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Phytoextraction of nickel and rhizosphere microbial communities under mono- or multispecies hyperaccumulator plant cover in a serpentine soil

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Abstract. The efficiency of nickel (Ni) phytoextraction by hyperaccumulating *Brassicaceae* was compared in two types of covers, namely, monoculture or mixed culture. The selected species were from the Pindus Mountains (Greece), including *Alyssum murale*, *Noccaea tymphaea*, *Leptoplax emarginata* and *Bornmuellera tymphaea*. After 4 months of culture in mesocosms using ultramafic soil (Ni = 1480 mg kg^{-1}), plant biomass yield and Ni concentrations in shoots and roots were recorded for each of six treatments (mixed-culture cover, four monoculture covers and unplanted soil). Microbial biomass carbon, the size of the cultivable rhizosphere bacterial community and its phenotypic structure (Biolog EcoPlates™), bacterial and fungal genetic structure (SSCP), as well as the potential production of auxin compounds, were also evaluated. Moreover, measurements of various microbial enzymes were performed. The biomass and shoot Ni concentration (albeit not significant) of *B. tymphaea* increased in co-cropping system. A slight acidification of the soil occurred and a strong correlation between pH and the size of the bacterial community was also observed. No significant change in enzyme activity was observed among the cover types, except in the case of arylsulfatase. The phenotypic structure of the bacterial communities and the bacterial and fungal genetic structures appeared to be specific to the type of cover, although the size of the culturable bacterial community did not show variation among treatments. Therefore, on the basis of the bioaccumulation coefficient and the translocation factor, our results showed that *B. tympheae*, and to a lesser extent *N. tympheae*, were the two species with the greatest Ni phytoextraction potential in co-culture systems.

Additional keywords: Biolog™, hyperaccumulating plants, molecular fingerprinting, rhizobacteria.

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Introduction

The term 'hyperaccumulator plant' was first proposed by Jaffré *et al*. (1976) when they observed the accumulation of nickel (Ni) in *Sebertia acuminata*. Such plants are able to accumulate metals in their tissues at concentrations at least 100 times above normal and with values greater than 1000 mg kg^{-1} (Brooks *et al.* 1977; van der Ent *et al.* 2013). For Ni, when concentrations in shoots exceed 10 000 mg kg^{-1}, hyperaccumulator plants are called 'hypernickelophores' (van der Ent *et al.* 2013), thus being excellent candidates for phytomining (Chaney *et al.* 2007). *Brassicaceae* is one of the most represented families among hyperaccumulators and contains hypernickelophores (van der Ent *et al.* 2013). Depending on the metal, some families, and even some plant species, will be more effective for given phytoextraction purposes (Prasad and Freitas 2003; Chaney *et al.* 2007; Tang *et al.* 2012).

Bioremediation techniques based on plants and associated rhizosphere microorganisms offer an environmentally friendly method of cleaning-up metal-contaminated soils (Chaney 1983; Baker *et al.* 1994; Lucisine *et al.* 2014). Among soil microorganisms, some promote Ni-hyperaccumulator plant germination, facilitate the development of root biomass via the production of hormone-like molecules and also promote the resistance of plants to the stress exerted by the pollutant. The

latter is due to the increased exudation of 1-aminocyclopropane-1-carboxylate (ACC) together with a decrease in the synthesis of ethylene (known as a plant response to stress due to the pollutant). This leads to better plant development parallel to favoured bacterial growth, since the ACC is a source of both carbon (C) and nitrogen (N) for the rhizosphere microflora (Glick 2005, 2010; Zhuang *et al*. 2007; Lebeau *et al*. 2008; Ma *et al*. 2011; Cabello-Conejo *et al*. 2014). Plant diversity and composition induce a variety of rhizodeposits (Zak *et al*. 2003; Benizri and Amiaud 2005), thus generating a range of soil bacterial communities and functional microbial groups closely related to the community and diversity of plants (Lavelle *et al*. 1995; Wardle *et al*. 2004; Benizri and Amiaud 2005; Gao *et al*. 2010). A few studies have attempted to relate the association of different plants to the efficiency of metal extraction, with the hypothesis that multi-species vegetation covers promote the development and activity of rhizosphere microorganisms (e.g. rape with alfalfa, Pan *et al*. 2008). Only a few studies have dealt with the effect of the combination of metal-hyperaccumulator plants with other species, such as, for example, *Sedum alfredii* + *Zea mays* (Wei *et al*. 2011), *Sedum alfredii* + *Alocasia macrorrhiza* (Wu *et al*. 2007), *Noccaea caerulescens* + *Lolium perenne* (Jiang *et al*. 2010), *Brassica juncea* + *Brassica chinensis* (Liu *et al*. 2007) and *Noccaea caerulescens* + *Hordeum vulgare* + *Lepidium heterophyllum* (Gove *et al*. 2002). Most of these experiments have shown that co-cropping with non-hyperaccumulator plants enhances the growth of the hyperaccumulator, increases metal extraction, and also often improves the living conditions of less metal-tolerant plants (Epelde *et al*. 2012).

To our knowledge, few studies have evaluated, exclusively, associations of hyperaccumulator plants. Recently, the effect of multi-hyperaccumulator species was tested on the efficiency of Ni phytoextraction through the enhancement of bacterial activity (Lucisine *et al*. 2014). The nature of the hyperaccumulating plant cover affected the size and the genetic structure of bacterial communities in the rhizosphere. The coexistence of multiple plant species improved biomass production by each species, and changed the rhizosphere microorganisms and the abiotic micro-environment, including metal bioavailability in rhizosphere soil. However, the duration of plant cultivation was limited and the number of replicates used did not allow a full understanding of the effects (Lucisine *et al*. 2014).

Therefore, the objective of the present work was to compare, over a period of 120 days under controlled conditions, the cropping of single hyperaccumulator plant species (*Alyssum murale*, *Noccaea tymphaea*, *Leptoplax emarginata* or *Bornmuellera tymphaea*) with co-cropping of all four species. With respect to earlier work (Lucisine *et al.* 2014), we introduced *B. tymphaea* because of its strong Ni-phytoextraction potential (Zhang *et al*. 2014) and because all four species can occur simultaneously in their natural habitat in Greece (Bani *et al*. 2009). The effect of all treatments on the efficiency of Ni extraction from a serpentine soil (i.e. naturally Ni-rich) was studied. The effects on soil physicochemical properties and on changes in microbial community (bacterial abundance, microbial biomass carbon, enzyme activities, and metabolic and genetic structures) colonising the rhizosphere were also evaluated.

