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Bacterially Induced Weathering of Ultramafic Rock and Its Implications for Phytoextraction

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The bioavailability of metals in soil is often cited as a limiting factor of phytoextraction (or phytomining). Bacterial metabolites, such as organic acids, siderophores, or biosurfactants, have been shown to mobilize metals, and their use to improve metal extraction has been proposed. In this study, the weathering capacities of, and Ni mobilization by, bacterial strains were evaluated. Minimal medium containing ground ultramafic rock was inoculated with either of two *Arthrobacter* strains: LA44 (indole acetic acid [IAA] producer) or SBA82 (siderophore producer, PO_4 solubilizer, and IAA producer). Trace elements and organic compounds were determined in aliquots taken at different time intervals after inoculation. Trace metal fractionation was carried out on the remaining rock at the end of the experiment. The results suggest that the strains act upon different mineral phases. LA44 is a more efficient Ni mobilizer, apparently solubilizing Ni associated with Mn oxides, and this appeared to be related to oxalate production. SBA82 also leads to release of Ni and Mn, albeit to a much lower extent. In this case, the concurrent mobilization of Fe and Si indicates preferential weathering of Fe oxides and serpentine minerals, possibly related to the siderophore production capacity of the strain. The same bacterial strains were tested in a soil-plant system: the Ni hyperaccumulator *Alyssum serpyllifolium* subsp. *malacitanum* was grown in ultramafic soil in a rhizobox system and inoculated with each bacterial strain. At harvest, biomass production and shoot Ni concentrations were higher in plants from inoculated pots than from noninoculated pots. Ni yield was significantly enhanced in plants inoculated with LA44. These results suggest that Ni-mobilizing inoculants could be useful for improving Ni uptake by hyperaccumulator plants.

Over the last 2 decades, there has been increased interest in the development of plant-based remediation techniques (phytoremediation) for the cleanup of polluted soils. These techniques are intended to provide a viable alternative to traditional and civil engineering methods. Phytoextraction uses plants that take up metals from the soil and accumulate them in their above-ground biomass. Recently, the incorporation of plant-associated bacteria into these systems has been suggested as a means of enhancing phytoextraction efficiency (1, 2). Microorganisms play an essential role in the global biogeochemical cycling of metals and nutrients. Their activity can either increase or reduce the mobility of these elements in soils (3). Bacteria influencing the availability of plant nutrients (such as N, Fe, or P) have been used as "biofertilizers" to enhance plant nutrient uptake and alleviate nutrient deficiencies (4). Inoculation of pine seedlings with *Burkholderia glathei* PML1(12) significantly improved plant growth and nutrition through the weathering of biotite and release of nutrients, such as K and Mg (5). Similarly, the use of metal-mobilizing bacteria to enhance plant metal uptake has been proposed by several authors as a promising method to increase metal bioavailability, which can be a limiting factor in phytoextraction processes (6–9). Such microbial inoculants offer an alternative to the controversial use of metal chelants, which can increase metal bioavailability but at the same time lead to environmental problems because of their limited biodegradability or to an enhanced leaching of metals into groundwater. Several microbial metabolites have been shown to enhance rock weathering through chemical interaction or oxidation-reduction reactions, leading to mineral dissolution and metal solubilization. These metabolites include inorganic acids (HNO_3 and H_2SO_4); organic acids (such as citric, oxalic, and gluconic acid); and metal-chelating ligands, such as iron-complexing siderophores or biosurfactants (10). Microbial activity was associated with an enhanced release of Co and Ni in ultramafic soils of New Caledonia (11). Quantin et al. (12) showed that bacterial reduction of oxides led to the solubilization of Fe, Mn, Ni, and Co and modified metal distribution in the soil. Li et al. (13) related the production of short-chain organic acids by rhizosphere bacteria to the mobilization of Cd and Zn. Cell-free culture filtrates of rhizobacterial strains have been shown to mobilize soil Ni (8, 9), Zn, and Cd (6, 14). This bacterially promoted solubilization of metals led, in some cases, to an increase in metal uptake by plants. For instance, inoculation with Ni-mobilizing rhizobacteria enhanced Ni uptake by the Ni hyperaccumulator *Alyssum murale* (9, 15) and by the nonhyperaccumulator *Brassica juncea* (16). Similarly, Zn/Cd-mobilizing bacteria enhanced metal accumulation in *Salix caprea* (14) and Zn concentration in *Thlaspi caerulescens* (6).

In this study, two bacterial strains isolated from the rhizosphere of the Ni hyperaccumulator *Alyssum serpyllifolium* (subsp. *lusitanicum* and subsp. *malacitanum*) were evaluated for their

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TABLE 1 Chemical composition of the rock used in batch culture experiments obtained by energy-dispersive X-ray fluorescence spectrometry (EDXRF)

Constituent	Amt
Major elements (% [wt/wt] oxides)	
SiO_2	42.79
Al_2O_3	1.49
Fe_2O_3	15.73
MnO	0.39
MgO	34.00
CaO	0.98
Na_2O	0.54
K_2O	0.05
Trace metals (mg kg^{-1})	
Ni	3,530
Cr	2,660
Co	130

weathering capacities and abilities to mobilize Ni from different mineral phases of ultramafic rock. The influence of these strains on metal availability and plant uptake was further evaluated in a pot experiment by growing plants in ultramafic soil and adding bacterial inoculants.

MATERIALS AND METHODS

Bacterial strains and preparation of inoculants. Two bacterial isolates identified as members of the genus *Arthrobacter* were selected for this study. These strains were isolated from the rhizosphere soil of two subspecies of the Ni hyperaccumulator *A. serpyllifolium* (8). They included the strains (identified by partial sequencing of 16S rRNA genes) *Arthrobacter nitroguajacolicus* LA44 (an indole acetic acid [IAA] producer) isolated from the rhizosphere of *A. serpyllifolium* subsp. *lusitanicum* (Melide, northwest Spain) and *Arthrobacter oxydans* SBA82 (a siderophore producer, PO_4 solubilizer, and IAA producer) isolated from *A. serpyllifolium* subsp. *malacitanum* (Sierra Bermeja, southern Spain).

To prepare the bacterial inoculants, the strains were cultivated in 869 medium (17) for 3 days, harvested by centrifugation (4,000 × *g*; 15 min; 4°C), washed, and resuspended in 10 mM $MgSO_4$ to an optical density of 1.0 at 600 nm (about 10^8 cells per ml).

