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Peer-reviewed author version

Rangel, Wesley M.; THIJS, Sofie; JANSSEN, Jolien; Oliveira Longatti, Silvia M.; Bonaldi, Daiane S.; Ribeiro, Paula R. A.; JAMBON, Inge; EEVERS, Nele; WEYENS, Nele; VANGRONSVELD, Jaco & Moreira, Fatima M. S. (2017) Native rhizobia from Zn mining soil promote the growth of Leucaena leucocephala on contaminated soil. In: INTERNATIONAL JOURNAL OF PHYTOREMEDIATION, 19(2), p. 142-156.

DOI: 10.1080/15226514.2016.1207600
Handle: http://hdl.handle.net/1942/23806

International Journal of Phytoremediation

ISSN: 1522-6514 (Print) 1549-7879 (Online) Journal homepage: http://www.tandfonline.com/loi/bijp20

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To cite this article: Wesley M. Rangel, Sofie Thijs, Jolien Janssen, Silvia M. Oliveira Longatti, Daiane S. Bonaldi, Paula R. Ribeiro, Inge Jambon, Nele Eevers, Nele Weyens, Jaco Vangronsveld & Fatima M. S. Moreira (2016): Native rhizobia from Zn-mining soil promotes the growth of Leucaena leucocephala on contaminated soil, International Journal of Phytoremediation

To link to this article: http://dx.doi.org/10.1080/15226514.2016.1207600

Accepted author version posted online: 13 Jul 2016.
Published online: 13 Jul 2016.

Native rhizobia from Zn-mining soil promotes the growth of *Leucaena leucocephala* on contaminated soil

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Abstract

Plants on contaminated mining soils often show a reduced growth due to nutrient depletion as well as trace elements (TE) toxicity. Since those conditions threat plant's survival, plant growth promoting rhizobacteria (PGPR) such as rhizobia, might be of crucial importance for plant colonization on TE contaminated soils. Native rhizobia from mining soils are promising candidates for bioaugmented phytoremediation of those soils as they are adapted to the specific conditions. In this work, rhizobia from Zn- and Cd-contaminated mining soils were *in vitro* screened for their plant growth promoting features (organic acids, indole-3-acetic-acid and siderophore production, 1-aminocyclopropane-1-carboxylate deaminase activity, and $Ca_3(PO_4)_2$ solubilization) and Zn- and Cd-resistance. In addition, some type and reference rhizobia strains were included in the study as well. The *in vitro* screening indicated that rhizobia and other native genera have great potential for phytoremediation purposes, by exerting, besides biological N_2 fixation, other plant growth promoting traits. *Leucaena leucocephala-Mesorhizobium* sp. (UFLA

01-765) showed multi-element tolerance and an efficient symbiosis on contaminated soil deacresing the activities of antioxidative enzymes in shoots. This symbiosis is a promising combination to be used for phytostabilization concern.

Keywords: Mining soils, rhizobia, plant-growth promoting, leguminous plants.

1 Introduction

Mining activity has a millennial origin and helps to understand human history, as boundary stones of their eras, as stone age (Paleolithic), polished stone age (Neolithic), and metals age (Copper, Bronze and Iron).

Nowadays the global economic development is strongly supported by different mining activities, which are closely linked to social development, generating assets and wealth. On the other hand, mining exploitation, especially metal mining, produces huge environmental impacts (Baker *et al.* 1994; Salomons, 1995; Vangronsveld, Colpaert, Van Tichelen 1996; Dias-Júnior *et al.* 1998). Those areas often are devoid of natural means of biotic regeneration, requiring the aid of human intervention for the revegetation of the soils (Vangronsveld *et al.* 2009). A really promising technology for *in situ* land reclamation is phytoremediation (Baker *et al.* 1994), which is showing satisfactory results for either organic or inorganic contamination (Cunningham *et al.* 1997; Vangronsveld *et al.* 2009; Weyens *et al.* 2011; Weyens *et al.* 2013a,b). Phytoremediation success is linked to microbiota, which benefit plant growth by performing essential biological processes in the rhizosphere or inside the plants (Croes *et al.* 2013; Weyens *et al.* 2013a,b). Among those processes, biological N_2 fixation (BNF), which is performed by a limited group of prokaryotes able to convert N_2 into to NH_4, is very significant. An important prokaryote group able to perform BNF is represented by rhizobia, which establish mutualistic symbiosis with leguminous plants. Nodulated leguminous plants incorporate C and N into soil, which is besides increasing nutrient uptake capacity, also improving their tolerance to environmental stresses (Franco and Faria 1997; Franco *et al.* 2000; Franco and Balieiro 2000; Melloni *et al.* 2006; Moreira, Carvalho, Siqueira 2010; Moreira *et al.* 2010; Carvalho and Moreira 2010; Moreira *et*

al. 2015). Therefore, the main objectives of this study were to isolate and select rhizobia from nodules of leguminous plants growing on contaminated mining soils, with plant growth promoting traits, and to unravel the potential of these rhizobia for phytoremediation purposes.

2 Materials and Methods

2.1 Capturing N_2-fixing nodulating bacteria by the indirect method using a trap plant

N_2 fixing and nodulating soil bacterial strains were isolated from a Zn mining area, contaminated with Zn and Cd, by the indirect method using a trap plant [*Leucaena leucocephala* (Lam.) de Wit.]. *L. leucocephala* was chosen as a trap plant because it is widespread at this Zn mining area. Soil chemical and physical parameters (0-20 cm) from the Zn-mining area contaminated with Cd and Zn are presented in Table 1. A set of five mixed samples was used, it means that five samples were taken, of which each was composed of five sub-samples.

Pre-germinated seeds were inoculated with 1 mL of diluted soil suspension (10^{-1}) in 0.85% NaCl solution. Plants were grown for 70 days in a greenhouse and were supplied with modified Hoagland solution (Hoagland and Arnon 1950) with low mineral nitrogen content (5.25 mg N L^{-1}).

All cultivable rhizobia strains from nodules of *L. leucocephala* were isolated on 79 solid medium (Fred and Waksman 1928), which is known as Yeast extract Mannitol Agar (YMA) (Vincent, 1970). Strains were isolated and morphologically characterized according to Moreira *et al.* (1993), and Jesus *et al.* (2005).

2.2 Genotypic characterization

Strains were identified by partial sequencing of the 16S rRNA gene (Table 2). DNA was extracted using the kit protocol ZR Fungal/Bacterial DNA (Zymo Research Corp).

PCR was performed using 50 ng of 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') (Lane, 1991), 1 U Taq DNA polymerase (Fermentas), 10x KCl buffer, and ultrapure sterile water. The amplification reaction was performed using a Eppendorf Mastercycler® under the following conditions: an initial denaturation step at 94°C for 5 min, 40 denaturation cycles at 94°C for 40 s, an annealing step at 55°C for 40 s, an extension step at 72°C for 1.5 min, and a final extension at 72°C for 7 min. The obtained PCR products were purified and sequenced by Macrogen (South Korea).

The bionumerics 6.5 program (Applied Maths, Sint-Martens-Latem, Belgium) was used to check the quality of the sequences after which they were submitted to BLAST (Basic Local Alignment Search Tool) by comparing them with 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 KT694174 to KT694192.

2.3 Phenotypic characterization

Plant growth promoting (PGP) traits such as production of organic acids (OA) (Cunningham and Kuiack 1992), and indole-3-acetic-acid (IAA) (adapted from Patten and Glick 2002), ACC deaminase activity (ACC) (Belimov *et al.* 2005), siderophore production (SID) (Schwyn and Neilands 1987), and $Ca_3(PO_4)_2$ solubilization (Nautiyal, 1999) (Table 2) were used for screening all purified bacterial strains. Moreover, their Cd and Zn tolerance were verified as well (Table 3) (Weyens *et al.* 2013; Croes *et al.* 2013).

2.4 Authentication test

The authentication test, *i.e.* the ability to establish symbiosis with the trap host plant, and the symbiotic efficiency of the 19 nitrogen-fixing bacteria strains isolated from *L. leucocephala* nodules were verified performing a greenhouse experiment (Table 4).

