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Use of endophytic and rhizosphere bacteria to improve phytoremediation of arsenic-contaminated industrial soils by autochthonous *Betula celtiberica*

Victoria Mesa[1*], Alejandro Navazas[2,3], Ricardo González-Gil[2], Aida González[2], Nele Weyens[3], Béatrice Lauga[4], Jose Luis R. Gallego[5], Jesús Sánchez[1], Ana Isabel Peláez[1].

[1] *Departamento de Biología Funcional - IUBA, Universidad de Oviedo, 33006 Oviedo, Spain*

[2] *Departamento de Biología de Organismos y Sistemas-IUBA, Universidad de Oviedo, 33006 Oviedo, Spain*

[3] *Centre for Environmental Sciences (CMK), Hasselt University, BE-3500 Hasselt, Belgium*

[4]*Equipe Environnement et Microbiologie (EEM), UMR IPREM 5254, Université de Pau et des Pays de l'Adour, IBEAS, 64013 Pau, France*

[5]*Departamento de Explotación y Prospección Minera-IUBA, Universidad de Oviedo, 33600 Mieres, Spain*

*Corresponding author: Victoria Mesa (email: victoriamesa@gmail.com)

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ABSTRACT

The aim of the study was to investigate the potential of indigenous arsenic-tolerant bacteria to enhance arsenic phytoremediation by autochthonous pseudometallophyte *Betula celtiberica*. The first goal was to perform an initial analysis of the entire rhizosphere and endophytic bacterial communities of the above-named accumulator plant, including the cultivable bacterial species. *B. celtiberica*'s microbiome was dominated by taxa related to Flavobacteriales, Burkholderiales, and Pseudomonadales, specially the *Pseudomonas* and *Flavobacterium* genera. A total of 54 cultivable rhizobacteria and 41 root endophytes,

mainly affiliated to the phyla Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria, were isolated and characterized with respect to several potentially useful features for metal plant accumulation, such as the ability to promote plant growth, metal chelation, and/or mitigation of heavy metal stress. Seven bacterial isolates were further selected and tested for in vitro arsenic plant-accumulation; four of them were finally assayed in field-scale bioaugmentation experiments. The exposure to arsenic in vitro caused increased total non-protein thiol compounds content in roots, suggesting a detoxification mechanism through phytochelatins complexation. In the contaminated field, the siderophore and IAA producers of the endophytic bacterial consortium enhanced As-accumulation in the leaves and roots of *Betula celtiberica*, whereas the rhizosphere isolate Ensifer adhaerens strain 91R mainly promoted plant growth. Field experimentation showed that additional factors, such as soil arsenic content and pH, influenced arsenic uptake in the plant, attesting to the relevance of field conditions in the success of phytoextraction strategies.

IMPORTANCE

Microorganisms and plants have developed several ways of dealing with arsenic, allowing them to resist and metabolize this metalloid. These properties form the basis of phytoremediation treatments and understanding the interactions of plants with soil bacteria is crucial for the optimization of As-uptake. To address this in our work, we initially performed a microbiome analysis of the autochthonous *Betula celtiberica* plants growing in As-contaminated soils, including endophytic and rhizosphere bacterial communities. We then proceeded to isolate and characterize the cultivable bacteria, potentially better suited to enhance phytoextraction efficiency. Eventually, we went to the field application stage. Our results corroborated that recovery of pseudometallophytes-associated bacteria adapted to a large historically contaminated site and their use in bioaugmentation technologies are affordable experimental approaches and potentially very useful for implementing effective phytoremediation strategies with plants and their indigenous bacteria.

INTRODUCTION

Arsenic (As) is a natural component of the earth's crust and is widely distributed throughout the environment (air, water, and land). Other sources of environmental arsenic are anthropogenic i.e. insecticides, mining, industrial processes, coal combustion, timber preservatives, etc. (1). High concentrations of As lead to environmental damage and health problems (2, 3). Arsenic exists in four oxidation states – predominantly arsenate (As^{V}) and arsenite (As^{III}), and to a lesser extent, as arsenic (As^{0}) and arsine (As^{-III}) (4, 5). As^{V} is a phosphate analogue and interferes with essential cellular processes, such as oxidative phosphorylation and ATP synthesis, whereas the toxicity of As^{III} is due to its tendency to bind to sulfhydryl groups, affecting general protein functioning (6).

A sustainable technology for cleaning As-contaminated soils is phytoremediation, which is defined as the use of plants to remove or reduce toxic concentrations of hazardous substances in the environment (7). As part of As detoxification, plants produce metabolites, like non-protein thiols (NPTs), cysteine, glutathione (GSH), or phytochelatins (PCs) synthesized from GSH, involved in plants' defense pathways against As-induced oxidative stress (8). PCs have been identified in plants and some microorganisms (9). Phytoremediation of As-, Cd-, and Pb-contaminated soils is more cost-effective, efficient, and less time-consuming than most other remediation technologies (10). Phytoremediation can reduce the available concentration of inorganic compounds through different processes such as phytoextraction, rhizofiltration, phytostabilization, or phytovolatilization (11, 12). Phytoextraction seeks to remove inorganic contaminants, especially heavy metals, metalloids, and radionuclides from contaminated soils through uptake by plants and accumulation in harvestable plant biomass (13).

Microbial processes play a major role in As cycling in the plant-soil-microbe system and effective phytoremediation of contaminated soils involves interactions with plant-associated microbes (14). Some bacterial mechanisms that enhance phytoremediation consist of plant growth by bacterial metabolites, such as indole-3-acetic acid (IAA), metal chelation by siderophores and organic acid production, soil

acidification, solubilization of metal phosphates, methylation, and moderation of heavy metal stress by bacterial 1-amino-cyclopropane-1-carboxylic acid deaminase (ACCD) (15, 16). Rhizobacteria that colonize the vicinity of roots accelerate metal mobility and plant availability by various processes including redox transformations and the release of protons and organic acids, whereas endophytic bacteria colonize the internal tissues of plants and promote plant growth through mechanisms such as phosphate solubilization, IAA and siderophore production, and/or supplying essential vitamins to plants (17, 18). In addition to their tolerance for heavy metal stress, plant-associated bacteria can act as biocontrol agents against certain pathogenic organisms and ensure nitrogen fixation and the production of growth regulators (19).

In Asturias (northeastern Spain), several areas are contaminated with arsenic as a result of recent industrial (chemical, metallurgical and siderurgical) dismantling. A representative example of a large-scale contaminated site is Nitrastur, a highly-contaminated abandoned fertilizer industry, located in the municipality of Langreo (20). The main waste found at this site that affects soil quality is pyrite ashes, comprised largely of iron oxides and hydroxides, and other metal(loid)s that were produced as a by-products of roasting sulphur ores. *Betula celtiberica* (Rothm. & Vasc.) is a deciduous tree, pseudometallophyte (21), and fast-growing high biomass plant with well-developed root systems colonizing the study area. Birches are widely used in repopulations and the genus *Betula* includes several pioneer species found in soils contaminated with heavy metals displaying high tolerance to Zn, Pb, and Cd (22, 23). Its distribution is restricted; *Betula* was only found in mountain ranges in the Sistema Central (one of the largest mountain ranges on the Iberian Peninsula) (24). Thus, the autoecology of this species suggests that it might be a suitable candidate to bioremediate contaminated soils in Asturias.