Batch culture experiment design. To evaluate the abilities of the bacterial strains to mobilize Ni, they were cultivated in minimal medium containing sterile ultramafic rock. The ultramafic rock was collected from the serpentinitic area of Morais (northeastern Portugal), where *A. serpyllifolium* subsp. *lusitanicum* is found growing. The chemical composition of this rock is given in Table 1. As is expected for an ultramafic rock, the SiO_2 content is less than 45% and the Al_2O_3, K_2O, and CaO contents are low, whereas the MgO content and total Ni, Co, and Cr concentrations are elevated. Mineral associations and element distributions were characterized with a scanning electron microscope (SEM) (EVO LS 15) equipped with energy-dispersive X-ray (EDX) microprobe analysis (Inca X-act; Oxford Instruments, United Kingdom) (Fig. 1).

A modified 284 medium (18) was used, with no trace of Ni, Mn, or Co and reduced concentrations of K and Fe. This modified 284 medium contained (per liter medium) 6.06 g Tris-HCl, 4.68 g NaCl, 0.015 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.20 g $MgCl_2 \cdot 6H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$, 0.04 g $Na_2HPO_4 \cdot 2H_2O$ plus oligoelements (0.3 mg H_3BO_4, 0.02 mg $CuSO_4 \cdot 5H_2O$, and 0.036 mg $Na_2MoO_4 \cdot 2H_2O$) adjusted to pH 7. The medium was supplemented with glucose (0.5 g $liter^{-1}$) and fructose (0.5 g $liter^{-1}$). Flasks containing 2 g of ground rock (<100 μm) were autoclaved three times with a 24-h interval between cycles. To each flask, 100 ml of modified 284 medium was added. Each flask was then inoculated with 1 ml of one of the two bacterial inoculants, LA44 or SBA82, or with 1 ml of 10 mM $MgSO_4$ (control treatment). The flasks were incubated in the dark for 2 weeks at 28°C and agitated on a horizontal shaker at 150 rpm. Five replicates were prepared for each treatment.

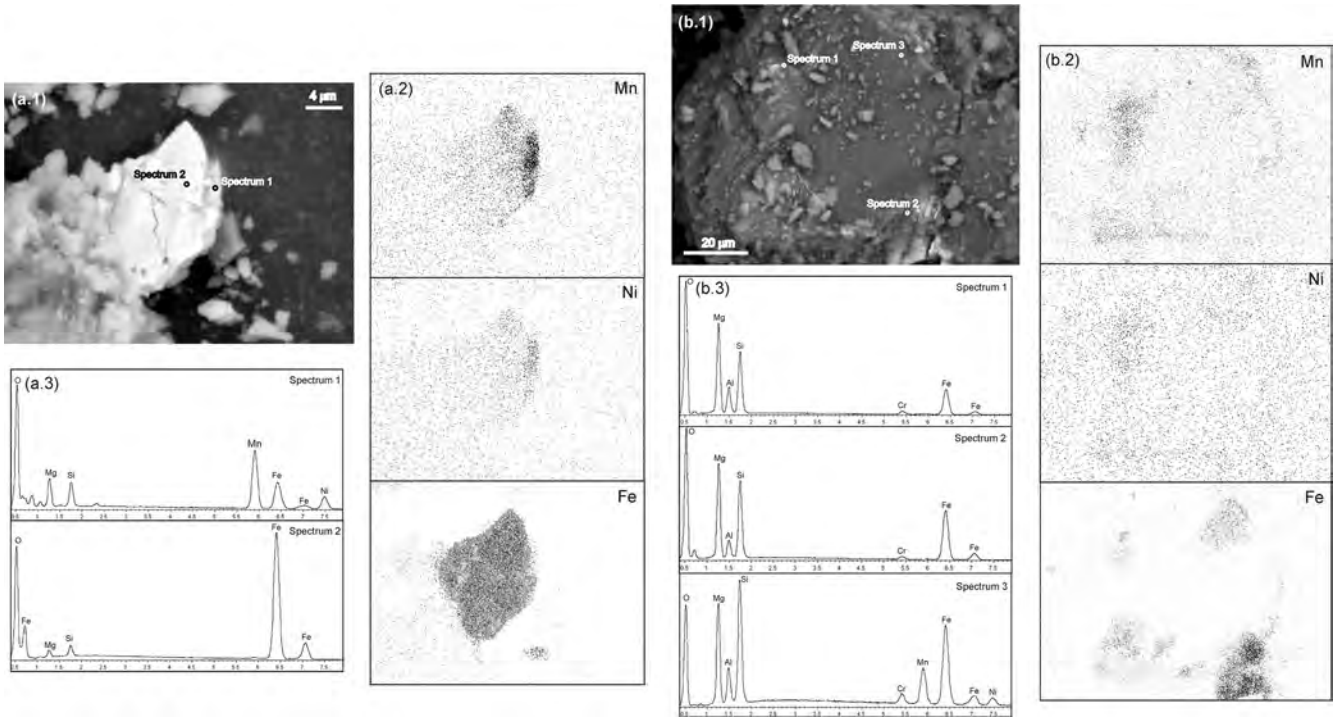

FIG 1 SEM photomicrographs taken from two areas of original ground ultramafic rock (a.1 and b.1), element mapping of these two areas (a.2 and b.2), and results of microprobe analysis from selected spots within each area (a.3 and b.3).

Aliquots (3 ml) were taken at different times (1, 2, 4, 7, 10, and 14 days). Samples were centrifuged, and the supernatant was decanted and filtered (0.22-μm pore size) and immediately frozen until analysis. Samples were analyzed for element concentrations, organic acids, and phenolic compounds. At the same sampling times, bacterial densities were determined by plating out serial dilutions of the samples on 10-fold-diluted 869 agar medium for three replicates of each treatment. At the end of the experiment (14 days), the medium pH was determined. The sterility of the control flasks was checked by plating on 1:10-diluted 869 agar medium. Finally, the cultivated bacterial strains (LA44 and SBA82) were compared with the original inoculants using BOX-PCR and following the methods described by Becerra-Castro et al. (8).

Analysis of supernatant samples. The concentrations of Al, Co, Cr, Fe, Mn, Ni, and Si in the culture medium were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Elan 9000 DRCe; PerkinElmer, Norwalk, CT) and K by emission spectrophotometry (atomic emission spectroscopy [AES]) (PerkinElmer 2380).

