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REGULAR ARTICLE

Improving nickel phytoextraction by co-cropping hyperaccumulator plants inoculated by plant growth promoting rhizobacteria

A. Durand · S. Piutti · M. Rue · J. L. Morel · G. Echevarria · E. Benizri

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Abstract
Aim Phytomining is the conception of agro-metallurgical production chains based on cropping hyperaccumulator plants on contaminated or naturally rich (ultramafic) soils to produce high value metal compounds. This study aimed to evaluate the effect of enhancing multispecies hyperaccumulator covers with Plant Growth Promoting Rhizobacteria (PGPR) on Ni phytoextraction.
Method Plant growth promoting rhizobacteria were isolated from the rhizosphere of two different plant associations (*Bornmuellera tymphaea* - *Noccaea tymphaea* (NB) and *Bornmuellera tymphaea* - *Alyssum murale* (AB)) collected from natural conditions. The screening of isolates from both plant associations for their PGPR traits revealed two PGPR strains (AB30 and NB24) affiliated to *Variovorax paradoxus*. Inoculation of mesocosms containing the same plant associations with these selected PGPR was performed after 5 months of culture. The biomass of the different plant parts and respective concentrations of nickel in the plants were recorded 1 month after inoculation.
Results Root biomass of the inoculated plant associations was significantly higher than the respective non-inoculated ones. The total biomass of the inoculated plant association *Bornmuellera tymphaea* - *Noccaea tymphaea* was significantly higher than the total biomass of all others. Similarly, shoot biomass of the inoculated plant association *B. tymphaea* - *N. tymphaea* was significantly higher than that of all other covers. Results showed an increase in Ni uptake when plants were inoculated with PGPR strains, when compared with uninoculated plant associations. According to the plant cover, the inoculation increased Ni amounts in roots of 105.8 and 66.4 % respectively in ABi and NBi covers, and 39.9 and 79.6 % in the shoots.
Conclusion The combination of the hyperaccumulator plants *N. tymphaea-B.tymphaea* inoculated by one of the two PGPR strains (strain NB24), isolated from the rhizosphere of this mixed cover, seemed to be an interesting option for an efficient Ni phytoextraction to be tested in the field.

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A. Durand · M. Rue · J. L. Morel · G. Echevarria · E. Benizri
Laboratoire Sols et Environnement, Université de Lorraine, UMR 1120, 2 avenue de la Forêt de Haye, TSA 40602, Vandoeuvre-lès-Nancy F-54518, France

A. Durand · M. Rue · J. L. Morel · G. Echevarria · E. Benizri (✉)
INRA, Laboratoire Sols et Environnement, UMR 1120, 2 avenue de la Forêt de Haye, TSA 40602, Vandoeuvre-lès-Nancy F-54518, France
e-mail: emile.benizri@univ-lorraine.fr

S. Piutti
Laboratoire Agronomie et Environnement, Université de Lorraine, UMR 1121, 2 avenue de la Forêt de Haye, TSA 40602, Vandoeuvre-lès-Nancy F-54518, France

S. Piutti
INRA, Laboratoire Agronomie et Environnement, UMR 1121, 2 avenue de la Forêt de Haye, TSA 40602, Vandoeuvre-lès-Nancy F-54518, France

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Introduction

In the last two decades, phytomining has been successfully developed using plants with high biomass potential in order to recover Ni in an economical way (Chaney et al. 2008; Bani et al. 2015; van der Ent et al. 2015). Recently, phytoextraction of metals has benefited from work on the role of microorganisms in the rhizosphere: remediation of soil contaminated by heavy metals through hyperaccumulating plants associated with plant growth promoting rhizobacteria (PGPR) (Zhuang et al. 2007; Ma et al. 2009; Lebeau et al. 2008; Glick 2010; Sessitsch et al. 2013; Cabello-Conejo et al. 2014). The increase both of the rate of metals accumulated by plants and the biomass yield of crops in low-fertile soils (a deficiency in essential nutrients such as N, P and K) could still be improved by engineering rhizosphere microorganisms (Lebeau et al. 2008; Cabello-Conejo et al. 2014). Among these microorganisms, some promote plant germination and facilitate the development of root biomass via the production of hormone-like molecules. Others also promote the resistance of plants to the stress exerted by the pollutant via the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, with a consequent decrease in the synthesis of ethylene in plant tissues. All these effects lead to better plant development (Zhuang et al. 2007; Lebeau et al. 2008; Glick 2005, 2010; Ma et al. 2011; Cabello-Conejo et al. 2014) and improve plant growth, biomass production and/or metal uptake and accumulation (Burd et al. 1998, 2000; Belimov et al. 2005; Abou-Shanab et al. 2006; Lebeau et al. 2008; Ma et al. 2009; Glick 2010; Rajkumar et al. 2012; Cabello-Conejo et al. 2014; Visioli et al. 2014). Moreover, it has been shown that rhizobacteria increase soil nickel (Ni) availability and hyperaccumulation of Ni in *Alyssum murale* (Abou-Shanab et al. 2003, 2006). Nevertheless, in many cases the effects of these plant-microbial associations have been shown to be plant-species specific (Becerra-Castro et al. 2012), which underlines the importance of the origin of strains and the choice of plants inoculated. Until now, PGPR selected for their effect in increasing Ni phytoextraction yields have only been screened from mono-specific hyperaccumulator covers (Abou-Shanab et al. 2006; Cabello-Conejo et al. 2014; Visioli et al. 2014). All these previous works only concerned the effects of PGPR on mono-species vegetation covers. But, it has been shown that multi-species vegetation covers both in associations of non-accumulator with hyperaccumulator plants (Gove et al. 2002; Liu et al. 2007; Wu et al. 2007; Jiang et al. 2010; Wei et al. 2011) or in association of hyperaccumulator species only (Lucisine et al. 2014; Rue et al. 2015) induced a better Ni phytoextraction than mono specific hyperaccumulator covers.

So, multi-species associations of hyperaccumulator plants exclusively, assisted by specific bacterial strains which present plant growth promoting (PGP) capacities could be a good alternative for soil phytomining, should the hypothesis that inoculation improves metal phytoextraction be proven. Our previous work (Rue et al. 2015) showed that the combination of two Ni hyperaccumulators (*Noccaea tymphaea* (Hausskn.) F. K. Mey and *Bornmuellera tymphaea* Hausskn) improved the efficiency of phytoextraction of this metal. This work underlined the fact that this multi-species cover also promoted the development and activity of rhizosphere microorganisms. In addition, field observations in 2013 (Katara Pass, Greece) demonstrated that such plant associations exist spontaneously on soils naturally rich in nickel. This is the case not only for the combination of *B. tymphaea* with *N. tymphaea,* but also for *B. tymphaea* with *A. murale* Waldst. & Kit.

