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Effect of hyperaccumulating plant cover composition and rhizosphere-associated bacteria on the efficiency of nickel extraction from soil

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ABSTRACT

Most plant species selected as appropriate candidates for phytoextraction have been studied as monocultures. However, alternative cropping patterns which include rhizosphere microbial communities can significantly influence the extraction of metals, as well as soil protection and quality. Therefore, the objective of this work was to study the effect of species-rich vegetation cover, which consisted of three hyperaccumulator plant species, on the efficiency of nickel extraction from a naturally mineralized ultramafic soil. An experiment was set up with three hyperaccumulator species (*Leptoplax emarginata*, *Noccaea tymphaea* and *Alyssum murale*). Plants were cultivated separately (monospecific cover), or in combination (multispecies cover) in mesocosms under controlled conditions, on a nickel-rich ultramafic soil. Plants were grown for 92 days in controlled conditions. Each plant produced more biomass when grown in multispecies cover than alone. *Noccaea* and *Alyssum* showed the highest shoot Ni concentrations but *Alyssum* had by far the lowest shoot biomass. So, in this soil, *Noccaea* and *Leptoplax* have greater potential for hyperaccumulation than *Alyssum*. The amount of nickel accumulated in total biomass of *Noccaea* alone and of the multispecies cover was higher than that accumulated in either the monospecies *Leptoplax*or *Alyssum*. Furthermore, the highest values of microbial biomass were obtained with the multispecies cover and a consistent production of auxin compounds by bacterial communities was measured, which emphasized the role of rhizosphere bacteria. The bacterial genetic structure also depended on the plant covers. A combination of the three species (multispecies cover), could be a good strategy for phytoremediation.

1. Introduction

Worldwide contamination of soils with heavy metals causes a serious threat to the ecosystem and to human health. In recent decades, increasing concern about metal contamination and its toxicity to microorganisms, plants and animals has been reported (Kidd et al., 2009). Removal of metals from contaminated soils is particularly challenging because heavy metals are non-biodegradable (Garbisu and Alkorta, 2001) and can accumulate in plant tissues. Bioremediation based on microorganisms, plants or other biological systems provides a cost-effective and environmentally friendly method for metal clean-up (Chaney et al., 2007). Also, the phytomining of metals (i.e. metallurgical recovery of metal hyperaccumulated in plant biomass) has been considerably developed and is now ready for field application with nickel (Bani et al., 2007; Chaney et al., 2007; Tang et al., 2012). To increase phytomining yield, plant hormone external application has been tested directly on hyperaccumulator growth and metal uptake but it gives contradictory results (Cassina et al., 2011; Cabello-Conejo et al., 2013).

For nearly 10 years, the use of PGPR (plant growth-promoting rhizobacteria) in the remediation of soil inorganic pollutants has increased steadily (Lebeau et al., 2008; Glick, 2010). It has been shown that, in pollutant-hyperaccumulating plants, PGPR promotes their germination, increases the root biomass and also facilitates the survival of plants, despite the stress exerted by the pollutant. 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, have also been observed (Glick, 2005). This results in a better plant

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development, although a bacterial growth was observed because ACC may act as a source of carbon (C) and nitrogen (N) for the rhizosphere microflora. In contrast, PGPR survival is hampered by nutrient-limiting conditions, unlike most polluted soils, underlining the important role of root exudates as a source of C and N for the microflora. Thus, many works have attempted to relate the association of different plants and the efficiency of inorganic pollutants extraction, with the hypothesis that these vegetation covers promote the development and the activities of some microorganisms, such as PGPR. Studies up to now, have mainly concerned crop associations. In particular, these studies have involved associations of tobacco in the presence of a legume such as clover (Liu et al., 2011), rice and maize in the presence of bean (Murakami and Ae, 2009), rape with alfalfa (Pan et al., 2008), co-cultures of various *Brassica* species (*B. junica*, *B. chinensis*) (Liu et al., 2007), or different tree species (Pulford and Watson, 2003). However, few studies have concerned the effect of the combination of metal hyperaccumulator plants with other species (Epelde et al., 2012). These experiments show that co-culture with non-hyperaccumulator plants could enhance the growth of the hyperaccumulator and increase the absorption of metals. Even fewer articles have concerned the effect of the combination of plants known to be hyperaccumulators (Whiting et al., 2001).

The objective of this work was to study the effect of species-rich vegetation cover, which only consisted of three hyperaccumulating plant species, on the efficiency of nickel extraction from a soil naturally rich in this metal. The effects on some soil physicochemical properties and on microbial communities colonizing the rhizosphere were also evaluated.

2. Materials and methods

2.1. Soil sampling and analysis

The soil used in this study had been collected from the top layers (A_1 and B_W horizons; 5–30 cm) of a natural forest ultramafic Hypermagnesic Hypereutric Cambisol (Vosges Mountains, eastern France, 07°06′42.2″ E, 48°11′03.7″ N).