Carboxylic acids were separated by reversed-phase liquid chromatography on a C_{18} column with 5-μm particle size (GraceSmart, RP18, and 2.1- by 150-mm column from Grace Davison Discovery Sciences, Deerfield, IL, USA) and analyzed by liquid chromatography–electrospray ionization-time of flight mass spectrometry (LC–ESI-TOF MS) (Agilent Technologies, Palo Alto, CA, USA) as described by Jaitz et al. (19). Quantification of selected phenolic compounds (caffeic acid, catechin, *p*-coumaric acid, 2.5-dihydroxybenzoic acid, epicatechin, ferulic acid, gallic acid, 4-hydroxy-3-methoxycinnamidaldeyhde, resveratrol [3,5,4′-trihydroxy-*trans*-stilbene], sinapic acid, syringic acid, and vanillic acid) was performed via LC–tandem MS (MS-MS) in negative ionization mode on a 6410 triple-quadrupole mass spectrometer from Agilent Technologies (Palo Alto, CA, USA) equipped with an ESI interface (20).

Analysis of rock residues. The ground rock remaining at the end of the experiment was recovered by centrifugation, washed 4 times with sterile deionized water, and air dried. A metal fractionation scheme was carried out on the residual rock samples according to the method of Zeien and Brümmer (21).

First, soil samples were shaken with 1 M NH_4NO_3 (unbuffered) for 24 h at room temperature, and the solution was separated by centrifugation (15 min; 938 × *g*), filtered, and stabilized with HNO_3 (14.4 N). This extracts the water-soluble and exchangeable metals (fraction 1 [F1]).

Second, the resulting residue was shaken for 24 h at room temperature with 1 M ammonium acetate (NH_4OAc; adjusted with 50% acetic acid [HOAc] to pH 6), centrifuged, filtered, and stabilized with HNO_3 (14.4 N). NH_4NO_3 (25 ml) was added to the remaining residue and shaken for 10 min. The solution was separated by centrifugation, filtered, and combined with the NH_4OAc extract. This step extracts easily mobilizable metals (F2).

Third, the residue was shaken with 0.1 M NH_2OH-HCl plus 1 M NH_4OAc (adjusted to pH 6 with diluted HCl) for 30 min, separated by centrifugation, filtered, and stabilized with HCl (12 N). NH_4OAc (25 ml; 1 M) was added to the residue and shaken for 10 min, centrifuged, filtered (repeated once), and combined with the above-mentioned solution. This extracts metals bound to Mn oxides (F3).

Fourth, 50 ml 0.2 M NH_4-oxalate (equivalent to 0.2 M diammonium oxalate monohydrate plus 0.2 M oxalic acid dihydrate adjusted to pH 3.3 with diluted NH_4OH) was added to the residue, and after shaking (overhead) for 4 h in the dark, the solution was separated by centrifugation (15 min; 938 × *g*) and filtered. A second volume of 25 ml 0.2 M NH_4-oxalate (pH 3.3) was added to the residue and shaken overhead in the dark for 10 min. The solution was separated by centrifugation (15 min; 938 × *g*) and filtered, and the two filtrates were combined. This step targets those metals bound to amorphous Fe oxides (F4).

Fifth, 50 ml 0.1 M ascorbic acid plus 0.2 M NH_4-oxalate [equivalent to 0.1 M L(+)-ascorbic acid plus 0.2 M NH_4-oxalate buffer-0.2 M oxalic acid dihydrate adjusted to pH 3.25 with diluted NH_4OH] were added, and after shaking horizontally in a water bath for 30 min at 96°C ± 3°C, the solution was separated by centrifugation (15 min; 938 × *g*) and filtered. The remaining solution was extracted with 25 ml 0.2 M NH_4-oxalate (pH 3.3) by shaking overhead in the dark for 10 min. The solution was separated by centrifugation (15 min; 938 × *g*) and filtered, and the filtrates were combined. This extracts metals bound to crystalline Fe oxides (F5).

Finally, the remaining residue was digested with aqua regia to determine the residual (silicate-bound) fraction (F6).

The concentrations of Al, Co, Fe, Mn, Ni, and Si were analyzed in the filtered supernatants of each extraction by ICP-optical emission spectrometry (OES) (Vista Pro; Varian Inc., Australia). In the case of Si, the residual fraction was obtained by subtracting the sum of all fractions from the total Si (obtained by energy-dispersive X-ray fluorescence spectrometry (EDXRF).

Rhizobox experiment. Ultramafic soil was collected from a serpentine site in Redschlag, Austria (22). The soils at this site present a slightly acidic pH (pH_{CaCl2} 6.55) with an organic C content of 13 g kg^{-1}, a predominance of Mg in the exchange complex, and a high concentration of total Ni (2,580 mg kg^{-1}). The soils were air dried and sieved to <2 mm before being poured into the rhizoboxes (bulk density, 1.2 g cm^{-3}). The rhizobox used in this study was based on the system of Fitz et al. (23). Root growth was restricted to a central compartment by a 30-μm-mesh-size nylon net (Labor Becker, Vienna, Austria) to avoid growth of root hairs into the adjacent 2-mm-thick root-free rhizosphere soil compartment. The root-free rhizosphere compartment was separated from bulk soil by the same 30-μm-mesh-size nylon net. The rhizoboxes were made of Perspex acrylic material (Evonik Industries AG, Darmstadt, Germany), allowing observation of root growth. The rhizoboxes were wrapped with aluminum foil during the experiment to avoid growth of photosynthetic soil organisms and weed germination.

For this experiment, the Ni-hyperaccumulating *A. serpyllifolium* subsp. *malacitanum* was selected. Surface-sterilized seeds were germinated on perlite and transplanted into rhizoboxes (2 seedlings per rhizobox). After 5 weeks, 20 ml of LA44 or SBA82 bacterial suspensions (prepared as described previously) was added to the rhizoboxes at the bases of the plants. The same amount of 10 mM $MgSO_4$ was added to noninoculated plants in control rhizoboxes. All treatments were replicated five times. The plants were grown for a further 2 months. At harvest, the shoots and roots of plants were separated, washed with pressurized tap water (and 0.05 M $CaCl_2$ in the case of roots) followed by deionized water, oven dried at 45°C, and ground. Shoot tissues (0.1 g) were digested in a 2:1 mixture of concentrated HNO_3-HCl on a hot plate at 160°C, and the Ca, Mg, K, P, Fe, and Ni concentrations were measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed on the basis of dry weight of plant material. The full recovery of plant roots was not possible, and only shoot dry weight was taken into account when assessing the effects of inoculation on plant biomass production or Ni yield.

Soil analyses were carried out on the <2-mm fraction of rhizosphere and bulk soil samples. Soil pH was measured in H_2O using a 1:2.5 soil/solution ratio. Exchangeable cations were extracted with 0.1 M $BaCl_2$, and Al, Ca, K, Mg, and Na concentrations were determined by ICP-OES (Vista Pro; Varian Inc., Australia). The water-soluble Ni concentration was analyzed by ICP-OES in soil extracts after 30 min of shaking using a 1:2.5 soil/H_2O ratio. $Ca(NO_3)_2$- and $Sr(NO_3)_2$-extractable Ni concentrations were determined by ICP-OES in soil extracts after 2 h of shaking using a 1:4 soil/extractant ratio for both extractants.

