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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<sub>2</sub>-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  $\alpha$ -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<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 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  $\beta$ -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<sub>2</sub> fixation, trace elements multi-resistance,  $\beta$ -lactam antibiotics resistance.

## 1 Introduction

Soil microorganisms delivering ecosystem services (*e.g.* biological N<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>-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  $\beta$ -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 ( $\text{HCl } 0.05 \text{ mol L}^{-1} + \text{H}_2\text{SO}_4 \text{ } 0.0125 \text{ mol L}^{-1}$ ); calcium, magnesium and aluminium were determined after KCl extraction ( $1 \text{ mol L}^{-1}$ ). The potential acidity ( $\text{H} + \text{Al}$ ) was estimated by SMP extraction, and organic matter was determined by oxidation using  $\text{Na}_2\text{Cr}_2\text{O}_7 + \text{H}_2\text{SO}_4$  (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 °C). 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<sup>T</sup>; *A. doebereineriae* – BR 5401<sup>T</sup>), *Mesorhizobium* (*M. plurifarum* – BR 3804), *Rhizobium* (*R. tropici* – CIAT 899<sup>T</sup>), *Burkholderia* (*B. cepacia* – LMG 1222<sup>T</sup>), 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. atterrimum* 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<sub>2</sub>, 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.

1           The seeds were scarified using  $\text{H}_2\text{SO}_4$  p.a. (*C. spectabilis* for 5 min and *S.*  
 2   *aterrimum* for 45 min), and placed on sterile Petri dishes containing moistened cotton  
 3   incubated at 28 °C until radicle emergence. The strains were grown in 79 liquid medium  
 4   shaking (125 rpm, 28 °C) for 120 h. At the moment of sowing, each seed was inoculated  
 5   with 1 mL of the bacterial inoculum containing about  $10^8$  cells. After inoculating the  
 6   seeds, a thin layer of the sterile mixture of sand-benzene-paraffin was disposed on the  
 7   top to avoid contamination. Two plants were grown in sterile Leonard jars for 45 days.  
 8   Sand and vermiculite (1:1 ratio) were used as substrate in the topmost portion of the  
 9   jars, and in the lower portion a four-fold diluted modified Hoagland nutrient solution  
 10   (22) was added. The inoculated plants and the non-inoculated control plants were  
 11   supplied with a low nitrogen concentration ( $5.25 \text{ mg}\cdot\text{L}^{-1}$ ) in the nutrient solution, which  
 12   is considered a starting dose for, and not an inhibitor of, the biological nitrogen fixation  
 13   process. The following quantities of the stock solutions were added to 4 L of water: 0.4  
 14   mL of  $236.16 \text{ g L}^{-1} \text{ CaN}_2\text{O}_6\cdot 4\text{H}_2\text{O}$ ; 0.1 mL of  $115.03 \text{ g L}^{-1} \text{ NH}_4\text{H}_2\text{PO}_4$ ; 0.6 mL of  
 15    $101.11 \text{ g L}^{-1} \text{ KNO}_3$ ; 2.0 mL of  $246.9 \text{ g L}^{-1} \text{ MgSO}_4\cdot 7\text{H}_2\text{O}$ ; 3.0 mL of  $87.13 \text{ g L}^{-1} \text{ K}_2\text{SO}_4$ ;  
 16   10 mL of  $12.6 \text{ g L}^{-1} \text{ CaH}_4\text{P}_2\text{O}_8\cdot \text{H}_2\text{O}$ ; 200 mL of  $1.72 \text{ g L}^{-1} \text{ CaSO}_4\cdot 2\text{H}_2\text{O}$ ; 1 mL of  $10 \text{ g L}^{-1}$   
 17    $\text{FeCl}_3$ , and 1 mL of micronutrients ( $2.86 \text{ mg L}^{-1} \text{ H}_3\text{BO}_3$ ;  $2.03 \text{ mg L}^{-1} \text{ MnSO}_4\cdot 4\text{H}_2\text{O}$ ;  
 18    $0.22 \text{ mg L}^{-1} \text{ ZnSO}_4\cdot 7\text{H}_2\text{O}$ ;  $0.08 \text{ mg L}^{-1} \text{ CuSO}_4\cdot 5\text{H}_2\text{O}$ , and  $0.09 \text{ mg L}^{-1} \text{ Na}_2\text{MoO}_4\cdot \text{H}_2\text{O}$ ).  
 19   In addition, a control treatment supplemented with a high inorganic nitrogen  
 20   concentration ( $52.5 \text{ mg}\cdot\text{L}^{-1}$ ) was also provided, composed by the following quantities of  
 21   the stock solutions added to 4 L of water: 4.0 mL of  $236.16 \text{ g L}^{-1} \text{ CaN}_2\text{O}_6\cdot 4\text{H}_2\text{O}$ ; 1.0  
 22   mL of  $115.03 \text{ g L}^{-1} \text{ NH}_4\text{H}_2\text{PO}_4$ ; 6.0 mL of  $101.11 \text{ g L}^{-1} \text{ KNO}_3$ ; 2.0 mL of  $246.9 \text{ g L}^{-1}$   
 23    $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ ; 1.0 mL of  $10 \text{ g L}^{-1} \text{ FeCl}_3$ , and 1.0 mL of micronutrients solution  
 24   (composition as described above). Besides the negative control treatments (low and high  
 25   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  $RE = [(inoculated\ SDW / high\ N\ SDW) \times 100]$  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<sup>-1</sup>), 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<sub>2</sub>HAsO<sub>4</sub>·7H<sub>2</sub>O) was used at concentrations of 50, 100, 150 and 200 mmol L<sup>-1</sup>, 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 $\beta$ -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<sup>T</sup>, *A. doebereineriae* BR 5401<sup>T</sup>, *Mesorhizobium plurifarum* BR 3804, *Rhizobium tropici* CIAT 899<sup>T</sup>, *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 colorimetric method (28), and  $\text{Ca}_3(\text{PO}_4)_2$  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<sup>T</sup> and ORS571<sup>T</sup>.