The soil's physicochemical properties were determined by the Soil Analysis Laboratory of INRA (Arras, France), (Table 1). The soil material was a clay loam, with a pH of 5.83 and an organic matter content of 14.1%, C/N 13.4 and 416, 391 and 193 g kg^{-1} clay, silt and sand, respectively. Developed on a serpentinized harburgite, this soil was naturally rich in nickel (Ni) and the total Ni content was 1170 mg kg^{-1}.

2.2. Plant characteristics

Three *Brassica* sp., *Leptoplax emarginata* (Bois) O.E. Schulz, *Noccaea tymphaea* (Hausskn.) F. K. Mey. and *Alyssum murale* Waldst. & Kit were selected for this study based on their demonstrated ability to accumulate substantial amounts of nickel in shoots (Bani et al., 2009, 2014). At flowering stage (maximal Ni accumulation), *L. emarginata* exceeds one meter, *A. murale* exceeds 60 cm and *N. tymphaea* barely reaches 20 cm. Seeds were collected in Greece. *Nocceae* seeds were taken July 19, 2011 from the Katara Pass (1700 m) in Greece (39°47′765″ N, 21°13′739″ E). *Leptoplax* and *Alyssum* seeds were taken on July 20, 2011 and come from a site near the village of Trigona (830 m) in Greece (N 39°4′223″, E 21°15′869″); (see characteristics of the three species in Bani et al., 2009).

2.3. Experimental design

Three kilograms of soil (on a DW basis) were placed in parallelepiped pots 13 cm × 24 cm × 16 cm (l × L × h). The mesocosms were planted with species used either separately (monospecific

Table 1
Chemical and physical properties[a] of the soil used.

pH	Clay	Silt	Sand	Organic carbon	Total nitrogen	P_2O_5	C.E.C.	Ca	Mg	K	$Fe_{Oxalate}$	Fe_{BCD}	Total Cr	Total Cu	Total Ni	Total Zn	Total Co	Ni_{DTPA}	Fe_{DTPA}	Mn_{DTPA}
(water)	(g kg^{-1})					(cmol + kg^{-1})					(g kg^{-1})		(mg kg^{-1})							
5.83	416	391	193	81.7	6.11	0.015	30	3.1	25.8	0.27	21.3	60.4	2680	13.4	1170	216	133	62.5	331	10.7

[a] Analyzed by INRA, Arras, France.

cover: *Leptoplax emarginata*, *Noccaea tymphaea* or *Alyssum murale*), or in combination (i.e. multispecies cover where 2 specimens of each species are planted together). Control mesocosms were without plants. The total plant density was 6 plants per mesocosm. The experiment had a completely randomized block design with four replications that had the following treatments: Lep: *Leptoplax*; Noc: *Noccaea*; Aly: *Alyssum*; Mix: mixture of the three species; SWP: soil without plants. The mesocosms were transferred to an environmental growth chamber (photoperiod 16 h, temperature 15 °C night and 20 °C day, relative humidity 70%, PPFD: 350 mmol m^{-2} s^{-1}) and watered to 70% of the soil water holding capacity. Thus, no leaching could take place.

2.4. Plant analysis

After 3 months of culture (92 days), plant roots, stems and leaves were collected and their dry weights were recorded. Ni concentration in shoots and roots was measured with an Atomic Emission Spectrometer (ICP-AES, Liberty II, Varian) after digestion with 8 mL HNO_3 and 4 mL H_2O_2 per 0.5-g plant sample.

2.5. Soil analysis after harvest

The total organic carbon from each mesocosm was determined with a TOC analyzer (Shimadzu TOC-VCSH). Ni in soil samples from each mesocosm was extracted with the DTPA–TEA solution (0.005 M DTPA, 0.01 M $CaCl_2$, 0.1 M triethanolamine, pH 7.3) according to Lindsay and Norvell (1978) or digested with Aqua regia (1/3 HNO_3–HCl, v/v) and Ni concentration in solution was measured with an atomic emission spectrometer (ICP-AES, Liberty II, Varian). Soil pH was measured using a pH meter in a soil/water solution mixture (soil water ratio 1:5).

2.6. Soil microbial analysis

The number of culturable bacteria as colony forming units (CFU) was determined by spread-plating soil suspensions onto TSA 10% (Tryptone Soy Agar, Difco). The soil microbial biomass carbon (SMB-C) was estimated using the fumigation extraction technique previously described by Vance et al. (1987). The determination of auxin-like compounds, from the soil samples, was adapted from the method described by Sarwar et al. (1992) and Wöhler (1997). ACC deaminase (ACCd) activity was determined by the production of α-ketobutyrate from ACC, based on the method described by Honma and Shimomura (1978) for measuring ACC deaminase in cell extracts. The genetic structures of the bacterial communities colonizing the rhizosphere were analyzed. For this, DNA was extracted from 0.5 g of soil with the FastDNA®SPIN®Kit (For Soil) (BIO 101, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. Comparison of the genetic structures of the bacterial communities was made by principal component analysis (PCA) of bacterial 16S rRNA profiles obtained by single strand conformation polymorphism (SSCP) (Vallance et al., 2009) with the primers 799f (AACMGGATTAGATACCCKG) and 1115r (AGGGTTGCGCTCGTTG) (Redford et al., 2010).