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

Changes in the culture medium composition (element and organic acid anion concentrations) were also analyzed by principal-component analysis (PCA). Values below detection limits (DL) were recorded as the

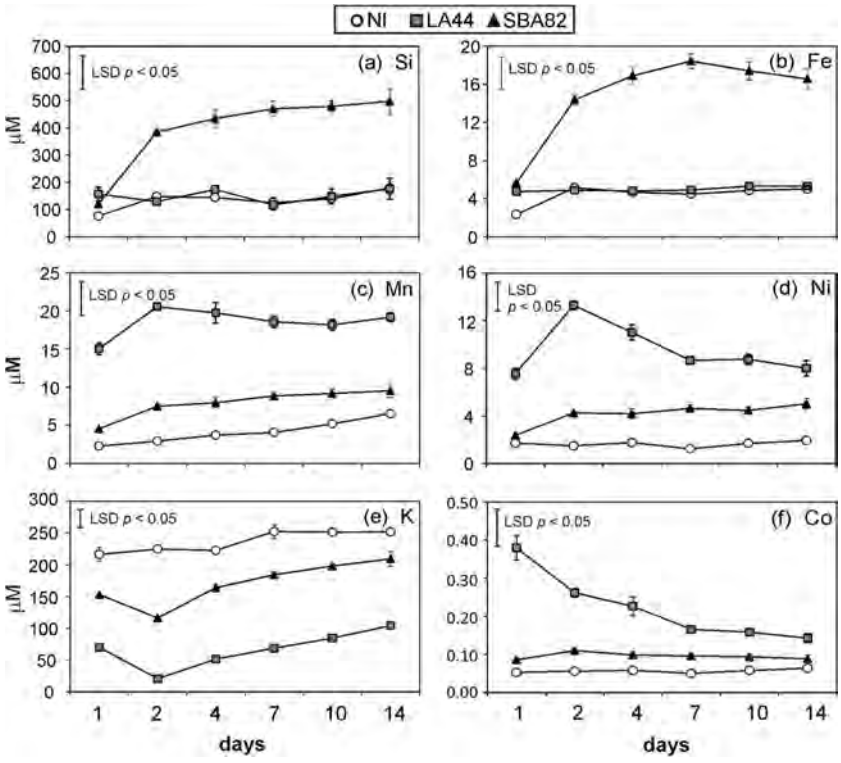

FIG 2 Concentrations of Si (a), Fe (b), Mn (c), Ni (d), K (e), and Co (f) in culture media during 14 days of incubation ($n = 5$). LSD, least significant difference. The error bars indicate standard errors.

1/2 DL for statistical analysis. A varimax rotation was applied to the PCAs in order to facilitate the interpretation of the extracted principal components.

Differences in plant biomass and plant metal/nutrient concentrations in the rhizobox-grown plants were determined using analysis of variance (ANOVA). A multiple comparison of means was determined by the *post hoc* least significance difference test. Data were log transformed where necessary to achieve homogeneity of variance. Comparison of means between bulk and rhizosphere soils was achieved by the Student *t* test for related means.

RESULTS

Bacterial growth in medium with ground rock. During the experiment, no significant differences were found in the densities of the two bacterial strains (data not shown). They presented similar growth rates, reaching a stable density of 1.1×10^8 to 6.4×10^8 CFU ml medium^{-1} by day 2. The identities of the recovered strains at the end of the experiment were confirmed by BOX-PCR to be the same as the originally inoculated strains.

Element solubilization from rock and release of organic compounds. Element concentrations in control flasks (noninoculated) varied slightly over time. They were generally lower than in experimental flasks (inoculated) (Fig. 2). Iron and K were added in low concentrations to the initial liquid medium (1.6 and 200 μM, respectively), while Co, Mn, and Ni were absent. Bacterial activity and growth led either to depletion of some elements (such as K) compared to the noninoculated control or, alternatively, to the release of some elements (such as Fe, Co, Mn, Ni, or Si) from the rock. At the end of the experiment, there was no difference in the pH of the culture medium between treatments. The repeated-measures ANOVA showed that the time, the inoculant treatment, and the time-inoculant interaction factors significantly affected changes in the medium composition ($P < 0.01$) (Table 2).

The K concentration in control flasks was similar to the initial K concentration in inoculated flasks and remained constant for the duration of the experiment; values varied from 216 μM on day

TABLE 2 Effects of inoculant treatment and time on element solubilization and organic acid production (results of eight separate 2-way rANOVAs)

		Si		Fe		Mn		Ni		K		Co		Oxalate		Succinate	
Parameter	df	MS[a]	*F*[b]	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Time	5	39.9	20.7[d]	0.1	18.5[d]	0.3	13.7[d]	0.1	17.2[d]	17.2	66.0[d]	1.4×10^{-4}	15.5[d]	422.4	44.9[d]	13.2	27.4[d]
Strain[c]	2	514.2	329.4[d]	2.9	298.5[d]	5.4	283.1[d]	1.8	336.0[d]	336.8	3,710.5[d]	1.5×10^{-4}	63.1[d]	7,169.1	585.9[d]	9.7	12.0[d]
Time × strain	10	23.2	12.1[d]	0.1	24.3[d]	0.1	4.6[d]	0.1	15.7[d]	1.8	6.8[d]	8.8×10^{-4}	16.0[d]	135.5	14.4[d]	15.4	32.0[d]

[a] MS, mean square.
[b] *F*, *F* statistic.
[c] Strain, inoculant treatment.
[d] $P < 0.01$.

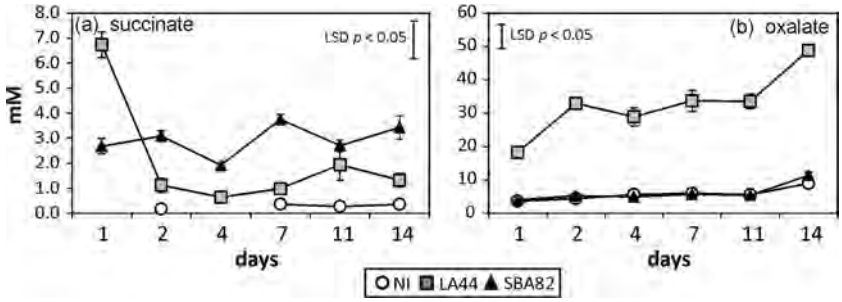

FIG 3 Succinate (a) and oxalate (b) concentrations in culture media during 14 days of incubation ($n = 5$). The error bars indicate standard errors.

