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Abstract.
The objective of this work was first to compare the cropping of single hyperaccumulator plant species (either *Alyssum murale*, *Noccaea tymphaea*, *Leptoplax emarginata* or *Bornmuellera tymphaea*) with co-cropping of all four species on the efficiency of Ni extraction from a Ni-rich ultramafic soil. Also, the effects on soil physicochemical properties and on microbial communities' changes colonizing the rhizosphere were evaluated. Secondly, this study aimed at evaluating the effect of selected rhizobacterial strains, isolated from the rhizosphere of one of the different natural plant associations (*B. tymphaea – N. tymphaea)*, on plant biomass production and nickel phytoextraction by these two hyperaccumulator plants. The screening of isolates from this plant association for their PGPR traits revealed one PGPR strain (NB24). The genetic characterization showed that it was similar to *Variovorax paradoxus*. After 6 months of culture, biomass and quantities of nickel in the plants were assessed. The results showed significant improvement of root growth and an increase of Ni uptake when plants were inoculated with this PGPR strain. The combination *Noccaea-Bornmuellera* inoculated by the PGPR strain *Variovorax* NB24, seemed to be a good choice for an efficient phytoextraction *in situ.*

Keywords. Nickel, phytomining, hyperaccumulor plants, rhizobacteria, rhizosphere, PGPR.

1 Introduction

Heavy metals, such as nickel (Ni), are non-biodegradable (Garbisu and Alkorta 2001) and bioremediation techniques based on plants and associated rhizosphere microorganisms offer an environmental friendly method for cleaning-up metal-contaminated soils and for extracting valuable metals (Chaney 1983; Baker et al. 1994; Lucisine et al. 2014). Plant diversity and composition induce a variety of rhizodeposits (Benizri and Amiaud 2005), rendering thus the diversity of soil bacterial communities and microbial functional groups closely related to the community and diversity of plants (Wardle et al. 2004; Benizri and Amiaud 2005). Few studies 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. Studies up to now mainly concerned crop associations (Murakami and Ae 2009; Liu et al. 2011). Moreover, a few studies dealt with the effect of the combination of metal hyperaccumulator plants with other species (Wu et al. 2007; Jiang et al. 2010). Most of these experiments showed that co-cropping with non-hyperaccumulator plants enhanced the growth of the hyperaccumulator and increased the metal extraction and often improve the living condition of less metal-tolerant plants.

Therefore, the objective of the present work was first to compare the cropping of single hyperaccumulator plant species (either *A. murale*, *N. tymphaea*, *L. emarginata* or *B. tymphaea*) with co-cropping of all four species. The effect of all treatments on the efficiency of Ni extraction from a serpentine soil (*i.e.* naturally nickel-rich) and on the rhizosphere microbial community changes was studied.

Secondly, this study aimed at evaluating the effect on plant biomass production and nickel phytoextraction, of selected rhizobacterial strains, isolated from the rhizosphere of one of this natural plant association (*B. tymphaea – N. tymphaea*). Sixty eight nickel resistant bacterial strains were isolated from the rhizosphere of a serpentine natural association of *B. tymphaea – N. tymphaea*. Then, bacterial isolates were screened for their PGP capacities. This hyperaccumulator plant association was inoculated with one selected bacterial isolate screened for its PGP capacities (IAA, siderophores, ACCd production). The effects of bacterial inoculants on soil metal availability, plant growth and Ni extraction were evaluated.

2 Materials and methods

2.1 Soil characteristics and experimental design

A 120-d 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-east France, 07°06'42.2"E, 48°11'03.7"N). 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·kg^{-1} N, a C/N ratio of 12.6, a Mg/Ca ratio of 8.6 and a phosphorus (P) content of 11 mg·kg^{-1}. Developed on a serpentinized harburgite, this soil was naturally rich in nickel (Ni) and the total Ni content was 1480 mg·kg^{-1}. The mesocosms (3 kg of soil per mesocosm) were planted with plant species considered separately (monospecific cover: *Leptoplax emarginata* (Bois) O.E. Schulz, *Noccaea tymphaea* (Hausskn.) F. K. Mey. *Bornmuellera tymphaea* Hausskn or *Alyssum murale* Waldst. & Kit) and combined (multispecies cover). Total plant density was 8 per mesocosm. Control mesocosms were not planted. The experiment had a completely randomized block design with seven replicates that had the following treatments: L: *Leptoplax*; N: *Noccaea*; A: *Alyssum*; B: *Bornmullera*; LNAB: a mixture of the four species; SWP: soil without plant. 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 adjusted to 75% of soil water holding capacity with water. Mesocosms were kept in the growth chamber for four months.

