to remove trace metals from the soil through their uptake and accumulation by plants; whereas phytostabilisation aims to establish a vegetation cover and promote *in situ* inactivation of metals. Metal-tolerant populations of common plant species (pseudometallophytes) are able to resist higher concentrations of metals compared with members of the same species growing on uncontaminated soils. On the other hand, true metallophytes have evolved over time on substrates derived from weathered mineral deposits (Whiting *et al.*, 2004). Metallicolous populations of temperate grasses, such as *Agrostis capillaris*, *Agrostis gigantea* and *Festuca rubra*, have shown a good ability to colonize Pb-, Zn- and Cu-contaminated soils and have been successfully applied in phytostabilisation (Mench *et al*., 2010).

Metalliferous soils do not only provide sources of interesting flora but also of metal-tolerant microorganisms (Schippers *et al.*, 1995; Prasad and Freitas, 1999; Batty, 2005; Epelde *et al.*, 2010). Like plants, these microorganisms have adapted to the extreme conditions and can aid the establishment and proliferation of colonising plant species (Hanbo *et al.*, 2004; Grandlic *et al.*, 2008). The plant growth-promoting bacteria (PGPB) include P and K solubilisers, the free living N_2 -fixing bacteria, rhizobia, and arbuscular mycorrhizal fungi (AMF) etc. A growing number of studies suggest that the phytostabilisation process can be more effective after inoculating plants with PGPB, due to an enhanced plant metal tolerance, growth and survival (Petrisor *et al.*, 2004; Wu *et al.*, 2006; Grandlic *et al.*, 2008). A successful vegetative cover on multi-metal-contaminated mine tailings was achieved after inoculating native plant species with PGPB in combination with soil amendments (Grandlic *et al.*, 2008). The use of inoculants also reduced the requirement for amendments and associated economic costs. A substantial improve-

ment in the growth of *Albizia lebbeck* was observed on gypsum mine soils due to inoculation with *Bradyrhizobium* sp. (Rao and Tak, 2001). Inoculating *Lupinus luteus* with a bacterial consortium of metal-resistant PGP rhizobacteria improved plant growth, and reduced plant metal accumulation, in a multi-metal-contaminated soil (Dary *et al.*, 2010).

Plant-associated microorganisms can also influence the trace metal mobility and availability to plants through the release of chelating agents, acidification, P solubilisation or redox changes. A microbial-mediated reduction in metal availability is particularly interesting from a phytostabilisation point of view. Pishchik *et al.* (2002) suggested that Cd-tolerant plant growth-promoting rhizobacteria (PGPR) (*Antrhobacter mysorens* 7, *Flavobacterium* sp. L30, *Klebsiella mobilis* CIAM 880) migrated from the rhizoplane to the rhizosphere where they bound soluble Cd in biologically unavailable complex forms. Metabolic processes of both plants and microorganisms can strongly affect trace metal behaviour in the rhizosphere (Kidd *et al.*, 2009). Exploiting interactions between (pseudo)metallophytes and their associated microflora in the rhizosphere could assist in the further development of phytoremediation strategies. To do this, further studies characterising plants and their associated microorganisms in metalliferous sites are necessary.

The overall objective of this study was to characterise the plant-microorganism-soil system associated with dominant pseudometallophytes colonizing a Pb/Zn-mining area. As part of this objective we (a), evaluated the bioavailability and chemical fractionation of Cd, Pb, and Zn in the rhizosphere, and the accumulation of these metals by plants; (b), obtained a collection of metal-tolerant rhizobacterial isolates with potential application in phytoremediation; and (c), assessed the effects of selected rhizobacteria on growth of two plant species (*Festuca pratensis* Huds. and *Salix viminalis* L.) commonly used in phytoremediation strategies.

4.2 Materials and methods

Study site and sampling

This study was carried out in the abandoned Pb-Zn mine of Rubiais in the Lugo province of NW Spain (UTM 29T 660781/4726800). Metal deposits are rich in sulphides, principally in the form of sphalerite (ZnS) and galena (PbS) in a 7 to 1 ratio (Arias Prieto, 1991). The mine operated from 1977 until the early 1990s, and during the 1980s the average annual production was 95000 t of zinc concentrates

(with 61 % Zn, 0.12 % Cd and 0.16 % Hg) and 15000 t of lead concentrates (with a 70 % Pb content) (Arias Prieto, 1991). The mine tailings cover a surface area of 30 ha, and large areas were re-planted with birch at the time of the mine closure. The surrounding area is characterised by the presence of sandstone, quartzite and slate, which alternate with limestone and dolomite (Arias Prieto, 1991). The most frequent natural soils in the area are Alumi-umbric Leptosols and Regosols (WRB, 2006). The climate of the region is Oceanic, with a mean annual precipitation of 2000 mm and annual temperature of 8-9 ºC (Carballeira *et al.*, 1983).

Plant and soil samples were taken at one of the most contaminated points of the mine tailings, where the vegetation cover is low with frequent bare areas (Diez Lázaro, 2008). The spontaneous vegetation is predominantly *Cytisus scoparius* and *Betula celtiberica* and some gramineae. Five individuals of *Cytisus scoparius* (L.) Link and *Betula celtiberica* Rothm. & Vasc., and seven individuals of *Festuca rubra* L. were sampled. All three plants had spontaneously grown at the site, and in the case of *C. scoparius* and *B. celtiberica* only young plants (approximately < 10 years) were sampled. Due to a severely stunted growth (< 50 cm height), it was possible to collect the whole plant including the root ball. The rhizosphere was defined as the soil attached to roots after gentle crushing of the root ball and shaking the root system. In addition, surface soil samples (0-10 cm) were collected from bare patches where no plants were found growing (non-vegetated soil).

Soil and plant analyses

Soil analyses were carried out on the air-dried, < 2 mm fraction of non-vegetated and rhizosphere soil samples. Soil pH (in H_2O and KCl), total C and N and exchangeable cations were determined as described in Chapter 1. Dissolved organic C (DOC) was measured in a 1:5 soil/H_2O extract (1 h shaking) using a TOC-5000 total carbon analyser (Model FLOWSYS, SYSFEA, Italy). The carbonate fraction was determined following the Schleiber method (Tatzber *et al.*, 2007). Soils (0.5 g) were digested in a 2:1 mixture of concentrated HNO_3:HCl in Teflon PFA vessels in a microwave accelerated reaction system (MarsXpress; CEM Corp., USA) and total concentrations of metals were analysed by atomic absorption spectroscopy (AAS) (Perkin-Elmer 2380, Norwalk, CT). Water-soluble Cd, Pb and Zn concentrations were analysed by AAS with graphite furnace as described in Chapter 1. A metal fractionation scheme was carried out following a modified BCR protocol (see chapter 2). Briefly, a first step was included in which soils were shaken at room temperature with 1 M NH_4Cl for 16h. This extracts the water-soluble and

exchangeable metal fraction (exchangeable). Second, the resulting residue was shaken at room temperature with 0.11 M CH_3COOH for 16 h. This step extracts the carbonate-bound metal fraction (carbonate). Third, the residue was shaken for 16 h at room temperature with 0.10 M $NH_2OH.HCl$ adjusted to pH 2.0 with high purity HNO_3. This extracts mainly iron and manganese oxide bound forms (reducible). Fourth, the residue was digested with 30 % H_2O_2, taken to dryness on a water bath heated to 85 ºC, and shaken with 1 M NH_4OAc adjusted to pH 2.0 with HOAc for 16 h. This step extracts primarily organically bound and sulphide metals (oxidisable fraction). Finally, the residual fraction (silicate-bound metals) was digested as above, and the concentration of Cd, Pb and Zn were analysed in the filtered supernatants of each extraction by AAS as above.

Shoots and roots of plants were separated, washed with pressurised tap water (and 0.05 M $CaCl_2$ in the case of roots) followed by deionised water, oven-dried at 45 ºC and ground. Plant tissues (0.1 g) were digested and the concentration of Cd, Pb and Zn were determined as described in Chapter 2. Shoot metal content, in the case of *B. celtiberica* individuals, refers to leaf concentrations. The transport factor was calculated as the ratio of metal concentrations in the shoots to that in the roots (TF).

Determination of soil culturable bacterial densities and isolation of rhizobacterial strains

Four grams of fresh rhizosphere or non-vegetated soil were suspended in 16 ml sterile sodium hexametaphosphate solution (1 %) and shaken for 30 min in an end-over-end shaker. Soil suspensions were diluted in 10-fold series and plated in duplicate onto modified 284 agar medium as described previously (see Chapter 2). Densities of culturable metal-tolerant bacteria were determined in 284 medium supplemented with either, 0.1 mM or 1 mM of Cd (as $CdSO_4.8/3H_2O$) or, with 1 mM or 3 mM of Zn (as $Zn(SO_4)_2.7H_2O$). After 7 days (28 °C), colony forming units were counted and calculated per gram soil (CFUs g^{-1} soil). The rhizosphere effect was calculated as the ratio of the number of microorganisms in the rhizosphere over the number of microorganisms in the non-vegetated soil (R/NV). Distinct metal-tolerant morphotypes (1-5 colonies) associated with each plant species were sub-cultured at least three times and cryopreserved at -70 ºC in culture medium supplemented with 15 % (v/v) glycerol.

Phenotypic characterisation of rhizobacterial isolates

Rhizobacterial strains were screened for metal resistance using 284 agar medium supplemented with Cd (0, 0.5, 1.0, 2.0, 4.0, 5.0, 6.0 mM Cd) or Zn (0, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0 mM Zn). The Maximal Tolerable Concentration (MTC) of Cd/Zn was recorded as the highest metal concentration in which the isolate grew. Rhizobacterial strains were also screened for the ability to produce biosurfactants, and for various PGP characteristics: phosphate solubilisation capacity, siderophore production and indoleacetic acid (IAA) production as described previously (see Chapter 2).

Genotypic characterisation of rhizobacterial strains

DNA was extracted from all purified bacterial strains as described previously and one µl of extracted DNA was used for BOX-PCR reactions as described previously (see Chapter 2). PCR products were separated by electrophoresis in agarose gels (1.8 %) and ethidium bromide-stained gel images were analysed using the Gel Compar Bionumerics programme (v5.1, Applied Maths, Belgium). Isolates were grouped according to their BOX-PCR profiles at a similarity level of 92 %.

Amplification targeting the 16S rDNA gene was carried out as described previously on those strains showing distinct BOX-PCR profiles (see Chapter 2). PCR products were purified and partially sequenced (approximately 600 bases) using the primer 16S-27F. Sequence data were checked using the Chromas v.1.45 software (Technelysium Pty. Ltd., Australia) and assessed for similarity with sequences of type strains in the Ribosomal Database Project (Cole *et al.*, 2009). Sequences are available in the EMBL database (www.ebi.ac.uk) under accession numbers HE585527-HE585572.

Influence of rhizobacteria on growth and metal uptake of *Festuca pratensis* and *Salix viminalis*

A selection of the rhizobacterial strains were used in a pot experiment to evaluate their influence on plant growth and metal uptake by *Festuca pratensis* (metal-tolerant population; Boisson *et al.*, 1998) and *Salix viminalis* (metal-tolerant population; unpublished data). The rhizobacterial strains were selected according to their MTC values and phenotypic traits (Table 4.3).

Two-week-old rooted cuttings of *S. viminalis* and seeds of *F. pratensis* were potted in perlite:quartz sand (2:1 v/v). After germination, *F. pratensis* were thinned out to four plants per pot. Plants were watered with half-strength Hoagland solu-

tion for two weeks until bacterial inoculation. The half-strength Hoagland's solution contains per liter deionised water 50 ml macroelements, 500 ml microelements and 300 ml Fe-EDTA (macroelements (g l^{-1}): 10.2 KNO_3, 7.08 $Ca(NO_3)_2.4H_2O$, 2.30 $NH_4H_2PO_4$, 4.9 $MgSO_4.7H_2O$; microelements (g l^{-1}): 2.86 H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.08 $CuSO_4.5H_2O$, 0.09 $H_2MoO_4.H_2O$, 0.22 $ZnSO_4.7H_2O$; Fe-EDTA (g l^{-1}): 5.00 EDTA-Na, 7.60 $FeSO_4.7H_2O$). Fresh cultures of bacterial strains were grown in 869 medium (see Chapter 2) for 3 days, harvested by centrifugation (4000 g, 15min) and resuspended in 10 mM $MgSO_4$ to an optical density of 1.0 at 660 nm (about 10^7 cells per ml). Each plant pot was inoculated with 9 ml of bacterial suspension. The same amount of sterile 10 mM $MgSO_4$ was added to non-inoculated pots. Seven replicates of each plant species were prepared for each inoculation treatment. After inoculation, plants were watered with half-strength Hoagland solution supplemented with 2 µM Cd and 100 µM Zn for seven weeks. Shoots and roots were then separated, washed in deionised water (0.05 M $CaCl_2$ followed by rinsing in the case of roots), oven-dried at 45 ºC, weighed and ground. Plants were grown in an environmentally controlled growth chamber (16/8h light/darkness, day/night temperature 26/20 ºC, PPFD 190 µmol m^{-2} s^{-1}) and solutions replenished every 2 days. Oven-dried leaves were digested in a 2:1 HNO_3:HCl mixture and P, K, Ca, Mg, Fe, Cd, Cu, Mn and Zn measured by ICP-OES (Vista Pro; Varian Inc., Australia).

Statistical analyses

Differences in soil physicochemical parameters, plant metal content and microbial densities were determined using analyses of variance (ANOVA). A multiple comparison of means was determined by the "post-hoc" Least Significance Difference test. Significant effects of bacterial strains on biomass production, nutrient and metal content in the plant inoculation experiment were determined using ANOVA followed by the "post-hoc" Dunnett's test.

4.3 Results

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

Non-vegetated soils presented alkaline pH (pH 8.3) with an important carbonate fraction (10.4 %), and low N content (< 0.1 %) (Table 4.1). Cation exchange capacity (CEC) was low (< 10 $cmol_c$ kg^{-1}) and dominated by Ca. Rhizosphere soils

presented higher CEC (up to 1.5-fold) and a higher DOC (up to 8-fold) compared to non-vegetated soil, but a similar total C content (1.5-2.0 %). DOC increased in the order *F. rubra* < *C. scoparius* < *B. celtiberica*. On the contrary, pH values were lower in rhizosphere soils (pH 7.5-8.0) (Table 4.1). Total Cd, Pb and Zn in non-vegetated soil were close to 50 mg kg^{-1}, 3000 mg kg^{-1} and 20000 mg kg^{-1}, respectively (Figure 4.1a). In contrast, metal concentrations in the rhizosphere were consistently lower (1.5 to 2-fold), decreasing in the order *B. celtiberica* > *C. scoparius* ≥ *F. rubra* (Figure 4.1a). Concentrations of water-soluble metals followed the same order as total concentrations but tended to be in the rhizosphere compared to non-vegetated soil (2.5 to 19-fold higher) (Figure 4.1b).

The residual fraction was dominant in non-vegetated soils, representing more than 60 % of total Cd and Zn, and almost 50 % of total Pb (Figure 4.1c). Of the non-residual fractions, Cd was detected in the exchangeable pool or oxidisable fraction (10 and 17 %, respectively). Carbonate-bound and reducible fractions represented in this case a minor percentage of the total concentration (4 and 5 %, respectively; Figure 1c). After the residual fraction, the carbonate and oxidisable pools were the most dominant for Pb and Zn (22 and 25 % for Pb, and 12 and 18

Table 4.1. Physicochemical properties of non-vegetated and rhizosphere soils. Within each row different letters indicate significant differences at $p < 0.05$.

	Non-vegetated soil	Rhizosphere soils		
		F. rubra	*C. scoparius*	*B. celtiberica*
pH_{H2O}	8.3 ± 0.1 *c*	8.0 ± 0.1 *b*	7.9 ± 0.1 *b*	7.5 ± 0.0 *a*
pH_{KCl}	7.6 ± 0.1 *b*	7.7 ± 0.1 *b*	7.6 ± 0.1 *b*	7.2 ± 0.1 *a*
Total organic C (mg l^{-1})	6.3 ± 0.6 *a*	23.6 ± 4.2 *b*	41.8 ± 11.9 *c*	51.9 ± 6.0 *c*
Carbonates (%)	10.4 ± 0.1	n.d.	n.d.	n.d.
C (%)	1.9 ± 0.3 *a*	1.9 ± 0.1 *a*	1.5 ± 0.4 *a*	2.0 ± 0.0 *a*
N (%)	< D.L.	< D.L.	< D.L.	< D.L.
Exchangeable cations (cmol$_c$ kg^{-1})				
Ca^{2+}	9.1 ± 0.1	12.3 ± 0.7	12.4 ± 2.1	10.1 ± 0.3
Mg^{2+}	0.3 ± 0.01	0.4 ± 0.03	0.9 ± 0.3	0.4 ± 0.04
Na^{+}	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.04	0.2 ± 0.03
K^{+}	0.01 ± 0.0	0.06 ± 0.01	0.2 ± 0.05	0.17 ± 0.03
CEC	9.4 ± 0.2 *a*	12.9 ± 0.7 *b*	13.7 ± 2.4 *b*	10.9 ± 0.3 *ab*
Ca/Mg	33 ± 1	30 ± 2	16 ± 3	23 ± 3

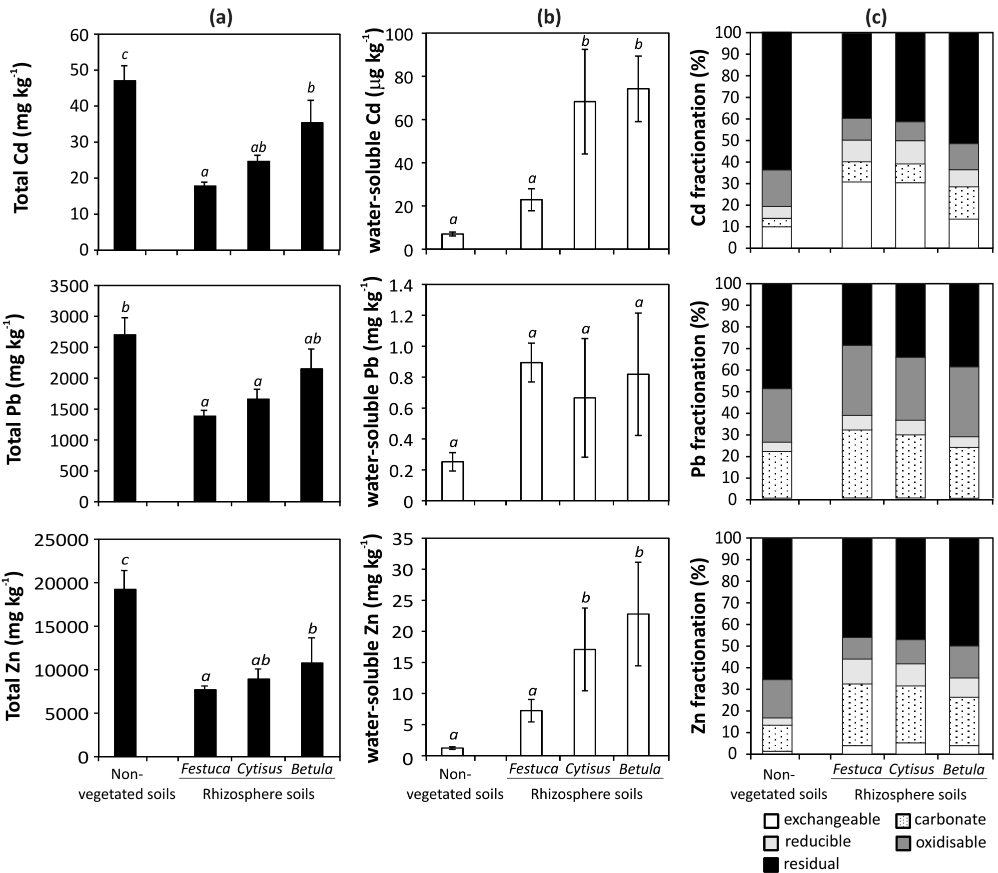

Figure 4.1. (a) Total concentrations (mean ± SE), (b) water-soluble concentrations (mean ± SE), and (c) chemical fractionation (percentage distribution) of Cd, Pb, and Zn in non-vegetated and rhizosphere soils. Different letters indicate significant differences ($p < 0.05$).

% for Zn, respectively). Despite representing less than 1 % of the total metal content, exchangeable Pb and Zn were important in terms of absolute values (20 mg kg^{-1} Pb and 257 mg kg^{-1} Zn).

In rhizosphere soils, the residual fraction was not always the dominant pool and the more labile metal fractions generally represented a higher percentage than in non-vegetated soil (Figure 4.1c). Metal fractionation was similar between the rhizosphere of the three species. Cd was principally in an exchangeable form, representing 30 % of total Cd in the rhizosphere of *F. rubra* and *C. scoparius*. In the rhizosphere of *B. celtiberica*, Cd was associated with the exchange complex or carbonate-bound (15 % of total Cd in both cases). Zn was mainly associated with carbonates (22-29 % of total Zn), while Pb was associated with both carbonates and the oxidisable fraction (representing 24-32 % and 29-32 % of total Pb, respectively; Figure 4.1c).

Shoot concentrations of Cd and Pb did not differ significantly between species, ranging from 5.8 to 6.9 mg kg^{-1} Cd and 69 to 139 mg kg^{-1} Pb (Figure 4.2). In the case of Zn, *B. celtiberica* accumulated significantly more Zn in leaves than either *C. scoparius* or *F. rubra* (1935 mg kg^{-1} compared to 1205 mg kg^{-1} and 1037 mg kg^{-1}, respectively). However, all three species excluded Cd, Pb and Zn from their shoot tissues (Figure 4.2). TF values were consistently less than 1, especially in the case of Pb (≤ 0.2). This excluder characteristic was particularly evident in *F. rubra*, which presented the highest root concentrations (34 mg kg^{-1} Cd, 1308 mg

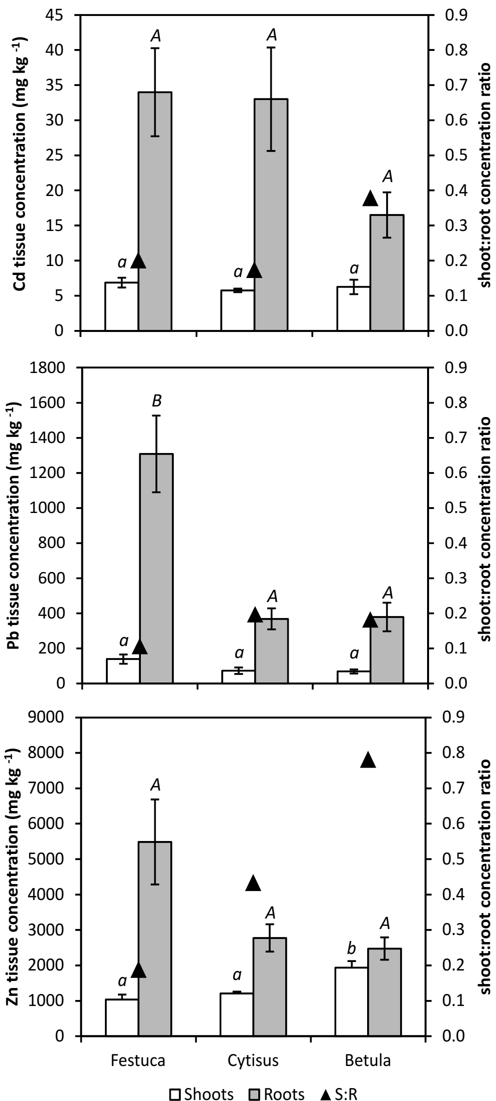

Figure 4.2. Metal concentrations (mean ± SE) in shoot and root tissues of plants and shoot:root metal concentration ratio. Different letters indicate significant differences ($p < 0.05$).

kg^{-1} Pb and 5485 mg kg^{-1} Zn) and lowest shoot:root ratios (≤ 0.2) for all 3 metals. In contrast, the highest TF values were observed in *B. celtiberica* (Cd (0.4) and Zn (0.8)).

Abundance of culturable bacteria, phylogenetic affiliation and phenotypic traits

In non-vegetated soil, a mean microbial density of 3.2 ± 0.1 log CFUs g^{-1} soil was determined. Densities of Cd- and Zn-tolerant bacteria (cultivated in medium with 0.1 mM Cd or 1.0 mM Zn) were lower and represented 22 % and 10 % of the total culturable abundance, respectively. This percentage was reduced to less than 5 % of the total population when the metal concentration was increased to 1.0 mM Cd or 3.0 mM Zn (Table 4.2).

Microbial densities were higher in the rhizosphere of all three plant species compared to non-vegetated soil (in many cases by one or two logarithmic units; Table 4.2). This was the case for both the total (R/NV ratios of 5-40) and metal-tolerant populations (R/NV ratios of 1-1265). *C. scoparius* hosted the highest bacterial densities in the rhizosphere. Abundance of bacteria cultivated in medium supplemented with 0.1 mM Cd tended to be higher than the total population in Cd-free medium. Bacterial densities were reduced at the higher Cd concentration (1.0 mM) or at both concentrations of Zn (1.0 and 3.0 mM) (Table 4.2). However, this toxic effect of metals was less evident in the plant rhizosphere than in non-vegetated soil. At 1.0 mM Cd, rhizobacterial densities represented 14, 19 and 25 % of the total population in *F. rubra*, *C. scoparius* and *B. celtiberica* respectively, compared to 4 % in non-vegetated soil. At 1.0 mM Zn, rhizobacterial densities represented 61, 39 and 34 % of the total population in *F. rubra*, *C. scoparius* and *B.*

Table 4.2. Microbial densities (log CFUs g^{-1} soil) of total and metal-tolerant culturable bacteria in non-vegetated and rhizosphere soils. Within each row different letters indicate significant differences at $p < 0.05$.

	Non-vegetated soil	Rhizosphere soils		
		F. rubra	*C. scoparius*	*B. celtiberica*
284	3.2 ± 0.1 *a*	3.9 ± 0.6 *ab*	4.8 ± 0.5 *b*	4.2 ± 0.4 *ab*
284 + Cd 0.1 mM	2.4 ± 0.2 *a*	4.4 ± 0.5 *b*	5.5 ± 0.2 *b*	4.6 ± 0.4 *b*
284 + Cd 1 mM	1.4 ± 0.5 *a*	3.2 ± 0.7 *b*	4.5 ± 0.4 *b*	3.6 ± 0.3 *b*
284 + Zn 1 mM	2.1 ± 0.2 *a*	3.8 ± 0.3 *a*	4.1 ± 0.8 *a*	2.8 ± 1.1 *a*
284 + Zn 3 mM	1.6 ± 0.1 *a*	2.6 ± 0.9 *a*	1.8 ± 1.0 *a*	1.5 ± 0.9 *a*

celtiberica respectively, compared to 10 % in non-vegetated soil. This percentage was reduced to < 7 % at 3.0 mM Zn in all three species, reaching values similar to those recorded in non-vegetated soil.

A total of 74 metal-tolerant rhizobacterial strains were isolated. According to their BOX-PCR profiles, isolates were allocated into 46 distinct groups (Table 4.3). Thirty-seven of these were represented by only one isolate. BOX-PCR groups containing more than one bacterial strain were generally composed of strains isolated from the same host plant (e.g. all 6 isolates from group B02 were from *F. rubra*, while in B26 all four isolates were from *C. scoparius*). In contrast, some groups (e.g. B15 and B30) included strains with the same BOX profile but isolated from different plant species i.e. these strains are associated with more than one plant species. Rhizobacterial strains were primarily affiliated with the Phylum *Actinobacteria*. At the genus level, the majority belonged to the genera *Streptomyces* (61 %), *Tsukamurella* (18 %) or *Pseudomonas* (18 %). In terms of Cd and Zn tolerance, the isolate collection can be divided into two main groups. In the first group (Box Groups 1 to 24) isolates showed a lower metal tolerance, with MTC values of < 0.5-2 mM for Cd, and < 0.5-2.5 mM for Zn. In the second group, (Box Groups 25 to 46) MTC values increased to 4-5 mM for Cd, and 2.5-25 mM for Zn. Isolates belonging to the *Pseudomonas* genera were all included in the first group with lower MTC values. On the contrary, all of the isolates belonging to the *Tsukamurella* genera were included in the second group with higher MTC values. Rhizobacterial isolates classified as *Streptomyces* were interspersed among both groups.

Thirty eight percent of the isolates presented at least one PGP trait and/or the ability to produce biosurfactants. Twenty percent of the isolates produced biosurfactants. Fifteen percent were able to solubilise inorganic PO_4, mainly identified as members of the genera *Pseudomonas*. Only 6 strains were siderophore-producers and none were isolated from *F. rubra*. Only two strains were IAA-producers (P87, *Massilia niastensis*, 98.0 % similarity; P30, *Rhodococcus erythropolis*, 99.2 % similarity).

Re-inoculation of metal-tolerant *Festuca pratensis* and *Salix viminalis*

Fourteen strains were selected (seven from each MTC group) for a re-inoculation experiment with *Festuca pratensis* and *Salix viminalis* plants (Table 4.3). In both plant species, those strains which had a positive (or negative) effect on shoot biomass had a similar effect on root biomass (R^2 0.87; $p < 0.01$). In general, inoculat-

Table 4.3. BOX-PCR fingerprints, partial 16S rDNA sequence identification, and phenotypic characteristics of bacterial isolates from the rhizosphere of *F. rubra* (F), *C. scoparius* (C), and *B. celtiberica* (B). Isolates used in the plant inoculation experiment are highlighted in bold.
Ht, host; Cd, MTC mM Cd; Zn, MTC mM Zn; P, phosphate solubilisation capacity; Sf, biosurfactant producer; Sd, siderophore producer; IAA, indoleacetic acid producer.