2.7. Statistical analysis

All data were analyzed by analysis of variance (one-way ANOVA with Tukey's test, $\alpha = 0.05$ or Kruskal–Wallis test, $\alpha = 0.05$) using StatBox software (StatBox ver.7, software, Grimmersoft, Paris, France, http://www.grimmersoft.com). Bacterial 16S rRNA profiles obtained by SSCP were analyzed using PCA (PCA, StatBox ver.7, software, Grimmersoft, Paris, France, http://www.grimmersoft.com).

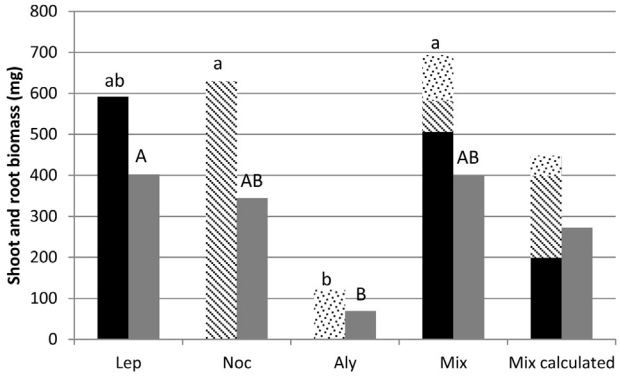

Fig. 1. Shoot (Lep Noc Aly) and root (greybar) biomass (mg) per pot. Bars with the same lower case letter are not significantly different at $P < 0.15$. Bars with the same capital case letter are not significantly different at $P < 0.05$. Mix calculated corresponded to the addition of 1/3 of the shoot biomass of each monoculture plants (shoot biomass of Mix calculated = 1/3 shoot biomass of Lep + 1/3 shoot biomass of Noc + 1/3 shoot biomass of Aly).

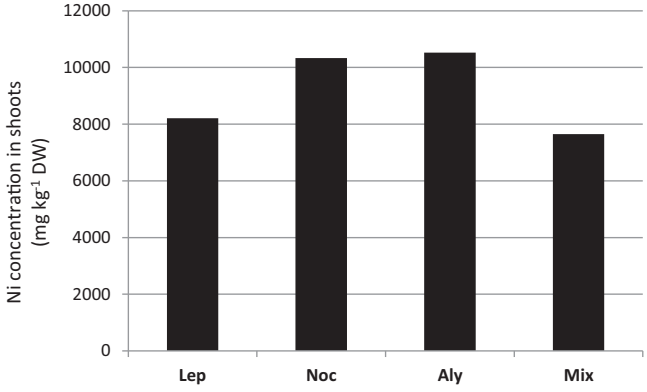

Fig. 2. Ni concentration in shoots (mg kg^{-1} DW) per pot. No significant differences at $P < 0.05$.

3. Results and discussion

3.1. Plant physiological aspects of co-cropping

At the end of the experiment, although differences were not statistically significant, the trend was a greater shoot biomass for the Mix treatment (Fig. 1). Aly shoot biomass was significantly lower than Lep and Noc. In monoculture, 6 plants of the same species were cultivated per pot; in mixture, two plants of each of the three species were cultivated per pot. Without any interaction between plants, the whole biomass (Mix calculated) would have been 197 + 210 + 40 = 447 mg of shoot biomass which is by far lower than what we observed (692 mg). The trend is thus confirmed by a strong synergetic effect of co-cropping on shoot biomass, mainly an increase of *Leptoplax* and *Alyssum* biomass.

Similarly, significantly higher values of Lep root biomass, and to a lesser extent for Noc and Mix, were observed as compared to Aly root biomass. Within plant associations, the intra and interspecific interactions, positive and negative, are known to be complex (Callaway and Lawrence, 1997). Prior to our work, little was known about the association of hyperaccumulator plants.