Seeds were scarified using H_2SO_4 *pro analysis* for 30 min, and placed on sterile Petri dishes containing moistened cotton incubated at 28°C until radicle emergence. Strains were grown in 79 liquid medium shaking (125 rpm, 28°C) for 72 h. Seeds were inoculated at the moment of planting (1 mL bacterial inoculum 10^8 cells/seed). One plant was grown in sterile plastic tube. Sand and vermiculite (1:1 ratio) were used as substrate and a four-fold dilution of modified Hoagland nutrient solution (Hoagland and Arnon 1950). This Hoagland was composed by 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} KNO3; 2.0 mL of 246.9 g L^{-1} $MgSO_4$ $7H_2O$; 3.0 mL of 87.13 g L^{-1} K_2SO_4; 10 mL of 12.6 g L^{-1} $CaH_4P_2O_8$ H_2O; 200 mL of 1.72 g L^{-1} $CaSO_4$ $2H_2O$; 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} $MnSO_4$ $4H_2O$; 0.22 mg L^{-1} $ZnSO_4$ $7H_2O$; 0.08 mg L^{-1} $CuSO_4$ $5H_2O$, and 0.09 mg L^{-1} Na_2MoO_4 H_2O) stock solutions, which were added to 4 L of distilled water. Inoculated plants and non-inoculated control plants received a low nitrogen concentration (5.25 mg·L^{-1}) in the nutrient solution, which is considered a starting dose for, and not an inhibitor of, the process of biological N_2 fixation. In addition, a control treatment supplemented with a high mineral nitrogen concentration (52.5 mg L^{-1}) was provided as well. Besides the negative control treatments without inoculation (low and high N content), a positive control treatment inoculated with *Sinorhizobium fredii* (BR 827 strain) (Moreira *et al.* 1993), which has been approved as inoculant for *L. leucocephala* by the Brazilian Ministry of

Agriculture, was also added. The assay was completely randomized and performed using 4 replicates. Plants were harvested after 65 days. The following parameters were determined: nodule number and dry weight (NN and NDW), shoot dry weight (SDW), and relative efficiency (RE%), which is also known as the symbiotic efficiency. The RE% of each inoculated treatment was calculated in relation to the shoot dry matter production by the control treatment supplied with high mineral nitrogen content, using the formula RE = [(inoculated SDW / high N SDW) x 100] where RE means relative, inoculated SDW means shoot dry weight of the inoculated treatments, and high N SDW means shoot dry weight of the control treatment supplied with high mineral nitrogen content. The data were analyzed by ANOVA using the statistical program SISVAR (Ferreira, 2011). The NN and NDW were transformed using the formula $(x+0.5)^{0.5}$. The averages of the treatments were grouped by the Scott-Knott test at 5% significance.

2.5 Native rhizobia from Zn-mining soil promoting the growth of *L. leucocephala* on a contaminated soil

2.5.1 Soil sampling and pots preparation

In order to evaluate the symbiosis of *in vitro* screened rhizobia with *L. leucocephala* on a contaminated soil free of native rhizobia able to establish symbiosis with *L. leucocephala*, a contaminated soil (0-20 cm top layer) from a former agricultural field at 500 m northeast of a Zn-smelter in Lommel (Belgium), was sampled (June 2014). The soil has a sand texture according to the USDA triangle (Meers *et al.* 2010), a $pH_{(H_2O)}$ of about 6.56 and $pH_{(KCl)}$ of about 5.87, and C (in % humus) of 1.58%. Pseudo-total metal concentrations were estimated by aqua regia digestion and are in the range of 6.9 mg Cd kg^{-1} soil, 429 mg Zn kg^{-1} soil and 217 mg Pb kg^{-1} soil (Ruttens *et al.* 2011). Plant available metal fractions were estimated by determining the

fractions exchangeable by 0.01 M $CaCl_2$; they are in the range of 0.43 mg Cd kg^{-1} soil, 21.2 mg Zn kg^{-1} soil and 0.30 mg Pb kg^{-1} soil (Meers *et al.* 2010; Van Slycken *et al.* 2013).

Before preparing the pots, the soil was homogenized and sieved with 4 mm sieve opening. The soil was placed in 1 kg pots. Sterilized distilled water was used for irrigation up to about 60% of the field capacity.

2.5.2 Confirming that the Zn-smelter contaminated soil is free of native rhizobia able to establish symbiosis with *L. leucocephala*

In order to verify that the Lommel soil is free of native rhizobia able to establish symbiosis with *L. leucocephala*, a most probable number (MPN) experiment was performed. The soil inoculum was diluted (10^{-1} to 10^{-6}) using sterile sand and 0.5 kg pots were filled with the soil dilution mixture. A positive control for nodulation was provided by inoculating *Mesorhizobium plurifarium* (BR 3804 strain) (de Lajudie *et al.* 1998). Furthermore, two treatments both of them without inoculation, a negative control for nodulation supplied with low mineral nitrogen concentration, and a positive control for nitrogen utilization supplied with high mineral nitrogen concentration were included as well.

2.5.3 Exploiting *L. leucocephala*-rhizobia symbiosis on a contaminated soil

Mesorhizobium sp. (UFLA 01-765 strain) and *Rhizobium huautlense* (UFLA 01-775 strain) isolated from a Zn mining area in the southeast of Brazil, using *L. leucocephala* as a trap plant, were previously screened *in vitro* and selected based on their plant-growth promoting abilities (production of organic acids, indole-3-acetic-acid, siderophores and 1-aminocyclopropane-1-carboxylate deaminase, and $Ca_3(PO_4)_2$ solubilization), and Zn- and Cd-resistance. The UFLA 01-765 strain has shown positive results for all above-mentioned plant-

growth promoting traits, whereas UFLA 01-775 strain did not produce OA and IAA *in vitro* (Table 2).

Seeds of *L. leucocephala* were surface sterilized using 70% ethanol for 30 s, and 2% sodium hypochlorite for 3 min, after which the seeds were scarified using warm water (100°C) for 3 min. Surface-sterilized seeds were germinated on Petri dishes containing moistened sterile cotton in a growth chamber at 28°C for 4 days, or until the radicle emerged.

Besides the UFLA 01-765 and UFLA 01-775 strains, the BR 3804 strain (*Mesorhizobium plurifarium*) was also included in the experiment as a positive control for nodulation. Strains were grown in 79 liquid medium (Fred and Waksman 1928) under 120 rpm shaking at 28°C for 72 h. Each seed was inoculated with 1 mL of inoculum containing about 10^7 cells. Two germinated seeds were planted per pot.

A completely randomised design (CRD) with a 4 (inoculation treatments) x 2 (nitrogen content) factorial arrangement and 16 replicates was used, including three inoculated treatments (UFLA 01-765, UFLA 01-775 and BR 3804) and one control without bacterial inoculation, and two nitrogen levels [low (15 mg N dm^{-3}) "starter" N and high N (150 mg N dm^{-3})]. NH_4NO_3 was used as the nitrogen source, and the application of the high N content was divided over two times, the first application was done at the time of planting and the second 15 days after planting.

Plants were grown in a greenhouse for 90 days (June until September 2014). During this experimental period, the numbers of fully expanded leaves were counted at 30, 60 and 90 days after planting. Before harvest the height of the plants was measured.

During harvest, shoots were sampled for determining the activities of antioxidant enzymes, plants were cut at ground level, and the shoots and roots were separated. Nodules were detached from roots, counted and their dry weight was determined.

2.5.4 Activity of enzymes involved in anti-oxidative defence

To determine the activity of stress-related enzymes, shoot samples (8 replicates for each condition) were harvested and immediately snap-frozen in liquid nitrogen before storage at -80 °C. The frozen shoot tissues were macerated and homogenized with an ice-cooled mortar and pestle using ice-cooled 0.1M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1mM dithiothreitol and 4% insoluble polyvinylpyrrolidone (1 ml buffer per 100 mg fresh weight). This homogenate was centrifuged for 10 min at 20,000 g and 4 °C. Glutathione reductase (GR, EC 1.8.1.7) and guaiacol peroxidase (GPOD, EC 1.11.1.7) activities, as markers for oxidative stress (Vangronsveld and Clijsters 1994), were determined spectrophotometrically in the supernatant at 25 °C. GR was determined at 340 nm and GPOD at 436 nm both according to Bergmeyer, Gawenn, Grassl (1974).

2.5.5 Zn, Cd and Pb concentrations in soil and in plant samples

Zn, Cd and Pb concentrations were determined in soil, roots and shoots using 0.150 g samples of ground dry material, digested according to the microwave-assisted 3051A protocol of the US Environmental Protection Agency (USEPA, 2007).

During harvest, fresh shoot and root samples (at least 8 replicates for each condition) were washed with tap water and kept in 0.1 M HCl solution for 1 min (Tu and Ma 2003). After vigorously rinsing with distilled water, root and shoot samples were oven-dried (72 h at 65 °C) and subsequently powdered to a fine powder, and wet digested in Pyrex tubes in a heating block.

After harvesting the plants, the pseudo-total Zn, Cd and Pb concentrations in the soil were accessed by aqua regia digestion and ICP-OES analysis.