1 to 252 μM on day 14 (Fig. 2e). In contrast, bacterial growth led to a significant decrease in the original K concentration of the medium. The K concentrations were lower in the inoculated flasks than in the controls throughout the experiment. Minimum values of 21 μM and 116 μM were detected on day 2 in LA44 and SBA82 cultures, respectively. From that time on, however, there was a steady release of K into the medium in the presence of either strain, although the concentrations never reached the values of the controls. On day 14, the K concentrations were 104 μM and 209 μM in the LA44 and SBA82 cultures, respectively.

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

In contrast to what was observed with the LA44 cultures and in the controls, the presence of strain SBA82 led to a significant release of Fe and Si from the ultramafic rock into the medium solution. By day 7, the concentrations of these two elements were up to 4.1- and 3.8-fold higher, respectively, than those detected in controls. The two elements followed similar patterns with time, with a higher rate of solubilization occurring during the initial days, which then stabilized between days 4 and 7 (Fig. 2a and b). Concentrations of Fe and Si in SBA82 cultures were significantly correlated ($R^2 = 0.93$; $P < 0.001$). Values for Ni and Mn in SBA82 cultures were also significantly higher than in controls (although lower than in LA44 cultures): by day 4, the concentrations of the two elements were up to 2.8- and 2.6-fold higher than in the control, respectively (Fig. 2c and d), and were also significantly correlated ($R^2 = 0.95$; $P < 0.001$). The presence of this strain also induced the release of Al into the medium; concentrations between 0.3 μM and 0.5 μM were detected. In contrast, Al was consistently below the detection limit in both controls and LA44 cultures.

None of the phenolic compounds were detected in the culture media. In contrast, detectable levels of malate, malonate, oxalate, and succinate were measured. Aconitate and citrate were below the detection limit. Malate concentrations in control and SBA82 cultures were detected in similar ranges and always remained below 0.70 μM. LA44 cultures showed somewhat higher values of the anion, between 0.20 and 1.15 μM. In the control treatments, the malonate concentration did not exceed 0.30 μM, and in SBA82 cultures, the values ranged from 0.20 to 0.55 μM. Higher concentrations of malonate, varying from 0.30 to 0.90 μM, were found in LA44 cultures (data not shown). Significant differences were observed in inoculated flasks with respect to oxalate and succinate concentrations ($P < 0.05$; repeated-measures ANOVA) (Table 2). In control cultures, succinate was detected only at some sampling times (2, 7, 11, and 14 days). With the exception of day 1, the succinate concentration in SBA82 cultures was generally higher (1.9 to 3.8 μM) than in LA44 cultures (0.6 to 1.9 μM) (Fig. 3a). With respect to oxalate, the difference in concentration between the two strains was more marked (Fig. 3b). The concentrations in SBA82 cultures were similar to those in control treatments (4.1 to 5.4 μM), whereas those in LA44 cultures increased from 18 μM on day 1 to close to 50 μM on day 14.

Element solubilization and organic acid production were subjected to PCA. The Kaiser-Meyer-Oklin value was 0.71, and the Barlett's test of sphericity reached statistical significance ($P < 0.05$), supporting the factorability of the correlation matrix. The PCA extracted two principal components that explained 70% of total variance (Fig. 4). The first component (PC1; 41%) was mainly represented by the Co, Ni, and Mn concentrations and by the malonate, oxalate, and malate concentrations. The second principal component (PC2; 29%) was related to the concentrations of Fe, Al, Si, and, to a lesser extent, to Cr. In the PCA plot, control samples are grouped together, with negative scores on both axes. SBA82 samples are mainly placed on the positive axis of the second component (PC2), represented by the Al, Fe, and Si concentrations in the medium, whereas LA44 samples are grouped on the positive axis of the first component (PC1), associated with Co, Mn, and Ni concentrations and organic acids (Fig. 4). The principal-component analysis confirmed the main differences observed between the two inoculants in the release of trace elements and organic acids into the medium.

Metal fractionation in rock samples. Figure 5 shows the element fractionation in the recovered rock from the control treatment, as well as the bacterially induced depletion/increase of each fraction relative to the control treatment. The residual fraction was the most important geochemical phase for Al, Cr, and Si (representing more than 85% of the total content). This phase was also dominant for Fe and Ni (representing more than 60%), but for these two elements, an important fraction was also associated with either amorphous or crystalline Fe oxides (32 to 35% in total). Mn was principally associated with the Mn oxide fraction (41% of the total Mn). EDX microprobe analysis confirmed the presence of Ni

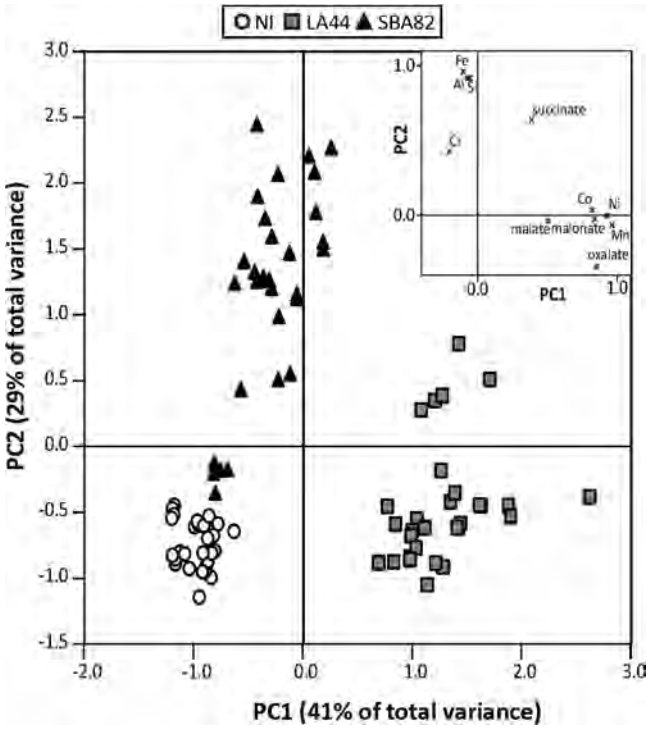

FIG 4 Score and loading (upper right corner) plots of PCA of elements and organic acid concentrations in media with LA44, SBA82, and noninoculated (NI) treatments.

associated with Mn oxides (Fig. 1), and Co was distributed among Mn oxide, amorphous Fe oxide, and residual fractions.