2.2 Biotic parameters

Ni concentrations in plant parts. After four months of culture, plant parts were collected, and respective dry weights were recorded. Ni concentration in shoots and roots was measured by ICP-AES (Liberty II, Varian) after acid digestion. A Bioaccumulation Coefficient (BAC) was employed to qualify heavy metal accumulation efficiency in plants (BAC = Cp/Cs, where, Cp and Cs are heavy metal concentrations in plant parts and in soil at the beginning of the experiment; ie.1480 mg.kg^{-1}), (Zayed et al. 1998). Heavy metal root-to-shoot translocation factor was calculated (TF = Cs/Cr, where, Cs and Cr are metal concentrations in the shoot and root), (Tappero et al. 2007).

Soil microbial analysis. At the end of the experiment, 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 (MB-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 Smaill *et al.* (2010). 1-aminocyclopropane-1-carboxylate deaminase (ACCd) activity was determined based on the method described by Honma and Shimomura (1978) for measuring ACC deaminase in cell extracts. ß-glucosidase (EC 3.2.1.21), arylsulphatase (EC 3.1.6.1), acid phosphatase (EC 3.1.3.2) and urease (EC 3.5.1.5) activities were determined according to Tabatabai (1982). Spectrophotometric determination of the hydrolysis of fluorescein diacetate (FDA) was also used to determine microbial activity in soil.

2.3 Abiotic parameters

Moisture content of the soil samples was determined at 105°C until a constant weight was achieved. 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) (Lindsay and Norvell, 1978) and Ni concentration in solution was measured by ICP-AES. Soil pH was measured using a pH meter in a soil/water solution mixture (soil water ratio 1:5).

2.4 Genetic and functional (ie. PGP capacities) characterization of nickel resistant rhizobacteria isolated from rhizosphere soil

Bacteria strains were isolated from the rhizosphere soil of the natural hyperaccumulator plant association: *B. tymphaea – N. tymphaea* (NB). This natural association was found at Katara Pass (1700 m) in Greece (39 ° 47'765" N, 21 ° 13'739" E). A sample (3 g) of the rhizosphere soil was used for the determination of the number of culturable bacteria as colony forming units (CFU) by spread-plating them onto TSA supplemented with various concentrations of Ni from a filter sterilized stock solution. Nickel was added as $NiSO_4$, $6H_2O$ at concentrations of 0.5, 5, 7.5 and 10 mM. Agar plates were then incubated in the dark at 27°C for 12 days. From the plates containing Ni concentration of 7.5 mM, we carried a collection of strains. Then, each isolate was cultured in 6 ml of NB medium for 15 hours on shaker table (120 rpm) at 30°C and finally stored individually in 20% sterile glycerol and kept in the freezer (-20 ° C). Then, each isolate was cultured in 8 ml of NB medium for 48 hours on shaker table (120 rpm) at 27°C. After centrifugation, DNA was extracted from the pellet with the FastDNA®SPIN Kit (MP Biomedicals) in accordance with the manufacturer's instructions. The 16S rDNA genes were amplified with universal primers (Oligold, Eurogentec) 27f (5'-GAGAGT TTG ATC CTG GCT CAG-3', positions 8–27 of *Escherichia coli* 16S rDNA) and 1492r (5'-CTA CGG CTA CCT TGT TAC GA-3', positions 1492–1513 of *E. coli* 16S rDNA) (Gürtler and Stanisich 1996). DNA amplification was carried out in a thermocycler (i-cycler, BioRad) using the following conditions: 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, plus an additional 15-min cycle at 72°C. All the 16S rDNA PCR products were sequenced by GATC Biotech. DNA sequences were edited and screened against those in the GenBank database using BLASTn (http://www.ncbi.nlm.nih.gov/). Each isolates were then screened for various plant growth promoting (PGP) characteristics: siderophore production, IAA and ACCd production.