Metal remediation capacity of *L. leucocephala* was determined as the remediation factor (RF%), which represents the percentage of metal accumulation in plant dry mass regarding to the soil metal content and its volume (Vyslouzilova, Tlustos, Szakova 2003; Saraswat and Rai 2011). It was calculated by:

$$RF(\%) = \frac{HMplant \times DMplant}{HMsoil \times Wsoil} \times 100$$

with HMplant: the content of metals in the plant (mg kg^{-1} DM)

DMplant: the total plant dry mass (g),

HMsoil: the total metal content (mg kg^{-1}) in soil

Wsoil: the weight (g) of the soil.

2.5.6 Statistical analysis

The results were statistically analysed by an analysis of variance (ANOVA), and a Tukey statistical test at 5% probability was applied using the statistical program SISVAR 5.7 version (Ferreira, 2011). Before performing the statistical analysis, the variables nodule number (NN) and nodule dry matter (NDM) were transformed by $(x + 0,5)^{0,5}$ formula.

3 Results and discussion

All purified bacterial strains were able to induce nodule formation authenticated on *L. leucocephala*, and were genetically identified by partial sequencing of the 16S rDNA gene (Table 2).

3.1 Genotypic characterization

All 19 cultivable bacterial strains isolated from *L. leucocephala* nodules were genetically identified by 16S rDNA gene sequencing and blasting (Table 2). Most of the strains (17) belong to the group of *α*-Proteobacteria like *Mesorhizobium* sp. (15), *Rhizobium* sp. (1) and *Rhizobium huautlense* (1). Two *Variovorax paradoxus*, belonging to the *β*-Proteobacteria, strains were isolated. To our best knowledge, up to now neither the ability of *V. paradoxus* to induce nodule formation nor to perform N_2 fixation in symbiosis with leguminous plants have been reported. So we report for the first time the ability of *V. paradoxus* to induce nodule formation and also its ability to perform N_2 fixation in symbiosis with *L. leucocephala*.

3.2 Phenotypic characterization

All strains isolated from the Zn- and Cd-contaminated soil were screened for their potential to assist their host plant for phytoremediation purposes by verifying their plant growth promoting traits and their TE tolerance.

In general, the number of strains equipped with plant-growth promoting traits is remarkably high (Table 2). The total percentages of positive strains for all plant growth-promoting features are higher than 50%: OA (84%), IAA (52%), ACC deaminase (100%), SID (73%) and solubilizing P (89%). Phosphorus solubilization was estimated by a halo produced by the bacteria (Nautiyal, 1999). However, there are a few strains (11%), which did not form a visible solubilization halo, but their growth was observed, after 15 days incubation. The Nautiyal (1999) protocol considers the extracellular oxidation of glucose via quinoprotein glucose dehydrogenase, which produces gluconic acid and mobilizes insoluble phosphates very efficiently. However, other mechanisms apart from gluconic acid production and excretion, such

as production of chelating substances, release of protons originated by NH^{4+} assimilation and production of inorganic acids, have also been proposed to explain phosphate solubilization by bacteria (Illmer and Schinner 1995). Therefore, those strains need to be studied more in detail to unravel the mechanisms they utilize to mobilize insoluble-P.

Croes *et al.* (2013) have shown that the selective pressure occurring on a Cd, Zn and Pb-contaminated field in Belgium is in favor of a bacterial community able to solubilize phosphorus, fix nitrogen and produce ACC deaminase and IAA.

Plant growth-promoting rhizobacteria (PGPR) may synthesize and provide their host plant with compounds such as fixed nitrogen or phytohormones, as indole-3-acetic acid (IAA). Moreover, P and Fe uptake may be facilitated, and enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene levels, controlling plant growth, may be synthesized (Glick *et al.* 2007).

Iron might be found in very low concentration in plants growing on TE contaminated soils, due to iron deficiency on those soils. Plants are able to deal with this threat by producing siderophores, which bind iron, making plants capable to take up iron. Moreover, plants may also take up bacterial Fe-siderophore complexes. Since these bacterial siderophores are more efficient for binding iron than plant siderophores, plants depend on bacterial siderophores for their Fe uptake. Thus, unless bacterial siderophores are present in a TE contaminated soil, a plant is unable to accumulate a sufficient amount of iron (Glick, 2003).

Biological N_2 fixation is a highly Fe demanding process (Tang, Robson, Dilworth 1990; Brear, Day, Smith 2013) since Fe is present in the nitrogenase enzyme complex, cytochromes, ferredoxins, and hydrogenases. High numbers of siderophores producing bacteria (73% of the

total) were isolated from the Zn- and Cd-contaminated soil. About 90% of them are rhizobia. Tang *et al.* (1990) have shown a strict relation between Fe deficiency and depressed leghemoglobin production by *Lupinus angustifolius* inoculated with *Bradyrhizobium lupini* WU425, confirming Fe essentiality for nodule formation. Nodules of plants well provided with optimal Fe-concentrations show higher leghemoglobin concentration than iron deficient plants (Tang *et al.* 1990). However, there are a few rhizobia strains that do not produce siderophores: UFLA 01-766, UFLA 01-771, UFLA 01-772, UFLA 01-773, and UFLA 01-774 (*Mesorhizobium* strains) (Table 2). Rhizobia strains that do not produce siderophores may synthesize outer membrane (OM) receptors instead. OM specific receptors bind siderophores-metal complexes, enabling ions chelates uptake (Small *et al.* 2009). Rhizobia may recognize Fe^{3+}-xenosiderophore complexes by different OM receptors depending on the siderophores. As an example, *Bradyrhizobium japonicum* USDA 110 and 61A152 strains were able to use ferrichrome and rhodotorulic acid as iron source, even given the fact that these siderophores are produced by fungi (Plessner, Klapatch, Guerinot 1993).

Plant growth may also be improved by organic acids (OA) produced by bacteria. The high percentage (84%) of OA producing strains isolated from the Zn- and Cd-contaminated soil is remarkable. By producing OA under these conditions, bacteria can enhance their uptake of essential mineral nutrients, which are normally limiting in mining soils. However, besides improving the availability of essential mineral nutrients, OA production may also increase TE availability for the plant by means of the same mechanisms.

About 52% of the strains isolated from the Zn- and Cd-contaminated soil were able to produce IAA, which is another important bacterial process involved in plant growth promotion

on contaminated mining soils. IAA production can enhance root growth and root length, as well as proliferation and elongation of root hairs (Taghavi *et al.* 2009). In this way, plants can better deal with nutrient depletion through the bigger soil volume that can be explored by the more extended root system (Weyens *et al.* 2011). IAA production and other mechanisms performed by rhizospheric, phyllospheric and endophytic bacteria can improve phytoremediation efficiency (Valls and de Lorenzo 2002; Lebeau, Braud, Jézéquel 2008; Kidd *et al.* 2009; Mastretta *et al.* 2009; Rajkumar, Ae, Freitas 2009; Taghavi *et al.* 2009; Van der Lelie *et al.* 2009; Weyens *et al.* 2009a,b,c; 2010; 2011; 2013b; Becerra-Castro *et al.* 2011; 2012; Croes *et al.* 2013). IAA can also induce ACC synthase transcription. This enzyme catalyzes the formation of 1-aminocyclopropane-1-carboxilic acid (ACC), and consequently controls ethylene production, since ACC is its immediate precursor. Ethylene present in a low concentration may improve plant growth, but at higher levels, ethylene inhibits growth and can even induce senescence. Therefore, it is important that plant-associated bacteria are able to regulate ACC and ethylene levels by producing ACC deaminase (Stearns and Glick 2003; Glick *et al.* 2007; Glick and Stearns 2011). The high number (100%) of strains isolated from the Zn- and Cd-contaminated mining soil that tested positive for ACC deaminase activity (Table 2) is remarkable. Croes *et al.* (2013) and Truyens *et al.* (2014) also found that TE contamination pressures the bacterial comunity selecting genotypes, which test positive for plant growth promoting traits such as TE tolerance, phosphorus solubilization, nitrogen fixation and ACC deaminase and IAA production.

Considering the rhizobial behavior in general, we isolated different strains from different rhizobia genera with high amounts of positive results for most of all plant-growth promoting features (Table 2). This rhizobia behavior makes us enthusiastic to continue rhizobia research in

the framework of phytoremediation. This intrinsic plant-growth promoting ability of rhizobia increases the number of legume plants that can be considered for phytoremediation purposes in different conditions of soil contamination (*e.g*. mining, smelting etc.).