Materials and methods

Soil characteristics and experimental design

A 120-day mesocosm study was carried out with soil collected from the top layer (10–40 cm) of a natural forest ultramafic hypermagnesic hypereutric Cambisol (Vosges Mountains, north-eastern France, 48°11′03.7″N, 07°06′42.2″E). This soil is believed to have never hosted any hyperaccumulator species in natural conditions. Immediately after collection, the soil was sieved to <5 mm and stored at 4°C for less than 7 days, before soil physicochemical and microbiological analyses. Soil physicochemical properties were determined by the Soil Analysis Laboratory of INRA (Arras, France). The soil contained 317, 377 and 306 $g \cdot kg^{-1}$ soil clay, silt and sand, respectively, with a pH of 5.49 and an organic matter content of 10.3%. The main soil chemical characteristics were 59.3 $g \cdot kg^{-1}$ organic C and 4.72 $g \cdot kg^{-1}$ N, a C : N ratio of 12.6, a magnesium (Mg) : calcium (Ca) ratio of 8.6 and a phosphorus (P) content of 11 $mg \cdot kg^{-1}$. Developed on a serpentinised harburgite, this soil was naturally rich in Ni and the total Ni content was 1480 $mg \cdot kg^{-1}$.

A quantity of 3 kg of soil (dry weight basis) was placed in each 13 × 24 × 16 cm (W × L × H) mesocosm. The mesocosms were planted with only one of the four plant species each (monospecific cover: *Leptoplax emarginata* (Bois) O.E.Schulz, *Noccaea tymphaea* (Hausskn.) F.K.Mey., *Bornmuellera tymphaea* Hausskn or *Alyssum murale* Waldst. & Kit) or a mixture of the four species (multispecies cover). Seeds were collected in Greece in 2011. *Noccaea* seeds were taken on 19 July 2011 from the Katara Pass (1700 m; 39°47′765″N, 21°13′739″E). *Leptoplax*, *Bornmuellera* and *Alyssum* seeds were taken on 20 July 2011 and came from a site near the village of Trigona (830 m asl; 39°47′223″N, 21°15′869″E; see characteristics of the three species in Bani *et al*. 2009). Control mesocosms were not planted. Total plant density was eight per mesocosm. The experiment had a completely randomised block design, with seven replicates, of the following treatments: *Leptoplax* cover (L), *Noccaea* cover (N), *Alyssum* cover (A), *Bornmuellera* cover (B), a mixed cover of the four species (LNAB) and soil without plants (SWP). Mesocosms were transferred to an environmental growth chamber (photoperiod 16 h, temperature 15°C night and 20°C day, relative humidity 70%, photosynthetic photon flux density 350 $mmol\ m^{-2}\ s^{-1}$) and adjusted to 75% of soil water-holding capacity with water. Mesocosms were kept in the growth chamber for 4 months.

Biotic parameters

Ni concentrations in plant parts

After 4 months of culture, plant roots, stems and leaves were collected, carefully washed with deionised water, oven-dried at 70°C for 72 h and their dry weights were recorded. Subsamples (0.5 g) of dry plant tissue were acid-digested at 95°C in 8 mL of concentrated HNO_3 (69%) and 4 mL of H_2O_2 (30%). The final solutions were filtered (0.45 µm DigiFILTER, SCP science, Canada) and completed up to 25 mL with deionised water. Ni concentration in the solution was measured with inductively coupled plasma–atomic emission spectroscopy (ICP–AES) using aiCAP6300 Duo (ThermoScientific, Waltham, MA, USA). A bioaccumulation coefficient (BAC) was employed to

quantify heavy metal-accumulation efficiency in plants, by comparing the concentration in the plant parts (roots, stems or leaves) and external medium (such as soil), using the following formula: BAC = Cp/Cs, where Cp and Cs are heavy metal concentrations in plant parts (mg kg^{-1}) and in soil, respectively, at the beginning of the experiment (1480 mg kg^{-1}; Zayed *et al.* 1998). Heavy-metal translocation from root to shoot in plants was calculated using the following formula: TF = Cs/Cr, where TF is a translocation factor, and Cs and Cr are metal concentrations (mg kg^{-1}) in the shoot and root, respectively (Huang *et al.* 1997; Tappero *et al.* 2007).

Quantification of total culturable bacteria

At the end of the experiment, a sample (3 g) of the rhizosphere soil, defined as the root-adhering soil from planted mesocosms, and of bulk soil from unplanted mesocosms, was dropped into a flask containing 30 mL phosphate-buffered saline (PBS, 8 g NaCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per litre, pH 7.2). All the flasks were then placed on an orbital shaker (17 rpm) for 30 min. An aliquot of 1 mL per sample was transferred into a sterile test tube previously filled with 9 mL PBS. Microbial suspensions were serially diluted and the dilutions (10^{-2}–10^{-4}) were used for the determination of the number of culturable bacteria as colony-forming units (CFU) by spread-plating them onto 10% tryptone soy agar (TSA, Difco, VWR, West Chester, PA, USA). Three replicates for each dilution were prepared. The agar plates were then incubated in the dark at 27°C for 2 days. The number of bacteria was expressed as log (CFU g^{-1} soil dry weight).

Soil microbial-biomass carbon

The soil microbial-biomass carbon (MB-C) was estimated using the fumigation extraction technique previously described by Vance *et al.* (1987). Extractable C was measured by the automated UV-persulfate oxidation method in a total organic carbon (TOC) analyser (TOC-VSCN equipment, Shimadzu, Kyoto, Japan). The MB-C was determined using the following formula: MB-C = $(C_{\text{fumigated extract}} - C_{\text{unfumigated extract}})$/Kc, where the calculated Kc factor of 0.45 was used to convert extractable C into MB-C (Jenkinson and Ladd 1981).

Determination of auxin-like compounds

The determination of auxin-like compounds from the soil samples was adapted from the method described by Sarwar *et al.* (1992), Wöhler (1997) and Smaill *et al.* (2010). Briefly, 3 g of soil was incubated with 6 mL of phosphate buffer–glucose solution (Na_2HPO_4 0.2 M, pH 7.5, with 1% of D-glucose) and 4 mL of a 1% L-tryptophan solution at 40°C for 48 h, on a shaker table (150 rpm). Then, 2 mL of 5% trichloroacetic acid solution and 1 mL of 0.5 M $CaCl_2$ solution were added. Last, the soil solutions were filtered (Whatman N° 2, Whatman, Dassel, Germany). Then, 0.5 mL of the filtrate was put in a test tube, and to this 1.5 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 37% sulfuric acid) was added. The mixture was allowed to stand for 30 min for colour development. The intensity of the colour developed was measured at 535 nm with a spectrophotometer (80-2088-64, Pharmacia Biotech, Novaspec, USA). The concentration of auxin-like compounds in soil was reported as indole-3-acetic acid (IAA-equivalents, mg kg^{-1} dry soil h^{-1}), using standard IAA solutions.