This study aimed at evaluating the effect of selected rhizobacterial strains, isolated from the native rhizosphere of the two plant associations in field conditions, both on plant biomass production and Ni phytoextraction by these hyperaccumulator plant associations (*B. tymphaea – N. tymphaea* and *B. tymphaea – A. murale)*. Firstly, 68 nickel-resistant bacterial strains were isolated from the rhizosphere of a natural serpentine association of *B. tymphaea – N. tymphaea* and *B. tymphaea – A. murale.* Secondly, bacterial isolates were screened for their PGP capacities and for genetic diversity. Thirdly, these two hyperaccumulator plant associations, *ie. B. tymphaea – N. tymphaea* and *B. tymphaea – A. murale* were grown in a naturally Ni-rich serpentine soil, which was inoculated with the best two selected bacteria screened for PGP capacities (indole-3-acetic acid (IAA), siderophores, 1-aminocyclopropane-1-carboxylate deaminase (ACCd) production). The effects of bacterial inoculants on soil metal availability, plant growth, Ni accumulation and extraction were assessed.

Material and methods

Genetic and functional (i.e., PGP capacities) characterization of nickel resistant rhizobacteria isolated from rhizosphere soil

Bacterial strains were isolated from the rhizosphere soil of two hyperaccumulator plant associations collected under natural field conditions: *B. tymphaea – A. murale* (AB, soil 1) and *B. tymphaea – N. tymphaea* (NB, soil 2) (Table 1). These soils have already been studied (total Fe 7.5 %, total Ni 1160 ppm, total Co 102 ppm and total Mn 1820 ppm) (Bani et al. 2009). These two natural associations were found at the Katara Pass (1700 m) in Greece (39 ° 47.765′ N, 21 ° 13.739′ E) and the rhizosphere soil, defined as the root adhering soil, was collected in July 2013. A sample (3 g) of the rhizosphere soil, for each plant association, was dropped into a flask containing 30 ml phosphate buffer (PBS: 8 g NaCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter, pH 7.2). All the flasks were then placed on an orbital shaker (17 rpm) for 45 min. A 1-ml aliquot per each sample was transferred into a sterile test tube previously filled with 9 ml PBS. Microbial suspensions were serially diluted and the dilutions (10^{-1}–10^{-3}) were used for the determination of the number of culturable bacteria (i.e., number of colony-forming units (CFU)), by spread-plating them onto TSA 10 % (Tryptone Soy Agar, Difco) supplemented with various concentrations of Ni from a sterilized stock solution. Nickel was added in the form of $NiSO_4$, $6H_2O$ at concentrations of 0.5, 5, 7.5 and 10 mM (Aboudrar et al. 2007). Three replicates for each dilution were prepared. The agar plates were then incubated in the dark at 27 °C for 12 days. The number of bacteria was expressed as log CFU g^{-1} of soil dry weight. The plates containing Ni concentrations of 7.5 mM were gathered into a collection of strains. For this, each colony was isolated and purified by subculture on TSA 10 % (amended with 7.5 mM $NiSO_4$, $6H_2O$). Then, each isolate was cultured in 6 ml of Nutrient Broth medium (VWR, France) for 15 h on a shaker table (120 rpm) at 30 °C. Finally, each isolate was stored individually in 20 % sterile glycerol and kept in the freezer (−20 ° C).

Each isolate was then cultured in 8 ml of NB medium for 48 h on a 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 from 50 ng of DNA in a final volume of 50 μl containing 5 μl of Taq Polymerase buffer 10U, 1.25 U of Taq Polymerase (MP Biomedicals, France), 200 μM of each dNTP (MP Biomedicals, France), 1.5 mM $MgCl_2$, 0.5 μM of each universal primer (Oligold, Eurogentec) 27f (5′-GAG AGT 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) under 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 the GenBank database using BLASTn (http://www.ncbi.nlm.nih.gov/). The sequences of the two strains AB30 and NB24 used for inoculation have been deposited in GenBank under accession numbers KP844879 and KP844880, respectively.

Each isolate was then screened for various plant growth promoting (PGP) characteristics, namely siderophore production, IAA and ACCd production. The IAA production of each bacterial strain was determined by the use of Salkowski's reagent (1 ml $FeCl_3$ 0.5 M in 50 ml $HClO_4$ 35 %) as described by Barillot (2012). In addition, to compare the

Table 1 Chemical and physical properties[a] of the rhizosphere soil of the natural mixed covers (*B. tymphaea – A. murale* (soil 1) and *B. tymphaea – N. tymphaea* (soil 2) from Katara Pass (Greece)

Soil	pH (water)	Organic carbon ($g.kg^{-1}$)	Total nitrogen	P_{Olsen}	$Fe_{Oxalate}$ %	Ni_{DTPA} ($mg.kg^{-1}$)	Fe_{DTPA}	Mn_{DTPA}	Ni Oxalate	Ni CaCl ($\mu g.kg^{-1}$)$_2$
Soil 1	6.84	24.9	2.08	0.04	1.14	153	65.4	13.7	685	9280
Soil 2	6.91	76.0	7.15	0.02	3.09	467	147	12.0	1490	18700

[a] (analyzed by INRA, Arras, FRANCE)

production of IAA by the different bacterial strains, the quantity of proteins in the bacterial pellet obtained after centrifugation was estimated (Bio-Rad Protein Assay) and for each bacterial strain, the result was expressed in mg IAA mg protein^{-1} h^{-1}. The siderophore production of each bacterial strain was determined by the use of chrome azurol sulfonate (CAS) agar medium (Schwyn and Neilands 1987; Alexander and Zuberer 1991). After 5 days of incubation on CAS medium at 30 °C, a red-orange halo around the colony demonstrated the production of siderophores. For each strain, the diameter of the halo was measured and expressed in millimeters. Rhizobacterial isolates were tested for their ability to utilize ACC as sole source of N by using 96-well plates. The OD values of ACC and $(NH_4)_2SO_4$ wells were compared to $MgSO_4$ to determine the ability of this rhizobacteria to utilize ACC from the medium as described by Jacobson et al. (1994) and Shahzad et al. (2010).

