WESLEY DE MELO RANGEL

METAL TOLERANT BACTERIA WITH PLANT GROWTH PROMOTING TRAITS ISOLATED FROM MINING AREAS

LAVRAS - MG

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Thesis submitted in fulfillment of the requirements for the joint degree of Doctor in Science. Areas of concentration: Agricultural Microbiology at UFLA, and Biology at UHasselt.

Prof. Dr. Fatima Maria de Souza Moreira
Promoter (UFLA)

Prof. Dr. Jaco Vangronsveld
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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Rangel, Wesley de Melo.

Metal tolerant bacteria with plant growth promoting traits isolated from mining areas / Wesley de Melo Rangel. – Lavras : UFLA, 2015.

184 p. : il.

Tese(doutorado)–Universidade Federal de Lavras, 2015.
Orientador(a): Fatima Maria de Souza Moreira.
Bibliografia.

1. Leguminosas. 2. Rizóbio nativo de solo contaminado. 3. Fixação Biológica de Nitrogênio. 4. Promoção do crescimento vegetal. 5. Fitorremediação. I. Universidade Federal de Lavras. II. Título.

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APPROVED on September 11th, 2015.

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A Deus, onipotente e misericordioso.

Não perca a esperança.
Há milhões de pessoas aguardando os recursos de que você já dispõe.
Não perca o bom humor.
Em qualquer acesso de irritação, há sempre um suicidiozinho no campo de suas forças.
Não perca a tolerância.
É muita gente a tolerar você naquilo que você ainda tem de indesejável.
Não perca a serenidade.
O problema pode não ser assim tão difícil quanto você pensa.
Não perca a humildade.
Além da planície, surge a montanha, aparece o horizonte infinito.
Não perca o estudo.
A própria morte é lição.
Não perca a oportunidade de servir ao semelhante.
Hoje ou amanhã, você precisará de concurso alheio.
Não perca tempo.
Os dias voltam, mas os minutos são outros.
Não perca a paciência.
Recorde a paciência inesgotável de Deus.
pelo espírito de ANDRÉ LUIZ, médium: Francisco Cândido Xavier

Existem pessoas que fazem parte da vida da gente e sem elas nada seria significativo.....
*A **Sebastião**, meu pai, exemplo de simplicidade, caráter e respeito para com o próximo.*

*A **Mariana**, minha mãe, pelo vínculo sublime na minha vida. Exemplo de simplicidade, força, perseverança e amor incondicional. Esteve ao meu lado a cada segundo desde o princípio.*

*A **William**, **Thaís** e **Ana Carolina**, meus irmãos, pelo apoio, amizade, carinho e compreensão.*

*A **Giovanna**, minha sobrinha querida, pela continuidade, espírito alegre, carinhoso, amoroso e repleto de esperança.*

DEDICO

A todos os mestres e doutores desta vida que possuem simplicidade, sinceridade, humildade, respeito e amor ao próximo.

OFEREÇO

ACKNOWLEDGMENT

All the work in this thesis is part of a long lasting concept which have pushed me forward in life. My aim was, and still is, to learn from all the difficulties and be able to be sussessful, even if perfection is not achieved. I must say that the faith in God and in yourself, the sense of humor when it's possible, the friendship, tolerance, cooperation and the capacity of recognizing and distinguishing right from wrong, are just a few features which made this work possible and for sure added to my life experience.

My sincere thanks to my promoter Professor Fatima Maria de Souza Moreira at Federal University of Lavras (UFLA), who has accepted me as a PhD student at the Laboratory of Biology, Microbiology and Biological Processes of UFLA. I must say that your professional skills inspire me. Even with many tasks as the head of the Graduate Program in Soil Science, she is still efficient on what she does.

Besides my co-promoter Dr. Nele Weyens, there is a person who is even busier as he has the task of being the head of the Department for Environmental Sciences (CMK) at Hasselt University in Belgium (UHasselt), who has accepted me as a PhD student under his own supervision. I thank Prof. Dr. Jaco Vangronsveld for being my promoter and being able to find some time and dedicate it to my PhD research project. I greatly appreciate the way he manages to carry out so many tasks as head of the Department and still be efficient on what he does. Their positiveness and friendly advices was really important for me when I was in Belgium. I also appreciate their prompt support and belief in my work. For certain, they will be an example and will add in my scientific and personal lives.

Many thanks to Prof. Luiz Roberto (Bebeto) for his collaboration with the mining companies and also his financial help for samplings. His professional skills and friendly soul are an example and will add in my scientific and personal lives.

Thanks to Prof. Dr. Jonathan van Hamme for sequencing the bacterial genome. Thanks to Prof. Dr. François Rineau for helping me with the bacterial genome annotation.

Thanks a lot to Prof. Dr. Fabio Lopes Olivares, Prof. Dr. Maria Rita S. Muzzi and Prof. Dr. Patrícia Gomes Cardoso for their valuble suggestions for improving this thesis.

Many thanks to Manoel A. Silva and Marlene A. Souza from the Laboratory of Biology, Microbiology and Biological Processes and all the technicians from Soil Science Department from UFLA and Ann Wijgaerts and Carine Put from UHasselt for their essential thecnical assistance.

My very special thanks to Niels Lambrichts from UHasselt for all his help with all administrative tasks and his friendly soul. My greetings of good luck and success in future life goes for his wife and son as well.

I would like to take this opportunity to express my sincere appreciation to all the colleagues and friends I have met at the Laboratory of Biology, Microbiology and Biological Processes at UFLA, their lively and nice friendship in the lab made all the difference during the period I spent in the lab. Especial thanks to Silvia Maria O. Longatti, Paulo A. A. Ferreira, Daiane Bonaldi, Romildo Jr., Amanda A. Guimarães, Jessé V. Santos, Márcia Rufini, Paula R. Ribeiro and Teotônio S. Carvalho who has directly helped me on preparing this thesis. Thanks to all colleagues and friends from Biology and Soil Science Departments. Thanks to all colleagues and friends from Associação dos Pós-Graduandos (APG) da UFLA. I must say that because of you I've got three memorable years during my PhD.

My sincere thanks also go to my colleagues and friends there in Belgium. Especial thanks to Sofie Thijs, Wouter Sillen, Blanca Montalbán, Jolien Janssen, Inge Jambon, Nele Eevers, Marijke Gielen, Sacha Truyens, Bram Beckers, Panos Gkorezis and his wife Vivi, Michelle Holtappels, Hanne Vercampt, Marijke Jozefczak and Rafaela A. dos Reis. I would not forget to acknowledge my desk mates at D5-office at UHasselt - Ariadna S. López, Evi Syranidou, Frank van Bellegen, Giselle T. Farradá, Iva Cholakova, Jordan Espenshade, Roberto Abbamondi and Valeria Imperato. I am deeply thankful to them all, our relation was wonderful, as we an international office could understand, tolerate and accept each other in the best way possible. That is the first major important aspect for anyone living abroad. That is what provides us with the energy required to overcome the 'difficulties' of not being in your home country. Thanks to the international support team from UHasselt. I must say that 2014, even Brazilian futebol team did not win world cup in Brazil, it was one of the best years I have lived, because I have met you all and learned a bit more about this wonderful world.

I would not forget to express my gratefulness to Liesbeth Oeyen, Jan Nys, Izabela Oliveira, Renata Yokota, Martin Otava, Trishanta Padayachee, Thiago Bastos, Andressa Cristina, Kênya Sodré, Johan van Ophem and to all

Brazilian community in Hasselt, in Belgium or even in Europe. I'm thankfull to all of them who have given me the credibility of friend and help in all respects. I would like to wish, not only those who are close friends, but to everyone there in Hasselt, in Belgium or in Europe good luck and a lots of success in their objectives and life.

Thanks to Prof. Anísio C. R. Fonseca, Prof. Dr. Leyser R. Oliveira and Prof. Dr. Pascoal Jr. from Centro Universitário de Formiga (UNIFOR-MG) who has given me input for doing my master. Six years after their encouragement I got my PhD. Many thanks!!!

Thanks to Fabiana Couto and Ludmila Oliveira for giving me the opportunity to being part of República Formigueiro.

My final thanks certainly go to my family and friends who has encouraged and send me good vibes for making this thesis possible. Thanks for providing me with great moments of entertainment. Sou muito grato aos meus pais Sebastião e Mariana, por terem proporcionado a mim excelentes condições de estudo. Tenham certeza que essa tese também é de vocês. Gratidão também manifesto ao incentivo e suporte recebido dos meus irmãos William, Thaís e Ana Carolina, e da minha sobrinha Giovanna, os quais compreendem a minha ausência durante todos esses anos longe da família, Muito Obrigado!!!

It is also acknowledged that my studies in Brazil and Belgium were made possible by financial support from the Brazilian Ministry of Higher Education (CAPES), the scholarship abroad were supported by its PDSE - Process BEX: 13079/2013-01.

RESUMO GERAL

A exploração mineral, em especial a mineração, é uma atividade essencial a sociedade moderna, sendo fonte de recursos para diversos setores econômicos imprencindíveis, como a indústria e a agricultura. No entanto, a mineração ocasiona um grande distúrbio no local e ambientes próximos à atividade. Ambientes sob interferência causada pela mineração, provavelmente estão desprovidos de meios naturais de regeneração biótica, sendo necessário o auxílio de ação antrópica para a revegetação do ambiente. A fitorremediação tem provado ser uma técnica bastante promissora para a reabilitação *in situ* dessas áreas. Neste trabalho foram estudas duas áreas distintas de mineração. Uma área de extração de ouro, contaminada com arsênio e a outra de extração de zinco contaminada com cádmio e zinco. Bactérias do solo, principalmente rizóbio, nativos destas áreas foram isolados e caracterizados geneticamente e funcionalmente *in vitro*. Além de testes para a avaliar a capacidade destas bactérias em promover o crescimento vegetal, a resistência delas aos metal(óides) também foi avaliada. A caracterização genotípica, pelo sequenciamento parcial do gene 16S rRNA, revelou que a maior parte das bactérias isoladas pertecem à classe α-Proteobacteria com indivíduos representando os gêneros *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Bosea*, *Inquilinus*, *Labrys*, *Starkeya* e *Methylobacterium*, sendo isolados também representantes da classe β- Proteobacteria pelos gêneros *Burkholderia*, e *Variovorax*. A maioria das espécies isoladas foram positivas em nodular sua respectiva leguminosa hospedeira no teste de autenticação da capacidade de nodulação. Somente os isolados dos gêneros *Bosea*, *Methylobacterium* e *Starkeya* não nodularam seus hospedeiros. Interessantemente, as estirpes *Inquilinus* sp. e *Labrys monachus* foram capazes de nodular *Crotalaria spectabilis*, e *Variovorax paradoxus* foi capaz de nodular *Leucaena leucocephala*. Embora essas estirpes sejam consideradas atípicas normalmente encontradas nodulando leguminosas. A caracterização funcional dessas bactérias revelou sua grande capacidade em desempenhar processos promotores do crescimento vegetal e elevada resistência a metal(óides). Além da resistência *in vitro* a Cd e Zn, *Mesorhizobium* sp. UFLA 01-765 confirmou sua tolerância em solo contaminado, estabelecendo simbiose, promovendo o crescimento vegetal e acúmulo de nitrogênio de *L. leucocephala*. Portanto, rizóbios nativos de solos de áreas de mineração são ferramentas promissoras para potencializar a revegetação destes solos.

Palavras-chave: Leguminosas. Rizóbio nativo de solo contaminado. Fixação Biológica de Nitrogênio. Promoção do crescimento vegetal. Fitorremediação.

SAMENVATTING

Het winnen van mineralen, meer in het bijzonder mijnbouw, levert belangrijke grondstoffen aan economische sectoren zoals de (maak)industrie en de landbouw en is daarom van essentieel belang in onze moderne samenleving. Anderzijds kan mijnbouw de lokale omgeving zo sterk verstoren dat natuurlijke biotische regeneratie niet optreedt. Om dergelijke gebieden opnieuw van een vegetatiedek te voorzien, is een menselijke ingreep bijgevolg noodzakelijk. Het is reeds gebleken dat fytoremediatie een veelbelovende technologie is voor het *in situ* herstel van zo'n gebieden. In dit werk bestuderen we 2 verschillende mijnbouw gebieden. Enerzijds een goudmijnbouw gebied dat gecontamineerd is met arseen en anderzijds een zinkmijnbouw gebied gecontamineerd met cadmium en zink. De rhizobiumbacteriën die van nature voorkomen in deze gebieden, werden geïsoleerd en gekarakteriseerd op genotypisch en functioneel niveau. Hiernaast werden de geïsoleerde stammen ook getest voor resistentie aan metalen en/of metalloïden en op plantengroeipromotie capaciteit. De genotypische karakterisering, gebruik makend van partiële 16SrDNA sequenering, maakte duidelijk dat de meeste bacteriële stammen behoren tot de klasse α-Proteobacteriën, met hierin volgende rhizobium genera vertegenwoordigd: *Bradyrhizobium*, *Mesorhizobium* en *Rhizobium*. Verder werden ook bacteriële stammen geïsoleerd van de genera *Bosea*, *Starkeya*, *Methylobacterium*, *Inquilinus* en *Labrys*, van de klasse β-Proteobacteriën, vertegenwoordigd door *Burkholderia* en *Variovorax*. Van de geïsoleerde stammen, testten de meerderheid positief voor de inductie van de vorming van nodules bij hun eigen leguminose gastheerplant. Gebruik makend van een authenticatietest test voor nodulatie capaciteit werden zelfs enkele atypische nodule vormende bacteriën gedetecteerd zoals *Inquilinus* sp., *Labrys monachus* en *Variovorax paradoxus*. Enkel isolaten van de genera *Bosea*, *Methylobacterium* en *Starkeya* waren niet in staat hun gastheerplant te noduleren. Opmerkelijk is dat *Inquilinus* sp. en *Labrys monachus* in staat waren om nodules te vormen bij *Crotalaria spectabilis* wortels, en *Variovorax paradoxus* bij *Leucaena leucocephala* wortels. Dit is opmerkelijk aangezien deze bacteriën behoren tot atypische genera met betrekking tot nodule vorming bij Leguminosen. Uit de functionele karakterisering van de geïsoleerde bacteriestammen bleek hun groot potentieel om de plantengroei te bevorderen en hun resistentie aan metalen en/of metalloïden. Naast de *in vitro* geteste resistentie aan Cd en Zn, werd voor de bacteriële stam *Mesorhizobium* sp. UFLA 01-765 de metaaltolerantie bevestigd op gecontamineerde bodem, waar deze stam een symbiose aanging met *L. leucocephala*, de plantengroei bevorderde en Ni accumuleerde. In conclusie kan gesteld worden dat rhizobia stammen die van nature voorkomen in mijnbouw gebieden veelbelovend zijn met het oog op herbegroeiing van deze bodems.