3.2. Practical considerations in phytoextraction

With regard to plant metal accumulation per pot, no significant differences were observed for the Ni concentrations between covers (Fig. 2). Nevertheless, in all cases, the concentrations of

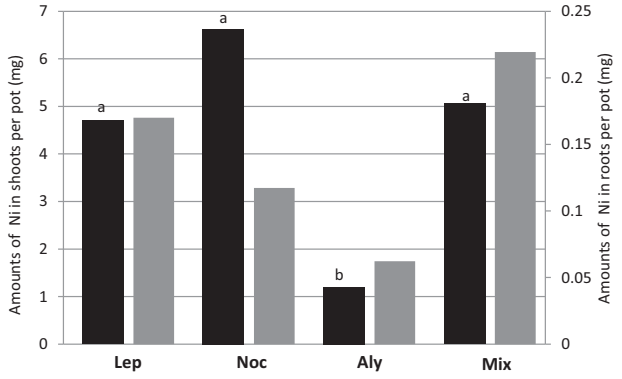

Fig. 3. Amount of Ni phytoextracted in shoots (blackbar) and roots (greybar) per pot (mg). Blackbars with the same letter are not significantly different at $P < 0.15$. No significant differences were noted for amount of Ni phytoextracted in roots (gray bar).

nickel in monospecific covers and in multispecies cover reached or exceeded the threshold value of 10,000 mg Ni kg^{-1} DW, a value recommended for species used in phytomining (van der Ent et al., 2013). With regard to plant metal accumulation per pot (shoot metal concentration × shoot biomass), no significant differences were observed for the Ni amounts in Mix, Noc and Lep shoots (Fig. 3). The highest values of Ni amounts in shoots were found in Noc pots (6.63 mg), followed by Mix (5.06 mg) and Lep (4.70 mg). The lowest values were recorded in Aly pots (1.20 mg). However, if we consider full plants (roots + shoots); the total amount of Ni (mg) in plants per pot was: Lep: 4.87 mg, Noc: 6.75 mg, Aly: 1.26 mg and Mix: 5.28 mg. It is worth noting that the Ni shoot to the root concentration ratio decreased when "Mix" (27.7) is compared to "Mix calculated" (35.1). Plants in combination seem to have a delayed translocation compared to monospecies.

Coinchelin et al. (2012) have shown that there is a time interval between root accumulation and shoot translocation of Ni. Moreover, Bani et al. (2014) have shown that the concentration of Ni in *Alyssum* shoots was maximal at flowering, while Aly plants from our experiment were harvested at the vegetative stage. A combination of these three species (Mix), despite these short-term findings, could be a good strategy for phytoremediation because the highest Ni concentration was recorded in Aly roots waiting for translocation (data not shown). Growing plants for a longer period would probably have given better results for Aly.

3.3. Soil properties

At the beginning of the experiment, the concentration of the total Ni in the soil was 1170 mg kg^{-1} dry soil after HF digestion. At the end of the experiment, the Aqua regia extractable nickel (usually 80–90% of total Ni in ultramafic soils) did not show significant differences among treatments at a mean value of around 930 mg Ni kg^{-1} dry soil. The mean value of DTPA-extractable nickel after plant treatments did not show significant differences and was in the order of 44.1 mg Ni kg^{-1} of dry soil, while it was 62.5 mg Ni kg^{-1} of dry soil before sowing plants (Table 2). The unplanted treatment also decreased after 92 days of incubation (no significant difference with the planted treatments).

The pH decreased during the 92 days from 5.83 to 5.2–5.55. Final pH was significantly lower for Aly and Mix cover (and also unplanted soil) than for Lep. These results seem contradictory with those reported by Kukier et al. (2004). On the contrary, the low pH value can explain the poor yield of Aly (Kukier et al., 2004). Finally, the strongest acidification concerns the Mix while Lep represents a significant fraction of the biomass produced. In addition, no obvious relationship appears between the quantities of extractable nickel

Table 2
Variation of pH and extractable nickel at initial time and after each treatment.

	pH	Ni DTPA (mg kg^{-1})	Ni phytoextracted (mg kg^{-1})
Soil T_0	5.83 a	62.5 a	
Lep	5.56 b	45.2 b	4.7 a
Noc	5.42 bc	42.6 b	6.6 a
Aly	5.29 c	44.6 b	1.2 b
Mix	5.25 c	44.0 b	5.1 a
Soil 92 days	5.30 c	44.4 b	

Values in a column with the same lower case letter are not significantly different at $P < 0.05$.

DTPA and the soil pH. It seems that the soil used in this study was buffered although a slight decrease in pH might have lowered DTPA-extractable Ni. However, a negative correlation was found between values of DTPA-extractable Ni concentrations from the soil and the amounts of the metal in the corresponding shoots ($R = -0.53$, $p < 0.05$); (Table 3).

3.4. Microbial properties

The size of the cultivable bacterial community appeared to be stable regardless of the soil type and showed no significant difference between mesocosms (mean values of 7.17 Log CFU g^{-1} dry soil). However, we noted lower values of about 0.2 Log unit sin the case of non-rhizosphere soil (SWP). Rhizodeposition was demonstrated for the first time in 1894 by Hiltner (Nguyen, 2003). Rhizodeposits were a source of C and energy for soil microorganisms involved in such important functions as nutrient cycles and pollutants. This release of exudates strongly affects the soil microbial composition and activity close to the roots, giving rise to the so-called rhizosphere effect (i.e. the ratio between the number of microorganisms in the rhizospheric soil (R) and the corresponding number of microorganisms in the root-free soil (S) – R/S ratio; Lynch and Whipps, 1990; Benizri et al., 2007). As part of our experiment, this ratio reached a value of 1.6 in the best case. This not very pronounced rhizosphere effect could result from too short a culture period, which did not allow significant bacterial growth.