Microbial enzymes

The activity of 1-aminocyclopropane-1-carboxylate deaminase (ACCd) was determined by the production of α-ketobutyrate from ACC, following the method described by Honma and Shimomura (1978) for measuring ACC deaminase in cell extracts. Briefly, 1 g of sieved soil (<2 mm) was placed in 50 mL of a solution containing 0.1 mol L^{-1} Tris buffer (pH 8.5) and 50 mmol L^{-1} ACC, and was incubated for 24 h at 30°C. After incubation, 1 mL of solution was removed, to which 1.8 mL of 0.56 mol L^{-1} hydrochloric acid was added to inhibit further production of α-ketobutyrate. A volume of 0.3 mL of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 mol L^{-1} hydrochloric acid was added, and the solution was incubated for 15 min at 30°C, after which 2 mL of 2 mol L^{-1} sodium hydroxide was added. The concentration of α-ketobutyrate in the solution was determined by absorbance at 540 nm with a spectrophotometer (80-2088-64, Pharmacia Biotech). The amount of ACCd was reported as μmol α-ketobutyrate g^{-1} dry soil h^{-1}, using standard α-ketobutyrate solutions.

Activities of β-glucosidase (EC 3.2.1.21), arylsulfatase (EC 3.1.6.1), acid phosphatase (EC 3.1.3.2) and urease (EC 3.5.1.5) were determined according to Tabatabai (1982). Spectrophotometric determination of the hydrolysis of fluorescein diacetate (FDA) was used to estimate microbial activity in soil (Nicolardot *et al.* 1982).

Microbial community-level physiological profiles (CLPPs)

Bacterial communities were characterised by their metabolic fingerprints, using Biolog Ecoplate™ microplates (Biolog Inc., Hayward, CA, USA). Biolog® system is an *in vitro* cultivation method used to discriminate heterotrophic microbial communities of environmental samples by comparing C-utilisation profiles (Garland and Mills 1991). The Biolog Ecoplate™ microplate contains 31 of the most useful C sources for soil-community analysis. Microplates were inoculated with 150 mL of the 10^{-2} soil suspension. This dilution was chosen so as to reduce the influence of soilborne C sources and soil particles on the colour development. Plates were incubated in the dark at 28°C. After incubation of the plates for 5 days, the colour development was automatically recorded at 590 nm with a microplate reader (Synergy™ HT, BioTk, Colmar, France). Absorbance values for the wells containing C sources were blanked against the control well. All absorbance values up to 0.25 were set to zero. Overall colour development expressed as average well colour development (AWCD) was calculated as the mean of the blanked absorbance values for all 31 wells. Each absorbance value of a plate was then divided by its AWCD, so as to minimise the influence of the differences in inoculum density among soils (Garland and Mills 1991). Richness (S), Shannon's diversity index (H) and evenness (E) (Magurran 2004) were calculated from data on CLPPs.

Genetic structure of bacterial and fungal communities

DNA was extracted from 0.5 g of soil with the FastDNA®SPIN®Kit (for soil) (BIO 101 Inc., Carlsbad, CA,

USA) in accordance with the manufacturer's instructions. Comparison of the genetic structures of the bacterial and fungal communities colonising the rhizosphere was performed by principal component analysis (PCA) of molecular fingerprintings obtained by single-strand conformation polymorphism (SSCP; Vallance *et al*. 2009; Lucisine *et al*. 2014). Pairs of primers recognising the mitochondrial large-subunit rDNA gene (ML1/ML2) for fungi and the 16S rDNA gene (799f/1115r) for bacteria were used for SSCP (Table 1). DNA was amplified by polymerase chain reaction (PCR) in an Epgradient Mastercycler (Eppendorf, Hamburg, Germany) in a reaction mixture (25 µL final volume) consisting of 1 µL of DNA template (10 ng µL^{-1}), 0.4 mM of each dNTP, 1 ng µL^{-1} of bovine serum albumin (BSA), 0.4 µM of each primer, Pfu Turbo buffer 1× and 0.05 units of Pfu Turbo DNA polymerase (Agilent Technologies). For fungi, the cycling parameters were 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. For bacteria, the cycling conditions were as follows: enzyme activation at 95°C for 2 min, 25 denaturation cycles at 95°C for 45 s, hybridisation at 54°C for 30 s, and an extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were visualised by 2% Tris-borate-EDTA (TBE) agarose gel electrophoresis before SSCP analysis.

Single-strand conformation polymorphism (SSCP) analyses were performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) equipped with four 36-cm-long capillaries. One microlitre of a PCR product was mixed with 18.8 µL formamide Hi-Di (Applied Biosystems) and 0.2 µL standard internal DNA molecular-weight marker Genescan 400 HD ROX (Applied Biosystems). The sample mixture was denatured at 95°C for 5 min and immediately cooled on ice, and then loaded onto the instrument. The non-denaturing polymer consisted of 5.6% POP conformational analysis polymer (Applied Biosystems), 10% glycerol, EDTA buffer 10× (Applied Biosystems) and water. The migration time was set to 2000 s, the voltage at 15 kV and the temperature was 32°C.

Samples were co-migrated with the fluorescent size standard (GeneScan-400 ROX, Life Technologies) to allow comparison of migration profiles among samples. Patterns were aligned with StatFingerprints (version 2.0, http://cran.r-project.org/web/packages/StatFingerprints/index.html) and studied by PCA with StatBox (StatBox version 7 software, Grimmersoft, Paris, France, http://www.statbox.com).

Abiotic parameters

Moisture content in the soil samples was determined by heating subsamples to 105°C until a constant weight was achieved. Ni in soil samples from each mesocosm was extracted with the diethylenetriaminepentaacetic acid (DTPA)– triethanolamine (TEA) solution (0.005 M DTPA, 0.01 M $CaCl_2$, 0.1 M TEA, pH 7.3) according to Lindsay and Norvell (1978) and Ni concentration in solution was measured with an ICP–AES. Soil pH was measured using a pH meter in a soil–water solution mixture (soil : water, ratio 1 : 5).

Statistical analysis

Variance analysis was carried out on all data (one-way ANOVA). Bacterial and fungal profiles obtained by SSCP were analysed using PCA. Biolog Ecoplates™ were submitted to PCA. Mean coordinates of individuals were calculated for the first two principal components (PC1, PC2) and compared by variance analysis (one-way ANOVA). The software used for all statistical analyses was StatBow software (Grimmersoft, Paris, France, http://www.statbox.com).

Results

Effect of co-cropping on plant physiology and Ni extraction

At harvest, shoot biomass was always greater than root biomass, regardless of the monospecific cover type (Fig. 1). The total biomass (shoot and root) of L and N was significantly higher than that of A. The total biomass of the mixed multispecies treatment was not greater than that of any of the monospecies treatments. Shoot biomass of L and A was significantly higher when these species were cultivated alone. However, B showed a higher shoot biomass when grown in mixed cover. Significantly higher values of root biomass were observed for L than for N, A and B.

With regard to plant metal accumulation per pot, A showed a significantly ($P < 0.10$, Kruskal–Wallis) lower total amount of Ni (shoot + root) than did the other cover treatments (data not shown). Moreover, when the amounts of Ni in the shoots were compared, no significant ($P > 0.05$, Wilcoxon) differences in the amount of Ni was found among L, N, B and LNAB treatments (Fig. 2). In contrast, significant differences in the quantity of Ni were noted in the roots. Indeed, plants in L and A had more Ni in the roots than did those in N and B ($P < 0.05$, Wilcoxon). The amount of Ni in the roots of plants in the mixed multispecies cover treatment was intermediate.