Since bacteria need to survive on TE-contaminated soils before they might be able to perform any of the above-mentioned ecosystem services (*e.g.* biological nitrogen fixation, phosphate solubilization, metal precipitation *etc.*), the rhizobacteria isolated from the Zn- and Cd-contaminated mining soil were *in vitro* evaluated for their Zn- and Cd-resistance. Moreover, it is important to know the multi-element resistance, since the bacterial strains we have investigated in this work came directly from mining soils (meaning they were exposed for many generations to a toxic environment and were forced to get shifts to survive under this selective pressure) and almost all mining soils may have a multi-element pollution.

The numbers of Zn- and Cd-resistant rhizobia strains are high (Table 3). However, differences concerning their growth were observed. Almost all rhizobial strains were resistant at high Zn and Cd concentrations, with the exception of only *Rhizobium huautlense* strain UFLA 01-775. Interestingly, almost all strains showed the same growth pattern under both toxic stresses (Zn and Cd), with the exception of *Variovorax paradoxus* strains UFLA 01-763 and UFLA 01-764, which showed an abundant growth and polysaccharide production on plate at both low and high Cd exposure.

Toxic concentrations of metal(oid)s and their different chemical forms and organometals notably threat microbial populations, affecting the microbial activity (Carneiro *et al.* 2008; Giller, Witter, McGrath 2009; Santos *et al.* 2013). However microbial resistance to metal(oid)s is widespread and ranges from low percentages in pristine environments to higher percentages in

heavily polluted environments (Trannin *et al.* 2001; Silver and Phung 2009; Croes *et al.* 2013). As we hypothesized, rhizobia isolated from Zn- and Cd-contaminated soil are highly Zn- and Cd-resistant, 100% strains resisted at low and 95% at high Zn- and Cd-concentrations. The only exception is *Rhizobium huautlense* strain UFLA 01-775, which is not resistant to high [Zn] and [Cd].

3.3 Authentication test

Concerning the authentication of the symbiosis and symbiotic efficiency of the strains isolated from *Leucaena leucocephala* nodules (Zn- and Cd-contaminated soil), no nodules were observed on the root system of non-inoculated control plants (supplied with 5.25 mg L^{-1} or 52.5 mg L^{-1} of mineral N) (Table 4). This confirms the absence of contamination and means that the experiment was performed under axenic conditions.

All bacterial strains, isolated from *L. leucocephala* as well as the inoculant control *Sinorhizobium fredii* strain BR 827 approved by MAPA nodulated the root system (Table 4). In total, eight strains are efficiently fixing N_2 in symbiosis with *L. leucocephala*, showing SDW and RE% values that are statistically similar to the control treatment supplied with high mineral N concentration (52.5 mg L^{-1}). Among those strains, five were *Mesorhizobium* spp. (Strains UFLA 01-761, UFLA 01-765, UFLA 01-768, UFLA 01-770, UFLA 01-772), two were *Variovorax paradoxus* (UFLA 01-763 and UFLA 01-764), and one was a *Rhizobium huautlense* (UFLA 01-775 strain). All those strains showed higher N fixation efficiency even comparing with *S. fredii* strain BR 827 approved by MAPA as *L. leucocephala* plant inoculant.

As mentioned above, there exist no earlier reports on the ability of *V. paradoxus* to nodulate and perform N_2 fixation in symbiosis with leguminous plants. However, the high

capacity of *V. paradoxus* to promote primary root elongation of spring wheat seedlings was already reported by Maimaiti *et al.* (2007). These authors have demonstrated that *V. paradoxus* promotes plant growth by producing ACC-deaminase and oxidizing H_2. The diversity in metabolic abilities of *V. paradoxus* is remarkable high (Davis, Doudoroff, Stanier 1969; Willems *et al.* 1991; Maimaiti *et al.* 2007).

As mentioned above, rhizobia and legume-rhizobia symbiosis can directly enhance phytoremediation success through biological nitrogen fixation and other plant growth promoting traits. These metal-resistant, plant growth promoting bacteria can play an essential role in phytoremediation of contaminated mining soils. Once the most appropriate legume-rhizobia symbiosis will be identified, they can be exploited. Moreover, most rhizobia are not pathogenic, which is another crucial point for exploiting the potential of those bacteria to improve plant growth in the framework of phytoremediation purposes

3.4 Native rhizobia from Zn-mining soil promoting the growth of *L. leucocephala* on a contaminated soil

No nodules were observed on the roots of *L. leucocephala* plants, which were inoculated with soil inoculum from Lommel site. Only control plants for nodulation feature, which were inoculated with BR 3804 strain showed nodules on their roots. This confirms that there are no rhizobia able to induce nodule formation on roots and to establish symbiosis with *L. leucocephala* in the Lommel soil (Figure S1). By consequence, the rhizobia strains that were inoculated in experiment 2 (see further) did not have to compete with native rhizobia for inducing nodule formation and establishing symbiosis with *L. leucocephala* growing on contaminated soil.

3.4.1 Exploiting *L. leucocephala*-rhizobia symbiosis on a contaminated soil

The numbers of fully expanded leaves on *L. leucocephala* at 30, 60 and 90 days after planting are shown in Figure 1.

Rhizobia and legume-rhizobia symbiosis have the potential to enhance phytoremediation success through biological N_2 fixation and other plant growth promoting traits. The effects depend on bacterial species and their origin, on legume species and on metal(oids) as well (Trannin, Moreira, Siqueira 2001; Matsuda, Moreira, Siqueira 2002a,b; Chaudhary, Dudeja, Kapoor 2004; Melloni *et al.* 2006; Ferreira *et al.* 2012; 2013; Rangel *et al.* 2014). Leguminous plants growing on soils contaminated with excessive trace elements concentrations usually show chlorosis, tissue damage, root browning and growth inhibition, further also affecting photosynthesis and symbiosis (Wani, Khan, Zaidi 2007; 2008).

Mesorhizobium sp. (UFLA 01-765) was able to induce nodule formation on roots, establish symbiosis and efficiently fix N_2, thereby promoting *L. leucocephala* growth on Zn- and Cd-contaminated soil. This symbiotic rhizobial strain increased the number of fully expanded leaves, plant height, and shoot N accumulation (Figure 1, 2A and 5C respectively). Interestingly it also decreased the activities of several enzymes involved in antioxidative defence (Figure 7). Figure 2C shows the remarkable low shoot dry weight loss by *L. leucocephala* after 90 days experiment. In average, *L. leucocephala* lost 50 mg of shoot per pot under high N content (150 mg N dm^{-3}) and 10 mg of shoot per pot under low N content (15 mg N dm^{-3}) during the 90 days experiment. This highlights the importance of inoculating *L. leucocephala* with rhizobia instead of supplying plants with N mineral fertilizers for phytoremediation purposes. High N mineral content increases plant growth and development very fast, by consequence increasing the loss of

shoot-associated metals. Figure S2 C-D highlight the contrast between plants inoculated under high N content (150 mg N dm^{-3}) and low N content (15 mg N dm^{-3}) showing that plants receiving N from an established symbiosis with rhizobia use N more efficiently, maintaining an intense green colour for a longer period than other plants (Figure S2 C-UFLA 01-765).

Biological nitrogen fixation is one of the most important processes in nature and it is performed by only a limited group of prokaryotic organisms. Rhizobia are a unique subset of this group, which can fix N_2 in symbiosis with legume plants. Therefore, the symbiosis between both legume plant and rhizobia tolerant to trace elements is really important for supplying plants with N when they are growing on contaminated soils. Since excessive amounts of trace elements are known to be toxic to most organisms and challenge their survival on contaminated soils, it is crucial that this N fixation and symbiosis can still be performed under trace elements stress. A picture of a nodule induced by *Mesorhizobium* sp. on *L. leucocephala* roots is presented in figure 3D. The intense red color inside the nodule is due to leghaemoglobin, which attests its activity. This illustrated that the UFLA 01-765 strain is able to efficiently fix N_2, even on contaminated soil, which was confirmed by shoot N accumulation presented in figure 3C. UFLA 01-765 strain increased the N accumulation of *L. leucocephala* with about 257% (2.5 times) in comparison to control plants that did not receive any rhizobia inoculum and were supplied with low N level. When control plants received high N levels, N accumulation increased about 218%.