In order to determine how the physiochemical and microbiological parameters measured in this study interact with each other and if, depending on the type of cover, differences can be detected, we generated a matrix in which the rows represent the cover types (Lep, Noc, Aly, Mix and SWP) and the columns, the various biotic and abiotic parameters. From this matrix, we carried out a Principal Component Analysis (PCA) (PCA, StatBox ver.7, software, Grimmersoft, Paris, France). The main plane of the PCA (PC1–PC2, Fig. 4a) explains 54% of the total variability. PC1, which explains 30% of the total variability, strongly discriminates between cover types; the unplanted mesocosms (SWP), (negative abscissa) are opposed to planted mesocosms, corresponding to mixed vegetal covers (Mix), (positive abscissa).

The mono-specific experimental units (Aly, Lep and Noc) showed an intermediate position along PC1. PC2, which represents 24% of the total variability, discriminates between mono-specific covers: Alymesocosms (positive ordinates) were opposed to those of Noc and Lep (negative ordinates).

Concerning the explanatory variables (Fig. 4b), the presence of a cover, whether single or multi-species, caused a reduction in the concentration of extractable Ni from the soil, probably due to active uptake of Ni by plants. In our case, this effect was most pronounced in the presence of mixed covers and mesocosms planted with *Leptoplax* and *Noccaea*. In these soils, the values of extractable Ni concentrations were the lowest, while the metal contents in the corresponding shoots were the highest (Table 3): the levels of extractable soil Ni were indeed significantly inversely correlated ($R = -0.53$) to the quantities of Ni present in the aerial parts of

Table 3
Correlation coefficients (r) for the relationships between physiochemical and microbiological parameters ($n = 20$).

	Auxins (mg eq IAA g^{-1} dry soil h^{-1})	CFU (Log CFU g^{-1} dry soil)	BM-C (mg kg^{-1} dry soil)	Soil moisture (%)	pH	ACC deaminase (n Mg^{-1} dry soil day^{-1})	Shoot biomass (mg)	Soil Ni concentration (mg kg^{-1} dry soil)	Shoot Ni content (mg)	TOC (mg kg^{-1} dry soil)
Auxins										
CFU	0.12									
BM-C	−0.11	0.38*								
Soil moisture	0.37*	0.08	−0.05							
pH	−0.23	−0.29	−0.47**	0.27						
ACC deaminase	0.45**	0.11	−0.35*	−0.03	−0.11					
Shoot biomass	0.11	0.43	0.23	0.47**	0.22	−0.07				
Soil Ni concentration	0.03	−0.23	−0.16	0.21	0.08	−0.21	−0.47**			
Shoot Ni content	0.19	0.45**	0.07	0.41*	0.16	0.02	0.92**	−0.53**		
TOC	−0.10	0.38*	0.99**	−0.01	−0.43*	−0.37*	0.24	−0.11	0.07	

* $p = 0.10$.
** $p = 0.05$.