Table 1. Pairs of universal primers used in single-strand conformation polymorphism (SSCP) analyses of rhizosphere/soil (SWP) fungal and bacterial communities

Rhizosphere/soil community	Gene	Primer	Primer sequence (5′→3′)	Amplicon size (bp)	Annealing temperature (°C)
Fungi	mt LSU rDNA	ML1[A,B]	6-FAM GTACTTTTGCATAATGGGTCAGC	253	58
		ML2[A,B]	6-FAM TATGTTTCGTAGAAAACCAGC		
Bacteria	16S rDNA	799f[C]	AACMGGATTAGATACCCKG	~350	54
		1115r[A,C]	6-FAM AGGGTTGCGCTCGTTG		

[A]Primers labelled with 6-FAM (6-carboxyfluoresceine).
[B]White *et al*. 1990.
[C]Redford *et al*. 2010.

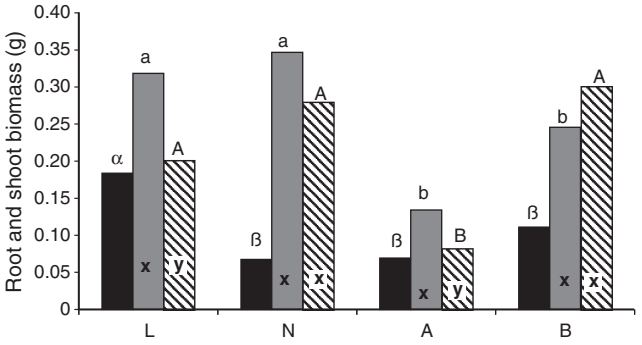

Fig. 1. Root (black bar) and shoot (grey bar) biomass (g) per plant in monospecific cover. Shoot (hatched bar) biomass (g) per plant in soil with multispecific cover. Mean values followed by different Greek (root biomass), lowercase (shoots biomass per plant in monospecies covers) or uppercase (shoot biomass per plant in multispecies covers) letters are significantly different at $P = 0.15$ (Kruskal–Wallis test; $n = 7$). Mean values followed by x or y (shoot biomass per plant in mono- and multispecies covers) are significantly different at $P = 0.15$ (Kruskal–Wallis test; $n = 7$).

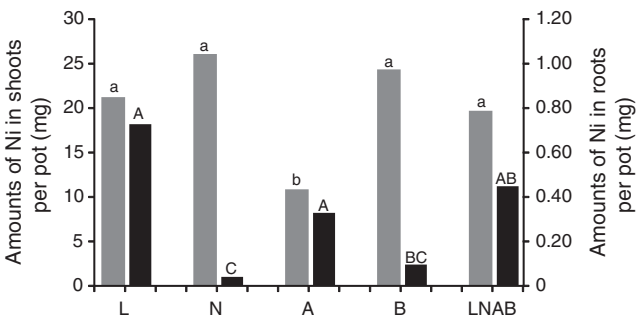

Fig. 2. Amounts of nickel (Ni) phytoextracted in shoots (grey bar) and roots (black bar) per pot (mg). Mean values followed by different lowercase (shoot) or uppercase (root) letters are significantly different at $P = 0.05$ (Wilcoxon test; $n = 7$).

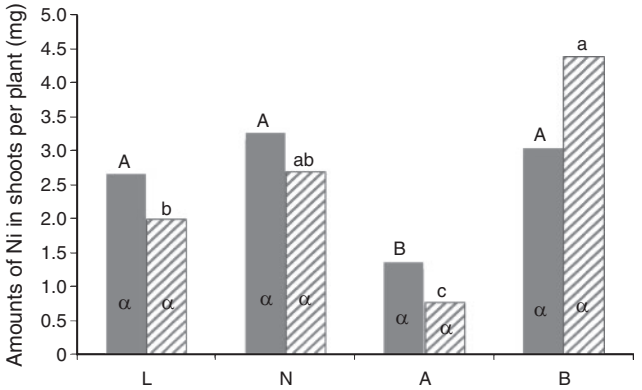

Fig. 3. Amounts (mg) of nickel (Ni) phytoextracted in shoots of monospecific cover (grey bar) and shoots of mixed cover (hatched bar) per plant. Mean values followed by different uppercase (monospecies cover), lowercase (multispecies cover) or Greek (mono- and multispecies covers) letters are significantly different at $P = 0.05$ (Wilcoxon test; $n = 7$).

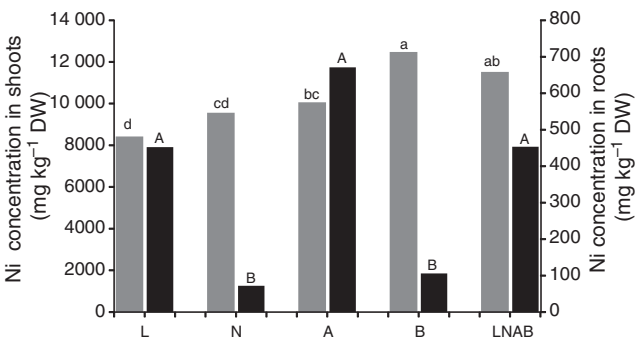

Fig. 4. Nickel (Ni) concentration (mg kg^{-1}) in shoots (grey bar) and roots (black bar). Mean values followed by different lowercase (shoot) or uppercase (root) letters are significantly different at $P = 0.05$ (Wilcoxon test; $n = 7$).

The amount of Ni in shoot per plant (in single-species or mixed multispecies cover) was calculated, for monospecific cover, by dividing the total amount of Ni present in the shoots by eight, and, for mixed multispecies cover, by dividing the quantity measured in the two identical plants by two (Fig. 3). The amount of Ni in shoot per plant showed no significant difference between the types of culture (monospecific or mixed multispecific). However, in monoculture, N, L and B had significantly ($P < 0.05$, Wilcoxon) more Ni in the shoots than did A, whereas in mixed multispecies cover, B contained more Ni than did L and A. B and N showed the highest values of Ni in shoots.

The Ni concentrations in roots per pot (Fig. 4, right scale) were much lower than those observed in shoots (Fig. 4, left scale). Plants in treatment A contained, in monospecific or mixed cover, less Ni in shoots than did those in the other treatments (Fig. 3). The concentration of Ni in the shoots of plants in A was not different from that in N, but was superior to that in L (Fig. 4). However, plants in B showed a Ni shoot concentration significantly ($P < 0.05$, Wilcoxon) greater than that of the species in the other treatments. Ni concentrations in the shoots were greater than 8000 mg kg^{-1} for the four species, whether grown in monoculture or as a mixed multispecies cover (Fig. 4). Overall, the concentration of Ni in the shoots seemed higher when plants were grown as a mixed multispecies cover than when they were grown as a monospecific cover (L, N and B); however, only B had a significantly higher Ni concentration when grown in mixed cover (data not shown). For roots, B and N showed significantly ($P < 0.05$, Wilcoxon) lower concentrations than did the other cover treatments (Fig. 4).