Sleutelwoorden: *Fabaceae*. Rhizobia die van nature voorkomen op metaal-verontreinigde bodem. Biologische stikstoffixatie. Plantengroei promoverende bacteriën. Fytoremediatie

GENERAL ABSTRACT

Mineral exploitation, particularly mining, is an essential modern society activity, providing resources for crucial economic sectors, such as industry and agriculture. However, mining has disturbing effects on the local environment. Environments under influence of mining are often devoid of natural means of biotic regeneration. The aid of human intervention is required for the revegetation of these environments. Phytoremediation has proven to be a very promising technique for *in situ* rehabilitation of these areas. In this work we studied two different mining areas. An area of gold mining, contaminated with arsenic and a zinc mining area contaminated with cadmium and zinc. Native soil rhizobacteria from these areas have been isolated and genetically and functionally characterized. Tests to evaluate the capacity of these bacteria to promote plant growth, besides its resistance to metal(oids), were also performed. Genotypic characterization by partial 16S rRNA gene sequencing revealed that most of the bacterial isolates belong to α-Proteobacteria class with individuals representing the rhizobial genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. Moreover, bacteria representing *Bosea*, *Starkeya*, *Methylobacterium*, *Inquilinus* and *Labrys* genera, and β-Proteobacteria class representants of *Burkholderia*, and *Variovorax* were also isolated. Most of the isolated species tested positive for inducing nodule formation on their respective host legume, through authentication test for nodulation capacity, even atypical nodule forming bacteria as *Inquilinus* sp., *Labrys monachus* and *Variovorax paradoxus*. Only isolates of *Bosea*, *Methylobacterium* and *Starkeya* genera did not nodulate their hosts. Interestingly *Inquilinus* sp. and *Labrys monachus* have induced nodule formation on *Crotalaria spectabilis* roots, and *Variovorax paradoxus* have induced nodule on *Leucaena leucocephala* roots. Although these bacteria are atypical genera normally found nodulating legume plants. The functional characterization of these bacteria showed their great ability to promote plant growth and resist metal(oid)s. In addition to *in vitro* resistance to Cd and Zn, *Mesorhizobium* sp. UFLA 01-765 strain confirmed its tolerance in contaminated soil, where it was able to establish symbiosis, promote plant growth and accumulate nitrogen when associated with *L. leucocephala*. In conclusion, native rhizobia from soil mining areas are promising tools to enhance revegetation of these soils.

Keywords: *Fabaceae*. Native rhizobia from metal-contaminated soils. Biological N_2 fixation. Plant-growth promoting bacteria. Phytoremediation.

SUMMARY

First part - Overview

1 General introduction

Nowadays the global economic development is mainly supported by different mining activities, which is strongly linked to social development, generating assets and wealth. On the other hand, mining exploitation, especially metal mining, causes huge environmental impacts (BAKER et al., 1994; DIAS JÚNIOR et al., 1998; SALOMONS, 1995; VANGRONSVELD; COLPAERT; VAN TICHELEN, 1996). Those areas often are devoid of natural means of biotic regeneration, requiring human intervention for the revegetation of the environment. 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; WEYENS et al., 2011, 2013). Phytoremediation success is linked to the microorganisms, which benefit plant growth by performing essential biological processes, either in the rhizosphere or inside the plant (CROES et al., 2013; WEYENS et al., 2013). 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 highlighted in this work. An important prokaryote group able to perform BNF is represented by rhizobia, which establish mutualistic symbiosis with legume plants. Nodulated legume plants incorporate C and N into soil, besides increasing their nutrients uptake capacity, improving their tolerance to environmental stresses (FRANCO; FARIA, 1997; FRANCO et al., 2000; FRANCO; BALIEIRO, 2000; MELLONI et al., 2006; MOREIRA; CARVALHO; SIQUEIRA, 2010; MOREIRA et al., 2010; MOREIRA et al., 2015). Therefore, this thesis reports the study of native, plant growth promoting rhizobia isolated from nodules of legume plants growing on contaminated

mining soils or used as a trap plant, to unravel the potential of rhizobia in function of phytoremediation purposes.

1.1 General objectives

1 – Isolate and characterize (genotypic and functional) native bacteria from *Crotalaria spectabilis* and *Stizolobium aterrimum* nodules, growing in an arsenic contaminated mining area (Chapter 3).

2 – Isolate and characterize (genotypic and functional) native rhizobia from a zinc mining area contaminated with Cd and Zn, and select strains with plant growth promoting traits for testing its potential on contaminated soil (Chapter 4).

3 – Unravel by genome mining the mechanisms performed by *Mesorhizobium* sp. UFLA 01-765, a multi-tolerant, efficient symbiont and plant-growth promoting rhizobia isolated from Zn-mining soil using *Leucaena leucocephala* as a trap plant (Chapter 5).

1.1.1 Specific objectives

1 – i) Evaluate the symbiotic efficiency and the multiple-tolerance of the native soil rhizobacteria to As, Cd and Zn, and *β*-lactam antibiotics, and ii) Check their plant growth promoting traits (Chapter 3).

2 – i) Evaluate the symbiotic efficiency and the tolerance of the native soil rhizobacteria to Cd and Zn, and ii) Check their *in vitro* plant growth promoting traits and iii) Confirm these plant growth promoting traits on Cd and Zn-contaminated soil (Chapter 4).

3 – i) Present the draft genome of *Mesorhizobium loti* UFLA 01-765 strain isolated from a Zn-mining soil using *Leucaena leucocephala* as a trap plant (Chapter 5).

This thesis provides a descriptive view of soil native bacteria able to induce nodule formation on roots of legume species, especially rhizobia, in two different mining areas. A gold mine area contaminated by arsenic and a zinc mine area contaminated by cadmium and zinc. In addition to demonstrating the capacity of these rhizobia to promote plant-growth, it provides an overview of the rhizobial symbioses with *L. Leucocephala* using an *in planta* approach on contaminated soil.

REFERENCES

BAKER, A. J. M. et al. The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. **Resources, Conservation and Recycling**, Amsterdam, v. 11, p. 41-49, 1994.

CROES, S. et al. Bacterial communities associated with *Brassica napus* L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. **Microbial Biotechnology**, London, v. 6, p. 371-384, 2013.

CUNNINGHAM, S. D. et al. Phytoremediation of contaminated water and soil. In: KRUGER, E. L.; ANDERSON, T. A.; COATS, J. L. (Ed.). **Phytoremediation of soil and water contaminants**. Washington: American Chemical Society, 1997. v. 664, p. 2-17.

DIAS JÚNIOR, H. E. et al. Heavy metals, microbial density and activity in a soil contaminated by wastes from a zinc industry. **Revista Brasileira de Ciência do Solo**, Viçosa, MG, v. 22, p. 631-640, 1998.

FRANCO, A. A.; BALIEIRO, F. C. The role of biological nitrogen fixation in land reclamation, agroecology and sustainability of tropical agriculture. In: ROCHA-MIRANDA, C. E. (Ed.). **Transition to global sustainability**: the contribuition of Brazilian Science. Rio de Janeiro: Academia Brasileira de Ciências, 2000. p. 209-233.

FRANCO, A. A. et al. The importance of biological nitrogen fixation on land rehabilitation. In: PEDROSA, F. O.; HUNGRIA, M.; YATES, G. (Ed.). **Nitrogen fixation**: from molecules to crop productivity. Dordrecht: Kluwer Academic, 2000. p. 569-570.

FRANCO, A. A.; FARIA, S. M. The contribution of N_2-fixing tree legumes to land reclamation and sustainability in the tropics. **Soil Biology and Biochemistry**, Elmsford, v. 29, p. 897-903, 1997.

MELLONI, R. et al. Efficiency and phenotypic diversity among nitrogen-fixing bacteria that nodulate cowpea [*Vigna unguiculata* (L.) WALP] and common bean (*Phaseolus vulgaris* L.) in bauxite-mined soils under rehabilitation. **Revista Brasileira de Ciência do Solo**, Viçosa, MG, v. 30, p. 235-246, 2006.

MOREIRA, F. M. S.; CARVALHO, T. S.; SIQUEIRA, J. O. Effect of fertilizers, lime, and inoculation with rhizobia and mycorrhizal fungi on the growth of four leguminous tree species in a low-fertility soil. **Biology and Fertility of Soils**, Berlin, v. 46, p. 771-779, 2010.

MOREIRA, F. M. S. et al. Bactérias fixadoras de N_2 e fungos micorrízicos arbusculares em espécies florestais: avanços e aplicações biotecnológicas. In: FIGUEIREDO, M. V. B. et al. (Org.). **Biotecnologia aplicada à agricultura**. Recife: Embrapa/IPA, 2010. v. 1, p. 439-477.

MOREIRA, F. M. S. et al. Symbioses of plants with rhizobia and mycorrhizal fungi in heavy metal-contaminated tropical soils. In: SHERAMETI, I.; VARMA, A. (Ed.). **Heavy metal contamination of soils**. [S.l]: Springer International Publishing, 2015. v. 44, p. 215-24. Disponível em: <http://dx.doi.org/10.1007/978-3-319-14526-6_12>. Access on April 22nd, 2015.

SALOMONS, W. Environmental impact of metals derived from mining activities: Processes, predictions, prevention. **Journal of Geochemical Exploration**, Amsterdam, v. 52, p. 5-23, 1995.

VANGRONSVELD, J.; COLPAERT, J. V.; van TICHELEN, K. K. Reclamation of a bare industrial area contaminated by non-ferrous metals: physicochemical and biological evaluation of the durability of soil treatment and revegetation. **Environmental Pollution**, Barking, v. 94, p. 131-140, 1996.

WEYENS, N. et al. Contrasting colonization and plant growth promoting capacity between wild type and a gfp-derative of the endophyte *Pseudomonas putida* W619 in hybrid poplar. **Plant and Soil**, The Hague, v. 356, p. 217-230, 2011.

WEYENS, N. et al. Plant-associated bacteria and their role in the success or failure of metal phytoextraction projects: first observations of a field-related experiment. **Microbial Biotechnology**, London, v. 6, p. 288-299, 2013.

2. Literature Review

2.1 Introduction

Native bacterial populations in soils with high levels of trace elements (TE) are well adapted to this kind of stress. By consequence, the study of the bacterial diversity under these conditions can provide important information on well adapted genotypes (YOUNG, 1994; TRANNIN et al., 2001). In addition, inoculating legume species with beneficial bacterial strains that are well-adapted to these conditions might be of high importance to both ecological and economic concern (COSTA et al., 2004; FRANCO et al., 1992; FRANCO; FARIA, 1997; STEPHENS; RASK, 2000).

Since Brazil has both, huge mining areas for the extraction of several minerals, as well as high plant and microbial diversity, it holds a huge potential for biotechnological applications, especially concerning the plant-growth promotion aspect. Studies on this topic may provide support for exploiting the microbial potential in sustainable technologies such as phytotechnology for revegetating mining areas.

Therefore, in this chapter, we give an overview of metal tolerant soil bacteria, especially rhizobia, and their importance for promoting plant-growth on TE contaminated soils, and thereby their biotechnological potential as a phytoremediation tool.

2.2 Soil microorganisms interacting with Trace Elements

Weathering minerals from rocks and anthropogenic activities are two major sources for TE entering soils. Ross (1994) classified the TE anthropogenic origination by 5 major groups: (1) metal mining and smelting – arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg), (2) industry – As, Cd, chromium (Cr), cobalt (Co), copper (Cu), Hg, nickel (Ni) and zinc (Zn), (3) atmospheric deposition - As, Cd, Cr, Cu, Pb, Hg and uranium (U), (4) agriculture - As, Cd,

Pb, Cu, selenium (Se), U and Zn, and (5) waste disposal - As, Cd, Cr, Cu, Pb, Hg and Zn.

Most of the TE interact with vital processes in microorganisms. Some TE, such as calcium (Ca), potassium (K), magnesium (Mg), iron (Fe), sodium (Na), Co, Cr, Cu, Ni and Zn are essential, since they are (micro)nutrients, they are utilized in redox processes and molecule stabilisation through electrostatic interactions, they are part of several enzymes and regulate osmotic pressure (BRUINS; KAPIL; OEHME, 2000). Several other TE do not have a known biological function, for instance silver (Ag), aluminium (Al), Cd, gold (Au), Pb and Hg. These TE are not essentials and present, even in low concentrations, potential toxicity to microorganisms. This toxicity may be caused through essential TE displacement from their binding sites or by binder interaction (BRUINS; KAPIL; OEHME, 2000; NIES, 1999). For example, Hg^{+2}, Cd^{+2} and Ag^{+2} tend binding to sulfhydryl groups (-SH), thereby inhibiting the activity of sensitive enzymes (NIES, 1999). Moreover, high levels from both essential and non-essential TE may cause damage to microbial cell membranes, shift enzymatic specificity, disturb the cellular functioning and cause harm to DNA structure (BRUINS; KAPIL; OEHME, 2000).

For both physiological and toxic effects, most of the metallic ions must enter into the microbial cell. Many divalent metal cations, such as Mn^{+2}, Fe^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2} and Zn^{+2} are highly similar at the structural level. Moreover, oxyanions structure, such as chromate is very similar to sulphate, as well as arsenate is similar to phosphate. Therefore, to differentiate between metallic ions that are structurally similar, absorption systems must be well regulated. Usually, microorganisms have solved this deadlock by using two different absorption systems for metallic ions. One is fast, nonspecific, and driven by a chemiosmotic gradient through the bacterial cytoplasmic membrane. Once this mechanism is used by many substrates, it is constitutively expressed (NIES, 1999). The second

absorption system has a high specificity for the substrate, and is slower, because it uses ATP as energy source and is activated by the cell only when needed, as in case of food deprivation or a special metabolic situation (NIES; SILVER, 1995).

Even in microorganisms holding this specific absorption system, high levels of non-essential TE can be carried into the cell through nonspecific systems that are constitutively expressed. This “open door” is the main reason for toxicity caused by metallic ions to microorganisms (NIES, 1999). Thereby, microorganisms have been forced to develop metal-ion homeostasis mechanisms, which are crucial for metal resistance (NIES; SILVER, 1995; NIES, 1999; BRUINS; KAPIL; OEHME, 2000). Metallic ions can not degraded or modified as organic compounds. In general, there are six possible mechanisms for TE resistance: 1) exclusion; 2-3) intracelular and extracelular sequestration; 4) active efflux pumps; 5) enzymatic reduction; and 6) reducing sensitivity of cellular targets to metal ions. (BRUINS; KAPIL; OEHME, 2000; JI; SILVER, 1995; NIES; SILVER, 1995; NIES, 1999; RENSING; GHOSH; ROSEN, 1999). One or more of these resistance mechanisms allow microorganisms performing their functions on TE contaminated environments.