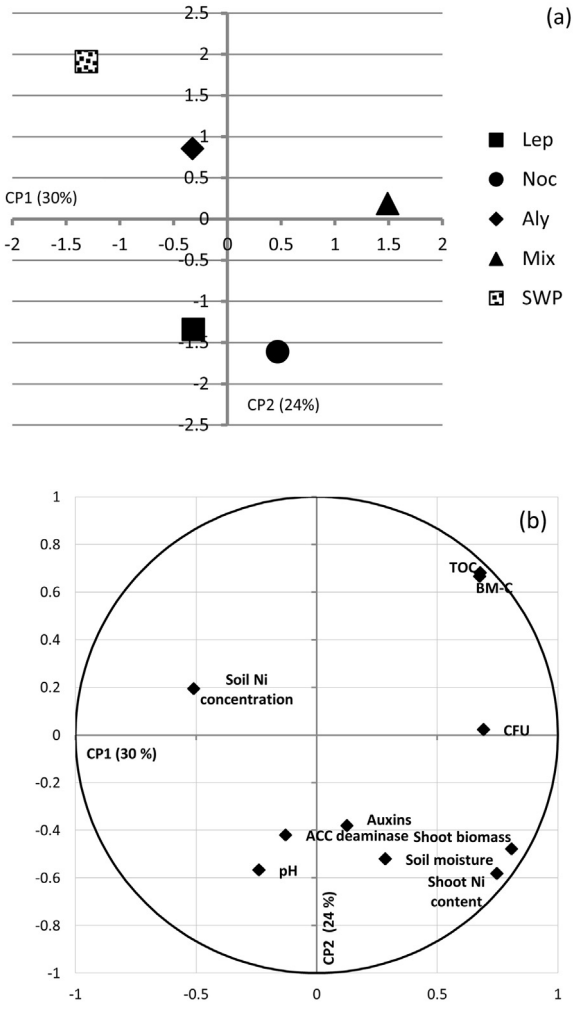

Fig. 4. (a) Ordination plot of soil samples, generated by principal components analysis of the physicochemical and microbiological parameters. Points represent means of four replicate samples (Lep: *Leptoplax*; Noc: *Noccaea*; Aly: *Alyssum*; Mix: mixture of the three species; SWP: unplanted soil). (b) Physicochemical and microbiological parameters involved in the discrimination of soil samples.

plants. Moreover, Aly and Mix rhizosphere showed the lowest pH values. Thus, significant contents of Ni should be expected to be measured in the aerial parts of Aly and Mix covers as pH controls Ni availability (Echevarria et al., 2006), to the extent that soil acidification would favor the passage of Ni from the soil's solid to its solution phase and then to hyperaccumulator biomass (Robinson et al., 1997). Yet the pH is not only acting in this way, but rather reversely (Kukier et al., 2004).

Other parameters should also be considered. Firstly, the presence of roots induces a release of exudates, which also play a role in the regulation of microbial activity (Benizri and Amiaud, 2005; Viketoft et al., 2005; Benizri et al., 2007) and act directly on the rhizosphere processes (Dakora and Phillips, 2002). In our case, in comparison with mono-specific covers, the highest density of the culturable bacterial community and the highest microbial biomass were obtained with the Mix covers. Furthermore, we noted a significant correlation between microbial biomass and the size of the cultivable bacterial community ($R = 0.38$). Thus, the presence of a cover, and in particular Mix covers, implies that microbial growth conditions are more favorable, allowing the bacteria to multiply. Moreover, the significant positive correlation ($R = 0.45$), between the size of the cultivable bacterial community and the amounts of Ni in the aerial plant parts, suggests that bacterial activity would promote the accumulation of nickel in plants. This may be due to the fact that root exudates also favor the activity of microbial communities (Nunan et al., 2005).

In fact, a significant correlation between ACC deaminase activity and the potential production of auxin-compounds ($R = 0.45$) was obtained too. This is consistent with the report of Smaill et al. (2010) who showed an increase in the production of auxin-compounds caused root proliferation that in turn could increase ACC release in the rhizosphere. It thus creates conditions which are favorable for the bacterial communities to proliferate. In our case the soils were not fertilized, the ACC could be an additional source of nutrients (e.g. nitrogen) and energy for soil microorganisms.

However, we did not observe any significant correlation between microbial activities and the amounts of Ni in shoots of different plant covers, even if these quantities showed higher values in the presence of Noc and to a lesser extent in Mix covers. This therefore provides evidence that the vegetation cover, especially that of co-culture, promotes the microbial growth and the specific activities of certain bacterial communities other than simply the production of auxins and ACC deaminase.

The 16S rDNA gene was used to compare the genetic structure of the bacterial communities colonizing the rhizosphere of the various plants (Fig. 5). PC1, represents 87.7% of the total variability,

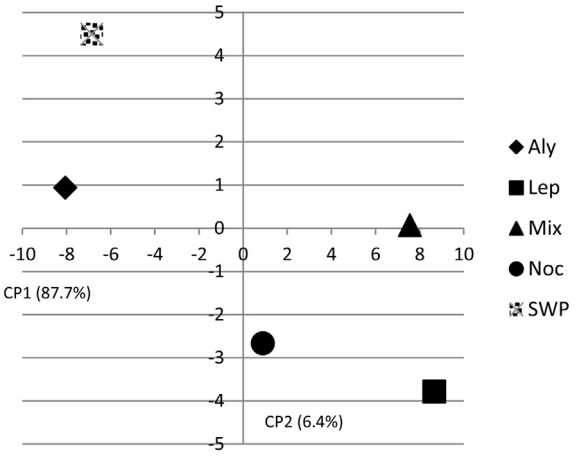

Fig. 5. Ordination plot of bacterial communities, generated by principal component analysis of SSCP matrices. Points represent means of four replicate samples (Lep: *Leptoplax*; Noc: *Noccaea*; Aly: *Alyssum*; Mix: mixture of the three species; SWP: unplanted soil).

discriminating between the bacteria communities in Mix mesocosms from those of the other plant covers, except for Lep. Note that SWP samples and Noc and Lep samples were separated on the second PCA axis. It is well-known that different plant species can be associated with microbial communities with unique characteristics probably due to the differences in the amount and quality of root exudates (Benizri et al., 2002; Nguyen, 2003; Nunan et al., 2005). The coexistence of multiple plant species enhances the complexity of soil microorganisms by increasing the heterogeneity of organic substrates produced by living roots (Broughton and Gross, 2000). These findings should explain the differences observed in the SSCP profiles between the three mono-cropping mesocosms.

