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Leguminosae native nodulating bacteria from a gold mine As-contaminated soil: multi-resistance to trace elements, and possible role in plant growth and mineral nutrition

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Abstract

Efficient N_2-fixing Leguminosae nodulating bacteria resistant to As may facilitate plant growth on As-contaminated sites. In order to identify bacteria possessing these features, 24 strains were isolated from nodules of the trap species *Crotalaria spectabilis* (12) and *Stizolobium aterrimum* (12) growing on an As-contaminated gold mine site. 16S rRNA gene sequencing revealed that most of the strains belonged to the group of *α*-Proteobacteria, being representatives of the genera *Bradyrhizobium*, *Rhizobium*, *Inquilinus*, *Labrys*, *Bosea*, *Starkeya* and *Methylobacterium*. Strains of the first four genera showed symbiotic efficiency with their original host, and demonstrated *in vitro* specific plant growth promoting traits (production of organic acids, indole-3-acetic-acid and siderophores, 1-aminocyclopropane-1-carboxylate deaminase activity, and $Ca_3(PO_4)_2$ solubilization), and increased resistance to As, Zn and Cd. In addition, these strains and some type and reference rhizobia strains exhibited a wide resistance spectrum to *β*-lactam antibiotics. Both, intrinsic plant-growth promoting abilities and multi-element resistance of rhizobia are promising for exploiting the symbiosis with different legume-plants on trace element contaminated soils.

Keywords: plant-growth promoting, biological N_2 fixation, trace elements multi-resistance, *β*-lactam antibiotics resistance.

1 Introduction

Soil microorganisms delivering ecosystem services (*e.g.* biological N_2 fixation - BFN) have been recognized as important allies for phytotechnologies. Their ability to improve the nutritional status of the plant, positively influence the tolerance of plants to excess of trace elements.

Symbiotic relationships between legume plants and rhizobia possess a great potential for phytostabilization of hostile environments (1-3) They are, for instance, (i) protecting soils, (ii) enriching ecosystems with N, (iii) supplying land cover, (iv) restoring soil functions, and (v) increasing diversity of flora and fauna (2,4). Moreover, the symbiosis rhizobia-legume can replace ammonium-based fertilizers, and thus reduce risks on soil acidification, which might increase availability of trace elements. Further, the presence of a vegetation cover prevents the dispersal of contaminated dusts through wind and water erosion from formerly bare or sparsely vegetated sites, and markedly decreases leaching of contaminants to groundwater. In fact, contamination is 'inactivated' in place, preventing further spreading and transfer into food chains (2,5,6,7,8). This all contributes to an attenuation of the impacts of the contaminants on site and on adjacent ecosystems (2,8).

Trace elements resistance has been demonstrated for different rhizobia genera (9-12). Moreover, the intrinsic plant growth-promoting features of rhizobia enlarge the horizons for exploiting the symbiosis with different legume plants on both single and multi-trace element contaminated soils (2,3,12,13).

Inoculating legume plants with, or stimulating native rhizobia strains, efficient in N_2 fixation and equipped with other plant growth-promoting traits, and well adapted to trace element-induced stress, features high relevance from both, ecological as well as economic points of view (1,2,12). Thus, investigating native rhizobia populations from

soils with high trace elements contents (like mining areas) can provide essential information concerning genetic or phenotypic resources that are better adapted to trace elements stress, in function of phytoremediation approaches (9,12,14).

Therefore, this study aimed to (a) isolate and characterize native N_2-fixing bacteria from nodules of *Crotalaria spectabilis* and *Stizolobium aterrimum* growing on an arsenic contaminated mining site; (b) identify them by the partial sequencing of their 16S rRNA genes; (c) evaluate their multi-resistance to As, Cd and Zn, and their resistance to *β*-lactam antibiotics; (d) investigate their *in vitro* plant growth promoting traits, and (e) evaluate their symbiotic efficiency with their original host plant.

2 Materials and Methods

2.1 Isolation and strain characterization

Bacteria were isolated from *S. aterrimum* (17° 10’59.88”S 46° 52’24.11”W) and *C. spectabilis* (17° 8’10.99”S 46° 51’31.75”W) nodules collected in a gold mine area contaminated with arsenic, in the northwest region of Minas Gerais, Brazil. Soil chemical and physical parameters (0-20 cm) from the As-contaminated gold mine area are presented in Table 1. Phosphorus and potassium were determined by Mehlich 1 extraction (HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}); calcium, magnesium and aluminium were determined after KCl extraction (1 mol L^{-1}). The potential acidity (H + Al) was estimated by SMP extraction, and organic matter was determined by oxidation using $Na_2Cr_2O_7$ + H_2SO_4 (10N) (15). According to the 5th Approach (Guidelines for use of lime and fertilizers in Minas Gerais) (16), the soil active acidity was chemically classified as medium acidity; the phosphorus availability considering the clay content and Prem value was classified as good; the soil fertility (based on organic matter and

cation exchange capacity) was classified as very good for P, low for potassium, calcium and magnesium, very low for aluminium, hydrogen + aluminium and organic matter content. The soil texture was determined by the pipette method as described by ref. 17, and according to the classification of the normative guideline number 2 from the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) October 9th 2008; the soil texture was identified as silt loam.

TABLE 1

Nodules were surface-sterilized according to ref. 18 and the nodule inhabiting bacteria were isolated on 79 solid culture medium (18,19) with bromothymol blue (pH 6.9, 28 ºC). After purification of the single colonies, the following characteristics of the colonies were evaluated: pH change of the culture medium, morphological features (diameter, form, edge, lifting, surface, light transmission, colour, and bromothymol blue absorption) and exopolysaccharide (EPS) production (20). The range of EPS production was classified as scarce, low, moderate and abundant.

Strains were clustered based on their characteristics including type or reference strains of the genera *Azorhizobium* (*A. caulinodans* – ORS571^{T}; *A. doebereinerae* – BR 5401^{T}), *Mesorhizobium* (*M. plurifarium* – BR 3804), *Rhizobium* (*R. tropici* – CIAT 899^{T}), *Burkholderia* (*B. cepacia* – LMG 1222^{T}), and strains of the genus *Bradyrhizobium* (*Bradyrhizobium* sp. – BR 2001 e BR 2811). The strain BR 2811 is the inoculum for *C. spectabilis* and *S. aterrimum* plant species, approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply. All strains were clustered considering 11 characteristics; a similarity dendrogram was composed using the WARD's minimum variance method, and assessing the binary distance by cluster package on R program (Figure S1).

2.2 Genotypic characterization

Genomic DNA was extracted from the bacterial cultures according to the extraction kit protocol ZR Fungal/Bacterial DNA (Zymo Research Corp). Strains were identified by sequencing the 16S rDNA genes.

PCR was performed using 50 ng of the extracted DNA, 45 µL PCR reaction mixture containing 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.2 µM 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.2 µM 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (21), 1 U Taq DNA polymerase (Fermentas), 10x KCl buffer, and ultrapure sterile water. The amplification was done using an Eppendorf Mastercycler® under the following conditions: initial denaturation step at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 40 s; annealing step at 55 °C for 40 s; extension step at 72 °C for 1.5 min; final extension at 72 °C for 7 min. The obtained PCR products were purified and sequenced by Macrogen (South Korea).

The quality of the sequences was verified using the Bionumerics 6.5 program (Applied Maths, Sint-Martens-Latem, Belgium), and they were blasted against the GenBank sequences (NCBI – National Center for Biotechnology Information).

Nucleotide sequence accession numbers - The sequences determined in this work were deposited in GenBank under accession numbers KT694150 to KT694173.

2.3 Strain authentication and symbiotic efficiency

The nodulation capacity (authentication), *i.e.* the ability to establish symbiosis with its original host, and the symbiotic efficiency of the 24 nitrogen-fixing bacterial strains isolated from nodules of the trap species *Crotalaria spectabilis* and *Stizolobium aterrimum* were examined in a greenhouse experiment for each trap species under axenic conditions.