Afer inoculation with UFLA 01-765 strain, means of 30 nodules on high N and 15 nodules on low N level were observed. Although plants on low N (starter N) have formed less root nodules, they were bigger than nodules from plants on high N, as it can be seen by nodule dry weight (figure 3B). The high N-mineral supply for soils with 150 mg N dm^{-3} content,

increased N-uptake by plants, by consequence decreasing the efficiency of BNF processes. Plants under high N content received the same amount of N at planting than plants under low N treatement. However, after 15 days, plants under high N treatment received an additional N application. So both, high and low N plants, started nodule formation under the same conditions. However, after 15 days, when the high N plants received an additional N application, these plants were exposed to a high N-mineral availability in soil (mostly as NO_3^-), disrupting nodulation processes, and by consequence BNF processes as well. It is widely accepted that high soil NO_3^- inhibits root infection, nodule development and nitrogenase activity (Atkins *et al.* 1984; Imsande, 1986; Eaglesham, 1989; Purcell and Sinclair 1990; Abdel-Wahab *et al.* 1996; Sanginga *et al.* 1996; Sodek and Silva 1996; Arreseigor *et al.* 1997). Sodek and Silva (1996) showed that NO_3^- inhibits both the establishment of nodules and their growth and development. Our results are in agreement with this observation. Since plants under low N content showed less nodules, but a similar nodule dry weight in comparison with plants under high N content. Many hypotheses have been proposed for answering the question on how NO_3^- affects nodule growth, *i.e.* carbohydrate privation in nodules, feedback inhibition by glutamine or asparagine products of nitrate metabolism, and a decreased O_2 diffusion into nodules which put a limit on respiration of bacteroids (Schuller, Minchin, Gresshoff 1988; Streeter, 1988; Vessey, Walsh, Layzell 1988; Vessey and Waterer 1992; Neo and Layzell 1997; Bacanambo and Harper 1996; Gordon *et al.* 2002). Kanayama and Yamamoto (1990) proposed that the formation of nitrosylleghemoglobin caused by NO binding, a product from NO_3^-, to the leghemoglobin disrupts O_2 binding activity.

Although the *Rhizobium huautlense* UFLA 01-775 strain induced nodule formation and showed a high N_2 fixing efficiency with *L. leucocephala* during the authentication and symbiotic

efficiency assay (Table 4), no nodules were induced by on *L. leucocephala* roots growing in the Zn-, Pb- and Cd-contaminated soil from Lommel (Figure 3A). The lack of nodulation in this experimental set-up might be due to the *in vitro* sensitivity that the UFLA 01-775 strain showed to high Zn and Cd concentrations (Table 3).

Figure 4 presents soil pH-H_2O and available metal content after 90 days, at the moment of plant harvesting. Soil pH was equally reduced by applying both high (150 mg N dm^{-3}) and low "starter" N level as NH_4NO_3 source, in comparison with the pH of the control soil, which did not receive any NH_4NO_3 content. Figure 4 shows that the available Cd, Pb and Zn contents in the soil are mainly related to soil pH. The decrease in soil pH could be induced by acidification caused by NH_4NO_3 application. Each two NH_4^+ molecules produce four H^+ by the reaction '$2NH_4^+ + 3O_2^- \rightarrow 2NO_2^- + 2H_2O + 4H^{+}$'. Moreover, H^+ extrusion by plants may also occur since assimilation of NH_4^+ stimulates H^+-ATPases to pump protons (maily H^+) out of cells, decreasing (extracellular) soil pH (Hedrich and Schoeder 1989). Moreover cations exchange (Cd, Zn and Pb) by NH_4^+ is another way to deacrease pH. This last mechanism can be confirmed by soil available Cd, Zn and Pb content after harvesting plants. The addition of high amounts of NH_4NO_3 (150 mg N dm^{-3}) to soil increased the available metals contents (Figure 4B-C-D). On the other hand, in case of low NH_4NO_3 content (15 mg N dm^{-3}), the amount of NH_4^+ in soil was not enough for exchanging cations. Moreover NO_3^- uptake occurs against an electrochemical gradient, which requires energy spending (Williams and Miller 2001), and the optimal pH for NO_3^- uptaking is below 6 due the higher H^+ availability for cotransport (Epstein and Bloom 2005). Through cotransport, both NO_3^- and H^+ are absorbed by the same transporter, and by consequence an increase of pH can be expected.

Apart from these above-mentioned processes affecting soil acidification, organic acids production by bacteria may also acidify soil. Organic acids production is a mechanism of reducing metal toxicity and increasing metal uptake by plants, since metals chelated by organic acids are less toxic as when they are in the non-chelated form.

Interestingly, on low N, all rhizobia strains increased soil pH, in comparison with the non-inoculated treatment. *Mesorhizobium* sp. UFLA 01-765 strain increased pH about 0.3 units, and caused a remarkable decrease in available Cd and Zn (Figure 4A-B and 4D). This potential of *Mesorhizobium* sp. UFLA 01-765 strain to reduce soil available Cd and Zn content might be explained by OA production (Glick, 2003; Bais *et al.* 2006, Rajkumar *et al.* 2013). Chelated metals might be taken up by plants, which can be confirmed by the RF of *Mesorhizbobium* sp. UFLA 01-765 (Figure 6).

The metal accumulation was influenced by the N level showing maximum values in plants grown on high N (150 mg N dm^{-3}) due to acidification caused by NH_4NO_3 supply, as already explained above. In general, Zn concentration was higher than Pb and Cd, and the accumulation of all metals was higher in roots than in shoots (Figure 5). This behavior of *L. leucocephala* was already reported by Saraswat and Rai (2011) and Ferreira *et al.* (2012).

In general, metal accumulation in the shoots on low N did not differ among treatments (Figure 5), whereas control plants without rhizobia and plants inoculated with *Mesorhizobium* sp. UFLA 01-765 strain on high N accumulated higher metal in shoots.

Considering the remediation factor (RF) in case of low N, after inoculation with *Mesorhizobium* sp. UFLA 01-765 strain, significantly higher remediation factors could be achieved for all metals (Figure 6). Besides promoting plant growth through biological N_2 fixation

in symbiosis with *L. leucocephala*, *Mesorhizobium* sp. UFLA 01-765 strain may also colonize the rhizosphere where it is performing other plant-growth promoting traits as well, *e.g.* OA production, which can improve metal uptake by *L. leucocephala* (Figure 6). Interestingly, at the same time inoculation with the UFLA 01-765 strain under low N application induced decreases in both guaiacol peroxidase (GPOD) and glutathione reductase (GR) activities (Figure 7) and showed a better growth (Figures 3A-B and 4) and N accumulation (Figure 3C). As mentioned by Rajkumar *et al.* (2013), OA chelating metals are a way to increase antioxidative defense mechanisms by plants, decreasing the oxidative damage caused by metals.

The reductions in the activities of antioxidative enzymes suggests that homo-phytochelatins, which are peptides homologous to phytochelatins but contain *β*-alanine instead of glycine (γ-Glu-Cys)$_n$-*β*-Ala (n=2-7), may take part in the antioxidative defense (Grill *et al.* 1986; Clemente *et al.* 2012).

Saraswat and Rai (2011) have shown the potential of *L. leucocephala* for a substantial sequestration of Zn and Cd from brass industry contaminated soils, besides their beneficial soil microbial and chemical characteristics including N content. In our study, *L. leucocephala* showed higher RF for Cd than Zn.

Our observations concerning the tolerance of the symbiosis between legume species and rhizobia on multi-contaminated soils are in agreement with Mahieu *et al.* (2011), Saraswat and Rai (2011), and Ferreira *et al.* (2012). Legume species in symbiosis with native rhizobia on metal contaminated soils may facilitate the colonisation by other plant species on mine soils (Frérot *et al.* 2006), which generally contain low organic matter contents. The metal tolerant and functional symbiosis can promote the development of a vegetation cover and stabilize metal-enriched soils.

4 Conclusions

In this study the high potential of PGP native rhizobia from metal contaminated mining soils in function of phytoremediation purposes was demonstrated by means of their N_2 fixation and other plant growth promoting traits. Our results are in agreement with our hypothesis that contaminated mining soils harbour rhizobia that are well adapted to these harsh conditions, and those native rhizobia have potential to be used for phytoremediation of those contaminated sites. Moreover, a tolerant and functional symbiosis, as *L. leucocephala* with *Mesorhizobium* sp. UFLA 01-765, may promote the development of a vegetation cover and stabilize metal-enriched soils.

Acknowledgments

The authors are very grateful to André Janssen and Jorik Janssen for their helpful assistance on soil sampling in Lommel, to Ariadna S. Lopez, Iva Cholakova, Hendrik Fourier and Ms Carine Put for helpful assistance on harvesting, to Ms Ann Wijgaerts and Ms Carine Put for their kindly support with enzymatic analysis, and Ann Sofie Stevens for helping with the pictures of the nodules. This research was supported by the National Council for the Scientific and Technological Development (CNPq), the Commission for Improvement of Higher Education Staff (CAPES) and the Foundation for Research of the State of Minas Gerais (FAPEMIG). W.M. Rangel thanks CAPES for the Doctoral training sandwich abroad (BEX: 13079/2013-01). F.M.S. Moreira thanks CNPq for the research productivity fellowship and grant. We also thank CNPq, FAPEMIG and CAPES for students' fellowship. This work also has been financially supported by the Hasselt University Methusalem project 08M03VGRJ. We gratefully acknowledge partial

funding collaboration from Rede de Pesquisas em Áreas Afetadas por Mineração (RECUPERAMINA) through its coordinator Luiz R. G. Guilherme.