Arsenic oxyanions, for instance, enter into the cell through transporters for other compounds. Bacteria take up arsenate As(V) through the phosphate specific transport (Pst) system spending ATP (RENSING; GHOSH; ROSEN, 1999). An entryway for arsenite As(III) is through aquaporin transporters GlpF polyol (glycerol transport proteins and other small uncharged molecules), present in bacteria and yeast (RENSING; GHOSH; ROSEN, 1999; LIU et al., 2002). Bacterial detoxification for As is based on inducible efflux systems, which decrease intracellular As content by active efflux, as ilustrated in figure 1 (JI; SILVER, 1995; NIES; SILVER, 1995; RENSING; GHOSH; ROSEN, 1999). Once bacterial cells drive anion efflux by a chemiosmotic gradient, simple As(III) efflux systems are formed by only one excluder protein (JI;

SILVER, 1995; NIES; SILVER, 1995; RENSING; GHOSH; ROSEN, 1999). Therefore, As(V) cannot be transported through this system. The solution for a As(V) efflux would be the enzyme arsenate reductase. This enzyme catalyses the reduction of a As(V) to As(III), which is the substrate for the excluder system (JI; SIVER, 1995; NIES; SILVER, 1995; RENSING; GHOSH; ROSEN, 1999). Thus, arsenate reductases magnify the As resistance spectrum, including both As(III) as well as As(V).

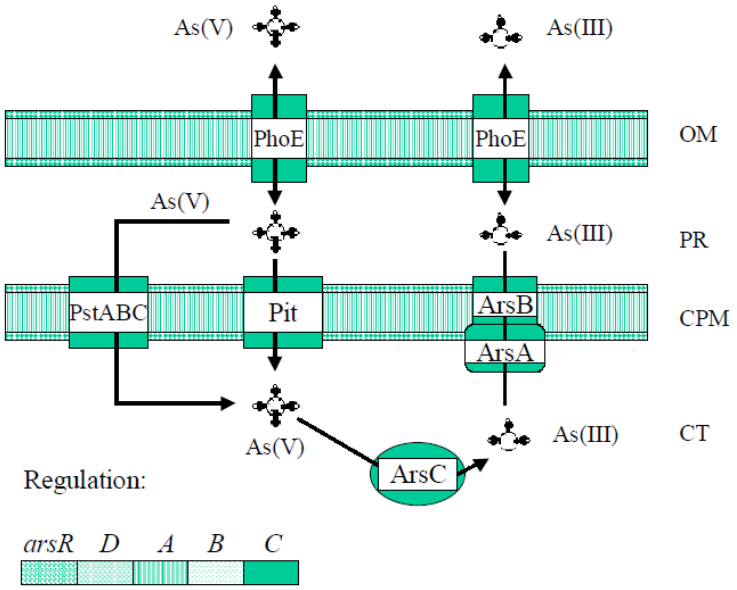

Figure 1. Transport and resistance to arsenate in *Escherichia coli*. Arsenate and phosphate enter the periplasmic space through the outer membrane porin, the PhoE protein. Both anions are transported into the cytoplasm by Pit proteins or the Pst system (which is more specific for phosphate, as it uses the PstS phosphate-binding protein and the PstABC ATPase complex for inner membrane uptake). Within the cell, arsenate is reduced to arsenite by the ArsC protein (dependent on glutaredoxin and glutathione) and arsenite is pumped out of the cell by the ArsAB efflux ATPase. The *arsRDABC* operon is regulated by the ArsR repressor protein and the ArsD co-regulator protein. OM - outer membrane, PR - periplasmic space, CPM - cytoplasmic membrane, CT - cytoplasmic space (NIES; SILVER, 1995).

The toxicity resistance mechanism for inorganic ions most commonly held by bacteria is pumping ions from the inside to the outside of the cell. Such

mechanism is energy-dependent for its operation, and is located on the membrane as efflux pump (SILVER; PHUNG, 2005) (Figure 2).

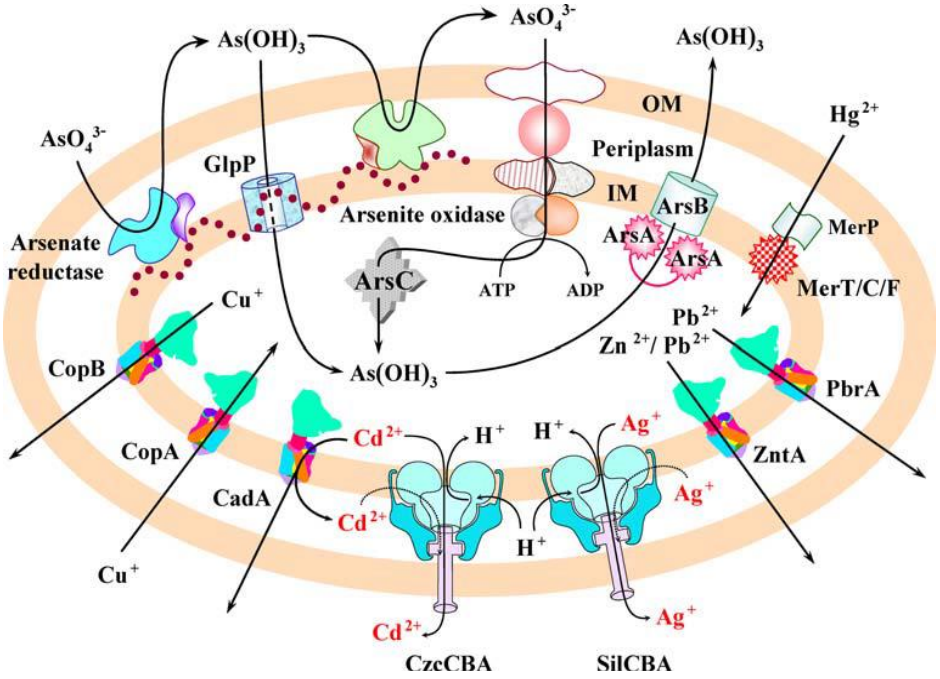

Figure 2. Overview of membrane-associated uptake, efflux, reduction and oxidation of metal ions. The complexity of several examples of some families (such as CopA/B and CadA P-type ATPases and three component chemiosmotic RND systems (CzcCBA and SilCBA) preclude a useful detailed listing here and readers are referred to the separate sections concerning each element. Arsenic is given more emphasis, as enzymes in the periplasm and cytoplasm are included as well as three classes of transporters (GlpP, the aquaglyceroporin; a multicomponent Pst-like ABC ATPase uptake system; and a two component ArsA/B ATPase efflux pump). The CBA efflux transport systems extend from the cytoplasm across the outer membrane of Gram negative bacteria. How the substrates of transport systems (influx and efflux) that do not have indicated associated outer membrane proteins function is not known (SILVER; PHUNG, 2005).

Cells respond to Zn^{2+} excess or Cd^{2+} and Pb^{2+} presence by activating resistance mechanisms inducible by the metals itself. Bacterial resistance to Zn^{2+}, Cd^{2+} and Pb^{2+} is mainly based on active efflux of metallic ions to protect cells from toxic effects. Resistance mechanisms to Zn^{2+} and Cd^{2+}, most often, are indistinguishable, because its resistance is coded by the same genes. Zn^{2+} and Cd^{2+} efflux is facilitated by P type ATPases, CBA transporters and chemiosmotic CDF transporters. Resistance to Pb^{2+} is still not well known, but it

is reported that the participation of P type ATPases and the detoxification occurs through sequestering (SILVER; PHUNG, 2005).

Concerning Cd efflux, three systems can be used by bacteria. The **CzcD** system, formed by one simple efflux pump, which is composed by a membrane chemiosmotic polypeptide, the **CzcCBA** system, formed by a complex of three chemiosmotic polypeptides including CzcA (a large inner membrane protein), CzcC (a smaller outer membrane protein) and CzcB (a periplasmic coupling protein connecting CzcA and CzcB and forming a continuous channel from the cytoplasm to the outside of the cell – Figure 3b), and the **CadA** system, a large unique polypeptide P-type ATPase (Figure 3a) (SILVER; PHUNG, 2005).

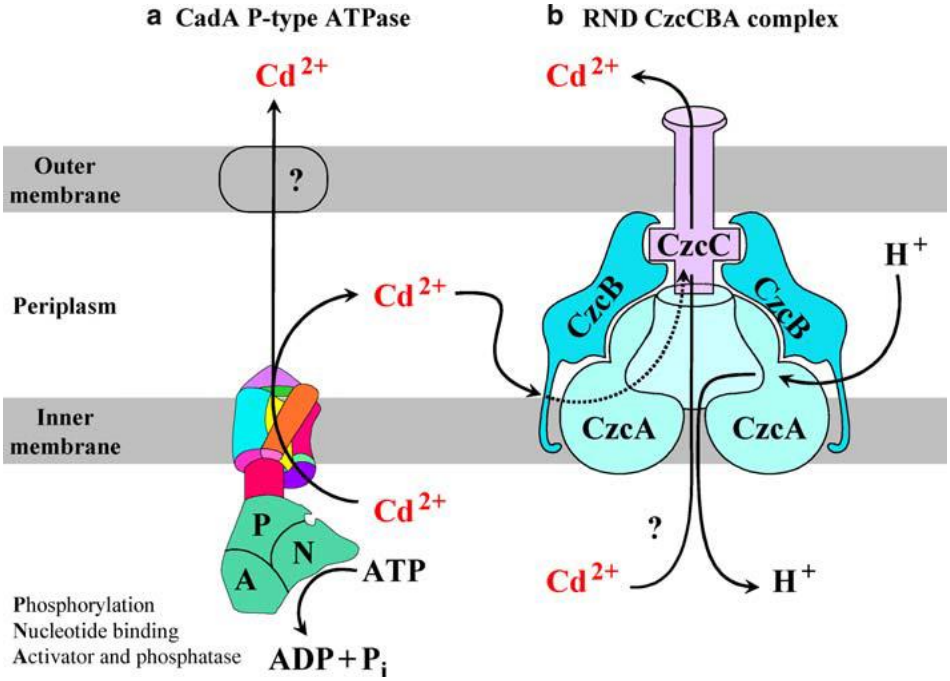

Figure 3. Molecular model of efflux systems a) P-type ATPase and b) RND chemiosmotic antiporter (SILVER; PHUNG, 2005).

The polypeptide chemiosmotic CzcCBA complex acts as a proton exchanger to efflux Cd^{2+}, Zn^{2+} and Co^{2+}. This complex is a member of the metal-resistance family, which is part of the superfamily of chemiosmotic pumps. This

superfamily was already found in rhizobia as *Cupriavidus metallidurans*[*] strains CH34 and 31A ([*]renamed from *Ralstonia metallidurans*), and some members are linked to the nodule formation by the bacteria. Thereby this superfamily of chemiosmotic pumps is called RND (Resistance, Nodulation and Division). Genes linked to RND related with cation efflux systems were identified on the above-mentioned rhizobia strains. Resistance to Cd^{2+} and Zn^{2+} was encoded by the *czr* gene, and to Co^{2+} and Ni^{2+} by *cnr* and *ncc* genes (HASSAN et al., 1999; LEGATZKI et al., 2003; MERGEAY et al., 2003; NIES, 2003). The cation diffusion facilitator, CzcD Cd^{2+} and Zn^{2+} efflux system, member of the family (CDF), was first described in *C. metallidurans*[*] (NIES, 2003; HANEY et al., 2005). Moreover, *C. metallidurans*[*] holds also ferrous iron efflux FieF, zinc efflux ZntA, nickel efflux Cnr and Ncc systems, and copper resistance conferred by periplasmic proteins PcoA, PcoE and PcoC, which bind Cu^{+} (ANTON et al., 2004; MERGEAY et al., 2003; NIES, 2003). *C. metallidurans*[*] CH34 was isolated from sediments of a decantation basin of a Zn factory in Belgium, near Liège (MERGEAY; HOUBA; GERITS, 1978). This rhizobia strain is highly resistant to Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, CrO_4^{2-}, Hg^{2+} and Pb^{2+}. Therefore, it highlights the importance and potential of rhizobia during phytoremediation studies.

2.3 Phytoremediation as a phytotechnology to revegetate trace elements contaminated soil

Several technologies identified by the US Environmental Protection Agency (EPA, 1997; EPA, 2002) are applicable to soil and waste contaminated by TE, such as solidification/stabilization, vitrification, soil washing/acid extraction, pyrometallurgical recovery (coverage), *in situ* leveling the ground electrokinetically, biological treatment, and phytoremediation. To facilitate comparison with the physical/chemical recovery systems, Cunningham, Berti

and Huang (1995) have redefined plants as "pumping and filtering systems driven by solar energy" that have "measurable skills to carry, degrade, and immobilize" TE. Roots are described as "exploratory liquid phase extractors that can find, change and/or translocate elements and compounds against large chemical gradients". Plants can also be an economical alternative to physical recovery systems, with much lower costs than necessary to cover physical and chemical remediation technologies.

Phytoremediation (from the Greek “phyto” means plant and the Latin “remedium” means cure or treatment) is a given name for a number of technologies (phytotechnologies), which use plants to control and/or mitigate contamination in compromised areas. The term is relatively new, proposed in 1991 (EPA, 1997; EPA, 2002), although the use of plants in order to prevent or control leakage of hazardous materials in landfills or treat the slurry has been performed over decades, being reported by Brix (1994) in Australian studies by Mackney (New South Wales) and German studies developed by Seidel in 1904 and 1953 (Max-Planck Institute).

Phytoremediation distinguishes itself from the other remediation methods due to its permanent nature, lower maintenance costs, improved soil structure, increased fertility, and soil protection from water and wind erosion. This latter characteristic is extremely important in case of soils contaminated with As, Cu, Cd, Cr, Ni, Pb and Zn, because wind dispersal is one of the main sources of contamination by these elements (COMPANHIA AMBIENTAL DO ESTADO DE SÃO PAULO - CETESB, 2012). Moreover, phytoremediation retrieves the aesthetics on the affected area and improves conditions for the development of soil microorganisms, which perform and participate in a large number of processes essential to the ecosystem, such as nitrogen-fixing bacteria (NFB) (CUNNINGHAM; OW, 1996; HARTLEY et al., 2009; LOPEZ, 2010; SANTOS, 2010).

Due to the relatively low cost, phytoremediation behaves like an attractive option for the remediation of TE contaminated soils, especially in developing countries where funding for environmental recovery is scarce (NASCIMENTO; AMARASIRIWARDENA; XING, 2006). Accioly and Siqueira (2000) believe that phytoremediation is a promising practice, with a guaranteed market in Brazil, considering the existence of numerous and extensive contaminated areas, mainly mining areas.

2.4 Nitrogen-fixing bacteria that nodulate Legume species, and plant-associated bacteria as essential helpers for phytoremediation

Although phytoremediation is a rather recent phytotechnology (being in its "twenties"), it is already known that the interaction with soil microorganisms plays an important role. These soil microorganisms might carry out important biological processes such as biological N_2 fixation (BNF), which can improve the nutritional status of plants, indirectly influencing their tolerance to TE.