Consequently, a question that arises when considering the role of As in the plant-bacteria relationship is how a natural mechanism, such as phytoremediation, can be enhanced in the autochthonous pseudometallophyte *Betula celtiberica.* Additionally, it is worth asking what the importance of the

97 associated bacteria is in this process. Thus, the objectives of this study were to assess how the abundance,
98 richness, community composition, and activities of *B. celtiberica*'s microbiome respond to long-term As
99 contamination in the abandoned fertilizer industry and subsequently, to evaluate the effect of
100 bioaugmentation with indigenous endophytic and rhizospheric bacteria on As accumulation in *Betula*;
101 leaves and roots were also studied. To achieve these goals, we analyzed the *Betula*-associated bacterial
102 community through DNA-dependent approaches (pyrosequencing of 16S rRNA genes), isolation and
103 characterization cultivable root endophytes and rhizosphere strains capable of augmenting As
104 phytoextraction, and finally testing their behavior *in vitro* and in field bioaugmentation experiments.
105

106 **MATERIALS AND METHODS**

107 **Plant material, rhizosphere soil sampling, and chemical analysis**

108 Details of the contaminated site are given in supplemental material. Five *B. celtiberica* trees growing on
109 contaminated sites in NC5 and NC6 plots of the Nitrastur industrial area (Fig. S1) were sampled in March
110 2013. Root samples were randomly collected from each tree and adherent rhizosphere soils were pooled
111 from around each tree. Rhizosphere-containing soil, defined as soil in the immediate vicinity of the roots,
112 was obtained by shaking the roots, thereby collecting the soil that had been attached to the roots. Soils
113 were air-dried and sieved to a grain size of up to 2 mm. A 250-mg representative of each sample was
114 digested in a microwave (Multiwave3000, Anton Paar) with *aqua regia* at 800 W for 15 min. The solutions
115 were diluted to 50 mL with ultrapure water and filtered through a 0.45-µm PTFE filter prior to analysis.
116 As, Cd, Zn, Cr, Ni, Cu, Pb, and Hg were quantified by an inductively coupled plasma mass spectrometer
117 (Agilent Technologies 7700 ICPMS) using IDA (isotopic dilution analysis) as previously described (25).

Collection of rhizospheric and endophytic fractions and isolation of cultivable *B. celtiberica*-associated bacteria

To obtain rhizospheric fractions, 5 g of rhizosphere soils were shaken in 10 mL of 0.1% sodium pyrophosphate (Sigma-Aldrich, USA). Soil particles were allowed to settle for 1 h. The supernatants were diluted to 10^{-8} and plated on two different media, 1/10 diluted tryptic soy agar (TSA) (26) and 1/10 diluted 869 solid media (27). The plates were incubated for 7 days at 30°C. The remaining supernatants were stored at -80 °C until DNA extraction. To obtain the endophytic fraction, roots were rinsed under running tap water. Root samples were surface-sterilized for 10 min in 2% active chloride solution supplemented with one droplet of Tween 80 (Merck, Germany) per 100 mL solution and subsequently rinsed three times for 1 min in sterile distilled water. After surface sterilization, root samples were macerated in 10 mL 10 mM $MgSO_4$ with a mortar. To isolate endophytes, the extracts obtained from the macerated roots were serially diluted to 10^{-5}, plated on the same medium, and incubated. Colonies with different morphotypes were selected and repeatedly streaked until axenic cultures were obtained (28). The identification and phylogenetic affiliation of cultivable *B. celtiberica*-associated bacteria are detailed in the corresponding section of the supplemental material. Methods of characterization of endosphere and rhizosphere isolates based on the production of Indole-3-Acetic Acid (IAA), 1-Amino-Cyclopropane-1-Carboxylic Acid Deaminase Activity (ACCD), siderophores and As resistance are included in the section "Characterization of Cultivable Isolates" of the supplemental material.

DNA extraction and 454 pyrosequencing

DNA samples were obtained from the 10 rhizospheric and endophytic fractions using the PowerSoil® DNA Isolation Kit (MoBio, USA). The V5-V7 region of the 16S rRNA gene was amplified using the primer set 799F (5'-AACMGGATTAGATACCCKG-3') (29) and 1391R (5'-GACGGGCGGTGWGTRCA-3') (30). Each 25-µl PCR reaction contained ~10 ng of DNA and was performed using the FastStart High Fidelity PCR System (Roche Applied Science, Mannheim, Germany), following the instructions provided by the manufacturer.

142 Cycling conditions included: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation
143 at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min; a final extension phase
144 was conducted at 72°C for 10 min. PCR amplicon pools were cleared from residual primers and primer
145 dimers by separating the PCR products on a 1.5% agarose gel. Bacterial amplicons were excised from the
146 gels using the QIAQuick gel extraction kit (Qiagen Benelux N.V., The Netherlands). Amplicon length of
147 sequences produced by primer set 799F-1391R was reduced by amplifying the samples with primer set
148 967F (5'-CAACGCGAAGAACCTTACC-3') (31) and 1391R in a second round. Forward primer was fused to
149 the Roche 454 pyrosequencing adaptor A and a sample-specific 10 bp barcode (multiplex identifiers,
150 MIDs) and reverse primer were fused to adaptor B (Roche Applied Science, Germany). PCR cycling
151 conditions were identical to the ones previously described, with the exception of the number of PCR
152 cycles, which was lowered to 25. Sequencing was carried out on one eighth of a Pico Titer Plate on a Roche
153 Genome Sequencer FLX+ using Titanium chemistry (Roche Appl Applied Science, Germany) by LGC
154 Genomics (Germany).

155 **Sequencing Data Analysis**

156 Sequences were analyzed using the Quantitative Insights into Microbial Ecology QIIME package (32).
157 Firstly, reads were assigned to samples based on their nucleotide barcode. This step also performed
158 quality filtering (minimum quality score of 30, eliminating reads with lengths >200 bp and 0 ambiguous
159 bases and mismatches in primer sequences). Chimeric sequences were checked and removed using
160 Usearch (33) and the resulting sequences obtained were clustered into operational taxonomic units
161 (OTUs) using a minimum identity of 97% based on their similarity according to UClust (34). Representative
162 sequences from each OTU were aligned with the SILVA databases v.119 (35), using PyNAST algorithm (36)
163 and singletons were excluded from the analysis. Sequences classified as chloroplast (0.3%) or
164 mitochondria (0.5%) were removed from the alignment. Alpha diversity was calculated through observed
165 species (observed OTUs), richness estimator (Chao1), and diversity indices (Shannon and Simpson) to

166 compare the diversity of the bacterial community within samples. Richness and diversity were estimated
167 using phyloseq R package (v 1.7.12) (37). Bacterial diversity, measured as OTU richness, was estimated by
168 rarefaction analysis and rarefaction curves were generated based on OTUs found in each sample using
169 vegan R package (v 2.4-0) (38).