From these concentrations, various ratios, including the BAC and TF, were calculated (Table 2). When the species were compared between the different types of cover, we observed that B had a significantly ($P < 0.05$, Wilcoxon) higher BAC than did the other three species, both when B was grown as a monospecific cover and multispecific cover. N and B showed the best TF, both in monoculture (142.5 and 133.4, respectively) and in a mixed cover (132.7 and 67.9, respectively) (Wilcoxon, $P < 0.05$). In contrast, the TF for A was significantly ($P < 0.10$, Wilcoxon) lower than that for other species, whether grown alone or in a mixed cover. There were no significant differences among the other species

between the types of cover (single-species or mixed-species cover).

Soil properties

At the beginning of the experiment, the concentration of total Ni in the soil was 1480 mg kg^{-1} dry soil after hydrofluoric acid (HF) digestion. The mean value of DTPA-extractable Ni after plant treatments showed significant differences and was in the order of 70.2 mg Ni kg^{-1} dry soil, whereas it was 62.7 mg Ni kg^{-1} dry soil before sowing (data not shown). The unplanted treatment had a significantly ($P < 0.05$, Wilcoxon) higher extractable Ni content (76.8 mg kg^{-1}) than did the planted soils (Fig. 5). The smallest concentration of extractable Ni (67.1 mg kg^{-1}) was on B cover.

There was no difference in soil pH among treatments and the mean was 5.77 ± 0.02; before culture treatments, pH was 6.14. Thus, the plant cover resulted in a significant acidification of the soil (data not shown).

Microbial properties

The ratio between the number of microorganisms in rhizosphere soils (R) and the corresponding number of microorganisms in the unplanted soils (S), the R : S ratio (Lynch and Whipps 1990; Benizri *et al*. 2007), was the highest, 1.34, for the LNAB cover treatment, and 1.23, 1.27, 1.24 and 1.23 for L, N, A and B treatments, respectively. The higher values in the mixed multispecies cover treatment (LNAB) than in non-rhizosphere soil (SWP) were noted despite a short culture period.

Table 2. Bioaccumulation coefficient (BAC) (mg Ni kg^{-1}) and nickel (Ni) translocation factor (TF) (mg Ni kg^{-1}) in plants in monospecific (*Leptoplax* (L), *Noccaea* (N), *Alyssum* (A), *Bornmuellera* (B)) and mixed multispecies (LNAB) covers

Mean values within a row followed by different lowercase letters are significantly different at $P = 0.05$ (Newman–Keuls test). Mean values within a column, for BAC, followed by different capital letters are significantly different at $P = 0.05$ (Newman–Keuls test). Mean values within a column, for TF, followed by different capital letters are significantly different at $P = 0.05$ (Newman–Keuls test)

Parameter	Cover	L	N	A	B
BAC	Monospecific	5.69cB	6.46bA	6.80bA	8.44aB
	LNAB	6.78bA	6.75bA	6.59bA	9.78aA
TF	Monospecific	25.1bA	142.5aA	19.9bA	133.4aA
	LNAB	13.7bA	132.7aA	5.2bB	67.9aB

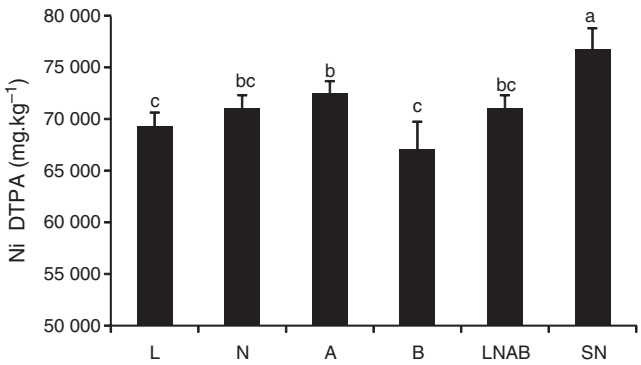

Fig. 5. Nickel (Ni) extracted with the diethylenetriaminepentaacetic acid–triethanolamine (DTPA-TEA). Mean values followed by different letters are significantly different at $P = 0.05$ (Wilcoxon test; $n = 7$).

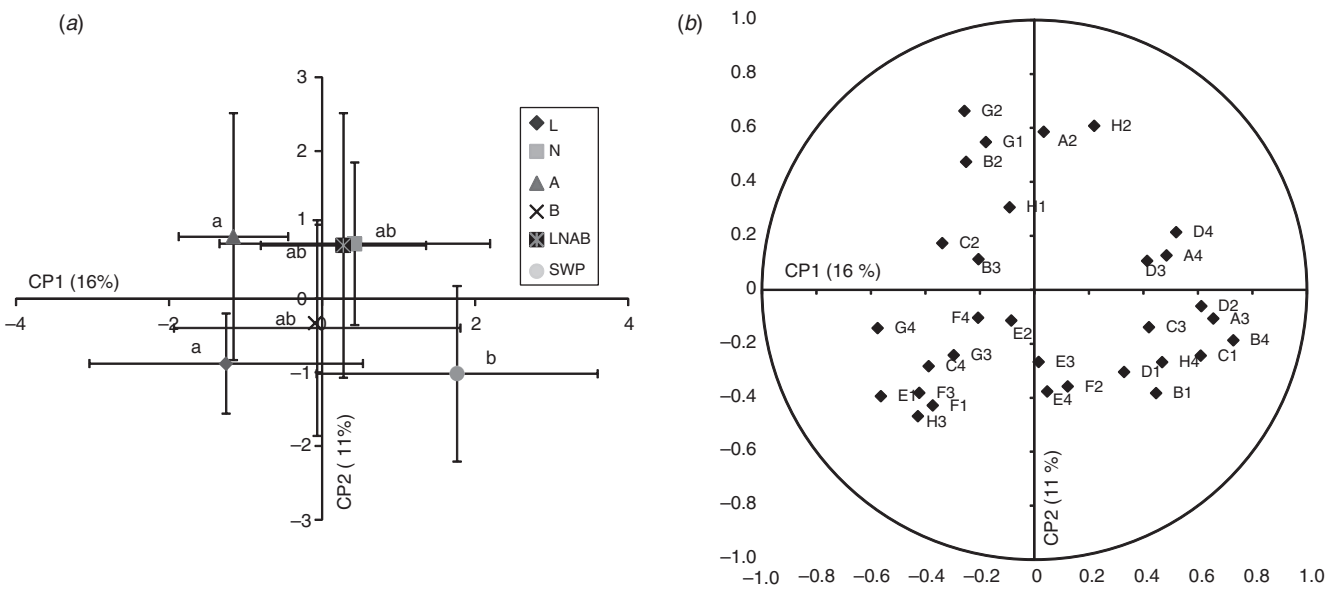

Fig. 6. (*a*) Ordination plot of soil samples generated by principal component analysis of the catabolic profiles of bacterial communities. Points represent means of seven replicate samples (*Leptoplax* cover (L), *Noccaea* cover (N), *Alyssum* cover (A), *Bornmuellera* cover (B), cover with a mixture of the four species (LNAB), soil without plants (SWP)). Points followed by different letters are significantly different at $P = 0.05$ (Newman–Keuls test; $n = 7$). (*b*) Substrates involved in the discrimination of bacterial communities (carbohydrates: B1, G1, H1, A2, B2, C2, D2, E2, G2, H2; carboxylic and acetic acids: F2, A3, B3, C3, D3, E3, F3, G3, H3; amino acids: A4, B4, C4, D4, E4, F4; amines and amides: G4, H4; polymers: C1, D1, E1, F1).