The mutualistic symbiosis between legumes and rhizobia has great potential for using in different situations such as restoration of mining areas and revegetation of contaminated areas (FRANCO et al., 1992; FRANCO et al., 1995; MELLONI et al., 2006). In these situations, legume plants will have several benefits such as soil protection, system enrichment with N, providing good soil coverage and providing diverse flora and wildlife recovery (SIQUEIRA; SOARES; SILVA, 2008). In addition to the N supply to plants, legume-rhizobia symbiosis can replace the ammoniacal fertilizer which acidifies the soil and by consequences increases the availability of many TE.

Studies searching for information about rhizobial behavior in TE contaminated soils have been developed by the Biology, Microbiology and Soil Biological Processes lab team at the Federal University of Lavras, since the end of the 90s. Mostasso (1997) conducted studies to investigate the development

and nodulation of different leguminous and rhizobia species. The experiment was performed using a multiple contaminated soil (Cd, Cu, Pb and Zn) from a Zn smelting site. Such contamination was accidentally caused by the leakage of metallurgical waste. The contents of these metals came to Zn-18,600, Pb-600, Cu-596 and Cd-135 mg dm^{-3}. Mostasso (1997) tested the potential of tamboril (*Enterolobium contortisiliquum* (Vell.) Morong.) inoculated with a *Bradyrhizobium japonicum* BR 4406 strain, sesbania (*Sesbania virgata* (L.) Merr.) inoculated with a *Azorhizobium* sp. BR 5401 strain (later identified as a strain of the species *Azorhizobium doebereinerae* (BR 5401^{T})), leucaena (*Leucaena* Benth. sp.) inoculated with a *Sinorhizobium* sp. BR 827 strain and siratro (*Macroptilium atropurpureum* Urb.) inoculated with the *Bradyrhizobium elkanii* INPA 173A strain, in soils with 0, 15, 30, 45 and 60% contamination.

Under these conditions the symbiosis between *E. contortisiliquum* and *B. japonicum* showed a better tolerance, while *M. atropurpureum* Urb. was the most affected plant. The authors attributed the better tolerance of *E. contortisiliquum* to the fact that this plant species is a tree species and has a slower growth rate than the others, providing it more time to better adapt to the toxic effects of metals.

Since the *M. atropurpureum* Urb. is an herbaceous plant, it presents fast growth, impeding its adaptation to adverse conditions. Regarding nodulation, the authors observed that the *B. japonicum* BR 4406 was the most tolerant on increasing levels of soil contamination with a greater number of nodules compared to the other rhizobia species.

This behavior was later confirmed by Trannin, Moreira and Siqueira (2001), who also found a high and even better tolerance for the symbiosis between *E. contortisiliquum* and *B. japonicum* BR 4406, than for the same plant species in symbiosis with other *Bradyrhizobium* strains (UFLA 01-457 and INPA 398) when grown in contaminated soil. Besides *Acacia mangium* in

symbioses with *Bradyrhizobium* BR 3617 and *S. virgata* in symbioses with *Azorhizobium* BR 5401; UFLA 01-483 and UFLA 01-515 strains. These results demonstrate that both the host plant and its microsymbiont might contain some mechanisms providing them tolerance to the metal-induced stresses.

Trannin et al. (2001) showed *in vitro* that rhizobia strains from contaminated soils are more tolerant to Cd, Cu and Zn, than rhizobia strains from non-contaminated environments. It is an indication that selecting rhizobia from contaminated areas might be a first step for revegetating those areas. Also in this study, the behavior of *Bradyrhizobium* and *Azorhizobium* genera was compared, showing higher metal tolerance for the former genus.

This behavior of the *Bradyrhizobium* genus was observed by Matsuda, Moreira and Siqueira (2002a) as well, who *in vitro* evaluated the tolerance of more rhizobia genera to Cd, Cu and Zn. Ranking the most metal-tolerant rhizobia, considering the maximum metal level, the authors found *Bradyrhizobium* > *Rhizobium* = *Mesorhizobium* = *Sinorhizobium* > *Azorhizobium*. Matsuda, Moreira and Siqueira (2002b) also assessed the behavior of the genera *Bradyrhizobium* and *Azorhizobium*, checking the number of viable cells after 28 days incubation in soil with different contamination levels, confirming the higher tolerance of *Bradyrhizobium* strains.

In addition to rhizobia belonging to the α-proteobacteria subclass, order Rhizobiales, there are bacteria belonging to the β-proteobacteria subclass, order Burkholderiales, like *Burkholderia* and *Cupriavidus* strains, capable of fixing N_2 and inducing nodules on legumes roots (CHEN et al., 2001; MOULIN et al., 2001). *Cupriavidus necator* was isolated by Florentino et al., (2009), who evaluated its symbiotic efficiency (FLORENTINO et al., 2012) as well as its ability to induce nodules on roots of different legume species (SILVA et al., 2012). Subsequently, Ferreira et al. (2012) evaluated *in vitro* the tolerance of *C. necator* to Cd, Cu, Pb and Zn, as well as the symbiotic efficiency of the most

tolerant strains associated with *L. leucocephala* (Lam.) R. de Wit, *E. contortisiliquum* (Vell.) Morong, *A. mangium* (Wild.), *M. caesalpiniifolia*, *M. pudica* L., *M. pigra* L. and *M. acutistipula* (Marth.) Benth. Regarding *in vitro* tolerance to metals, from the 35 strains tested 91% were able to grow up to 2.5 mmol L^{-1} of all metals tested. Only five strains were able to grow at 10 mmol L^{-1} of Zn and Cu, and just one strain grew up to 7.5 mmol L^{-1} of Pb. No strain grew at concentrations above 2.5 mmol L^{-1} of Cd. Ranking metal toxicity of the strains followed the order Cd > Pb > Cu = Zn.

The genetic and biochemical plasticity of prokaryotes is an extremely interesting feature to be explored, especially in situations of contamination by metals, where the organisms are threatened by conditions that allow them to use up all resources that they have to survive in adversity. Concerning the efficiency of the most tolerant strains to metals *in vitro*, Ferreira et al. (2012) observed that *C. necator* strains are symbiotically efficient with legume trees. UFLA 02-71 strain was effective in symbiosis with *M. caesalpiniifolia*, and UFLA 01-659 strain demonstrated its symbiotic efficiency with the *M. pudica* and *L. leucocephala*. Both strains were highly competitive with soil native rhizobia, improving plant growth.

Several legume species have shown potential when used for land recovering and phytoremediation practices since they satisfactorily grow in TE contaminated soil (FERREIRA et al., 2012; MARQUES; MOREIRA; SIQUEIRA, 2000; TRANNIN; MOREIRA; SIQUEIRA, 2001; RANGEL et al., 2014). For example *Acacia mangium*, *Copaifera langsdorffii*, *Crotalaria juncea*, *E. contortisiliquum*, *Hymenaea courbaril*, *L. leucocephala*, *M. caesalpiniifolia*, *M. pudica*, *Platypodium gonoacantha* and *Stizolobium aterrimum* among others. The rhizobia and legume species diversity in Brazil (MOREIRA et al., 1993) represents important genetic resources for selecting genotypes that are well adapted to adverse conditions such as TE contaminated soils.

Plant-associated bacteria resistant to TE have been considered a promising tool to assist plants in their nutrient acquisition, reduce TE toxicity, immobilize/mobilize TE in the soil and recycle nutrients in soils where fertility is compromised by mineral extraction and/or processing of metals (CHERIAN et al., 2013; SESSITSCH et al., 2013; WEYENS et al., 2013). The term plant-associated bacteria comprise endophytic, phyllosphere and rhizosphere bacteria. Besides plant-associated bacteria, mycorrhizal fungi may enhance plant biomass production and influence metal tolerance and uptake by plants (CABRAL et al., 2015; WEYENS et al., 2013). Since this thesis will focus on plant-associated bacteria (rhizosphere), mycorrhizal mechanisms to enhance plant growth will not be discussed.

Plant-associated bacteria might increase phytoremediation efficiency on TE contaminated soils through two complementary ways (Figure 4): i) direct promotion of phytoremediation, in which plant-associated bacteria might enhance TE uptake and/or translocation, facilitating phytoextraction, or they might reduce TE mobility/availability, enhancing phytostabilization, and ii) indirect promotion of phytoremediation, in which plant-associated bacteria confer tolerance to TE to the plants and/or increase biomass production, facilitating TE removal/accumulation/stabilisation (RAJKUMAR et al., 2013; WEYENS et al., 2013.).

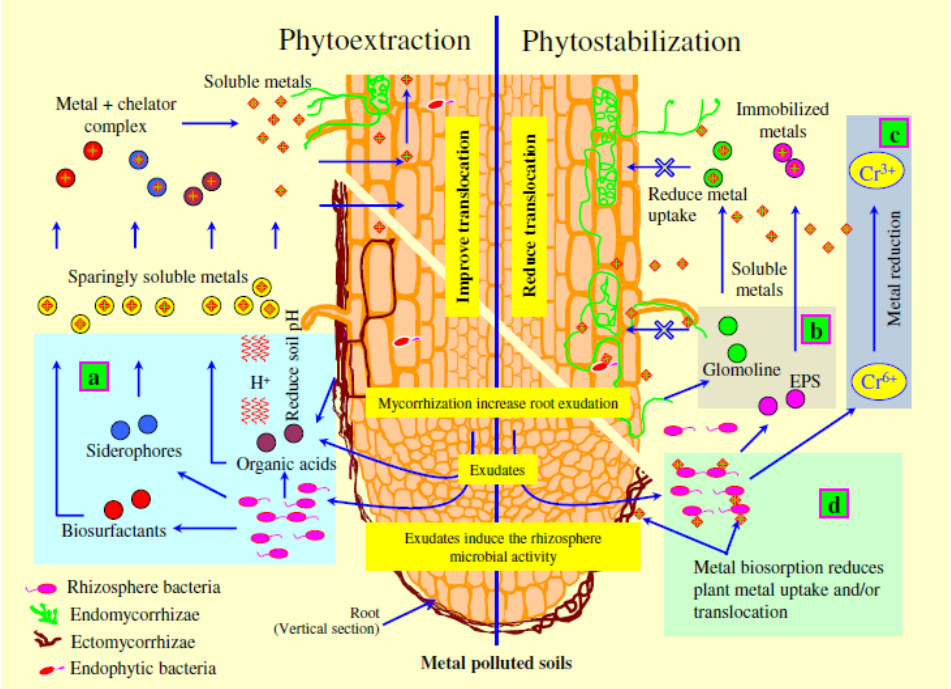

Figure 4. Plant-associated microbes can accelerate the phytoremediation process in metal contaminated soils by enhancing metal mobilization/immobilization. (a) Plant-associated microbes improve plant metal uptake by producing metal mobilizing chelators. Plant-associated microbes reduce plant metal uptake and/or translocation through (b) producing metal immobilizing metabolites, extracellular polymeric substances (EPS) (c) metal reduction and/or (d) metal biosorption. (RAJKUMAR et al., 2013).

Plant-associated bacteria can affect plant growth (mainly) in a positive or a negative way. Some causes diseases, impairing plant growth, whereas others may directly promote plant growth through a variety of mechanisms such as biological N_2 fixation, solubilizing insoluble phosphates, siderophore production, plant growth regulators and enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACC) production (Figure 5). Kloepper and Schroth (1978) have defined the rhizospheric bacterial community having beneficial effects for plant growth as ‘plant growth-promoting rhizobacteria (PGRP)’. Soil

microorganisms that promote plant growth may have different effects, since they depend on several rhizosphere conditions including soil organic matter, pH, temperature, nutrients and pollutants (BAIS et al., 2006; GLICK, 2003).

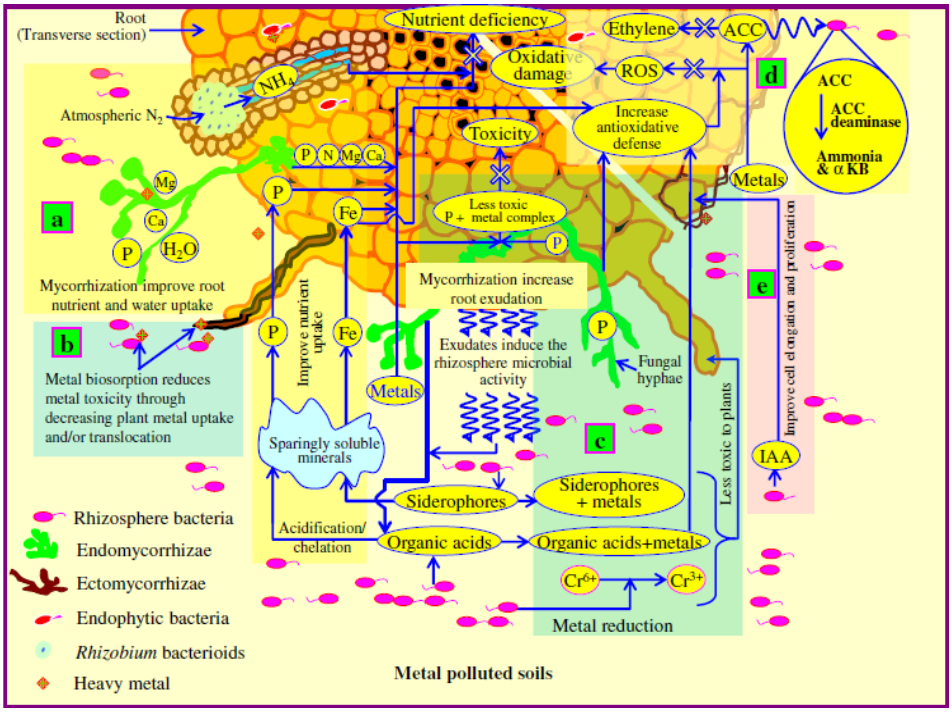

Figure 5. Schematic overview of direct plant growth promotion by plant-associated microbes in metal contaminated soils. (a) Plant-associated microbes improve plant nutrients and water uptake. Microbial metabolites reduce metal toxicity through (b) metal biosorption, (c) metal reduction and complexation reactions. Plant-associated microbes reduce heavy metal stress in plants through (d) increasing antioxidative defense and/or producing ACC deaminase and (e) improve the plant growth by producing plant growth regulators. Abbreviations: indole-3-acetic acid (IAA), reactive oxygen species (ROS), 1-aminocyclopropane-1-carboxylate (ACC), α ketobutyrate (α KB) (RAJKUMAR et al., 2013).