170 **Propagation of plant clones and bacterial inoculation under *in vitro* arsenic exposure experiments**

171 The micropropagated birch clones BC-K (*B. celtiberica)* were used. This clone has been selected because
172 of its great biomass, high tolerance to Cd accumulation, and well-developed root system (39). Birches
173 were micropropagated *in vitro* from apical shoot segments ~ 10-15 mm in a slightly modified Murashige
174 and Skoog medium (MS) (40) in which macronutrients were diluted by half and 30 g L^{-1} of saccharose and
175 7 g L^{-1} of agar were added. Medium pH was adjusted to 5.7. Plants were grown for two months in a growth
176 chamber with a 16-h photoperiod at 25°C. Bacteria were grown separately in 500 mL Erlenmeyer flasks
177 containing 100 mL of GAE (glucose, asparagine, yeast extract) medium (41) with continuous shaking at
178 30°C to reach 10^8-10^9 cells per mL (24-48 h). Cells were recovered by centrifugation (8,000 X g, 10 min,
179 4°C) and cell pellets were resuspended in MS liquid medium to avoid changes in the state of plants. For
180 the bacterial inoculation, plants were placed in falcon tubes containing 10 mL bacterial suspensions (see
181 below) for 20 min, ensuring proper immersion of the roots.
182 Seven treatments were tested with six biologically independent replicates per treatment: i) non-
183 inoculated plants were used as controls (C), ii) plants inoculated with endophytic strain 29E, iii) plants
184 inoculated with endophytic strain 32E, iv) plants inoculated with an endophytic bacterial consortium (BC)
185 composed of strains 28EY and 28EW (inoculum containing equal numbers of each strain), v) plants
186 inoculated with rhizospheric strain 44R, vi) plants inoculated with rhizospheric strain 89R and vii) plants
187 inoculated with rhizospheric strain 91R. Treated and control plants were transferred to polycarbonate
188 magenta vessels (Magenta Corp., USA) containing 80 mL of MS liquid medium supplemented with 150 μM
189 of As^V (arsenic is provided as Na_2HAsO_4) and 1 mL of each bacterial suspension was added to the medium

of each plant (except for the control). Plants were randomly placed in the growth chamber and after 30 days of As^V exposure, the leaves and roots of each plant were separated, rinsed with doubly deionized water (Milli-Q 185 Plus System). Increases in the fresh weight of plants were determined (FWI, defined as FWI = (final plant fresh weight – initial plant fresh weight) / (initial plant fresh weight)). Fresh plant material from three individual plants was reserved to estimate the total non-protein thiol compounds. The other three plants were individually dried at 40°C for 72 h and dry weights of leaves and roots were determined. Dried plant material was used for further chemical analyses, arsenic content, and speciation in plant tissues.

Determination of total non-protein thiol (NPT) compounds by HPLC, arsenic content and speciation in plant tissues

NPTs content was analyzed in the leaves and roots of each treatment (42) with slight modifications (43). Chemical As was determined in leaves and roots by ICP-MS. As speciation was quantified in leaves, roots, and in the culture medium plants grew in (44). Details of these methods are provided in the corresponding section of Supplemental Methods.

Field experiments

Field trials were performed on a 600-m^2 experimental plot located within the Nitrastur industrial zone (Fig. S1) from July to October 2014 with 216 *B. celtiberica* plants. This experimental plot was divided into 2 X 2-m subplots with at least 1 m between plots to minimize near-neighbour effects. Plants were transferred to the field when they were 9 cm tall and cultivated in 12 different plots (6 treatments x 2 replicate plots for each treatment x 18 plants in each plot). Based on results obtained in the previous study from *in vitro B. celtiberica* inoculation, four bacterial strains were selected for inoculation in the field. The experimental design included six treatments: i) non-inoculated plants were used as control (C), ii) non-inoculated plants supplemented with NPK-fertilizer (N:P:K, 6:8:15, Phenix, Italpollina) were used as fertilized control (FC), iii) plants inoculated with endophytic strain 32E (E), iv) plants inoculated with

rhizospheric strain 91R (R), v) plants inoculated with the endophytic BC, and vi) plants inoculated with endophytic BC and supplied with the NPK-fertilizer (BC+F). At the end of the experiment, plant heights were measured. Six plants per treatment were harvested and roots and shoots were sampled separately. They were washed with tap water, rinsed with deionized distilled water, and oven-dried at 40°C for 72 h. The dried plant material was ground in a cutting mill (Pulverisette Fritsch, Germany) to a size of up to 1 mm. As content was determined in dried plant material (leaves and roots). The effects of inoculations were evaluated by the increase in plant height (plant height index, defined as PHI = (final height – initial height) /(initial height) and the estimation of biomass through shoot dry weight. Root dry weights were not determined given the difficulty of uprooting entire roots. As concentration and pH measurement in the different plots, and the successive stages of *in vitro* propagation and acclimatization of the plants in greenhouse before their final planting in the field are detailed in the "Field experiments" section of Supplemental Methods. Determination of As phytoextraction efficiency indices is also described in the corresponding section of Supplemental Methods.

Statistical analysis

The analysis of variance (ANOVA) and Tukey's mean grouping were applied in *in vitro* experiments to determine the significance of the interactions between treatment means. Analyses were performed with SPSS software package version 22.0 (SPSS, Inc., Chicago, IL). We also used linear regression techniques to investigate the correlation between fresh weight index (FWI), defined as (final plant fresh weight – initial plant fresh weight) / initial plant fresh weight or leaf and root dry weights, and leaves and roots As concentrations in the *B. celtiberica* clones harvested in *in vitro* conditions and inoculated with different bacterial strains.

For the field experiment, data were processed by ANOVA and differences between specific pairs of mean values were evaluated using Tukey's test (SPSS, Inc., Chicago, IL). In contrast to *in vitro* experiments, the soil conditions among the plots where the *B. celtiberica* clones were exposed to different treatments could

potentially have varied in terms of soil pH and As concentrations. This might affect As concentrations in leaves and roots, leaf dry weight, and PHI. Thus, we had to account for this medium condition variability before exploring the effect of leaf and root As content on the leaves dry weight and PHI or find a possible treatment effect on these variables. To do so, we first conducted a model selection using the Akaike Information Criterion (AIC) for each variable comparing three different models:

$a)\ y = a + pH\ + \varepsilon$

b) $y = a + As\ soil\ + \varepsilon$

c) $y = a + As\ soil\ + pH + \varepsilon$

in which *y* could be the leaf or root As concentration, leaf dry weight, or PHI; *a* represents the intercept, and *ε* was the error term or residual component. From the selected best model, we extracted the residual part for each variable and studied the relationship between the residuals for leaf or root As concentration and those for leaf dry weight or PHI. For each variable, we also inspected the existence of significant differences in these residuals between treatments using Tukey's HSD test.