2.5 Influence of Ni resistant PGPR on plant growth and Ni uptake

After 4 months of culture of the hyperaccumulator plant association *B. tymphaea-N. tymphaea* (inoculated 'NBi' or not inoculated 'NBni'), plant parts were collected and their dry weights were recorded. Ni concentration was measured by ICP-AES after acid digestion. Bioaccumulation Coefficient (BAC), Bioconcentration factor (BFC) and heavy metal translocation (TF) were

estimated (Tappero et al. 2007).

2.6 Statistical analysis

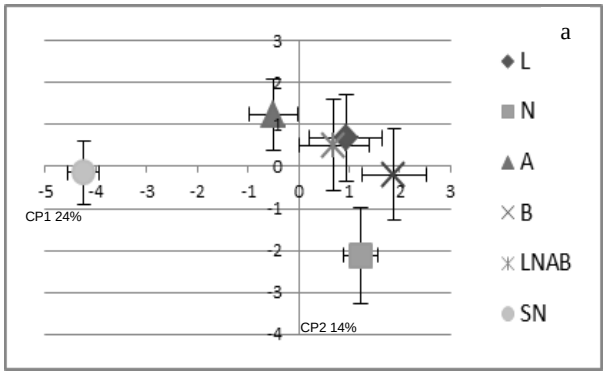
Variance analysis was carried out on all data (one-way ANOVA). All data were submitted to principal component analysis (PCA). Mean coordinates of individuals were calculated for the first two principal components (PC1, PC2) and compared by variance analysis (one-way ANOVA). The software used for all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

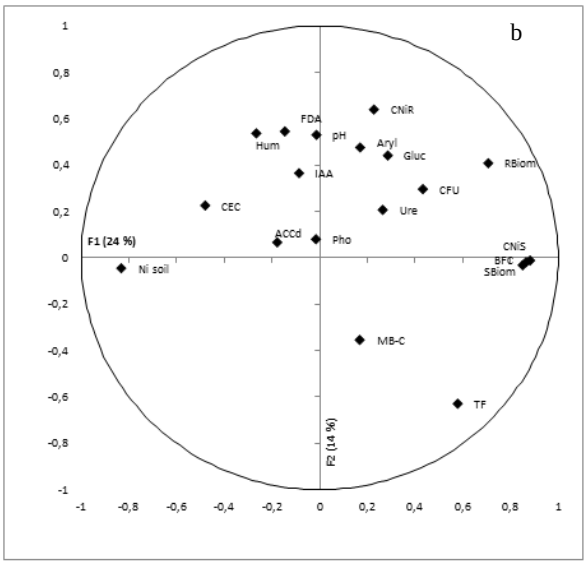
3 Results

3.1 Effect of mono- and multispecies covers on physicochemical and microbiological parameters

To consider all parameters together, we carried out another Principal Component Analysis (PCA) based on physicochemical and biological variables (Figure 1). Axis 1, which explained 24% of total variability, strongly discriminated unplanted soils (SWP), (negative abscissa), from planted mesocosms (positive abscissa) (Figure 1a). Thus, SWP were clearly opposed to the different cover types and in particular those of B and N. Axis 2, which represented 14% of the total variability, clearly discriminated monospecific cover of N from the other covers (A, L, B, LNAB). If we focus on the explanatory variables (Figure 1b), it appears that the presence of a cover, either single or multi-species, caused a decrease in the concentration of extractable soil Ni (PC1). Correspondingly, we noted that the concentrations of Ni in shoots ('CNiS') were significantly inversely correlated (R = -0.70, p <0.05) to the quantities of Ni present in soil. B, which had accumulated the highest amount of Ni in shoot, was the species for which the rate of Ni in soil was the lowest. In addition, it is clear from our analysis that the more important the plant biomass, the greater the concentrations of Ni in shoots (R = 0.56, p<0.05). This was especially marked in order of importance for B> N> L and LNAB> A. PC2 seems to discriminate vegetation cover according to the pH values of the different soils. The N rhizosphere showed the lowest pH values unlike all the other covers. We noted, however, a significant inverse correlation between the concentration of Ni in the roots (C Ni R) and translocation factor TF (R = -0.42, p<0.05). We also observed a positive correlation between pH and CFU (R = 0.32, p<0.05) and a significant correlation between CFU and most measured microbial activities (Ure, Pho, Gluc, R = 0.28, p<0.10). Among the microbial activities measured, ACCd activity was significantly correlated to the concentration of Ni in the roots (R = 0.29, p<0.10). Finally, the size of cultivable bacterial communities appeared to be favored by the presence of a vegetation cover and positively correlated with root biomass (R Bio, R = 0.35, p<0.05).