Also production of phytohormones that are mainly controlling plant growth, such as auxins, cytokinins, gibberellins and ethylene, is attributed to the presence of different plant-associated bacteria strains (FORCHETTI et al., 2007;

PERRIG et al., 2007; WEYENS et al., 2009). Plant-associated bacteria may indirectly improve plant growth by producing antibiotics, which may control pathogenic and harmful microorganisms (SIKORA; SCHAFER; DABABAT, 2007), besides suppressing growth or activity of pathogens by competing for space and nutrients (BACKMAN; SIKORA, 2008; WEYENS et al., 2009), inducing plant systemic resistance, producing hydrolytic enzymes that can lyse fungal cell walls or inhibiting enzymes or toxins produced by pathogenic microorganisms (GLICK; KARATUROVIC; NEWELL, 1995; GLICK et al., 2007; WEYENS et al., 2009). Therefore, to reduce TE toxicity and promote plant growth, extensive research efforts are needed to explore the microbial diversity, as well as its distribution and function on contaminated soil. Recently, Weyens et al. (2013) have reported the first observations of a field-related experiment concerning plant-associated bacteria and their role in the success or failure of metal phytoextraction projects in Belgium. A genotypic and phenotypic characterisation of the bacteria associated with two willow clones showed a more diverse and more metal-resistant plant growth promoting endophytic bacterial population associated with the clone with a twice as high metal accumulation than the endophytic population associated with the willow clone with a moderate metal extraction capacity.

2.5 Conclusions and perspectives

Native bacterial populations present in contaminated soils represent a source of well-adapted genotypes. On contaminated soils, especially mining soils, plant growth is threatened. It is well known that plant-associated bacteria are crucial and are able to enhance plant survival and growth on contaminated soils through several mechanisms. Therefore, using the microbial potential to support sustainable technologies such as phytotechnologies for revegetating mining areas might be of high interest. The challenge for the future is to further

unravel these complex interactions between plants and microbes, but also their interaction with the different kind of metal(oids) in the field.

REFERENCES

ACCIOLY, A. M. A.; SIQUEIRA, J. O. Contaminação química e biorremediação do solo. In: NOVAIS, R. F.; ALVAREZ, V. H.; SCHAEFER, C. E. (Ed.). **Tópicos em ciência do solo**. Viçosa, MG: Sociedade Brasileira de Ciência do Solo, 2000. v. 1, p. 299-352.

ANTON, A. et al. Characteristics of zinc transport by two bacterial cation diffusion facilitators from *Ralstonia metallidurans* CH34 and *Escherichia coli*. **Journal of Bacteriology**, Washington, v. 186, p. 7499-7507, 2004.

BACKMAN, P. A.; SIKORA, R. A. Endophytes: an emerging tool for biological control. **Biological Control**, Orlando, v. 46, p. 1-3, 2008.

BAIS, H. P. et al. The role of root exudates in rhizosphere interactions with plants and other organisms. **Annual Review of Plant Biology**, Palo Alto, v. 57, p. 233-266, 2006.

BRIX, H. Use of constructed wetlands in water pollution control: historical development, present status, and future perspectives. **Water Science and Technology**, Oxford, v. 30, p. 209-223, 1994.

BRUINS, M. R.; KAPIL, S.; OEHME, F. W. Microbial resistance to metals in the environment. **Ecotoxicology and Environmental Safety**, New York, v. 45, p. 198-207, 2000.

CABRAL, L. et al. Arbuscular mycorrhizal fungi in phytoremediation of contaminated areas by trace elements: mechanisms and major benefits of their applications. **World Journal of Microbiology and Biotechnology**, Oxford, 2015. DOI 10.1007/s11274-015-1918-y. Epub ahead of print.

CHEN, W. M. et al. *Ralstonia taiwanensis* sp. nov. isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 51, p. 1729-1735, 2001.

CHERIAN, S. et al. Phytoremediation of trace element-contaminated environments and the potential of endophytic bacteria for improving this process. **Critical Reviews in Environmental Science and Technology**, Boca Raton, v. 42, p. 2215-2260, 2012.

COMPANHIA AMBIENTAL DO ESTADO DE SÃO PAULO. Informações toxicológicas. 2012. Available at: <http://www.cetesb.sp.gov.br/tecnologia-ambiental/laboratorios/109-informacoes-toxicologicas>. Access on March 28th, 2013.

COSTA, G. S. et al. Nutrient input through litter in a degraded área revegetated with legume trees. **Revista Brasileira de Ciência do Solo**, Viçosa, MG, v. 28, p. 919-927, 2004.

CUNNINGHAM, S. D.; BERTI, W. R.; HUANG, J. W. Phytoremediation of contaminated soils. **Trends in Biotechnology**, Amsterdam, v. 13, p. 393-397, 1995.

CUNNINGHAM, S. D.; OW, D. W. Promises and prospects of phytoremediation. **Plant Physiology**, Bethesda, v. 110, p. 715-719, 1996.

FERREIRA, P. A. A. et al. Symbiotic efficiency of *Cupriavidus necator* strains tolerant to zinc, cadmium, copper and lead. **Pesquisa Agropecuária Brasileira**, Rio de Janeiro, v. 47, p. 85-95, 2012.

FLORENTINO, L. A. et al. Physiological and symbiotic diversity of *Cupriavidus necator* strains isolated from nodules of Leguminosae species. **Scientia Agricola**, Piracicaba, v. 69, p. 247-258, 2012.

FLORENTINO, L. A. et al. *Sesbania virgata* stimulates the occurrence of its microsymbiont in soils but does not inhibit microsymbionts of other species. **Scientia Agricola**, Piracicaba, v. 66, p. 667-676, 2009.

FORCHETTI, G. et al. Endophytic bacteria in sunflower (*Helianthus annuus* L.): isolation, characterization, and production of jasmonates and abscisic acid in culture medium. **Applied Microbiology and Biotechnology**, Berlin, v. 76, p. 1145-1152, 2007.

FRANCO, A. A. et al. **Revegetação de Solos Degradados**. Rio de Janeiro: EMBRAPA-CNPAB, 1992. (Comunicado Técnico, n. 9).

FRANCO, A. A. et al. Use of nodulated and mycorrhizal legume trees for revegetation of residues from bauxite mining. In: INTERNATIONAL SYMPOSIOM ON SUSTAINABLE AGRICULTURE FOR THE TROPICS – THE ROLE OF NITROGEN FIXATION, 1995, Angra dos Reis. **Abstracts**... Seropédica: EMBRAPA-CNPAB; UFRRJ; Brazilian Academy of Sciences, 1995. p. 80.

FRANCO, A. A.; FARIA, S. M. The contribution of N_2-fixing tree legumes to land reclamation and sustainability in the tropics. **Soil Biology and Biochemistry**, Elmsford, v. 29, p. 897-903, 1997.

GLICK, B. R. et al. Promotion of plant growth by bacterial ACC deaminase. **Critical Reviews in Plant Sciences**, Boca Raton, v. 26, p. 227-242, 2007.

GLICK, B. R.; KARATUROVIC, D. M.; NEWELL, P. C. A novel procedure for rapid isolation of plant-growth promoting pseudomonads. **Canadian Journal of Microbiology**, Ottawa, v. 41, p. 533-536, 1995.

GLICK, B. R. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. **Biotechnology Advances**, New York, v. 21, p. 383-93, 2003.

HANEY, C. J. et al. New developments in the understanding of the cation diffusion facilitator family. **Journal of Industrial Microbiology and Biotechnology**, Hampshire, v. 32, p. 215-226, 2005.

HARTLEY, W. et al. Arsenic mobility in brownfield soils amended with green waste compost or biochar and planted with *Miscanthus*. **Environmental Pollution**, Barking, v. 157, p. 2654-2662, 2009.

HASSAN, M. T. et al. Identification of a gene cluster, czr, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. **Gene**, Amsterdam, v. 238, p. 417-425, 1999.

JI, G.; SILVER, S. Bacterial resistance mechanisms for heavy metals of environmental concern. **Journal of Industrial Microbiology**, Amsterdam, v. 14, p. 61-75, 1995.

KLOEPPER, J. W.; SCHROTH, M. N. Plant growth-promoting rhizobacteria on radishes. In: PROCEEDINGS OF THE 4TH INTERNATIONAL CONFERENCE ON PLANT PATHOGENIC BACTERIA, 2., 1978, Angers. **Abstracts**... Angers: INRA, 1978. p. 879-882

LEGATZKI, A. et al. Interplay of the Czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. **Journal of Bacteriology**, Washington, v. 185, p. 4354-4361, 2003.

LIU, Z. et al. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 99, p. 6053-6058, 2002.

LOPEZ, M. V. **Microbiota edáfica como indicadora da reabilitação de áreas contaminadas por elementos-traço**. 2010. 60 p. Dissertação (Mestrado em Microbiologia Agrícola) - Universidade Federal de Lavras, Lavras, 2010.

MARQUES, T. C. L. L. S. M.; MOREIRA, F. M. S.; SIQUEIRA, J. O. Growth and metal concentration of seedlings of woody species in a heavy metal contaminated soil. **Pesquisa Agropecuária Brasileira**, Rio de Janeiro, v. 35, p. 121-132, 2000.

MATSUDA, A.; MOREIRA, F. M. S.; SIQUEIRA, J. O. Tolerance of rhizobia genera from different origins to zinc, copper and cadmium. **Pesquisa Agropecuária Brasileira**, Brasília, v. 37, p. 343-355, 2002a.

MATSUDA, A.; MOREIRA, F. M. S.; SIQUEIRA, J. O. Survival of *Bradyrhizobium* and *Azorhizobium* in heavy metal contaminated soil. **Revista Brasileira de Ciência do Solo**, Viçosa, MG, v. 26, p. 249-256, 2002b.

MERGEAY, M. et al. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. **FEMS Microbiology Reviews**, Amsterdam, v. 27, p. 385-410, 2003.

MERGEAY, M.; HOUBA, C.; GERITS, J. Extrachromosomal inheritance controlling resistance to cadmium, cobalt, copper and zinc ions: evidence from curing in a *Pseudomonas* [proceedings]. **Archives internationales de physiologie et de biochimie**, Liege, v. 86, p. 440-442, 1978.

MOREIRA, F. M. S. et al. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacylamide gel electrophoresis of their total proteins. **Systematic and Applied Microbiology**, Stuttgart, v. 16, p. 135-146, 1993.

MOSTASSO, F. L. **Crescimento e nodulação de leguminosas em solo contaminado com metais pesados**. 1997. 50 p. Dissertação (Mestrado em Ciência do Solo) - Universidade Federal de Lavras, Lavras, 1997.

MOULIN, L. et al. Nodulation of legumes by members of the β-subclass of Proteobacteria. **Nature**, London, v. 411, p. 948-950, 2001.

NASCIMENTO, C. W. A.; AMARASIRIWARDENA, D.; XING, B. Comparison of natural organic acids and synthetic chelates at enhancing phytoextraction of metals from a multimetal contaminated soil. **Environmental Pollution**, Barking, v. 140, p. 114-123, 2006.

NIES, D. H. Efflux-mediated heavy metal resistance in prokaryotes. **FEMS Microbiol Review**, Amsterdam, v. 27, p. 313-339, 2003.

NIES, D. H. Microbial heavy-metal resistance. **Applied Microbiology and Biotechnology**, Berlin, v. 51, p. 730-750, 1999.

NIES, D. H.; SILVER, S. Ion efflux systems involved in bacterial metal resistances. **Journal of Industrial Microbiology**, Amsterdam, v. 14, p. 186-199, 1995.

PERRIG, D. et al. Plant-growth promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. **Applied Microbiology and Biotechnology**, Berlin, v. 75, p. 1143-1150, 2007.

RAJKUMAR, M. et al. Perspectives of plant-associated microbes in heavy metal phytoremediation. **Biotechnology Advances**, New York, v. 30, p. 1562-1574, 2013.

RANGEL, W. M. et al. Phytoprotective Effect of Arbuscular Mycorrhizal Fungi Species against Arsenic Toxicity in Tropical Leguminous Species. **International Journal of Phytoremediation**, Philadelphia, v. 16, p. 840-858, 2014.

RENSING, C.; GHOSH, M.; ROSEN, B. Families of soft-metal-ion-transporting ATPases. **Journal of Bacteriology**, Washington, v. 181, p. 5891-5897, 1999.

ROSS, S. **Toxic metals in soil-plant systems**. Chichester: J. Wiley, 1994. 484 p.

SANTOS, J. V. **Biomassa e atividade microbiana como indicadoras da reabilitação de áreas contaminadas por elementos-traço**. 2010. 63 p. Dissertação (Mestrado em Microbiologia Agrícola) - Universidade Federal de Lavras, Lavras, 2010.

SESSITSCH, A. et al. The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. **Soil Biology and Biochemistry**, Elmsford, v. 60, p. 182-194, 2013.

SIKORA, R. A.; SCHAFER, K.; DABABAT, A. A. Modes of action associated with microbially induced in planta suppression of plant–parasitic nematodes. **Australasian Plant Pathology**, Murdoch, v. 36, p. 124-134, 2007.

SILVA, K. et al. *Cupriavidus necator* isolates are able to fix nitrogen in symbiosis with different legume species. **Systematic and Applied Microbiology**, Stuttgart, v. 35, p. 175-182, 2012.

SILVER, S.; PHUNG, L.T. A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. **Journal of Industrial Microbiology and Biotechnology**, Hampshire, v. 32, p. 587-605, 2005.

SIQUEIRA, J. O.; SOARES, C. R. F. S.; SILVA, C. A. Matéria orgânica em solos de áreas degradadas. In: SANTOS, G. A. et al. (Ed.). **Fundamentos da Matéria Orgânica do Solo**: ecossistemas tropicais e subtropicais. 2. ed. Porto Alegre: Metrópole, 2008. 654 p.

STEPHENS, J. H. G.; RASK, H. M. Inoculant production and formulation. **Field Crops Research**, Amsterdam, v. 65, p. 249-258, 2000.

TRANNIN, I. C. B. et al. Tolerance of *Bradyrhizobium* and *Azorhizobium* strains and isolates to copper, cadmium and zinc "in vitro". **Revista Brasileira de Ciência do Solo**, Viçosa, MG, v. 25, p. 305-316, 2001.

TRANNIN, I. C. B.; MOREIRA, F. M. S.; SIQUEIRA, J. O. Growth and nodulation of *Acacia mangium*, *Enterolobium contortisiliquum* and *Sesbania virgata* in heavy metal contaminated soil. **Revista Brasileira de Ciência do Solo**, Viçosa, MG, v. 25, p. 743-753, 2001.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. **Arsenic Treatment Technologies for Soil, Waste, and Water**. Office of Solid Waste and Emergency Response. EPA-542-R-02-004, 2002. Available at: <http://www.clu-in.org/download/remed/542r02004/arsenic_report.pdf>. Access on August 5th, 2010.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. **Introduction to Phytoremediation**. Office of Research and Development. EPA-600-R-99-107, 2002. Available at: <http://clu-in.org/download/remed/introphyto.pdf>. Access on August 5th, 2010.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. **Recent Development for In-situ Treatment of Metal Contaminated Soils**. Office of Solid Waste and Emergency Response. EPA-542-R-97-004, 1997. Available at: <https://clu-in.org/download/remed/metals2.pdf>. Access on August 5th, 2010.