The seeds were scarified using H_2SO_4 p.a. (*C. spectabilis* for 5 min and *S. aterrimum* for 45 min), and placed on sterile Petri dishes containing moistened cotton incubated at 28 °C until radicle emergence. The strains were grown in 79 liquid medium shaking (125 rpm, 28 °C) for 120 h. At the moment of sowing, each seed was inoculated with 1 mL of the bacterial inoculum containing about 10^8 cells. After inoculating the seeds, a thin layer of the sterile mixture of sand-benzene-paraffin was disposed on the top to avoid contamination. Two plants were grown in sterile Leonard jars for 45 days. Sand and vermiculite (1:1 ratio) were used as substrate in the topmost portion of the jars, and in the lower portion a four-fold diluted modified Hoagland nutrient solution (22) was added. The inoculated plants and the non-inoculated control plants were supplied with a low nitrogen concentration (5.25 $mg \cdot L^{-1}$) in the nutrient solution, which is considered a starting dose for, and not an inhibitor of, the biological nitrogen fixation process. The following quantities of the stock solutions were added to 4 L of water: 0.4 mL of 236.16 g L^{-1} $CaN_2O_6 \cdot 4H_2O$; 0.1 mL of 115.03 g L^{-1} $NH_4H_2PO_4$; 0.6 mL of 101.11 g L^{-1} KNO_3; 2.0 mL of 246.9 g L^{-1} $MgSO_4 \cdot 7H_2O$; 3.0 mL of 87.13 g L^{-1} K_2SO_4; 10 mL of 12.6 g L^{-1} $CaH_4P_2O_8 \cdot H_2O$; 200 mL of 1.72 g L^{-1} $CaSO_4 \cdot 2H_2O$; 1 mL of 10 g L^{-1} $FeCl_3$, and 1 mL of micronutrients (2.86 mg L^{-1} H_3BO_3; 2.03 mg L^{-1} $MnSO_4 \cdot 4H_2O$; 0.22 mg L^{-1} $ZnSO_4 \cdot 7H_2O$; 0.08 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and 0.09 mg L^{-1} $Na_2MoO_4 \cdot H_2O$). In addition, a control treatment supplemented with a high inorganic nitrogen concentration (52.5 $mg \cdot L^{-1}$) was also provided, composed by the following quantities of the stock solutions added to 4 L of water: 4.0 mL of 236.16 g L^{-1} $CaN_2O_6 \cdot 4H_2O$; 1.0 mL of 115.03 g L^{-1} $NH_4H_2PO_4$; 6.0 mL of 101.11 g L^{-1} KNO_3; 2.0 mL of 246.9 g L^{-1} $MgSO_4 \cdot 7H_2O$; 1.0 mL of 10 g L^{-1} $FeCl_3$, and 1.0 mL of micronutrients solution (composition as described above). Besides the negative control treatments (low and high N content) a positive control treatment inoculated with *Bradyrhizobium* sp. Strain BR

2811, which has been approved as inoculant for both plant species by the Brazilian Ministry of Agriculture, was also included. The assays were fully randomized and four replicates were implemented for each treatment. Harvesting was performed after 45 days incorporating the following measurements: nodule number (NN) and nodule dry weight (NDW), shoot dry weight (SDW), and relative efficiency (RE%). The RE% of each inoculated treatment was calculated in relation to the shoot dry matter production by the control treatment supplied with high inorganic nitrogen content, using the formula RE = [(inoculated SDW / high N SDW) x 100] where RE means relative efficiency, inoculated SDW means shoot dry weight of the inoculated treatment, and high N SDW means shoot dry weight of the control treatment supplied with high inorganic nitrogen content.

The data were analysed by one-way ANOVA using the statistical program SISVAR (23). The NN and NDW were transformed using the formula $(x+0.5)^{0.5}$. The average of the treatments was grouped by the Scott-Knott test at 5% significance.

2.4 Phenotypic characterization

2.4.1 Arsenic Minimum Inhibitory Concentration (As MIC)

Bacterial strains representative for the different “cultural” groups, which were formed after colony characterization, were selected for the As MIC assay. Seven type or reference strains belonging to the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Burkholderia* were also included in this assay.

Strains were grown in 30 mL of 79 liquid medium at pH 6.9, using an orbital shaker (125 rpm) at 28 °C. Subsequently, 1 mL of each strain containing about 10^8 cells was transferred to sterile microtubes (1.5 mL), which were centrifuged at 8,000 g at 25 °C for 4 min. The supernatant was discarded, the cells were resuspended in 1 mL sterile

NaCl (8.5 g L^{-1}), and centrifuged again. This ¨washing¨ procedure was repeated three times. After that, 20 µL aliquots of the cell suspension were inoculated on 79 solid media containing different As concentrations. Arsenic ($Na_2HAsO_4 \cdot 7H_2O$) was used at concentrations of 50, 100, 150 and 200 mmol L^{-1}, in addition to a control treatment without As. After adding As to the medium, pH was adjusted to 6.9 using HCl (0.5 M). The susceptibility of the strains to As was examined by determining the minimum inhibitory concentration (MIC), which was defined as the lowest concentration at which there are no colony-forming units (CFU) on the medium after 9 days of incubation at 28 °C. Each treatment (strains and controls) was evaluated in three replicates.

After composing the similarity dendrogram using the colony characteristics of the strains, the frequencies of resistant individuals at different As-concentrations within the groups formed by the characterization of the colonies were analysed using the chi-squared test at 5% significance.

2.4.2 Pattern of *β*-lactam antibiotics resistance

Bacteria were grown on 79 liquid medium for 72 h at 28 °C, after which 0.1 mL aliquots of the bacterial inoculum were spread on 79 solid medium using a Drigalski spatula. Susceptibility was determined using the disk diffusion method (Cecon-Sensobiodisc) for amoxicillin (AMO) (10 µg), ampicillin (AMP) (10 µg), cefadroxil (CFD) (30 µg), ceftriaxone (CEFT) (30 µg), oxacillin (OXA) (1 µg), and vancomycin (VAN) (30 µg) (24). The strains were defined as “sensitive” in case a radius zone was observed or “resistant” in case no radius zone was formed after 48 h at 28 °C. Strains were grown in triplicate.

2.4.3 Plant growth promoting traits

Besides the native bacterial strains isolated from nodules of both *Crotalaria spectabilis* and *Stizolobium aterrimum*, growing on a gold mine area contaminated with arsenic, five type or reference strains, *Azorhizobium caulinodans* ORS571^T, *A. doebereinerae* BR 5401^T, *Mesorhizobium plurifarium* BR 3804, *Rhizobium tropici* CIAT 899^T, *Burkholderia* sp. BR 11340, were also investigated *in vitro* for their plant-growth promoting (PGP) traits. Bacterial organic acid (OA) production was assessed based on a colorimetric method (25), indole-3-acetic-acid (IAA) production capacity was tested using the Salkowski assay (adapted from ref. 26), ACC deaminase activity was evaluated using a slightly modified protocol according to ref. 27, siderophore production was qualitatively evaluated by a widely used colorimetrical method (28), and $Ca_3(PO_4)_2$ phosphate solubilization ability was evaluated in solid medium (29) (Table 2). Their multi-element resistance was also studied including Cd and Zn (Table 4) (30,31). Resistance was appraised visually examining growth and polysaccharide (mucus) production on the plate. In case of exopolysaccharide production, the same classification is used as for the characterization of the colonies (*i.e.* scarce, low, moderate and abundant).

3 Results

3.1 Isolation, strain characterization, and identification by 16S rRNA gene partial sequencing

All strains isolated from nodules of *C. spectabilis* (12) and *S. aterrimum* (12) were characterized and clustered together with reference strains for the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Burkholderia*. In general, strains clustered in two main groups (Figure S1 - Table S1), using the Ward's hierarchical clustering method.

TABLE S1

Group A was formed by strains that alkalinized the 79 solid medium, and showed low or scarce exopolysaccharide (EPS) production. Six strains isolated from nodules of *C. spectabilis*, identified as *Bradyrhizobium* sp., and three strains isolated from nodules of *S. aterrimum*, identified as *Bosea* sp., *Starkeya novella* and *Methylobacterium* sp. clustered into this group (Table 2), as well as the two reference strains for the genus *Azorhizobium*, BR 5401^T and ORS571^T.

FIGURE S1

Within group A, two small subgroups were formed. One subgroup was represented by strains belonging to the Bradyrhizobiaceae family (*Bradyrhizobium* sp. and *Bosea* sp. strains). The second subgroup was composed of strains belonging to the Bradyrhizobiaceae family (*Bradyrhizobium* sp.), in addition to strains from the Methylobacteriaceae (*Methylobacterium* sp.), the Xanthobacteraceae (*Starkeya novella*), and the reference strains *A. caulinodans* - ORS571^T and *A. doebereinereae* - BR 5401^T. The strains belonging to the Bradyrhizobiaceae family in group A exhibited low EPS production (Table S1).

The nucleotide sequence of the 16S rRNA gene of the *Bosea* sp. strains isolated in our study, showed 100% similarity with the sequence of *Bosea* sp. S41RM2, deposited in GenBank with the accession number GU731243.1. The origin of that strain is also an As-contaminated soil (32).

TABLE 2

Group B consisted of strains with very different colony characteristics (Figure S1; Table S1). Some of these strains acidified or alkalinized the 79 solid medium, others did not affect the pH, leaving it neutral. Also the EPS production in this group was very diverse, showing low, moderate or abundant EPS production (Table S1). Most of the

strains of group B belonged to the Bradyrhizobiaceae family. These strains demonstrated moderate or abundant EPS production. Besides the Bradyrhizobiaceae family, group B also included representatives of the Rhizobiaceae, Phyllobacteriaceae, Xanthobacteraceae, Rhodospirillaceae and Burkholderiaceae. Even a strain belonging to the Bacillaceae family, phylum Firmicutes was isolated (Figure S1; Table 2). The following type or reference strains also clustered in group B: *Bradyrhizobium* sp. (BR 2001 and BR 2811), *Mesorhizobium plurifarium* (BR 3804), *Rhizobium tropici* (CIAT 899^{T}) and *Burkholderia cepacia* (LMG 1222^{T}).

The 16S rRNA gene sequencing revealed representatives of two phyla, Firmicutes and Proteobacteria (Table 2). Most of the strains belonged to the *α*-Proteobacteria such as *Bradyrhizobium*, *Rhizobium*, *Bosea*, *Inquilinus*, *Labrys*, *Starkeya* and *Methylobacterium*. A few strains belonged to the *β*-Proteobacteria.