Comparison of C-utilisation patterns of the different communities of soil bacteria extracted was performed by PCA of Biolog Ecoplates™. The main plot represented 27% of the total variability (Fig. 6*a*). There was a clear discrimination of communities between the unplanted soils (SWP) and the different rhizosphere soils (whatever the plant combination). Along the first axis (PC1), the communities from rhizospheres of L and A treatments were significantly ($P < 0.05$, Newman–Keuls) different from those of SWP, and showed different trophic abilities. Communities from the rhizospheres of N, B and LNAB treatments showed an intermediate position. In contrast, bacterial communities could not be consistently separated through the second axis (PC2). Bacteria living in the rhizospheres of A, N and LNAB treatments catabolised carbohydrate substrates more intensely than did bacteria from other soils (Fig. 6*b*). Similarly, bacteria from the rhizosphere of L seemed generally to prefer both carboxylic acids and polymers. Finally, we found that the communities from SWP showed the most diverse catabolic abilities, without any preference.

Different diversity and AWCD indices were calculated from Biolog Ecoplates™ data and are presented in Table 3. The number of substrates (richness, S), with absorbance >0.25, was also the lowest for SWP. This value was significantly lower than that calculated for L and, to a lesser extent, also those for the other cover treatments (N, A, B, LNAB). Thus, it appears that the presence of a cover induced an increased metabolic capacity of the soil bacterial communities (S cover > S unplanted soil). In contrast, we observed no significant difference, regardless of the treatment, in either the Shannon diversity index (H') or the evenness (E), suggesting that the different substrates were used in the same way. Finally, AWCD was the lowest in SWP. Indeed, the average optical density of plates inoculated with bacterial communities from SWP was lower, corresponding to the fact that the number of oxidised substrates was also lower. In contrast, bacterial communities from the rhizosphere of A treatment showed the highest AWCD, although the number of substrates oxidised was low.

A PCA was carried out to compare the genetic structure of the bacterial and fungal communities colonising the rhizosphere of the various plants (Fig. 7*a*). Concerning bacterial genetic structure, PC1 represented 90% of the total variability, discriminating the bacterial communities in LNAB, N and B mecososms from those of the other plant covers (A and L). They were also separated on the second PCA axis. Interestingly, a similar discrimination was observed concerning the genetic structure of fungal communities (Fig. 7*b*). Indeed PC1, which represented 57% of the total variability, discriminated fungal communities in LNAB, N and B mesocosms from those of the other plant-cover mesocosms.

To consider all parameters together, we performed another PCA on the basis of physicochemical and biological variables (Fig. 8). Axis 1, which explained 24% of the total variability, strongly discriminated unplanted soil (SWP) (negative abscissa) from soil from planted mesocosms (positive abscissa) (Fig. 8*a*). Thus, SWP was clearly different from

Table 3. Average well colour development (AWCD), richness (S), Shannon index (H') and evenness (E) for soils covered with a single species, a mixture of species or with no plant cover

A, *Alyssum* cover; B, *Bornmuellera* cover; L, *Leptoplax* cover; N, *Noccaea* cover; LNAB, covered with mixture of the four species; SWP, non-rhizosphere soil (no plant cover). Mean ± s.e. values followed by different letters are significantly different at $P = 0.05$ (Newman–Keuls test; $n = 7$)

Parameter	AWCD	S	H'	E
L	1.08 ± 0.11ab	24.00 ± 1.42a	4.00 ± 0.09a	0.93 ± 0.11a
N	1.05 ± 0.13ab	23.29 ± 1.70ab	4.00 ± 0.15a	0.88 ± 0.02a
A	1.16 ± 0.08a	23.57 ± 1.12ab	3.97 ± 0.08a	0.87 ± 0.01a
B	0.99 ± 0.11b	22.29 ± 1.11ab	3.88 ± 0.13a	0.87 ± 0.02a
LNAB	1.07 ± 0.05ab	22.57 ± 1.20ab	3.93 ± 0.05a	0.88 ± 0.02a
SWP	0.96 ± 0.09b	21.14 ± 1.17b	3.85 ± 0.12a	0.93 ± 0.10a

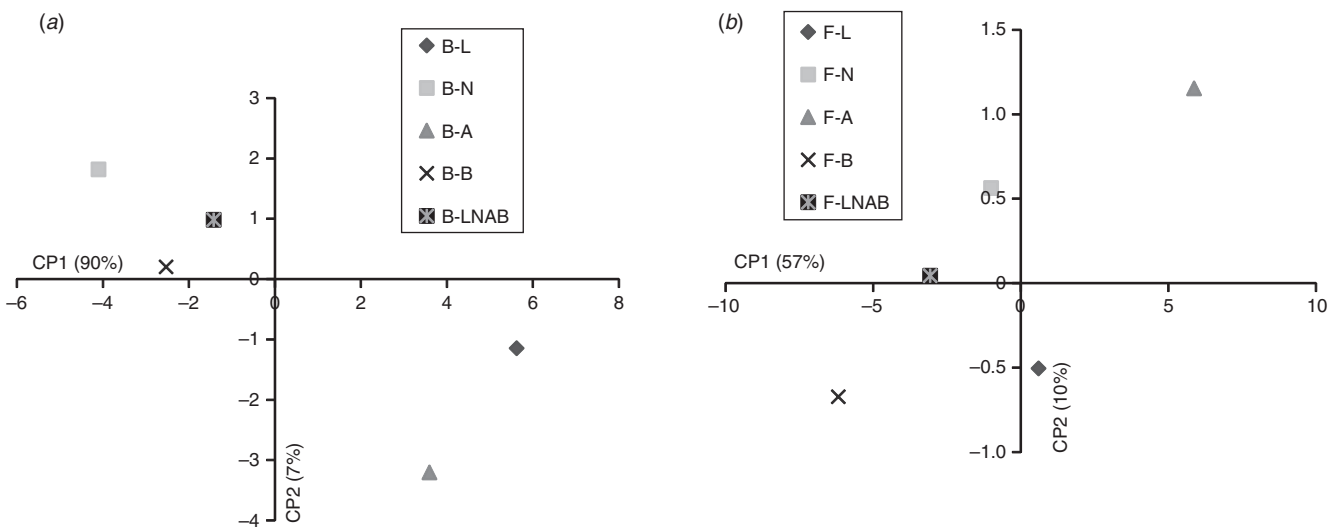

Fig. 7. Ordination plot of (*a*) bacterial and (*b*) fungal communities, generated by principal component analysis of single-strand conformation polymorphism (SSCP) matrices. Points represent means of seven replicate samples (*Leptoplax* cover (L), *Noccaea* cover (N), *Alyssum* cover (A), *Bornmuellera* cover (B), cover with a mixture of the four species (LNAB), soil without plants (SWP)).