In the bacterial diversity analysis, an ANOVA was applied to estimate potential differences in microbial community diversity between the rhizosphere and endosphere of the five *B. celtiberica* trees. Simple linear regression analyses were then used to study the effect of rhizosphere pH and As concentration on this microbial diversity (in particular on the Chao1 diversity index). Non-parametric analyses of variance on 16S data were performed to evaluate differences between bacterial communities with permutation-based hypothesis tests, ANOSIM (namely analysis of similarities), and adonis (permutational multivariate analysis of variance) with 99 and 999 permutations, respectively (45). All the above-mentioned statistical analyses were conducted with R (v 3.2.4) (46). Plots were made using the ggplot2 package for R (v 2.1.0) (47).

RESULTS

Rhizosphere soil characterization

The total As content of the rhizosphere soils varied from 865 to 3349 mg kg^{-1} with a mean value of 1900 mg kg^{-1}, which was 9.5-fold higher than the Spanish regulatory limits for total As content in industrial soils (48). Furthermore, the samples also had Pb concentrations exceeding Spanish regulatory limits for industrial soils. Organic matter was 9-16.36%, whereas texture was found to be similar in all samples. In contrast, pH of 6.01 - 6.97 in the close vicinity with the roots was relatively consistent among plants (Table S1).

Bacterial diversity of rhizosphere and endosphere communities associated with *Betula celtiberica* growing in Nitrastur

High-throughput 16S rRNA gene sequencing of the total community DNA was performed on the endophytic and rhizospheric fractions and was subsequently used to analyze cultivable bacteria. The raw sequence data from the samples consisted of 20,098 and 72,730 sequences from endosphere and rhizosphere, respectively. The number of OTUs based on a 97% similarity threshold was smaller in the endosphere than in the rhizosphere for all trees sampled (119-595 compared to 798-1463; Table S2).

Among trees, microbial communities exhibited greater diversity in the rhizosphere (average observed OTUs, Chao1, Shannon, and Simpson were 1068, 1122, 7.5, and 0.96, respectively) than in the endosphere (average observed OTUs, Chao1, Shannon, and Simpson were 296, 322, 5.49 and 0.87, respectively). Bacterial diversity was higher in the rhizosphere than in the endosphere for the different alpha diversity indices (Fig. 1A). Rarefaction curves revealed that endophytic bacterial communities were less diverse than rhizosphere communities. In the current analysis, the asymptotic shape of the curves indicates that sequencing depth was sufficient to capture the entire bacterial diversity (Fig. 1B). In addition, microbial community diversities from the endosphere and rhizosphere, estimated with the Chao1 index, were negatively affected by the soil As content, but correlated positively with soil pH (Fig. 1C).

From the classifiable sequences, the bacterial community composition was analyzed at three different taxa levels (phylum, order, and genus) considering abundant OTUs (sequences present in more than one sample and representing more than 1% of the total sequences). Some sequences could not be assigned to any taxa at the phylum level (0.9-21.3% in the endosphere and 7.3-15.5% in the rhizosphere). At the phylum level, six bacterial phyla were overrepresented for both communities. *Proteobacteria* was the most abundant phylum (accounting for 64% of total reads), followed by *Bacteroidetes* (17%), *Actinobacteria* (7.9%), *Firmicutes* (1.8%), and *Chlamydiae* (1.3%). Proteobacteria were represented by *Alphaproteobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria* (Fig. 2A). On closer inspection, some OTUs belonging to *Sphingobacteriales, Rhizobiales, Xanthomonadales* orders and *Aquicella, Flavobacterium, Burkholderia,* and *Pseudomonas* genera were shared by both endosphere and rhizosphere communities. In the rhizosphere, *Pseudomonas* (37.07%), *Burkholderiales* (*Oxalobacteraceae* and *Xanthomonadacea*e, 9.62%), and *Flavobacterium* (7.64%) were the dominant OTUs. In the endosphere, *Flavobacterium* (14.99%), *Pseudomonas* (13.83%), and *Burkholderiales* (*Comamonadaceae* and *Oxalobacteraceae,* 11.39%) were the most frequently observed OTUs (Fig. 2B). Consistent with these observations, the non-parametric analyses of variance on 16S data confirmed the similarity of the rhizo- and endophytic communities (ANOSIM: $p = 0.20$, $R^2 = 15\%$; adonis: $p = 0.11$, $R^2 = 13\%$).

Isolation and identification of autochthonous cultivable bacteria associated with *Betula celtiberica*

To explore the diversity of cultivable root endophytes (E) and rhizobacteria (R) associated with *B. celtiberica,* an isolation was performed on non-selective media. The number of colony-forming units (CFUs) on TSA and 869 media varied in samples taken from different trees. The total numbers of cultivable endophytes and rhizosphere strains ranged from $11.7 \times 10^4 \pm 3.16 \times 10^4$ CFU g^{-1} fresh weight of plant material to $15.8 \times 10^7 \pm 6.2 \times 10^7$ CFU g^{-1} fresh weight of rhizosphere soil, respectively. Ninety-five bacterial strains were isolated as axenic cultures: 41 endophytic strains and 54 rhizospheric strains. The phylogeny of the *B. celtiberica*- associated cultivable bacteria (based on the 16S rRNA genes) separated clearly into

four strongly supported phyla: *Actinobacteria, Bacteroidetes, Firmicutes*, and *Proteobacteria*. Each of these phyla accounted for 7.3%, 7.3%, 36.6%, and 48.8% of endophytic strains and 38.9%, 13%, 20.3%, and 27.8% of rhizospheric strains, respectively (Fig. 3). Based on 16S rDNA sequences, redundant strains were eliminated to end up with 39 endophytes and 46 rhizobacteria (Table S3). Strains from the endosphere belonged to 19 genera; among the most predominant were *Bacillus* (24%), *Rhizobium* (11%), *Flavobacterium,* and *Pseudomonas* (8% each), while rhizobacteria were principally *Streptomyces* (23%), *Bacillus* (17%), *Flavobacterium* (9%), and *Pseudomonas* (10%).

Characterization and selection of endosphere and rhizosphere isolates based on their IAA, ACCD, and siderophore production, and As resistance

The isolated strains were evaluated for their plant growth-promoting traits and resistance to As. Isolates produced varying amounts of IAA. Of the 39 endophytic isolates, 68% were able to produce more than 10 µg mL^{-1} of IAA, 46 rhizobacteria (53%) were able to produce more than 10 µg mL^{-1} IAA. High IAA production was exhibited by rhizospheric strain 91R (closely related to *Ensifer adhaerens* Sx1) (30.01 µg mL^{-1}) and root endophyte 89R (closely related to *Aminobacter aminovorans LZ1304-3-1*) (23.92 µg mL^{-1}). Another important plant growth promoting (PGP) trait, 1-amino-cyclopropane-1-carboxylic acid deaminase (ACCD) activity, was detected in 30% rhizospheric and 5% endophytic bacteria. Siderophore production was found in 32% of the endophytes and 36% of the rhizobacteria. MIC of As^{V} for 30.7% of the endophytic strains and 34% of the rhizobacteria were 100 mM As^{V}, while 10% endophytes and 8.7% rhizobacteria grew up to 50 mM As^{V}. More than one in five (20.5%) of the endophytes were sensitive to the lowest As^{V} concentration, while 6.5% of the rhizobacteria were sensitive to As. The As^{III} MIC was 20 mM for 12.8% of endophytes and 6.5% for rhizobacteria but 15% of endophytes and 13% of rhizobacteria were able to grow between 10 and 15 mM As^{III}. Table S3 illustrates that 30.7% and 41% of endophytes and rhizobacteria were sensitive to the lowest As^{III} concentration, respectively.