Soil characteristics and experimental design

A 6 months mesocosm study was carried out with soil collected from the top layer (10–40 cm) of a natural forest ultramafic Hypereutric Cambisol Hypermagnesic (Vosges Mountains, east France, 07°06′42.2″E, 48°11′ 03.7″N), which does not host any hyperaccumulator plant. Immediately after collection, the soil was sieved to <5 mm and stored at 4 °C for less than 7 days until soil physicochemical and microbiological analysis were carried out. Soil physicochemical properties were determined by the Soil Analysis Laboratory of INRA (Arras, France). Developed on a serpentinized harburgite, this soil was naturally rich in nickel (Ni) and the total Ni content was 1480 mg·kg^{-1}. It contained 317 g·kg^{-1} soil clay, with an organic carbon content of 59.3 g·kg^{-1}, it had 4.72 g·kg^{-1} N, a C/N ratio of 12.6, a Mg/Ca ratio of 8.6 and an available phosphorus content (Olsen P) of 11 mg·kg^{-1}. The soil pH was 6.14.

Hyperaccumulator seeds had been collected in Greece: *Noccaea* seeds were taken on July 19, 2011 at the Katara Pass (1700 m; 39°47′765″N, 21°13′739″E). *Alyssum* and *Bornmuellera* seeds were taken on July 20, 2011 near the village of Trigona (830 m; 39°47′223″N, 21°15′869″E). Each mesocosm (13×24×16 cm; l×L×h) containing 1717 g of soil (on the DW basis) were sown with the two following plant associations: *Noccaea tymphaea* with *Bornmuellera tymphaea (ie.* NB cover) and *Alyssum murale* with *B. tymphaea (ie.* AB cover). Total plant density was 8 individuals per mesocosm. Control mesocosms consisted in soil without plant association (soil without plant: SWP). Just before sowing, a nitrogen supply equivalent to 50 kg ha^{-1} was provided by adding a solution of NH_4NO_3.

The experiment had a completely randomized block design with four replicates of the following seven treatments: ABi and ABni: AB association being respectively inoculated or non-inoculated with a PGPR isolated from the rhizosphere of this natural hyperaccumulator plant association; NBi and NBni: NB association respectively inoculated or non-inoculated with a PGPR isolated from the rhizosphere of this natural hyperaccumulator plant association; SWPiAB and SWPiNB: control soil without plant respectively inoculated with a PGPR isolated from the rhizosphere of the corresponding natural hyperaccumulator plant association AB or NB; SWPni: control soil without plant and without inoculation. Mesocosms were transferred to a 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 daily to 75 % of soil water holding capacity. Mesocosms were kept in the growth chamber for 6 months.

Inoculation of all inoculated treatments was achieved 1 month before harvest: ABi and SWPiAB mesocosms were inoculated with a PGPR AB (accession number KP844879) and NBi and SWPiNB mesocosms were inoculated with a PGPR NB (accession number KP844880). Fresh cultures of each bacterial strain (AB or NB) were grown overnight in 30 ml NB medium. Then, 5 ml of the culture were transferred to 300 ml of Nutrient Broth medium for 24 h, harvested by centrifugation (7000*g*, 10 min). The bacterial pellet was rinsed three times in sterile PBS to remove all traces of culture medium. Each mesocosm was inoculated with 16 ml (2 ml of the bacterial suspension near the base of the stem of each plant) of a bacterial suspension (about 10^8 cells per ml). Thus, each mesocosm was inoculated with 9.3 10^5 CFU g^{-1} dry soil.

Influence of Ni resistant PGPR on physicochemical and biological parameters, plant growth and Ni uptake

One month after PGPR inoculation, plants were harvested and different parameters were measured on plant parts and rhizosphere soil.

Physicochemical parameters

The 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 a 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) and the Ni concentration in solutions was measured with an Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES, Liberty II, Varian) and expressed in mg per kg dry soil. Soil pH was measured using a pH meter in a soil/water solution mixture (soil water ratio: 1/5). CEC was determined using samples that had previously been air-dried and sieved (2-mm mesh), following French standard method (ISO 23470:2007) which is measured without adjusting soil pH. Briefly, 2.5 g of dry soil were mixed with 20 ml of hexamminecobalt trichloride solution (Co $(HN_3)_6$ Cl_3, 0.0167 mol.l^{-1}). The mixture was stirred 1 h, then filtered and finally measured with a spectrometer (Pharmacia Biotech, NovaspecII) at 475 nm.

Microbiological parameters

Determination of auxin-like compounds and production of ACC deaminase 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). The amount of auxin-like compound content in soil was reported as IAA-equivalents (mg kg^{-1} dry soil h^{-1}) using standard IAA solutions. ACC deaminase 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 amount of ACCd was reported as μmol α-ketobutyrate g^{-1} dry soil h^{-1}, using standard α-ketobutyrate solutions.

Microbial community-level physiological profiles (CLPPs) Bacterial communities were characterized by their metabolic fingerprints, using Biolog Ecoplate™ microplates (Biolog Inc., Hayward, CA.). The Biolog Ecoplate™ microplate contains 31 of the most useful carbon sources for soil community analysis. Microplates were inoculated with 150 ml of the 10^{-2} soil suspension using a 12-channel repeating pipette. After incubation of the plates for 5 days, the color development was automatically recorded at 590 nm by a microplate reader (Synergy™HT, BioTk). Absorbance values for the wells containing carbon sources were blanked against the control well. All absorbance values up to 0.25 were set to zero. Overall color development expressed as average well color 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 in order to minimize the influence of the inoculum density differences between soils (Garland and Mills 1991). Richness (S), Shannon's diversity index (H) and Evenness (E) (Magurran 2003) were calculated from data on CLPPs.

Plant growth and Ni uptake

At harvest, plant roots, stems and leaves were collected, separated and carefully washed with deionized water, oven-dried at 70 °C for 72 h; then dry weights were recorded. For roots, the root system of the association was collected as a whole (the roots of the two plant species could not be separated in our experiment) whereas for shoots, stems and leaves were collected individually for each species of the association. 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) and completed to 25 ml with deionized water. Ni concentration in solution was measured with an Atomic Emission Spectrometer (ICP-AES, Liberty II, Varian). The Bioconcentration factor (BCF total) can be employed to qualify heavy metal accumulation efficiency in plants by comparing the concentration in the plant parts (roots, stems or leaves) and external medium such as soil (Zayed et al. 1998). The Bioconcentration factor (BCF DTPA-Ni) was estimated by the following ratio: Ni concentration in shoot/soil Ni-DTPA. Heavy metal translocation (TF) from root to shoot in plants was calculated as described by Tappero et al. (2007). Further, TF>1 means that plants translocate heavy metals from roots to the shoots.