3.2 Strain authentication and symbiotic efficiency

In the greenhouse experiment, no nodulation was observed for the control treatments (without inoculation and supplied with 5.25 mg L^{-1} or 52.5 mg L^{-1} of inorganic N) of both, *C. spectabilis* and *S. aterrimum* plants.

Taking this into account, it was possible to verify the authentication of the symbiosis and the symbiotic efficiency of the isolated strains. *C. spectabilis* plants established symbiosis with all strains tested, including BR 2811 (Table 3). Without exception all strains exhibited N_2 fixation in symbiosis with *C. spectabilis*, showing NAS and RE% higher or similar to the control treatment supplied with the high concentration of inorganic N (52.5 mg L^{-1}). Only the UFLA 05-01 (*Bradyrhizobium* sp.) strain displayed a lower SDW production in comparison with the high inorganic N treatment, but it was still higher when compared with the low concentration of inorganic N and inoculation with the BR 2811 strain. The efficiency of all strains was higher even

in comparison with strain BR 2811 that was approved by MAPA as an inoculant for *C. spectabilis*. The strains UFLA 05-03 (*Bradyrhizobium* sp.) and UFLA 05-09 (*Bradyrhizobium* sp.) were able to provide more N to the plants, even more than in case of application of the high concentration of inorganic N (52.5 mg L^{-1}). Moreover, the two above-mentioned strains and UFLA 05-14 (*Bradyrhizobium* sp.) and UFLA 05-07 (*Inquilinus* sp.) also demonstrated higher RE% than the control treatment supplied with the high concentration of inorganic N (52.5 mg L^{-1}) (Table 3). Interestingly, this latter strain and also UFLA 05-08 (*Labrys monachus*) are atypical genera in nodulating legume plants. Although these strains were able to efficiently nodulate and fix N_2 in symbiosis with *C. spectabilis*, a further study examining for specific genes for those processes needs to be performed.

TABLE 3

Eight strains out of the 12 isolated from nodules of *S. aterrimum* were able to establish symbiosis and induced nodule formation on the roots of this plant species (Table 3). The strains UFLA 05-11 (*B. elkanii*), UFLA 05-12 (*Bradyrhizobium* sp.), UFLA 05-13 (*Bradyrhizobium* sp.), UFLA 05-17 (*Bradyrhizobium* sp.), UFLA 05-18 (*Bradyrhizobium* sp.), UFLA 05-19 (*B. elkanii*), UFLA 05-20 (*Bradyrhizobium* sp.) and UFLA 05-24 (*Bradyrhizobium* sp.) induced nodule formation in *S. aterrimum* plants, in addition to the control strain BR 2811. The strains UFLA 05-11 (*B. elkanii*) and UFLA 05-12 (*Bradyrhizobium* sp.) showed higher NN, even in comparison to the control strain BR 2811, but this feature did not increase their RE%. Unlike the other strains, which showed low NN but high NDW, strains UFLA 05-13 (*Bradyrhizobium* sp.), UFLA 05-17 (*Bradyrhizobium* sp.), UFLA 05-18 (*Bradyrhizobium* sp.), UFLA 05-19 (*B. elkanii*), UFLA 05-20 (*Bradyrhizobium* sp.) and UFLA 05-24 (*Bradyrhizobium* sp.), were more efficient in N_2-fixation and showed higher RE%. Interestingly, these strains

demonstrated higher efficiencies than the control strain BR 2811, which is approved as inoculant for *C. spectabilis*. Among the six strains that showed higher RE% than the high inorganic N treatment, four - UFLA 05-18 (*Bradyrhizobium* sp.), UFLA 05-19 (*B. elkanii*), UFLA 05-20 (*Bradyrhizobium* sp.) and UFLA 05-24 (*Bradyrhizobium* sp.) – showed SDW productions statistically similar to the control treatment supplied with the high concentration of inorganic N (52.5 mg L^{-1}). Although the SDW productions by the other 2 strains, UFLA 05-13 (*Bradyrhizobium* sp.) and UFLA 05-17 (*Bradyrhizobium* sp.), were lower than the control treatment with high inorganic N, these strains displayed higher RE% than the control treatment with high inorganic N (52.5 mg L^{-1}). The atypical genera, *Bosea* sp., *Methylobacterium* sp. and *Starkeya novella*, did not induce nodule formation on *S. aterrimum* roots in the authentication test (Table 3).

3.3 Phenotypic characterization

3.3.1 Arsenic minimal inhibitory concentration (MIC)

The MIC screening revealed 8 As-resistant strains (Figure 1). The strains UFLA 05-21 (*Methylobacterium* sp.) and UFLA 05-23 (*Starkeya novella*), isolated from nodules of *S. aterrimum*, tolerated up to 150 and 200 mmol As L^{-1}, respectively. Among the strains isolated from nodules of *C. spectabilis*, only UFLA 05-16 (*Rhizobium tropici*) tolerated the highest As concentration tested. Other strains isolated from *S. aterrimum* and *C. spectabilis* did not survive any of the As concentrations applied and were therefore considered to be sensitive to As. Among the type and reference strains, *A. caulinodans* ORS571^T, *Mesorhizobium plurifarium* BR 3804, *Rhizobium tropici* CIAT 899^T and *Burkholderia cepacia* LMG 1222^T were resistant to As, since they could grow until the highest As concentration tested. The strain *A. doebereinereae* BR 5401^T was also As-resistant, but it survived only until 100 mmol As L^{-1}. On the other hand,

Bradyrhizobium sp. BR 2001 and BR 2811 strains were considered to be As-sensitive, since they did not tolerate any of the As concentrations applied (Figure 1).

FIGURE 1

3.3.2 Pattern of *β*-lactams antibiotics resistance

The patterns of *β*-lactams antibiotics resistance indicated that most of the As-resistant strains had a similar resistance spectrum to AMO/AMP/CEFT/OXA and the As-sensitive strains had a similar resistance spectrum to VAN/OXA/AMO/AMP (Figure 2). The As-resistant strains UFLA 05-21 (*Methylobacterium* sp.), BR 5401^{T} (*A. doebereinereae*) and ORS571^{T} (*A. caulinodans*) showed the same pattern of *β*-lactams antibiotics resistance to AMO, AMP, CEFT, OXA and VAN, and sensitivity to CFD. The strains UFLA 05-16 (*R. tropici*) and CIAT 899^{T} (*R. tropici*) showed the same pattern of *β*-lactams antibiotic resistance to AMO, AMP, CFD, CEFT and OXA, and sensitivity to VAN. Strain BR 3804 (*M. plurifarium*) demonstrated a similar pattern, being resistant to the same *β*-lactams: AMO, AMP, CFD, CEFT and OXA, but it was sensitive to CFD, and VAN as well. Strain LMG 1222^{T} (*B. cepacia*) was resistant to all *β*-lactams studied: AMO, AMP, CFD, CEFT, OXA and VAN. On the other hand, the strain UFLA 05-23 (*S. novella*) was only resistant to CFD, and sensitive to all the other *β*-lactams under investigation.

FIGURE 2

3.3.3 Plant growth promoting traits

Alongside the native bacterial strains isolated from nodules of both, *Crotalaria spectabilis* and *Stizolobium aterrimum*, five type or reference strains, *Azorhizobium caulinodans* ORS571^{T}, *A. doebereinerae* BR 5401^{T}, *Mesorhizobium plurifarium* BR 3804, *Rhizobium tropici* CIAT 899^{T} and *Burkholderia* sp. BR 11340 were also screened

for their *in vitro* PGP traits (Table 2), and multi-resistance to Zn and Cd (Table 4). In general, the number of strains demonstrating potential PGP traits in *in vitro* tests, such as production of organic acids (OA), IAA, ACC deaminase, siderophores and solubilisation of P, is remarkably high (Table 2). The total percentage of strains that tested positive for production of OA was 26%, for IAA production 52%, for ACC deaminase activity 73%, for production of SID 69% and for solubilisation of P 73%. Without any exception, all strains isolated from As-contaminated soil tolerated the low and high Zn and Cd concentrations and were therefore considered to be Zn- and Cd-resistant (Table 4).

TABLE 4

4 Discussion

Phytostabilization of trace elements contaminated soils has been proposed as a sustainable and low cost technology (33). Symbiotic interactions between legume plants and rhizobia possess a great potential to improve the efficiency and sustainability of phytostabilization (1-3).

The interactions between plants with their associated microorganisms indeed are highly important to enhance the success of phytostabilization. Plant-associated microorganisms can perform several essential biological processes, such as biological N_2-fixation and improving and promoting plant growth (30). Both, the selection of the appropriate plant species as well as the most beneficial associated microorganisms are crucial steps in phytoremediation projects (10,11,30,34-36).

In this study bacterial strains were isolated from nodules of *C. spectabilis* and *S. aterrimum* plants growing on As-contaminated soils of a gold mine area. The isolated strains were characterized phenotypically (Figure S1; Table S1) and genotypically

(Table 2). In previous experiments, using the same soil, both plant species, *C. spectabilis* (37) and *S. aterrimum* (38), showed potential for phytostabilization of As-contaminated soils.

N_2-fixing bacteria have been isolated from several soils contaminated with different trace elements (11,30,31,39-41). Two main mechanisms for adaptation of bacteria to increased As concentrations have been proposed. The first mechanism comprises the reduction of arsenate to arsenite, using the Ars system, and the second mechanism would be activated at the same time, to oxidize arsenite on the respiratory system, producing energy required for growth.