## 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<sup>T</sup> and *A. doebereinereae* - BR 5401<sup>T</sup>. 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<sup>T</sup>) and *Burkholderia cepacia* (LMG 1222<sup>T</sup>).

The 16S rRNA gene sequencing revealed representatives of two phyla, Firmicutes and Proteobacteria (Table 2). Most of the strains belonged to the  $\alpha$ -Proteobacteria such as *Bradyrhizobium*, *Rhizobium*, *Bosea*, *Inquilinus*, *Labrys*, *Starkeya* and *Methylobacterium*. A few strains belonged to the  $\beta$ -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<sup>-1</sup> or 52.5 mg L<sup>-1</sup> 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<sub>2</sub> 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<sup>-1</sup>). 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<sup>-1</sup>). 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<sup>-1</sup>) (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<sub>2</sub> 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<sub>2</sub>-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<sup>-1</sup>). 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<sup>-1</sup>). The atypical genera, *Bosea* sp., *Methylobacterium* sp. and *Starkeya novella*, did not induce nodule formation on *S. atterrimum* 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. atterrimum*, tolerated up to 150 and 200 mmol As L<sup>-1</sup>, 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. atterrimum* 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<sup>T</sup>, *Mesorhizobium plurifarum* BR 3804, *Rhizobium tropici* CIAT 899<sup>T</sup> and *Burkholderia cepacia* LMG 1222<sup>T</sup> were resistant to As, since they could grow until the highest As concentration tested. The strain *A. doebereinereae* BR 5401<sup>T</sup> was also As-resistant, but it survived only until 100 mmol As L<sup>-1</sup>. 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 $\beta$ -lactams antibiotics resistance

The patterns of  $\beta$ -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<sup>T</sup> (*A. doebereineriae*) and ORS571<sup>T</sup> (*A. caulinodans*) showed the same pattern of  $\beta$ -lactams antibiotics resistance to AMO, AMP, CEFT, OXA and VAN, and sensitivity to CFD. The strains UFLA 05-16 (*R. tropici*) and CIAT 899<sup>T</sup> (*R. tropici*) showed the same pattern of  $\beta$ -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  $\beta$ -lactams: AMO, AMP, CFD, CEFT and OXA, but it was sensitive to CFD, and VAN as well. Strain LMG 1222<sup>T</sup> (*B. cepacia*) was resistant to all  $\beta$ -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  $\beta$ -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<sup>T</sup>, *A. doebereineriae* BR 5401<sup>T</sup>, *Mesorhizobium plurifarium* BR 3804, *Rhizobium tropici* CIAT 899<sup>T</sup> 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<sub>2</sub>-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<sub>2</sub>-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<sup>T</sup> (*A. caulinodans*), BR 3804 (*Mesorhizobium plurifarum*), CIAT 899<sup>T</sup> (*Rhizobium tropici*) and LMG 1222<sup>T</sup> (*Burkholderia cepacia*) tolerated up to 200 mmol As L<sup>-1</sup>, the strain UFLA 05-21 (*Methylobacterium* sp.) up to 150 mmol As L<sup>-1</sup>, and the type strain BR 5401<sup>T</sup> (*A. doebereineriae*) tolerated up to 100 mmol As L<sup>-1</sup>. These results emphasize the As resistance of these strains. All strains that could cope with the highest As concentration (200 mmol L<sup>-1</sup>) that was used were members of  $\alpha$ -Proteobacteria.