Stastistical analyses

Variance analysis was carried out on all data (one-way ANOVA). The software used for all statistical analyses was StatBox software (Grimmersoft, Paris, France, http://www.statbox.com).

Results

Isolation of Ni-resistant bacteria and screening of plant growth-promoting features of Ni-resistant bacteria

For both natural rhizosphere soils (NB or AB rhizosphere), a decrease in the number of culturable bacteria was noted when the concentration of Ni in the medium increased (data not shown). Independently of the rhizosphere soil studied, this decrease was significant at every concentration step tested (between 0.5 and 5, 5 and 7.5, 7.5 and 10 mM). Thus, for NB rhizosphere soil, the size of the nickel-resistant bacteria decreased from 7.28 log $CFUg^{-1}$ dry soil at $NiSO_4$ concentration of 0.5 mM to 5.51 at 10 mM. Similarly, for AB rhizosphere soil, we observed almost the same significant decrease in the number of culturable bacteria from 7.39 log CFU g^{-1} dry soil at 0.5 mM of $NiSO_4$ to 5.35 at 10 mM. A total of 68 bacterial isolates (27 from AB rhizosphere and 41 from NB rhizosphere) was picked randomly among the nickel-resistant colonies grown on TSA 10 % (amended with 7.5 mM $NiSO_4$, $6H_2O$).

All the 27 Ni resistant strains isolated from AB rhizosphere displayed a positive siderophore activity, as indicated by the development of an orange-colored zone on CAS agar plates. The diameter of the zone varied from 9 to 25.3 mm (data not shown). Among these strains, we selected only those which were able to produce a zone larger than 18.3 mm (Table 2). Similarly, only 8 of the 41 Ni-resistant strains isolated from NB rhizosphere showed an orange-colored zone larger than 18.3 mm. Further screening of the production of IAA by Ni-resistant bacteria, indicated that among the bacterial strains previously selected, only 5 AB strains and 3 NB strains produced more than 80 $mg.mg^{-1}$ protein.h^{-1}. In addition to IAA production, among the 8 selected bacterial strains (5 AB strains and 3 NB strains), all grew on minimum salt medium with ACC as the sole source of nitrogen. Finally, the two strains with the best profile in relation with plant growth promoting potential were recorded as AB30 and NB24. These two strains both belonged to *Variovorax paradoxus*.

Influence of Ni resistant PGPR on soil physicochemical parameters

Soil pH was 5.97 before sowing (ST0) (Table 3). At harvest, we found, regardless of treatments, a statistically significant but light decrease in pH of about 0.15 pH unit in the case of planted soils and more pronounced (0.31 unit) in unplanted soils, independently of the PGPR inoculation. Thus, this acidification was significantly more pronounced in the case of unplanted soils.

The unplanted treatment had a slightly higher cation exchange capacity (CEC) (Table 3) in comparison with planted soils, whether inoculated or not.

Unplanted soils had a significantly higher concentration of DTPA-extractable nickel (87.0, 89.5 and 87.7 $mg.kg^{-1}$ for SWPiAB, SWPiNB and SWPni, respectively) than planted soils independently of the plant association and the inoculation (ABi: 72.7 $mg.kg^{-1}$; ABni: 76.1 $mg.kg^{-1}$; NBi: 69.8 $mg.kg^{-1}$ and NBni: 73.9) (Table 3). Whatever the soil (planted or unplanted), no significant effect of inoculation was evidenced on the concentration of DTPA-extractable Ni.

Influence of Ni-resistant PGPR on microbiological parameters

Diversity index were calculated with data from Biolog Ecoplates™ (Table 4). In the case of AWCD, this was significantly lower in the case of non-planted soils, inoculated or not. The number of substrates, for which absorbance was recorded above 0.25, was higher with planted soils. Accordingly, there was a significantly lower Richness (*R*) in unplanted than in planted soils: the presence of plant associations induced an increase in the metabolic capacity of soil bacterial communities (*R* cover > *R* soil without plant). In detail, there was no significant difference between plant associations, inoculated or not. The effect of inoculation on the number of substrates oxidized by the bacterial community isolated from rhizosphere soil, was not significant. However, the bacterial communities from the unplanted non-inoculated soils (SWPni) showed a Shannon index diversity *H'* value significantly below those of bacterial communities in planted soils. The presence of a cover and / or inoculation produced a higher *H'* value suggesting a modification of the bacterial community structures. Nevertheless, we found no significant differences between treatments for Eveness (*E*), thus suggesting that the different substrates were used in the same way, regardless of cover type, planted or not and inoculated or not.

There was no significant difference between mesocosms regarding the amounts of auxin compounds. The quantities varied from 10 to 11 μg IAA g^{-1} dry soil h^{-1} for all treatments (data not shown). For ACC

Table 2 Phenotypic characteristics of plant growth-promoting Ni-resistant rhizobacterial strains selected for the screening test

Ni-resistant isolate[a]	Sid[b] ≥18.3 mm	IAA[c] mg.mg^{-1} protéine.h^{-1} ≥ 80 mg	ACCd[d] OD[ACC/$(NH_4)_2SO_4$]
AB1	21.0		
AB12	19.0	84.9	0.62
AB14	19.7	90.5	0.57
AB15	19.3	153	0.72
AB20	25.3		
AB21	22.0		
AB22	21.0		
AB30	**23.3**	**101**	**0.70**
AB31	22.3		
AB33	22.3		
AB34	23.0		
AB35	22.3		
AB36	21.0		
AB39	21.0	139	0.61
AB42	20.0		
NB3	19.0	83.3	0.60
NB4	19.0		
NB5	20.0		
NB11	20.5		
NB22	19.5		
NB24	**18.5**	**109**	**0.80**
NB25	19.0	81.0	0.62
NB42	21.0		

Strains shown in bold type were used for the soil experiment

[a]AB: strains isolates from *Alyssum-Bornmuellera* rhizosphere; NB: strains isolates from *Noccaea-Bornmuellera* rhizoshere

[b]Sid: diameter of red-orange halo around the colony on CAS medium

[c]IAA: indolacetic acid production

[d]ACCd: ratio of the OD values of ACC and $(NH_4)_2SO_4$

deaminase activity, in the case of inoculated soils, the unplanted soils had significantly lower activity compared to inoculated plant associations (ABi and NBi) (Fig. 1). Also, there was no significant effect of inoculation on ACC deaminase activity in none of both associations.