Figure 1. (a) Ordination plot of soil samples, generated by Principal Component Analysis of the physicochemical and microbiological parameters. Points represent means of seven replicate samples (L: *Leptoplax*; N: *Noccaea*; A: *Alyssum*; B: *Bornmuellera*; LNAB: mixture of the four species; SWP: soil without plant). **(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. 2h^{-1}), Gluc: ß-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 (%), CNiR et CNiS: 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 (Unity forming colony) (log10 cfu.g^{-1}dry soil), TF: Translocation factor, BCF: Bioconcentration factor).



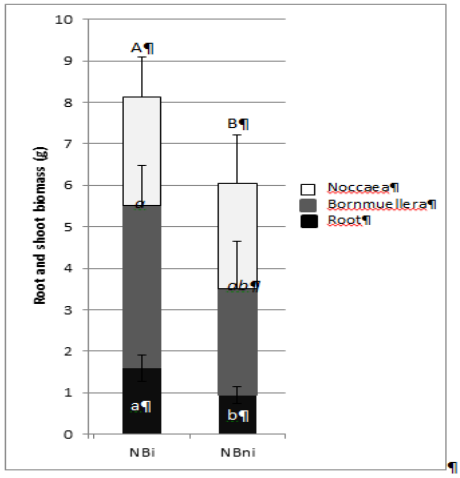


3.2 Influence of Ni resistant PGPR on plant growth and Ni uptake

Root biomass of the cover NBi was significantly higher than the non-inoculated cover (NBi: 1.6 g; NBni: 0.9 g) (Figure 2). Shoot biomass was always higher than root biomass. The total biomass of the inoculated cover NBi was significantly higher than the total biomass of non-inoculated cover. There was no significant difference between inoculated and non-inoculated shoot biomass, but we observed an increasing trend of shoot biomass

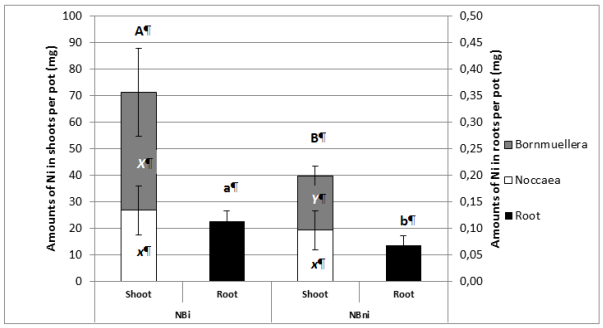
following inoculation (NBi: 6.5 g; NBni 5.1 g). Moreover, it appeared that the inoculation promoted preferentially the development of one of the two species in the plant association. Indeed, the shoot biomass of B (in the association NBi) were higher than B (in the association NBni).

Figure 2. Root (black bar) and shoot (grey and white bars) biomass (g) per cover. Mean values followed by different lowercase (root), italic (shoot) or capital letters (total biomass) are significantly different at $p<0.05$ (Newman-Keuls test), (n = 4).



The amount of Ni in NBi root was significantly higher than that in NBni root (Figure 3). In the case of aerial parts, it appeared that the amount of Ni in the shoots of the inoculated plant association was also significantly higher than that in non-inoculated cover. Moreover, the inoculation increased Ni phytoextraction in particular by B.

Figure 3. Amounts of Ni phytoextracted in shoots (grey and white bars) and roots (black bar) per pot (mg). Mean values followed by different lowercase (root), lower italic (*Noccaea* shoot), upper italic (*Bornmuellera* shoot) or uppercase (total shoot biomass) are significantly different at $p<0.05$ (Kruskal test), (n = 4).



4 Conclusion

This study identified an efficient candidate strain which could be useful for future field-based trials. Indeed, we have seen that plant growth-promoting effects by associated bacteria, mainly the PGPR *Variovorax* NB24 isolated from a natural nickel-rich soil, can significantly improve plant association performance (e.g. *Noccaea* and *Bornmuellera*) and result in higher amounts of phytoextracted Ni.

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