In this study, 13 strains representative for the different "cultural" groups were investigated *in vitro* for their resistance to As (Figure 1). The strains UFLA 05-16 (*Rhizobium tropici*) and UFLA 05-23 (*Starkeya novella*), and reference or type strains ORS571^T (*A. caulinodans*), BR 3804 (*Mesorhizobium plurifarium*), CIAT 899^T (*Rhizobium tropici*) and LMG 1222^T (*Burkholderia cepacia*) tolerated up to 200 mmol As L^{-1}, the strain UFLA 05-21 (*Methylobacterium* sp.) up to 150 mmol As L^{-1}, and the type strain BR 5401^T (*A. doebereinereae*) tolerated up to 100 mmol As L^{-1}. These results emphasize the As resistance of these strains. All strains that could cope with the highest As concentration (200 mmol L^{-1}) that was used were members of α-Proteobacteria.

Our results confirm that the α-Proteobacteria contain a high diversity of As-resistant bacteria, including several rhizobia genera like *Azorhizobium*, *Mesorhizobium*, *Rhizobium*, *Burkholderia* and *Starkeya* (Table 2). This fact makes the use of biological nitrogen fixation (BNF) for phytostabilization purposes more promising, since it increases the number of host legume species for these genera of rhizobia that can be tested in the field.

Already in 1982, Mobley and Rosen (42) demonstrated that As resistance is genetically conferred, and the genes are located on a plasmid. The resistance mechanism is an energy dependent efflux system, linked to the cellular membrane (43). Plasmids that contain resistance to trace elements may also confer resistance to *β*-lactams antibiotics (44). Different resistance mechanisms to *β*-lactams antibiotics have been well characterized, such as reduction of membrane permeability to trace elements and antibiotics, drug and trace elements inactivation and modification, and rapid efflux of the trace elements and antibiotics (45-47). In this study, most of the As-resistant strains also showed a pattern of multiple resistance to *β*-lactam antibiotics (Figure 2). The production of *β*-lactamase, an enzyme capable of modifying and inactivating the *β*-lactams antibiotics, is part of the resistance mechanism to *β*-lactams antibiotics (48). In Gram-negative bacteria, *β*-lactamases are produced constitutively, even when the antibiotic is not present (49). In contrast to Gram-positive bacteria, Gram-negative bacteria retain this enzyme within the periplasmic space, which results in a more efficient resistance mechanism. This fact might explain why the strains sensitive to As (Figure 1) also showed a pattern of multiple resistance to *β*-lactams antibiotics (Figure 2), since most of them are Gram-negative. Most of the As-sensitive bacteria (Figure 1; Table 2) belonged to the genus *Bradyrhizobium,* which is known for producing and using outer membrane proteins as an uptake system for siderophore-metal complexes (50). Overall, these observations might suggest that the *β*-lactams antibiotics resistance and As resistance are related only by the permeability of the membrane for trace elements and antibiotics. Both, trace elements and antibiotics, may be taken up through the same outer membrane proteins (porins), which are responsible for increasing or decreasing membrane permeability (45,47). However, the resistance to As depends on the presence of arsenate reductases and related enzymes (46).

Plant growth-promoting rhizobacteria (PGPR) can have different direct beneficial effects on the growth of their host plant. PGPR may synthesize and provide their host plant with fixed nitrogen or phytohormones such as indole-3-acetic acid (IAA), they may facilitate uptake of nutrients such as P and Fe, or may synthesize enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene levels and in this way may affect plant growth (51).

The total percentage of P solubilizing strains was 73%, which is high (Table 2). The Nautiyal protocol (29) is based on extracellular oxidation of glucose via quinoprotein glucose dehydrogenase, which produces gluconic acid and mobilizes insoluble phosphates very efficiently. Moreover, apart from the production and excretion of gluconic acid, other mechanisms such as the production of chelating substances, the release of protons originating from NH^{4+} assimilation and the production of inorganic acids, have also been proposed to be responsible for phosphate solubilization by bacteria (52).

Plants growing on trace elements contaminated soils might become severely deficient in the amount of available iron (53). Fortunately, plants can produce siderophores that bind iron which allows them to take up more iron. Moreover, plants can also assimilate complexes of iron with bacterial siderophores. Even though plants can produce siderophores themselves, the affinity of plant siderophores for iron is considerably lower than that of bacterial siderophores (53). Therefore, plants are considered to depend to a great extend on bacterial siderophore production. In trace elements contaminated soils plants are often unable to accumulate sufficient amounts of iron unless bacterial siderophores are present (53). A high number of our bacterial strains (69%) produced siderophores (Table 2). This high amount of siderophores producing strains was not surprising since they were isolated from legume-nodules and

most of the isolated strains were rhizobia (Table 2) that perform biological N_2 fixation (BNF). The latter process is highly Fe demanding (54,55) since not only the nitrogenase enzyme complex, but also cytochromes, ferredoxins, and hydrogenases contain iron. Moreover, iron is highly important for nodule formation. Nodules of iron adequate plants contained more than the double of leghemoglobin in comparison to iron deficient plants (54). These authors demonstrated that the leghemoglobin production by *Lupinus angustifolius* inoculated with *Bradyrhizobium lupini* WU425 was depressed under iron deficiency. Even if BNF and symbiosis between leguminous plants and rhizobia is highly iron demanding, there are some *Bradyrhizobium* strains which do not produce siderophores: UFLA 05-10, UFLA 05-11, UFLA 05-12, UFLA 05-13 and UFLA 05-18 (Table 2). This can partly be explained by the fact that rhizobia strains that do not produce siderophores do synthesize outer membrane receptors instead. These outer membrane specific receptors are able to bind siderophore-metal complexes, allowing the uptake of ion chelates (56). In rhizobia, Fe^{3+}-siderophore complexes are recognized by different outer membrane receptors depending on the siderophores. Such siderophores are referred to as xenosiderophores, since they are used by one organism but are synthesized and secreted by other organisms. An example was reported by Plessner et al. (50) who have shown that *Bradyrhizobium japonicum* USDA 110, reclassified as *B. diazoefficiens* (57), and 61A152 strains are able to use ferrichrome and rhodotorulic acid, siderophores of fungal origin, as iron source.

Organic acids (OA) production by bacteria is another way to promote plant growth, especially in trace elements contaminated soils, where plant growth is often inhibited. By producing OA, bacteria can improve plant uptake of essential mineral nutrients (30,58), which are usually limiting in mining soils (12,37,38). However, by

enhancing the availability of essential mineral nutrients, OA production may also increase the availability of potentially toxic trace elements for the plants.

The low number (26%) of OA producing bacterial strains isolated from the As-contaminated soil is remarkable (Table 2). Interestingly, most of the strains which did not produce OA were able to solubilize $Ca_3(PO_4)_2$. Therefore, the $Ca_3(PO_4)_2$ solubilization by these strains is most likely not due to release of OA. We hypothesize that these strains do not produce OA in As-contaminated soil in order to avoid As solubilization since OA release may cause arsenopyrite (FeAsS) dissolution, even if little efficient (59).

Plants growing on contaminated mining soils often show growth inhibition as a consequence of nutrient deficiency as well as exposure to toxic amounts of potentially toxic trace elements. Since these conditions threaten the survival of the plants, the plant growth promotion potential of bacterial strains might be of high importance. The auxin IAA is involved in enhancing root growth and root length, as well as in proliferation and elongation of root hairs (60). A more extended root system due to bacterial IAA production might reduce nutrient deficiency as a result of the bigger soil volume that can be explored by the roots (61). Several studies have shown that rhizosphere bacteria, phyllosphere bacteria and endophytes can improve phytoremediation efficiency by different mechanisms, including IAA production (14,31,36,41,60-64). 52% of the isolated bacterial strains are able to produce IAA *in vitro* (Table 2). Given the high number of native IAA-producing strains isolated from mining soils, we hypothesize that this trait might be of high importance for plant growth and development on these contaminated soils.

Besides directly affecting plant growth, IAA can also induce the transcription of ACC synthase, which catalyzes the formation of 1-aminocyclopropane-1-carboxilic acid

(ACC) (53). ACC is the immediate precursor of ethylene. At high levels, ethylene inhibits plant growth and induces early senescence. Therefore, plant-associated bacteria that are able to cleave ACC by ACC deaminase act as a sink for plant ACC, reducing the amount of ethylene released in the plant tissues, and thus also the consequences of high ethylene levels for plant growth and development (51,65). The high percentage (73%) of ACC deaminase producing strains isolated from As-contaminated soils is noteworthy. Also Croes et al. (31) and Truyens et al. (66) reported that trace element contamination presses the plant-associated bacterial community to trace element resistant, phosphorus solubilizing, nitrogen fixing and ACC deaminase and IAA producing genotypes.

Along the remarkable potential of the native rhizobia isolated from As-contaminated soils, also the results for the type or reference rhizobia strains that were included in this study are noteworthy (Table 2). Several strains belonging to different rhizobia genera exhibited positive results for most of the plant-growth promoting traits we tested for *in vitro*.