Table 3 Physicochemical parameters (pH, CEC and Ni DTPA) of the soil from Vosges (France) used for the culture of mixed covers, at the end of the experiment. Mean values followed by different letters are significantly different at $p<0.05$ (Newman Keuls test), ($n=4$)

	pH	CEC ($cmol^+.kg^{-1}$)	Ni DTPA ($mg.kg^{-1}$)
ABi	5.81±0.05 a	18.6±0.4 c	72.7±4.5 b
ABni	5.80±0.04 a	19.7±0.7 ab	76.1±8.3 b
NBi	5.82±0.04 a	18.9±0.2 bc	69.8±6.0 b
NBni	5.85±0.05 a	19.7±0.5 ab	73.9±5.4 b
SWPiAB	5.68±0.04 b	20.2±0.6 a	87.0±2.3 a
SWPiNB	5.63±0.10 b	20.7±0.5 a	89.5±1.9 a
SWPni	5.66±0.05 b	20.2±0.4 a	87.7±2.4 a

Table 4 Average Well Colour Development (AWCD), Richness (*R*), Shannon index (*H'*) and Eveness (*E*) in inoculated (i) or non-inoculated (ni) plant covers

	AWCD	*R*	*H'*	*E*
ABi	1.1±0.2 ab	22.7±1.7 a	3.8±0.2 ab	0.8±0.04
ABni	1.2±0.1 a	23.5±1.7 a	3.9±0.2 a	0.8±0.02
NBi	1.0±0.2 ab	22.2±1.8 a	3.8±0.2 ab	0.9±0.03
NBni	0.9±0.2 b	21.0±2.9 a	3.8±0.2 ab	0.9±0.01
SWPiAB	0.4±0.1 c	17.0±2.4 b	3.5±0.15 abc	0.8±0.02
SWPiNB	0.4±0.1 c	15.7±1.5 b	3.4±0.2 bc	0.8±0.06
SWPni	0.4±0.1 c	14.5±3.3 b	3.1±0.3 c	0.8±0.03

AB Alyssum-Bornmuellera, *NB Noccaea-Bornmuellera*, *SWP* soil without plant

Mean values±standard errors followed by different letters are significantly different at $p<0.05$ (Newman Keuls test), ($n=4$)

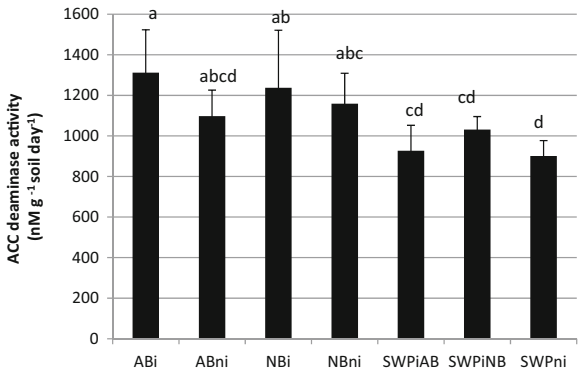

Fig. 1 ACC deaminase activity. Mean values followed by different letters are significantly different at $p<0.05$ (Newman Keuls test), ($n=4$)

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

Root biomasses of the inoculated covers ABi and NBi were significantly higher than the respective non-inoculated covers (ABi: 1.2 g; ABni 0.9 g and NBi: 1.6 g; NBni: 0.9 g) (Fig. 2). Shoot biomass was always higher than root biomass. The total biomass of the inoculated NBi cover was significantly higher than the total biomass of all other covers (AB, ABi and NBni). Similarly, NBi shoot biomass was significantly higher than that of ABi and ABni, NBni showing an intermediate shoot biomass. There was a significant increase in shoot biomass of the NB association following inoculation, when the analysis of variance was performed on the NB treatments only. It was not significant anymore when performed on both plant association treatments. The inoculation of AB associations had not significant effect on shoot biomass.

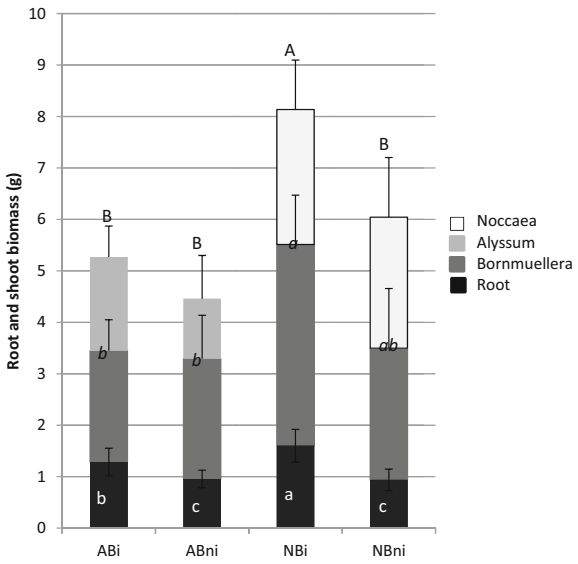

Fig. 2 Root (*black bar*) and shoot (*grey* and *white bars*) biomass (g) per cover. Mean values followed by different lowercase (root), italic (shoot) or capital letters (total biomass) are significantly different at $p<0.05$ (Newman-Keuls test), ($n=4$)

The Ni concentrations in roots (Fig. 3, right scale) were much lower than those observed in shoots (Fig. 3, left scale). For roots, ABi showed significantly higher concentrations (213.7 mg kg^{-1}) compared to other covers (146.0, 71.7 and 71.5 mg kg^{-1}, respectively for ABni, NBi and NBni). In the case of aerial parts, for each plant cover, inoculated or not, Ni concentrations were always higher in *Bornmuellera* shoots. Moreover, the concentration of Ni in the shoots of inoculated plant associations (shoots of ABi and NBi) was significantly higher than that in non-inoculated covers (ABni and NBni).