WEYENS, N. et al. Exploiting plant-microbe partnerships to improve biomass production and remediation. **Trends in Biotechnology**, Amsterdam, v. 27, p. 591-598, 2009.

WEYENS, N. et al. Planta-associated bacteria and their role in the success or failure of metal phytoextracyion projects: first observations of a field-related experiment. **Microbial Biotechnology**, London, v. 6, p. 288-299, 2013.

YOUNG, J. P. W. Sex and the single cell: The population ecology and genetics of microbes. In: RITZ, K.; DIGHTON, J.; GILLER, K. E. (Ed.). **Beyond the biomass**. Chichester: J. Wiley, 1994. p. 101-107.

Second part - Papers

PAPER 1 - LEGUMINOSAE NODULATING BACTERIA ISOLATED FROM A GOLD MINE AS-CONTAMINATED SOIL ARE ABLE TO PROMOTE PLANT GROWTH

According to *International Journal of Phytoremediation*

Leguminosae nodulating bacteria isolated from A gold minE As-contaminated soil are able to promote plant growth

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Abstract

Efficient N_2-fixing Leguminosae nodulating bacteria tolerant to As can contribute to phytoremediation of As contaminated areas. In order to identify bacteria with these features twenty-four strains were isolated from nodules of *Crotalaria spectabilis* (12) and *Stizolobium aterrimum* (12), which were growing on an As-contaminated soil in a gold mine area. Partial sequencing of the 16S rDNA gene revealed that most of those strains belong to the group of α-Proteobacteria, being representatives of the genera *Bradyrhizobium*, *Rhizobium*, *Bosea*, *Inquilinus*, *Labrys*, *Starkeya* and *Methylobacterium*. These strains were characterized symbiotically under axenic conditions with their original host, and screened *in vitro* for their plant growth promoting traits (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 studied as well. 33.3% of the strains isolated from nodules of *C. spectabilis* showed a higher relative nitrogen

fixing efficiency (RE%) than the control treatment supplied with high mineral nitrogen concentration (HN), and 58.3% demonstrated a RE% statistically similar to the control treatment HN. 50% of the strains isolated from nodules of *S. aterrimum* exhibited a RE% statistically similar to the control treatment HN, highlighting their symbiotic efficiency for supplying legume plant species with nitrogen. After the phenotypic characterization, strains representative for phenotypic groups were formed, in addition to 7 reference or type strains belonging to the genera *Azorhizobium* (BR 5401^T, ORS 571^T), *Bradyrhizobium* (BR 2001, BR 2811), *Mesorhizobium* (BR 3804), *Rhizobium* (CIAT 899T) and *Burkholderia* (LMG 1222^T). They were evaluated for their As tolerance on 79 solid medium, supplemented with As concentrations ranging from 0 to 200 mmol L^{-1}. The most tolerant strains were also evaluated for their resistance to different *β*-lactam antibiotics. UFLA 05-16 strain (*Rhizobium tropici*) tolerated the highest As concentration tested and exposed a wide resistance spectrum to *β*-lactam antibiotics. It further showed also efficient in fixing N_2 when in symbiosis with *Crotalaria spectabilis* and tested positive for all plant-growth promoting traits evaluated. Thus, all these features together highlighted its potential for phytoremediation purposes.

Keywords: plant-growth promoting, biological N_2 fixation, metal(oid) multi-resitance, *β*-lactam antibiotics resistance.

1 Introduction

Soil microorganisms performing biological services such as biological N_2 fixation (BFN), have been recognized as important allies for phytoremediation because they can improve the nutritional status of the plant, positively influencing tolerance to the excess of metal(oid)s.

The symbiotic relationship between legume plants and rhizobia has a great potential for reclaiming mining areas (Franco et al., 1992; Franco et al., 1995), due to many advantages, for instance: i) soil protection, ii) ecosystem enrichment with N, iii) great land cover supply, iv) soil restoration, and v) supply of flora and fauna diversity (Siqueira et al., 2008). Moreover, the symbiosis rhizobia-legume can replace ammoniacal fertilizers, which acidify the soil increasing trace elements availability.

Many authors have demonstrated trace elements tolerance for different rhizobia genera (Purchase et al., 1997; Trannin et al., 2001; Matsuda et al., 2002a; Carrasco et al., 2005). Currently most of the research is focused on strains genetically engineered to use them in bioremediation processes (Valls et al., 2000; Sriprang et al., 2002; Wu et al., 2006). However, native bacterial populations from soils with high metal(oid) content are probably most adapted to metal(oid)-induced stress. Therefore the isolation of bacteria surviving in these conditions can provide important information concerning genetic resources more adapted to metal(oid) stress (Young, 1994; Trannin et al., 2001; Weyens et al., 2009a). Moreover, inoculating legume plants with strains efficient in N_2

fixation, and well adapted to these environmental conditions features great relevance from an ecological as well as economical viewpoint (Franco et al., 1995; Franco & Faria, 1997; Stephens & Rask, 2000; Costa et al., 2004).

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

2 Materials and Methods

2.1 Isolation and cultural characterization

Bacteria were isolated from legume nodules collected in a gold mine area contaminated with arsenic, in the northwest region of Minas Gerais, Brazil. Nodules were sampled from *S. aterrimum* (17° 10’59.88”S 46° 52’24.11”W) and *C. spectabilis* plants (17° 8’10.99”S 46° 51’31.75”W). Soil chemical and physical parameters (0-20 cm) from

mining area contaminated with As are presented in Table 1. Phosphorus and potassium were determined by Mehlich 1 extraction (HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}), calcium, magnesium and aluminium were determined by KCl extraction (1 mol L^{-1}). The potential acidity (H + Al) was estimated by SMP extraction, organic matter was determined by oxidation using $Na_2Cr_2O_7$ + H_2SO_4 (10N) (EMBRAPA, 2011). According to 5th Approach (Guidelines for lime and fertilizers use in Minas Gerais) (Ribeiro et al., 1999), 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 (Ribeiro et al., 1999). The soil texture was determined by the pipette method according to Day (1965), 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 silt loam.

Table 1 – Soil chemical and physical parameters (0-20 cm) from mining area contaminated with As.

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

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

[1]Chemical parameters: pH – H_2O pH (ratio 1:2,5); P-rem (remaining phosphorus); [2]P (phosphorus), [2]K (potassium) - Mehlich 1 extractor (HCl 0,05 mol L^{-1} + H_2SO_4 0,0125 mol L^{-1}); [3]Ca (calcium), [3]Mg (magnesium), [4]Al (aluminium) – KCl extractor 1 mol L^{-1}; [4]H + Al (hydrogen + aluminium) – SMP extractor; [5]OM (Organic matter) – oxidation using $Na_2Cr_2O_7$ + H_2SO_4 10N (EMBRAPA, 2011); Arsenic available[6] and semitotal[7] content.

Nodules were surface disinfected according to Vincent (1970) and the bacteria were isolated on 79 solid culture medium (Fred & Waksman, 1928; Vincent, 1970) with bromothymol blue (pH 6.9, 28 ºC). After purification of the single colonies, the following characteristics were evaluated: pH change of the culture medium, growth rate, colonies' characteristics (diameter, form, edge, lifting, surface, light transmission, colour, and bromothymol blue absorption) and exopolysaccharide (EPS) production. The characterization was done according to Moreira et al., (1993) and Jesus et al., (2005). The onset of the colonies was observed considering the following ranges of days: 2-3 days fast growth; 4-5 days intermediary growth; 6-9 days slow growth. The range of EPS production was classified as scarce, little, moderate and abundant.

Strains were clustered considering the cultural characterization together with type or reference strains of the genera: *Azorhizobium* (*A. caulinodans* – ORS571^{T}; *A. doebereinerae* – BR 5401^{T}), *Mesorhizobium* (*M. plurifarium* – BR 3804), *Rhizobium* (*R. tropici* – CIAT 899^{T}), *Burkholderia* (*B. cepacia* – LMG 1222^{T}), and strains of the genus *Bradyrhizobium* (*Bradyrhizobium* sp. – BR 2001 e BR 2811). The strain BR 2811 is the inoculum for *C. spectabilis* and *S. aterrimum* plant

species, approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply. All the strains were clustered considering 11 cultural characteristics and the similarity dendrogram was made using the WARD's minimum variance method, and assessing the binary distance by cluster package on R program (Figure S1).

2.2 Genotypic characterization

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

The PCR was performed using 50 ng of the extracted DNA, 45 µL PCR reaction mixture containing 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.2 µM 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.2 µM 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991), 1 U Taq DNA polymerase (Fermentas), 10x KCl buffer, and ultrapure sterile water. The amplification was performed using an Eppendorf Mastercycler® under the following conditions: the initial denaturation step at 94 °C for 5 min, 40 denaturation cycles at 94 °C for 40 s, the annealing step at 55 °C for 40 s, the extension step at 72 °C for 1.5 min,

and the final extension at 72 °C for 7 min. The obtained PCR products were purified and sequenced by Macrogen (South Korea).

The sequences' quality was checked using the Bionumerics 6.5 program (Applied Maths, Sint-Martens-Latem, Belgium), and they were submitted to the BLAST (Basic Local Alignment Search Tool) by comparing them with the GenBank sequences (NCBI – National Center for Biotechnology Information).

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 bacteria strains isolated from the trap species *Crotalaria spectabilis* and *Stizolobium aterrimum* nodules were checked in a greenhouse experiment for each trap species under axenic conditions.

The seeds were scarified using H_2SO_4 PA (*C. spectabilis* for 5 min and *S. aterrimum* for 45 min), and placed on sterile Petri dishes containing moistened cotton incubated at 28 °C until radicle emergence. The strains were grown in 79 liquid medium shaking (125 rpm, 28 °C) for 120 hours. At the moment of planting, each seed was inoculated with 1 mL of the bacterial inoculum containing about 10^8 cells. After planting

and inoculating the seeds, a thin layer of the sterile mixture of sand-benzene-paraffin was disposed on the top to avoid contamination. Two plants were grown in sterile Leonard jars for 45 days. Sand and vermiculite (1:1 ratio) were used as substrate in the top portion of the jars, and in the lower portion a four-fold dilution of modified Hoagland nutrient solution (Hoagland & Arnon, 1950) was added. The inoculated plants and the non-inoculated control plants had a low nitrogen concentration (5.25 $mg \cdot L^{-1}$) in the nutrient solution, which is considered a starting dose for, and not an inhibitor of, the biological nitrogen fixation process. The following quantities of the stock solutions were added to 4 L of water: 0.4 mL of 236.16 g L^{-1} $CaN_2O_6 \cdot 4H_2O$; 0.1 mL of 115.03 g L^{-1} $NH_4H_2PO_4$; 0.6 mL of 101.11 g L^{-1} KNO_3; 2.0 mL of 246.9 g L^{-1} $MgSO_4 \cdot 7H_2O$; 3.0 mL of 87.13 g L^{-1} K_2SO_4; 10 mL of 12.6 g L^{-1} $CaH_4P_2O_8 \cdot H_2O$; 200 mL of 1.72 g L^{-1} $CaSO_4 \cdot 2H_2O$; 1 mL of 10 g L^{-1} $FeCl_3$, and 1 mL of micronutrients (2.86 mg L^{-1} H_3BO_3; 2.03 mg L^{-1} $MnSO_4 \cdot 4H_2O$; 0.22 mg L^{-1} $ZnSO_4 \cdot 7H_2O$; 0.08 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and 0.09 mg L^{-1} $Na_2MoO_4 \cdot H_2O$). In addition, a control treatment supplemented with a high mineral nitrogen concentration (52.5 $mg \cdot L^{-1}$) was also provided. Besides the negative control treatments (low and high

N content) a positive control treatment inoculated with BR 2811 strain (*Bradyrhizobium* sp.), which has been approved as inoculant for both plant species by the Brazilian Ministry of Agriculture, was also added. The assays were completely randomized and performed using 4 replicates. The harvest was done after 45 days including the following measurements: nodule number and dry weight (NN and 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 mineral nitrogen content, using the formula RE = [(inoculated SDW / high N SDW) x 100] where RE means relative efficiency, inoculated SDW means shoot dry weight of the inoculated treatment, and high N SDW means shoot dry weight of the control treatment supplied with high mineral nitrogen content.

The data were analysed by ANOVA using the statistical program SISVAR (Ferreira, 2011). The NN and NDW were transformed using the formula $(x+0.5)^{0.5}$. The average of the treatments were 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 obtained cultural groups 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 with pH 6.9, using an orbital shaker (125 rpm) at 28 °C. After growth, 1 mL of each cultural strain containing about 10^8 cells was transferred to sterile microtubes (1.5 mL), which were centrifuged at 8,000 g at 25 °C for 4 min. The supernatant was discarded, the cells were resuspended in 1 mL sterile NaCl (8.5 g L^{-1}), and centrifuged again. This ¨washing¨ procedure was repeated three times. After that, a 20 µL aliquot of the cell suspension was inoculated on 79 solid medium with different As concentrations. Arsenic ($Na_2HAsO_4 \cdot 7H_2O$) was used at concentrations of 50, 100, 150 and 200 mmol L^{-1}, in addition to a control treatment without As. After adding As to the medium, the pH was adjusted to 6.9 using HCl (0.5 mol L^{-1}). The susceptibility of the strains to As was analysed by determining the minimum inhibitory concentration (MIC), which is 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 constructing the similarity dendrogram using the strains' cultural characteristics, the frequency of tolerant individuals at different As-concentrations within the groups formed by the cultural characterization was analysed using the chi-squared test at 5% significance.

2.4.2 Pattern of *β*-lactam antibiotics resistance

Bacteria were grown on 79 liquid medium for 72 h at 28 °C, after which a 0.1 mL aliquot of the bacterial inoculum was spread over 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) (Bauer et al., 1966). The strains were defined as sensitive if the radius zone was observed or resistant if any radius zone was formed after 48 hours at 28 °C. The strains were grown in triplicate.

2.4.3 Plant growth promoting traits

Purified bacterial strains have been screened for their plant growth promoting (PGP) traits such as production of organic acids (OA) and indole-3-acetic-acid (IAA), ACC deaminase activity (ACC), siderophores production (SID), and $Ca_3(PO_4)_2$ phosphate solubilization (Table 2). Moreover their multi-element resistance was also studied by checking tolerance to Cd and Zn (Table 4) (Weyens et al., 2013; Croes et al., 2013).

Phosphate solubilization ability was evaluated in solid medium (Nautiyal, 1999), 25 µl aliquots of inoculum were inoculated in holes (Ø: 0.5 cm). Strains that produced a clear zone around the hole were considered positive. Siderophore production was qualitatively evaluated by the universal colorimetrical method of Schwyn and Neilands (1987) using the blue chromium-azurol S (CAS) reagent. 50 µl inoculum were inoculated in 800 µl of selective 284 medium with a carbon mix (CMIX) and 0, 0.25 and 3 mM Fe (respectively deficient, optimal and over supply of Fe). Bacterial organic acid production was detected according to the colorimetric method of Cunningham and Kuiack (1992) by adding the alzarine red S pH indicator. 20 µl inoculum were inoculated in 800 µl of sucrose tryptone (ST) medium. Bacterial IAA production capacity was tested by using the Salkowski assay (adapted from Patten and Glick,

2002). ACC deaminase activity was evaluated by using a slightly modified protocol according to Belimov et al. (2005).