Our results confirm that the  $\alpha$ -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  $\beta$ -lactams antibiotics (44). Different resistance mechanisms to  $\beta$ -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  $\beta$ -lactam antibiotics (Figure 2). The production of  $\beta$ -lactamase, an enzyme capable of modifying and inactivating the  $\beta$ -lactams antibiotics, is part of the resistance mechanism to  $\beta$ -lactams antibiotics (48). In Gram-negative bacteria,  $\beta$ -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  $\beta$ -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  $\beta$ -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).

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

7 The total percentage of P solubilizing strains was 73%, which is high (Table 2).  
8 The Nautiyal protocol (29) is based on extracellular oxidation of glucose via  
9 quinoprotein glucose dehydrogenase, which produces gluconic acid and mobilizes  
10 insoluble phosphates very efficiently. Moreover, apart from the production and  
11 excretion of gluconic acid, other mechanisms such as the production of chelating  
12 substances, the release of protons originating from  $\text{NH}_4^+$  assimilation and the production  
13 of inorganic acids, have also been proposed to be responsible for phosphate  
14 solubilization by bacteria (52).

15 Plants growing on trace elements contaminated soils might become severely  
16 deficient in the amount of available iron (53). Fortunately, plants can produce  
17 siderophores that bind iron which allows them to take up more iron. Moreover, plants  
18 can also assimilate complexes of iron with bacterial siderophores. Even though plants  
19 can produce siderophores themselves, the affinity of plant siderophores for iron is  
20 considerably lower than that of bacterial siderophores (53). Therefore, plants are  
21 considered to depend to a great extent on bacterial siderophore production. In trace  
22 elements contaminated soils plants are often unable to accumulate sufficient amounts of  
23 iron unless bacterial siderophores are present (53). A high number of our bacterial  
24 strains (69%) produced siderophores (Table 2). This high amount of siderophores  
25 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<sub>2</sub> 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 iron chelates (56). In rhizobia, Fe<sup>3+</sup>-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  $\text{Ca}_3(\text{PO}_4)_2$ . Therefore, the  $\text{Ca}_3(\text{PO}_4)_2$  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 ( $\text{FeAsS}$ ) 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-carboxylic 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 *Inquilius* sp. UFLA 05-07 also showed high Zn and Cd resistance.

## 5 Conclusions

The group of  $\alpha$ -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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## Tables

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

Chemical parameters <sup>(1)</sup>									
pH H <sub>2</sub> O	P-rem	P <sup>(2)</sup>	K <sup>(2)</sup>	Ca <sup>2+(3)</sup>	Mg <sup>2+(3)</sup>	Al <sup>3+(4)</sup>	H+Al <sup>(4)</sup>	OM <sup>(5)</sup>	As
	mg L <sup>-1</sup>	mg dm <sup>-3</sup>			cmol <sub>c</sub> dm <sup>-3</sup>			dag kg <sup>-1</sup>	Mehlich <sup>(6)</sup> USEPA <sup>(7)</sup>
									mg kg <sup>-1</sup>
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 <sup>-1</sup>		
160	760	80	Silt loam

<sup>(1)</sup>Chemical parameters: pH – H<sub>2</sub>O pH (ratio 1:2,5); P-rem (remaining phosphorus); <sup>(2)</sup>P (phosphorus), <sup>(2)</sup>K (potassium) - Mehlich 1 extractor (HCl 0,05 mol L<sup>-1</sup> + H<sub>2</sub>SO<sub>4</sub> 0,0125 mol L<sup>-1</sup>); <sup>(3)</sup>Ca (calcium), <sup>(3)</sup>Mg (magnesium), <sup>(4)</sup>Al (aluminium) – KCl extractor 1 mol L<sup>-1</sup>; <sup>(4)</sup>H + Al (hydrogen + aluminium) – SMP extractor;

<sup>(5)</sup>OM (Organic matter) – oxidation using Na<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> + H<sub>2</sub>SO<sub>4</sub> 10N (15); Arsenic available<sup>(6)</sup> and semitotal<sup>(7)</sup> content.