Seven isolates revealing resistance to high concentrations of As and/or potential promotion of growth by at least one PGP trait were selected (Table 1). Five bacteria were tested separately and two endophytic bacteria were tested in a consortium (28EY and 28EW), after evaluating their compatibility by cultivating the strains together on GAE medium and plating serial dilutions of the culture. All selected bacteria were resistant to high concentrations of As^{V} (100 mM), whereas only one, strain 44R (closely related to *Rhodococcus erythropolis)* was resistant to a high concentration of As^{III} (20 mM). In addition, endophytic strains 29E, 32E, 28EY, and 28EW (closely related to *Neorhizobium alkalisoli, Rhizobium herbae, Variovorax paradoxus,* and *Phyllobacterium myrsinacearum*, respectively) produced siderophore and IAA. Rhizosphere strains 44R, 89R (closely related to *Rhodococcus erythropolis and Aminobacter aminovorans*, respectively) and 91R (closely related to *Ensifer adhaerens*) produced siderophore, IAA, and ACC deaminase.

In vitro evaluation of arsenic uptake by non-inoculated and inoculated _Betula celtiberica_ plants

The seven above selected strains were used to inoculate *B. celtiberica* plants. Plant performances were evaluated under *in vitro* cultivation (n = 6 per treatment). After 30 days, no visible symptoms of As toxicity were observed in any *B. celtiberica* plants; however, As affected plant biomass ($P < 0.05$) (Table S6). Regarding As accumulation in plant tissues, root As concentration was higher and had a greater effect on leaf and root biomass than leaf As concentration, which showed almost no effect (Fig. 4). Moreover, the effect of root As concentration was positive on leaf biomass but negative on root biomass. Leaf and root As accumulation negatively impacted FWI, although root As concentration exerted a more pronounced effect. Fig. 4 also illustrates that only *B. celtiberica* clones inoculated with *Ensifer adhaerens* strain 91R and *Rhizobium herbae* strain 32E showed both root As concentration and root biomass above the mean. Furthermore, plants inoculated with the *Rhizobium herbae* strain 32E were the only ones with root As concentration and FWI above the mean.

354 Total NPTs content estimation would aid in analyzing their hypothetical role in As toxicity mitigation (Fig.
355 5). Different NPTs analyses in the absence of arsenic quantified higher concentrations of NPTs in leaves
356 than in roots in the different treatments (represented as ratio Leaf/Root NPTs = L/R) (Table S4). The
357 exception to this was plants inoculated with *Rhizobium herbae* strain 32E, the only treatment yielding an
358 L/R ratio >1 with the particularity of higher GSH content in roots than leaves. Exposed to arsenic, NPTs
359 concentrations were higher in roots than in leaves; hence, the ratio was reversed (L/R < 1). *Neorhizobium*
360 sp. strain 29E and *Aminobacter aminovorans* strain 89R treatments are the exceptions to this trend and
361 displayed higher contents in leaves than roots.
362 Overall, in the presence of arsenic, both leaves and roots had increased total content of NPTs, except in
363 the leaves of *Rhodococcus erythropolis* strain 44R- and *Ensifer adhaerens* strain 91R- treated plants (Fig.
364 5A). With these treatments, leaf NPTs contents decreased slightly compared to non-inoculated plants (C),
365 albeit had higher levels of phytochelatin 4 (PC_4). *De novo* PC synthesis was found in roots treated with
366 *Rhizobium herbae* strain 32E (8.62 nmol g^{-1} fw) and *Rhodococcus erythropolis* strain 44R (9.06 nmol g^{-1} fw)
367 under As exposure. In leaves, PCs were also detected after inoculation with BC (*Variovorax paradoxus*
368 strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW) (10.54 nmol g^{-1} fw), with *Rhizobium herbae*
369 strain 32E (13.21 nmol g^{-1} fw), *Neorhizobium* sp. strain 29E (12.82 nmol g^{-1} fw), *Rhodococcus erythropolis*
370 strain 44R (14.13 nmol g^{-1} fw), and *Ensifer adhaerens* strain 91R (13.30 nmol g^{-1} fw).
371 Based on controlled plants, the As speciation analysis of the culture medium (Fig. 5B) enables us to
372 conclude that *B. celtiberica* metabolism was responsible for some 20% of the reduction of As^{V} to As^{III}
373 present in the medium. Bacterial inocula with *Neorhizobium alkalisoli* strain 29E, *Aminobacter*
374 *aminovorans* strain 89R, or *Ensifer adhaerens* strain 91R would thereby oxidize this 20% of As^{III} generated
375 by plants, resulting in about 100% As^{V} in the medium. No further reduction was seen with any of the
376 strains in these culture conditions. In plant tissues (Fig. 5C), inoculation with *Aminobacter aminovorans*
377 strain 89R resulted in As^{III} oxidation of 16% in leaves and 35% in roots versus controls. Furthermore,

treatment with *Ensifer adhaerens* strain 91R led to 11% of As^{III} oxidation in leaves and 13% of As^{V} reduction in roots. The BC treatment oxidized 17% of As^{III} in leaves and reduced 26% of As^{V} in roots. Finally, As^{III} levels in roots were higher after inoculation with *Ensifer adhaerens* strain 91R, *Rhodococcus erythropolis* 44R, *Rhizobium herbae* strain 32E, and with BC; these treatments were also characterized by high NPTs content.

Field-scale evaluation of arsenic uptake by non-inoculated and inoculated *Betula celtiberica* plants

Bacterial strains were chosen for field testing based on *in vitro* As uptake and physiological traits. Another important feature was their ability to grow in the laboratory media, since high biomass is a factor of great practical relevance for field-scale bioaugmentation experiments. Four strains were selected: the siderophore and IAA- and ACC deaminase-producing *Ensifer adhaerens* strain 91R (As uptake in roots > 700 μg g^{-1} dw, As uptake in leaves > 10 μg g^{-1} dw) (treatment R), the siderophore and IAA-producing *Rhizobium herbae* strain 32E (As uptake in roots > 600 μg g^{-1} dw, As uptake in leaves > 15 μg g^{-1} dw) (treatment E), and the endophytic BC that included the siderophore and IAA-producing strains *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW (As uptake in roots > 500 μg g^{-1} dw, As uptake in leaves > 15 μg g^{-1} dw). Soil fertilization was also evaluated in the field for the BC inoculation (BC+F) and for the control (FC). Soil sample analyses performed prior to the experiment on the different phytoextraction field sub plots detected a wide range of pH values (2.56 -6.04), As (1219-3034 mg kg^{-1}), and Zn and Pb levels (Table S5). Inoculations affected *B. celtiberica* plant heights and shoot biomass ($P < 0.05$) (Table S6) in the field after 90 days of cultivation. In general, soil conditions such as As concentration and pH in the different plots could account for a large proportion of the variance in As concentration in roots and leaves, shoot biomass, and PHI (Fig. 6). Correlations between variables can be positive or negative according to independent variables (soil As concentration or pH) and the type of dependent variable (As concentration in leaves and roots, shoot biomass, or PHI). However, the effect of soil As and pH varies from one variable to the next; while both roots and leaves As content were positively

or negatively correlated with soil As concentration or pH, respectively, the opposite occurred for shoot biomass and PHI.