Most similar type strain (% similarity)	Group and Box Profile	Isolate	Ht	Cd	Zn	P	Sf	Sd	IAA
Massilia niastensis EU808005 (98.0)	B01	**P87**	F	0.5	1	-	-	-	+
Pseudomonas costantinii AF374472 (99.7)	B02	P36	F	0.5	< 0.5	+	+	-	-
		P39	F	0.5	0.5	-	+	-	-
		P28	F	0.5	< 0.5	+	+	-	-
		P29	F	0.5	0.5	+	+	-	-
		P31	F	0.5	2.5	+	+	-	-
		P33	F	0.5	0.5	+	+	-	-
Pseudomonas costantinii AF374472 (99.7)	B03	P34	F	0.5	1	+	+	-	-
Pseudomonas lini AY035996 (98.9)	B04	P24	F	< 0.5	0.5	+	+	-	-
Pseudomonas koreensis AF468452 (98.8)	B05	**P35**	F	0.5	0.5	+	-	-	-
Pseudomonas lurida AJ581999 (98.3)	B06	**P42**	B	1	5	+	-	-	-
		P41	B	0.5	0.5	+	-	+	-
		P48	B	1	2.5	-	-	-	-
Pseudomonas reinekei AM293565 (99.0)	B07	P18	C	1	0.5	-	-	+	-
Acinetobacter ursingii AJ275038 (99.8)	B08	P23	F	2	2.5	-	-	-	-
Streptomyces canus AY999775 (100)	B09	P84	F	< 0.5	2.5	-	-	-	-
Streptomyces umbrinus AB184305 (96.2)	B10	**P65**	C	1	2.5	-	-	+	-
Streptomyces canus AY999775 (99.3)	B11	P66	C	1	2.5	-	-	-	-
Streptomyces alboniger AY845349 (97.2)	B12	P86	F	< 0.5	1	+	-	-	-
Pseudomonas rhizosphaerae AY152673 (98.5)	B13	P38	F	0.5	1	+	+	-	-
Streptomyces canus AY999775 (99.2)	B14	P83	F	1	5	+	-	-	-
Streptomyces canus AY999775 (99.4)	B15	P80	F	1	1		-	-	-
		P45	B	1	2.5	-	-	-	-
		P73	C	0.5	2.5	-	-	-	-
		P68	C	1	2.5	-	-	-	-
		P69	C	1	2.5	-	-	-	-
		P67	C	0.5	2.5	-	-	-	-
		P40	B	2	2.5	-	-	-	-
Streptomyces canus AY999775 (99.0)	B16	P70	C	1	2.5	-	-	-	-
Streptomyces canus AY999775 (99.7)	B17	P49	B	0.5	1	-	+	-	-
Streptomyces canus AY999775 (99.6)	B18	P63	C	0.5	2.5	-	-	+	-
Streptomyces canus AY999775 (99.2)	B19	P89	F	0.5	2.5		-	-	-
Streptomyces canus AY999775 (98.2)	B20	P85	F	< 0.5	2.5	+	-	-	-
		P82	F	1	2.5		-	-	-
Streptomyces canus AY999775 (99.6)	B21	P81	F	1	2.5	-	-	-	-
Streptom. phaeochromogenes AB184738 (99.5)	B22	P37	F	2	5	-	-	-	-

Most similar type strain (% similarity)	Group and Box Profile		Isolate	Ht	Cd	Zn	P	Sf	Sd	IAA
Streptomyces floridae AB184656 (99.7)	B23		**P12**	C	1	25	-	-	-	-
			P13	C	2	25	-	-	-	-
Streptomyces floridae AB184656 (100)	B24		P50	C	2	25	-	+	-	-
Streptomyces coelescens AF503496 (97.7)	B25		P79	F	1	2.5	-	-	-	-
			P76	F	4	5	-	-	-	-
Streptomyces afghaniensis AB184847 (100)	B26		P07	C	5	5	-	-	-	-
			P08	C	1	2.5	-	-	-	-
			P09	C	5	5	-	-	-	-
			P10	C	5	5	-	-	-	-
Streptomyces arenae AJ399485 (97.9)	B27		P46	B	4	5	-	-	-	-
Streptomyces arenae AJ399485 (99.0)	B28		P43	B	4	5	-	-	-	-
Streptomyces arenae AJ399485 (97.6)	B29		P55	B	4	5	-	-	-	-
Streptomyces coelescens AF503496 (98.1)	B30		P51	B	4	5	-	-	-	-
			P56	B	4	25	-	-	+	-
			P25	F	4	25	-	-	-	-
			P88	F	6	15	-	-	-	-
Streptomyces coelescens AF503496 (97.7)	B31		P32	F	4	5	-	-	-	-
Streptomyces coelescens AF503496 (98.6)	B32		P53	B	4	25	-	-	-	-
Streptomyces canus AY999775 (99.5)	B33		P15	C	4	5	-	-	-	-
Streptomyces coelescens AF503496 (98.0)	B34		P52	F	4	5	-	-	-	-
Streptomyces coelescens AF503496 (99.0)	B35		**P71**	C	4	10	-	+	-	-
Streptomyces coelescens AF503496 (97.0)	B36		**P54**	B	4	25	-	-	-	-
Streptomyces coelescens AF503496 (98.7)	B37		**P26**	F	4	25	-	-	-	-
Streptomyces coelescens AF503496 (98.2)	B38		**P64**	C	5	5	-	-	+	-
Rhodococcus erythropolis X79289 (99.2)	B39		**P30**	F	4	2.5	-	-	-	+
Tsukamurella tyrosinosolvens AY238514 (99.9)	B40		P14	C	4	10	-	-	-	-
Tsukamurella spumae Z37150 (99.9)	B41		P17	C	4	10	-	-	-	-
Tsukamurella spumae Z37150 (99.6)	B42		P11	C	4	10	-	-	-	-
Tsukamurella paurometabola AF283280 (98.0)	B43		P16	C	5	25	-	-	-	-
Tsukamurella strandjordii AF283283 (100)	B44		P27	F	5	25	-	-	-	-
			P74	F	4	10	-	+	-	-
			P75	F	5	25	-	+	-	-
			P78	F	4	15	-	+	-	-
			P90	F	4	15	-	-	-	-
			P20	F	5	25	-			-
			P21	F	5	25	-	-	-	-
Tsukamurella strandjordii AF283283 (99.8)	B45		P19	F	5	25	-	-	-	-
Tsukamurella strandjordii AF283283 (100)	B46		P22	F	6	25		-	-	-

ing *F. pratensis* plants with the rhizobacterial strains improved plant growth (Figure 4.3a). Strains with a positive effect increased shoot biomass by 1.3 to 1.9-fold, and root biomass by 1.3 to 2.6-fold, compared to non-inoculated plants. Plants inoculated with the strain P87 (*Massilia niastensis*; 98.0 % similarity) showed the highest shoot dry weight, whereas those inoculated with strains P12 or P42 (*Streptomyces floridae*; 99.7 % similarity; and *Pseudomonas lurida*; 98.3 % similarity, respectively) showed the highest root biomass. Strain P54 (*Streptomyces coelescens*; 97.0 % similarity) was the only strain which negatively affected growth of *F. pratensis* (both shoot and root biomass; Figure 3b). Bacterial inoculants did not significantly influence leaf concentrations of macronutrients, such as Ca, Mg, and K: values ranged from 54.1-68.9 g K kg^{-1}, 5.8-7.2 g Ca kg^{-1} and 5.1-6.1 g Mg kg^{-1}(Figure 4.4). On the other hand, inoculants tended to reduce leaf Fe content (all inoculants except P30), and in some cases P (P12, P26, P42, P64, P75, P87) and Mn (P42 and P87) (although not significantly). Cd leaf concentrations varied from 7.0–10.2 mg kg^{-1} and Zn from 241–359 mg kg^{-1}. In general, plants tended to accumulate less Cd and Zn in shoots compared to non-inoculated plants.

In the case of *S. viminalis*, the majority of strains reduced growth compared to non-inoculated plants (Figure 4.3b). This was observed for 9 bacterial strains (P12, P26, P29, P41, P54, P56, P65, P71). On the other hand, strains P30 (*Rhodococcus erytrhopolis*; 99.2 % similarity), P35 (*Pseudomonas koreensis*; 98.8 % similarity), P42 (*Pseudomonas lurida*; 98.3 % similarity), P64 (*Streptomyces coelescens*; 98.2 % similarity) and P87 (*Massilia niastensis*; 98.0 % similarity) enhanced growth of *S. viminalis* (only significant for P87). As for *F. pratensis*, the bacterial inoculants did not have a significant effect on the nutrient content of *S. viminalis* (leaf tissues). However, they tended to increase Ca, Mg, Mn and in some cases P contents (Figure 4.5). Cd and Zn bioaccumulation tended to be similar or slightly lower than in non-inoculated plants. Cd leaf concentrations varied from 9.0 -15.8 mg kg^{-1} and Zn from 217–324 mg kg^{-1}.

4.4 Discussion

In accordance with the presence of sulphides (principally galena and sphalerite) the sampling site was highly polluted with Cd, Zn and Pb. Total metal contents around this site have previously shown a high level of heterogeneity (Diez Lázaro, 2008), a characteristic which is also typical of many mine tailings (García-Sánchez *et al.*, 1999; Epelde *et al.*, 2010; Barrutia *et al.*, 2011). Diez Lázaro (2008) found values ranging from 2-95 mg kg^{-1} for Cd, 46-6100 mg kg^{-1} for Pb, and 340-52000 mg kg^{-1}

for Zn. The concentrations of Cd, Pb and Zn, exceeded official threshold values established by the Galician government (Decreto, 60/2009). Although these thresholds are based on total concentrations, in the case of Cd even the more plant-available fractions exceeded permitted values. In addition, since a considerable amount of Pb and Zn are associated with carbonates these two metals are also potentially bioavailable.

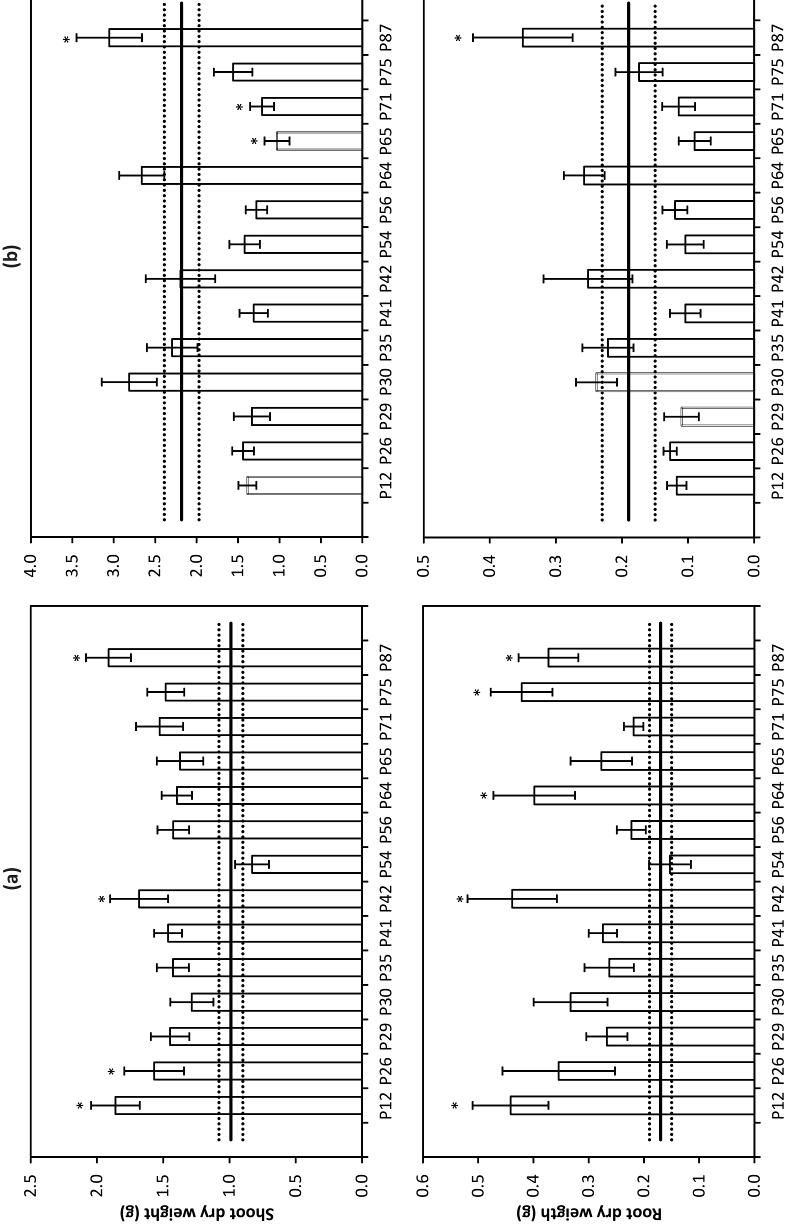

Figure 4.3. Shoot and root dry weight (mean ± SE) of (a) *Festuca pratensis* and (b) *Salix viminalis*. Values of non-inoculated controls are indicated by a continuous line (± SE (broken lines)). Asterisks indicate significant differences from the control ($p < 0.05$).

Total metal concentrations in rhizosphere soils were always lower than in non-vegetated soil. Obviously, this does not indicate that the plants are able to reduce total metal concentrations, but rather that they are capable of colonising "less" contaminated spots whenever possible. Of the three species, *B. celtiberica* appears to be the most tolerant. Nonetheless, all three species are capable of growing in highly contaminated soils (20-40 mg kg^{-1} Cd, 1500-2500 mg kg^{-1} Pb and 7000-10000 mg kg^{-1} Zn). Although all three species behaved as excluder plants, the shoot metal concentrations exceeded phytotoxic concentration thresholds proposed by Kabata-Pendias and Pendias (2001) (5-30 mg kg^{-1} Cd; 30-300 mg kg^{-1} Pb and 100-400 mg kg^{-1} Zn). In fact, the plants showed visible signs of toxicity in the field, such as an extremely stunted growth. Shoot metal concentrations were in the same range as those obtained by other authors in native vegetation of Pb-Zn mine spoils (Yoon *et al.*, 2006; Chehregani *et al.*, 2009; Moreno-Jiménez *et al.*, 2009; Ha *et al.*, 2011). Metal accumulation did not differ between plant species, except for Zn which was more accumulated in the leaves of *B. celtiberica*. In agreement, water-soluble concentrations of this element were also highest in the rhizosphere of this species. The relative increase in plant-available metal pools in the rhizosphere is presumably an indirect consequence of root exudation of organic compounds, the reduction in soil pH and dissolution of mineral phases (e.g. carbonates). The genus *Betula* includes several pioneer species which are often found on soils contaminated with trace metals (Eltrop *et al.*, 1991). In contrast, water-soluble metal concentrations were not significantly increased in the rhizosphere of *F. rubra*, and the TF values of this species suggest it to be efficient at excluding metals from its aboveground biomass. This population could be a promising candidate for application in phytostabilisation trials combining distinct soil amendments and/or bacterial inoculants.

Numerous studies have shown a reduction in the density, metabolic activity and diversity of microbial communities after long-term exposure to trace metals (Giller *et al.*, 1998; Kozdrój and van Elsas, 2000; Dell'Amico *et al.*, 2005; Lorenz *et al.*, 2006). Toxic concentrations of metals typically induce a shift in species composition and the selection of metal-tolerant microorganisms. The density of culturable bacteria observed in the non-vegetated spots at this sampling site was extremely low and, as expected, the rhizosphere always harboured higher bacterial densities. This rhizosphere effect is probably due to the higher concentration of DOC in rhizosphere soil compared to non-vegetated soil. Moreover, all three plant species hosted higher metal-tolerant populations, and these rhizobacteria tolerated

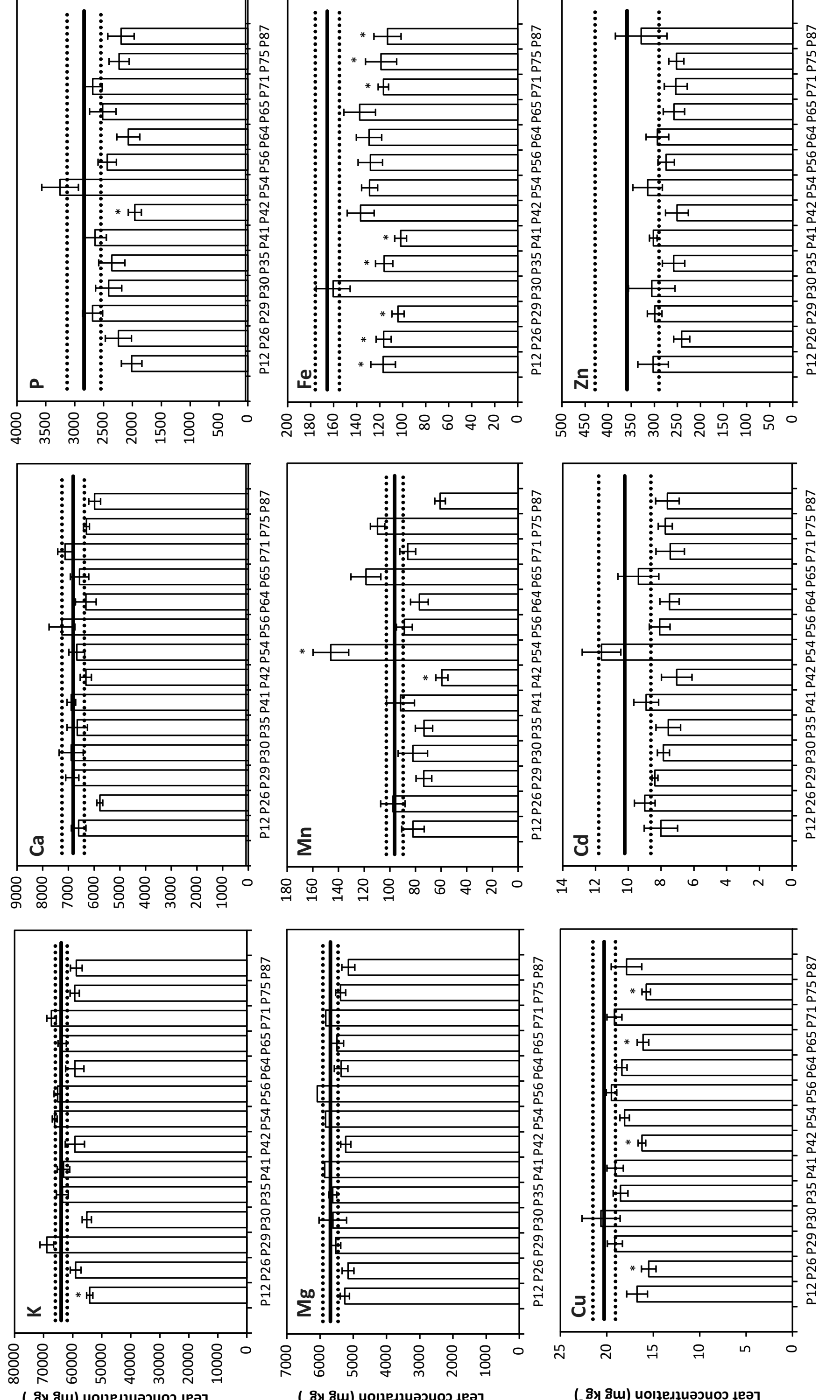

Figure 4.4. Concentrations of metals and nutrients in shoots (mean ± SE) of *Festuca pratensis*. Values of non-inoculated controls are indicated by a continuous line (± SE (broken lines)). Asterisks indicate significant differences from the control ($p < 0.05$).

higher concentrations of bioavailable metals. Some isolates resisted up to 6 mM Cd and 25 mM Zn, concentrations which are similar to those tolerated by rhizobacteria and endophytes associated with *Salix caprea* trees from a Zn/Pb mining site in Austria (Kuffner *et al.*, 2010). Although there are contradictions, several studies have also found Gram-positive bacteria, and in particular the *Actinobacteria*, to dominate culturable bacterial collections from trace metal-contaminated soils (Gremion *et al.*, 2003; Hanbo *et al.*, 2004; Bååth *et al.*, 2005). Over 90 % of the studied culturable bacteria in Pb-Zn mine-soils were affiliated with the *Arthrobacter* genus (Hanbo *et al.*, 2004). In the present study only metal-tolerant strains were sub-cultured for identification which is likely to have led to an underestimation of bacterial diversity. Moreover, since the culturable microbial community represents < 1 % of the actual diversity further studies using culture-independent approaches would be useful.

For the re-inoculation experiment plant species were selected to represent either phytoextraction or phytostabilisation scenarios. Metal-accumulating *Salix* species have been shown to be ideal extractor plants due to their high biomass production and extensive root system, whereas numerous *Festuca* species have been successfully implemented in long-term field trials of phytostabilisation (Kuffner *et al.*, 2010; Mench *et al.*, 2010). Here, the effects of the bacterial strains were dependent on the plant species. One of the most contrasting effects was in the case of strain P12 which improved shoot biomass of *F. pratensis* but had the opposite effect on *S. viminalis*. Grandlic *et al.* (2008) found the effect of bacterial strains on the biomass of *Atriplex lentiformis* and *Buchloe dactyloides* was both plant- and substrate-dependent. Nonetheless, five of the tested strains (P30, P35, P42, P64 and P87) had a positive effect on the growth of both plant species. For phytostabilisation, inoculating a metal-excluding population of gramineae with these rhizobacteria could achieve a healthy vegetation cover in a shorter period of time. On the other hand, in the case of phytoextraction a simple increase in biomass production leads to an increase in the overall metal yield. Strains P87, P30 and P64 did not induce a higher accumulation of Cd or Zn in leaves of *S. viminalis* but the microbial-induced increase in biomass production results in an increase in the total metal phytoextracted. Total Cd and Zn extracted by *S. viminalis* (metal accumulated in leaves x leaf biomass production) tended to be higher after inoculating with P87 (mean phytoextracted Cd increased from 59 to 69 µg, and Zn from 1426 to 1768 µg). These results suggest that some of the plant-associated bacteria isolated from this site could be exploited for improving plant growth and performance in

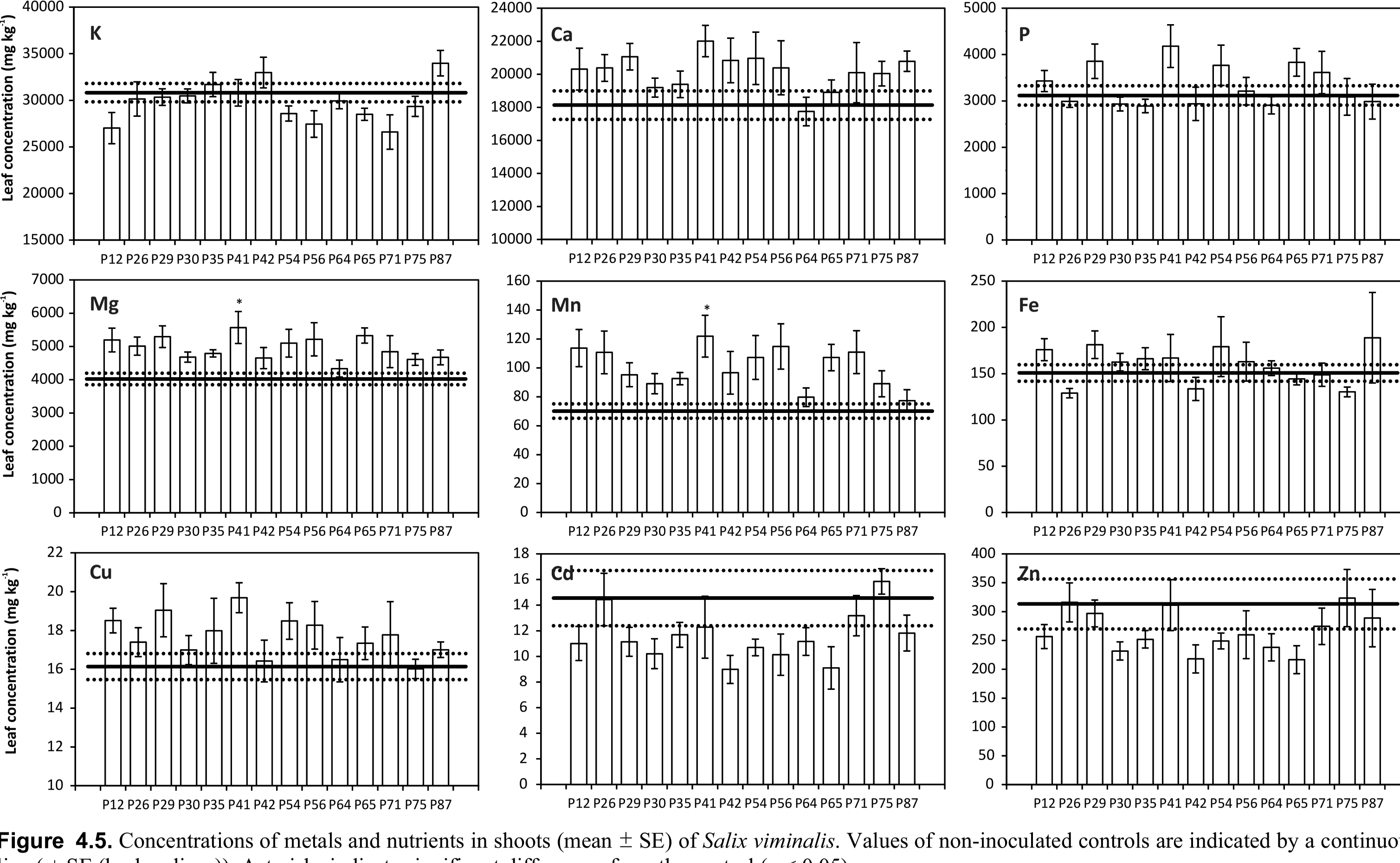

Figure 4.5. Concentrations of metals and nutrients in shoots (mean ± SE) of *Salix viminalis*. Values of non-inoculated controls are indicated by a continuous line (± SE (broken lines)). Asterisks indicate significant differences from the control ($p < 0.05$).

metal-contaminated soils. However, further studies are necessary to ascertain their effect on plant growth, nutrient status and metal accumulation in real-life metal-contaminated soils. Furthermore, the ability of these strains to modify soil metal mobility needs to be evaluated. Some of the PGP traits tested here could influence metal bioavailability in soils. Siderophores can complex a variety of heavy metal ions, and biologically-produced surfactants have recently been shown to enhance metal removal from contaminated soils (Mulligan, 2005). The ability to produce biosurfactants was the most common trait observed in the isolate collection. Rhizosphere bacteria associated with *S. caprea* have been shown to mobilise or immobilise metals, influencing plant metal accumulation or exclusion (Kuffner *et al.*, 2008; 2010).

Only a few isolates were able to produce siderophores, and none of these were isolated from *F. rubra*. This is perhaps not surprising since gramineae are known producers of phytosiderophores and recruiting siderophore-producing bacteria in their rhizosphere may not be advantageous (Morrissey and Guerinot, 2009). However, this trait could be valuable in this type of mine-soil due to limited Fe availability at alkaline pH values. The production of IAA is also known to significantly improve plant growth and biomass production. Many authors have attributed bacterial-induced increases in plant growth in the presence of metals due to the production of this phytohormone (Shilev *et al.*, 2006; Dell'Amico *et al.*, 2008). Only two strains were capable of producing IAA (P30 and P87), and both enhanced biomass production in *F. pratensis* and *S. viminalis*. However, microbial-induced improvements in plant growth could not always be related to the isolate PGP characteristics. For example, both isolates P56 and P64 are siderophore-producers, and both belong to the genus *Streptomyces*, but they caused contrasting effects in *S. viminalis* growth. Similarly, isolates P41 and P42 are P-solubilisers, but again induced contradicting effects on the growth of this species. It is evident that mechanisms other than those studied here must be involved in this growth enhancement. The environmental conditions to which an inoculant is exposed will influence whether or not certain PGP traits are activated. The screening method used here allows for a rapid selection of interesting strains, however in a hydroponic system (with an adequate nutrient supply) mechanisms of P or Fe acquisition may not be induced. These traits are more likely to be induced in a plant-microbe-soil system, and particularly in mine-soils with nutrient deficiency and toxic concentrations of metals. In such a system the tendencies towards biomass enhance-

ment or the improvement in plant nutrient status which were observed here may be more pronounced.

In conclusion, the pseudometallophytes studied were identified as metal excluders: of the three species, *B. celtiberica* tolerated the highest soil metal concentrations and *F. rubra* was the most efficient metal excluder. An increase in labile metal concentrations in the rhizosphere was associated with a higher metal-tolerant bacterial population. We obtained a collection of rhizobacterial isolates, and several of these strains could be potentially useful for improving plant growth and establishment in trace metal-contaminated soils. This plant growth promotion could lead to a healthy vegetation cover in the phytostabilisation of heavily contaminated soils, or alternatively may be an important parameter in improving the metal extraction capacities of plants in the phytoextraction of metal-contaminated soils.