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Table 1 – Physico-chemical parameters (0-20 cm) of the Zn- and Cd-contaminated mining soil.

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]**	**Cd**	**Zn**	**Cd**	**Zn**
									Mehlich[6]		USEPA[7]	
	mg L^{-1}	_mg dm^{-3}_		_____$cmol_c$ dm^{-3}_____				dag kg^{-1}	____mg kg^{-1}____			
5.9	5.9	3.5	14.8	0.9	0.7	0.4	2.2	0.0	0.53	144	1.6	530
Soil physical size group and texture[8]												
Sand		**Silt**		**Clay**		**Soil texture**						
_____g kg^{-1}_____												
14.5		**29.5**		**56**		**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 (EMBRAPA, 2011); Cadmium and Zinc available[6] and semitotal[7] content. [8]Soil physical and texture (Day, 1965).

Table 2 - Original host legume species, most similar sequence (accession number) available in NCBI and qualitative plant growth promoting traits of the strains isolated from Zn- and Cd-contaminated mining soil.

Host species	**Strains**	**bp[*] of 16S rDNA**	**Identity**	**Most similar sequence (accession number)[#]**	**Phylum/class**	**Phenotypical tests**				
						OA[**]	**IAA[**]**	**ACC[**]**	**SID[**]**	**$Ca_3(PO_4)_2$ Sol[***]**
Leucaena leucocephala	UFLA 01-761	550	95%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	++	+	+	++++	L
Leucaena leucocephala	UFLA 01-762	959	100%	*Mesorhizobium* sp. (EU130444.1)	*α-Proteobacteria*	+	-	+	++++	I
Leucaena leucocephala	UFLA 01-763	925	99%	*Variovorax paradoxus* (HQ219937.1)	*β-Proteobacteria*	-	-	+	+++	I
Leucaena leucocephala	UFLA 01-764	910	99%	*Variovorax paradoxus* (HQ219937.1)	*β-Proteobacteria*	-	-	+	+++	I
Leucaena leucocephala	UFLA 01-765	747	99%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	+	+	+	++	I
Leucaena leucocephala	UFLA 01-766	725	99%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	+++	-	+	-	L
Leucaena leucocephala	UFLA 01-767	1101	100%	*Mesorhizobium* sp. (EU130444.1)	*α-Proteobacteria*	+++	+	+	++	L
Leucaena leucocephala	UFLA 01-768	446	98%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	++	++++	++++++	++++	L
Leucaena leucocephala	UFLA 01-769	1045	100%	*Rhizobium* sp. (HQ589024.1)	*α-Proteobacteria*	++	++++	+++	++++	L
Leucaena leucocephala	UFLA 01-770	666	100%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	++	++++	+	++++	L
Leucaena leucocephala	UFLA 01-771	908	100%	*Mesorhizobium* sp. (EU130444.1)	*α-Proteobacteria*	+++	-	+	-	L
Leucaena leucocephala	UFLA 01-772	738	99%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	+++	-	+	-	L
Leucaena leucocephala	UFLA 01-773	792	99%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	+++	+	+	-	I
Leucaena leucocephala	UFLA 01-774	1048	99%	*Mesorhizobium* sp. (EU130444.1)	*α-Proteobacteria*	++	-	+	-	GNFH[§]
Leucaena leucocephala	UFLA 01-775	596	98%	*Rhizobium huautlense* (JQ670240.2)	*α-Proteobacteria*	-	-	+	+	GNFH
Leucaena	UFLA	415	95%	*Mesorhizobium* sp.	*α-*	+	-	+	++	I

leucocephala	01-776			(HF931067.1)	*Proteobacteria*					
Leucaena leucocephala	UFLA 01-777	1094	99%	*Mesorhizobium* sp. (EU130444.1)	*α-Proteobacteria*	+	+	+	++++	L
Leucaena leucocephala	UFLA 01-778	1094	99%	*Mesorhizobium* sp. (EU130444.1)	*α-Proteobacteria*	++	+	+	++++	L
Leucaena leucocephala	UFLA 01-779	742	99%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	++	+	+++	++++	L
				Percentage of positive strains		52%	52%	85%	71%	89%
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 cepacia*						+++	++	+	+	GNFH

[*]bp – base pairs of 16S rDNA sequence. [#]Identification based on 16S rDNA sequences using forward primer 27F. [**]Classification conferred according to the color intensity. OA: organic acid production; IAA: indole-3-acetic acid production; ACC: 1-aminocyclopropane-1-carboxylate deaminase activity. [***]Based on the $Ca_3(PO_4)_2$ solubilisation index, the strains were classified as Low (L) with solubilisation 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 – Cadmiun and zinc tolerance in 284 medium of rhizobia isolated from Zn-mining soil using *Leucaena leucocephala* as trap plant.

		Cadmiun		Zinc	
Strains	**Closest related strain by NCBI**	**0.4 mM**	**0.8 mM**	**0.6 mM**	**1.0 mM**
UFLA 01-761	*Mesorhizobium* sp.	+++Moderate	++Low	+++Moderate	++Low
UFLA 01-762	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-763	*Variovorax paradoxus*	+++Moderate	++Low	++++Abundant	++++Abundant
UFLA 01-764	*Variovorax paradoxus*	+++Moderate	++Low	++++Abundant	++++Abundant
UFLA 01-765	*Mesorhizobium* sp.	+++Moderate	++Low	+++Moderate	++Low
UFLA 01-766	*Mesorhizobium* sp.	+++Moderate	++Low	+++Moderate	++Low
UFLA 01-767	*Mesorhizobium* sp.	+++Moderate	++Low	+++Moderate	++Low
UFLA 01-768	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-769	*Rhizobium* sp.	++++Abundant	++Low	++++Abundant	++Low
UFLA 01-770	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-771	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-772	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-773	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-774	*Mesorhizobium*sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-775	*Rhizobium huautlense*	++Low	-No growth	++Low	-No growth
UFLA 01-776	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-777	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-778	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
UFLA 01-779	*Mesorhizobium* sp.	++Low	+Scarce	++Low	+Scarce
Type or Reference rhizobia strains					
CIAT 899^{T} – *Rhizobium tropici*		++Low	+Scarce	++Low	+Scarce
BR 3804 – *Mesorhizobium plurifarium*		++Low	+Scarce	++Low	+Scarce
ORS 571^{T} – *Azorhizobium caulinodans*		++Low	+Scarce	++Low	+Scarce
BR 5401^{T} – *Azorhizobium doebereinerae*		++Low	+Scarce	++Low	+Scarce
BR 11340 – *Burkholderia* sp.		+Scarce	+Scarce	+Scarce	+Scarce

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

Table 4 – Authentication and symbiotic efficiency of rhizobacteria isolated from nodules of *Leucaena leucocephala* used as a trap plant to access native rhizobia on Zn, Cd-contaminated soil.

Strains	Closest related strain by NCBI	NN	NDW	SDW	Relative efficiency
			---------g/pot---------		
UFLA 01-761	*Mesorhizobium* sp.	37.00 a	0.04 a	0.52 a	89.4 a
UFLA 01-762	*Mesorhizobium* sp.	17.75 b	0.03 b	0.44 b	76.1 b
UFLA 01-763	*Variovorax paradoxus*	29.00 a	0.04 a	0.54 a	93.1 a
UFLA 01-764	*Variovorax paradoxus*	23.50b	0.05 a	0.58 a	100.6 a
UFLA 01-765	*Mesorhizobium* sp.	21.25 b	0.04 a	0.66 a	111.9 a
UFLA 01-766	*Mesorhizobium* sp.	32.75 a	0.04 a	0.47 b	81.5 b
UFLA 01-767	*Mesorhizobium* sp.	26.25 a	0.04 a	0.48 b	83.8 a
UFLA 01-768	*Mesorhizobium* sp.	28.25 a	0.04 a	0.50 a	87.5 a
UFLA 01-769	*Rhizobium* sp.	26.00 a	0.04 a	0.46 b	77.7 b
UFLA 01-770	*Mesorhizobium* sp.	39.50 a	0.04 a	0.56 a	96.1 a
UFLA 01-771	*Mesorhizobium* sp.	33.25 a	0.03 b	0.44 b	75.3 b
UFLA 01-772	*Mesorhizobium* sp.	33.25 a	0.04 a	0.51 a	88.7 a
UFLA 01-773	*Mesorhizobium* sp.	19.25 b	0.007 d	0.39 c	67.2 b
UFLA 01-774	*Mesorhizobium*sp.	19.00 b	0.005 d	0.37 c	63.6 b
UFLA 01-775	*Rhizobium huautlense*	33.50 a	0.05 a	0.55 a	92.8 a
UFLA 01-776	*Mesorhizobium* sp.	22.75 b	0.02 c	0.37 c	63.7 b
UFLA 01-777	*Mesorhizobium* sp.	3.00 c	0.03 b	0.46 b	78.8 b
UFLA 01-778	*Mesorhizobium* sp.	28.75 a	0.01 c	0.46 b	72.9 b
UFLA 01-779	*Mesorhizobium* sp.	28.50 a	0.03 b	0.44 b	75.7 b
BR 827 – *Sinorhizobium fredii*		19.25 b	0.02 c	0.42 b	63.4 b
5.25 mg N L^{-1}		0.00 c	0.00 d	0.26 d	46.2 b
52.5 mg N L^{-1}		0.00 c	0.00 d	0.60 a	100.0 a
CV (%)		18.26	0.72	13.49	22.69

Values followed by the same letter on the column comparing strains do not differ by Scott-Knott test, p<0,05.