The total amount (shoot+root) of Ni was significantly higher for NBi (71.3 mg) than for the other three covers (ABi: 51.2 mg; NBni: 39.7 mg; and ABni: 36.5 mg) (Fig. 4). In detail, we found that the amount of Ni in roots (of the order of a tenth of mg) was significantly lower than in aerial parts (of the order of tens of mg) (Fig. 4). Moreover, the amount of Ni in roots of ABi (0.28 mg) was significantly greater than in roots of the other three covers (ABni: 0.13 mg; NBi: 0.11 mg; NBni: 0.07 mg). The amount of Ni between NBi and NBni roots was not significantly different. In the case of aerial parts, the amount of Ni in the shoots of the inoculated plant associations (shoots of ABi and NBi) was significantly higher than that in non-inoculated covers (ABni and NBni). When comparing the amounts of nickel in shoots and roots between inoculated and non-inoculated plants, after 6 months of culture, we found that amounts of Ni taken up increased from 66.4 to 105.9 % in roots, respectively for NB and AB covers and from 39.9 to 79.6 % in shoots, respectively for AB and NB covers. Inoculation had a greater effect on Ni taken up in roots for the AB cover and in shoots for the NB cover.

From the concentrations and quantities previously measured, we were able to calculate the BCF in relation to total soil Ni (BCF_{total}), BCF in relation to DTPA-Ni ($BCF_{DTPA\text{-}Ni}$) and TC coefficients to evaluate the efficiency of Ni extraction by inoculated or non-inoculated plant associations (Table 5). NBi had a significantly higher $BCF_{DTPA\text{-}Ni}$ than NBni (160 and 109,

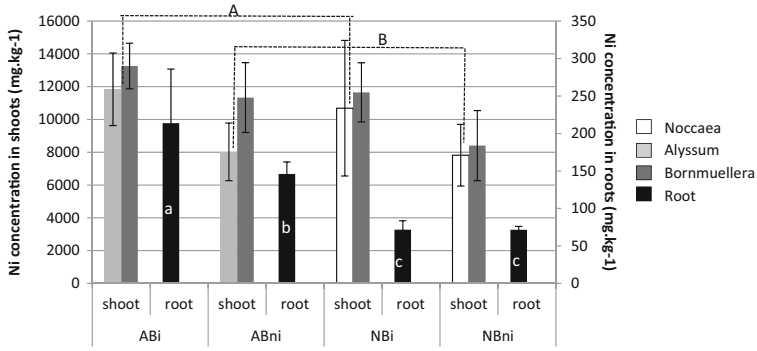

Fig. 3 Ni concentration in shoots (*light* and *dark grey* and *white bars*) and roots (*black bar*) (mg.kg^{-1}). Mean values followed by different lowercase (root) or capital letters (shoot) are significantly different at $p<0.05$ (Wilcoxon test), ($n=4$)

respectively) but inoculation induced no significant increase in BCF $_{DTPA\text{-}Ni}$ for ABi. With or without inoculation, AB had a significantly lower TF than NB. In contrast, the inoculation of NB involved a significant increase in TF (NBi: 158 and NBni: 114).

In order to consider all parameters together, we carried out a global Principal Component Analysis (PCA) on physicochemical and microbiological variables (Fig. 5). Axis 1, which explains 32 % of the total variability, strongly discriminated non-inoculated (ABni and NBni) (negative abscissa) from inoculated plant associations (ABi and NBi; positive abscissa) (Fig. 5a). Axis 2, which represented 18 % of the total variability, clearly discriminated AB covers (inoculated or not; positive ordinate) from NB covers (inoculated or not; negative ordinate). If we focus on the explanatory variables (Fig. 5b), it appears that the inoculation induced a decrease in the concentration of extractable soil Ni and in CEC (PC1). Correspondingly, we noted that the quantities of Ni in shoots (Q Ni shoot) were inversely correlated to the quantities of Ni present in soil (Ni DTPA) ($R=-0.60$) and to the CEC. NBi accumulated the highest Ni amount in shoot while ABi had the highest Ni quantity in roots. In addition, it is clear that the greater the biomass of shoots and roots, the higher the Ni they contain ($R=0.80$ and 0.40, respectively). Indeed, ABi showed the greatest root biomass, whilst NBi the highest shoot biomass. We noted a significant inverse correlation between the quantity of Ni in the roots (Q Ni Root) and the translocation factor TF ($R=-0.56$,). Moreover, NBi showed the highest TF (i.e., a more efficient translocation of Ni from roots to shoots). Among the microbial activities measured, IAA production appeared to be correlated to root biomass.

Discussion

We isolated bacteria from the rhizosphere of natural mixed hyperaccumulator plant covers (*Alyssum-Bornmuellera* and *Noccaea-Bornmuellera*), growing on Ni-rich soils. The choice of focusing on these covers was based on earlier works (e.g., Rue et al. 2015), which showed that the association *Noccaea+Bornmuellera* improved phytoextraction compared to the same species at same density. Also Gao et al. (2012) showed that the coexistence of different plant species increased the size of the microbial community by 15-20 % compared to a monoculture and correlatively a stimulation of certain functions of these microbes. In the two collections of

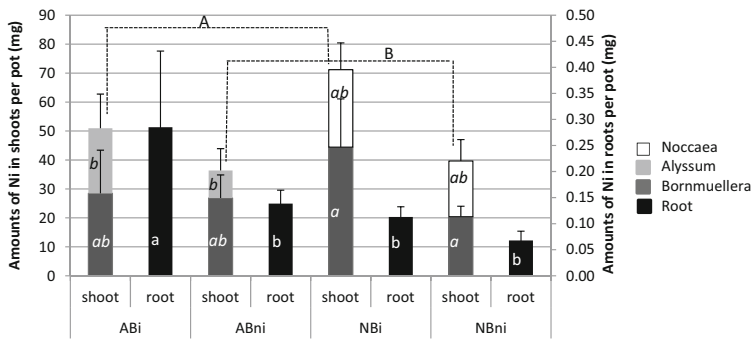

Fig. 4 Amounts of Ni phytoextracted in shoots (*light* and *dark grey* and *white bars*) and roots (*black bar*) per pot (mg). Mean values followed by different lowercase (root), italic (shoot) or capital letters (total shoot biomass) are significantly different at $p<0.05$ (Newman-Keuls test), ($n=4$)

Table 5 Bio-concentration factor (BCF DTPA-Ni), Ni translocation factor (TF) and Bio-concentration factor (BCF total) in inoculated (i) or non-inoculated (ni) plant covers