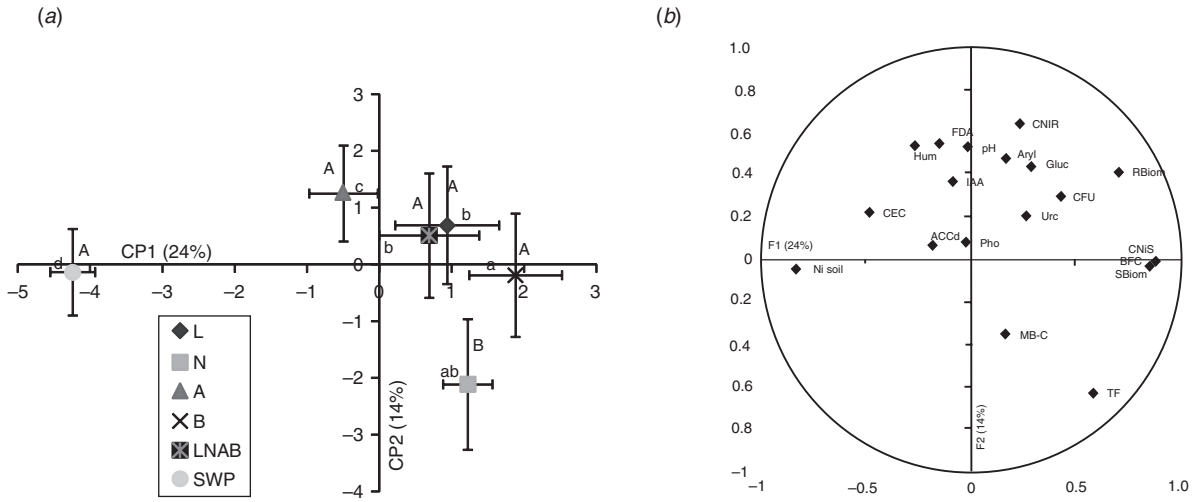

Fig. 8. (*a*) Ordination plot of soil samples, generated by principal component analysis of the physicochemical and microbiological parameters. Points represent means of seven replicate samples (*Leptoplax* cover (L), *Noccaea* cover (N), *Alyssum* cover (A), *Bornmuellera* cover (B), cover with a mixture of the four species (LNAB), soil without plants (SWP)). (*b*) Physicochemical and microbiological parameters involved in the discrimination of soil samples. Ni soil, DTPA-extractable nickel from soil (mg kg^{-1}); CEC, cation exchange capacity (cmol kg^{-1}); ACCd, 1-aminocyclopropane-1-carboxylic acid deaminase (nM g^{-1} dry soil day^{-1}); pho, phosphatase acid (µg p-nitrophenol g^{-1}dry soil h^{-1}); aryl, arylsulfatase (µg p-nitrophenol sulfate g^{-1} dry soil h^{-1}); ure, urease (µg NH_4-N g^{-1} dry soil (2 h)$^{-1}$); gluc, β-glucosidase (µg p-nitrophenol g^{-1} dry soil h^{-1}); FDA, fluorescein di-acetate (µg FDA g^{-1} dry soil h^{-1}); IAA, auxin compounds (mg g^{-1} dry soil h^{-1}); hum, soil humidity (%); C Ni R et C NI S, root and shoot nickel concentrations (mg kg^{-1}); RBiom et SBiom, root and shoot biomass (g); MB-C, microbial biomass carbon (mg C g^{-1} dry soil); CFU (colony forming units) (log10 cfu g^{-1} dry soil); TF, translocation factor; BCF, bioconcentration factor.

the other cover types and, in particular, from B and N. Other covers (L, A and LNAB) showed an intermediate position along PCA. Axis 2, which represented 14% of the total variability, clearly discriminated the N cover treatment (negative ordinates) from the other covers (A, L, B, LNAB) (positive ordinates). If we focus on the explanatory variables (Fig. 8*b*), it appears that the presence of a cover, whether single- or multi-species, caused a decrease in the concentration of extractable soil Ni (PC1). In the presence of plants, the values of Ni extractable from soil (Ni soil) were the lowest. Correspondingly, we noted that the concentrations of Ni in shoots (C Ni S) were significantly inversely correlated ($r = -0.70$, $P < 0.05$) with the quantities of Ni present in soil (Ni soil). Cover B, where the plants had accumulated the highest amount of Ni in shoots, was the cover where the rate of Ni in soil was the lowest, whereas SWP had the highest concentration of Ni in soil. In addition, it is clear that the greater the plant biomass, the higher the concentration of Ni in shoots ($r = 0.56$, $P < 0.05$). This was especially marked in the order of importance for B > N > L and LNAB > A. Thus, all of the plants used in this experiment were able to grow on a naturally Ni-rich soil and accumulated this metal in the shoots. PC2 discriminated vegetation cover according to soil pH, even if pH showed only a slight variation between the different mesocosms. Nevertheless, the rhizosphere in the N treatment had the lowest pH (negative ordinates), being unlike all the other covers (positive ordinates). However, we noted a significant inverse correlation between the concentration of Ni in the roots (C Ni R) and TF ($r = -0.42$, $P < 0.05$). This appeared logical because TF corresponded to the ratio of the concentration of Ni in the shoots to that in the roots. Anyway, PC2 clearly separated N from the other cover treatments, although pH may not be the only parameter responsible for this discrimination. There was correlation among pH, the size of the cultivable bacterial community and microbial enzymatic activities. More specifically, we observed a positive correlation between pH and CFU ($r = 0.32$, $P < 0.05$) and a significant correlation between CFU and most of the measured microbial activities (Ure, Pho, Gluc, $r = 0.28$, $P < 0.10$). Among the microbial activities measured, ACCd activity was significantly positively correlated with the Ni concentration in the roots ($r = 0.29$, $P < 0.10$) and inversely correlated with urease activity. Finally, the size of the cultivable bacterial community appeared to be favoured by the presence of a vegetation cover, and positively correlated with root biomass (R Bio, $r = 0.35$, $P < 0.05$).

Discussion

The objective of the present work was to study the effect of plant cover, which consisted exclusively of four Ni-hyperaccumulating plants, on the efficiency of Ni extraction from ultramafic soil. We hypothesised that a hyperaccumulating-plant cover composed of various species of plants participates in the establishment of a microflora involved in phytoextraction of metal pollutants and that this would be more effective than the microflora associated to plant species grown alone.