A model selection showed that both soil As content and pH must be considered when accounting for the effect of soil variability among plots on leaves and roots As concentration, shoot biomass, and PHI. In fact, a model using soil As and pH as independent variables resulted in the lowest AIC, or was at least fairly similar to the other candidate models (i.e. $\Delta AIC < 2$, Table S7; see more details about the statistical analysis in Material and Methods). The residuals from this model for leaf As concentration were uncorrelated with those from shoot biomass and PHI (Fig. S2). However, the shoot biomass and PHI residuals correlated somewhat negatively with the root As concentration residuals. This coincides with those from the *in vitro* experiments, where root As concentration also impacted plant growth more than leaf As concentration. We also observed that only those *B. celtiberica* clones inoculated with *Ensifer adhaerens* strain 91R (R) and BC showed positive residuals means for PHI, shoot biomass, and root As concentration (Fig. S2A and S2B).

The indices involved in plant-trace metal interactions (bioconcentration factor, BCF, mobility ratio, MR, and translocation factor, TF; Table S8) and determination of indices involved in As phytoextraction efficiency in the supplemental material) were all lower than 1. Arsenic BCF was increased in plants inoculated with BC compared to non-inoculated plants. Likewise, the mobility ratio (MR) was also increased in plants inoculated with the bacterial consortium (BC) with an average value of 0.009. In general, a mobility ratio < 1 indicates that the plants exclude or have a low As uptake rates. BCF and MR rose from 10% and 33% respectively, in plants inoculated with BC with respect to non-inoculated plants. TF exhibited low translocation rates of As from roots to leaves.

DISCUSSION

Microorganisms and plants have developed several constitutive or adaptive mechanisms to cope with As enabling them to resist and metabolize it. Phytoextraction has emerged as an alternative remediation

approach to restore contaminated sites (10). Moreover, the use of common native plants appears to be a good approach to guarantee phytoextraction success, since it limits plant competition and ensures autoecological plant requirements (49). In this context, the recovery of pseudometallophyte-associated bacteria adapted to a large, historically contaminated site may improve phytoremediation through bioaugmentation technologies (50). In Asturias, the location of this study, the endemic *B. celtiberica* prospers in numerous places and has naturally colonized the Nitrastur industrial area. On this site *B. celtiberica* trees grow in soils containing varying amounts of As (up to 3349 mg kg^{-1}). This, together with birch autoecology, make this species an ideal candidate to investigate its phytoextraction capabilities. Our approaches include analyzing the *B. celtiberica* endosphere and rhizosphere microbiome via culture-independent and culture-dependent techniques as a first step to later proceed to field application using these bacteria in bioaugmentation-assisted phytoextraction of *B. celtiberica* clones.

Culture-independent techniques indicated that *B. celtiberica*'s microbiome was dominated by *Bacteroidetes*, *Betaproteobacteria,* and *Gammaproteobacteria*, represented by the taxa related to *Flavobacterium, Burkholderiales,* and *Pseudomonas.* At the genus level, *Pseudomonas* displays the most promising levels of colonization and ability to persist in the endophytic niche, probably due to its wide spectrum of fatty acid and carbon source utilization (18). Predominant bacterial strains in the rhizosphere are gram-negative, and *Pseudomonas* and *Flavobacterium* are among the most represented genera. This may be attributed to the efficiency of gram-negative bacteria to utilize the root exudates and organic compounds released by living plant roots into their surrounding environment (51, 52). The number of OTUs in the endophytic bacterial communities (296 ± 183) was much lower than in the rhizosphere (1068 ± 283); similar results have been described for mature poplar trees growing in natural ecosystems (53). Interestingly, root endophytic and rhizospheric communities differ in richness estimators and diversity indices, while they do not differ in community composition. Both communities share many bacterial species and the non-parametric analyses of variance on 16S data bore out these similarities. Regarding

450 the impact of microbial diversity in As-contaminated soils, our results show that the diversity of the
451 rhizosphere and endosphere microbial communities negatively correlated to As soil contents. Hence,
452 arsenic affected microbial diversity, not only of the rhizospheric fraction, but also of the endophytic
453 fraction of roots as previously noted by Hu et al. (54). Likewise, soil pH appeared also to be an important
454 factor impacting the structure of the bacterial community in Nitrastur soils. At lower pH, lower microbial
455 diversity was reported in differing environmental contexts (55, 56).
456 Cultured bacteria were predominantly represented by *Actinobacteria, Bacteroidetes, Firmicutes*,
457 *Alphaproteobacteria,* and *Gammaproteobacteria*, among which the main genera were *Streptomyces,*
458 *Flavobacterium, Bacillus, Rhizobium,* and *Pseudomonas*. Many endophytes are members of common soil
459 bacterial genera, such as *Pseudomonas* and *Bacillus. Rhizobia, Pseudomonas,* and *Bacillus* and have been
460 reported to be typical rhizobacteria (18, 51). The composition of the cultivable bacterial community of *B.*
461 *celtiberica* at the phylum level, appears to be similar to the root microbiome of other plants (17, 57, 58).
462 Although more strains were present in the total communities than in the cultivable bacterial community,
463 the cultivated bacterial strains gave a good overview of the most dominant genera present in *B. celtiberica*
464 plants. However, *Actinobacteria* and *Firmicutes* were more represented in our culture-dependent
465 isolation method than in the pyrosequencing surveys. As expected, the population density of cultivable
466 bacteria was considerably higher in the rhizosphere than in the endosphere recovered from root tissues
467 (59). Rhizosphere soils are indeed rich environments, due to nutrients exuded from the roots of most
468 plants which are accustomed to supporting bacterial growth and metabolism. In addition, rhizosphere
469 soils are described as mesotrophic, favoring suitable conditions for microbial growth (12, 60). As in
470 previous reports (17), we found the ability to produce IAA mainly among endophytes, whereas
471 siderophore and ACCD production was more common among rhizobacteria, which were also the most
472 resistant to high concentrations of arsenate and arsenite.