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Chapter 5

Endophytic and rhizoplane bacteria associated with *Cytisus striatus* growing on hexachlorocyclohexane-contaminated soil: isolation and characterisation

ABSTRACT

Inoculation of plants with their associated microorganisms is a promising strategy for improving phytoremediation of organic contaminants. Isolation and characterisation of these organisms from plants growing in contaminated sites will permit the identification of candidate strains for re-inoculation studies. The diversity of culturable endophytic and rhizoplane bacteria found in association with *Cytisus striatus* plants growing at a hexachlorocyclohexane (HCH)-contaminated site was studied. A total of 97 strains of endophytic bacteria were isolated from the root, stem and leaf tissues, and 49 from the rhizoplane. They were further characterised genotypically (BOX-PCR, 16S rDNA sequencing, presence of *linA* and *linB* genes) and phenotypically (trace metal tolerance, capacity to produce biosurfactants and plant growth promoting (PGP) traits). *Proteobacteria* and *Actinobacteria* dominated the isolate collection, and taxonomic diversity was strongly tissue-specific. The *linA* and *linB* genes were not detected in the isolate collection. The majority of isolates had at least one of the PGP traits tested, whereas biosurfactant-producing strains were less frequent. Resistance to more than one trace metal was generally restricted to endophytes isolated from shoot tissues. The PGP characteristics found in an important number of the bacterial isolates obtained in this study could be particularly useful for exploiting the phytoremediation potential of *C. striatus*.

This study formed part of the following publication:

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5.1 Introduction

Soil remediation using phytoremediation techniques are considered to be less invasive, more cost-effective and restorative of soil structure and function compared to conventional, civil-engineering methods (such as leaching, electrokinetical treatment, chemical oxidation/reduction of pollutant, excavation). These techniques appear to be particularly promising in the case of organic contaminants which can be degraded by the cooperation of plants and their associated microorganisms. Interactions between plants and their associated bacteria have been studied for many years, and both beneficial and pathogenic effects to their host have been described (Raaijmakers *et al.*, 2009). Dense populations of bacteria have been shown to inhabit the rhizosphere, rhizoplane or phyllosphere (surface-dwelling epiphytes) and the internal tissues of the plant (endophytes) (Chanway, 2002; Lindow and Brandl, 2003). Endophytic bacteria colonise the internal tissues of plants without causing symptoms of infection or negative effects on their host (Schulz and Boyle, 2006). They appear to be ubiquitous in plant species: bacteria from 82 genera have been found in a broad range of species including woody plants and arable crops, with *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* being the most commonly isolated bacterial genera (Lodewyckx *et al.*, 2002; Mastretta *et al.*, 2006). Traditional interest in endophytic microorganisms was aimed towards their commercial potential as biocontrol agents of plant pathogens and for increasing plant yield and nutritive value of agricultural crops (Berg 2009; James 2000). A more novel application is the use of these plant-endophyte associations to enhance *in situ* phytoremediation of organic pollutants: these associations have been the focus of several recent studies (Barac *et al.*, 2004; Germaine *et al.*, 2009; Huang *et al.*, 2004a; 2004b; Weyens *et al.*, 2009a; 2009b; 2009c). Exploiting these plant-microbial partnerships is generally based on the capacity of the bacteria to (1) improve plant growth and survival in stress conditions of contaminated soils (plant growth promoting bacteria (PGPB)) and/or (2) degrade contaminants or alter contaminant bioavailability (Ryan *et al.*, 2008; Weyens *et al.*, 2009b; 2009c). Assuring plant growth in the presence of toxic levels of contaminants is a key element for successful phytoremediation. Inoculating plants with strains of PGPB can increase their tolerance to contaminants and improve growth. Direct plant growth promoting mechanisms include the production of plant growth regulators (such as auxins (e.g. indoleacetic acid), cytokinins, gibberellins), suppression of stress ethylene production, or, enhancing nutrient and water uptake (e.g. N_2 fixers, P-solubilisers,

siderophore-producers) (Glick 2003). Indirect mechanisms include the production of antibiotics or cell wall lytic enzymes such as chitinases to inhibit pathogens, and the induction of plant defence mechanisms (Weyens *et al.*, 2009b; 2009c).

Inoculating plants with PGPB has been shown to improve plant growth and establishment in soils contaminated with both trace metals and organic pollutants (Zhuang *et al.*, 2007). Growth of *Brassica napus* in Cd-contaminated soils was improved after inoculation with PGP strains (*Pseudomonas tolaasii* ACC23, *P. fluorescens* ACC9 and *Mycobacterium* sp. ACC14). Total Cd accumulation was increased due to the increase in plant biomass and not due to any direct influence on Cd uptake by the plant (Dell'Amico *et al.*, 2008). Poplar cuttings grown in hydroponic solutions and inoculated with the endophyte *Pseudomonas putida* W619 (equipped with the pTOM plasmid coding for TCE degradation) showed improved plant growth and reduced TCE phytotoxicity compared to non-inoculated cuttings (Weyens *et al.*, 2010a). Given the diversity of soil, rhizosphere, and endophytic microorganisms, the opportunity to find new and beneficial plant-microbial associations is considerable. The isolation and characterisation of bacteria associated with plants growing in different contaminated sites will allow for the identification and selection of potentially useful candidate species. Further knowledge of their physiology and ecology will also help towards their practical application in phytoremediation techniques.

The organochlorine 1,2,3,4,5,6 hexachlorocyclohexane (HCH) is a broad-spectrum insecticide that was used on a large-scale worldwide since the 1940s, and is available in two formulations: technical-grade HCH (a mixture of different isomers, mainly α- (60-70 %), β- (5-12 %), γ- (10-15 %), and δ-HCH (6-10 %)) and lindane (almost pure γ-HCH, the only isomer with insecticidal properties). All HCH isomers are acutely toxic to mammals, due to their mutagenic, teratogenic and carcinogenic properties. Due to its widespread use, and strong persistence, residues of lindane and other HCH isomers are found all over the world in air, water, sediments and soils (Willet *et al.*, 1998). Heavily contaminated sites have been reported from different locations such as Germany, Greece, Spain, The Netherlands, Brazil, Canada, the United States, China and India (Lal *et al.*, 2010). Several bacterial strains (predominantly *Sphingomonas* and *Sphingobium* strains) capable of degrading HCH isomers under aerobic conditions have been isolated from contaminated soils. Most of these degraders when analysed at a genetic level have been shown to harbour the genes *linA* and *linB*, which encode the first two enzymes of the only upstream HCH aerobic degradation pathway elucidated to date

(see reviews by Lal *et al.*, 2010 and Nagata *et al.*, 2007). In previous studies, the leguminous plant *Cytisus striatus* was shown to grow spontaneously in HCH-contaminated sites and has been proposed as a candidate species for the clean-up of this type of contaminant (Calvelo-Pereira *et al.*, 2006; Kidd *et al.*, 2008). In a greenhouse experiment, enhanced dissipation of α- and δ-HCH isomers in the rhizosphere of this species was attributed to the selective enrichment of HCH-degrading microorganisms (Kidd *et al.*, 2008). Inoculating these plants with PGPB strains could potentially improve their growth in such contaminated sites and at the same time enhance HCH dissipation. The diversity of endophytic communities associated with plants growing on HCH-contaminated soils has not to date been studied.

The objectives of this study were (a) to assess the diversity of culturable endophytic and rhizoplane bacterial communities associated with *Cytisus striatus* from an HCH-contaminated site; (b) to assess the spatial compartmentalisation of the endophytic isolate collection; (c) to identify those strains with plant growth promoting abilities; (d) to assess the presence of genes encoding enzymes involved in HCH degradation; and, (e) to identify those strains able to produce biosurfactants and evaluate their metal resistance. In this study we focus on the culturable bacterial community since the global aim is to obtain an isolate collection which can be used in re-inoculation studies for improving remediation of HCH-contaminated soils.

5.2 Materials and methods

Study area and sample collection

Samples of root, stem and leaves were collected from *Cytisus striatus* (Hill) Rothm. (Portuguese broom) growing in a hexachlorocyclohexane (HCH)-contaminated site (around 3560 m^2) in Porriño (Pontevedra), NW Spain, where residues from lindane fabrication were disposed of during the 1950s and 1960s. This leguminous shrub grows spontaneously, and is one of the most abundant plant species in the area. Soil concentrations of Σ-HCH (calculated as the sum of α-HCH, β-HCH, δ-HCH and γ-HCH) at this site typically range between 2 and 100 mg kg^{-1}, although at some local points concentrations of up to 20000 mg kg^{-1} are found (Calvelo-Pereira *et al.*, 2006). Total HCH concentration in plant tissues varies from 1.6 to 63 mg kg^{-1} (median concentrations of total HCH in roots, stems and leaves were 6, 10, and 22 mg kg^{-1}, respectively; Calvelo-Pereira *et al.*, 2006).

Root samples collected from five *C. striatus* plants, and leaf and stem samples collected from 15-20 individuals (selected at random), were pooled together and divided into three subsamples for isolation of both rhizoplane and endophytic bacteria. In the zone where plant samples were taken soil HCH concentrations ranged from 12 to 61 mg kg^{-1} (Concha-Graña *et al.*, 2006). Nodules were not removed from root tissues, and the root endophytic population includes nodule-occupying (nitrogen-fixing) bacteria.

Isolation of culturable bacteria associated with *C. striatus*

To isolate rhizoplane bacteria, adhering soil was removed from roots and 1 g of root tissue was shaken in 10 ml sterile 1 % sodium hexametaphosphate solution for 30 min in an end-over-end shaker. Suspensions were diluted in 10-fold series and plated in duplicate onto 1/10 strength 869 agar medium (see Chapter 2) supplemented with 100 µg ml^{-1} of the fungicide cycloheximide. After 7 days incubation at 28 ºC, colony forming units (CFUs) were counted and calculated per gram of root tissue.

To isolate the endophytic bacteria, tissue samples (0.2-0.3 g (roots) or 0.7-0.8 g (stems and leaves)) were surface sterilised for 10 (roots) or 3 (stems and leaves) min in 2 % (roots), 1 % (stems) or 0.1 % (leaves) NaClO solution supplemented with one droplet Tween 80 per 100 ml solution, and were subsequently rinsed three times for 1 min in sterile deionised water. The surface-sterilisation process was checked by plating aliquots of the third rinsing solution on 869 medium (if no growth was observed after 7 days, surface sterilisation was considered to be successful). The concentrations of NaClO and the sterilisation times were empirically determined in previous studies. After surface sterilisation, the leaf, stem or root tissue was macerated in 10 ml of 10 mM $MgSO_4$ using a Polytron PT1200E homogeniser (Kinematica AG). Ten-fold serial dilutions were plated in duplicate on 1/10 strength 869 agar medium, CFUs determined as above and calculated per gram fresh plant weight (FW). Distinct colony morphotypes were subcultured at least three times to ensure purity and cryopreserved at -70 ºC in 1/10 strength 869 medium supplemented with 15 % (v/v) glycerol.

Genotypic characterisation of rhizobacterial strains

DNA was extracted from all purified bacterial strains as described previously and amplified by PCR using the BOX A1R as described previously (see Chapter 2). PCR products were separated by electrophoresis in agarose gels (1.8 %) and eth-

idium bromide-stained gel images were analysed using the Gel Compar Bionumerics programme (v5.1, Applied Maths, Belgium). Isolates were grouped according to their BOX-PCR profiles at a similarity level of 92 %. Genetic diversity of isolates was assessed for each microbial group (rhizoplane, root, stem and leaf endophytes) using the Shannon Diversity Index ($H = -\sum(x_i/x_0)\ln(x_i/x_0)$) where x_i is the number of strains per BOX-PCR group (determined according to 92 % similarity), and x_0 is the total number of strains.

Amplification targeting the 16S rDNA gene was carried out as described previously on those strains showing distinct BOX-PCR profiles (see Chapter 2). PCR products were purified and partially sequenced (approximately 750 bases) using the primer 16S-27F. Sequence data were checked using the Chromas v.1.45 software and assessed for similarity with sequences of type strains in the Ribosomal Database Project (Cole *et al.*, 2009). Sequences were aligned using Clustal W (Thompson *et al.*, 2007) and phylogenetic analyses were performed using MEGA version 4 (Tamura *et al.*, 2007). The neighbour-joining tree was constructed based on the Maximum Composite Likelihood method (Tamura *et al.*, 2004). Bootstrap values were calculated from 2000 replicated analyses. The sequences used for identification of the cultivable strains are available in the EMBL database (www.ebi.ac.uk) under accession numbers FN796796 through FN796876.

Phenotypic characterisation and amplification of *linA* and *linB* genes

Trace metal resistance of the strains was tested using 284 agar medium (see Chapter 2) supplemented with 1 mM Cd, Co, Ni or Zn (added as $CdSO_4.8/3H_2O$, $CoCl_2.6H_2O$, $NiSO_4.6H_2O$ and $ZnSO_4.7H_2O$) and incubated at 28 ºC for 7 d. The following carbon sources were added: lactate (0.7 g l^{-1}), glucose (0.5 g l^{-1}), gluconate (0.7 g l^{-1}), fructose (0.5 g l^{-1}) and succinate (0.8 g l^{-1}). Rhizobacterial strains were also screened for the ability to produce biosurfactants, and for various PGP characteristics: phosphate solubilisation capacity, siderophore production and indoleacetic acid (IAA) production as described previously (see Chapter 2).

Bacterial strains were tested for their ability to grow in minimal medium with different C sources. Strains were plated on solid 284 medium supplemented with either, lactate (0.7 g l^{-1}), glucose (0.5 g l^{-1}), gluconate (0.7 g l^{-1}), fructose (0.5 g l^{-1}), or succinate (0.8 g l^{-1}) and incubated at 28 ºC for 7 d.

PCR amplifications targeting the *linA* and *linB* genes were carried out on all strains using the primers LinA-1F (5'-ATGAGTGATCTAGACAGACTT-3') and LinA-411R (5'-GGTGAAATAGTTCGTGCATG-3'), and LinB-18F

(5'-GCCATTTGGCGAGAAGAA-3') and LinB-729R (5'-GGCGTTGATGAAGAGTTT-3') (Kumari *et al.*, 2002; Thomas *et al.*, 1996). PCR reactions were performed in a total volume of 20 µl containing: 1x Taq buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1.75U Taq polymerase (Invitrogen), 0.4 µM of each primer, and 1 µl of extracted DNA. Thermocycling conditions for *linA* were: 2 min at 94 °C; 30 cycles of 1 min at 94 °C; 1 min at 55 °C and 2 min at 72 °C; and 1 cycle of 10 min at 72 °C. For *linB*, PCR cycles were identical but with an annealing temperature of 52 °C. The obtained PCR products were separated by gel electrophoresis in 0.8 % agarose. A lindane-degrading strain was used as a positive control.

5.3 Results

Abundance of culturable endophytic and rhizoplane bacteria

Densities of culturable bacterial endophytes were highest in root tissues (2.3 x 10^6 CFU g^{-1} FW tissue), around 3 orders of magnitude greater than shoot communities. In contrast, a similar number of endophytic bacteria were recovered from stems and leaves (1.9 x 10^3 and 4.3 x 10^3 CFU g^{-1} FW tissue, respectively). Densities of rhizoplane bacteria were of a similar magnitude to root endophytes (2.6 x 10^6 CFU g^{-1} FW tissue). Morphologically different colonies were selected for purification: a total of 49 strains were isolated from the rhizoplane, and 97 endophytic strains were isolated from leaves (29), stems (19) and roots (49) of *C. striatus*.

Identification and phylogenetic analysis of culturable endophytic and rhizoplane bacteria

The diversity of culturable rhizoplane and endophytic bacteria was estimated using BOX-PCR genomic fingerprints and 16S rDNA sequencing. BOX-PCR profiles for all rhizoplane and endophytic bacteria isolated in the present study are presented in Table 5.1. The highest diversity was observed in root-associated bacteria: at a similarity of 92 %, BOX-PCR profiles of isolates were allocated into 32 or 29 groups for rhizoplane strains or root endophytes, respectively (Table 5.1). Diversity was similar between stem and leaf endophytes: BOX-PCR profiles were allocated into 8 (stem endophytes) and 12 (leaf endophytes) groups (Table 5.1). Similarly, the Shannon diversity index values (H) indicated a reduction in bacterial diversity from roots to shoots: rhizoplane (H = 3.34)~ root (H = 3.19) > leaf (H = 2.15) ~ stem (H = 1.84).

The 16S rDNA of one representative isolate per BOX-PCR group was partially sequenced. In general, Gram-positive bacteria were amply represented, and in particular the actinomycetes. Five taxonomic classes, *Actinobacteria*, *Bacilli*, *Alpha*-, *Beta*- and *Gammaproteobacteria* (and one member of the *Flavobacteria*), were represented in the root (rhizoplane or endophytes) while the culturable bacterial community present in the leaves was composed of members of the *Actinobacteria*, *Alpha*- and *Gammaproteobacteria*, and in the stem was dominated by members of the *Actinobacteria* and *Bacilli* (and one member of the *Alphaproteobacteria*) (Figures 5.1 and 5.2).

Isolates belonging to the phylum *Proteobacteria* were found in all plant compartments (rhizoplane, root/stem/leaf endophytes) (Figure 5.1). In many cases an important percentage of the isolate collection was affiliated with this phylum: 69 % of leaf endophytes, 72 % of root endophytes, and 41 % of rhizoplane bacteria were *Proteobacteria*. In contrast, only 5 % of isolates were affiliated with *Proteobacteria* in the stem endophytic community (all of which were members of the genus *Methylobacterium*). The diversity of *Proteobacteria* was higher in the root endophyte and rhizoplane communities (Figures 5.1, 5.2a and 5.2b). In the roots and stems the *Proteobacteria* were dominated by *Alphaproteobacteria*, whereas in the leaves *Gammaproteobacteria* are dominant. In rhizoplane and root endophytic communities this class was mainly represented by members of the genus *Rhizobium* (23 % and 25 %, respectively). The phylogenetic tree shows two distinct groups of *Rhizobium*, one of which was solely represented by root endophytes closely related to *Rhizobium pisi*, *R. phaseoli* and *R. alamii* (Figure 5.2a). Within the root endophytes another important group of isolates were those belonging to the family Bradyrhizobiaceae, with genera such as *Bradyrhizobium* and *Rhodopseudomonas* (Figure 5.1). *Alphaproteobacteria* in the stem and leaf endophyte community were only represented by isolates of the genus *Methylobacterium* (accounting for 5 and 21 % of isolates, respectively). Isolates affiliated with *Betaproteobacteria* were only found in the roots, and were all identified as *Burkholderia* sp. Members of this genus were predominant in the root endophytic community (representing 15 % of isolates). *Gammaproteobacteria* were isolated from the rhizoplane and the interior of root and leaf tissues. At the root level, the genera most represented were *Dyella* and *Stenotrophomonas*. Strains affiliated with *Dyella* were particularly abundant inside the root. In the leaves, the *Gammaproteobacteria* isolates belonged to the genera *Pantoea* and *Pseudoxanthomonas* (Figures 5.1 and 5.2b).

Table 5.1. BOX-PCR fingerprints, partial 16S rDNA sequence identification, and phenotypic characteristics of bacterial isolates from the leaves (EF), stems (ET), roots (ER) and rhizoplane (RP) of *C. striatus*. BOX- PCR groups: leaf endophytes (GF), stem endophytes (GT), root endophytes (GR), and rhizoplane bacteria (GP). Sd, siderophore-producer; P, P-solubiliser; IAA, indoleacetic acid producer; Gc, glucose; Gt, gluconate; Fr, fructose; Sc, succinate.

Most similar type strain (% similarity)	Group and Box profile	Isolate	Sd	P	IAA	Gc	Gt	Fr	Sc
Brachybacterium paraconglomeratum AJ415377 (98.8)	GF1	EF07	-	-	-	+	+	+	+
		EF16	+	-	+	+	+	+	+
		EF18	+	-	-	+	+	+	+
Curtobacterium flaccumfaciens AJ312209 (100)	GF2	EF28	-	-	+	-	-	-	-
Curtobacterium flaccumfaciens AJ312209 (99.9)	GF3	EF46	-	-	-	+	+	+	-
Curtobacterium flaccumfaciens AJ312209 (99.9)	GF4	EF57	-	-	-	+	+	+	-
Curtobacterium flaccumfaciens AJ312209 (99.8)	GF5	EF24	-	-	+	+	+	+	-
		EF25	-	-	+	-	-	-	-
Curtobacterium flaccumfaciens AJ312209 (100)	GF6	EF37	-	-	+	-	-	-	-
Methylobacterium radiotolerans D32227 (100)	GF7	EF56	-	-	-	-	-	-	-
		EF59	-	-	-	-	-	-	-
		EF19	+	-		+	+	+	+
Methylobacterum radiotolerans D32227 (99.5)	GF8	EF04	-	-	-	-	-	-	-
Methylobacterum radiotolerans D32227 (99.3)	GF9	EF55	-	-	+	+	+	+	-
Methylobacterium oryzae AY683045 (99.5)	GF10	EF58	-	-	-	-	-	-	-
Pseudoxanthomonas koreensis AY550263 (99.9)	GF11	EF34	-	-	-	-	-	-	-
		EF38	-	-	-	-	-	-	-
		EF20	-	-	+	-	-	-	-
		EF21	-	-	-	-	-	-	-
		EF03	-	-	-	-	-	-	-
		EF60	+	-	+	+	+	+	+
Pantoea agglomerans AJ233423 (98.5)	GF12	EF17	+	-	+	+	+	+	+
		EF44	+	-	+	+	+	+	+
		EF48	-	-	+	+	+	+	+
		EF54	+	-	+	+	+	+	+
		EF42	+	-	+	+	+	+	+
		EF09	+	-	+	+	+	+	+
		EF11	+	-	+	+	+	+	+
		EF41	-	-	+	+	+	+	+
Arthrobacter protophormiae X80745 (99.9)	GT1	ET21	-	+	-	+	+	+	+
		ET53a	-	+	-	+	+	+	+
		ET54a	-	-	+	+	+	+	+
		ET52	-	-	+	+	+	+	+
Arthrobacter protophormiae X80745 (100)	GT2	ET51a	-	-	+	+	+	+	+
Dermacoccus nishinomiyasensis X87757 (99.2)	GT3	ET11	-	-	-	+	+	+	+

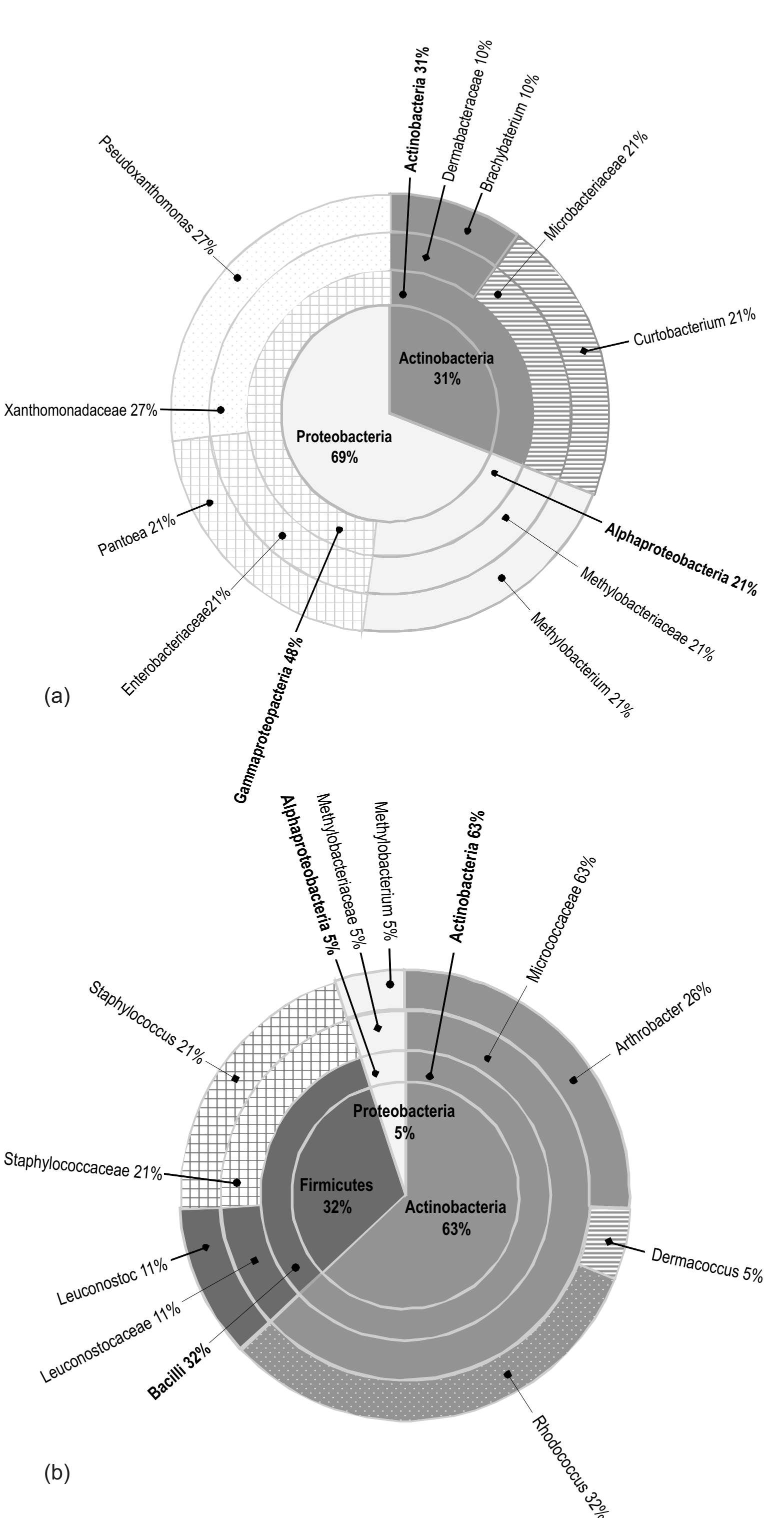
Most similar type strain (% similarity)	Group and Box profile	Isolate	Sd	P	IAA	Gc	Gt	Fr	Sc
Rhodococcus erythropolis X81929 (100)	GT4	ET56b	+	-	-	+	+	+	+
		ET57b	+	-	-	+	+	+	+
		ET53b	+	-	-	+	+	+	+
		ET54b	+	-	-	+	+	+	+
		ET51b	+	-	-	+	+	+	+
		ET41b	+	-	-	+	+	+	+
Leuconostoc citreum AF111948 (99.7)	GT5	ET61	-	-	-	-	-	-	-
		ET62	-	-	-	-	-	-	-
Staphylococcus epidermidis D83363 (100)	GT6	ET55	-	-	-	-	-	-	-
		ET57c	-	-	-	-	-	-	-
		ET56c	-	-	-	-	-	-	-
Staphylococcus cohnii subps. cohnii D83361 (100)	GT7	ET41a	-	+	-	-	-	-	-
Methylobacterium oryzae AY683045 (100)	GT8	ET71	-	-	-	-	-	+	-
Amycolatopsis vancoresmycina AJ508240 (99.7)	GR1	ER14	-	-	-	+	+	+	+
		ER60	-	-	+	+	+	+	+
Amycolatopsis vancoresmycina AJ508240 (99.7)	GR2	ER13	-	-	+	+	+	+	-
Leucobacter komagatae D45063 (99.3)	GR3	ER19	-	-	-	-	-	-	-
		ER20	-	-	-	-	-	-	+
Chryseobacterium shigense AB193101 (97.9)	GR4	ER45	-	-	-	-	-	+	-
Bacillus cereus AE016877 (99.9)	GR5	ER71	-	-	-	+	+	+	-
		ER73	-	-	-	+	+	-	-
Bacillus subtilis subsp. subtilis AJ276351 (99.9)	GR6	ER64	+	-	+	+	+	+	+
Bacillus subtilis subsp. subtilis AJ276351 (99.9)	GR7	ER02	+	-		+	+	+	+
		ER24	+	-	+	+	+	+	+
		ER65	+	-	+	+	+	+	+
		ER66	+	-	+	+	+	+	+
Bacillus weihenstephanensis AB021199 (99.4)	GR8	ER67		+	+	+	+	+	+
Brevundimonas diminuta AB021415 (99.5)	GR9	ER27		-	-	-	-	-	-
Bradyrhizobium japonicum U69638 (99.6)	GR10	ER22	-	-	-	+	+	+	+
		ER28		-	-	-	-	+	-
Bradyrhizobium japonicum U69638 (99.6)	GR11	ER33	-	-	+	+	-	+	+
Pseudochrobactrum saccharolyticum AM180484 (99.1)	GR12	ER26		-	+	-	-	+	+
Mesorhizobium amorphae AF41442 (99.8)	GR13	ER40	-	-	+	+	+	+	+
Rhizobium miluonense EF035074 (100)	GR14	ER25	-	-	+	+	+	+	+
		ER35	-	-	+	+	+	+	+
		ER36	-	-	-	+	+	+	+
		ER37	-	+	+	+	+	+	+
Rhizobium haianense U71078 (100)	GR15	ER68a	-	+	-	+	+	+	+
		ER68b	-	+	-	+	+	+	+
Rhizobium multihospitium EF035074 (100)	GR16	ER17	+	-	-	+	+	+	+
		ER18	+	+	-	+	+	+	+
		ER12	-	-	+	+	+	+	+
		ER04	-	-	+	+	+	+	+