For testing the trace element tolerance, all strains were plated on selective 284 medium (Weyens et al., 2013) with a carbon mix (CMIX) (per litre of medium: 0.54 g of fructose, 0.66 g of gluconate, 0.52 g of glucose, 0.7 g of lactate and 0.81 g of succinate) and 0, 0.4 and 0.8 mM $CdSO_4$ or 0, 0.6 and 1.0 mM $ZnSO_4$. An aliquot of 20 µl was used and six repetitions were performed per plate. Tolerance was rated visually checking the growth and the polysaccharide (mucus) production on the plate. In this case, the same exopolysaccharide production pattern applied to rhizobia cultural characterization was used (i.e. scarce, low, moderate and abundant).

3 Results

3.1 Isolation, cultural characterization and identification of the strains by 16S rDNA 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*. Strains clustered in two main groups (Figure S1 - Table S1), using the Ward's hierarchical clustering method.

Table S1 – Main cultural characteristics of groups A and B formed by cluster dendrogram.

Strains	Main cultural characteristics of group A[(1)]			Strains	Main cultural characteristics of group B[(1)]		
	1	2	3		1	2	3
UFLA 05-22	2-3	Alkaline	Little amount	UFLA 05-13	6-9	Neutral	Abundant
UFLA 05-10	2-3	Alkaline	Little amount	UFLA 05-12	6-9	Neutral	Moderate
UFLA 05-03	2-3	Alkaline	Little amount	UFLA 05-11	6-9	Alkaline	Moderate
UFLA 05-01	2-3	Alkaline	Little amount	UFLA 05-19	6-9	Alkaline	Moderate
UFLA 05-02	2-3	Alkaline	Little amount	UFLA 05-20	6-9	Alkaline	Moderate
UFLA 05-21	2-3	Alkaline	Little amount	BR 2811	6-9	Alkaline	Abundant
UFLA 05-09	2-3	Alkaline	Little amount	BR 2001	6-9	Alkaline	Abundant
UFLA 05-14	2-3	Alkaline	Little amount	UFLA 05-18	4-5	Alkaline	Abundant
UFLA 05-23	2-3	Alkaline	Little amount	UFLA 05-17	6-9	Alkaline	Little amount
BR 5401^{T}	2-3	Alkaline	Scarce	UFLA 05-24	6-9	Alkaline	Little amount
ORS 571^{T}	2-3	Alkaline	Scarce	UFLA 05-15	2-3	Acid	Little amount
				BR 3804	4-5	Acid	Abundant
				UFLA 05-16	2-3	Acid	Abundant
				CIAT 899^{T}	2-3	Acid	Abundant
				UFLA 05-05	2-3	Acid	Little amount
				UFLA 05-07	2-3	Acid	Moderate
				UFLA 05-04	2-3	Neutral	Moderate
				LMG 1222^{T}	2-3	Neutral	Moderate
				UFLA 05-06	2-3	Acid	Moderate
				UFLA 05-08	2-3	Alkaline	Moderate

[(1)]Main cultural strain characteristics – 1 (Growth rate in days: 2-3 fast growth; 4-5 intermediary, and 6-9 slow); 2 (pH on 79 medium after growth); 3 (Exopolysaccharides production).

Group A was formed by strains that alkalinize the 79 medium, with fast growth, and exopolysaccharide (EPS) production classified as little or scarce. 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. were clustered into this group (Table 2), as well as the two reference strains for the genus *Azorhizobium*, BR 5401^{T} and ORS571^{T}.

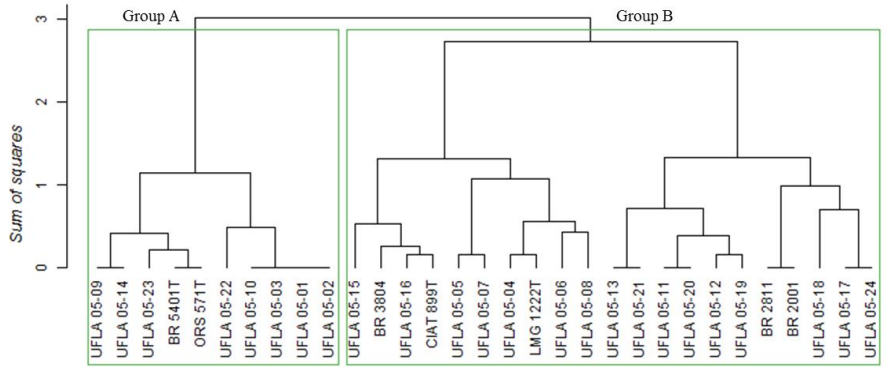

Figure S1 - Dendrogram based on bacterial phenotypic characteristics of strains isolated from nodules of *S. aterrimum* and *C. spectabilis* plants growing on As-contaminated soil. Reference and type strains are *Azorhizobium* (BR 5401^T and ORS 571^T), *Bradyrhizobium* (BR 2001 and BR 2811), *Mesorhizobium*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

Within group A, two small groups were formed, one represented by strains belonging to the Bradyrhizobiaceae family (*Bradyrhizobium* sp. and *Bosea* sp. strains), and the second group composed of strains belonging to the Bradyrhizobiaceae family (*Bradyrhizobium* sp.), in addition to strains from the Methylobacteriaceae family (*Methylobacterium* sp.) and the Xanthobacteraceae family (*Starkeya novella* and the representative species *A. caulinodans* - ORS571^T, and *A. doebereinereae* - BR 5401^T). The strains belonging to the Bradyrhizobiaceae family in group A showed fast growth and little EPS production (Table S1).

The nucleotide sequence of 16S rDNA of the species *Bosea* sp., isolated in our study, showed 100% similarity with the sequence of the *Bosea* sp. S41RM2 species deposited in GenBank, with the access number GU731243.1. The origin of that species is also an As-contaminated soil (Sultana et al., 2012).

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

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

***bp – base pairs of 16S rDNA sequence. #Identification based on 16S rDNA sequences using forward primer 27F or reverse primer 1392R(R). **Classification conferred according to the color intensity. OA: organic acid production; IAA: indole-3-acetic acid production; ACC: 1-aminocyclopropane-1-carboxylate deaminase activity; SID: siderophores production. ***Based on the $Ca_3(PO_4)_2$ 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.**

Group B consisted of strains with very different cultural characteristics (Figure S1; Table S1). Into this group, there are strains that acidify or alkalinize the pH of the 79 medium, as well as strains that don't change the pH, leaving it neutral. The growth rate and EPS production characteristics are also very different into this group, since strains with fast, intermediate and slow growth, and with little, moderate and abundant EPS production are clustering together (Table S1). Most strains in group B belong to the Bradyrhizobiaceae family, these strains showed slow growth rates, and a moderate or abundant EPS production. Besides the Bradyrhizobiaceae family, group B includes representatives of Rhizobiaceae, Phyllobacteriaceae, Xanthobacteraceae, Rhodospirillaceae and Burkholderiaceae families as well. Even a strain representative for the Bacillaceae family, phylum Firmicutes was isolated (Figure S1; Table 2). The following type or reference strains were also clustered in group B: *Bradyrhizobium* sp. (BR 2001 and BR 2811), *Mesorhizobium plurifarium* (BR 3804), *Rhizobium tropici* (CIAT 899^{T}) and *Burkholderia cepacia* (LMG 1222^{T}).

The 16S rDNA partial sequencing showed representatives for two phyla, Firmicutes and Proteobacteria (Table 2). The phylum

Proteobacteria showed representatives of two groups, *α* and *β*-Proteobacteria. Most of the strains belong to the *α*-Proteobacteria genera such as *Bradyrhizobium*, *Rhizobium*, *Bosea*, *Inquilinus*, *Labrys*, *Starkeya* and *Methylobacterium*.

3.2 Strain authentication and symbiotic efficiency

In the greenhouse experiment, nodulation was not observed for the control treatments (without inoculation and supplied with 5.25 mg L^{-1} or 52.5 mg L^{-1} of mineral N), confirming the absence of contamination, in both experiments for *C. spectabilis* and *S. aterrimum* plants.

Taking this into account, it's possible to check the authentication of symbiosis and the symbiotic efficiency of the isolated strains. *C. spectabilis* plants established symbiosis with all strains tested, including the inoculant strain BR 2811 (Table 3). Without exception all the strains were efficient to fix N_2 symbiotically with *C. spectabilis*, showing NAS and RE% higher or similar to the control treatment supplied with high mineral N concentration (52.5 mg L^{-1}). Concerning the SDW production, only UFLA 05-01 (*Bradyrhizobium* sp.) strain showed a lower SDW in comparison with the high mineral N concentration treatment, but it is still higher when compared with the low mineral N concentration and the BR

2811 strain treatments. The efficiency of all the strains was higher even in comparison with the strain BR 2811 approved by MAPA as *C. spectabilis* plant inoculant. 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 the high mineral N concentration (52.5 mg L^{-1}) was supplied. Moreover these two above-mentioned strains and UFLA 05-14 (*Bradyrhizobium* sp.) and UFLA 05-07 (*Inquilinus* sp.) also showed a higher RE% than the control treatment supplied with high mineral N concentration (52.5 mg L^{-1}) (Table 3). Interestingly this latter strain and also UFLA 05-08 (*Labrys monachus*) are atypical genera normally found nodulating legume plants. Although these strains were able to efficiently nodulate and fix N_2 in symbiosis with *C. spectabilis* a further study for checking specific genes for those processes need to be performed.

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

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

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

Concerning the strains isolated from nodules of *S. aterrimum*, 8 strains of 12 were able to establish symbiosis, inducing the 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. UFLA 05-11 (*B. elkanii*) and UFLA 05-12 (*Bradyrhizobium* sp.) strains showed higher NN, even in comparison with the control strain BR 2811, but this feature was not able to increase their RE%. Differently than the other strains, which have shown low NN but high NDW, 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 the N_2-fixing process and showed higher RE%. Interestingly, those strains were more efficient than the control strain BR 2811, approved as inoculant to *C. spectabilis*. Among the six strains that showed higher RE% than the high mineral 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 production statistically similar to the control treatment supplied with high mineral N concentration (52.5 mg L^{-1}). Although the SDW production by the other 2 strains UFLA 05-13 (*Bradyrhizobium* sp.) and UFLA 05-17 (*Bradyrhizobium* sp.) had been lower than the control treatment supplied with high mineral N concentration, those strains have shown higher RE% than the control treatment supplied with high mineral N concentration (52.5 mg L^{-1}). The atypical genera did not induced nodule formation on *S. aterrimum* roots in the authentication test (Table 3).

3.3 Phenotypic characterization

3.3.1 Arsenic minimal inhibitory concentration (MIC)

The As-MIC screening showed 8 tolerant strains (Figure 1). The strains UFLA 05-21 (*Methylobacterium* sp.) and UFLA 05-23 (*Starkeya novella*), isolated from nodules of *S. aterrimum*, were tolerant up to 150 and 200 mmol As L^{-1}, respectively. Among the strains isolated from nodules of *C. spectabilis*, only UFLA 05-16 (*Rhizobium tropici*) was tolerant until the highest As concentration tested. Other strains isolated from *S. aterrimum* and *C. spectabilis* showed to be sensitive to As, as

they did not grow in any As concentration studied. Among the type and reference strains, *A. caulinodans* ORS571^{T}, *Mesorhizobium plurifarium* BR 3804, *Rhizobium tropici* CIAT 899^{T} and *Burkholderia cepacia* LMG 1222^{T} were tolerant to As, since they grew until the highest As concentration tested. The strain *A. doebereinereae* BR 5401^{T} was also As-tolerant, but it just grew up to 100 mmol As L^{-1}. On the other hand *Bradyrhizobium* sp. BR 2001 and BR 2811 strains were As-sensitive, since they did not grow in any As concentration studied (Figure 1).

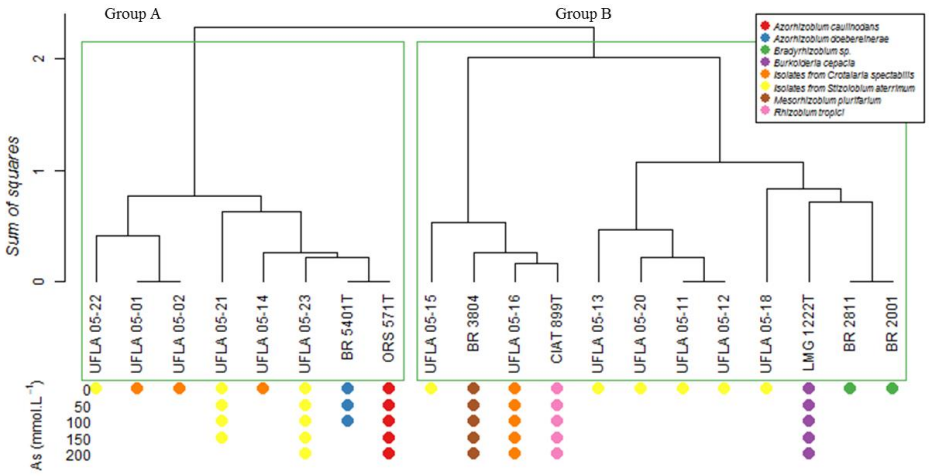

Figure 1 – Dendrogram based on the bacterial phenotypic characteristics of strains isolated from nodules of *S. aterrimum* and *C. spectabilis* plants growing on As-contaminated soil. Reference and type strains *Azorhizobium* (BR 5401^{T} and ORS 571^{T}), *Bradyrhizobium* (BR 2001 and BR 2811), *Mesorhizobium* (BR 3804), *Rhizobium* (CIAT 899^{T}), and *Burkholderia* (LMG 1222^{T}). Relation between cultural groups and bacterial As tolerance.