Bacterial activity significantly influenced element fractionation in the rock, and these changes were dependent on the bacterial strain (Fig. 5). After incubation with strain LA44, all elements associated with the Mn oxide fraction were significantly reduced compared to the control. In the cases of Mn and Ni, concentrations were reduced from 492 to 393 mg kg^{-1} and from 108 to 87 mg kg^{-1}, respectively (Fig. 5d and f). These metals were then redistributed among the more labile fractions (F1 and F2) and amorphous Fe oxides (F4). In F1, Ni concentrations increased from 16.9 to 40.0 mg kg^{-1} and Mn concentrations from 26.6 to 72.8 mg kg^{-1}. Corresponding shifts in F2 were less pronounced: Ni increased from 31.6 to 35.5 mg kg^{-1} and Mn from 21.9 to 37.0 mg kg^{-1}. Although not always statistically significant, a similar pattern was observed for Co (Fig. 5g): an increase in the first two fractions and a decrease in Co associated with the Mn oxide fraction. This effect of bacterial activity on Mn oxides was either not detectable or far less pronounced in rock samples that were incubated with strain SBA82 (Fig. 5).

In the case of SBA82, the presence of the bacterial strain led to a significant increase in soluble and exchangeable concentrations of the major elements Al (from 1.5 to 1.8 mg kg^{-1}), Si (from 399 to 840 mg kg^{-1}), and Fe (from 17.8 to 48.2 mg kg^{-1}) (Fig. 5a to c). The strain also induced a significant increase in all elements associated with amorphous Fe oxide fractions compared to controls.

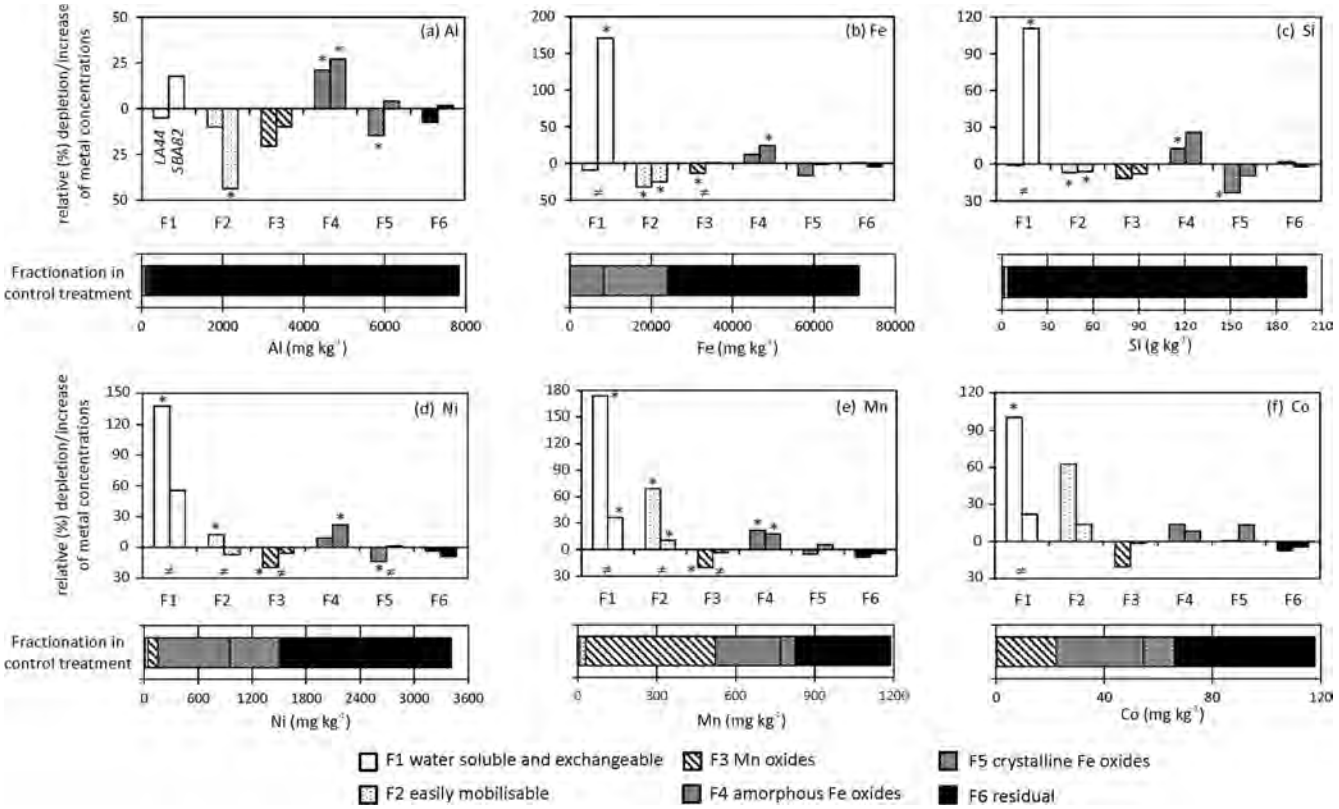

FIG 5 Element fractionation in the recovered rock from the control treatment and mean relative (percent) bacterially induced depletion/increase of each fraction relative to the control treatment ($n = 5$). The depletion/increase was calculated by subtraction of the amounts of metals in each fraction at bacterial treatment (LA44 or SBA82) from the amount in the noninoculated treatment. A significant depletion/increase in the metal concentration is denoted with an asterisk ($P < 0.05$). Differences between bacterial treatments are denoted with ≠.

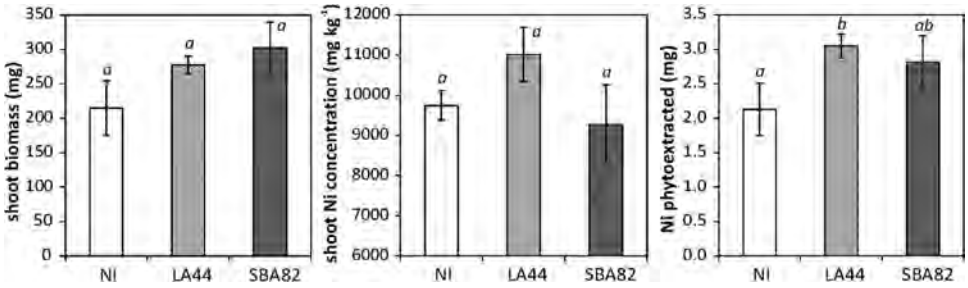

FIG 6 Plant biomass, shoot Ni concentration, and Ni phytoextracted (means ± standard errors [SE]) of *A. serpyllifolium* subsp. *malacitanum* ($n = 5$). Bars with different letters indicate significant differences ($P < 0.05$).