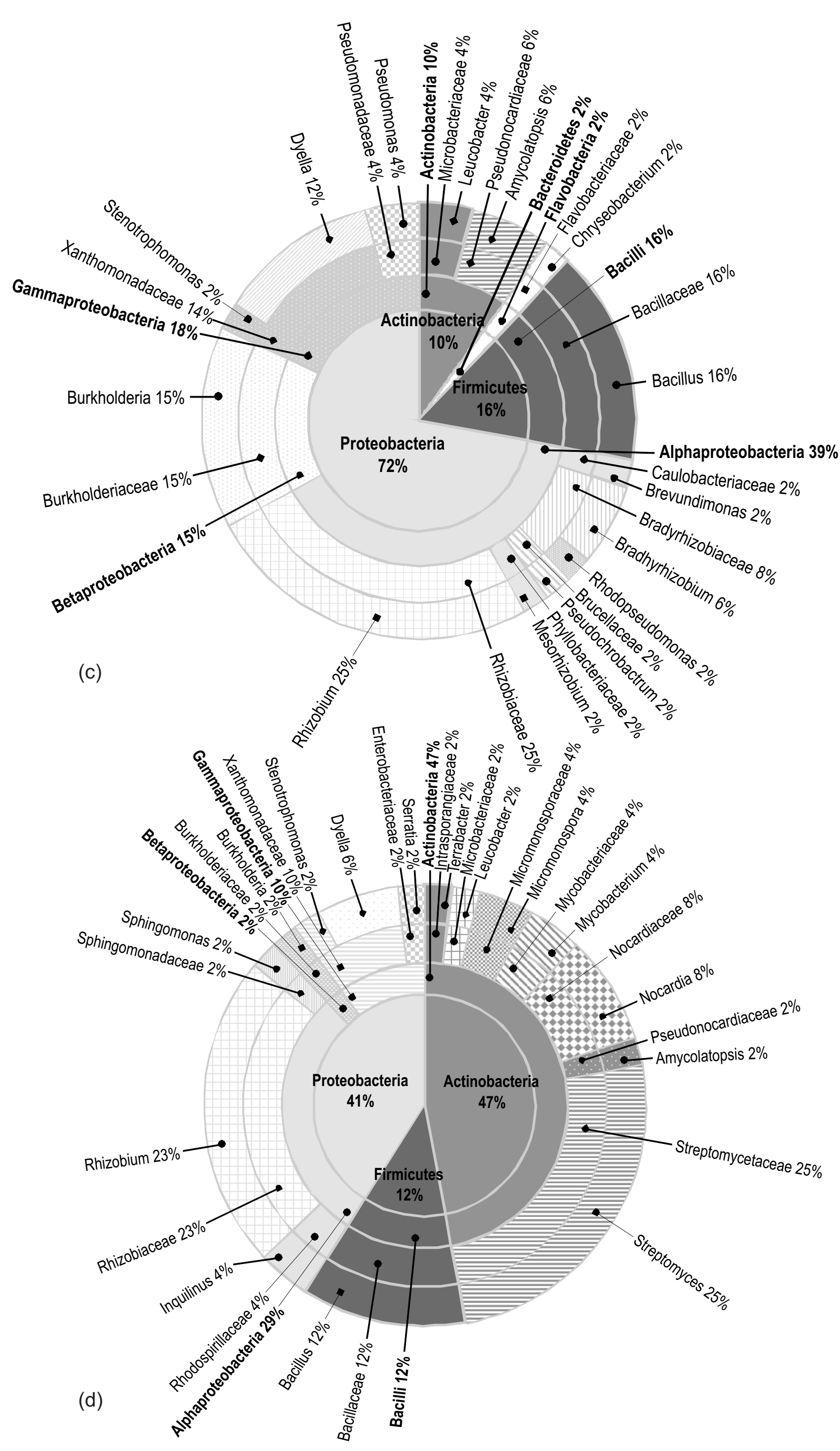
Most similar type strain (% similarity)	Group and Box profile	Isolate	Sd	P	IAA	Gc	Gt	Fr	Sc
Rhizobium alamii AM931436 (99.7)	GR17	ER29	-	-	+	+	+	+	+
Rhizobium pisi AY509899 (98.6)	GR18	ER50	-	+	+	+	-	+	+
Rhodopseudomonas rhenobacensis AB087719 (98.0)	GR19	ER58	-	-	-	+	+	+	+
Burkholderia terrae AB201285 (100)	GR20	ER06	+	+	+	+	+	+	+
		ER16	+	+		+	+	+	+
Burkholderia terrae AB201285 (100)	GR21	ER15	+	+	-	+	+	+	+
Burkholderia graminis U96939 (99.8)	GR22	ER38	+	+	+	+	+	+	+
Burkholderia caledonica AF215704 (99.7)	GR23	ER03	+	+		+	+	+	-
Burkholderia caledonica AF215704 (100)	GR24	ER09	-	+	+	+	+	+	+
Burkholderia graminis U96939 (99.8)	GR25	ER07	+	+	-	+	+	+	+
Stenotrophomonas maltophlia AB021406 (100)	GR26	ER30		-	-	-	-	+	-
Dyella yeojuensis DQ181549 (99.1)	GR27	ER56	-	-	+	-	-	-	-
		ER57	-	-	+	-	-	-	-
		ER46	-	-	+	+	-	-	-
		ER47	-	+	+	-	-	-	-
		ER43		+	+	-	-	-	-
Dyella koreensis AY884571 (98.4)	GR28	ER48	-	+	+	-	-	-	-
Pseudomonas graminis Y11150 (99.4)	GR29	ER42	+	+	+	+	+	+	+
		ER53	+	+	+	+	+	+	+
Amycolatopsis sulphurea AJ293756 (98.2)	GP1	RP90	+	-	-	+	+	+	+
Leucobacter aridicollis AJ781047 (99.6)	GP2	RP27*	-	-	-	+	+	+	+
Micromonospora chaiyaphumensis AB196710 (99.8)	GP3	RP24	-	-	-	-	-	-	-
		RP64	+	-	-	+	+	+	+
Mycobacterium hodleri X93184 (99.8)	GP4	RP61	-	-	-	+	+	+	+
Mycobacterium hodleri X93184 (99.8)	GP5	RP62	+	-	-	+	+	+	+
Nocardia ninae DQ235687 (99.0)	GP6	RP58	-	-	-	+	+	+	+
		RP59	-	-	-	+	+	+	+
		RP57	-	-	-	+	+	+	+
Nocardia transvalensis AF430047 (99.5)	GP7	RP32	+	-	+	+	+	+	+
Streptomyces rhizosphaericus AB249941 (99.9)	GP8	RP30	-	-	-	+	+	+	+
		RP78	-	+	+	+	+	+	+
		RP89	-	-	-	-	-	-	-
Streptomyces rhizosphaericus AB249941 (99.9)	GP9	RP60	+	-	+	+	+	+	+
Streptomyces prunicolor DQ26659 (99.8)	GP10	RP50	+	-	-	+	+	+	+
		RP51	+	-	-	+	+	+	+
Streptomyces bungoensis AB184696 (99.5)	GP11	RP31	-	-	-	-	+	-	-
Streptomyces costaricanus AB249939 (99.9)	GP12	RP69	+	-	-	+	+	+	+
		RP70	+	+	-	+	+	+	+
		RP92	+	-	+	+	+	+	+
Streptomyces costaricanus AB249939 (99.9)	GP13	RP63	+	-	-	+	+	+	+
Streptomyces thermocarboxydovorans U94489 (99.4)	GP14	RP33	+	-	-	+	+	+	+
Terrabacter terrae AY944176 (99.5)	GP15	RP54	-	-	-	+	+	+	+
Bacillus barbaricus AJ422145 (100)	GP16	RP53	-	-	-	-	-	-	-

Most similar type strain (% similarity)	Group and Box profile		Isolate	Sd	P	IAA	Gc	Gt	Fr	Sc
Bacillus cereus AE016877 (100)	GP17		RP72	-	-	-	+	+	+	+
Bacillus subtilis subsp. subtilis AJ276351 (99.9)	GP18		RP01	+	-	+	+	+	+	+
			RP21	+	-	+	-	-	+	+
			RP22	+	-	+	+	+	+	+
			RP23	+	-	+	+	+	+	+
Inquilinus limosus AY043374 (98.8)	GP19		RP68	-	+	-	+	+	+	+
			RP76	-	+	+	+	+	+	-
Rhizobium lusitanum AY738130 (100)	GP20		RP08	-	-		+	+	+	+
Rhizobium multihospitium EF35074 (100)	GP21		RP06	+	+	+	+	+	+	+
			RP07	+	+	+	+	+	+	+
Rhizobium haianense U71078 (100)	GP22		RP03	-	+		+	+	+	+
			RP75	-	+	-	+	+	+	+
Rhizobium miluonense EF061096 (99.9)	GP23		RP74	+	-	-	+	+	+	+
Rhizobium miluonense EF061096 (100)	GP24		RP05	-	+	-	+	+	+	+
Rhizobium miluonense EF061096 (100)	GP25		RP56	+	-		+	+	+	+
			RP80	-	-	-	+	+	+	+
			RP46	-	-	-	+	+	+	+
Rhizobium rhizogenes D14501 (99.8)	GP26		RP83	-	-	-	+	+	+	+
Sphingomonas asaccharolytica Y09639 (100)	GP27		RP17	-	-		+	-	-	-
Burkholderia caledonica AF215704 (99.1)	GP28		RP04	-	+	-	+	+	+	+
Dyella marensis AM939778 (100)	GP29		RP47	-	-	+	-	-	-	-
Stenotrophomonas maltophilia AB294553 (99.2)	GP30		RP13	-	-	+	+	+	+	+
			RP14	-	-	-	+	+	+	+
Stenotrophomonas maltophilia AB294553 (99.2)	GP31		RP49	+	-	-	+	+	+	+
Serratia grimesii AJ233430 (99.7)	GP32		RP48	+	+	+	+	+	+	+

Bacteria belonging to the phylum *Actinobacteria* (class *Actinobacteria*) were also found in all plant compartments, accounting for 10, 63 or 31 % of root, stem or leaf endophyte diversity, and 47 % of rhizoplane isolates (Figure 5.1). The highest diversity within this group was found in the rhizoplane community. Isolates corresponding to *Streptomyces* spp. were the most numerous (representing 25 % of isolates) (Figure 5.2c). Root endophytes affiliated with *Actinobacteria* included representatives of only two genera, *Leucobacter* and *Amycolatopsis*, members of both were also observed in the rhizoplane community. Shoot endophytic isolates belonging to the phylum *Actinobacteria* were represented by genera which were not found to be associated with the roots (Figure 5.2c). Leaves and stems did not share isolates from the same genera of *Actinobacteria*. *Actinobacteria* in the leaf endophytes were affiliated with two genera, *Curtobacterium* (21 %) and *Brachybacterium* (10 %). In the case of stem endophytes the genera represented were *Rhodococcus* (32 %), *Arthrobacter* (26 %) and *Dermacoccus* (5 %). The *Ac-*

Figure 5.1. Taxonomic breakdown of 16s rDNA gene sequences of the culturable (a) leaf, (b) stem and (c) root endophytic and (d) rhizoplane bacterial community composition isolated from *Cytisus striatus*. The central pie shows percentages by phyla; each outer ring progressively breaks these down to finer taxonomic levels (phyla, class, family, genera).





Proteobacteria 72%
Actinobacteria 10%
Firmicutes 16%
Actinobacteria 10%
Microbacteriaceae 4%
Leucobacter 4%
Pseudonocardiaceae 6%
Amycolatopsis 6%
Bacteroidetes 2%
Flavobacteria 2%
Flavobacteriaceae 2%
Chryseobacterium 2%
Bacilli 16%
Bacillaceae 16%
Bacillus 16%
Alphaproteobacteria 39%
Caulobacteriaceae 2%
Brevundimonas 2%
Bradyrhizobiaceae 8%
Bradhyrhizobium 6%
Rhodopseudomonas 2%
Brucellaceae 2%
Pseudochrobactrum 2%
Phyllobacteriaceae 2%
Mesorhizobium 2%
Rhizobiaceae 25%
Rhizobium 25%
Betaproteobacteria 15%
Burkholderiaceae 15%
Burkholderia 15%
Gammaproteobacteria 18%
Xanthomonadaceae 14%
Stenotrophomonas 2%
Dyella 12%
Pseudomonadaceae 4%
Pseudomonas 4%
(c)
Proteobacteria 41%
Actinobacteria 47%
Firmicutes 12%
Enterobacteriaceae 2%
Serratia 2%
Actinobacteria 47%
Intrasporangiaceae 2%
Terrabacter 2%
Microbacteriaceae 2%
Leucobacter 2%
Micromonosporaceae 4%
Micromonospora 4%
Mycobacteriaceae 4%
Mycobacterium 4%
Nocardiaceae 8%
Nocardia 8%
Pseudonocardiaceae 2%
Amycolatopsis 2%
Streptomycetaceae 25%
Streptomyces 25%
Bacilli 12%
Bacillaceae 12%
Bacillus 12%
Alphaproteobacteria 29%
Rhodospirillaceae 4%
Inquilinus 4%
Rhizobiaceae 23%
Rhizobium 23%
Sphingomonadaceae 2%
Sphingomonas 2%
Betaproteobacteria 2%
Burkholderiaceae 2%
Burkholderia 2%
Gammaproteobacteria 10%
Xanthomonadaceae 10%
Stenotrophomonas 2%
Dyella 6%
(d)

tinobacteria were separated phylogenetically in three main groups, and shoot endophytic isolates tended to cluster together in one group (Figure 5.2c).

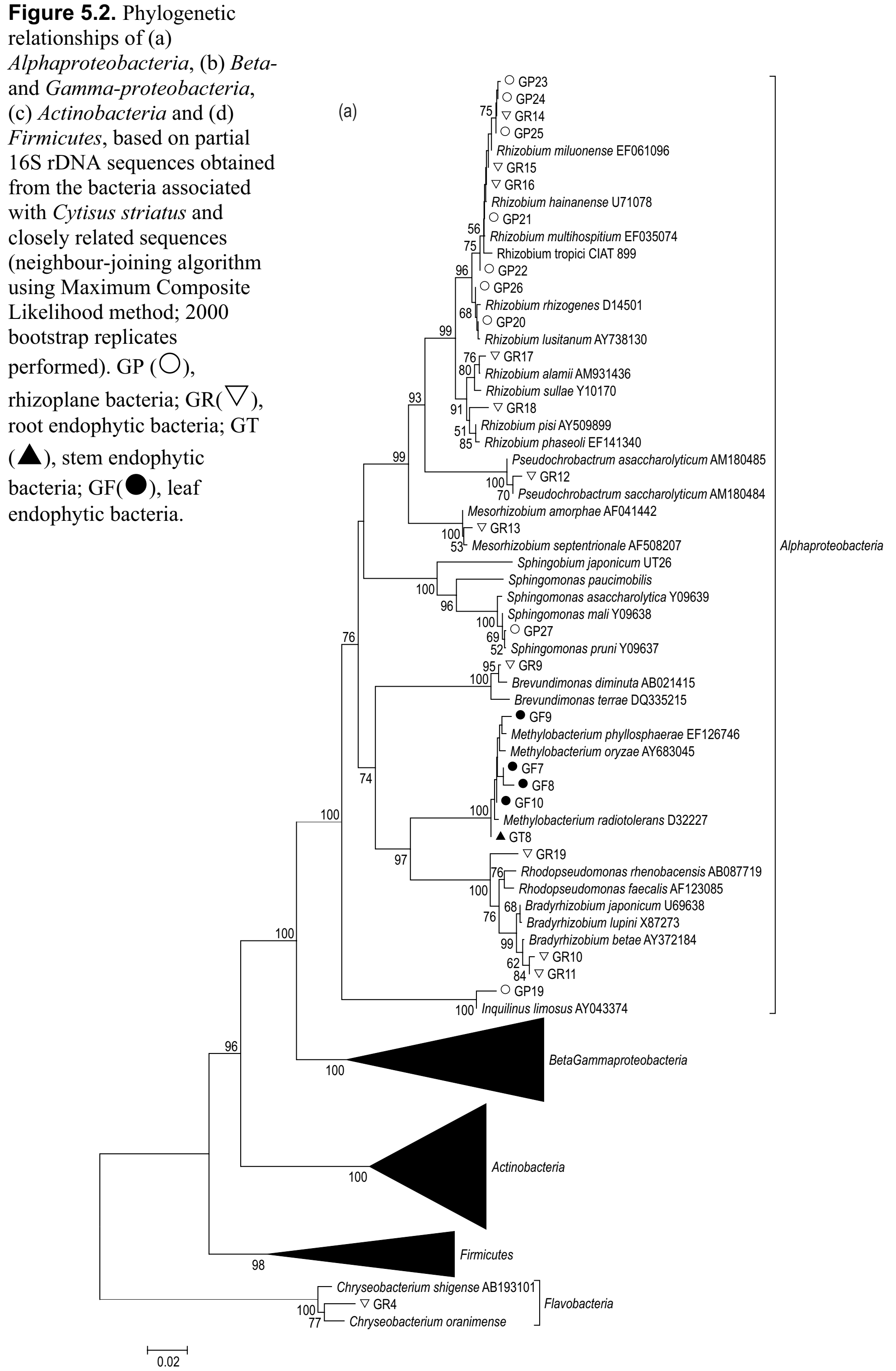
Members of the phylum *Firmicutes* (class *Bacilli*) were found in both root isolate collections (rhizoplane and root endophytes) and among the stem endophytic isolates. In roots this phylum was represented by isolates of the genus *Bacillus* (either rhizoplane (12 %) or root endophytes (16 %)), while stem endophytes included members of the genera *Leuconostoc* (11 %) and *Staphylococcus* (21 %) (Figure 5.1). In the phylogenetic tree there is no clear compartmentalization of *Bacillus* spp. isolated from the two root compartments (Figure 5.2d).

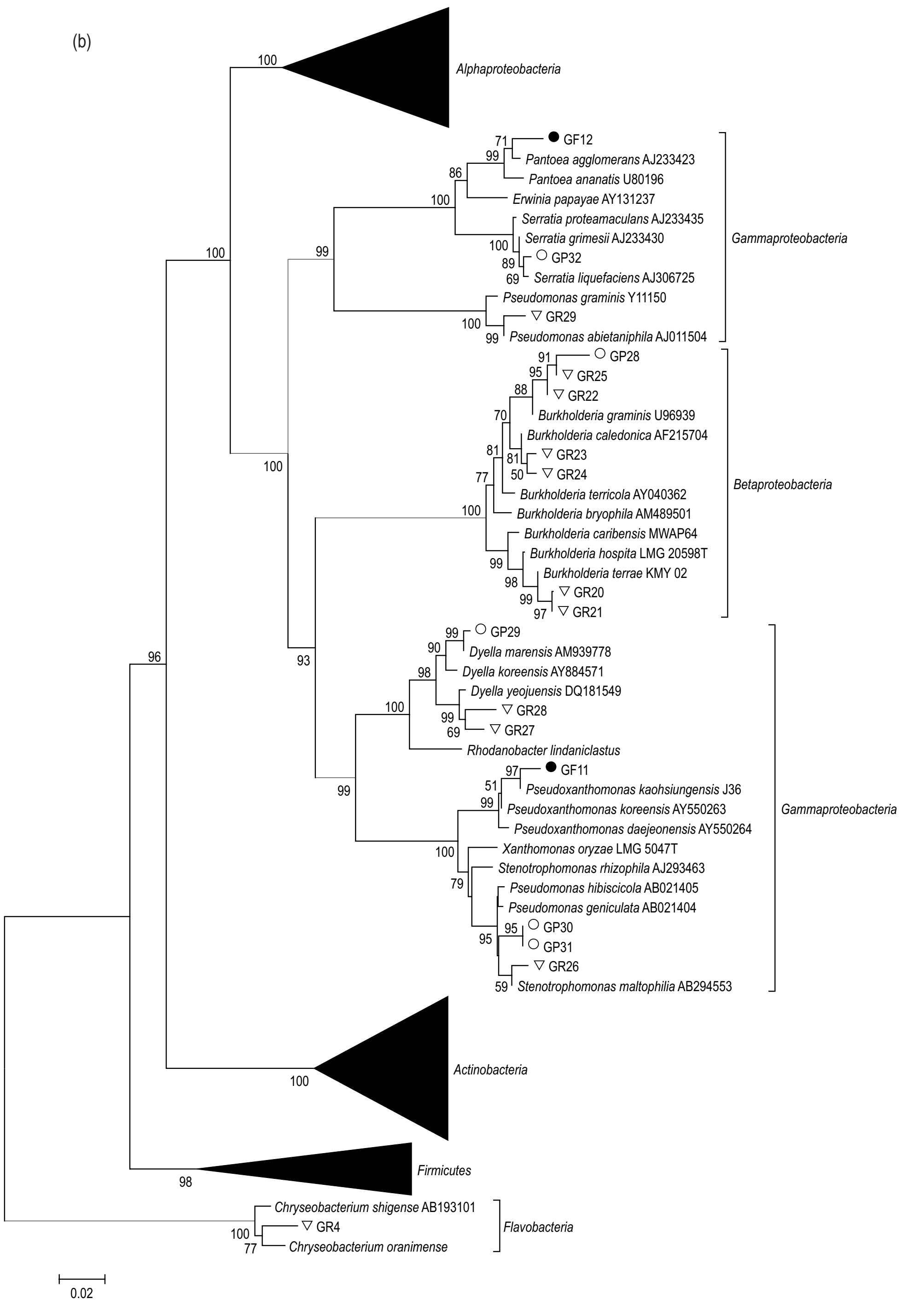
Plant growth-promoting characteristics

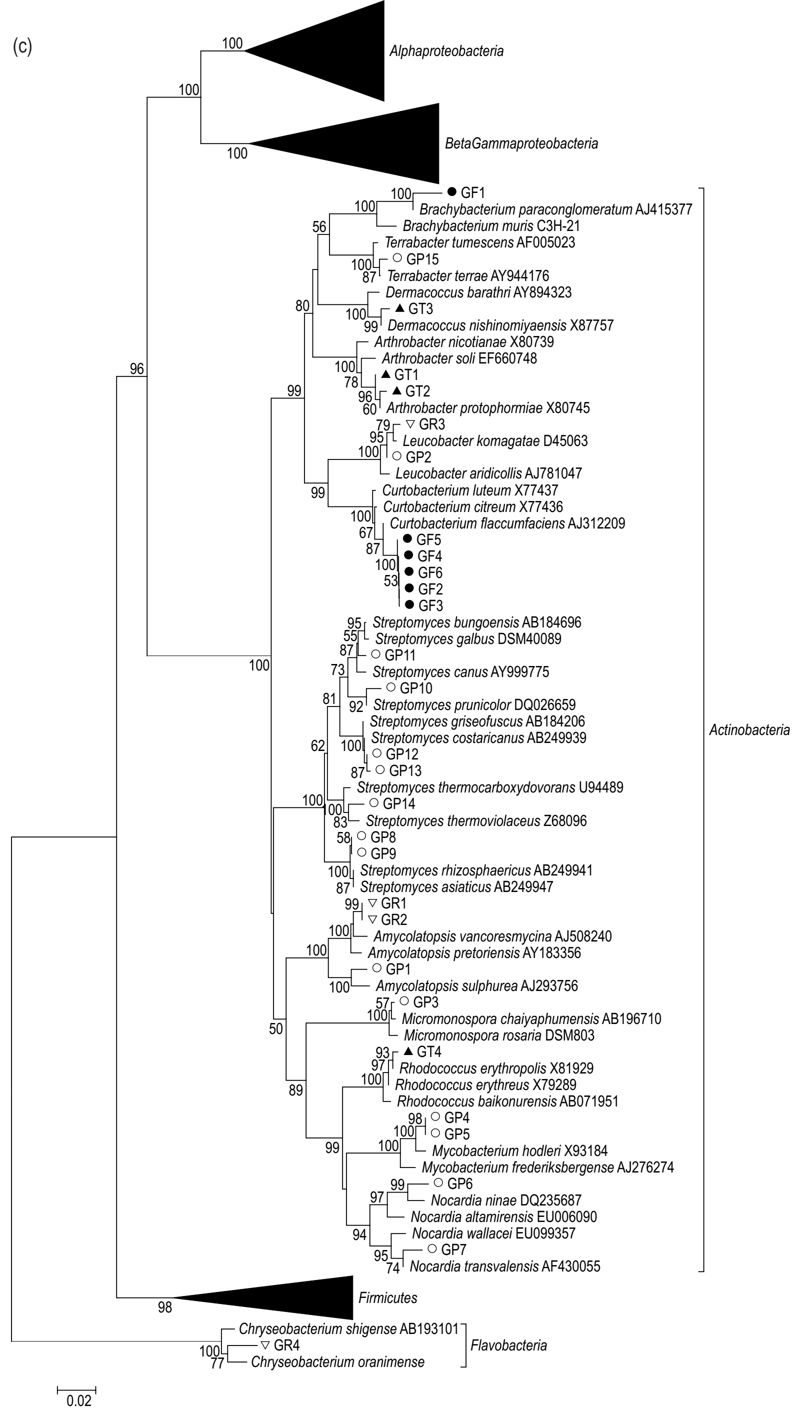
Over 80 % of root endophytes and rhizoplane bacteria were able to grow in agar medium with at least one of the four different C sources tested. This percentage was lower in the case of stem and leaf endophytes, but still 46 to 68 % of isolates were able to use at least one of the different C sources tested (Table 5.1).

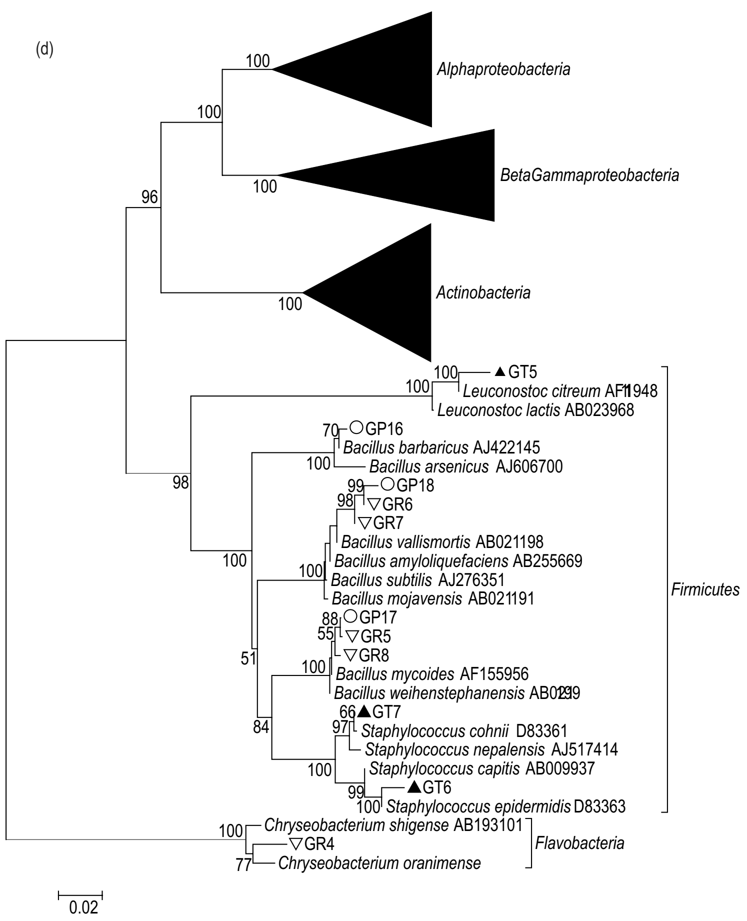
The majority of isolates had at least one of the PGP traits tested (over 60 % of isolates). Table 5.1 shows that many of the isolates tested were able to produce indoleacetic acid (45 % of all isolates tested), to secrete siderophores into the growth medium (38 % of isolates) or to solubilise mineral phosphate (22 % of isolates). P-solubilisers are predominantly associated with the roots of *C. striatus*, and in particular with root endophytes (35 % of isolates were able to solubilise mineral phosphate). These isolates belonged principally to the *Proteobacteria*, most of them affiliated with genera such as *Burkholderia*, *Dyella*, and *Rhizobium*. No strains of leaf endophytes were able to solubilise mineral phosphate, and only three strains of stem endophytes presented this trait (*Arthrobacter* spp. strains ET21/ET53a and *Staphylococcus* sp. strain ET41a) (Table 5.1). IAA-producers were more common amongst leaf and root endophytes (this characteristic was present in 59 % and 61 % of leaf and root endophytic isolates, respectively). IAA formation was particularly common in the leaf endophytic strains identified as members of the *Pantoea* (8 isolates) and *Curtobacterium* (4 isolates). IAA-producing root endophytes were present amongst the rhizobia, and members of the genera *Burkholderia*, *Dyella* (the 6 isolates obtained produced IAA), *Amycolatopsis* and *Bacillus* (Table 5.1). The production of IAA by rhizoplane isolates was less frequent and was detected in some members of the *Actinobacteria*, rhizobia and in 4 isolates identified as *Bacillus*. In stem endophytes only 3 stains (all closely related to species of the genus *Arthrobacter*) were IAA-producers. Siderophore-producing

Figure 5.2. Phylogenetic relationships of (a) *Alphaproteobacteria*, (b) *Beta-* and *Gamma-proteobacteria*, (c) *Actinobacteria* and (d) *Firmicutes*, based on partial 16S rDNA sequences obtained from the bacteria associated with *Cytisus striatus* and closely related sequences (neighbour-joining algorithm using Maximum Composite Likelihood method; 2000 bootstrap replicates performed). GP (○), rhizoplane bacteria; GR(▽), root endophytic bacteria; GT (▲), stem endophytic bacteria; GF(●), leaf endophytic bacteria.