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

Strains			Strains		
Colony characteristics of the strains of the group A			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 <sup>T</sup>	Alkaline	Scarce	UFLA 05-24	Alkaline	Little amount
ORS 571 <sup>T</sup>	Alkaline	Scarce	UFLA 05-15	Acid	Little amount
			BR 3804	Acid	Abundant
			UFLA 05-16	Acid	Abundant
			CIAT 899 <sup>T</sup>	Acid	Abundant
			UFLA 05-05	Acid	Little amount
			UFLA 05-07	Acid	Moderate
			UFLA 05-04	Neutral	Moderate
			LMG 1222 <sup>T</sup>	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.

Host species	Strains	bp* of 16S rDNA	Identity	Most similar sequence (accession number) <sup>#</sup>	Phylum/class	Phenotypical tests				
						OA**	IAA**	ACC**	SID**	Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> Sol***
<i>Crotalaria spectabilis</i>	UFLA 05-01	688	100%	<i>Bradyrhizobium</i> sp. (FR872439.1)	<i>α-Proteobacteria</i>	-	-	-	++++	I****
<i>Crotalaria spectabilis</i>	UFLA 05-02	557	100%	<i>Bradyrhizobium</i> sp. (FR872439.1)	<i>α-Proteobacteria</i>	-	-	+	++++	GNFH <sup>†</sup>
<i>Crotalaria spectabilis</i>	UFLA 05-03	505	100%	<i>Bradyrhizobium</i> sp. (FR872439.1)	<i>α-Proteobacteria</i>	-	-	-	++++	I
<i>Crotalaria spectabilis</i>	UFLA 05-04	516	100%	<i>Bradyrhizobium</i> sp. <sup>(8)</sup> (FR872439.1)	<i>α-Proteobacteria</i>	-	-	-	++++	I
<i>Crotalaria spectabilis</i>	UFLA 05-06	953	99%	<i>Burkholderia</i> sp. JPY321 (FN543702.1)	<i>β-Proteobacteria</i>	-	-	+	++++	L****
<i>Crotalaria spectabilis</i>	UFLA 05-07	773	100%	<i>Inquilinus</i> sp. MG-2011-30-BD (FR872493.1)	<i>α-Proteobacteria</i>	-	-	+	++++	L
<i>Crotalaria spectabilis</i>	UFLA 05-08	992	99%	<i>Labrys monachus</i> (NR_025581.1)	<i>α-Proteobacteria</i>	+	+	+	++++	L
<i>Crotalaria spectabilis</i>	UFLA 05-09	653	100%	<i>Bradyrhizobium</i> sp. (FR872439.1)	<i>α-Proteobacteria</i>	-	+	+	++++	I
<i>Crotalaria spectabilis</i>	UFLA 05-10	888	100%	<i>Bradyrhizobium</i> sp. <sup>(8)</sup> (AB601666.1)	<i>α-Proteobacteria</i>	-	+	++	-	GNFH
<i>Stizolobium aeterrimum</i>	UFLA 05-11	1096	100%	<i>Bradyrhizobium elkanii</i> (HQ231447.1)	<i>α-Proteobacteria</i>	-	+	+	-	GNFH
<i>Stizolobium aeterrimum</i>	UFLA 05-12	512	100%	<i>Bradyrhizobium</i> sp. UFLA 03-143 (JX284230.1)	<i>α-Proteobacteria</i>	++	++++	+	-	L
<i>Stizolobium aeterrimum</i>	UFLA 05-13	835	99%	<i>Bradyrhizobium</i> sp. UFLA 03-174 (JX284219.1)	<i>α-Proteobacteria</i>	-	-	+	-	L
<i>Crotalaria spectabilis</i>	UFLA 05-14	414	98%	<i>Bradyrhizobium</i> sp. (DQ202330.1)	<i>α-Proteobacteria</i>	-	-	-	++++	GNFH
<i>Stizolobium aeterrimum</i>	UFLA 05-15	553	99%	<i>Bacillus</i> sp. DB170 (HM566884.1)	<i>Firmicutes</i>	-	-	-	++++	I
<i>Crotalaria spectabilis</i>	UFLA 05-16	952	100%	<i>Rhizobium tropici</i> CIAT 899 (NR_102511)	<i>α-Proteobacteria</i>	+	+	++++	++++	I
<i>Stizolobium aeterrimum</i>	UFLA 05-17	378	100%	<i>Bradyrhizobium</i> sp. UFLA 03-182 (JX284238.1)	<i>α-Proteobacteria</i>	-	+	+	++++	I
<i>Stizolobium aeterrimum</i>	UFLA 05-18	642	100%	<i>Bradyrhizobium</i> sp. UFLA 03-140 (JX284229.1)	<i>α-Proteobacteria</i>	-	-	+	-	L
<i>Stizolobium aeterrimum</i>	UFLA 05-19	295	100%	<i>Bradyrhizobium elkanii</i> IAR12 (JQ809927.1)	<i>α-Proteobacteria</i>	++	++++	+	++++	L
<i>Stizolobium aeterrimum</i>	UFLA 05-20	1097	99%	<i>Bradyrhizobium</i> sp. CCBAU 23005 (GU433446.1)	<i>α-Proteobacteria</i>	++	++++	+	++++	I
<i>Stizolobium aeterrimum</i>	UFLA 05-21	1068	99%	<i>Methylobacterium</i> sp. AMS19 <sup>(6)</sup> (AB600008.1)	<i>α-Proteobacteria</i>	-	++++	+	++++	I
<i>Stizolobium aeterrimum</i>	UFLA 05-22	794	100%	<i>Bosea</i> sp. S41RM2 (GU731243.1)	<i>α-Proteobacteria</i>	-	-	+++	-	GNFH
<i>Stizolobium aeterrimum</i>	UFLA 05-23	578	98%	<i>Starkeya novella</i> DMS 506 (CP002026.1)	<i>α-Proteobacteria</i>	-	+	-	-	GNFH
<i>Stizolobium aeterrimum</i>	UFLA 05-24	307	100%	<i>Bradyrhizobium</i> sp. LmjM3 (JX514883.2)	<i>α-Proteobacteria</i>	++	++++	+	++++	I
Percentage of positive strains						26%	52%	73%	69%	73%
<b>Type or Reference rhizobia strains</b>										
CIAT 899 <sup>†</sup> – <i>Rhizobium tropici</i>						+	+++	+++	++++	L
BR 3804 – <i>Mesorhizobium plurifarum</i>						++	++	+++	+	GNFH
ORS 571 <sup>†</sup> – <i>Azorhizobium caulinodans</i>						-	+++	+++++	++++	L
BR 5401 <sup>†</sup> – <i>Azorhizobium doebereineriae</i>						-	++	++++	-	GNFH
BR 11340 – <i>Burkholderia</i> sp.						+++	++	+	+	GNFH