The selection criteria for the most promising strains to improve phytoextraction efficiency were As resistance, suppression of stress ethylene production (due to ACC deaminase activity), stimulation in plant growth and biomass, phytohormones (such as IAA) production, or improvement in plant nutrition, due to the presence of siderophore-producers (61). Considering all the properties studied, the strains selected were those closely related to *Neorhizobium alkalisoli* ZY-4s, *Rhizobium herbae* CCBAU 83011, *Rhodococcus erythropolis* TS-TYKAKK-12, *Aminobacter aminovorans* LZ1304-3-1, and *Ensifer adhaerens* Sx1. Moreover, strains that were highly similar to *Variovorax paradoxus* S110 and *Phyllobacterium myrsinacearum* NBRC 100019 were also tested in a consortium. In an initial phase, bioaugmentation was performed under sterile conditions (*in vitro* experiment) to exclusively determine the effect of the inoculated strain. The *in vitro* inoculations of *B. celtiberica* clones showed a positive effect of the siderophore-, IAA-, and ACCD-producing *Ensifer adhaerens* strain 91R and the siderophore- and IAA-producing *Rhizobium herbae* strain 32E on growth and root As concentration.

To minimize the toxic effects of metals accumulated inside the plant, internal tolerance mechanisms include: (i) sequestration of the trace elements, i.e. transport to cell components not involved in physiological processes (vacuole, cell wall), and (ii) complexation with metal-binding peptides, i.e. metallothioneins and PCs (61). PCs are cysteine-rich peptides synthesized enzymatically from glutathione (GSH) by PC synthase under metal exposure (9). From the analysis of components involved in thiol metabolism (total NPTs), the *in vitro* evaluation showed that the concentration of GSH in roots with respect to leaves was reduced to undetectable levels, probably due to their involvement in the synthesis of long-chain compounds responsible for As detoxification (62). Under exposure to arsenic, the content of NPTs in leaves of plants inoculated with *Rhodococcus erythropolis* strain 44R or *Ensifer adhaerens* strain 91R decreased in comparison to non-inoculated plants (C), but showed increased levels of phytochelatin 4 (PC_4). This might point to a defense mechanism for As chelation by PCs. Additionally, we found that As exposure caused increased NPTs content in roots, suggesting that As was complexed in the roots through

497 enhanced synthesis of NPTs (62). Among the conditions with high As accumulation in roots are
498 inoculations with *Rhodococcus erythropolis* 44R (750 mg kg^{-1}) and with *Ensifer adhaerens* 91R (up to 790
499 mg kg^{-1}). Likewise, these conditions showed the highest NPTs values in roots, 89 and 87 nmol g^{-1} fw,
500 respectively.
501 Arsenic accumulation in roots correlated positively with NPTs content, indicating that As, in the form of
502 As^{V} introduced in the culture media, was complexed in roots through enhanced synthesis of NPTs. Indeed,
503 we found a positive relation between NPTs and As^{III} concentration in roots of the treatments inoculated
504 with *Ensifer adhaerens* strain 91R, *Rhodococcus erythropolis* 44R, *Rhizobium herbae* strain 32E, and with
505 BC. It has been demonstrated that the presence of As^{III} as a dominant As species indicates the prevailing
506 of PC-based As detoxification mechanism in plants (63).
507 In a next step toward a field trial, species of *Ensifer*, *Rhizobium*, *Variovorax,* and *Phyllobacterium* taxa were
508 selected, based on *in vitro* evaluation results. Interestingly previous studies indicated that these strains
509 exhibit traits of interest for phytoextraction. Thus, species of the *Ensifer* genus were found in association
510 with *Medicago* plants where inoculated plants exhibited a reduction in the stress response, thereby
511 indicating the protective effect of As-tolerant rhizobia (64). It has been demonstrated that *Rhizobium*
512 species promoted plant growth in *Lolium multiflorum* Lam. and on Cd uptake in *Glycine max* (L.) Merr.
513 (65). Furthermore, a *Variovorax strain* with siderophore- and IAA-producing traits was found in the
514 rhizosphere of *Pteris vittata* where it contributed to increased arsenic accumulation in fronds upon
515 inoculation (14). Additionally, *Phyllobacterium* has been described as a plant growth-promoting bacterium
516 (PGPB) that stimulates *Brassica napus* root morphogenesis (66).
517 In the field trials conducted on the Nitrasur site, the plants inoculated with the endophytic BC (composed
518 of the siderophore- and IAA-producing strains, *Variovorax paradoxus* strain 28EY and *Phyllobacterium*
519 *myrsinacearum* strain 28EW) achieved a high As uptake (11 ± 2 s.d. mg kg^{-1} in leaves and 595 ± 52 s.d. mg
520 kg^{-1} in roots). The effect of the fertilizer was reflected by an increase in shoot biomass and the treatment

supplemented with fertilizer (BC+F) increased shoot biomass (9 ± 4 s.d. g dw) compared to non-fertilized plants inoculated with BC (3.45 ± 2 s.d. g dw).

Usually, the highest BCFs are observed in soils with decreased contamination; however, BCFs also vary depending on the metal and plant species, irrespective of bioaugmentation (67). For As, the translocation from roots to shoots in most plant species is generally not very effective, supporting the high As concentrations found in roots and low As translocation by *B. celtiberica* (63). We can hypothesize that arsenate in the roots is rapidly reduced to arsenite, then complexed to PCs and subsequently sequestered in root vacuoles, thus limiting its translocation to the leaves (68).

Furthermore, the amount of metals extracted by plants from the soil, as well as their extraction performance, are directly related to bioavailability, which, in turn, was influenced by soil characteristics, such pH (67). In our study, soil conditions for BC treatment were pH 2.74 and 2527 mg kg^{-1} As (As uptake in leaves of 11.11 ± 2.21 s.d. g dw, and roots of595.15 ± 52 s.d. g dw), while soil characteristics for the BC+F treatment were pH 3.64 and 1484 mg kg^{-1} As (As uptake in leaves of 3.65 ± 1.29 s.d. g dw, and roots of 302.18 ± 78 s.d. g dw) (Figure 6). Thus, As uptake might be influenced by soil conditions. The effect on As accumulation in the BC inoculation would be expected, given the lower pH (61). However, the statistical model without the effect of the pH variable (Fig. S2) suggests that pH was not the sole factor that determines the accumulation and we propose that the bacteria inoculation had a decisive influence. Consequently, our data highlight the difficulty in conducting bioaugmentation experiments under field conditions and underline the necessity to take into account all the environmental variables and their subsequent consideration in the interpretation of final results (69). This also applies for the effects on plant growth of rhizophytic *Ensifer* upon metal exposure. We observed an increase of some 1.5-fold in plant height in the presence of *Ensifer adhaerens* strain 91R compared to non-inoculated plants; nonetheless, the control pH and assay plots with the bacterium were more similar (Fig. 6). These findings