(b)
Alphaproteobacteria
GF12
Pantoea agglomerans AJ233423
Pantoea ananatis U80196
Erwinia papayae AY131237
Serratia proteamaculans AJ233435
Serratia grimesii AJ233430
GP32
Serratia liquefaciens AJ306725
Pseudomonas graminis Y11150
GR29
Pseudomonas abietaniphila AJ011504
Gammaproteobacteria
GP28
GR25
GR22
Burkholderia graminis U96939
Burkholderia caledonica AF215704
GR23
GR24
Burkholderia terricola AY040362
Burkholderia bryophila AM489501
Burkholderia caribensis MWAP64
Burkholderia hospita LMG 20598T
Burkholderia terrae KMY 02
GR20
GR21
Betaproteobacteria
GP29
Dyella marensis AM939778
Dyella koreensis AY884571
Dyella yeojuensis DQ181549
GR28
GR27
Rhodanobacter lindaniclastus
GF11
Pseudoxanthomonas kaohsiungensis J36
Pseudoxanthomonas koreensis AY550263
Pseudoxanthomonas daejeonensis AY550264
Xanthomonas oryzae LMG 5047T
Stenotrophomonas rhizophila AJ293463
Pseudomonas hibiscicola AB021405
Pseudomonas geniculata AB021404
GP30
GP31
GR26
Stenotrophomonas maltophilia AB294553
Gammaproteobacteria
Actinobacteria
Firmicutes
Chryseobacterium shigense AB193101
GR4
Chryseobacterium oranimense
Flavobacteria
0.02


(c)
Alphaproteobacteria
BetaGammaproteobacteria
GF1
Brachybacterium paraconglomeratum AJ415377
Brachybacterium muris C3H-21
Terrabacter tumescens AF005023
GP15
Terrabacter terrae AY944176
Dermacoccus barathri AY894323
GT3
Dermacoccus nishinomiyaensis X87757
Arthrobacter nicotianae X80739
Arthrobacter soli EF660748
GT1
GT2
Arthrobacter protophormiae X80745
GR3
Leucobacter komagatae D45063
GP2
Leucobacter aridicollis AJ781047
Curtobacterium luteum X77437
Curtobacterium citreum X77436
Curtobacterium flaccumfaciens AJ312209
GF5
GF4
GF6
GF2
GF3
Streptomyces bungoensis AB184696
Streptomyces galbus DSM40089
GP11
Streptomyces canus AY999775
GP10
Streptomyces prunicolor DQ026659
Streptomyces griseofuscus AB184206
Streptomyces costaricanus AB249939
GP12
GP13
Streptomyces thermocarboxydovorans U94489
GP14
Streptomyces thermoviolaceus Z68096
GP8
GP9
Streptomyces rhizosphaericus AB249941
Streptomyces asiaticus AB249947
GR1
GR2
Amycolatopsis vancoresmycina AJ508240
Amycolatopsis pretoriensis AY183356
GP1
Amycolatopsis sulphurea AJ293756
GP3
Micromonospora chaiyaphumensis AB196710
Micromonospora rosaria DSM803
GT4
Rhodococcus erythropolis X81929
Rhodococcus erythreus X79289
Rhodococcus baikonurensis AB071951
GP4
GP5
Mycobacterium hodleri X93184
Mycobacterium frederiksbergense AJ276274
GP6
Nocardia ninae DQ235687
Nocardia altamirensis EU006090
Nocardia wallacei EU099357
GP7
Nocardia transvalensis AF430055
Actinobacteria
Firmicutes
Chryseobacterium shigense AB193101
GR4
Chryseobacterium oranimense
Flavobacteria
0.02



isolates were almost equally distributed between the different plant compartments (varying from 32 % to 45 % of isolates). Isolates showing this characteristic were predominantly identified as *Burkholderia* spp. or *Bacillus* spp. amongst the root endophytes, and as *Streptomyces* spp. amongst rhizoplane bacteria. Isolates from stem and leaf endophytic communities which were able to produce siderophores were closely related to *Brachybacterium paraconglomeratum* (98.8 % similarity), *Pantoea agglomerans* (98.5 % similarity), and *Rhodococcus erythropolis* (100 % similarity) (Table 5.1).

Six isolates showed all three PGP traits (*Rhizobium* sp. strain RP6/RP7, *Serratia* sp. strain RP48, *Burkholderia* sp. strain ER6, *Burkholderia* sp. stain ER38, *Pseudomonas* sp. strain ER42), and all of them were members of *Proteobacteria* and associated with roots.

Resistance to trace metals and production of biosurfactants

In general, more than half the strains isolated from each plant compartment showed resistance to at least one of the trace metals tested (Figure 5.3). This was most pronounced in bacteria associated with the roots of *C. striatus*: 86 % of rhizoplane bacteria and 76 % of root endophytes were able to grow in the presence of at least one metal. However, in both cases these results primarily reflected the high number of root-associated bacteria that were able to tolerate Zn (76 % of rhizoplane bacteria and 65 % of root endophytic strains were Zn-resistant). Only one strain from each root compartment showed resistance to three of the four metals tested (Figure 5.3): the root endophyte strain ER42 identified by 16S rDNA as *Pseudomonas graminis* (99.4 % similarity) (Cu, Ni- and Zn-resistant), and the rhizoplane bacteria identified as *Leucobacter aridicollis* strain RP27 (99.6 % similarity) (Cd-, Ni-, and Zn-resistant) (Table 5.1).

The number of strains showing resistance to more than one metal was significantly higher in bacteria associated with the shoots of *C. striatus*: 58 % of strains isolated from the stems, and 17 % of strains isolated from leaves (Figure 5.3). In fact, the majority of stem endophytes found to be metal-resistant tolerated three metals, and only one strain was characterised as mono-resistant (Zn; GT3; *Dermacoccus nishinomiyaensis* strain ET11, 99.2 % similarity). Five isolates grouped together within BOX-PCR GT4 and identified as *Rhodococcus erythropolis* (ET56b, ET57b, ET53b, ET51b, ET41b) were Zn-, Cd-, and Cu-resistant; three strains identified as *Arthrobacter protophormiae* and *Rhodococcus erythropolis* were Zn-, Cd-, and Ni-resistant (ET54a and ET52 (GT1) and ET54b (GT4)); and finally, *Arthrobacter protophormiae* strain ET51a placed in GT2 was able to grow in media supplemented with either Zn, Ni or Cu. Five isolates of leaf endophytes, showed resistance to three metals: *Pseudoxanthomonas koreensis* strains EF34 and EF3, *Methylobacterium radiotolerans* strain EF56, and *Pantoea agglomerans* strain EF48 were Cu-, Ni- and Zn-resistant; and *Curtobacterium flaccumfaciens* strain EF24 was Cd-, Cu- and Zn-resistant.

The ability to produce biosurfactants was a rare characteristic: only 17 strains were able to produce biosurfactants. Rhizoplane bacteria strains showing this characteristic belonged to three BOX-PCR groups: GP29, with only one isolate identified as *Dyella marensis* (strain RP17, 100 % similarity); GP7, with only one isolate identified as *Nocardia transvalensis* (strain RP32, 99.5 % similarity); and GP18, with four isolates, all of which produced biosurfactants and were identified as *Bacillus subtilis* (strains RP01, RP21, RP22, RP23; 99.9 % similarity).

Biosurfactant producing root endophytes were found within the BOX-PCR groups, GR7 (with four isolates, strains ER02, ER24, ER65, ER66) and GR6 (with only one isolate, strain ER64), all of which were closely related to *Bacillus subtilis* (99.9 % similarity). This trait was only observed in six isolates of stem endophytes clustered together in one group (GT4), and identified as *Rhodococcus erythropolis* (strains ET56b, ET57b, ET53b, ET54b, ET51b, ET41b; 100 % similarity).

Presence of linA and linB genes

The *linA* genes were not detected in any of the isolates. In some isolates, fragments of a similar size to the *linB* gene were amplified. However, posterior sequencing of these fragments showed no similarity with *linB* sequences found in the EMBL database.

5.4 Discussion

Culturable bacteria were isolated from the rhizoplane, root, stem and leaf tissues of *C. striatus*. The bacterial counts of endophytic bacteria isolated from the different tissues of *C. striatus* were of a similar magnitude to those published for other species (herbaceous plants and woody tree species) (Kuklinsky-Sobral *et al.*, 2004; Sharma *et al.*, 2005; Sun *et al.*, 2010; Weyens *et al.*, 2009d). Germida *et al.* (1998) found higher densities of culturable bacteria in the rhizoplane compared to the root interior of canola or wheat. Similarly, populations of soil-extract agar-culturable bacteria were higher in the rhizoplane of barley, wheat and canola than in the root interior (Lupwayi *et al.*, 2004). Higher bacterial densities in the rhizoplane compared to endophytes are expected due to the release of nutrients and organic compounds into the rhizosphere, however densities of root endophytes associated with *C. striatus* were similar to those of rhizoplane bacteria. Similarly, microbial densities at the rhizoplane or in the root interior were not found to vary significantly during maize growth (Cavaglieri *et al.*, 2009).

The fact that densities of culturable root endophytes were significantly greater than either stem or leaf endophytes supports the theory that these bacteria are thought to colonise plants primarily through the root system via natural and artificial wound sites, root hairs and at epidermal junctions (Pan *et al.*, 1997; Porteous-Moore *et al.*, 2006; Weyens *et al.*, 2009d). Members of some genera were common to both root compartments, such as *Amycolaptosis*, *Bacillus*, *Burkholderia*, *Dyella*, *Leucobacter* and *Rhizobium*. The similarity in BOX-PCR fingerprints of GR7 and GP18 suggests that these could be the same strain of *Bacillus subtilis*.

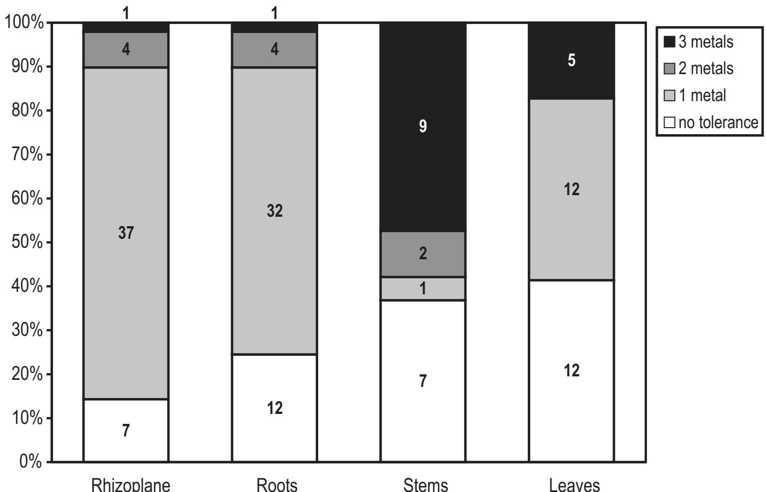


Figure 5.3. Percentage of bacterial strains isolated from *Cytisus striatus* (rhizoplane strains and root/stem/leaf endophytes) which tolerate 0, 1, 2 or 3 trace metals. Trace metal resistance of the strains was tested using 284 agar medium supplemented with 1 mM Cd, Co, Ni or Zn (added as $CdSO_4.8/3H_2O$, $CoCl_2.6H_2O$, $NiSO_4.6H_2O$ and $ZnSO_4.7H_2O$) and incubated at 28 ºC for 7 d.

Similarly, BOX-PCR fingerprints of GR16 and GP21 could represent the same strain of *Rhizobium multihospitium*. The phylogenetic trees also indicated a close relationship between rhizoplane and root endophytic isolates (Figure 5.2).

Strains isolated from the rhizoplane of *C. striatus* included members of genera commonly found in the soil environment, such as *Bacillus*, *Burkholderia*, *Streptomyces* and *Sphingomonas*. The prevalence of rhizobia (*Rhizobium, Bradyrhizobium, Mesorhizobium*) amongst the root-associated bacteria was also expected given that *C. striatus* is a leguminous species. These species are of interest in remediation projects for their N_2-fixing capacity, especially since contaminated soils are often nutrient-limiting. Dissipation of chrysene in soils planted with both white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) was enhanced after inoculation with rhizobia (*Rhizobium leguminosarum* bv. *trifolii*) due to the improved plant vigour and presumable stimulation in rhizospheric microflora (Johnson *et al.*, 2004). Most of the endophytic strains isolated in this study also included genera previously isolated from a range of plant tissues (e.g. *Arthrobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium* etc.; see reviews (Chanway, 2002; Lodewyckx *et al.*, 2002; Weyens *et al.*, 2009c). *Proteobacteria* (in particular, *Alpha*- and *Gammaproteobacteria*) and *Actinobacteria* dominated the total endophytic isolate collection (comprising 49 % and 35 % of isolates, respectively). Bacterial endophytes isolated from cultivars of *Populus* and *Salix*, or *Fraxinus*

excelsior, were dominated by members of the *Gammaproteobacteria*, with a high representation of the genus *Pseudomonas* (Porteous-Moore *et al.*, 2006; Taghavi *et al.*, 2009; Weyens *et al.*, 2009d). In contrast, the culturable endophytic community associated with *Quercus robur* was dominated by *Actinobacteria* (Weyens *et al.*, 2009d).

The results of BOX-PCR profiling and 16S rDNA sequencing indicate that taxonomic diversity of endophytic bacterial communities was tissue-specific. Most taxa only occurred in a specific plant tissue (Figure 5.1; e.g. *Arthrobacter*, *Staphylococcus*, *Curtobacterium*, *Amycoltopsis*, *Bradhyrhizobium*), while others (such as *Bacillus*, *Burkholderia*, *Leucobacter*, *Rhizobium*) were found both inside and outside the plant. Porteous Moore *et al.* (2006) studying endophytic bacteria isolated from two cultivars of *Populus* (cv. Hazendans and cv. Hoogvorst) also found marked spatial compartmentalisation within the plant, indicating the likelihood of species-specific and non-specific associations between bacteria and plants. This was also the case for *Quercus robur*: several taxa were found exclusively inside the plant (*Frigobacterium*, *Okibacterium*, *Curtobacterium*, *Aeromicrobium*, *Enterobacter*, and *Erwinia*) (Weyens *et al.*, 2009d). These authors suggested that this tissue-specificity is indicative of an endophytic community composed of both facultative endophytes colonising the plant via the roots and obligate endophytes transferred from one generation to the other through the seeds. Indeed, the same strain of *Rhodococcus* (GT4) isolated in this study was also isolated from the seeds of *C. striatus* (unpublished data).

Bacterial diversity (number of different genera) was similar in rhizoplane and root endophytic bacteria of *C. striatus* (although *Actinobacteria* were more prominent in the rhizoplane collection of isolates) and decreased in stem and leaf endophytes. Leaf endophytic communities have been proposed to be a combination of those translocated from the stem and those entering through leaf wounds or stomata (Porteous-Moore *et al.*, 2006). The fact that the taxonomic affiliation of leaf and stem isolates of *C. striatus* was not in common with that of the strains found at the root level points to an aerial route of entry. Furthermore, members of genera commonly cited as being found in the leaf phyllosphere were also detected here (e.g. *Methylobacterium*, *Pantoea* or *Curtobacterium*; Lindow and Brandl, 2003), and their presence in the shoot tissues but not the roots further supports this additional route of entry into the plant. Although the possibility that shoot endophytes enter the plant through the roots but then re-distribute within the plant to their preferred niche cannot be discarded.

In several recent studies, endophytes isolated from plants growing in contaminated areas have shown a natural capacity for xenobiotic degradation. Siciliano *et al.* (2001) showed that plants growing in soils contaminated with petroleum hydrocarbons and nitroaromatics naturally recruited root endophytes with the necessary contaminant-degrading genes. Van Aken *et al.* (2004) described a methylotrophic endophytic bacterium isolated from hybrid poplar trees (*Populus deltoides* X *Populus nigra* DN34) that was capable of degrading explosives (TNT, RDX and HMX). Germaine *et al.* (2006) reported that when pea plants were inoculated with *Pseudomonas* endophytes, isolated from hybrid poplars (*Populus trichocarpa* X *Populus deltoides* cv. Hoogvorst) and capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), the pea plants showed no accumulation of 2,4-D in their tissues and little or no signs of phytotoxicity when compared to uninoculated controls. Weyens *et al.* (2009d) isolated and characterised bacterial communities (bulk soil, rhizosphere, root, stem and leaf) associated with oak and ash trees growing on a TCE-contaminated groundwater plume. The majority of isolated bacteria showed increased tolerance to TCE and toluene, and TCE degradation capacity was observed in some rhizosphere strains. Most of the known degraders of HCH isomers are sphingomonads and those analysed at a genetic level have been shown to harbour the genes *linA* and *linB*, which encode the first two enzymes of the only upstream HCH aerobic degradation pathway elucidated to date (see reviews by Lal *et al.*, 2010 and Nagata *et al.*, 2007). However, in the present study neither the *linA* nor *linB* gene were amplified by PCR from the DNA extracted from the isolates, not even in strain GP27 which was identified as a *Sphingomonas* sp. The PCR primers used show a perfect match with most corresponding *linA* and *linB* in five HCH-degrading *Sphingomonas* strains isolated from the same contaminated soil as well as with most homologous sequences found in the EMBL database. Moreover, tests carried out in liquid cultures to analyse the degradation of HCHs by some of the isolates did not successfully demonstrate any degradation of these compounds. HCHs show a low water solubility (particularly the beta and alpha isomers, which are also the predominant isomers found in the contaminated sampling site), while the intermediates of their degradation show a much higher solubility and may be absorbed by the plant more efficiently than HCH isomers. Metabolites such as pentachlorocyclohexene (PCCH) and cyclopentiltrichloroethene were identified in soil samples taken from the same HCH-contaminated site as that sampled in this study, and in root, stem and leaf tissues of *C. striatus* growing at this site (Barriada-Pereira *et al.*, 2005; Concha-

Graña *et al.*, 2006). Further studies are underway to ascertain the tolerance and/or degradative ability of selected endophytic strains isolated from *C. striatus* to possible intermediates produced during the degradation of HCH isomers (e.g. PCCH, 1,2,4-trichlorobenzene, 2,5-dichlorophenol, 2,5 dichlorohydroquinone).

A major limiting factor for phytoremediation of recalcitrant organic pollutants is often their low bioavailability. Biosurfactant-producing microorganisms can increase contaminant availability, plant uptake and/or microbial degradation (Schwitzguébel *et al.*, 2002). The few isolates shown to produce biosurfactants (e.g. *Bacillus* spp., *Rhodococcus* spp.) will be studied further to ascertain their effect on the bioavailability of HCH isomers. *Rhodococcus* sp. strain ET54b has been shown to tolerate HCH isomers in the growth medium (unpublished data). Taghavi *et al.*, (2009) also observed a significant number of *Rhodococcus* spp. (12 % of culturable strains) in the endophytic community of poplar trees.

In field situations phytoremediation processes will need to be applied to sites co-contaminated with organic and metal pollutants. Remediation of co-contaminated sites is a very complex problem. The presence of toxic metals can, for example, affect the biodegradation of a variety of organic pollutants through impacting both the physiology and ecology of degrading microorganisms (Lin *et al.*, 2006; Weyens *et al.*, 2010b). In addition, tolerance to a trace metal is advantageous for re-isolation in controlled conditions (Weyens *et al.*, 2009d). In this study, isolates which were able to grow in 284 medium supplemented with 1 mM of Cd, Co, Ni or Zn were considered resistant. A concentration of 1 mM has been used to indicate metal resistance in previous studies (Nieto *et al.*, 1987). In terms of metal-resistance, the most common phenotype was a single resistance to Zn (74 % of all isolates tested were able to grow on 284 medium supplemented with 1 mM Zn). Some strains showed co-resistance (e.g. *Arthrobacter*, *Pseudoxanthomonas*, *Pantoea*, *Rhodococcus*), and one strain of *Pseudomonas* (ER42) showed co-resistance (Cu, Ni, Zn) and PGP characteristics (siderophore producer, P solubiliser and IAA producer). The PGP characteristics found in an important number of the bacterial isolates obtained in this study could be particularly useful for improving the phytoremediation potential of *C. striatus*. For example, the root endophyte strains, ER43, ER53 and ER6, which showed all three of the PGP characteristics tested in this study will be tested for their ability to re-colonise *C. striatus* tissues, this will be necessary if they are to be of potential use in field-scale trials. PGP strains shown to successfully colonise plants will be used in re-inoculation experiments to evaluate their ability to improve seed germination and plant establish-

ment in HCH-contaminated soils and degradation of HCH isomers and/or metabolites.

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Chapter 6

Improving performance of *Cytisus striatus* on substrates contaminated with hexachlorocyclohexane (HCH) isomers using bacterial inoculants: developing a phytoremediation strategy

ABSTRACT

Microbe-assisted phytoremediation is particularly effective for organic pollutants. The leguminous shrub *Cytisus striatus* (Hill) Rothm. has been proposed as a candidate species for the rhizoremediation of hexachlorocyclohexane (HCH)-contaminated sites. The aim of this study was to improve the performance of this species using microbial inoculants. *C. striatus* was grown in substrates contaminated with 0, 10 and 35 mg HCH kg^{-1} for eight weeks. Plants were either not inoculated (NI), or inoculated with the endophyte *Rhodococcus erythropolis* ET54b and the HCH-degrader *Sphingomonas* sp. D4 (isolated from a HCH-contaminated soil) on their own or in combination (ET, D4 and ETD4). Inoculation with both bacterial strains (ETD4) resulted in decreased HCH phytotoxicity and improved plant growth. HCH-exposed plants inoculated with ETD4 presented a 120-160 % increase in root, and 140-160 % increase in shoot biomass, and led to a decrease in the activities of enzymes involved in anti-oxidative defence. APOD activity was reduced by up to 37 % in shoot tissues and 25 % in root tissues, and corresponding activities of SOD were reduced by up to 35 % and 30 %. HCH dissipation was enhanced in the presence of *C. striatus* but no significant effect of microbial inoculants was observed. Inoculating *C. striatus* with this combination of bacterial strains is a promising approach for the remediation of HCH-contaminated sites.

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6.1 Introduction

The organochlorine 1,2,3,4,5,6-hexachlorocyclohexane (HCH) is considered a persistent organic pollutant (POP) due to its acute toxicity and strong persistence in the environment (UNEP, 2009; Vijgen *et al.*, 2011). HCH exists as eight possible isomers, which differ in the axial or equatorial orientations of their chlorine atoms. Technical-grade HCH, also known as technical lindane, is a mixture of HCH isomers and was widely used as an insecticide from the 1940s until relatively recently. After it was discovered that the active ingredient was in fact γ-HCH (or lindane), this isomer was frequently isolated from technical HCH for commercial purposes (Breivik *et al.*, 1999). As a result, large quantities of by-products (mixtures of other HCH isomers) were dumped around production facilities leading to the contamination of many sites worldwide: for each ton of lindane produced, 8-12 tonnes of waste are generated (generally enriched in the alpha and beta isomers) (Breivik *et al.*, 1999; Vijgen *et al.*, 2011). The recent inclusion of the α-, β- and γ-HCH isomers in the Stockholm Convention list of POPs has led to a renewed interest in these contaminants and the remediation of affected sites (UNEP, 2009).

Phytoremediation techniques based on the interactions between plants and their associated microorganisms have been proposed as cost-efficient and eco-friendly methods to clean up polluted soils with POPs and organochlorine pesticides (Gerhardt *et al.*, 2009; Kidd *et al.*, 2009; Weyens *et al.*, 2009a). Recent studies demonstrate significant enhanced dissipation and/or mineralisation of POPs at the root-soil interface or rhizosphere. This effect is generally attributed to an increase in microbial density, diversity and/or metabolic activity due to the release of plant rhizodeposits (Chaudhry *et al.*, 2005; Gerhardt *et al.*, 2009). The natural capacity to degrade organic compounds presented by many microorganisms is a characteristic that can be exploited in phytoremediation methods. In the case of HCH, several bacterial strains (predominantly belonging to the genera *Sphingomonas* or *Sphingobium*) are capable of degrading the different isomers of HCH (see review by Lal *et al.*, 2010). However a careful selection of tolerant plant species and optimising plant growth are vital since the phytotoxic nature of these contaminants can inhibit plant performance, reducing the overall efficiency of the remediation process (Huang *et al.*, 2004; Weyens *et al.*, 2009b). Overcoming this limitation will therefore be a key element for successful phytoremediation. One way of achieving this is by inoculating plants with strains of plant growth promot-

ing bacteria (PGPB) which increase plant tolerance to contaminants and improve growth (Huang *et al.*, 2004; Weyens *et al.*, 2009b). A prominent mechanism used by many PGPB to facilitate plant growth is through the production of phytohormones (such as indoleacetic acid (IAA), cytokinins) or the production of the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick *et al.*, 1998). Other known mechanisms to stimulate plant growth include enhancing nutrient (e.g. N_2 fixers, siderophore producers) and water uptake, altering root morphology, inhibiting pathogens, and the induction of plant defence mechanisms (Van Loon and Bakker, 2003). The presence of plant-colonising, naphthalene-degrading bacteria were shown to protect their host plants against the toxic effects of naphthalene exposure (Germaine *et al.*, 2009; Kuiper *et al.*, 2002). Likewise, the TCE-degrading poplar endophyte *Pseudomonas putida* W619-TCE promoted plant growth and reduced TCE phytotoxicity in inoculated poplar cuttings (Weyens *et al.*, 2010).

The leguminous species *Cytisus striatus* grows spontaneously on HCH-contaminated sites and has been proposed as a candidate species for the clean-up of this type of contaminant (Calvelo-Pereira *et al.*, 2006; Kidd *et al.*, 2008). In a previous study, the culturable endophytic and rhizoplane bacterial communities associated with *Cytisus striatus* from an HCH-contaminated site were characterised (see chapter 5). Due to their high hydrophobicity HCH isomers are thought to be poorly taken up inside plant tissues. Phytodegradation is therefore less likely to be an effective remediation option for this contaminant, whereas rhizoremediation or phytostimulation could be promising alternatives. The objective of this study was to evaluate the influence of two bacterial strains on the growth and survival of *C. striatus* growing in HCH-contaminated substrates, and the plant-microbial effects on the dissipation and/or degradation of HCH isomers.

6.2 Materials and methods

Experimental design and inoculation of *Cytisus striatus* plants

Two bacterial strains were chosen from previous studies to inoculate *Cytisus striatus* (Hill) Rothm. (Portuguese broom). These included *Rhodococcus erythropolis* ET54b, an endophyte isolated from shoot tissues of *C. striatus* growing at a HCH-contaminated site (Porriño, NW Spain) (chapter 5; Calvelo-Pereira *et al.*, 2006). This strain is a siderophore- and biosurfactant-producer, shows resistance to Cd, Cu and Zn, and is able to grow in liquid culture in the presence of lindane residue.

The second strain, *Sphingomonas* sp. D4, was previously isolated from soil collected at the same contaminated site and is able to grow in the presence of α-, β-, γ – and δ-HCH isomers as sole C sources. To prepare the bacterial inoculants, strains were cultivated in 869 medium (see chapter 2) for 3 days, harvested by centrifugation (4000 g, 15 min) and re-suspended in 10 mM $MgSO_4$ to an optical density of 1.0 at 660 nm (about 10^7 cells per ml). A mixture containing equal volumes of the ET54b and D4 suspensions (OD_{660} 1.0) was also prepared.

Seeds of *C. striatus* were collected from plants growing at the HCH-contaminated site, surface-sterilized in 1 % NaClO for 1 min, rinsed five times in sterile deionized water and dried on sterile filter paper. Seeds (in lots of 30) were germinated in the dark on a moistened perlite:quartz sand mixture (2:1 v/v) to which 10 ml of bacterial inocula (prepared as described above) were added: strain ET54b (ET), strain D4 (D4), or strains ET54b and D4 in combination (ETD4). The same amount of sterile 10 mM $MgSO_4$ was added to control seeds (non-inoculated control, NI). One-week-old seedlings were then transferred into pots filled with the same perlite:sand mixture (2:1 v/v) (one seedling per pot) and contaminated with HCH. Residues produced during the fabrication of γ-HCH were used to contaminate the substrates. The mean composition of the residue was: 77 % α-, 16 % β-, 5 % γ- and 2 % δ-HCH (Calvelo-Pereira *et al.*, 2006). The residues were dissolved in acetone and thoroughly mixed with quartz sand; thereafter the acetone was allowed to evaporate. Appropriate amounts of contaminated and non-contaminated sand were added to perlite to prepare three levels of HCH contamination: 0, 10 and 35 mg kg^{-1}.

After transferring to pots, seedlings were re-inoculated with the bacterial suspensions prepared as described above (10 ml suspension/pot), which were applied to the substrate close to the stem of each seedling. Ten ml of sterile 10 mM $MgSO_4$ was applied to non-inoculated control pots. For each inoculant/HCH condition 16 replicates were prepared. Unplanted inoculated and non-inoculated pots for each level of HCH contamination were also prepared (4 replicates of each combination of HCH and inocula). During the assay, planted and unplanted pots were watered with half-strength Hoagland solution (see chapter 4). Solutions were buffered at pH 6.0 with 1 mM MES (2-(N-morpholino)ethanesulfonic acid) half neutralised with NaOH.