Altogether, our promising results are encouraging to continue rhizobia research in the framework of phytostabilization. These intrinsic plant-growth promoting abilities of rhizobia increase the number of legume plant species that can be considered for phytoremediation purposes in different soil contamination conditions (*e.g.* mining, smelting etc.).

In addition to their capacity to promote plant growth on trace element contaminated soils, it is interesting that these bacteria possess multi-element resistance since almost all mining soils contain a multi-element pollution. The bacteria we have investigated in this work originated from mining soils. This implies that they were exposed for many generations to a toxic and hostile environment and thus were forced

to adapt in order to survive under this selective pressure. Without exception, all bacterial strains isolated from the As-contaminated soil were resistant to both, low and high Zn and Cd concentrations (Table 4). Among those strains, we highlight the Zn and Cd resistant *Rhizobium tropici* UFLA 05-16 strain, which also showed high As resistance (Figure 1). *Labrys monachus* strain UFLA 05-08 and *Inquilinus* sp. UFLA 05-07 also showed high Zn and Cd resistance.

5 Conclusions

The group of α-Proteobacteria harbours a high diversity of strains resistant to As, Cd and Zn;

The symbiosis between UFLA 05-16 (*R. tropici*) and *C. spectabilis* plants has potential to be used on As-contaminated soils for phytostabilization purposes;

The potential plant-growth promoting abilities together with the multi-element resistance of rhizobia are promising for exploiting the symbiosis with different legume species on trace element contaminated soils.

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References

[1]Franco AA, Dias LE, Faria SM, Campello EFC, Silva EMR. Use of nodulate and mycorrhizal forest leguminous trees as agents of recovery and maintenance of soil life: a technological model. Oecol Bras. 1995;1:459–467.

[2]Dary M, Chamber-Pérez MA, Palomares AJ, Pajuelo E. "In situ" phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. J Hazard Mater. 2010;177:323–330.

[3]Hao X, Taghavi S, Xie P, Orbach MJ, Alwathnani HA, Rensing C, Wei G. Phytoremediation of heavy and transition metals aided by legume-rhizobia symbiosis. Int J Phytoremediation. 2014;16:179–202.

[4]Siqueira JO, Soares CRFS, Silva CA. Matéria orgânica em solos de áreas degradadas. In: Santos GA, Silva LS, Canellas LP, Camargo FAO, editors. Fundamentos da Matéria Orgânica do Solo: ecossistemas tropicais e subtropicais. 2nd ed. Porto Alegre: Metrópole; 2008. p. 495–524.

[5]Vangronsveld J, Van Assche F, Clijsters H. Reclamation of a bare industrial area contaminated by non ferrous metals – in situ metal immobilization and revegetation. Environ Pollut. 1995a;87:51–59.

[6]Vangronsveld J, Sterckx J, Van Assche F, Clijsters H. Rehabilitation studies on an old non-ferrous waste dumping ground: effects of revegetation and metal immobilization by beringite. J Geochem Explor. 1995b;52:221–229.

[7]Vangronsveld J, Colpaert J, Van Tichelen K. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environ Pollut. 1996;94:131–140.

[8]Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D, Mench M. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ Sci Pollut Res. 2009;16:765–794.

[9]Trannin ICB, Siqueira JO, Moreira FMS, Lima AS. Tolerância de Estirpes e Isolados de *Bradyrhizobium* e de *Azorhizobium* a Zinco, Cádmio e Cobre "In Vitro". R Bras Ci Solo. 2001;25(2): 305–316.

[10]Matsuda A, Moreira FMS, Siqueira JO. Tolerância de rizóbios de diferentes procedências ao zinco, cobre e cádmio. Pesq Agropec Bras. 2002a;37(3): 343–355.

[11]Carrasco JA, Armario P, Pajuelo E, Burgos A, Caviedes MA, Lopes R, Chamber MA, Palomares AJ. Isolation and characterisation of symbiotically effective *Rhizobium* resistant to arsenic and heavy metals after the toxic spill at the Aznalcóllar pyrite mine. Soil Biol Biochem. 2005;37(6):1131–1140.

[12]Rangel WM, Thijs S, Janssen J, Oliveira Longatti SM, Bonaldi DS, Ribeiro PRA, Jambon I, Eevers N, Weyens N, Vangronsveld J, Moreira FMS. Native rhizobia from Zn-mining soil promotes the growth of *Leucaena leucocephala* on contaminated soil. Int J Phytoremediation. 2016. doi:10.1080/15226514.2016.1207600.

[13]Ma Y, Prasad MNV, Rajkumar M, Freitas H. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnol Adv. 2011;29:248–258.

[14]Weyens N, van der Lelie D, Taghavi S, Newman L, Vangronsveld J. Exploiting plant–microbe partnerships for improving biomass production and remediation. Trends Biotechnol. 2009a;27(10):591–598.

[15]Empresa Brasileira de Pesquisa Agropecuária–EMBRAPA. Manual de métodos de análise de solos. 2nd ed. Rio de Janeiro: EMBRAPA-CNPS; 2011.

[16]Ribeiro AC, Guimarães PTG, Alvarez VVH. Recomendações para o uso de corretivos e fertilizantes em Minas Gerais – 5ª aproximacão. Viçosa: UFV; 1999.

[17]Day RP. Pipette method of particle size analysis. In: Methods of soil analysis. Agronomy 9. ASA USA; 1965. p. 553–562.

[18]Vincent JM. A manual for the practical study of root-nodule bacteria. Oxford: Blackwell Scientific Publications; 1970.

[19]Fred EB, Waskman SA. Laboratory Manual of General Microbiology. New York and London: McGraw-Hill Book; 1928.

[20]Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacylamide gel electrophoresis of their total proteins. Syst Appl Microbiol. 1993;16(1):135–146.

[21]Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in Bacterial Systematics. New York; 1991. p. 115–148.

[22]Hoagland DR, Arnon DL. The water culture methods for growing plants without soil. Berkeley: California Agriculture Experiment Station; 1950.

[23]Ferreira DF. SISVAR: A computer statistical analysis system. Ciênc Agrotec. 2011;35:1039–1042.

[24]Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493–496.

[25]Cunningham JE, Kuiack C. Production of citric and oxalic acids and solubilization of calcium-phosphate by *Penicillium bilaii*. Appl Environ Microbiol. 1992;58(5):1451–1458.

[26]Patten C, Glick B. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl Environ Microbiol. 2002;68(8):3795–3801.

[27]Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem. 2005;37(2):241–250.

[28]Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem. 1987;160(1):47–56.

[29]Nautiyal CS. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett. 1999;170(1):265–270.

[30]Weyens N, Beckers B, Schellingen K, Ceulemans R, Croes S, Janssen J, Haenen S, Witters N, Vangronsveld J. Plant-associated bacteria and their role in the success or failure of metal phytoextraction projects: first observations of a field-related experiment. Microb Biotechnol. 2013a;6(3):288–299.

[31]Croes S, Weyens N, Janssen J, Vercampt H, Colpaert JV, Carleer R, Vangronsveld J. Bacterial communities associated with *Brassica napus* L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microb Biotechnol. 2013;6(4):371–384.

[32]Sultana M, Vogler S, Zargar K, Schmidt AC, Saltikov C, Seifert J, Schlömann M. New clusters of arsenite oxidase and unusual bacterial groups in enrichments from arsenic-contaminated soil. Arch Microbiol. 2012;194(7):623–635.

[33]Vangronsveld J, Cunningham SD. Introduction to the concepts. In: Vangronsveld J, Cunningham SD, editors. Metal-Contaminated Soils i*n situ* Inactivation and Phytorestoration. Berlin: Springer-Verlag; 1998. p. 1–16.

[34]Matsuda A, Moreira FMS, Siqueira JO. Sobrevivência de *Bradyrhizobium* e *Azorhizobium* em misturas de solo contaminadas com metais pesados. R Bras Ci Solo. 2002b;26(1):249–256.

[35]Pajuelo E, Rodríguez-Llorente ID, Dary M, Palomares AJ. Toxic effects of arsenic on *Sinorhizobium* e *Medicago sativa* symbiotic interaction. Environ Pollut. 2008;154(2):203–211.

[36]Weyens N, van der Lelie D, Taghavi S, Vangronsveld J. Phytoremediation: plant–endophyte partnerships take the challenge. Curr Opin Biotechnol. 2009b;20(2):248–254.

[37]Lopes G, Ferreira PAA, Pereira FG, Curi N, Rangel WM, Guilherme LRG. Beneficial use of industrial by-products for Phytoremediation of an arsenic-rich soil from a gold mining area. Int J Phytoremediation. 2015;28:0. doi: 10.1080/15226514.2015.1131240.

[38]Rangel WM, Schneider J, Costa ETS, Soares CRFS, Guilherme LRG, Moreira FMS. Phytoprotective Effect of Arbuscular Mycorrhizal Fungi Species Against Arsenic Toxicity in Tropical Leguminous Species. Int J Phytoremediation. 2014;16(7-12):840–858.

[39]Drewniak L, Matlakowska R, Sklodowska A. Arsenite and Arsenate Metabolism of *Sinorhizobium* sp. M14 living in the Extreme Environment of the Zloty Stok Gold Mine. Geomicrobiol J. 2008;25(7-8):363–370.