544 are a potent indicator of indigenous rhizospheric bacterium's ability to promote plant growth, which is
545 consistent with its capacity to produce high levels of IAA, the highest production among all isolates.
546 In this study, bacterial responses to long-term As contamination were investigated in an abandoned
547 industrial area. Arsenic content and pH of soils affected diversity of the entire bacterial community. The
548 effect of plant inoculation with *Rhodococcus erythropolis* and *Ensifer adhaerens* tested *in vitro* caused an
549 increase in total NPTs content in roots, suggesting a detoxification mechanism through phytochelatin
550 complexation. Likewise, bacterial inoculation affected the As-speciation in plants. The field trials
551 suggested that inoculation with *Ensifer adhaerens* can increase plant growth and that inoculation with the
552 consortium consisting of *Variovorax paradoxus* and *Phyllobacterium myrsinacearum* enhances As
553 accumulation in roots. Extraction performance is directly related to and influenced by soil characteristics
554 such as pH. From these results, we conclude that the inoculation of plants with indigenous bacteria
555 exhibiting As-resistance, producing growth-promoting factors, and having the capacity to reduce As^{V} to
556 As^{III}, thereby facilitating As detoxification, can improve As-phytoextraction efficiency by
557 pseudometallohyte species such as *Betula*. Additionally, the use of autochthonous plants and indigenous
558 bacteria alleviated autoecological requirement of both partners, ensuring successful plant establishment
559 and site remediation. This approach appears to be particularly useful for metal(loid)- contaminated sites
560 produced as a by-product of roasting sulphur ores at the Nitrastur industrial site researched in this
561 investigation.
562

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Table 1. Bacterial strains selected for inoculation in *in vitro* culture. Phylogenetic affiliations are based on sequence analysis of about 800 bp of 16S rRNA genes. The letters of the strains indicate origin: E, endosphere; R, rhizosphere.

Strain	Most closely related species	MIC (mM)		IAA (± SD) (µg mL^{-1})	SID[a]	ACCD[b]
		As^{V}	As^{III}			
29E	*Neorhizobium alkalisoli* ZY-4s	> 100	<2	13.26 (±0.38)	++	-
32E	*Rhizobium herbae* CCBAU 83011	> 100	<2	13.06 (±0.43)	++	-
28EY	*Variovorax paradoxus* S110	> 100	<2	10.29 (±0.51)	+	-
28EW	*Phyllobacterium myrsinacearum* NBRC 100019	> 100	2	13.77 (±0.22)	+	-
44R	*Rhodococcus erythropolis* TS-TYKAKK-12	> 100	20	12.39 (±0.36)	++	++
89R	*Aminobacter aminovorans* LZ1304-3-1	> 100	<2	23.92 (±0.91)	++	++
91R	*Ensifer adhaerens* Sx1	> 100	5	30.01 (±0.82)	++	+

[a]Siderophore production

[b]1-amino-cyclopropane-1-carboxylic acid deaminase (ACCD) activity

Figure legends

Figure 1. (**A**) Values of several alpha diversity metrics for the endosphere (E) and rhizosphere (R) microbial populations of five *B. celtiberica* trees (colorful dots) and their corresponding E and R means and 95% confidence intervals (solid black dots and vertical error bars). The significance level of the difference between E and R means for each alpha metric resulting from ANOVA tests are also indicated (p-value = 0.050 – 0.100, 0.001 – 0.010, or 0.000 – 0.001 corresponding respectively to ms, which denotes marginally significant, ** or ***) (**B**) Rarefaction curves at the 3% distance cut-off of microbial communities (**C**) Relationship between microbial community diversity of E and R and As concentration or pH of the rhizosphere soils. The shaded areas represent the 95% confidence intervals of each linear relationship (black solid lines). The slope (b) and the R^2 (proportion of variance explained) of each relationship are also shown.

Figure 2. Microbial community analysis plots based on 16S rDNA pyrosequencing in endosphere and rhizosphere. (**A**) Bar chart showing the relative abundance of major bacterial phyla (over 1%) (**B**) Heatmap showing the distribution of bacterial order and genus level (over 1%).

Figure 3. Phylogenetic correlations of endophytic and rhizospheric strains isolated from *B. celtiberica* trees (Endosphere n=41; Rhizosphere n=54). Neighbor-joining tree inferred using MEGA v6.0. Species associated with rhizosphere and roots were used as reference strains. The scale bars indicate the number of substitutions per nucleotide, 323 substitutions per site.

Figure 4. *In vitro* evaluation under arsenic (150 µM) exposure. Relationship between mean leaf and root biomass (g dry weight) (n = 3) or mean plant fresh weight index (FWI) of *B. celtiberica* plants (n = 6) and their mean leaf or root arsenic concentration (µg g^{-1} dry weight, n = 3). Dot labels indicate whether mean values correspond to non-inoculated (C, Control) or inoculated (*Neorhizobium* sp. strain 29E, *Rhizobium herbae* strain 32E, *Rhodococcus erythropolis* strain 44R, *Aminobacter aminovorans* strain 89R, *Ensifer adhaerens* strain 91R, or bacterial consortium (BC) including *Variovorax paradoxus* strain 28EY and

804 *Phyllobacterium myrsinacearum* strain 28EW) *B. celtiberica* plants. The shaded areas represent the 95%
805 confidence intervals of the linear relationships (black solid lines). The dashed lines mark the mean for each
806 variable. The slope (b) and the R^2 (proportion of variance explained) of the linear correlations are also
807 shown. Significant relationships (p-value = 0.01-0.05) are denoted with *.
808 **Figure 5.** (**A**) Effect of As^V (150 μM) on NPTs, GSH and PCs and percentage of As^V and As^{III} found in (**B**)
809 medium and different parts of the plant (**C**) leaves and roots by ICP-MS. Treatments: non-inoculated
810 (Control) or inoculated *B. celtiberica* plants (*Neorhizobium alkalisoli* strain 29E, *Rhizobium herbae* strain
811 32E, *Rhodococcus erythropolis* strain 44R, *Aminobacter aminovorans* strain 89R, *Ensifer adhaerens* strain
812 91R, or bacterial consortium (BC) integrated by *Variovorax paradoxus* strain 28EY and *Phyllobacterium*
813 *myrsinacearum* strain 28EW, respectively).
814 **Figure 6.** Field evaluation. Correlation between leaf and root As concentration (mg kg^{-1} dry weight), shoot
815 biomass (g dry weight), or PHI of *B. celtiberica* plants and their soil As concentration (mg kg^{-1}) or pH of
816 their corresponding plot. Dot labels indicate whether plants were inoculated (E, with *Rhizobium herbae*
817 strain 32E; R, with *Ensifer adhaerens* strain 91R; BC, with the bacterial consortium including *Variovorax*
818 *paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW and BCF, with the same
819 consortium, but with fertilizer) or not (C and FC for control and fertilized control, respectively). The shaded
820 areas represent 95% confidence intervals of the linear correlations (black solid lines). The slope (b) and
821 the R^2 (proportion of variance explained) of each regression are also shown. The significance level of the
822 correlations is also indicated (p-value = 0.010 – 0.050, 0.001 – 0.010 or 0.000 – 0.001 correspond to *, **
823 or ***, respectively).
824