Plants were grown for 8 weeks under greenhouse conditions. At harvest root and shoot fresh weight was determined. A sub-sample of each tissue was immediately frozen in liquid nitrogen and conserved at -80 ºC for enzymatic analyses, and

a second sub-sample of fresh tissues was used for microbial analyses. The remaining plant material was oven-dried at 45 ºC, weighed and ground (for analysis of nutrient and HCH content).

Plant nutritive status and determination of enzymatic activities

The plant nutritive status was determined in four independent replicates per treatment. Shoot tissues were digested in a 2:1 HNO_3:HCl mixture and P, K, Ca, Mg, Fe, Cu, Mn and Zn measured by ICP-OES (Vista Pro; Varian Inc., Australia).

The activity of plant enzymes involved in anti-oxidative defence was determined in 10 independent replicates per treatment. Frozen leaf or root tissue was homogenized in ice-cooled 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 4 % insoluble polyvinylpyrrolidone (1 ml buffer per 100 mg fresh weight). The homogenate was squeezed through a nylon mesh and centrifuged at 20,000 g and 4 ºC for 10 min. Enzyme activities were measured spectrophotometrically in the supernatant at 25 ºC. Catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2) and guaiacol peroxidase (GPOD, EC 1.11.1.7) activities were determined at 240, 340 and 436 nm, respectively according to Bergmeyer *et al.* (1974). Ascorbate peroxidase (APOD, EC 1.11.1.11) activity was measured at 298 nm according to Gerbling *et al*. (1984). Analysis of superoxide dismutase (SOD, EC 1.15.1.1) activity was based on the inhibition of cytochrome c at 550 nm following the method of McCord and Fridovich (1969).

Microbiological analyses

Endophytic bacterial densities were determined in the plant tissues (roots, stems and leaves) of *C. striatus* after 8 weeks of growth. This was performed on three replicates per treatment. For each replicate sub-samples from 2 independent plants were pooled together and bacterial densities were determined as described earlier (see chapter 5). Briefly, root, stem and leaf tissues were surface-sterilised in respectively 2 %, 1 % or 0.1 % NaClO solution supplemented with Tween 80. After surface sterilisation, tissues were rinsed in sterile deionised water, macerated in 10 mM $MgSO_4$ and plated on 1:10 diluted 869 agar medium (Mergeay *et al.*, 1985). Bacterial densities in the plant growth substrates (perlite:sand mixtures) were also determined by extracting cells with 1 % sodium hexametaphosphate and plating out serial dilutions on 1:10 diluted 869 agar medium. After 7 days incubation at 28 ºC, colony forming units (CFUs) were counted and calculated per gram of substrate or plant tissue.

An attempt was made to recover the inoculated bacterial strains. For this, the serial dilutions of bacterial endophytes and plant growth substrates (described above) were plated on selective media for *R. erythropolis* ET54b (284 medium supplemented with 0.8 mM Cd (as $CdSO_4.8/3H_2O$)) and for *Sphingomonas* sp. D4 (284 medium sprayed with γ-HCH as sole C source; modified from Kiyohara *et al.*, 1982). In the case of *R. erythropolis*, the following carbon sources were added: lactate (0.7 g l^{-1}), glucose (0.5 g l^{-1}), gluconate (0.7 g l^{-1}), fructose (0.5 g l^{-1}) and succinate (0.8 g l^{-1}). BOX-PCR profiling was used to confirm the identity of these two strains (ET54b and D4) following the methods described in chapter 2. When the strain could not be recovered by culture-dependent methods, molecular methods were used. For this reason, the survival of *Sphingomonas* sp. D4 strain was also checked using Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA amplified fragments. For DGGE analysis, at the end of the experiment total DNA was extracted from the perlite/sand substrates using the kit Power Soil DNA isolation kit (MoBio Inc.) following the manufacturer instructions. Fragments of *Sphingomonas* sp. 16S rDNA gene were amplified using a nested PCR approach. In the first PCR round the *Sphingomonas* sp. specific primer Sphingo108f (5´-GCGTAACGCGTGGGAATCTG-3) (Leys *et al.*, 2004) and the universal primer 1492R (5'- TACGGYTACCTTGTTACGACT T-3') (Lane, 1991) were used. The 25 µl PCR mixtures contained 1 µl of extracted DNA, 1× *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP's, 5 % (vol/vol) dimethyl sulfoxide (DMSO), 0.2 µM of each primer and 1.25U TrueStart^{TM} Hot Start *Taq* DNA Polymerase (Fermentas, Germany) and the reaction conditions consisted of an initial denaturation step at 94 ºC for 7 min, followed by 25 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, and a final extension step of 7 min at 72°C. Amplified fragments were re-amplified in a second PCR round with the primer 984F-GC, carrying a 40–mer GC clamp linked to 5´end, and the primer 1401R (Nübel *et al.*, 1996). In this second round the 25 µl reaction mixtures contained 1 µl of first round PCR product 1× *Taq* buffer (which contains 2.5 mM $MgCl_2$), 0.1 mM dNTP's, 0.4 µM of each primer and 1.25U DreamTaq DNA Polymerase (Fermentas, Germany) and the amplification conditions were as described above except for the number of cycles (30 cycles) and the duration of the final extension (10 min). As a positive control, 16S rDNA was also amplified from genomic DNA of isolate D4 (extracted with the FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer instructions and following the nested protocol detailed above.

DGGE was carried out with a IngenyPhorU apparatus (Ingeny, The Netherlands). The denaturing gels were prepared with a double gradient of denaturant (46.5 to 65 % denaturant, 100 % denaturant was defined as 7 M urea and 40 % formamide) and acrylamide (6.2 to 9 %). Aliquots of 4-5 µl of PCR products were loaded on the gels and run in 1 × Tris-acetate-EDTA buffer (pH 8) at a constant voltage of 140 V for 17 h at 58°C. The gels were fixed with ethanol–acetic acid (10 %/0.5 % (v/v)) and stained with silver nitrate (0.2 % wt/v). The developing was performed with NaOH and formaldehyde (1.5 %/0.3 % v/v) and the reaction was stopped with $NaCO_3$ (0.75 % wt/v).

Bands with a migration profile identical to that of D4 were excised from the gel and placed overnight in 100 µl sterile H_2O at 4 ºC. Five µl of this eluate were used for amplification with the primers 984F (without GC-clamp) and 1401R as described above. Amplified products were cloned using the InsTAclone Cloning Kit (Fermentas, Germany) and the fragment present in 5 of the clones was sequenced. The obtained sequences were compared with that of D4 using Clustal W (Thompson *et al.*, 1994). Sequence data are deposited in the EMBL database under accession numbers HE681122 and HE681123.

Determination of HCH concentrations in growth substrates and plant tissues

The concentration of HCH isomers (α-, β-, δ- and γ-HCH) were measured in the initial plant growth substrates and at the end of the experiment in planted (six replicates per treatment) and unplanted pots (four replicates per treatment). Two g of air-dried substrate were extracted with 20 ml of 1:1 (v/v) hexane:acetone in an ultrasonic bath for 30 min. Extracts were dried with anhydrous Na_2SO_4 and filtered through a glass fibre filter.

HCH concentrations were also determined in root and shoot tissues of 4-5 plants per treatment. Sub-samples of 0.2 g of ground plant material were extracted with 10 ml of 80:20 (v/v) hexane:ethyl acetate using a laboratory microwave with a built-in magnetic stirrer and the following program: 20 min ramp from ambient temperature to 115 ºC, followed by a 15 min hold at 115 ºC (MARS, CEM Corp.). Extracts were filtered (through a glass fibre filter) and a clean-up step was carried out using solid phase extraction with Florisil (Sep-Pak Vac Florisil (5 g) cartridge) and deactivated alumine (2 g) as adsorbents. HCH was eluted with 10 ml of 80:20 (v/v) hexane:ethyl acetate and the extract was made up to 25 ml with the same solution. All the substrate and plant extracts were stored at 4 ºC until analysis.

Identification and quantification of the different HCH isomers was carried

out by a gas chromatograph (Model 450GC, Varian Inc., Australia) equipped with a CP-8400 autosampler and coupled to an ion-trap mass spectrometer (Model 220MS, Varian Inc., Australia). The chromatographic separations were performed on a FactorFour capillary column (30 m length × 0.25 mm inner diameter, 0.25 µm film thickness; Varian Inc., Australia). The oven temperature program was 70°C (held for 1.5 min), increased by 25°C min^{-1} to 180 °C (held for 2 min), then by 10° C min^{-1} to 230°C, and then by 25°C min^{-1} to 300°C (held for 5 min). Helium was used as carrier gas at a constant flow-rate of 1 ml min^{-1}. The injector temperature was kept at 280 ºC and samples (1 µl) were injected in the splitless injection mode. The MS/MS was operated in electronic impact (EI) mode. Manifold, ion source trap and transfer line temperatures were set at 80, 220 and 280 ºC, respectively. The calibration curve data was obtained by injection of standard solutions of a mixture of the four HCH isomers in hexane.

Statistical analysis

All datasets were statistically compared using analyses of variance (ANOVA) and Least Significance Difference post-hoc comparison test. Comparison of HCH concentrations between planted and non-planted substrates was achieved by the Students t-test for independent means. Transformations were applied when necessary to approximate normality and/or homoscedasticity.

6.3 Results

Densities of culturable bacteria and recovery of inocula

Bacterial densities were consistently higher in planted than unplanted substrates (by one or two logarithmic units) (Table 6.1). There were no significant differences between inoculated and/or non-inoculated treatments; Table 6.1 presents the mean CFUs g^{-1} substrate for each HCH concentration. HCH contamination did not significantly influence bacterial abundance in planted pots but had a negative effect (although not significant) in unplanted controls at the higher HCH concentration (Table 6.1). Densities of culturable bacterial endophytes decreased from the root to the leaves but were not significantly affected by either the inoculant or HCH concentration in the substrate (Table 6.1).

At the end of the growth period, the *R. erythropolis* ET54b strain was re-isolated from both the perlite:sand substrates and plant tissues by cultivation in Cd -enriched medium, which allows the selective growth of this strain. BOX-PCR

Table 6.1. Colony forming units per gram fresh weight (CFU g^{-1} ± SE) of substrate or plant tissues (n = 3). Within each column, different letters indicate significant differences ($p < 0.05$).

	Substrate		Plant tissue		
	Non planted	Planted	Roots	Stems	Leaves
0 mg kg^{-1}	$8.0 \pm 1.1 \times 10^5$ *a*	$4.6 \pm 1.4 \times 10^6$ *a*	$4.1 \pm 0.5 \times 10^4$ *a*	$1.2 \pm 0.5 \times 10^4$ *a*	$6.5 \pm 3.0 \times 10^2$ *a*
10 mg kg^{-1}	$9.6 \pm 1.2 \times 10^5$ *a*	$1.9 \pm 0.5 \times 10^6$ *a*	$3.0 \pm 1.1 \times 10^4$ *a*	$5.1 \pm 2.0 \times 10^3$ *a*	$1.5 \pm 0.8 \times 10^3$ *a*
35 mg kg^{-1}	$1.2 \pm 1.2 \times 10^5$ *a*	$2.3 \pm 0.4 \times 10^6$ *a*	$4.7 \pm 1.2 \times 10^4$ *a*	$4.6 \pm 1.4 \times 10^3$ *a*	$1.2 \pm 0.5 \times 10^3$ *a*

profiling was used to confirm the identity of this strain. At the end of the experiment, the *Sphingomonas* sp. D4 strain was not re-isolated on selective media but its survival could be confirmed on the basis of the DGGE technique. The DGGE profiles corresponding to treatments D4 or ETD4 showed a band with a migration behaviour identical to that of the fragment amplified from *Sphingomonas* sp. D4. This band was absent in profiles corresponding to NI or ET54b treatments. The DNA sequences of these bands excised from DGGE gels confirmed the presence of D4 in the inoculated pots at the end of the experiment.

Plant status: biomass production, enzyme activities and nutrient content

Exposure of non-inoculated plants to HCH was toxic: a reduction in root and shoot biomass (only significant for shoots) was observed with increasing HCH concentration (Figure 6.1). The effect on plant growth depended on both the inoculant and the HCH concentration ($p < 0.05$). In the absence of HCH, both of the bacterial strains when inoculated individually (ET and D4) had a negative effect on plant growth (root and shoot biomass production). This trend was repeated to some extent at 10 mg HCH kg^{-1}, particularly for plants inoculated with ET54b (Figure 6.1). At the highest HCH level (35 mg kg^{-1}) inoculation with ET54b did not affect plant growth and inoculation with D4 tended to have a positive effect on shoot growth. However, inoculation with both strains together (ETD4) resulted in a significant increase in the biomass production of *C. striatus* compared to non-inoculated controls, and this effect was observed in both uncontaminated- and HCH-contaminated substrates (at 35 mg HCH kg^{-1} shoot biomass was up to 1.6-fold greater than non-inoculated plants) (Figure 6.1). The increase in shoot biomass was always significant ($p < 0.05$), while root biomass was only significantly increased in plants growing in 35 mg HCH kg^{-1}.

In general, the plant nutritional status was not affected by either the microbial inoculants or by the presence of HCH, in almost all cases nutrient concentrations did not vary between treatments (data not shown). Plant Fe content was the

only nutrient found to decrease in the presence of HCH. Moreover, in these substrates inoculation of *C. striatus* with the siderophore-producer ET54b (either on its own or in combination with D4) led to a slight increase in shoot Fe content (Figure 6.2).

In general, inoculation of plants growing in uncontaminated substrates resulted in an increase in the activity of stress-related enzymes, and this was most pronounced in the roots (Figure 6.3). On the other hand, HCH-exposure also induced an enzymatic response in this plant species. In non-inoculated plants, an increment in antioxidative enzyme activities was observed in the presence of HCH, and this was again particularly evident in the roots. This was more evident at the higher HCH level of 35 mg kg^{-1} and particularly for APOD, GR and GPOD, where activities increased by 1.2- to 3.4-fold (Figure 6.3). In HCH-exposed plants, inoculation with ET54b or D4 either had no effect, or led to an increase, in enzyme activities. The most noteworthy effect of microbial inoculation was the reduction in the activity of some antioxidative enzymes in plants inoculated with both bacterial strains (ETD4) and growing at the highest HCH concentration, indicating a lower level of stress. For example, APOD and SOD activities in these plants were reduced to 60 % (in shoots) and 75 % (in roots) of corresponding activities in non-inoculated plants (Figure 6.3b and 8.3d).

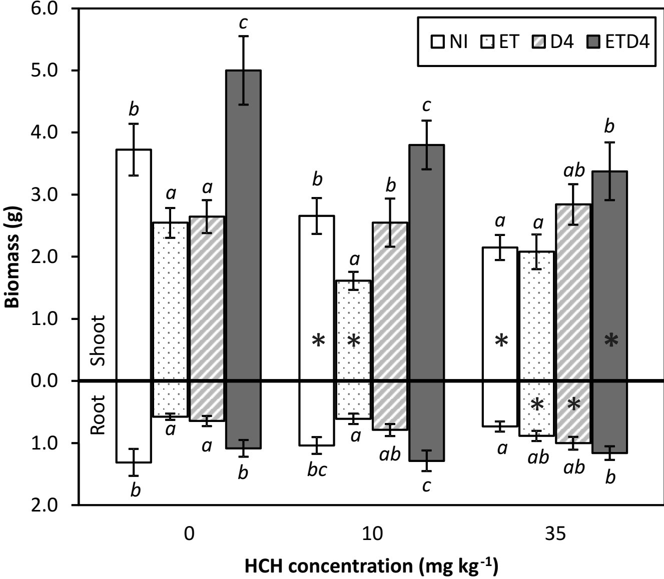

Figure 6.1. Shoot and root fresh weight (mean ± SE) of inoculated and non-inoculated *Cytisus striatus* plants (n = 16). Bars with different letters in each contamination level indicate significant differences ($p < 0.05$). Bars with asterisks indicate significant differences compared to the same inoculation treatment at 0 HCH mg kg^{-1} ($p < 0 .05$).

HCH concentrations in substrates and plant tissues

In practically all of the samples analysed (substrates or plants), the γ- and δ-HCH isomers were below the level of detection. For this reason, only results of α- and β-HCH isomers are presented, and total-HCH (or ∑-HCH) refers to the sum of these two isomers.

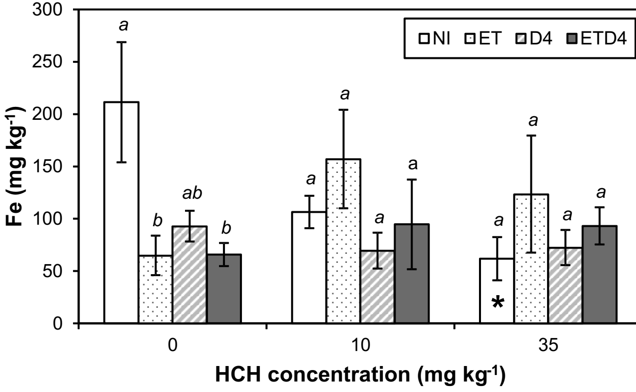

Figure 6.2. Fe concentration (mean ± SE) in shoots of inoculated and non-inoculated *Cytisus striatus* plants (n = 4). Bars with different letters in each contamination level indicate significant differences ($p < 0.05$). Bars with asterisks indicate significant differences compared to the same inoculation treatment at 0 HCH mg kg^{-1} ($p < 0.05$).

Concentrations of α- and β-HCH isomers (extracted with hexane:acetone) before plant growth were 8.3 and 28.2 mg α-HCH kg^{-1}, and 2.4 and 6.9 mg β-HCH kg^{-1}, in the 10 and 35 mg kg^{-1} HCH contamination levels, respectively. At the end of the experiment, the remaining concentration of α-HCH in the substrates contaminated with 10 mg kg^{-1} total HCH were extremely low, between 0.3 and 0.4 mg kg^{-1} (Figure 6.4a). There were no significant differences in this treatment between inoculants, or between unplanted and planted substrates (mean α-HCH concentrations were 0.32 ± 0.02 and 0.34 ± 0.02, respectively). Dissipation of β-HCH was less pronounced (approximately 45 %) and final concentrations of this isomer varied between 1.5 and 2.0 mg kg^{-1} (Figures 6.4a and 6.5). As with the α-HCH isomer, the bacterial inocula did not significantly affect the final concentration of β-HCH. However, dissipation of β-HCH was enhanced in the presence of *C. striatus*: the mean concentration of this isomer was significantly lower in planted substrates (1.53 ± 0.04 mg kg^{-1} β-HCH) compared to unplanted substrates (1.79 ± 0.06 mg kg^{-1} β-HCH) ($p < 0.05$; Figure 6.4a). Beta-HCH dissipation attributed to plant activity was about 14 % of the initial concentration, compared to less than 1

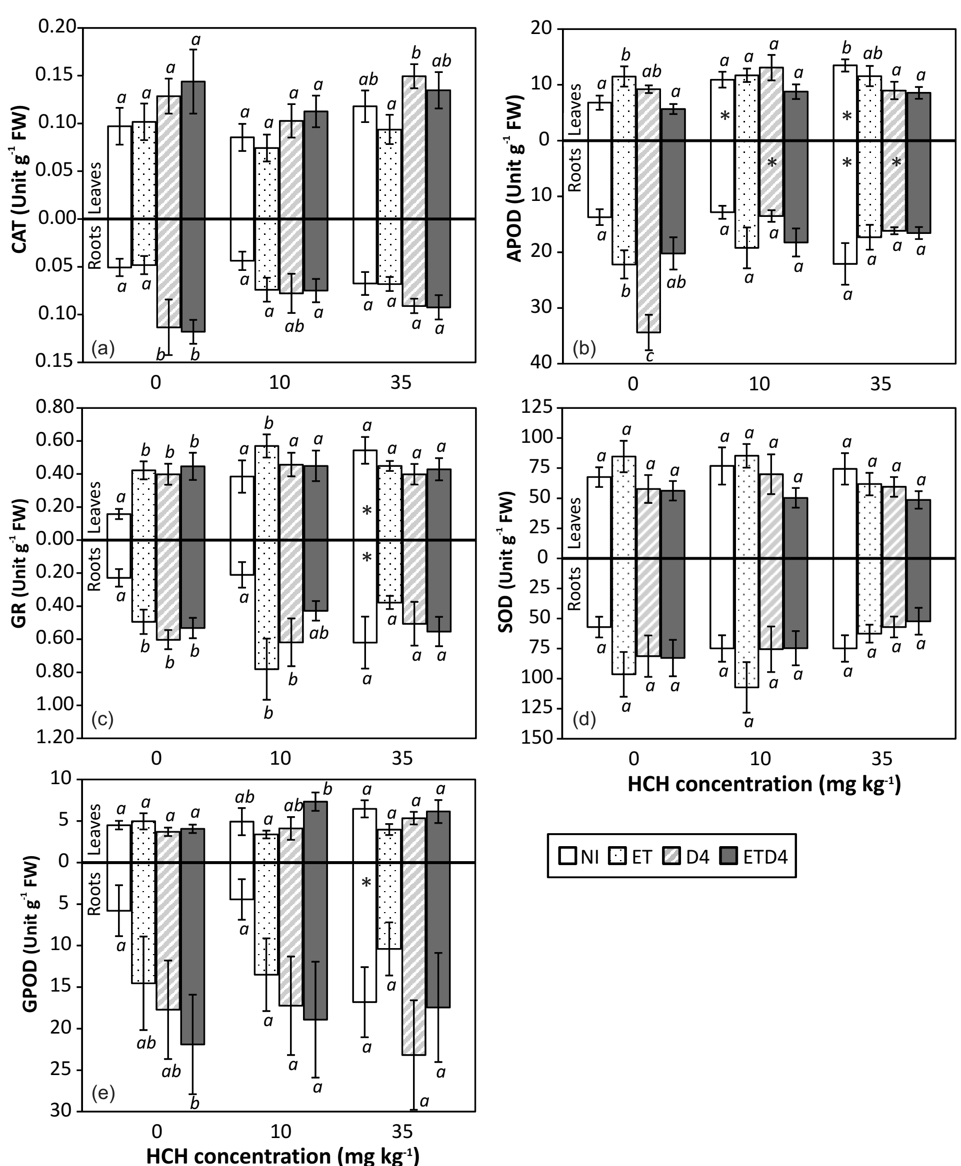

Figure 6.3. Antioxidative enzyme capacities (mean ± SE) in leaves and roots of inoculated and non-inoculated *Cytisus striatus* plants (n = 10). Bars with different letters in each contamination level indicate significant differences ($p < 0.05$). Bars with asterisks indicate significant differences compared to the same inoculation treatment at 0 HCH mg kg^{-1} ($p < 0.05$).

% in the case of α-HCH (Figure 6.5).

At the higher contamination level (35 mg kg^{-1}), HCH dissipation was again more pronounced for α-HCH than β-HCH (93 % dissipation compared to 40 %; Figure 6.5). As observed in the substrates contaminated with 10 mg HCH kg^{-1}, there was no significant effect of bacterial inoculants on HCH dissipation, but at

this higher concentration the positive effect of the plant in the reduction of HCH isomers was more evident (Figures 6.4b and 6.5). At the end of the experiment, the mean concentration of α-HCH in planted substrates (2.00 ± 0.13 mg kg^{-1}) was approximately half that detected in unplanted substrates (4.15 ± 0.42 mg kg^{-1}) (p < 0.05). This effect of the plant was also observed in the case of the β isomer: the final concentration of β-HCH in planted substrates (4.87 ± 0.10 mg kg^{-1}) was significantly lower than in unplanted substrates (5.63 ± 0.11 mg kg^{-1}) (p < 0.05). Once again, β-HCH dissipation attributed to plant activity was higher (13 %) than in the case of α-HCH (7 %) (Figure 6.5).

The mean concentration of total HCH in plants was 17 mg kg^{-1} in shoots and 46 mg kg^{-1} in root tissues, irrespective of the initial HCH concentration in the substrate. Plants inoculated with D4 tended to accumulate higher concentrations of HCH, although in the majority of cases this difference was not statistically significant (Table 6.2). However, HCH accumulated in plant tissues accounted for a minimal proportion of total HCH, between 1.2 and 3.4 % (Figure 6.5). Plant accumulation of HCH was isomer-dependent. In the case of root tissues, α-HCH was the dominant isomer (58 % of the total HCH), although β-HCH also represented an important percentage (42 % of the total HCH). In contrast, β-HCH was the domi-

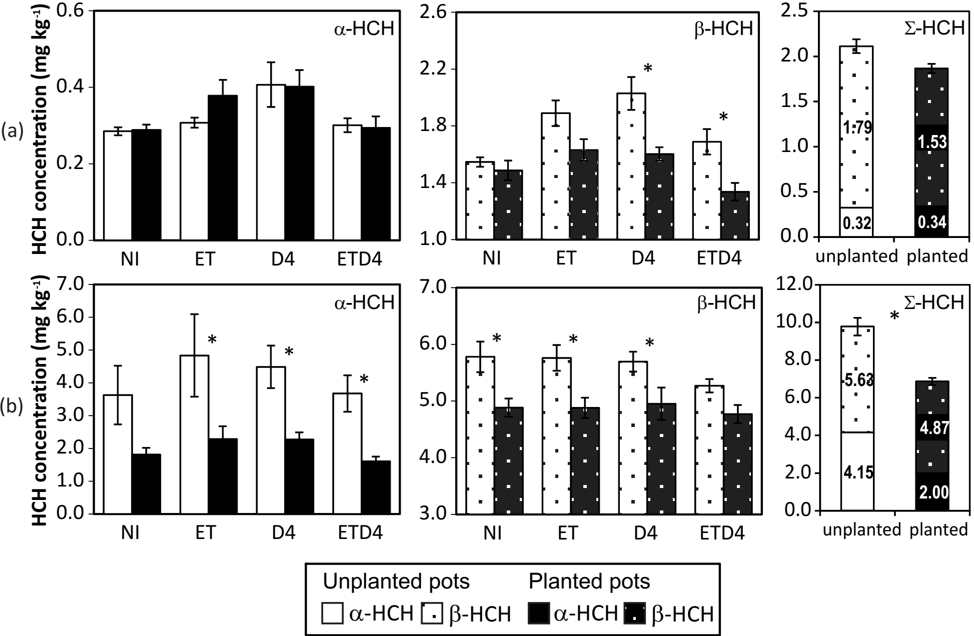

Figure 6.4. α-, β- and total HCH concentrations (mean ± SE) in substrates of planted (n = 6) and unplanted (n = 4) pots with an initial concentration of (a) 10 mg kg^{-1} and (b) 35 mg kg^{-1}. Asterisks indicate significant differences between planted and unplanted pots (p < 0.05).

nant isomer detected in the aerial plant tissues, representing 80 % of the total HCH concentration. HCH was also detected in the aerial biomass, but not in the roots, of plants growing in uncontaminated substrates (approximately 3 mg kg^{-1} β-HCH was detected in the shoot tissues of control plants).

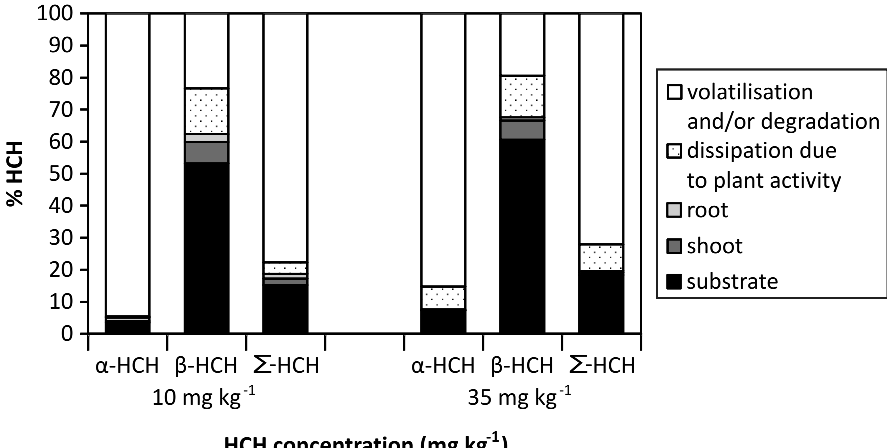

Figure 6.5. Mass balance of HCH for 10 mg kg^{-1} and 35 mg kg^{-1} HCH treatments, taking into account the remaining substrate HCH concentration, HCH concentration in plant tissues, HCH dissipation due to either, plant activity or volatilization and/or degradation. Balances are presented for total-HCH, α-HCH and β-HCH.

6.4 Discussion

Hexachlorocyclohexane was clearly phytotoxic to *C. striatus*, exposure led to a reduction in biomass production and induced oxidative-stress. In addition, there was some indication that HCH exposure provoked nutrient imbalance: Fe contents of shoot tissues were significantly reduced with increasing HCH concentration in substrates. The phytotoxicity of HCH has been observed previously by other authors (Abhilash and Singh, 2010a; 2010b; Calvelo Pereira *et al.,* 2010). However, inoculation of *C.striatus* with both bacterial strains *Rhodococcus erythropolis* ET54b and *Sphingomonas* sp. D4 in combination led to an improvement in plant development and tolerance, reflected through an increase in biomass production and a reduced level of oxidative stress in substrates contaminated with HCH. The antioxidative enzyme activities (SOD and APOD) were significantly reduced in HCH-exposed plants that were inoculated with both bacterial strains. These two bacterial inoculants in combination seem to represent a mutualistic association with high promise for developing phytoremediation strategies aimed at the clean-up of HCH-contaminated sites. Aiding plant establishment and growth in these unfavourable growth conditions will have a direct bearing on the efficiency of the

phytoremediation strategy employed. For example, Weyens *et al.* (2010) have shown that inoculation of poplar cuttings with the bacterial strain *Pseudomonas putida* W619-TCE results in a higher plant biomass and a reduced TCE phytotoxicity. Huang *et al.* (2004) have obtained similar results in which inoculated grass species grow better on creosote-contaminated soils.