\*bp – base pairs of 16S rRNA gene sequence. <sup>#</sup>Identification based on 16S rRNA gene sequences using forward primer 27F or reverse primer 1392R<sup>(R)</sup>. \*\*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<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 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 <sup>-1</sup> -----		mg pot <sup>-1</sup>	%
<i>Crotalaria spectabilis</i>					
UFLA 05-01 <i>Bradyrhizobium</i> sp.	304±28b	0.19±0.03a	3.9±0.6c	97.5±0.04c	65.6±8.5b
UFLA 05-02 <i>Bradyrhizobium</i> sp.	312±42b	0.17±0.01a	5.2±0.4b	148.7±4.5b	80.2±1.1b
UFLA 05-03 <i>Bradyrhizobium</i> sp.	309±49b	0.24±0.04a	8.8±0.03a	242.5±12.1a	123.6±3.3a
UFLA 05-04 <i>Bradyrhizobium</i> sp.	262±30b	0.16±0.03a	6.9±1.0b	150.5±12.37b	79.2±7.9b
UFLA 05-05 <i>Burkholderia</i> sp.	422±87a	0.19±0.04a	7.8±0.1a	180.0±10.9b	94.6±7.4b
UFLA 05-06 <i>Burkholderia</i> sp.	215±13b	0.05±0.01b	5.4±1.2b	132.6±24.7b	70.1±14.2b
UFLA 05-07 <i>Inquilinus</i> sp.	308±59b	0.14±0.003a	6.9±0.7b	174.9±10.0b	106.2±9.9a
UFLA 05-08 <i>Labrys monachus</i>	330±58b	0.07±0.03b	6.2±0.1b	163.5±7.4b	85.6±1.9b
UFLA 05-09 <i>Bradyrhizobium</i> sp.	418±17a	0.21±0.003a	9.1±0.6a	226.6±5.7a	138.8±17.5a
UFLA 05-10 <i>Bradyrhizobium</i> sp.	481±26a	0.17±0.08a	6.1±0.3b	151.0±10.5b	92.7±13.5b
UFLA 05-14 <i>Bradyrhizobium</i> sp.	303±39b	0.22±0.05a	8.5±0.5a	189.3±7.4b	116.6±17.5a
UFLA 05-16 <i>Rhizobium tropici</i>	362±31a	0.23±0.01a	6.7±1.7b	143.0±26.3b	93.0±31.8b
BR 2811 <i>Bradyrhizobium</i> sp.	299±36b	0.03±0.01b	1.3±0.01d	13.6±2.5d	8.0±0.7c
5.25 mg N L <sup>-1</sup>	0c	0c	1.6±0.4d	20.8±5.8d	12.0±2.0c
52.5 mg N L <sup>-1</sup>	0c	0c	5.3±0.2b	169.0±22.5b	100.0b
<i>Stizolobium aterrimum</i>					
UFLA 05-11 <i>Bradyrhizobium elkanii</i>	348±42a	0.30±0.03c	3.2±0.2c	102.2±8.5c	105.5±3.6c
UFLA 05-12 <i>Bradyrhizobium</i> sp.	317±45a	0.34±0.04c	3.6±0.3c	131.2±11.4c	128.7±2.9c
UFLA 05-13 <i>Bradyrhizobium</i> sp.	93±15b	0.50±0.02a	6.3±0.4b	271.9±36.8a	311.2±21.1b
UFLA 05-15 <i>Bacillus</i> sp.	0d	0d	4.4±0.5c	61.0±7.2d	60.6±4.6d
UFLA 05-17 <i>Bradyrhizobium</i> sp.	30±4c	0.44±0.1b	7.2±0.8b	266.5±29.3b	275.3±49.8b
UFLA 05-18 <i>Bradyrhizobium</i> sp.	35±5c	0.52±0.04a	7.8±0.3a	305.9±17.3b	356.3±25.7a
UFLA 05-19 <i>Bradyrhizobium elkanii</i>	35±1c	0.60±0.03a	9.0±0.3a	343.6±15.7a	347.3±28.9a
UFLA 05-20 <i>Bradyrhizobium</i> sp.	45±5c	0.52±0.1a	8.2±0.9a	284.3±22.8b	338.5±22.8a
UFLA 05-21 <i>Methylobacterium</i> sp.	0d	0d	4.8±0.7c	62.7±6.8d	55.9±7.8d
UFLA 05-22 <i>Bosea</i> sp.	0d	0d	4.3±0.5c	58.7±9.3d	52.5±7.9d
UFLA 05-23 <i>Starkeya novella</i>	0d	0d	4.9±0.2c	69.1±3.2d	65.2±6.6d
UFLA 05-24 <i>Bradyrhizobium</i> sp.	42±8c	0.50±0.05a	7.9±0.4a	289.9±8.2b	296.1±34.7b
BR 2811 <i>Bradyrhizobium</i> sp.	91±10b	0.41±0.01b	4.7±0.2c	64.5±5.0d	59.0±1.6d
5.25 mg N L <sup>-1</sup>	0d	0d	5.1±0.3c	66.1±5.1d	57.5±3.1d
52.5 mg N L <sup>-1</sup>	0d	0d	7.6±0.4a	101.4±10.6c	100.0c