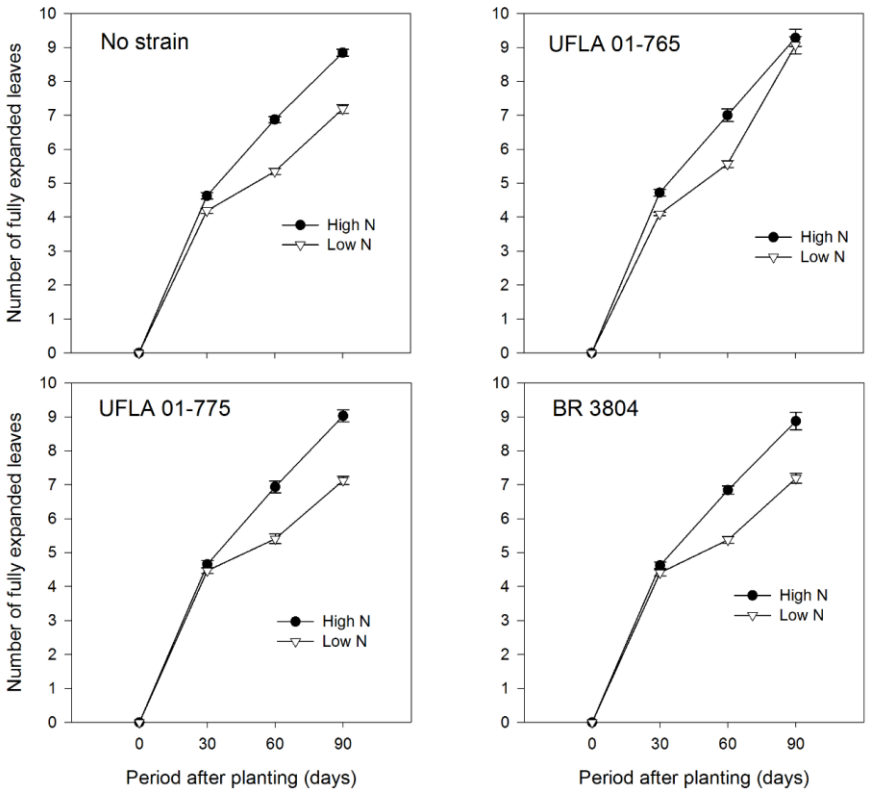

Figure 1. Increasing of the number of fully expanded leaves during the experimental period. $p<0.05$.

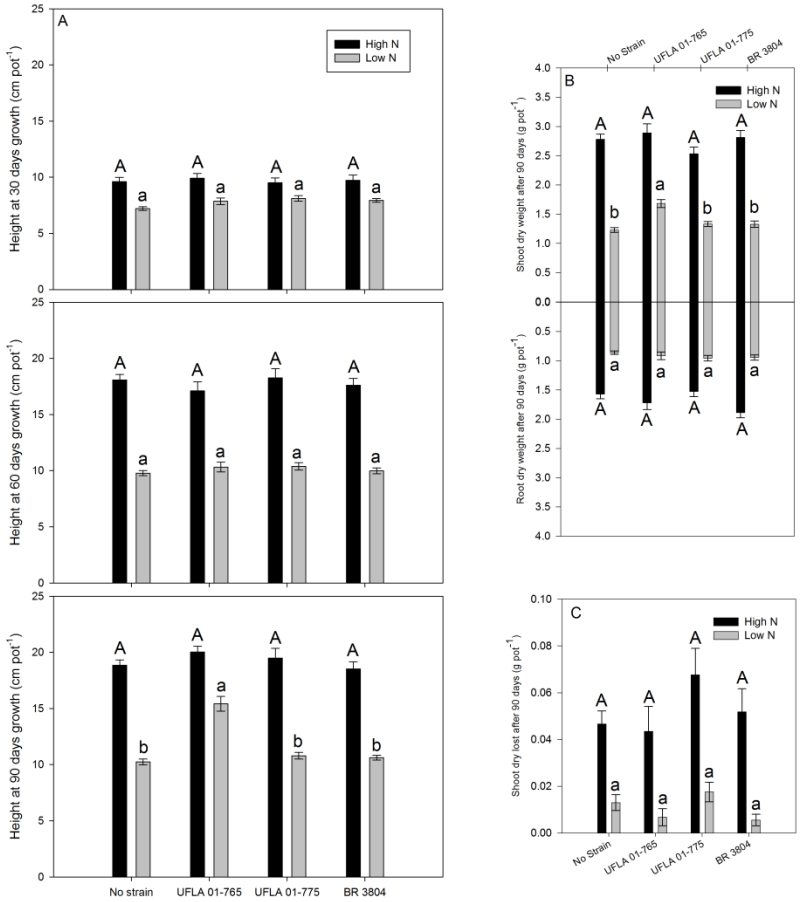

Figure 2. ***Mesorhizobium*** **sp. (UFLA 01-765) isolated from Zn-mining soil contaminated with Zn and Cd is able to promote** ***Leucaena leucocephala*** **growth on Zn-smelting soil contaminated with Zn, Pb and Cd. A- Height. B- Dry biomass production (dry shoot and root weight). C- Dry shoot biomass lost after 90 days pot-experiment. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.**

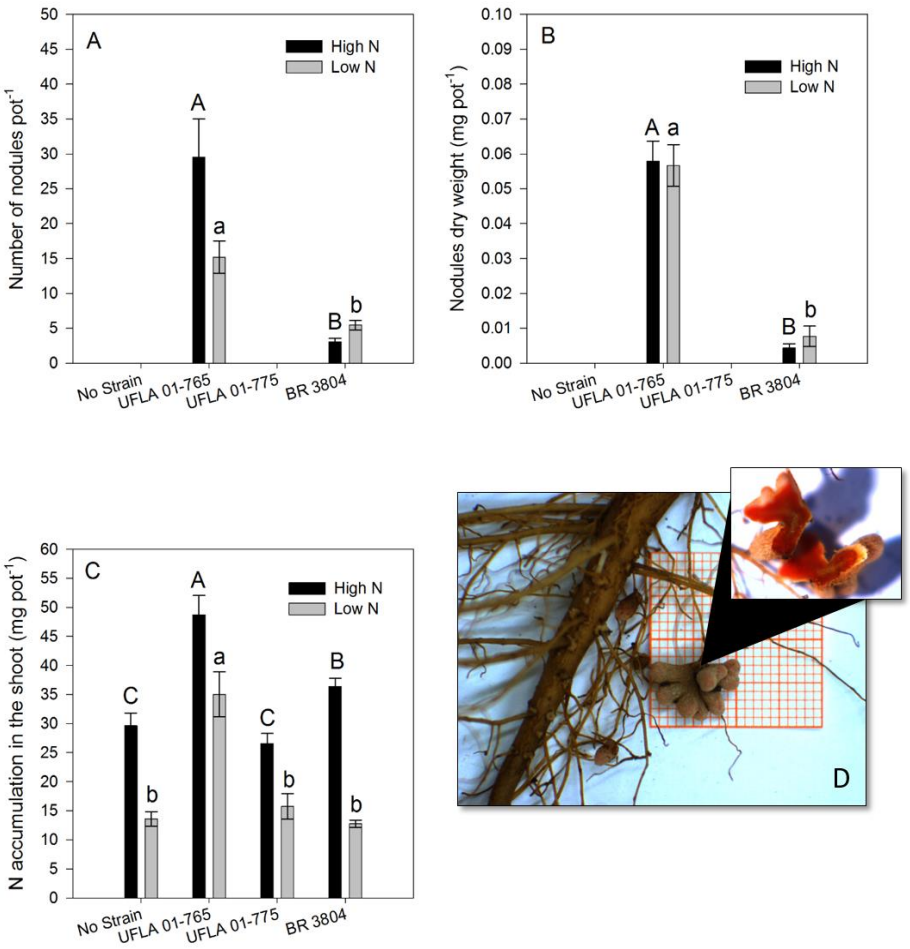

Figure 3. ***Mesorhizobium*** **sp. (UFLA 01-765) isolated from Zn-mining soil contaminated with Zn and Cd is able to efficiently fix N_2 in symbiosis with** ***Leucaena leucocephala*** **on Zn-smelting soil contaminated with Zn, Pb and Cd. A- Number of nodules. B- Nodules dry weight. C- N accumulation in shoot after 90 days experiment. D-** ***L. leucocephala*** **nodule induced by UFLA 01-765 under low N content (15 mg N dm^{-3}) highlighting its red color conferred by leghaemoglobin, which attests N_2 fixing active into nodule by this rhizobia strain. Bars with the same upper or lower case letters do not differ by Scott-Knott test, $p<0.05$.**