	BCF DTPA-Ni	TF	BCF total
ABi	178±26 a	65.1±19.2 c	8.9±0.9 a
ABni	136±17 ab	71.6±13.7 c	7.2±0.8 ab
NBi	160±12 a	158±19 a	7.8±1.1 ab
NBni	109±26 b	114±36 b	5.6±1.4 b

AB Alyssum-Bornmuellera, *NB Noccaea-Bornmuellera*

Mean values±standard errors followed by different letters are significantly different at $p<0.05$ (Newman Keuls test). ($n=4$)

strains isolated from both mixed covers, the ratios of Ni-resistant bacteria to total bacteria steadily decreased from 81 % at 0.5 mM of Ni to 0.76 % at 10 mM and were in accordance with a previous study (Aboudrar et al. 2007). Such Ni concentrations might seem very elevated (400–1000 times more) compared with natural Ni concentrations in ultramafic soils pore water (Bani et al. 2015) but, it is known that a significant amount of added Ni is adsorbed onto agar in Petri dishes. But the main reason was that we wanted to be as selective as all previous studies (Schlegel et al. 1991; Abou-Shanab et al. 2003; Aboudrar et al. 2007; Turgay et al. 2012;

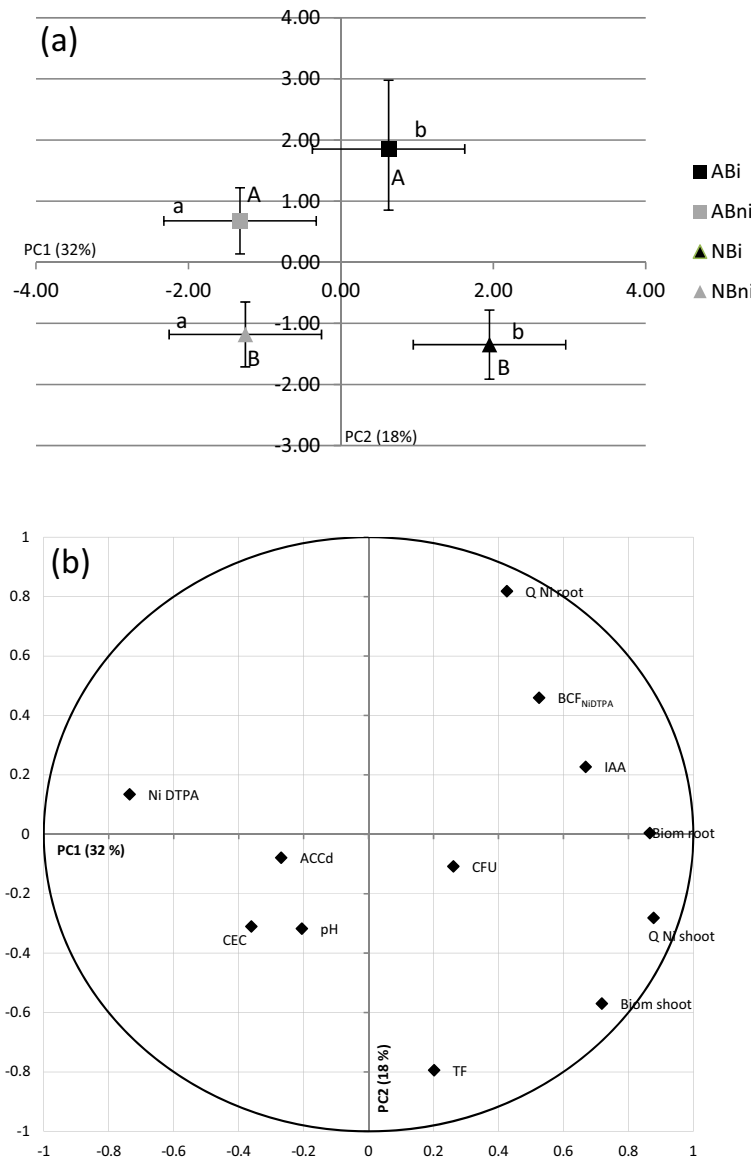

Fig. 5 Ordination plot of planted mesocoms, generated by Principal Components Analysis of the physicochemical and microbiological parameters. **a** Points represent means of four replicate samples with standard errors. Means with different lower and capital letters have significantly ($P<0.05$) different scores on PC 1 and PC2, respectively. **b** Physicochemical and microbiological parameters involved in the discrimination of mesocosms (*Ni DTPA* 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}), *IAA* auxin compounds ($mg.g^{-1}$dry soil.h^{-1}), *Q Ni root and Q Ni shoot* root and shoot nickel quantities (mg), *Biom root and Biom shoot* root and shoot biomass (g), *CFU* colony-forming units (log10 $cfu.g^{-1}$dry soil), *TF* Translocation factor, $BCF_{Ni\text{-}DTPA}$ Bio-concentration factor)

Cabello-Conejo et al. 2014) in the choice of Ni resistant strains using the same or lower ranges of Ni concentrations.

From this collection, we selected two strains of interest, isolating at 7.5 mM Ni, according to i) their ability to grow on agar medium containing Ni, ii) their ability to produce auxin compounds, siderophores and ACC deaminase - these characteristics described strains identified as PGPR. The strains were AB30 (from *Alyssum-Bornmuellera* rhizosphere) and NB24 (from *Noccaea-Bornmuellera* rhizosphere). These two strains belong to the genus *Variovorax*. Many soil bacteria affiliated to the genus *Variovorax* are able to show resistance to various trace elements such as B (Miwa et al. 2008), and Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn. This suggests that these bacteria are able to adapt to a wide variety of soil environments. Furthermore, it has been shown previously that *Variovorax paradoxus*, had PGPR properties (Belimov et al. 2005; Jiang et al. 2012).

Strains AB30 and NB24 both were able to produce siderophores, auxin compounds and ACCd at high concentrations. Comparison of the efficiency of PGPR properties with those obtained by other authors was difficult, insofar as the units used for the quantification of the different compounds were not always the same. However, both bacteria considered in this study were selected as strongly Ni-resistant such as those selected by Abou-Shanab et al. (2003) ($NiCl_2$), Cabello-Conejo et al. (2014) ($NiPO_4$) and Aboudrar et al. (2012) ($NiSO_4$). Both strains also showed a siderophore production with values in the same range than that found by Ma et al. (2009), as well as for the production of auxins.