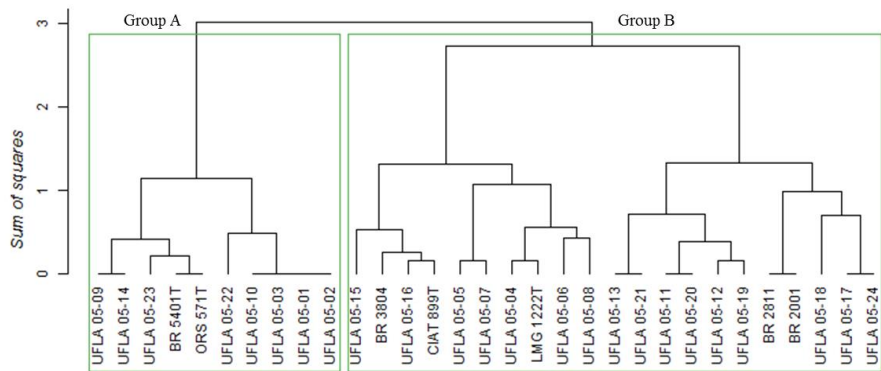
3 NN – number of nodules, NDW – nodule dry weight, SDW – shoot dry weight, NAS – nitrogen accumulation in shoot,  
 4 RE – relative efficiency. Values followed by the same letter on the column comparing strains do not differ by Scott-  
 5 Knott test, p<0,05.