Interestingly, a relatively similar discrimination was observed between hyperaccumulating plant covers, based on physical and biochemical parameter analysis (Fig. 4a) and the genetic structure of the rhizosphere associated bacteria, based on SSCP analysis (Fig. 5).

4. Conclusions

The nature of hyperaccumulating plant covers affects the size and the genetic structure of the bacterial communities in the rhizosphere. Indeed, the coexistence of multiple plant species improves biomass production by each species, changes rhizosphere microorganisms and the abiotic micro-environment. Metal bioavailability did not change between treatment despite changes shown for some microbial data. A combination of different hyperaccumulator plants appears promising in phytoremediation practices, but further research is needed to unravel the links between aboveground hyperaccumulating plants, the belowground rhizosphere microbial communities in metaliferous soils and the implication of these microbial communities in the survival of plants and their ability to extract Nickel. In particular, associations of plants should be tested in pairs, to define the plant cover providing the best phytoextraction.

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References

Bani, A., Echevarria, G., Sulçe, S., Morel, J.L., Mullai, A., 2007. *In-situ* phytoextraction of Ni by a native population of *Alyssum murale* on an ultramafic site (Albania). Plant Soil 293, 79–89.
Bani, A., Echevarria, G., Mullaj, A., Reeves, R.D., Morel, J.L., Sulçe, S., 2009. Nickel hyperaccumulation by Brassicaceae in serpentine soils of Albania and Northwest Greece. Northeast. Nat. 16, 385–404.
Bani, A., Echevarria, G., Sulçe, S., Morel, J.L., 2014. Improving the agronomy of *Alyssum murale* for extensive phytomining: a five-year field study. Int. J. Phytoremediation, http://dx.doi.org/10.1080/15226514.2013.862204.
Benizri, E., Dedourge, O., Di Battista-Leboeuf, C., Nguyen, C., Piutti, S., Guckert, A., 2002. Effect of maize rhizodeposits on soil microbial community structure. Appl. Soil Ecol. 21, 261–265.
Benizri, E., Amiaud, B., 2005. Relationship between plant and soil microbial communities in fertilized grasslands. Soil Biol. Biochem. 37, 2055–2064.
Benizri, E., Nguyen, C., Piutti, S., Slezack-Deschaumes, S., Philippot, L., 2007. Additions of maize root mucilage to soil changed the structure of the bacterial community. Soil Biol. Biochem. 39, 1230–1233.
Broughton, L.C., Gross, K.L., 2000. Patterns of diversity in plant and soil microbial communities along a productivity gradient in a Michigan old field. Oecologia 125, 420–427.
Callaway, R.M., Lawrence, R.W., 1997. Competition and facilitation: a synthetic approach to interactions in plant communities. Ecology 78, 1958–1965.
Cassina, L., Tassi, E., Morelli, E., Giorgetti, L., Remorini, D., Massai, R., Chaney, R.L., Barbafieri, M., 2011. Exogenous cytokinin treatments of a Ni hyper-accumulator, *Alyssum murale*, grown in a serpentine soil: implications for phytoextraction. Int. J. Phytoremediation 13 (S1), 90–101.
Cabello-Conejo, M.I., Centofanti, T., Kidd, P.S., Prieto-Fernández, A., Chaney, R.L., 2013. Evaluation of plant growth regulators to increase Ni phytoextraction by *Alyssum* species. Int. J. Phytoremediation 15 (4), 365–375.
Chaney, R.L., Angle, J.S., Broadhurst, C.L., Peters, C.A., Tappero, R.V., Donald, L.S., 2007. Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. J. Environ. Qual. 36, 1429–1443.
Coinchelin, D., Bartoli, F., Robin, C., Echevarria, G., 2012. Ecophysiology of nickel phytoaccumulation: a simplified biophysical approach. J. Exp. Bot. 63 (2), 5815–5827.
Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245, 35–47.
Echevarria, G., Massoura, S., Sterckeman, T., Becquer, T., Schwartz, C., Morel, J.L., 2006. Assessment and control of the bioavailability of Ni in soils. Environ. Toxicol. Chem. 25, 643–651.
Epelde, L., Becerril, J.M., Blanco, F., Kowalchuck, G.A., Garbisu, C., 2012. Links between pseudomettalophytes and rhizosphere microbial communities in metalliferous soil. Pedobiologia 55, 219–225.
Garbisu, C., Alkorta, I., 2001. Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. Bioresour. Technol. 77, 229–236.
Glick, B.R., 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol. Lett. 25, 11–17.
Glick, B.R., 2010. Using soil bacteria to facilitate phytoremediation. Biotechnol. Adv. 28, 367–374.
Honma, M., Shimomura, T., 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agric. Biol. Chem. 42 (10), 1825–1831.
Kidd, P., Barceló, J., Pilar Bernal, M., Navari-Izzo, F., Poshenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behavior at the root-interface: implications in phytoremediation. Environ. Exp. Bot. 67, 243–259.
Kukier, U., Peters, C.A., Chaney, R.L., Scott Angle, J., Roseberg, R.J., 2004. The effect of pH on metal accumulation in two *Alyssum* species. J. Environ. Qual. 33, 2090–2102.
Lebeau, T., Braud, A., Jézéquel, K., 2008. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: a review. Environ. Pollut. 153, 497–522.
Lindsay, W.L., Norvell, W.A., 1978. Development of DTPA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42, 421–428.
Liu, L., Li, Y., Tang, J., Hu, L., Chen, X., 2011. Plant coexistence can enhance phytoextraction of cadmium by tobacco (*Nicotiana tabacum* L.) in contaminated soil. J. Environ. Sci. 23, 453–460.
Liu, Y.G., Ye, F., Zeng, G.M., Fan, T., Meng, L., Yuan, H.S., 2007. Effects of added Cd on Cd uptake by oilseed rape and pai-tsai co-cropping. Trans. Nonferrous Met. Soc. China 17, 846–852.
Lynch, J.M., Whipps, J.M., 1990. Substrate flow in the rhizosphere. Plant Soil 129, 1–10.
Murakami, M., Ae, N., 2009. Potential for phytoextraction of copper, lead, and zinc by rice (*Oryza sativa* L.), soybean (*Glycine max* [L.] Merr.), and maize (*Zea mays* L.). J. Hazard. Mater. 162, 1185–1192.
Nguyen, C., 2003. Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie 23, 375–396.
Nunan, N., Daniell, T.J., Singh, B.K., Papert, A., McNicol, J.W., Prosser, J.I., 2005. Links between plant and rhizoplane bacterial communities in grassland soils characterized using molecular techniques. Appl. Environ. Microbiol. 71, 6784–6792.
Pan, S.W., Wei, S.Q., Yuan, X., Cao, S.X., 2008. The removal and remediation of phenanthrene and pyrene in soil by mixed cropping of alfalfa and rape. Agric. Sci. China 7, 101–110.

Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by Trees – a review. Environ. Int. 29, 529–540.
Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y., Fierer, N., 2010. The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environ. Microbiol. 12, 2885–2893.
Robinson, B.H., Chiarucci, A., Brooks, R.R., Petit, D., Kirkman, J.H., Gregg, P.E.H., De Dominicis, V., 1997. The nickel hyperaccumulator plant *Alyssum bertolonii* as a potential agent for phytoremediation and phytomining of nickel. J. Geochem. Explor. 59, 75–86.
Sarwar, M., Arshad, M., Martens, D.A., Frankenberger Jr., W.T., 1992. Tryptophan-dependent biosynthesis of auxins in soil. Plant Soil 147, 207–215.
Smaill, S.S., Leckie, A.C., Peter, W., Clinton, P.W., Hickson, A.C., 2010. Plantation management induces long-term alterations to bacterial phytohormone production and activity in bulk soil. Appl. Soil Ecol. 45, 310–314.
Tang, Y.-T., Deng, T.-H.-B., Wu, Q.-H., Qiu, R.-L., Wei, Z.-B., Guo, X.-F., Wu, Q.-T., Lei, M., Chen, T.-B., Echevarria, G., Sterckeman, T., Simonnot, M.-O., Morel, J.-L., 2012. Designing cropping systems adapted to metal contaminated sites: a review. Pedosphere 22, 470–488.
Vallance, J., Le Floch, G., Déniel, F., Barbier, G., Lévesque, C.A., Rey, P., 2009. Influence of *Pythium oligandrum* biocontrol on fungal and oomycete population dynamics in the rhizosphere. Appl. Environ. Microbiol. 75, 4790–4800.
Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19, 703–707.
van der Ent, A., Baker, A.J.M., Reeves, R.D., Pollard, A.J., Schat, H., 2013. Hyperaccumulators of metal and metalloid trace elements: facts and fiction. Plant Soil 362, 319–334.
Viketoft, M., Palmborg, C., Sohlenius, B., Huss-Danell, K., Bengtsson, J., 2005. Plant species effects on soil nematode communities in experimental grasslands. Appl. Soil Ecol. 30, 90–103.
Whiting, S.N., Leake, J.R., McGrath, S.P., Baker, A.J., 2001. Hyperaccumulation of Zn by *Thlaspi caerulescens* can ameliorate Zn toxicity in the rhizosphere of *Thlaspi arvense*. Environ. Sci. Technol. 35, 3237–3241.
Wöhler, I., 1997. Auxin-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography. Microbiol. Res. 152, 399–405.