The presence of plant cover, inoculated or not, changed the phenotypic structure of bacterial community. The latter was quite different from that of communities isolated from unplanted soils. Furthermore, the presence of a plant cover increased the metabolic capacity of soil bacterial communities, and finally, microbial activity - such as ACC deaminase - was significantly higher in the rhizosphere of inoculated plant associations compared to unplanted soil. Thus, rhizodeposits might have had a strong impact on indigenous rhizosphere microbial communities through the expression of particular functions and specific catabolic activities. These results are in accordance with previous studies in which, depending on plant species, microbial communities were different (Ibekwe and Kennedy 1999). It is commonly accepted that qualitative and quantitative differences in exudate composition are the main factors of changes in microbial community structure (Benizri and Amiaud 2005).

Concerning the effect of inoculation, we observed a significant increase in whole root biomass for both plant associations with PGPR strains *Variovorax* AB30 and NB24. This effect was particularly pronounced for NBi root biomass. Such increase in root biomass induced by inoculation may result from the production of ACCd or IAA by both strains. ACCd activity was significantly higher in the presence of a plant cover, but even more after inoculation with PGPR. The production of this enzyme by PGPR thus leads to a decrease in ACC concentration in root tissue and therefore to a reduction of ethylene concentration (Glick et al. 1998). Moreover, production of ACCd was shown to enhance root hairs growth (Shaharoona et al. 2006).

The shoot biomass of inoculated covers was also greater than for non-inoculated covers (only significant in the case of NBi). This again confirms that the NB24 strain appeared to be more efficient. In addition, for each type of cover (AB and NB), inoculation showed a greater effect on one of the two partners of the association. AB30 appeared to promote specifically the development of *A. murale* shoot biomass (compared to *B. tymphaea*), and NB24 appeared to promote more efficiently *B. tymphaea* than *N. tymphaea*.

The total dry biomass of each cover (shoot and root parts) was significantly greater after inoculation of the plant association NB. Inoculation of NB24 thus appeared to be significantly effective in our short-term culture conditions. Moreover, bacteria used in our study produced significant IAA concentrations, which were correlated to biomass production, and several authors (Teixeira et al. 2007; Cabello-Conejo et al. 2014) showed that much lower concentrations of IAA produced by PGPR (of the order of 1 to 25 $mg.l^{-1}$), were sufficient to significantly increase plant growth. The increase in the growth of metal-exposed plants has often been attributed to the production of this hormone (Dell'Amico et al. 2008).

In our study, the two bacteria showed the ability to produce siderophores. Soils contaminated with metals are often associated with induced iron deficiency in plants (Ma and Nomoto 1993; Burd et al. 2000). But in our serpentine soil, available iron in the soil used was estimated to be sufficient to provide plants with iron, despite the high level of Ni.

A number of previous studies showed that bacterial treatments (PGPR) increased plant nutrient contents in leaves. In particular, Orhan et al. (2006) found an

increase in P content up to 433 % of control. In our study, the inoculation of the plant association NB only induced a non-significant increase of 10 % of the total P content in shoot (498 and 452 mg kg^{-1}, respectively for NBi and NBni). No significant effect was recorded on the P content in shoot of AB. Thus, two hypothesis could be emitted: these strains showed absolutely no effect on P solubilisation compared with other studies or plant covers had enough P to fulfill their requirements (Shallari et al. 2001). So, the inoculated bacterial strains were capable of producing siderophores and slightly improving phosphate nutrition for NBi cover, the main PGPR properties in our study, would rather be the production of IAA and ACCd.

Concerning physicochemical parameters, we found a lower cation exchange capacity (CEC) in the presence of a plant than in unplanted soils; CEC decreased in the case of inoculated plant associations. In contrast, pH levels increased significantly in planted soils, as already shown by Blossfeld et al. (2009) in the presence of *Noccaea caerulescens*. This could be due to the fact that Brassicaceae absorbs many anions (NO_3^- and PO_4^{3-}) to balance the high cation uptake with perhaps a consequent partial release of OH^- ions, which in turn increases pH. Ni concentration was significantly higher in unplanted soils than in rhizosphere soils. Finally, extractable Ni-DTPA was lowest in inoculated planted soils due to higher uptake by plants. All the plants used in our study accumulated at least 1000 mg.kg^{-1} of Ni in their shoot and therefore met the threshold for hyperaccumulator plants (van der Ent et al. 2013). Both the amount and concentration of Ni in the shoots were significantly higher after inoculation (NBi and ABi>NBni and ABni). In addition, in the case of roots, inoculation significantly increased both the amount and the concentration of Ni under AB cover and we also noted a better accumulation in roots under NB cover. Studies by Cabello-Conejo et al. (2014) and Visioli et al. (2014) showed that inoculation of PGPR on Ni hyperaccumulating plants increased concentrations of Ni in both shoots and roots, as confirmed by our results. In all planted soils, shoot:root Ni transport ratios were far above 1, confirming the ability of the selected plant associations to translocate this element into the aboveground biomass. The TF under NB cover was significantly enhanced by inoculation. This was not the case for AB cover (inoculated or not) due to a high Ni concentration in the roots. *A. murale* is known to transfer Ni from the roots to the shoot mainly during flowering (Bani et al. 2015; Estrade et al. 2015) which was not reached in case of our experiment. Regarding other factors, BCF_{total} and $BCF_{DTPA\text{-}Ni}$ were increased for both inoculated covers (ABi and NBi). Thus, inoculation of the two covers by the selected bacteria improved phytoextraction of Ni. Finally, it was obvious from our study that for an efficient phytoextraction, the establishment of the hyperaccumulator plant association such as *N. tymphaea*+*B. tymphaea* inoculated with the PGPR strain *Variovorax* NB24 was the best alternative.

Conclusion

This study underlined the importance of using multi-species covers of hyperaccumulator plants, exclusively to improve Ni phytoextraction. It identified candidate strains, which could be useful for future field-based trials. Plant growth-promoting effects by associated bacteria, mainly PGPR *Variovorax* NB24 isolated from the natural nickel-rich rhizosphere soil of the mixed cover of *N. tymphaea* and *B. tymphaea*, can significantly improve plant association performance. They also result in larger amounts of phytoextracted Ni (+66.4 and +79.6 %, respectively in roots and in shoots). The PGPR effect could be attributed to the production of ACCd and IAA compounds. These experiments were performed over 6 months in controlled conditions and the performance of this PGPR *Variovorax* NB24 strain remains to be investigated in field trials for full growth experiments up to flowering plant.

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