The concentrations of the major elements (Al, Fe, and Si) associated with this phase were increased by 20 to 27%, while the trace metals, Ni and Cr, increased from 797 to 971 mg kg^{-1} and from 13.9 to 18.0 mg kg^{-1}, respectively. In parallel, a reduction in the residual Ni fraction was observed (from 2,322 to 2,115 mg Ni kg^{-1}). These effects were far less pronounced in the LA44 cultures (Fig. 5).

Pot experiment. After 3 months growth, plants grown in inoculated pots presented a higher biomass than plants from noninoculated pots, although the difference was not significant for either inoculant (Fig. 6a). Inoculation did not significantly influence nutrient contents in shoot tissues (Table 3). However, some trends could be observed; for instance, plants grown in inoculated pots tended to have higher shoot Fe and Ca contents than plants grown in noninoculated pots. Furthermore, inoculation with SBA82 also tended to increase shoot P content (Table 3).

No differences were observed in root Ni concentrations due to inoculation: values were in the range of 806 to 849 mg Ni kg^{-1} for all three treatments. In contrast, strain LA44 tended to increase Ni accumulation in shoots (reaching 11,873 mg kg^{-1}) compared to plants grown in noninoculated pots (mean Ni concentration, 9,700 mg kg^{-1}), although this increase was not significant (Fig. 6b). Moreover, inoculation with both LA44 and SBA82 increased the total Ni phytoextracted by 1.4- and 1.3-fold, respectively, compared to plants grown in noninoculated pots. This increase was statistically significant in the case of plants grown in LA44-inoculated pots ($P < 0.05$) (Fig. 6c).

Plant growth induced few changes in physicochemical soil properties, and no significant effects of bacterial inoculation were observed. In plants grown in noninoculated pots, the rhizosphere soil pH was slightly higher than the bulk soil pH: 8.4 ± 0.1 compared to 8.3 ± 0.1. As expected, the cation-exchange complex (CEC) was dominated by Mg (8.1 to 9.4 centimoles of charge [$cmol_c$] kg^{-1}). The CECs were 10.3 and 10.9 $cmol_c$ kg^{-1} in bulk and rhizosphere soils, respectively, and Ca/Mg quotients were consistently less than 1 (around 0.2). The water-soluble Ni concentration was significantly higher ($P < 0.01$) in the rhizosphere than in bulk soil (0.12 and 0.06 mg kg^{-1}, respectively). In contrast, $Sr(NO_3)_2$- or $Ca(NO_3)_2$-extractable Ni concentrations tended to be depleted in the rhizosphere (falling from 0.49 to 0.44 mg kg^{-1} and 6.44 to 5.89 mg kg^{-1}, respectively). Bacterial inoculation did not lead to any significant changes in these general physicochemical properties.

TABLE 3 Shoot nutrient concentrations in *A. serpyllifolium* subsp. *malacinatum*

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

[a] For each element, the same letter indicates no significant difference ($P < 0.05$).
[b] NI, noninoculated.

DISCUSSION

The influence of two bacterial strains on the weathering and solubilization of elements from an ultramafic rock was assayed. Bacterial activity was found to have a significant influence on mineral weathering, as indicated by the release of structural elements (Al, Fe, and Si) or adsorbed or interlayer cations (such as Mn, Ni, and Co) into the medium solution and their redistribution among the different geochemical compartments. The release of elements into the medium was most pronounced during the first 2 days of incubation, coinciding with the period of maximum bacterial growth. Furthermore, based on the differences observed between the two bacterial strains and on the PCA, our results suggest that the strains act preferentially upon different mineral phases.

In the case of SBA82, the corelease of Al, Fe, and Si into the culture medium suggests a preferential weathering of Ni-rich ferromagnesium silicates. Serpentine and chlorite have been identified as the dominant primary minerals in these rocks (unpublished data). Most Fe was included in primary silicate minerals and crystalline Fe oxides and appears to be rendered amorphous during microbial weathering. Other elements released during weathering were also incorporated into amorphous Fe oxide phases (Al, Si, Co, Cr, Mn, and Ni) or released into soluble and exchangeable pools. Amorphous Fe oxides have a high specific surface area and can act as important sorbents of trace metals (24). Our results are in line with previously described weathering sequences of serpentine minerals (25). Since strain SBA82 is a siderophore producer, its mechanism of weathering probably involves siderophore-induced mineral dissolution. Siderophores are iron-chelating secondary metabolites that are known to be produced under Fe-limiting conditions. The reduced Fe concentration of the culture medium used in this study presumably induced siderophore production by strain SBA82. Previous studies have demonstrated siderophore-promoted dissolution of goethite and hornblende and consequent release of Fe, Si, and Al (26, 27). Although siderophores have a high affinity for Fe(III), they are known to form complexes with other trace elements (28, 29). Their presence in the culture medium would therefore maintain

elements such as Al, Fe, and Si in solution (which was the case in SBA82 cultures). Siderophore-mediated Fe solubilization from Fe-rich minerals could also lead to the solubilization and redistribution of their companion trace metals (Cr and Ni).

On the other hand, since LA44 is not a siderophore producer, the concentration of major elements (Al, Fe, and Si) was no different from that in controls. However, the possibility that strain LA44 is able to alter ferromagnesian minerals by other means cannot be completely ruled out. The strain could alter ferromagnesian minerals by attack with oxalic acid and/or other metabolites that it produces. Bacterial secretion of organic acids has been related to the weathering of silicates and Fe (hydr)oxides (30). Extracellular polymers produced by bacteria have also been shown to affect mineral solubility (31). Nonetheless, the strong mobilization of Co, Mn, and Ni into the culture, combined with a general reduction of elements associated with Mn oxides, suggests that the LA44 strain principally acts on this mineral phase. Although Ni associated with Mn oxides is not the principal Ni fraction in this rock, these oxides seem to be important in determining Ni availability. The analysis of rock samples by SEM confirmed an association between Mn and Ni in the rock. The role of Mn oxides in determining Ni availability has also been demonstrated by other authors in ultramafic areas. In a study evaluating Ni and Cr extractability in ultramafic soils, Quantin et al. (24) concluded that Ni behavior appeared to be partly controlled by pH and partly by Mn oxides. Antić-Mladenović et al. (32) suggested that the dissolution and precipitation of Fe/Mn oxides, organic matter transformations, and adsorption on solids were important processes controlling Ni solubility in ultramafic soils. Similarly, a study carried out in the ultramafic region of Morais, in the same serpentine outcrop where the rock sample used in this study was collected, revealed that the Ni bioavailability in these soils is linked to the Mn oxide fraction (33). The strong release of Ni and Mn, and to a lesser degree of Co, in LA44 cultures seems to be related to the release of organic acids by this bacterial strain. Low-molecular-weight organic acids, and in particular oxalic acid, are most often cited as the main agent in biogeochemical weathering of silicate minerals. These organic ligands can attack minerals directly by complexing with ions at the surface, weakening metal-oxygen bonds, or catalyzing dissolution reactions. Indirectly, they affect weathering rates by complexing ions in solution, thus lowering the solution saturation rate (34). The production of organic acid anions by bacterial strains was related to the weathering of hornblende (35) and the mobilization of metals from carbonates and oxides (13). In the present study, oxalate production seems to have an important role in the solubilization of Mn and Ni. Oxalate-promoted dissolution of Mn oxides by reduction of Mn(IV) to Mn(II) has been proposed by several authors (36–38). The higher concentrations of Ni released into the culture medium (or the redistribution of Ni toward labile phases) may be related to the ability of strain LA44 to create reducing conditions. Although no differences in E_h were detected in culture media inoculated with either strain, LA44 may generate redox microgradients at the rock surface that were not detected. Solubilization of Ni in the case of SBA82 came mainly from serpentine group minerals, although a possible influence of the strain on Mn oxides cannot be discounted. Siderophores can also interact with Mn oxides (39) and could therefore explain, at least partially, the solubilization of Mn induced by SBA82. In fact, Mn oxides were also reduced after incubation with the strain, although its influence on this phase was far less pronounced than for the LA44 strain.