Table 6.2. Concentrations of α-and β-HCH (mg kg^{-1} ± SE) in root and shoot tissues of *Cytisus striatus* plants (n = 4). For each tissue and within each column, different letters indicate significant differences ($p < 0.05$).

		10 mg kg^{-1}			**35 mg kg^{-1}**		
		α-HCH	**β-HCH**	**∑-HCH**	**α-HCH**	**β-HCH**	**∑-HCH**
Shoot	**NI**	2.8 ± 0.3 *a*	12.6 ± 1.7 *a*	15.4 ± 2.0 *a*	3.2 ± 0.2 *a*	12.2 ± 1.3 *a*	15.4 ± 1.2 *a*
	ET	3.1 ± 0.1 *a*	11.9 ± 1.5 *ab*	15.0 ± 1.5 *a*	3.1 ± 0.3 *ab*	12.5 ± 1.4 *a*	15.6 ± 1.7 *a*
	D4	4.2 ± 0.6 *ab*	14.2 ± 1.9 *b*	18.4 ± 2.2 *a*	4.5 ± 1.8 *b*	16.6 ± 1.3 *a*	21.1 ± 2.9 *b*
	ETD4	4.2 ± 0.6 *b*	16.7 ± 1.1 *b*	20.9 ± 1.2 *a*	2.7 ± 0.7 *ab*	13.3 ± 1.2 *a*	16.0 ± 0.6 *ab*
Root	**NI**	26.0 ± 3.5 *a*	20.4 ± 2.3 *a*	46.4 ± 4.8 *a*	23.6 ± 3.2 *a*	18.4 ± 1.5 *a*	41.9 ± 4.4 *a*
	ET	22.8 ± 3.2 *a*	22.7 ± 3.4 *a*	42.3 ± 5.3 *a*	23.8 ± 4.5 *a*	19.4 ± 3.5 *a*	43.2 ± 7.6 *a*
	D4	30.2 ± 2.5 *a*	19.0 ± 1.2 *a*	49.2 ± 3.5 *a*	39.7 ± 7.3 *a*	22.3 ± 2.8 *a*	56.0 ± 9.0 *a*
	ETD4	29.8 ± 4.7 *a*	18.8 ± 0.4 *a*	48.6 ± 4.8 *a*	22.3 ± 3.4 *a*	19.6 ± 1.9 *a*	41.9 ± 4.5 *a*

The highest concentrations of HCH in plant tissues were found in the roots, although the majority of the HCH is likely to be adsorbed on the roots surface. This retention of organic contaminants at the root level has also been demonstrated for other POPs (Gao and Zhu, 2004). The hydrophobic nature of HCH isomers (log K_{ow} 3.7-4.1; Willet *et al.*, 1998) makes root uptake and translocation of these compounds to aerial tissues highly unlikely. The detectable levels of β-HCH observed in the shoots of plants grown in uncontaminated substrates can be explained by the adsorption of volatilized HCH on shoot tissue surfaces. In fact, similar results have also been reported by Abhilash and Singh (2010b) and Calvelo Pereira *et al.* (2008), and also for other contaminants such as pyrene and phenanthrene (Gao and Zhu, 2004). However, the possibility that a certain proportion of HCH in the shoot tissues of HCH-exposed plants was a result of root uptake and translocation cannot be completely ruled out. For example, Dettenmaier *et al.* (2009) have found a ratio between the concentration of tetrachloroethene (log K_{OW} 3.4) in the xylem to that in the solution adjacent to the roots (transpiration stream concentration factor, TSCF) of 0.3. Recent studies have shown that some plants (primarily those belonging to the Cucurbitaceae family) are capable of taking up

significant amounts of POPs, such as DDE (log K_{ow} 6.51), and accumulating them in their tissues despite their high hydrophobicity (Chhikara *et al.,* 2010; White, 2010). Nonetheless, the concentration of HCH in the plant represented at most 3.4 % of total HCH, and clearly demonstrated that plant accumulation of this contaminant does not represent an important route of HCH decontamination. Our results coincide with other authors in that the most viable approach for the remediation of this type of contaminant will be based on rhizoremediation or rhizodegradation techniques (Kidd *et al.*, 2008; Schwitzguébel *et al.*, 2006).

In this study, no correlation was found between HCH concentrations in plant tissues and in substrates. This result contradicts the findings of other authors working with HCH or other POPs. For example, γ-HCH plant concentrations were positively correlated with soil concentrations for the species *Whitania somnifera* and *Sesamum indicum* (Abhilash and Singh, 2010a; 2010b). Similarly, a correlation has also been reported between soil pyrene and phenanthrene concentrations and their accumulation in *Brassica parachnensis* (Gao and Zhu, 2004). The results obtained in this study might be due to the fact that the two HCH concentrations tested did not differ greatly, and hence no significant differences in plant bioaccumulation were observed. On the other hand, the results obtained are not surprising considering that the amount of HCH taken up by roots is very likely a passive phenomenon directly related to the contaminant concentration in the root-zone (Marschner, 1995; Shone and Wood, 1974) and also dependent on the amount of water transpired by the plant. Since HCH isomers present a low water solubility (1.6 and 0.3 mg l^{-1} for α- and β-HCH, respectively; Mackay *et al.*, 1997); the solution in contact with the roots was likely to have been saturated in HCH throughout the duration of the experiment. This implies that HCH entry into the plant due to this transpiration effect should have been similar in both treatments. No relationship was observed between HCH tissue concentrations and plant biomass or enzyme activities. On the other hand a reduction in biomass and increase in antioxidant enzyme activities with increasing HCH concentration in the substrates was observed. These results suggest that it was the concentration of HCH in the substrate and possibly also root adsorbed HCH, and not the internal HCH concentration in the plant tissues, which were the determining factors influencing the plant's level of stress.

The remaining concentration of HCH in the substrates at the end of the experiment was not significantly affected by the bacterial inoculant treatment, and this was the case for both levels of HCH contamination. It was expected to see an

increase in HCH dissipation due to degradation by *Sphingomonas* sp. D4 which is able to grow on α-HCH, γ-HCH or HCH residues as the sole C source (unpublished results) but unfortunately this was not observed. It is possible that this inoculant did not survive the full duration of the experiment. However, the significant differences observed between inoculant treatments on the growth of *C. striatus* and tolerance to HCH indicate that the inoculants were active, at least for part of the growth period. Numerous studies have demonstrated an enhanced dissipation and/or mineralisation of POPs in the presence of plants (Chekol *et al.*, 2002; Shaw and Burns, 2005; White, 2001). Plants can improve the physical and chemical properties of contaminated soils, their root system can help spread bacteria through the soil and increase contact between microbes associated with the roots and the contaminants, and finally, root exudation can increase contaminant bioavailability and/or stimulate degrading-bacterial populations. In this study, dissipation of α- and β-HCH was enhanced in the presence of *C. striatus*. This plant-induced effect was more pronounced at the higher level of HCH contamination and for α-HCH. These results confirm the recalcitrance of the β-HCH isomer which has been demonstrated previously (Kidd *et al.*, 2008; Singh, 2003). Kidd *et al.* (2008) have also observed a higher dissipation of α-HCH (but not β-HCH) in the rhizosphere of this plant species. The differences between these two studies could be due to the different growth substrates used: contaminated perlite/sand mixture compared to spiked soils. In this study, the lack of organic matter in the substrate was likely to have led to a higher availability of HCH and as a result, a higher dissipation. There is also no guarantee that the positive effects of both bacterial strains, when inoculated in combination, will also occur in soil conditions. The exact mechanisms by which these two bacterial strains in combination reduce HCH phytotoxicity for the plant are unclear. Since ET54b is a biosurfactant-producer, it is possible that this strain increases HCH availability, which in turn is then more susceptible to degradation by D4. Although differences in HCH dissipation were not significant, there was a slight tendency to an increased dissipation of HCH in this inoculant treatment. This effect may be more evident in soil grown plants where adsorption by organic matter is likely to limit the bioavailability of this contaminant. Furthermore, root exudates from *C. striatus* could present dehalogenase activity or could also improve pollutant bioavailability through the exudation of surfactant compounds (Wenzel *et al.*, 1999). Clearly, it will be necessary to test the plant growth promotion and the protective effect of this inoculant combination (D4 and ET54b) in plants grown in HCH-contaminated soils. The

effects of these inocula on plant development and stress and HCH dissipation, the influence of soil type and properties (such as organic matter content, clay minerals) on HCH availability, as well as the survival of inoculated strains, will need to be evaluated.

In conclusion, substrates planted with *C. striatus* showed a higher dissipation of HCH isomers (including the more recalcitrant β-HCH isomer). Inoculation of this plant species with two bacterial strains in combination (*Rhodococcus erythropolis* ET54b and *Sphingomonas* sp. D4) led to an increase in shoot and root biomass, and protected the plants against the toxic effects of the contaminant. Inoculating *C. striatus* with this combination of bacterial strains could therefore be a promising approach for the remediation of HCH-contaminated sites.

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Chapter 7

Phytoremediation of hexachlorocyclohexane (HCH)-contaminated soils using *Cytisus striatus* and bacterial inoculants in soils with distinct organic matter content

ABSTRACT

The tolerant plant *Cytisus striatus* (Hill) Rothm. has been shown to enhance the dissipation of the organochlorine hexachlorocyclohexane (HCH) which was widely used as an insecticide. In a previous pot experiment with inert growth substrates microbial inoculants were shown to improve plant growth after exposure to HCH. The aim of this study was to test the performance of *Cytisus* in association with different microbial inoculant treatments, and their effects on HCH dissipation, under soil conditions. Soil samples were collected from the A and B horizon (A and B soil) and contaminated with 0 or 65 mg HCH kg^{-1}. The soils mainly differed in their organic matter content. Plants were either not inoculated (NI), or inoculated with the endophyte *Rhodococcus erythropolis* ET54b and the HCH-degrader *Sphingomonas* sp. D4 on their own or in combination (ET, D4 and ETD4), and grown for 4 months. Growth of *C. striatus* was poorer on the B soil and HCH exposure reduced growth further. Microbial inoculants did not significantly promote plant growth, however some inoculant treatments tended to improve either root or shoot biomass production and/or reduce the activities of enzymes involved in anti-oxidative defence. Nevertheless, microbial inoculants significantly modified HCH dissipation, although the effects were soil-dependent and the observed results suggest that this was likely to be due to the retention of HCH by organic matter. In both soils, inoculation with both bacterial strains in combination (ETD4) led to an enhanced HCH dissipation compared to non-inoculated plants or other inoculant treatments. Inoculating *C. striatus* with this combination of bacterial strains is a promising approach for the remediation of HCH-contaminated sites but needs to be further studied under realistic field conditions.

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7.1 Introduction

Recent evidence indicates that the phytoremediation of soils contaminated by organic compounds can greatly benefit from plant-bacterial associations. The rhizosphere and plant endosphere host plant growth-promoting bacteria and bacteria showing the capacity to degrade organic contaminants and/or modify their bioavailability (Siciliano *et al.*, 2001; Andria *et al.*, 2009; Weyens *et al.*, 2009a). These bacterial associates can be applied so as to aid plant establishment and growth under stress conditions through the production of phytohormones (such as indoleacetic acid (IAA), cytokinins or auxins) and enzymes involved in the metabolism of growth regulators such as ethylene (1-aminocyclopropane-1-carboxylic acid deaminase, ACCD), or via the release of essential nutrients (N_2-fixers, phosphate-solubilisers, siderophore-producers), or the induction of plant defence mechanisms (Glick *et al.*, 1998; Glick, 2003; Weyens *et al.*, 2009b). Reports of enhanced contaminant remediation have been related to microbial-induced transformation and degradation of organic compounds in the rhizosphere and in the plant interior. At the root-soil interface or rhizosphere, microbial density, diversity and/or metabolic activity is enhanced, catabolic enzymes are induced, contaminants with similar structures to rhizodeposits can be co-metabolised, and the number and activity of contaminant degraders can be selectively increased (Anderson *et al.*, 1993; Siciliano and Germida, 1999; Kuiper *et al.*, 2004; Shaw and Burns, 2005; Dzantor, 2007). Phenolic compounds such as naringen, coumarin or catechin, released by roots of certain plants have been shown to support the growth of rhizospheric PCB -degrading bacteria (Donnelly *et al.*, 1994; Chaudhry *et al.*, 2005). Shifts in microbial activity in the rhizosphere of grasses and legumes led to an increased degradation of petroleum hydrocarbons (Nichols *et al.*, 1997; Miya and Firestone, 2001; Corgié *et al.*, 2003; Muratova *et al.*, 2003; Phillips *et al.*, 2006). Huang *et al.* (2004) attributed a more effective remediation of creosote-contaminated soil to the beneficial effects of PGP rhizobacteria on root proliferation of three perennial grasses. Inoculating the tolerant plant *Withania somnifera* with the lindane-degrading rhizobacteria *Staphyylococcus cohnii* subsp. *urealyticus* led to an enhanced dissipation of this organochlorine and improved plant growth (Abhilash *et al.*, 2011).

More recent studies have proposed the bioaugmentation of phytoremediation with endophytic bacteria; these bacteria reside within living plant tissues without causing substantive harm to the host plant. Degrading endophytes have

been shown to be active once inside the plant, contributing to the detoxification of the pollutant and/or better survival of the plant under toxic conditions (Andria *et al.*, 2009). The *in planta* metabolism of TCE and toluene was strongly improved in plants inoculated with endophytes equipped with the appropriate degradation pathway, resulting in a diminished phytotoxicity and a reduced evapotranspiration of the contaminants to the ambient air (Barac *et al.*, 2004; Taghavi *et al.*, 2005; Weyens *et al.*, 2009c). Endophytic *Enterobacter ludwigii* strains in association with Italian ryegrass and alfalfa were capable of hydrocarbon degradation and efficiently colonised both the rhizosphere and plant interior (Yousaf *et al.*, 2011). Diesel contamination led to a strong reduction in shoot and root biomass, however, inoculation had a significant alleviating effect.

Soil properties such as texture, organic matter content, pH and cation exchange capacity influence the behaviour of pollutants, and are critical factors determining their bioavailability. Organic compounds, and especially hydrophobic compounds (HOCs), are principally retained or sorbed within the soil by organic matter and clay minerals (Müller *et al.*, 2007). In general, contaminant sequestration by soil components leads to a decrease in bioavailability and hinders remediation and/or degradation. Manilal and Alexander (1991) found that the mineralisation of phenantrene was lower in an organic soil and higher in a mineral soil which had a lower organic matter content. Davis and Madsen (1996) concluded that degradation of toluene was affected by soil type, soil organic matter content and inorganic nitrogen availability: toluene degradation in a sandy soil was significantly slower due to a low organic content (0.8 %) compared to sandy loam and clay soils containing high organic contents (4 % and 5.5 %, respectively). The nature and quantity of organic matter are important factors influencing these soil-compound interactions. Chung and Alexander (1998) studied the sequestration of phenanthrene and atrazine in sterilized samples of 16 soils that differed greatly in their physical and chemical properties. In general, the sequestration of both compounds increased with time, however, the rate and extent of this sequestration varied markedly among the soils and also between soils with similar organic matter contents. Rodríguez Garrido (2009) evaluated the retention of hexachlorocyclohexane (HCH) isomers in contrasting soil types and soil colloids. Organic matter was the principal factor determining the fate of HCH, and this affinity for organic matter was isomer-specific. In the latter two studies the authors highlighted the importance of the type and quality of the organic matter in determining contaminant fate. In addition to influencing contaminant bioavailability, the dominating

soil properties also affect the survival and activity of plants and microorganisms (Scherr *et al.*, 2007; Afzal *et al.*, 2011). Studies carried out by Afzal *et al.* (2011) showed that soil type influenced plant growth and also microbial activity, plant colonization and degradation capacity of the alkane-degrading strains *Pseudomonas* sp. ITRI53 and *Pantoea* sp. BTRH79. Sandy soil negatively influenced the expression and abundance of degrading genes, as well as plant colonization, compared to loamy and loamy sandy soils, and this led to a lower hydrocarbon dissipation.

In a previous study, a promising combination of bacterial strains was identified for the rhizoremediation of HCH-contaminated soils together with the tolerant leguminous shrub, *Cytisus striatus* (see chapter 6). This bacterial combination consisted of the endophyte *Rhodococcus erythropolis* ET54b and a soil HCH-degrader *Sphingomonas* sp. D4. The inoculation of *Cytisus striatus* plants showed a beneficial effect on the growth and development of plants growing on HCH-contaminated substrates, but this effect was only seen when plants were inoculated with both bacterial strains in combination. Since the previous study was carried out in inert substrates (perlite/sand mixtures) in this study, the influence of the same bacterial inocula was tested on plants growing in two natural soils spiked with HCH isomers. The soils mainly differed in their organic matter content.

7.2 Materials and methods

Experimental design and inoculation of *Cytisus striatus* plants

The bacterial inoculants (strain ET54b (ET), strain D4 (D4), or strains ET54b and D4 in combination (ETD4)) and inoculation of surface-sterilised seeds of *C. striatus* was carried out as described previously (see chapter 6). Sterile 10 mM $MgSO_4$ was added to control seeds (non-inoculated control, NI).

Soil samples were collected from the A (0-30 cm) and B (30-60 cm) horizon of an alumi-umbric Cambisol in the surroundings of Santiago de Compostela (Galicia, NW Spain) and contaminated with HCH. The soils are hereafter referred to as the A or B soil. Some general physicochemical properties of the soils are given in Table 7.1. They showed typical characteristics of Galician soils: low pH (4.5 -5), low cation exchange capacity (CEC < 5 $cmol_c\ kg^{-1}$) and a sandy loam texture. The main difference was in their organic matter content (4.2 % in soil A compared to < 0.5 % in soil B). The soils were fertilised at rates equivalent to 150 kg P ha^{-1}, 200 kg K ha^{-1} with KH_2PO_4 and 100 kg N ha^{-1} with NH_4NO_3 before the experi-

Table 9.1. Some physicochemical properties of the soils used in this study.

Properties	A soil	B soil
pH_{H2O}	4.6	5.0
pH_{KCl}	4.2	4.0
C (%)	4.23	0.39
N (%)	0.25	0.11
C:N	16.9	3.5
CEC ($cmol_c\ kg^{-1}$)	3.47	2.56
Particle size (%)		
sand	68.6	69.3
silt	19.9	23.4
clay	11.6	7.3

CEC, cation exchange capacity

ment. Residues produced during the fabrication of γ-HCH were used to contaminate the soils. The mean composition of the residue was: 77 % α-, 16 % β-, 5 % γ- and 2 % δ-HCH (Calvelo-Pereira *et al.*, 2006). The residues were dissolved in acetone and thoroughly mixed with quartz sand; thereafter the acetone was allowed to evaporate. Appropriate amounts of contaminated sand were added to either soil A or B to achieve a HCH concentration of 65 mg kg^{-1}. The same amount of non-contaminated sand was added to aliquots of the same soils to prepare non-contaminated controls (0 HCH). One-week-old seedlings were transferred into pots filled with non-contaminated or HCH-contaminated soil (one seedling per pot).

After transferring to pots, seedlings were re-inoculated with the bacterial suspensions as previously (10 ml suspension/pot). Ten ml of sterile 10 mM $MgSO_4$ was applied to non-inoculated control pots. For each inoculant/HCH condition 12 replicates were prepared. During the assay, plants were watered with deionized water.

Plants were grown for 4 months under greenhouse conditions. At harvest root and shoot fresh weight was determined. A sub-sample of root tissue was immediately frozen in liquid nitrogen and conserved at -80 ºC for enzymatic analyses, and a second sub-sample of fresh root tissue was used for microbial analyses. The remaining plant material was oven-dried at 45 ºC, weighed and ground (for analysis of nutrient and HCH content).

Plant nutritive status and determination of enzymatic activities

The plant nutritive status was determined in four independent replicates per treatment. Shoot tissues were digested in a 2:1 HNO_3:HCl mixture and P, K, Ca, Mg,

Fe, Cu, Mn and Zn measured by ICP-OES (Vista Pro; Varian Inc., Australia). The activity of plant enzymes involved in anti-oxidative defence was determined in 10 independent replicates per treatment as described previously. Frozen root tissue was homogenized in ice-cooled 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 4 % insoluble polyvinylpyrrolidone (1 ml buffer per 100 mg fresh weight). The homogenate was squeezed through a nylon mesh and centrifuged at 20,000 g and 4 ºC for 10 min. Enzymes activities were measured spectrophotometrically in the supernatant at 25 ºC. Glutathione reductase (GR, EC 1.6.4.2), guaiacol peroxidase (GPOD, EC 1.11.1.7) and syringaldazine peroxidase (SPOD, EC 1.11.1.7) activities were determined at 340, 436 and 530 nm, respectively according to Bergmeyer *et al.* (1974). Ascorbate peroxidase (APOD, EC 1.11.1.11) activity was measured at 298 nm according to Gerbling *et al.* (1984). Analysis of superoxide dismutase (SOD, EC 1.15.1.1) activity was based on the inhibition of cytochrome c at 550 nm following the method of McCord and Fridovich (1969). Plant nutritive status and enzymatic activities were not determined for plants inoculated with *R. erythropolis* ET54b growing on B soil due to their low biomass.

Microbiological analyses

Three replicates (combinations of two independent plants) per treatment were used to determine endophytic bacterial densities in root tissues as described earlier (see chapter 5). Bacterial densities were also determined in soils by plating out serial dilutions on 1;10 diluted 869 agar medium (see chapter 2). After 7 days incubation at 28 ºC, colony forming units (CFUs) were counted and calculated per gram of soil or plant tissue. An attempt to recover the inoculated bacterial strains was made. For this, one ml of the 10^{-1} dilutions of plant and soil suspensions were cultivated in two selective liquid media: 284 medium supplemented with 0.8 mM Cd, which is selective for *R. erythropolis* ET54b; and 284 medium (without C sources) supplemented with γ-HCH as a sole C source, which is selective for *Sphingomonas* sp. D4. After 5 days growth, serial dilutions were plated on selective media as described in chapter 6. BOX-PCR profiling was used to confirm the identity of the strains following the methods described in chapter 2.

Determination of HCH concentrations in growth substrates and plant tissues

The concentration of HCH isomers (α-, β-, γ- and δ-HCH) were measured in the initial contaminated soils and at the end of the experiment (six replicates per treat-

ment). For this, 0.5 g of soil were mixed with diatomaceous earth (0.2 g) and quartz sand (14 g), placed in a stainless steel cell (11 ml) and extracted under pressure in an ASE® 200 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) with hexane and a single extraction cycle, at 2000 psi and 100°C for 5 min. All of the extracts obtained were stored at -18°C for posterior analysis by gas chromatography. HCH concentrations were also determined in root and shoot tissues of 4-5 plants per treatment using a laboratory microwave as described in chapter 6. Identification and quantification of the different HCH isomers was carried out by a gas chromatograph (Model 450GC, Varian Inc., Australia) equipped with a CP-8400 autosampler and coupled to an ion-trap mass spectrometer (Model 220MS, Varian Inc., Australia) using the same analytical conditions as described in chapter 6. Plant HCH content was not determined in plants inoculated with *R. erythropolis* ET54b and grown on the B soil due to their low biomass.

Statistical analysis

All datasets were statistically compared using analyses of variance (ANOVA) and Bonferroni post-hoc comparison testing with the SPSS software v19.0. Comparisons between the two HCH concentrations for each inoculation treatment were achieved by the Students t test for independent means. Significant reductions in soil HCH concentrations from the initial HCH concentration were determined using ANOVA followed by the post-hoc Dunnet test. Transformations were applied when necessary to approximate normality and/or homoscedasticity.

7.3 Results

Densities of culturable bacteria and recovery of inocula

B soils showed significantly higher bacterial densities than the more organic A soils ($p < 0.05$). Soil bacterial densities in A soils ranged from $5.6 \pm 0.3 \times 10^6$ to $5.9 \pm 0.7 \times 10^6$ CFUs g^{-1}, while mean bacterial densities in B soils varied between $2.8 \pm 0.6 \times 10^7$ and $1.6 \pm 3.1 \times 10^7$ CFUs g^{-1}. However, the presence of HCH did not affect bacterial densities in either soil, and there were no significant differences between inoculant treatments. Similarly, densities of cultivable root endophytes were unaffected by HCH contamination or the bacterial inoculant. Root endophytes reached densities of $5.9 \pm 1.2 \times 10^4$ and $8.2 \pm 1.2 \times 10^4$ CFUs g^{-1} in plants grown in HCH-contaminated and non-contaminated A soil, respectively. Corresponding densities observed in plants grown in B soils were similar to those

of plants from A soils: 5.2 ± 1.0 x 10^4 and 7.7 ± 3.1 x 10^4 in HCH-contaminated and non-contaminated soil, respectively.

At the end of the growth period, the *R. erythropolis* ET54b strain was re-isolated from both soils and from plant tissues by enrichment cultures in Cd-enriched medium. Similarly, the *Sphingomonas* sp. D4 strain was re-isolated from soils by enrichment cultures with γ-HCH as a sole C source. BOX-PCR profiling was used to confirm the identity of both strains (data not shown).

Plant status: biomass production, enzyme activities and nutrient content

Plant growth was significantly influenced by both the soil type (A or B soil) and the HCH concentration (0 or 65 mg kg^{-1}) (Table 7.2; $p < 0.01$). Interaction effects reached statistical significance in the case of the combined effect of HCH and soil type or inoculant (Table 7.2; $p < 0.001$). Plant biomass was significantly higher in the A soil than the B soil ($p < 0.05$), and this was irrelevant of whether or not the soil was contaminated with HCH (Figure 7.1). However, exposure of non-inoculated plants to HCH was toxic, and the resulting reductions in plant biomass were more accused in those plants growing in the B soil (Figure 7.1). In the A soil, exposure of non-inoculated plants to HCH led to a significant decrease in root biomass ($p < 0.05$) but had no effect on shoot biomass (Figure 7.1a). On the other hand, in the B soil the presence of HCH led to a reduction in both shoot and root biomass of non-inoculated plants (by 2.5- to 2.6-fold, respectively) (Figure 7.1b).

In the absence of HCH, inoculation with either D4 on its own or the ETD4 combination had a negative effect on the growth of plants in both the A and B soil (shoot and root biomass production) (Figures 7.1a and 7.1b). In HCH-contaminated A soil, inoculation with the strains individually (ET or D4 treatment) tended

Table 7.2. Effects of soil type (soil), bacterial inoculum (inoc) and HCH contamination (HCH) on plant biomass (shoot + root weight) (3-way ANOVA).

	Plant biomass		
	df	MS	F
soil	1	31	107.4^{***}
inoc	3	0.7	2.4
HCH	1	3	10.5^{**}
soil * inoc	3	0.3	1.1
soil * HCH	1	4.6	15.9^{***}
inoc * HCH	3	3.6	12.7^{***}
soil * inoc * HCH	3	0.3	1.2

$^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$

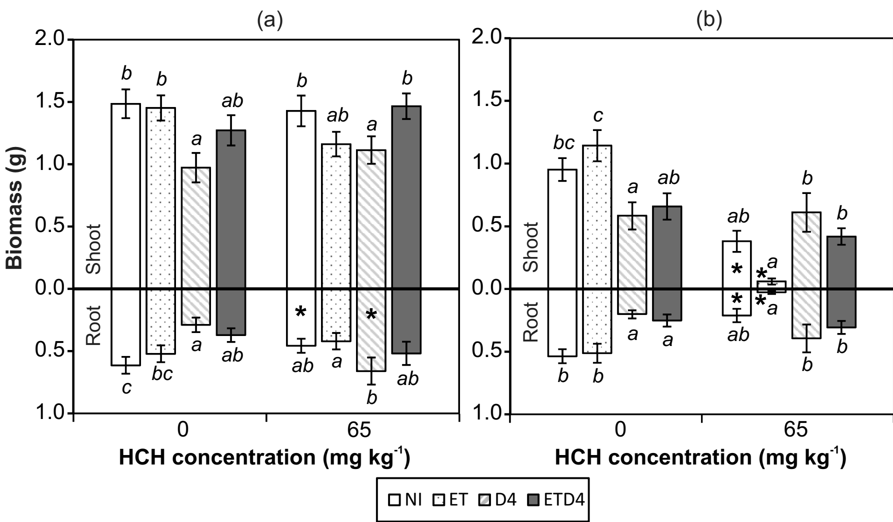

Figure 7.1. Shoot and root biomass (mean ± SE) of *Cytisus striatus* growing on the A soil (a) or B soil (b) (n = 12). Bars with different letters in each contamination level indicate significant differences ($p < 0.05$). Bars with asterisks indicate significant differences compared to the same inoculation treatment at 0 mg HCH kg^{-1} ($p < 0.05$).

to reduce shoot biomass, and this was signifcant in the case of D4 ($p < 0.05$). In contrast, the same inoculant (D4) led to a significant increase in root biomass. In this soil no significant effect was observed in the combination (ETD4) treatment (Figure 7.1a). In HCH-contaminated B soil, the effect of inoculation was drastic in the case of ET54b. In this treatment, survival of plants was dramatically decreased (only 6 plants were alive at the end of the experiment), and the biomass of surviving plants was significantly reduced compared to the other inoculum treatments (Figure 7.1b). In this soil, inoculation with D4 or the combination (ETD4) treatment tended to increase both shoot and root weights although not significantly (Figure 7.1b).