[40]Oliveira A, Pampulha ME, Neto MM, Almeida AC. Enumeration and Characterization of Arsenic-Tolerant Diazotrophic Bacteria in a Long-Term Heavy-Metal-Contaminated Soil. Water Air Soil Pollut. 2009;200(1):237–243.

[41]Becerra-Castro C, Kidd PS, Prieto-Fernández A, Weyens N, Acea MJ, Vangronsveld J. Endophytic and rhizoplane bacteria associated with *Cytisus striatus* growing on hexachlorocyclohexane contaminated soil: isolation and characterisation. Plant Soil. 2011;340(1):413–433.

[42]Mobley HL, Rosen BP. Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. Proc Natl Acad Sci. 1982;79(20):6119–6122.

[43]Messens J, Silver S. Arsenate Reduction: Thiol Cascade Chemistry with Convergent Evolution. J Mol Biol. 2006;362(1):1–17.

[44]Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends Microbiol. 2006;14(4):176–182.

[45]Silver S. Bacterial heavymetal resistance: new surprises. Ann Rev Microb. 1996;50:753–789.

[46]Mukhopadhyay R, Rosen BP. Arsenate reductases in prokaryotes and eukaryotes. Environ Health Perspect. 2002;110(5):745–748.

[47]Wright GD. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv Drug Deliv Rev. 2005;57(10):1451–1470.

[48]Williams JD. β-lactamases and β-lactamase inhibitors. Inter J Antimicrob Agents. 1999;12(1):3–7.

[49]Marchou B, Bellido F, Charnas R, Lucain C, Pechere J. Contribution of β-Lactamase Hydrolysis and Outer Membrane Permeability to Ceftriaxone Resistance in *Enterobacter cloacae*. Antimicrob Agents Chemother. 1987;31(10):1589–1595.

[50]Plessner O, Klapatch T, Guerinot ML. Siderophore utilization by *Bradyrhizobium japonicum*. Appl Environ Microbiol. 1993;59(5):1688–1690.

[51]Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B. Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci. 2007;26(5-6):227–242.

[52]Illmer P, Schinner F. Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biol Biochem. 1995;27(3):257–263.

[53]Glick BR. Phytoremediation: Synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv. 2003;21(5):383–393.

[54]Tang C, Robson AD, Dilworth MJ. The role of iron in nodulation and nitrogen fixation in *Lupinus angustifolius* L. New Phytol. 1990;114(2):173–182.

[55]Brear EM, Day DA, Smith PMC. Iron: an essential micronutrient for the legume-rhizobium symbiosis. Front Plant Sci. 2013;4: 359.

[56]Small SK, Puri S, Sangwan I, O'Brian MR. Positive Control of Ferric Siderophore Receptor Gene Expression by the Irr Protein in *Bradyrhizobium japonicum*. J Bacteriol. 2009;191(5):1361–1368.

[57]Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E, Melo IS, Martínez-Romero E, Hungria M. Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. Int J Syst Evol Microbiol. 2013;63:3342–3351.

[58]Braud A, Jézéquel K, Bazot S, Lebeau T. Enhanced phytoextraction of an agricultural Cr-, Hg-, and Pb-contaminated soil by bioaugmentation with siderophore producing bacteria. Chemosphere. 2009;74:280–286.

[59]Drewniak L, Rajpert L, Mantur A, Sklodowska A. Dissolution of Arsenic Minerals Mediated by Dissimilatory Arsenate Reducing Bacteria: Estimation of the Physiological Potential for Arsenic Mobilization. BioMed Research International. 2014; Article ID 841892. doi:10.1155/2014/841892.

[60]Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl Environ Microbiol. 2009;75(3):748–757.

[61]Weyens N, Boulet J, Adriaensen D, Timmermans J-P, Prinsen E, Van Oevelen S, D'Haen J, Smeets K, van der Lelie D, Taghavi S, Vangronsveld J. Contrasting colonization and plant growth promoting capacity between wild type and a gfp-derative of the endophyte *Pseudomonas putida* W619 in hybrid poplar. Plant Soil. 2011;356(1):217–230.

[62]Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Gonnelli C, Barac T, Boulet J., Weyens N, Vangronsveld J. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. Int J Phytoremediation. 2009;11(3):251–267.

[63]Weyens N, van der Lelie D, Artois T, Smeets K, Taghavi S, Newman L, Carleer R, Vangronsveld J. Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation. Environ Sci Technol. 2009c;43(24):9413–9418.

[64]Weyens N, Schellingen K, Beckers B, Janssen J, Reinhart C, van der Lelie D, Taghavi S, Carleer R, Vangronsveld J. Potential of willow and its genetically engineered associated bacteria to remediate mixed Cd and toluene contamination. J Soils Sediments. 2013b;13(1):176–188.

[65]Glick BR, Stearns JC. Making Phytoremediation Work Better: Maximizing a Plant's Growth Potential in the Midst of Adversity. Int J Phytoremediation. 2011;13(1):4–16.

[66]Truyens S, Jambon I, Croes S, Janssen J, Weyens N, Mench M, Carleer R, Cuypers A, Vangronsveld J. The effect of long-term Cd and Ni exposure on seed endophytes of *Agrostis capillaris* and their potential application in phytoremediation of metal-contaminated soils. Int J Phytoremediation. 2014;16(7-12):643–659.

Tables

Table 1. Soil chemical and physical parameters (0-20 cm) from the As-contaminated mining area in Minas Gerais.

Chemical parameters[1]										
pH H_2O	**P-rem**	**P[2]**	**K[2]**	**Ca^{2+}[3]**	**Mg^{2+}[3]**	**Al^{3+}[4]**	**H+Al[4]**	**OM[5]**	**As**	
									Mehlich[6]	USEPA[7]
	mg L^{-1}	_mg dm^{-3}_		_____ $cmol_c$ dm^{-3}_____				dag kg^{-1}	____mg kg^{-1}____	
5.5	61.36	40.1	25.4	0.68	0.22	0.1	0.9	0.5	13.2	395.9

Soil physical size group and texture			
Sand	**Silt**	**Clay**	**Soil texture**
_____g kg^{-1}_____			
160	**760**	**80**	**Silt loam**

[1]Chemical parameters: pH – H_2O pH (ratio 1:2,5); P-rem (remaining phosphorus); [2]P (phosphorus), [2]K (potassium) - Mehlich 1 extractor (HCl 0,05 mol L^{-1} + H_2SO_4 0,0125 mol L^{-1}); [3]Ca (calcium), [3]Mg (magnesium), [4]Al (aluminium) – KCl extractor 1 mol L^{-1}; [4]H + Al (hydrogen + aluminium) – SMP extractor; [5]OM (Organic matter) – oxidation using $Na_2Cr_2O_7$ + H_2SO_4 10N (15); Arsenic available[6] and semitotal[7] content.

Table S1. Main colony characteristics of the strains of groups A and B formed by cluster dendrogram.

Strains	Colony characteristics of the strains of the group A		Strains	Colony characteristics of the strains of the group B	
	1	2		1	2
UFLA 05-22	Alkaline	Little amount	UFLA 05-13	Neutral	Abundant
UFLA 05-10	Alkaline	Little amount	UFLA 05-12	Neutral	Moderate
UFLA 05-03	Alkaline	Little amount	UFLA 05-11	Alkaline	Moderate
UFLA 05-01	Alkaline	Little amount	UFLA 05-19	Alkaline	Moderate
UFLA 05-02	Alkaline	Little amount	UFLA 05-20	Alkaline	Moderate
UFLA 05-21	Alkaline	Little amount	BR 2811	Alkaline	Abundant
UFLA 05-09	Alkaline	Little amount	BR 2001	Alkaline	Abundant
UFLA 05-14	Alkaline	Little amount	UFLA 05-18	Alkaline	Abundant
UFLA 05-23	Alkaline	Little amount	UFLA 05-17	Alkaline	Little amount
BR 5401^{T}	Alkaline	Scarce	UFLA 05-24	Alkaline	Little amount
ORS 571^{T}	Alkaline	Scarce	UFLA 05-15	Acid	Little amount
			BR 3804	Acid	Abundant
			UFLA 05-16	Acid	Abundant
			CIAT 899^{T}	Acid	Abundant
			UFLA 05-05	Acid	Little amount
			UFLA 05-07	Acid	Moderate
			UFLA 05-04	Neutral	Moderate
			LMG 1222^{T}	Neutral	Moderate
			UFLA 05-06	Acid	Moderate
			UFLA 05-08	Alkaline	Moderate

1 - pH on 79 solid medium after growth; 2 - Exopolysaccharides production.

Table 2. Original host species, most similar sequence 16S rRNA gene available in NCBI and qualitative plant growth promoting traits of the strains isolated from As-contaminated mining soil.