Metal availability in the soil or the replenishment of labile metal pools from solid soil phases is a key element in successful phytoextraction. There has been considerable debate as to whether hyperaccumulating plants are able to access metal fractions not available to nonaccumulator plants, thus increasing metal uptake (1, 40, 41). This study demonstrates the capacity of rhizobacterial strains associated with hyperaccumulating species to mobilize metals such as Ni from rocks. By increasing soil labile metal fractions, these bacterial inoculants could also potentially increase metal uptake by metal-(hyper)accumulating plants. In phytoextraction (or phytomining), this would lead to an overall improvement in the efficiency of the process. In the present study, we tested the effects of strains LA44 and SBA82 on metal uptake by the hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* in a rhizobox experiment.

In rhizospheric studies of hyperaccumulators, some authors show a depletion of labile metal fractions in the rhizosphere (attributed to plant uptake), while others indicate an increase in such fractions in the rhizosphere. In either case, the differences in the concentrations of labile metal fractions do not explain the extreme metal uptake by these plants. It therefore continues to be a point of controversy whether these plants are able to access metal fractions not available to nonaccumulating plants (thus increasing metal uptake) or if their root activity leads to faster replenishment of soluble metal pools. In this study, water-soluble Ni concentrations increased in the rhizosphere of *A. serpyllifolium* subsp. *malacitanum* compared to bulk soil. The same effect, surprisingly, was not seen in $Sr(NO_3)_2$- or $Ca(NO_3)_2$-extractable Ni concentrations. However, a similar increase in water-soluble Ni and a decrease in labile Ni in the rhizosphere has been observed with other Ni hyperaccumulators, such as *Thlaspi goesingense* (22, 42) and *A. serpyllifolium* subsp. *lusitanicum* (*Alyssum pintodasilvae*) (43). Wenzel et al. (22) and Puschenreiter et al. (42) suggested a more intense weathering of Ni-rich minerals in the rhizosphere of the hyperaccumulator *T. goesingense* and a concurrent release of labile Ni.

After 14 weeks growth, the plant biomass of plants grown in inoculated pots tended to be higher than that of plants grown in noninoculated pots: shoot biomass was up to 1.3- or 1.4-fold greater in plants grown in pots inoculated with LA44 or SBA82, respectively (although the differences were not statistically significant). The increase in shoot biomass could be related to the ability of both of these strains to produce IAA (2). Shoot nutrient concentrations, such as Ca, Fe, and P, also tended to be higher in plants grown in inoculated pots than in plants grown in noninoculated pots, especially in the case of inoculation with strain SBA82. This could be due to bacterially induced mineral weathering; SBA82 is a phosphate solubilizer and a siderophore producer, which could lead to an improvement in the plant P and Fe status. Both characteristics have previously been related to an improvement in plant nutrition (44). This improvement could also be associated with the observed increase in biomass production.

Shoot Ni concentrations were far above the criteria given for Ni hyperaccumulation ($>$1,000 mg Ni kg^{-1}) (45) and were similar to concentrations found in field-collected plants of hyperaccumulating subspecies of *A. serpyllifolium* (46). Shoot Ni concentrations tended to be higher in plants grown in LA44-inoculated pots. Although the activity of either bacterial strain did not significantly

influence either the biomass or the Ni concentration, the combined effect led to an increase in Ni phytoextracted, and this was significant in the case of plants grown in LA44-inoculated pots. Similar results were obtained by Cabello-Conejo et al. (M. I. Cabello-Conejo, C. Becerra-Castro, C. Monterroso, Á. Prieto-Fernández, M. Mench, and P. Kidd, presented at the 11th International Conference on the Biogeochemistry of Trace Elements, Firenze, Italy, 2011) when they inoculated *A. pintodasilvae* with the same *Arthrobacter* strain LA44 and grew the plants in ultramafic soil from Trás-os-Montes (northeastern Portugal). The positive influence of specific bacterial strains on metal uptake by hyperaccumulator plants has been shown by several authors. For instance, inoculation with Ni-mobilizing rhizobacteria enhanced Ni uptake by the Ni hyperaccumulator *A. murale* (9, 15) and by the nonhyperaccumulator *B. juncea* (16). The increase in shoot Ni concentration observed with the LA44 treatment could be due to the ability of the strain to act on Mn oxides through the production of organic acids and consequent release of associated Ni. This could effectively help to replenish metals in the more labile fractions and enhance metal uptake by the plant.

In conclusion, the activities of two bacterial strains promoted the weathering of ultramafic rock in *in vitro* batch cultures. The two bacterial strains studied acted on distinct mineral phases, and the mechanisms involved in this process were isolate specific. Further studies should be carried out using pure mineral phases (e.g., olivine and Mn oxides) to study the different mechanisms operating in more detail. Nonetheless, bacterial activity led to an increase in the availability of metals such as Mn, Ni, and Co. Inoculation with either bacterial strain had a positive, although not significant, effect on the plant growth and shoot Ni concentration of *A. serpyllifolium* subsp. *malacitanum*. Moreover, a significant increase in phytoextracted Ni was observed with the bacterial inoculum, which was able to solubilize Ni associated with Mn oxides, a fraction which has previously been associated with Ni bioavailability in serpentine soils. This type of plant-associated bacteria could potentially be applied in phytoextraction (phytomining) systems as a means of improving their efficiency.

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