In general, the plant nutritional status was not significantly affected by the microbial inoculation, and in almost all cases nutrient concentrations did not vary between inoculant treatments (Table 7.3). Differences in plant nutrient content seemed to be more influenced by the soil type. Thus, plants grown on the B soil presented a significantly lower content of P, Ca and Fe than plants from the A soil. For instance, P content in non-contaminated and non-inoculated plants decreased from 998 ±70 mg kg^{-1} in the A soil to 630 ± 95 mg kg^{-1} in the B soil. Ca and Fe content from non-contaminated and non-inoculated plants grown in the A soil was 1450 ± 158 mg kg^{-1} and 101 ± 10 mg kg^{-1}, respectively, while in the B soil corre-

sponding values dropped to 481 ± 32 mg kg^{-1} and 85 ± 13 mg kg^{-1}, respectively. The presence of HCH led to a significant reduction in P shoot content (Table 7.3): the mean P concentration in plants grown in the non-contaminated A and B soil was 1040 ± 51 mg kg^{-1} and 586 ± 47 mg kg^{-1}, repestively, while corresponding values in HCH-contaminated soils were 669 ± 35 mg kg^{-1} and 409 ± 33 mg kg^{-1}, respectively.

In general, inoculation of plants grown in uncontaminated substrates resulted in an increase in the activity of stress-related enzymes in both soils, which was often significant in the case of D4 or ETD4 treatments (Figure 7.2). HCH-exposure also induced an enzymatic response in *C. striatus*. In non-inoculated plants, an increment in antioxidative enzyme activities was observed in presence of HCH, with the only exception being APOD in plants grown in the A soil. In the B soil, enzyme activities were increased by 1.1- to 1.7-fold (Figure 7.2). In the A soil, increases were especially evident for GPOD, SPOD and GR, where activities aumented by 1.4- to 1.8-fold (Figure 7.2). Enzyme activities in inoculated plants grown in HCH-contaminated soils tended to be similar to corresponding treatments in non-contaminated soils or lower (Figure 7.2).

Table 7.3. Effects of soil type (soil), bacterial inoculum (inoc) and HCH contamination (HCH) on plant nutritional status (results of six independent 3-way ANOVAs).

		K		P		Ca	
	df	MS	F	MS	F	MS	F
soil	1	27.35	3.15	2.14	79.24***	16.16	81.60***
inoc	3	8.12	0.94	0.07	2.52	0.02	0.11
HCH	1	14.54	1.68	1.09	40.47***	0.25	1.28
soil * inoc	3	5.22	0.6	0.04	1.54	0.18	0.89
soil * HCH	1	10.22	1.18	0.09	3.49	0.51	2.57
inoc * HCH	3	23.82	2.75	0.01	0.31	0.27	1.35
soil * inoc * HCH	2	1.79	0.21	0.2	7.47**	0.01	0.01

		Mg		Mn		Fe	
	df	MS	F	MS	F	MS	F
soil	1	0.28	0.97	0.01	0.01	4667.4	4.62*
inoc	3	0.18	0.61	0.83	0.85	2881.6	2.85
HCH	1	0.05	0.16	1.43	1.47	6502.2	6.44*
soil * inoc	3	0.41	1.42	0.36	0.37	1150.7	1.14
soil * HCH	1	0.17	0.58	0.06	0.06	281.5	0.28
inoc * HCH	3	0.41	1.41	1.91	1.95	3223.8	3.19*
soil * inoc * HCH	2	0.78	2.7	0.94	0.96	120.4	0.12

$^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$

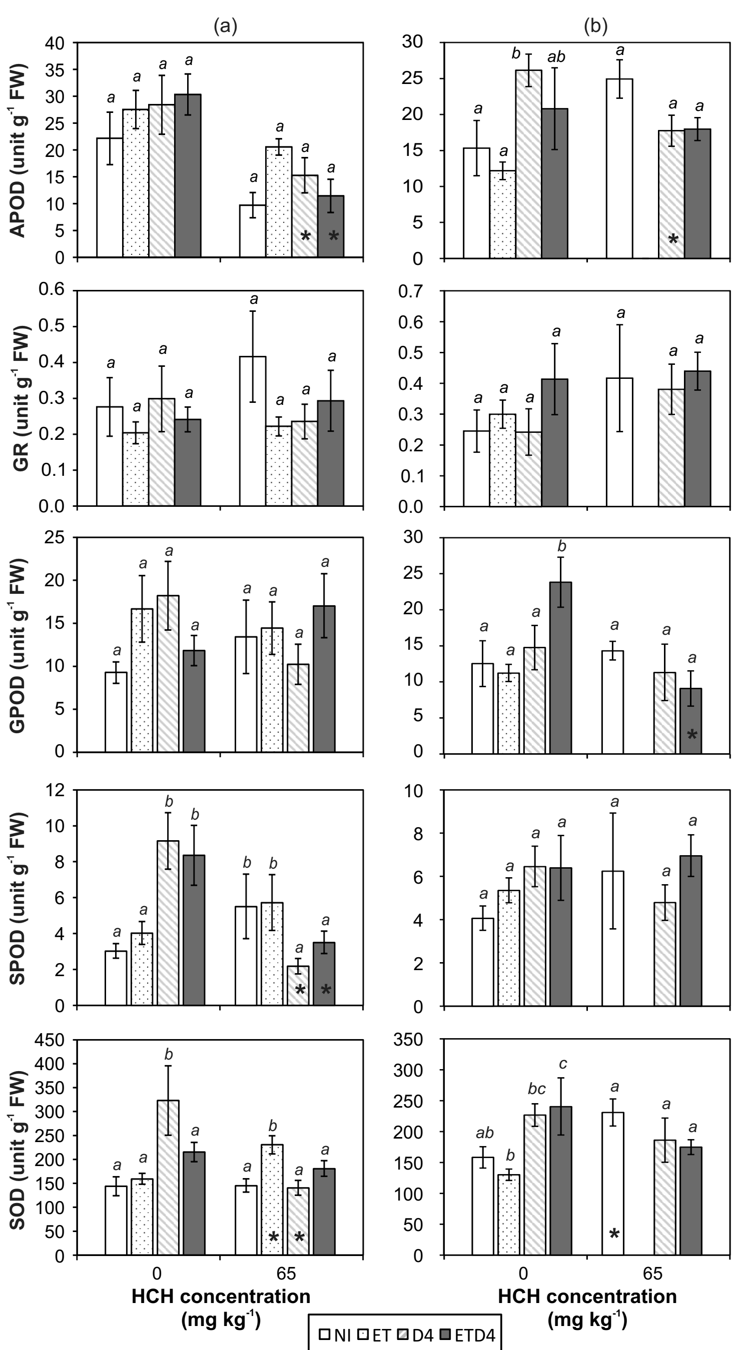

Figure 7.2. Antioxidative enzyme activities (mean ± SE) in leaves and roots of inoculated and non-inoculated *Cytisus striatus* plants (n = 10) growing in the A soil (a) and B soil (b). Bars with different letters in each contamination level indicate significant differences ($p < 0.05$). Bars with asterisks indicate significant differences compared to the same inoculation treatment at 0 mg HCH kg^{-1} ($p < 0.05$). Enzymatic activities were not determined for ET inoculated plants growing on B soil due to their low biomass.

HCH concentrations in soils and plant tissues

Concentrations of total-HCH (sum of the α-, β-, γ- and δ-isomers) before plant growth were 65.8 ± 2.2 and 62.3 ± 5.1 mg HCH kg^{-1} in the A and B soil, respectively. In general after 4 months growth the concentration of total-HCH in the soils was significantly reduced, and this dissipation of HCH was more pronounced in the A soil than the B soil. In the A soil with non-inoculated plants, total-HCH was reduced to a final concentration of 52.4 ± 3.5 mg kg^{-1} (Figure 7.3a). In the B soil, non-inoculated plants also tended to reduce total-HCH (final concentration of 57.2 ± 4.0 mg kg^{-1}) but this was not statistically significant (Figure 7.3b). Inoculation had a significant effect on HCH dissipation and differences were observed between the three inoculant treatments, but these were soil-dependent. Nonetheless, in both soils the greatest reductions in soil HCH concentrations were always obtained with ETD4-inoculated plants (Figure 7.3). In the A soil, plants inoculated with either ET or the ETD4 combination reduced soil HCH to 33 % and 53 % of the initial concentration, respectively, and in both cases HCH dissipation was enhanced compared to non-inoculated plants (Figure 7.3a). Curiously in this soil, no significant decrease in total-HCH was observed in the pots of plants inoculated with the HCH-degrader D4. In contrast, plants in the B soil inoculated with the HCH-degrader D4 or ETD4 reduced HCH to 43 % and 37 % of the initial concentration, respectively (Figure 7.3b). Again remaining concentrations of HCH in these treatments were significantly lower than those in soils where non-inoculated plants were grown (Figure 7.3b).

The isomers γ- and δ-HCH were detected at low concentrations in both soils (around 2.8 and 1.5 mg kg^{-1}, respectively in the A soil and 1.4 and 1.5 mg kg^{-1}, respectively in the B soil). Generally, changes in the concentrations of the two main isomers (α- and β-HCH) followed the same tendencies as observed for total-HCH (Figure 7.3). HCH dissipation was more pronounced for α-HCH than for β-HCH. Non-inoculated plants led to a dissipation of 22 % of α-HCH compared to 16 % of β-HCH in the A soil and to 14 % of α-HCH compared to 0 % of β-HCH in the B soil (Figure 7.3). For both isomers and for both soils the greater reductions were again obtained with ETD4-inoculated plants. In the A soil, α- and β-HCH concentrations in the ETD4 treatment were reduced to 55 % and 50 % of the initial concentrations, respectively. In the B soil, ETD4-inoculated plants led to a dissipation of 48 % and 32 % of α- and β-HCH, respectively (Figure 7.3).

HCH concentrations in plant tissues were highly variable and the observed differences between inocula were not statistically significant (Table 7.4). Plants

grown in the B soil showed significantly higher HCH concentrations than plants from the A soil ($p < 0.05$). Root and shoot HCH concentrations of plants grown in the B soil were in fact 1.7- and 2.1-fold higher than in the A soil, respectively. Mean concentrations found in plants from the A soil (irrelevant of the inoculation treatment) were 42 ± 3 mg kg^{-1} total-HCH in shoots (ranging from 14 to 72 mg HCH kg^{-1}) and 111 ± 19 mg kg^{-1} in roots (ranging from 20 to 302 mg HCH kg^{-1}).

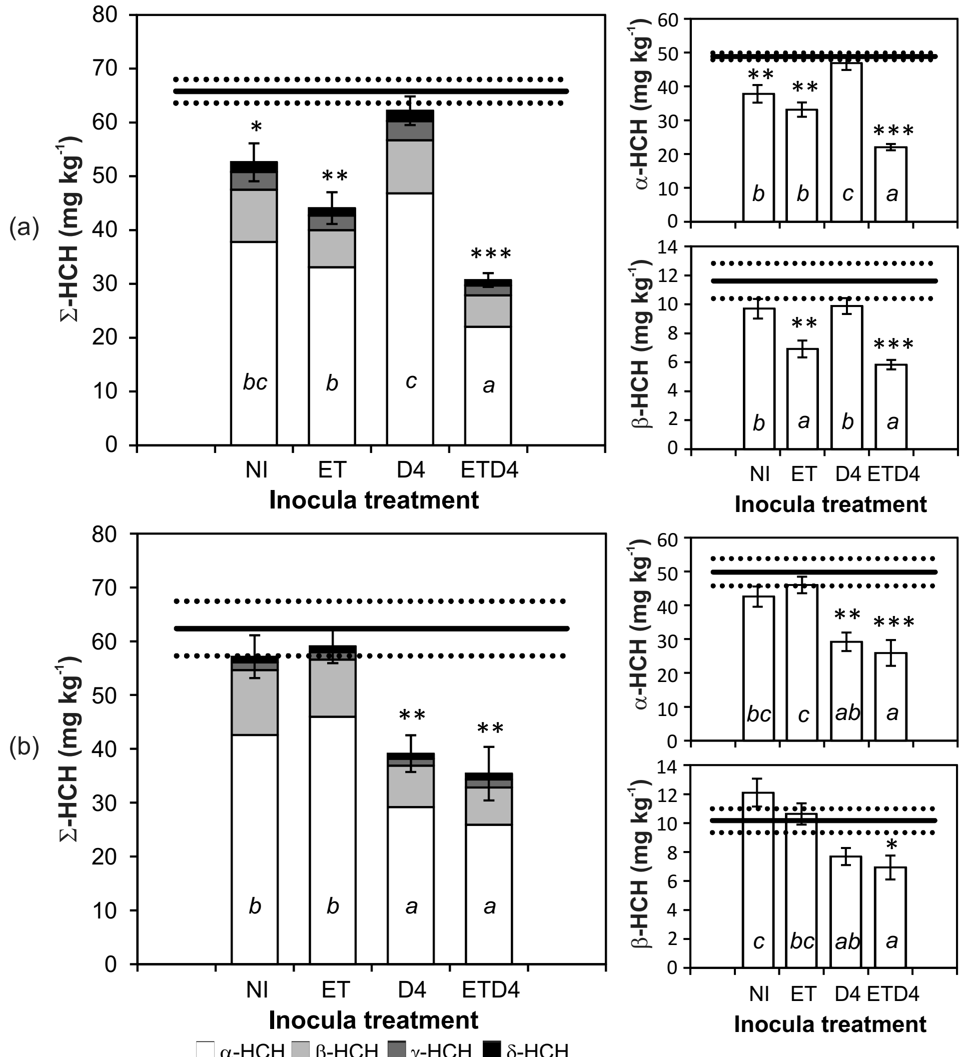

Figure 7.3. Concentrations of ∑-HCH (mean ± SE) and of the two main isomers (α- and β-HCH) in the A soil (a) and B soil (b). Initial HCH concentrations are indicated by a continuous line (± SE (broken lines)). Bars with different letters indicate significant differences ($p < 0.05$). Asterisks indicate significant differences with the initial HCH concentration (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Table 7.4. Concentration of HCH (mean ± SE) in root and shoot tissues of *Cytisus striatus* plants (n = 4-5).

		A soil (mg kg^{-1})				
		α-HCH	**β-HCH**	**γ-HCH**	**δ-HCH**	**Σ-HCH**
Shoot	**NI**	15.9 ± 1.8	21.4 ± 2.2	3.3 ± 0.4	2.7 ± 0.4	43.3 ± 4.2
	ET	23.3 ± 3.7	20.2 ± 3.0	3.9 ± 0.4	2.7 ± 0.3	50.1 ± 7.4
	D4	20.2 ± 1.4	16.3 ± 1.1	4.1 ± 0.3	2.8 ± 0.2	43.4 ± 3.0
	ETD4	16.9 ± 4.9	12.1 ± 3.0	2.6 ± 0.6	1.6 ± 0.4	33.2 ± 8.7
Root	**NI**	91.4 ± 21.1	16.3 ± 2.9	8.8 ± 1.8	4.1 ± 0.7	120.5 ± 26.3
	ET	79.7 ± 21.3	13.3 ± 3.0	6.9 ± 1.6	3.3 ± 0.7	103.3 ± 26.4
	D4	135.9 ± 42.0	24.5 ± 6.8	12.4 ± 3.5	6.2 ± 1.6	179.0 ± 53.4
	ETD4	31.5 ± 11.5	7.6 ± 2.1	3.2 ± 1.1	2.0 ± 0.6	44.4 ± 15.0
		B soil (mg kg^{-1})				
		α-HCH	**β-HCH**	**γ-HCH**	**δ-HCH**	**Σ-HCH**
Shoot	**NI**	19.3 ± 5.1	28.1 ± 2.3	9.4 ± 2.1	13.9 ± 1.8	70.8 ± 10.3
	ET[a]					
	D4	28.0 ± 3.6	28.0 ± 2.0	10.6 ± 1.1	9.8 ± 1.0	76.5 ± 4.8
	ETD4	17.8 ± 4.8	19.5 ± 4.3	6.9 ± 1.7	6.3 ± 1.4	50.5 ± 11.8
Root	**NI**	186.4 ± 50.2	37.7 ± 4.3	42.9 ± 10.7	32.1 ± 4.1	299.0 ± 68.1
	ET[a]					
	D4	216.3 ± 53.0	35.3 ± 8.2	31.8 ± 9.0	16.0 ± 4.0	299.3 ± 73.7
	ETD4	171.2 ± 37.6	29.5 ± 2.7	23.7 ± 3.6	12.5 ± 1.1	237.0 ± 44.4

[a] Plant HCH content was not determined for this soil in ET treatment due to the low biomass of the plants

In the B soil, mean total-HCH concentrations in shoots tissues were 70 ± 4 mg HCH kg^{-1} (ranging from 48 to 96 mg HCH kg^{-1}) and in roots 238 ± 25 mg HCH kg^{-1} (ranging from 108 to 494 mg HCH kg^{-1}). HCH concentrations in roots tissues were 2.7- and 3.4-fold higher than in shoot tissues in A and B soils, respectively. Furthermore, α-HCH was the dominant isomer detected in root tissues (75 % and 66 % of total HCH in A and B soil, respectively). While, this percentage tended to decrease in the case of shoot tissues, principally due to the relative increase in β-HCH (Table 7.4). In shoot tissues α-HCH represented 45 % and 31 % of total HCH and β-HCH 41 % and 38 % of total HCH, in A and B soil, respectively. Plants grown in non-contaminated soils consistently showed HCH levels below the detection limits in both root and shoot tissues.

7.4 Discussion

Hexachlorocyclohexane has been shown to be phytotoxic to the growth of a diverse array of plant species, including monocotyledons and dicotyledons (Calvelo Pereira *et al.*, 2010). Nonetheless, several plant species have been identified as potentially useful candidates for the phytoremediation of HCH-contaminated soils

(Kidd *et al.*, 2008; Abhilash *et al.*, 2009; Abhilash and Singh, 2010a; 2010b; Álvarez *et al.*, 2012). Among these is the leguminous shrub *Cytisus striatus* which has been shown to grow spontaneously on HCH-contaminated sites (Calvelo-Pereira *et al.*, 2006), and was proposed by Kidd *et al.* (2008) as a suitable candidate for application in rhizoremediation strategies. HCH dissipation has been shown to be enhanced in the rhizosphere of this plant species. Although *C. striatus* can tolerate the presence of HCH in its growth substrate, exposure to this contaminant can have a toxic effect on plant performance. In this study, HCH exposure led to a reduction in the plant's biomass production, provoked a nutrient imbalance (particularly of P) and induced oxidative-stress. This confirms the results obtained in previous studies where the same species was grown on inert substrates and soils spiked with different concentrations of HCH (chapter 6; Kidd *et al.*, 2008).

The phytotoxic effect of HCH was more accused in the B soil, where plants showed a reduced growth and poorer nutritive status. The higher phytotoxic effect of HCH in this soil could be a result of two main factors. Firstly, the B soil is a sandy soil with a low content of C, N and other plant nutrients and in general a poorer soil for plant growth. In accordance, plants grown on the non-contaminated B soil presented a lower biomass and poorer nutritive status than those of the A soil. Since plant growth conditions were clearly not optimum the phytotoxic effects of HCH were even more pronounced. A second explanation could be related to the bioavailability of HCH, which is presumably higher in the B soil than in the A soil due to its lower organic matter content. Hydrophobic organic compounds are well known to associate with the organic carbon fraction of soils (Alexander, 2000; Reid *et al.*, 2000). Rodríguez Garrido *et al.* (2010) found a strong negative influence of soil organic matter content on the degradation of HCH. A higher HCH bioavailibility could also explain the higher HCH content found in the shoot and root tissues of plants grown in the B soil than those in the A soil. Optimal plant growth is known to be a critical factor that directly affects phytoremediation processes, influencing plant performance, bacterial colonization and rhizodegradation efficiency (Wenzel, 2009; Afzal *et al.*, 2011).

There was a drastic reduction in plant biomass and plant survival in ET-inoculated plants grown in the HCH-contaminated B soil. This negative effect of *R. erythropolis* ET54b on plant survival was not observed in either the A soil or the non-contaminated B soil. These results suggest that this bacterial strain had a stronger effect on the behaviour of this contaminant in the B soil. Since ET54b is a biosurfactant-producer it is possible that this strain increases the soil bioavailable

fraction of HCH, and at the same time its toxicity to the plant. These results reinforce the important influence of soil properties on phytoremediation processes, and the fact that the development of a phytoremediation strategy has to be site-specific.

In the previous perlite assay (chapter 6), inoculation of *C. stiatus* with the two bacterial strains *R. erythropolis* ET54b and *Sphingomonas* sp. D4 in combination reduced HCH phytotoxicity and improved plant growth. In contrast, in this study the ETD4 bacterial combination did not have a clear plant growth promoting effect. However, plant root biomass tended to increase in ETD4-inoculated soils in the B soil where plant growth was more severely affected, suggesting that this combination aided plant growth on this soil to a certain extent. It is possible that this effect would have become more evident with time. The D4 inoculant also improved plant growth under HCH exposure in this soil. In some cases (depending on the soil and inoculants) inoculated plants also tended to present lower activities of antioxidative enzymes, again suggesting that the bacterial inoculants can have a protective effect on plant growth in the presence of HCH.

In this study there was a clear microbial-induced enhancement in HCH dissipation, an effect which was not observed in the previous perlite assay. Inoculation with the combination of both strains led to a significant reduction in HCH concentration: up to 53 % in the A soil and 43 % in the B soil. Furthermore, and at least in the A soil, this *Cytisus*-ETD4 association induced a significantly higher HCH dissipation than in any of the other treatments (NI, ET, or D4). A possible explanation for which the combination treatment enhanced HCH dissipation in the soil experiment could be related to the phenotypical characteristics of the two strains. As is the case for other organic contaminants, the availability of HCH is considered a critical factor affecting its degradation in the soil. The capacity of the ET54b strain to produce biosurfactants could lead to an increase in HCH bioavailability, which would also make it more available for microbial degradation by the HCH-degrader D4. In the B soil, the ETD4 treatment also led to an important decrease in the soil HCH concentration but in this case HCH dissipation was similar to that observed in the D4 treatment. Degradation of HCH by the D4 bacterial strain in this soil was presumably less limited by the contaminant availability due to its low organic matter content (and lower retention), thus explaining why both bacterial inoculant treatments (ETD4 and D4) led to a similar decrease in the soil HCH content. Biosurfactants produced by *Pseudomonas aeruginosa* WH-2 have been shown to increase the partitioning of HCH-isomers (Sharma, 2009). Similar-

ly, the use of biosurfactants produced by *P. aeruginosa* UG2 effectively mobilized pyrene from soil (Lafrance and Lapointe, 1998). Zhang *et al.* (1997) revealed that biosurfactants increased the solubility and enhanced the rate of phenanthrene biodegradation. The degradation of HCH isomers by *Sphingomonas* sp. NM05 has also been enhanced in the presence of biosurfactants in both liquid medium and soil slurry systems (Manickam *et al.*, 2012). Afzal *et al.* (2012) also found higher hydrocarbon degradation when Italian ryegrass was inoculated with a combination of three alkane-degrading strains *Pantoea* sp. ITSI10, *Pantoea* sp. BTRH79 and *Pseudomonas* sp. MixRI75 compared to when the bacterial strains were inoculated individually.

In conclusion, the inoculation of *Cytisus striatus* with the combination of the two strains, the endophyte *Rhodococcus erythropolis* ET54b and the HCH-degrader *Sphingomonas* sp. D4, resulted in an enhanced HCH dissipation in two soils (differing in their organic matter content) compared to the non-inoculated treatment. The combination of these two selected strains show a promising future for the phytoremediation of HCH-contaminated soils, although soil properties such as organic matter content can influence their efficiency and this should to be taken in account before they are applied. The successful implementation of these plant-microbial systems in rhizoremediation techniques will depend on the ability of the inoculated bacterial strain(s) to establish themselves within the indigenous microbial community. In this study, the inoculated strains were recovered at the end of the experiment but their survival and competitive ability will need to be evaluated under realistic field conditions.

7.5 References

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Final synthesis and conclusions

The Ni hyperaccumulator *Alyssum serpyllifolium* subsp. *lusitanicum* hosts higher proportions of Ni-resistant bacteria in the rhizosphere compared to the metal-excluder *Dactylis glomerata*, but this difference was only significant in the case of the Portuguese populations. Furthermore, in these populations a significant positive correlation between densities of Ni-tolerant bacteria and concentrations of water-soluble Ni in soils was observed.

A collection of rhizobacteria (346 isolates) associated with three Iberian subspecies of *A. serpyllifolium*, two Ni-hyperaccumulating subsp. (subsp. *lusitanicum* and subsp. *malacitanum*) and a closely related non-hyperaccumulator (subsp. *serpyllifolium*) was established. The characterisation of these rhizobacterial strains allowed the identification of several potential plant-growth promoting isolates. The most common plant-growth promoting traits detected were the secretion of organic acids and production of IAA (37–39% of isolates), followed by siderophore production (24% of isolates).

Amongst the isolates, Ni resistance was only observed in strains originating from ultramafic soils. Additionally, the screening of bacterial strains to identify Ni-mobilising/-immobilising isolates permitted the selection of potentially useful strains for phytoextraction/phytostabilisation processes. The majority of isolates that significantly change soil Ni extractability are members of the phylum *Actinobacteria*, and most of them are affiliated with the genus *Arthrobacter*.

The activity of selected bacterial strains (associated with the Ni-hyperaccumulator) was shown to influence the weathering of ultramafic rock and the mechanisms involved in this process were isolate-specific. The siderophore-producing strain *Arthrobacter oxydans* SBA82 appeared to preferentially alter Ni-rich ferromagnesium silicates, while the strain *A. nitroguajacolicus* LA44 principally acted on Mn oxides, and this was related to the secretion of oxalate. Moreover, inoculation of *A. serpyllifolium* subsp *malacitanum* with these bacterial strains led to an increase in phytoextracted Ni. This increase was higher in the case of the bacterial inoculum which was able to solubilise Ni associated with Mn oxides, a fraction which increased in the rhizosphere of *Alyssum* and which has previously been associated with Ni bioavailability in these serpentine soils.

In the Pb/Zn-mining area, the three pseudometallophytes studied were identified as metal excluders. Of the three species *Betula celtiberica* tolerated the highest soil metal concentrations and *Festuca rubra* was the most efficient metal excluder. These metal-tolerant plant species hosted a higher density of heterotrophic bacteria and greater proportion of metal-tolerant bacteria in their rhizosphere compared to non-vegetated soil. The establishment of a collection of metal-tolerant rhizobacteria (74 isolates) allowed the identification of potentially useful strains for application in phytoremediation processes. The collection was dominated by members of the phylum *Actinobacteria* and many of them were affiliated with the genus *Streptomyces*. Almost half of the isolates showed tolerance to Cd and Zn (4–5 mM for Cd, and 2.5–25 mM for Zn) and the most common potential plant-growth promoting traits found were the solubilisation of P and the production of biosurfactants (15% and 20% of the isolates, respectively).

Inoculation of contrasting plant species with selected isolates from this collection allowed the identification of useful plant-bacterial associations which could aid plant establishment and growth in metal-contaminated soils. The results of these experiments showed that bacterial-induced effects on plant growth are plant species-specific, and cannot always be related to the plant growth promoting traits of the inoculated strain.

Densities of culturable bacteria associated with *Cytisus striatus* growing at the hexachlorocyclohexane (HCH)-contaminated site were higher in root (rhizoplane and root interior) than in shoot tissues. The majority of isolates presented at least one of the plant growth promoting traits tested, and the most common traits found were IAA (45%) and siderophore (38%) production. The capacity to produce biosurfactants was the rarest characteristic found among this isolate collection. None of the isolated strains harboured the genes coding two key enzymes involved in the elucidated HCH aerobic degradation pathway.

The presence of *C. striatus* enhanced the dissipation of HCH isomers (including the more recalcitrant β-HCH isomer) in perlite/sand substrates spiked with lindane residues. Furthermore, inoculation of this plant species with two bacterial strains in combination (*Rhodococcus erythropolis* ET54b and *Sphingomonas* sp. D4) led to an increase in shoot and root biomass, and protected the plants against the toxic effects of the contaminant. When *C. striatus* was grown in natural soils spiked with lindane residues and differing in their organic matter content, inoculation with the same bacterial strains resulted in an enhanced dissipation of HCH isomers. The efficiency of this inoculant combination depended on the soil.

As a result of this doctoral thesis the following general conclusions can be drawn:

- The isolation and characterisation of bacteria associated with tolerant plants growing spontaneously at contaminated sites is a valuable and necessary means of identifying bacterial strains, as well as plant-bacteria associations, with potential application in phytoremediation processes: aiding plant-growth and survival or influencing contaminant behaviour.
- Rhizobacteria can produce metabolites that influence soil metal extractability, as well as modify soil metal fractionation and increase the weathering of ultramafic rocks.
- Bacterial inoculation of plants can significantly affect:
  - the survival and growth of plants, although the effects cannot always be related to the plant growth promoting characteristics of the isolate, and they seem to be species-specific.
  - the uptake and accumulation of metals by plants, and in consequence the efficiency of phytoextraction processes.
  - the dissipation of HCH isomers and in consequence the rhizoremediation of HCH-contaminated soils.

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