						Phenotypical tests				
Host species	**Strains**	**bp* of 16S rDNA**	**Identity**	**Most similar sequence (accession number)#**	**Phylum/class**	**OA****	**IAA****	**ACC****	**SID****	**$Ca_3(PO_4)_2$ Sol*****
Crotalaria spectabilis	UFLA 05-01	688	100%	*Bradyrhizobium* sp. (FR872439.1)	*α-Proteobacteria*	-	-	-	++++	I*****
Crotalaria spectabilis	UFLA 05-02	557	100%	*Bradyrhizobium* sp. (FR872439.1)	*α-Proteobacteria*	-	-	+	++++	GNFH§
Crotalaria spectabilis	UFLA 05-03	505	100%	*Bradyrhizobium* sp. (FR872439.1)	*α-Proteobacteria*	-	-	-	++++	I
Crotalaria spectabilis	UFLA 05-04	516	100%	*Bradyrhizobium* sp.(R) (FR872439.1)	*α-Proteobacteria*	-	-	-	++++	I
Crotalaria spectabilis	UFLA 05-06	953	99%	*Burkholderia* sp. JPY321 (FN543702.1)	*β-Proteobacteria*	-	-	+	++++	L****
Crotalaria spectabilis	UFLA 05-07	773	100%	*Inquilinus* sp. MG-2011-30-BD (FR872493.1)	*α-Proteobacteria*	-	-	+	++++	L
Crotalaria spectabilis	UFLA 05-08	992	99%	*Labrys monachus* (NR_025581.1)	*α-Proteobacteria*	+	+	+	++++	L
Crotalaria spectabilis	UFLA 05-09	653	100%	*Bradyrhizobium* sp. (FR872439.1)	*α-Proteobacteria*	-	+	+	++++	I
Crotalaria spectabilis	UFLA 05-10	888	100%	*Bradyrhizobium* sp.(R) (AB601666.1)	*α-Proteobacteria*	-	+	++	-	GNFH
Stizolobium aterrimum	UFLA 05-11	1096	100%	*Bradyrhizobium elkanii* (HQ231447.1)	*α-Proteobacteria*	-	+	+	-	GNFH
Stizolobium aterrimum	UFLA 05-12	512	100%	*Bradyrhizobium* sp. UFLA 03-143 (JX284230.1)	*α-Proteobacteria*	++	++++	+	-	L
Stizolobium aterrimum	UFLA 05-13	835	99%	*Bradyrhizobium* sp. UFLA 03-174 (JX284219.1)	*α-Proteobacteria*	-	-	+	-	L
Crotalaria spectabilis	UFLA 05-14	414	98%	*Bradyrhizobium* sp. (DQ202330.1)	*α-Proteobacteria*	-	-	-	++++	GNFH
Stizolobium aterrimum	UFLA 05-15	553	99%	*Bacillus* sp. DB170 (HM566884.1)	*Firmicutes*	-	-	-	++++	I
Crotalaria spectabilis	UFLA 05-16	952	100%	*Rhizobium tropici* CIAT 899 (NR_102511)	*α-Proteobacteria*	+	+	++++	++++	I
Stizolobium aterrimum	UFLA 05-17	378	100%	*Bradyrhizobium* sp. UFLA 03-182 (JX284238.1)	*α-Proteobacteria*	-	+	+	++++	I
Stizolobium aterrimum	UFLA 05-18	642	100%	*Bradyrhizobium* sp. UFLA 03-140 (JX284229.1)	*α-Proteobacteria*	-	-	+	-	L
Stizolobium aterrimum	UFLA 05-19	295	100%	*Bradyrhizobium elkanii* IAR12 (JQ809927.1)	*α-Proteobacteria*	++	++++	+	++++	L
Stizolobium aterrimum	UFLA 05-20	1097	99%	*Bradyrhizobium* sp. CCBAU 23005 (GU433446.1)	*α-Proteobacteria*	++	++++	+	++++	I
Stizolobium aterrimum	UFLA 05-21	1068	99%	*Methylobacterium* sp. AMS19(R) (AB600008.1)	*α-Proteobacteria*	-	++++	+	++++	I
Stizolobium aterrimum	UFLA 05-22	794	100%	*Bosea* sp. S41RM2 (GU731243.1)	*α-Proteobacteria*	-	-	+++	-	GNFH
Stizolobium aterrimum	UFLA 05-23	578	98%	*Starkeya novella* DMS 506 (CP002026.1)	*α-Proteobacteria*	-	+	-	-	GNFH
Stizolobium aterrimum	UFLA 05-24	307	100%	*Bradyrhizobium* sp. LmjM3 (JX514883.2)	*α-Proteobacteria*	++	++++	+	++++	I
				Percentage of positive strains		26%	52%	73%	69%	73%
Type or Reference rhizobia strains										
CIAT 899^T – *Rhizobium tropici*						+	+++	+++	++++	L
BR 3804 – *Mesorhizobium plurifarium*						++	++	+++	+	GNFH
ORS 571^T – *Azorhizobium caulinodans*						-	+++	++++++	++++	L
BR 5401^T – *Azorhizobium doebereinerae*						-	++	++++	-	GNFH
BR 11340 – *Burkholderia* sp.						+++	++	+	+	GNFH

***bp – base pairs of 16S rRNA gene sequence. #Identification based on 16S rRNA gene sequences using forward primer 27F or reverse primer 1392R(R). **Classification conferred according to the color intensity. OA: organic acid production; IAA: indole-3-acetic acid production; ACC: 1-aminocyclopropane-1-carboxylate deaminase activity; SID: siderophores production. ***Based on the $Ca_3(PO_4)_2$ solubilization index, the strains were classified as Low (L) with solubilization index < 2.00, Intermediate (I) 2.00 ≤ SI < 4.00 or High (H) SI ≥ 4.00. §Grown but did not form a halo (GNFH) by the 15th day.

Table 3. Authentication and symbiotic efficiency of native bacteria isolated from nodules of legume species growing on As-contaminated soil.

Bacteria and control treatments	NN	NDW	SDW	NAS	RE
		---------g pot^{-1}---------		mg pot^{-1}	%
		Crotalaria spectabilis			
UFLA 05-01 *Bradyrhizobium* sp.	304±28b	0.19±0.03a	3.9±0.6c	97.5±0.04c	65.6±8.5b
UFLA 05-02 *Bradyrhizobium* sp.	312±42b	0.17±0.01a	5.2±0.4b	148.7±4.5b	80.2±1.1b
UFLA 05-03 *Bradyrhizobium* sp.	309±49b	0.24±0.04a	8.8±0.03a	242.5±12.1a	123.6±3.3a
UFLA 05-04 *Bradyrhizobium* sp.	262±30b	0.16±0.03a	6.9±1.0b	150.5±12.37b	79.2±7.9b
UFLA 05-05 *Burkholderia* sp.	422±87a	0.19±0.04a	7.8±0.1a	180.0±10.9b	94.6±7.4b
UFLA 05-06 *Burkholderia* sp.	215±13b	0.05±0.01b	5.4±1.2b	132.6±24.7b	70.1±14.2b
UFLA 05-07 *Inquilinus* sp.	308±59b	0.14±0.003a	6.9±0.7b	174.9±10.0b	106.2±9.9a
UFLA 05-08 *Labrys monachus*	330±58b	0.07±0.03b	6.2±0.1b	163.5±7.4b	85.6±1.9b
UFLA 05-09 *Bradyrhizobium* sp.	418±17a	0.21±0.003a	9.1±0.6a	226.6±5.7a	138.8±17.5a
UFLA 05-10 *Bradyrhizobium* sp.	481±26a	0.17±0.08a	6.1±0.3b	151.0±10.5b	92.7±13.5b
UFLA 05-14 *Bradyrhizobium* sp.	303±39b	0.22±0.05a	8.5±0.5a	189.3±7.4b	116.6±17.5a
UFLA 05-16 *Rhizobium tropici*	362±31a	0.23±0.01a	6.7±1.7b	143.0±26.3b	93.0±31.8b
BR 2811 *Bradyrhizobium* sp.	299±36b	0.03±0.01b	1.3±0.01d	13.6±2.5d	8.0±0.7c
5.25 mg N L^{-1}	0c	0c	1.6±0.4d	20.8±5.8d	12.0±2.0c
52.5 mg N L^{-1}	0c	0c	5.3±0.2b	169.0±22.5b	100.0b
		Stizolobium aterrimum			
UFLA 05-11 *Bradyrhizobium elkanii*	348±42a	0.30±0.03c	3.2±0.2c	102.2±8.5c	105.5±3.6c
UFLA 05-12 *Bradyrhizobium* sp.	317±45a	0.34±0.04c	3.6±0.3c	131.2±11.4c	128.7±2.9c
UFLA 05-13 *Bradyrhizobium* sp.	93±15b	0.50±0.02a	6.3±0.4b	271.9±36.8a	311.2±21.1b
UFLA 05-15 *Bacillus* sp.	0d	0d	4.4±0.5c	61.0±7.2d	60.6±4.6d
UFLA 05-17 *Bradyrhizobium* sp.	30±4c	0.44±0.1b	7.2±0.8b	266.5±29.3b	275.3±49.8b
UFLA 05-18 *Bradyrhizobium* sp.	35±5c	0.52±0.04a	7.8±0.3a	305.9±17.3b	356.3±25.7a
UFLA 05-19 *Bradyrhizobium elkanii*	35±1c	0.60±0.03a	9.0±0.3a	343.6±15.7a	347.3±28.9a
UFLA 05-20 *Bradyrhizobium* sp.	45±5c	0.52±0.1a	8.2±0.9a	284.3±22.8b	338.5±22.8a
UFLA 05-21 *Methylobacterium* sp.	0d	0d	4.8±0.7c	62.7±6.8d	55.9±7.8d
UFLA 05-22 *Bosea* sp.	0d	0d	4.3±0.5c	58.7±9.3d	52.5±7.9d
UFLA 05-23 *Starkeya novella*	0d	0d	4.9±0.2c	69.1±3.2d	65.2±6.6d
UFLA 05-24 *Bradyrhizobium* sp.	42±8c	0.50±0.05a	7.9±0.4a	289.9±8.2b	296.1±34.7b
BR 2811 *Bradyrhizobium* sp.	91±10b	0.41±0.01b	4.7±0.2c	64.5±5.0d	59.0±1.6d
5.25 mg N L^{-1}	0d	0d	5.1±0.3c	66.1±5.1d	57.5±3.1d
52.5 mg N L^{-1}	0d	0d	7.6±0.4a	101.4±10.6c	100,0c

NN – number of nodules, NDW – nodule dry weight, SDW – shoot dry weight, NAS – nitrogen accumulation in shoot, RE – relative efficiency. Values followed by the same letter on the column comparing strains do not differ by Scott-Knott test, p<0,05.