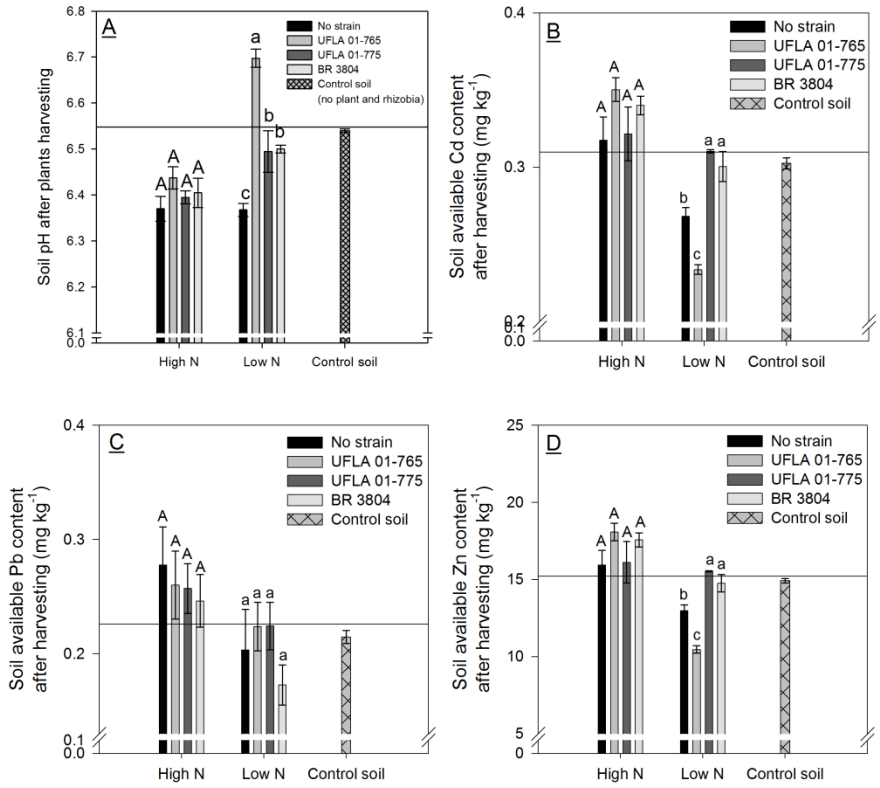

Figure 4. Soil pH-H_2O and available metal content after plant harvesting at 90 days and pH and metal content of the control soil without plant, rhizobia and NH_4NO_3. A- Soil pH-H_2O after plant harvesting. B- Cd available, C- Pb available and D- Zn available after plant harvesting. Bars with the same upper or lower case letters do not differ by Scott-Knott test, $p<0.05$.

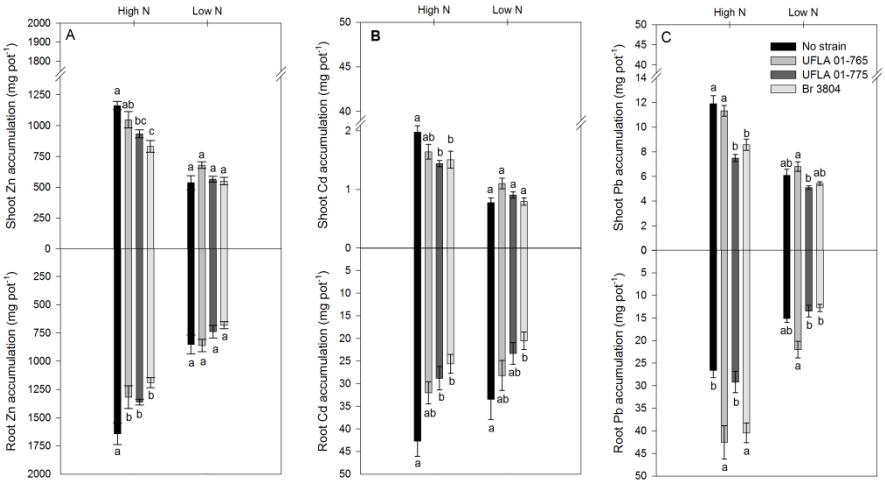

Figure 5. Zn, Cd and Pb accumulation by *Leucaena leucocephala* on a smelting Zn-, Pb- and Cd-contaminated soil after 90 days pot-experiment. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.

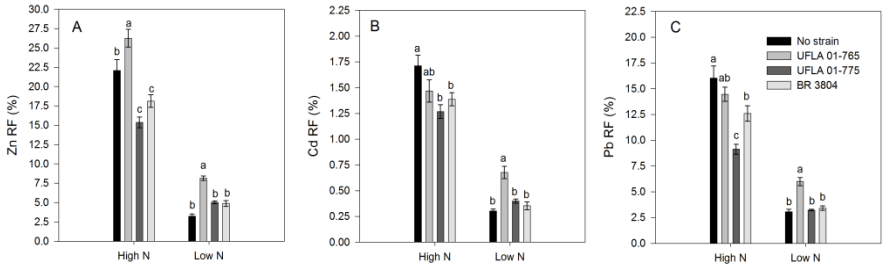

Figure 6. Zn, Cd and Pb remediation factor (RF) by *Leucaena leucocephala* on a smelting Zn-, Pb- and Cd-contaminated soil after 90 days pot-experiment. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.

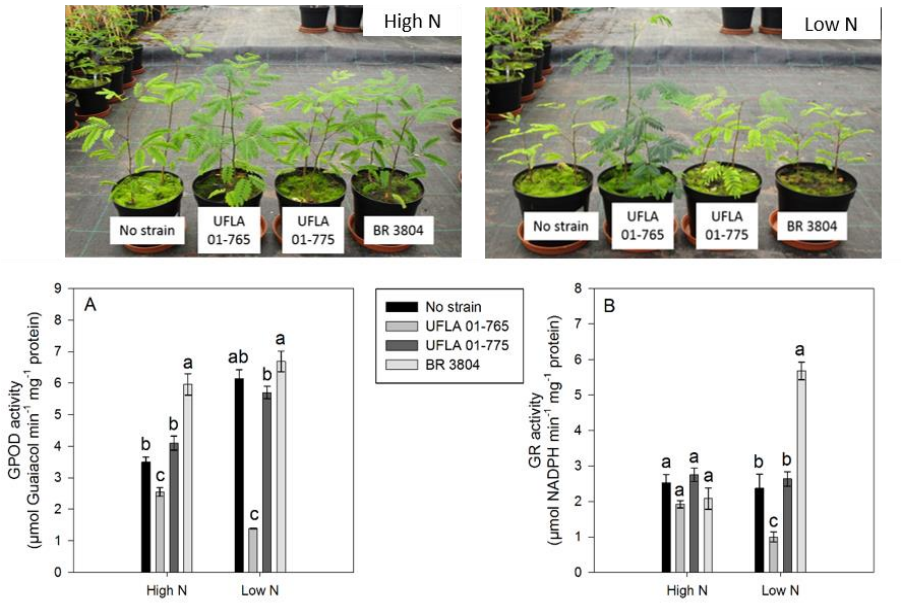

Figure 7. Shoot enzymatic activity of *Leucaena leucocephala* inoculated with rhizobia, and control without rhizobia, after 90 days pot-experiment on a Zn-, Pb- and Cd-contaminated soil. A- Guaiacol peroxidase (GPOD) activity. B- Glutathione reductase (GR) activity. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.