Concerning the efficiency on Ni accumulation by the different cover treatments, our results were consistent with the definition of a Ni hyperaccumulator, accumulating at least 1000 mg kg^{-1} of Ni in dry matter (Reeves 1993). Indeed, we measured concentrations of Ni in the shoots of the order of 8000, 9000, 10 000 and 12 000 mg kg^{-1}, respectively, for L, N, A and B covers, with the concentration exceeding 14 000 mg kg^{-1} in for B when this

species was grown in a mixed culture (multispecies cover). Moreover, *Bornmuellera* is known to be able to accumulate up to 3% of Ni in the dry aerial parts (Reeves *et al*. 1983). These four hyperaccumulator species also have a high TF. However, the Ni concentrations and biomass of the plant parts varied among the species. The plants in B (i.e. *Bornmuellera tymphaea*), despite not having a dry biomass superior to that of the other species, had a high TF, similar to that of the plants in N (i.e. *Noccaea tymphaea*), and seemed to be the most effective of the four hyperaccumulator species tested because of the high Ni concentration in its shoots. The plants in A (i.e. *Alyssum murale*), despite having a very high Ni concentration, produced, in our conditions, a low biomass and therefore extracted a smaller amount of metal than did the other species. Conversely, the plants in L (i.e. *Leptoplax emarginata*) showed a high biomass, but accumulated a lesser amount of Ni and had a low TF. As for *Alyssum murale* (Treatment A), *Leptoplax emarginata* (Treatment L) had a root Ni concentration significantly higher than did *Noccaea tymphaea* (Treatment N) and *Bornmuellera tymphaea* (Treatment B), and, in consequence, presented low TF values. The amounts of Ni found in plant parts in LNAB were not superior to those in all single-species covers. Indeed, in a mixed cover, *Leptoplax emarginata* and *Alyssum murale* often had the lowest values, whereas *Noccaea tymphaea* and *Bornmuellera tymphaea* had the highest. The influence of the phenological stage of the four species probably explains the differences in metal translocation. *Bornmuellera tymphaea* and *Noccaea tymphaea* need probably a lesser time period to achieve full metal translocation. These are both high-altitude species, as opposed to *Alyssum murale*, *Leptoplax emarginata*, which can occur down to sea level (Bani *et al.* 2009).

We found differences in structure, both in the analyses of the catabolic abilities of the bacterial community and those of the genetic structure of the fungal and bacterial communities, that depended on the conditions, i.e. control soil or plants. Concerning Biolog fingerprints, several authors have shown that the presence of a plant cover induces modifications to the trophic ability of microbial communities. Epelde *et al*. (2008) showed, by comparing the catabolic profiles of bacterial communities from soil without plants with those from the rhizosphere of *Noccaea caerulescens*, that, depending on the presence of the plant cover, certain substrates were specifically used. Because of the presence of easily degraded compounds, i.e. root exudates (such as e.g. sugars, amino compounds, organic acids; Curl and Truelove 1986; Benizri *et al*. 2007), planted soils generally had greater activity and microbial biomass than did soils without plants. In line with these previous papers, in the present experiment, we observed that S and AWCD, as well as BM-C, showed significantly lower values in unplanted soil. Hernández-Allica *et al*. (2006) and Wang *et al*. (2006) also found significantly greater biological activity in the rhizosphere of *Noccaea caerulescens* than in non-rhizosphere soil. In our experiment, we found also that more of the carbohydrates present in the Biolog plates were oxidised by bacterial communities from rhizosphere soil (8 of 10 carbohydrates) than by those from soil without plants (2 of 10 carbohydrates).

Furthermore, some authors have investigated the catabolic profiles of bacterial communities from the rhizosphere of plants growing on metalliferous soils. For instance, Epelde *et al*. (2012) showed that the bacterial communities from the rhizosphere of *Noccaea caerulescens*, *Rumex acetosa* and *Festuca rubra* growing on Zn-rich soil were characterised by different catabolic profiles. In our case, we also observed that the catabolic profiles of bacterial communities in the rhizospheres of A and L cover treatments were different from those in the rhizospheres of B, N and LNAB treatments. As highlighted by Zak *et al*. (2003), it appeared that plant communities influence microbial communities mainly through root exudates rather than through the diversity of plant cover itself. This observation is consistent with our results. Indeed, it seems that even if plants used in our experiments were all *Brassicaceae*, each cover selected a specific bacterial community in terms of catabolic abilities. This effect was stronger for communities in A and L treatments than for those in B, N and LNAB treatments.

We also found differences in the SSCP profiles among the different mesocosms. Interestingly, a relatively similar discrimination was observed for the microflora colonising hyperaccumulating plant-cover treatments when their catabolic abilities and SSCP profiles were analysed. Indeed, for the genetic structure of both bacterial and fungal communities, we observed a discrimination between A and L covers on one hand, and B, N and the mixed cover on the other. So it seems that there was a link between the modification of the genetic structure and changes in the trophic ability of the microbial communities. Apparently, these changes induced a clear discrimination between B, N and LNAB covers, and the other two covers (A and L). Moreover, it transpired, on the basis of the C-utilisation patterns and SSCP analysis, that in the mixed cover, *Bornmuellera* and *Noccaea* had the greatest influence on all the physicochemical and biological parameters. Clearly, this would explain why the mixed cover was close to these two monospecies covers. However, it did not seem that the culture of these plants in a mixture, and the subsequent effect on the communities of soil microorganisms, significantly improved their ability to hyperaccumulate Ni, or even biomass, except for *Bornmuellera*, for which the mixed cover seemed to have a beneficial effect.

Conclusions

We showed that the association of four hyperaccumulator plants (multispecies cover) did not allow a greater total biomass (shoot + root) to be obtained, nor more Ni to be concentrated, in particular in aerial parts. However, effective phytoextraction must provide the production of significant biomass and possess the ability to extract the metal from the soil to the roots and from the roots into shoots. Thus, the mixed cover, as we observed, did not appear to be the optimal combination promoting rhizosphere processes either in terms of extraction of the pollutant, or again, in terms of functional microbial diversity. The mixture of four selected species probably involved synergistic relationships, but also certainly engendered competition among plants and among rhizosphere bacterial communities.

Bacterial communities from *Noccaea tymphaea* and mixed-cover rhizospheres were characterised by close trophic abilities, but were different from those of other covers. Similarly, the genetic structure of bacterial and fungal communities from *Bornmuellera*, *Noccaea* and LNAB rhizospheres differed from those of other covers. We noted, moreover, that greater shoot

biomass concerned *Noccaea* and *Leptoplax* covers and that higher Ni concentrations referred to *Bornmuellera* cover. It seemed that mixed culture both promoted *Bornmuellera* shoot biomass and the quantities of Ni accumulated in the aerial parts of this plant. The translocation factor values were highest for species such as *Noccaea tymphaea* and *Bornmuellera tymphaea*. Thus, it seems that N and B were the best two candidates for an improved phytoextraction efficiency at such a time scale. Further experiments based on the association of these two hyperaccumulator plants will confirm these observations.

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