Table 4. Cadmium and zinc resistance (in 284 medium) of strains isolated from nodules of legume species growing in As-contaminated mining soil.

		Cadmiun		Zinc	
Strains	Closest related strain by NCBI	0.4 mM	0.8 mM	0.6 mM	1.0 mM
UFLA 05-01	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-02	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-03	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-04	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-06	*Burkholderia* sp. JPY321	+	+	+	+
UFLA 05-07	*Inquilinus* sp. MG-2011-30-BD	+++	+++	+++	+++
UFLA 05-08	*Labrys monachus*	++++	++++	++++	++++
UFLA 05-09	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-10	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-11	*Bradyrhizobium elkanii*	++	+	++	+
UFLA 05-12	*Bradyrhizobium* sp. UFLA 03-143	++	+	+	+
UFLA 05-13	*Bradyrhizobium* sp. UFLA 03-174	++	+	++	+
UFLA 05-14	*Bradyrhizobium* sp.	+	+	+	+
UFLA 05-15	*Bacillus* sp. DB170	++	+	++	++
UFLA 05-16	*Rhizobium tropici* CIAT 899	+++	+++	+++	+++
UFLA 05-17	*Bradyrhizobium* sp. UFLA 03-182	++	+	+	+
UFLA 05-18	*Bradyrhizobium* sp. UFLA 03-140	+++	+++	++	+
UFLA 05-19	*Bradyrhizobium elkanii* IAR12	++	+	++	++
UFLA 05-20	*Bradyrhizobium* sp. CCBAU 23005	+	+	+	+
UFLA 05-21	*Methylobacterium* sp. AMS19	+	+	+	+
UFLA 05-22	*Bosea* sp. S41RM2	+	+	+	+
UFLA 05-23	*Starkeya novella* DMS 506	++	+	+	+
UFLA 05-24	*Bradyrhizobium* sp. LmjM3	+	+	++	++
Type or Reference rhizobia strains					
CIAT 899^{T} – *Rhizobium tropici*		++	+	++	+
BR 3804 – *Mesorhizobium plurifarium*		++	+	++	+
ORS 571^{T} – *Azorhizobium caulinodans*		++	+	++	+
BR 5401^{T} – *Azorhizobium doebereinerae*		++	+	++	+
BR 11340 – *Burkholderia* sp.		+	+	+	+

+Scarce, ++Low, +++Moderate and ++++Abundant - Growth plus polysaccharide production under *in vitro* contamination.

Figures

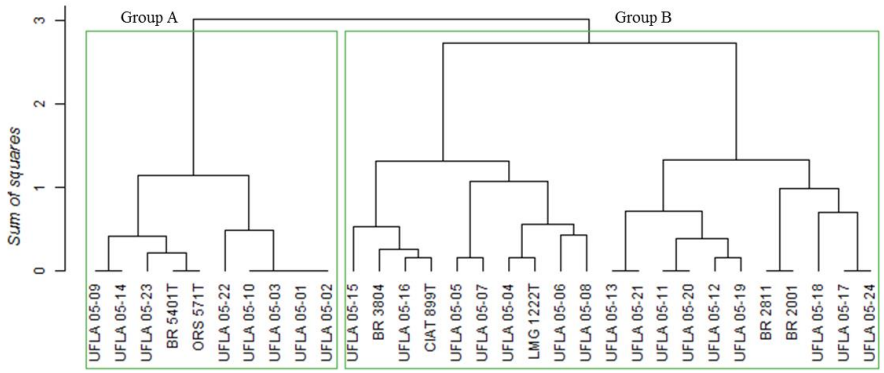

Figure S1. Dendrogram based on colony characteristics of strains isolated from nodules of *S. aterrimum* and *C. spectabilis* plants growing on As-contaminated soil. Reference and type strains are *Azorhizobium* (BR 5401^{T} and ORS 571^{T}), *Bradyrhizobium* (BR 2001 and BR 2811), *Mesorhizobium* (BR 3804), *Rhizobium* (CIAT 899^{T}), and *Burkholderia* (LMG 1222^{T}).

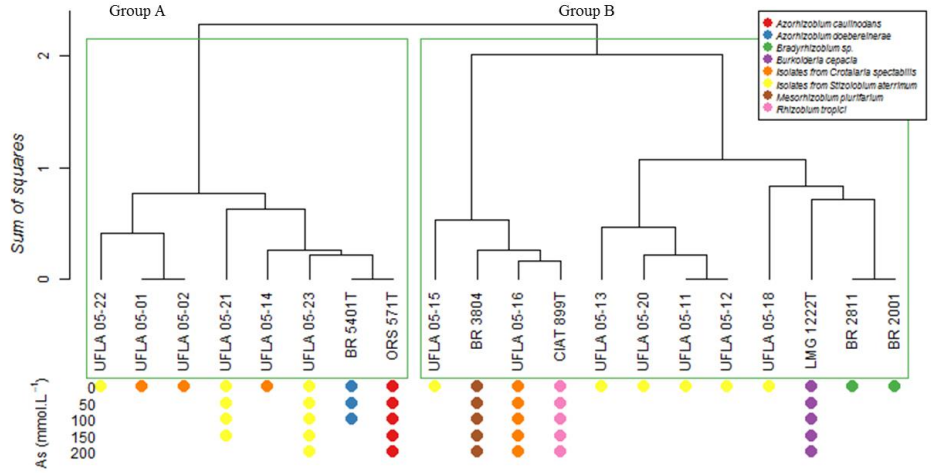

Figure 1. Dendrogram based on colony characteristics and As resistance of the strains isolated from nodules of *S. aterrimum* and *C. spectabilis* species, growing on As-contaminated soil. UFLA 05-01, UFLA 05-02, UFLA 05-12, UFLA 05-13, UFLA 05-14, UFLA 05-18 e UFLA 05-20 (*Bradyrhizobium* sp.), UFLA 05-11 (*Bradyrhizobium elkanii*), UFLA 05-16 (*Rhizobium tropici*), UFLA 05-22 (*Bosea* sp.), UFLA 05-21 (*Methylobacterium* sp.), UFLA 05-23 (*Starkeya novella*), UFLA 05-15 (*Bacillus* sp.). Reference and type strains *Azorhizobium* (BR 5401^{T} and ORS 571^{T}), *Bradyrhizobium* (BR 2001 and BR 2811), *Mesorhizobium* (BR 3804), *Rhizobium* (CIAT 899^{T}), and *Burkholderia* (LMG 1222^{T}).

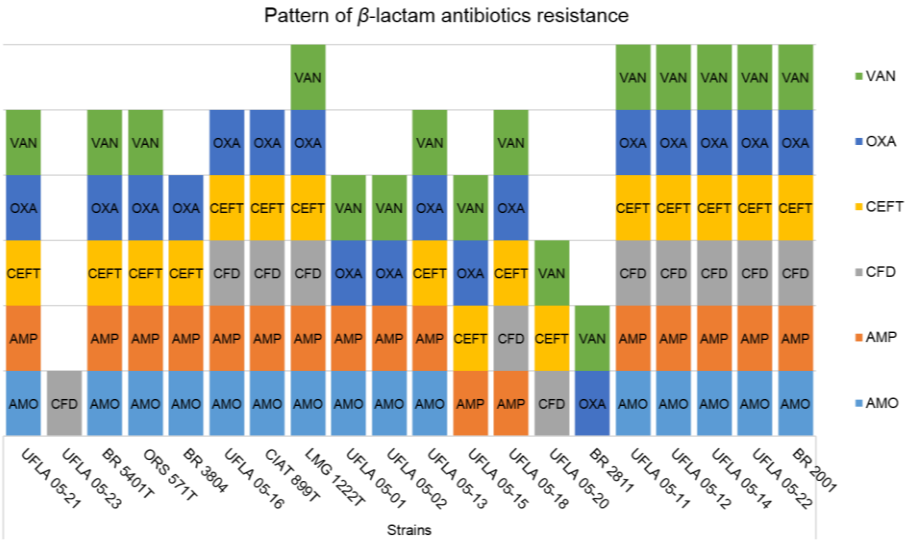

Figure 2. Pattern of *β*-lactam antibiotics resistance of arsenic resistant strains by disk diffusion method. AMO: Amoxicillin, AMP: Ampicillin, CFD: Cefadroxil, CEFT: Ceftriaxone, OXA: Oxacillin, and VAN: Vancomycin.