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

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

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

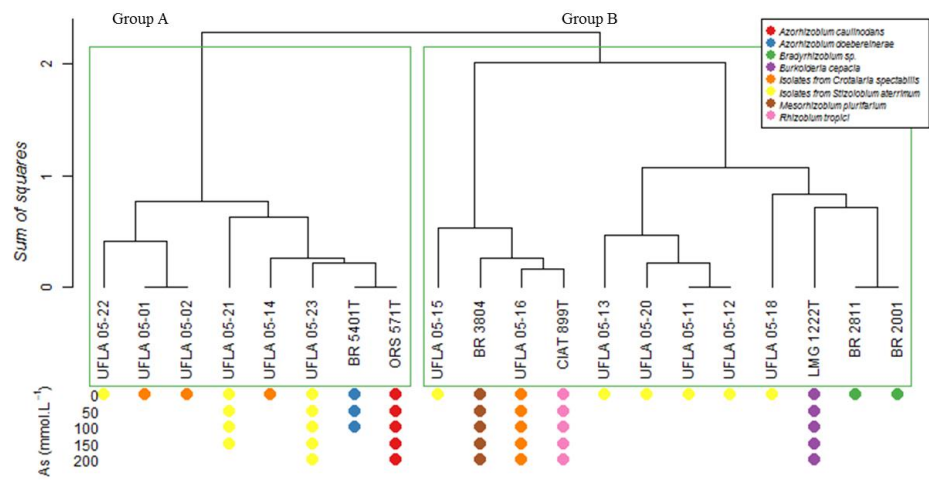
1 **Figures**



2

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

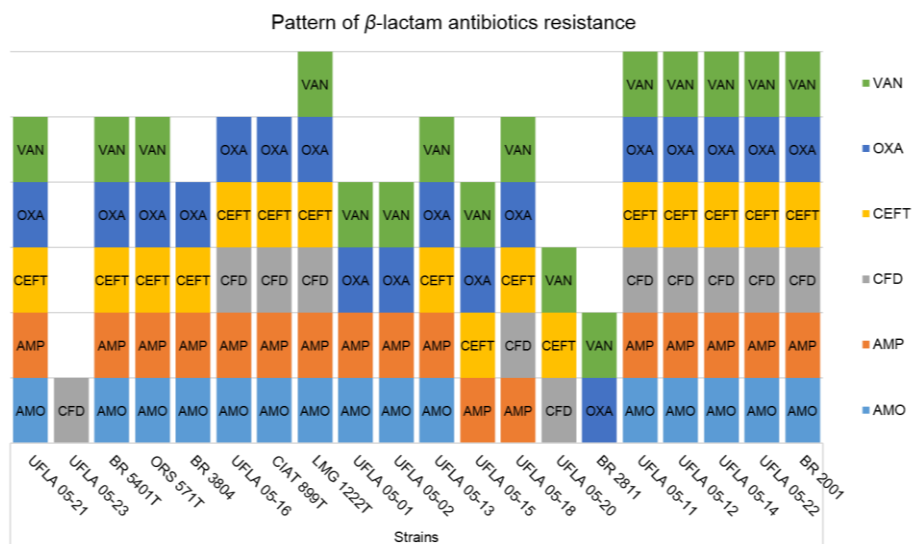
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8

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

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