3.3.2 Pattern of *β*-lactams antibiotics resistance

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

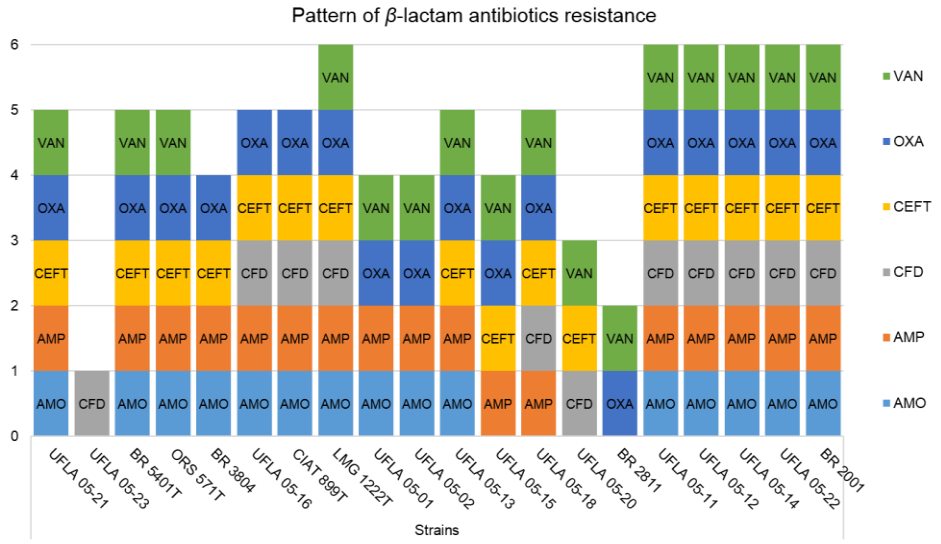

Figure 2 – Pattern of *β*-lactam antibiotics resistance of arsenic tolerant strains by disk diffusion method. AMO: Amoxicillin, AMP: Ampicillin, CFD: Cefadroxil, CEFT: Ceftriaxone, OXA: Oxacillin, and VAN: Vancomycin.

3.3.3 Plant growth promoting traits

All strains were screened for their potential plant growth promoting traits (Table 2) and multi-resistance to Zn and Cd to check their multi-element resistance potential (Table 4). In general, the amount of bacteria with traits to promote plant growth by producing organic acids (OA), IAA, ACC deaminase, siderophores and solubilizing P is remarkably high (Table 2). The total percentage of positive strains for OA production is 26%, IAA 52%, ACC deaminase 73%, SID 69% and solubilizing P 73%. Concerning their multi-element resistance, without

exception, all bacteria isolated from As-contaminated soil are Zn- and Cd-resistant at both low and high [Zn] and [Cd] (Table 4).

Table 4 – Cadmiun and zinc tolerance in 284 medium of strains isolated from nodules of legume species growing in As-contaminated mining soil.

		Cadmiun		Zinc	
Strains	Closest related strain by NCBI	0.4 mM	0.8 mM	0.6 mM	1.0 mM
UFLA 05-01	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-02	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-03	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-04	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-06	*Burkholderia* sp. JPY321	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-07	*Inquilinus* sp. MG-2011-30-BD	+++Moderate	+++Moderate	+++Moderate	+++Moderate
UFLA 05-08	*Labrys monachus*	++++Abundant	++++Abundant	++++Abundant	++++Abundant
UFLA 05-09	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-10	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-11	*Bradyrhizobium elkanii*	++Low	+Scarce	++Low	+Scarce
UFLA 05-12	*Bradyrhizobium* sp. UFLA 03-143	++Low	+Scarce	+Scarce	+Scarce
UFLA 05-13	*Bradyrhizobium* sp. UFLA 03-174	++Low	+Scarce	++Low	+Scarce
UFLA 05-14	*Bradyrhizobium* sp.	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-15	*Bacillus* sp. DB170	++Low	+Scarce	++Low	++Low
UFLA 05-16	*Rhizobium tropici* CIAT 899	+++Moderate	+++Moderate	+++Moderate	+++Moderate
UFLA 05-17	*Bradyrhizobium* sp. UFLA 03-182	++Low	+Scarce	+Scarce	+Scarce
UFLA 05-18	*Bradyrhizobium* sp. UFLA 03-140	+++Moderate	+++Moderate	++Low	+Scarce
UFLA 05-19	*Bradyrhizobium elkanii* IAR12	++Low	+Scarce	++Low	++Low
UFLA 05-20	*Bradyrhizobium* sp. CCBAU 23005	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-21	*Methylobacterium* sp. AMS19	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-22	*Bosea* sp. S41RM2	+Scarce	+Scarce	+Scarce	+Scarce
UFLA 05-23	*Starkeya novella* DMS 506	++Low	+Scarce	+Scarce	+Scarce
UFLA 05-24	*Bradyrhizobium* sp. LmjM3	+Scarce	+Scarce	++Low	++Low
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.

4 Discussion

To obtain an efficient and successful phytoremediation process on metal(oids) contaminated soils, the interaction with plant-associated microorganisms is highly relevant. These plant-associated microorganisms perform several essential biological processes, such as biological N_2-fixation, improving and promoting plant growth (Weyens et al., 2013).

Phytoremediation of metal(oids) contaminated soils has been proposed as a sustainable and low cost phytotechnology (Vangronsveld and Cunningham, 1998). Until recently, scientific research on that subject mainly focused on the search for metal(oids) hyperaccumulator plants. Indeed, many metal(oids)-hyperaccumulator plants have been described, belonging to about 45 plant families, representing herbaceous plants, shrubs and trees (Kramer et al., 2000; Ma et al., 2001; McGrath and Zhao, 2003; Pulford and Watson, 2003; Sagiroglu et al., 2006; Srivastava et al., 2006; Meers et al., 2007; Wieshammer et al., 2007; Li et al., 2009; Bissonnette et al., 2010; Carvalho et al., 2013). However, the potential of most of them for field applications is limited due to slow growth rates, low biomass production, and their occurrence in habitats with specific

features, in addition to the limited knowledge of their agronomic characteristics, as in the case of pteridophyte species As-hyperaccumulators (Ma et al., 2001; Srivastava et al., 2006).

The selection of appropriate plants as well as of their associated microorganisms is a crucial step in phytoremediation projects (Matsuda et al., 2002a-b; Carrasco et al., 2005; Pajuelo et al., 2008; Weyens et al., 2009b; Weyens et al., 2013).

In this study bacterial strains were isolated from nodules of *C. spectabilis* and *S. aterrimum* plants growing on As-contaminated soil, in a gold mine area, and the isolated strains were characterized phenotypically (Figure S1; Table S1) and genotypically (Table 2),. In previous assays, using the same soil, both plant species *C. spectabilis* (Lopes et al. – data unpublished) and *S. aterrimum* (Rangel et al., 2014), showed potential for As phytostabilization.

Several researchers have isolated N_2-fixing bacteria from soils contaminated with different metal(oids) (Carrasco et al., 2005; Jackson et al., 2005; Oliveira et al., 2009; Becerra-Castro et al., 2011; Croes et al., 2013; Weyens et al., 2013). Carrasco et al. (2005) isolated 96 rhizobia strains from nodules of five legume plant species, which grew on a soil

contaminated with As, Cd, Co, Cr, Cu, Ni, Pb and Zn. The authors showed the high tolerance of *Sinorhizobium meliloti* to As, at genetic level. This rhizobium strain contains the ArsB protein, which is involved in As tolerance in other Proteobacteria strains. Drewniak et al. (2008) have isolated a rhizobium strain, identified as *Sinorhizobium* sp. M14, extremely As tolerant, from As-contaminated soil in a gold mining area. This strain grew on up to 250 mmol L^{-1} arsenate, and 20 mmol L^{-1} arsenite. The authors have proposed two mechanisms for adaptation to As used by the strain. The first mechanism would be 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 its growth. Recently, the pSinA plasmid of *Sinorhizobium* sp. M14 was genetically and functionally characterized by Drewniak et al (2013). This strain has its tolerance to As compromised without the pSinA plasmid, and it is not able to grow on minimal medium containing arsenite as energy source. In addition, to demonstrate the functionality of the pSinA plasmid, the horizontal transfer capability and the stability of the plasmid were also presented. The horizontal transfer of the plasmid on metal(oids) contaminated soils

is relevant, since the plasmid contains genes that confer tolerance to metal(oid)s and can be received by other taxonomically distant groups, assisting in increasing metal(oid) tolerance within the bacterial community, consequently increasing the functionality of this community.

In this study, 12 strains were studied *in vitro* for their tolerance to As (Figure 1). The As tolerance of the strains, could be related to their growth rate (Table S1, Figure S1). The strains UFLA 05-16 (*Rhizobium tropici*) and UFLA 05-23 (*Starkeya novella*), and reference or type strains ORS571^{T} (*A. caulinodans*), BR 3804 (*Mesorhizobium plurifarium*), CIAT 899^{T} (*Rhizobium tropici*) and LMG 1222^{T} (*Burkholderia cepacia*) tolerated up to 200 mmol As L^{-1}, the strain UFLA 05-21 (*Methylobacterium* sp.) up to 150 mmol As L^{-1}, and the type strain BR 5401^{T} (*A. doebereinereae*) tolerated up to 100 mmol As L^{-1}. These results illustrate the high tolerance to arsenate of those strains. All strains that tolerated the maximum As concentration (200 mmol L^{-1}) are members of *α*-Proteobacteria class, the same class of the strain *Sinorhizobium* sp. M14 isolated by Drewniak et al. (2008), which tolerated up to 250 mmol L^{-1}, and is equipped with the pSinA plasmid, encoding for As tolerance (Drewniak et al., 2013).

Our results show that the α-Proteobacteria group contains a high diversity of As-tolerant bacteria, belonging to several rhizobia genera (*Azorhizobium*, *Mesorhizobium*, *Rhizobium*, *Burkholderia* and *Starkeya*). That fact makes the BNF use for phytoremediation purposes more promising, since it increases the number of host legume species for those rhizobia genera that can be tested in the field.

In 1982, Mobley and Rosen showed that As tolerance is genetically conferred, and the genes are located on a plasmid. The tolerance mechanism is an energy dependent efflux system, linked to the cellular membrane (Ji and Silver, 1992; Nies and Silver, 1995; Rosen et al., 1999; Wang et al., 2000; Messens and Silver, 2006). Plasmids that confer tolerance to metal(oid)s, may also confer resistance to β-lactams antibiotics (Millar et al., 1987; Baker-Austin et al., 2006). Different resistance mechanisms to β-lactams antibiotics have been well-characterized such as reduction of membrane permeability to metal(oid)s and antibiotics, drug and metal(oids) inactivation and modification, and rapid efflux of the metal(oid)s and antibiotics (Silver, S., 1996; Ruiz, N., 2003; Mukhopadhyay & Rosen, 2002; Wright, G. D., 2005; Nies, D.H., 2003; Levy, S. B., 2002). In this study, most of the As-tolerant strains

also showed a pattern of multiple resistance to *β*-lactam antibiotics (Figure 2). The production of *β*-lactamase, an enzyme capable of modifing and inactivating the *β*-lactams antibiotics, is part of the resistance mechanism to *β*-lactams antibiotics (Williams, 1999). In Gram-negative bacteria, *β*-lactamases are produced consitutively, even when the antibiotic is not present (Marchou et al., 1987), and 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 explains why As-sensitive strains also showed a pattern of multiple resistance to *β*-lactams antibiotics (Figure 2), since most of them are Gram-negative. Most of all As-sensitive bacteria are representants of the *Bradyrhizobium* genus, which is known for producing and using outer membrane proteins as an uptake system for siderophores-metal complexes (Plessner et al., 1993). Thus, taken together, these observations might suggest that the *β*-lactams antibiotics resistance and As tolerance are related only by membrane permeability for metal(oid)s and antibiotics. Both metal(oid)s and antibiotics may be taken up by the same outer membrane proteins (porins), which are responsible for increasing or decreasing membrane permeability (Silver, S., 1996; Mata

et al., 2000; Aendekerk et al., 2002; Nies, D.H., 2003; Wright, G. D., 2005). However, the resistance to As is only conferred by arsenate reductases and related enzymes (Mukhopadhyay & Rosen, 2002).

Plant growth-promoting rhizobacteria (PGPR) can directly exert different beneficial effects on the growth of their host plant. PGPR may synthesize and provide their host plant with compounds such as fixed nitrogen or phytohormones, such as indole-3-acetic acid (IAA), they may facilitate nutrients uptake such as P and Fe, or even may synthesize enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase which lowers plant ethylene levels and may afftect plant growth (Glick et al., 2007).

The total percentage of P solubilizing bacterial strains was high (73%). The Nautiyal (1999) methodology is based on extracellular oxidation of glucose via quinoprotein glucose dehydrogenase, which produces gluconic acid and mobilizes insoluble phosphates very efficiently. Moreover, other mechanisms apart from the production and excretion of gluconic acid, such as production of chelating substances, the release of protons originated by NH^{4+} assimilation and the production of

inorganic acids, have also been proposed to explain phosphate solubilization by bacteria (Illmer and Schinner, 1995).

Plants growing on TE contaminated soils might become severely depleted in the amount of available iron. Fortunately, plants can produce siderophores that bind to iron and allow them to take up iron. Moreover plants can also take up complexes of iron and bacterial siderophores. Plants are dependent on bacterial siderophore production because even though they can produce siderophores themselves, the affinity of plant siderophores is much lower than that of bacterial siderophores. So in a TE contaminated soil, a plant is unable to accumulate a sufficient amount of iron unless bacterial siderophores are present (Glick, 2003). A high number of bacterial strains (69%) produced siderophores. This high number of siderophores producing bacteria was not a surprise since the bacteria were isolated from legume-nodules and most of the isolated bacteria are rhizobia and perform biological N_2 fixation (BNF). This biological process is highly Fe demanding (Tang et al., 1990; Brear et al., 2013) for synthesizing the nitrogenase enzyme complex, as well as cytochromes, ferredoxin, and hydrogenase. Moreover, iron is highly important for nodule formation. Nodules of iron adequate plants contain

more than double the amount of leghemoglobin than iron deficient plants (Tang et al., 1990). These authors have shown that the leghemoglobin production by *Lupinus angustifolius* inoculated with *Bradyrhizobium lupini* WU425 is 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 (OM) receptors instead. These OM specific receptors are able to bind siderophores-metal complexes, allowing the uptake of ion chelates (Small et al., 2009). In rhizobia, Fe^{3+}-siderophore complexes are recognized by different OM receptors depending on the siderophores. Such siderophores are referred to as xenosiderophores, since they are used by one organism and synthesized and secreted by other organisms. An example was given by Plessner et al. (1993) who has shown that *Bradyrhizobium japonicum* USDA 110 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 TE-contaminated soils, where the plant growth is normally decreased. By producing OA, bacteria can improve uptake of essential mineral nutrients, which are normally limiting in mining soils. However, by improving the availability of essential mineral nutrients, OA production may also increase availability of toxic TE for the plant by means of the same mechanisms.

The low number (26%) of OA producing bacterial strains isolated from the As-contaminated soil is remarkable (Table 2). Interestingly, most of the strains which did not produce OA were able to solubilize $Ca_3(PO_4)_2$. Probably the $Ca_3(PO_4)_2$ solubilization by those bacteria is not due to OA release. We hypothesize that those bacteria, which do not produce OA in As-contaminated soil, have this feature to avoid As solubilization since OA release during bacterial cells lysis may cause arsenopyrite (FeAsS) dissolution, even if little efficient (Drewniak et al., 2014). This feature requires more detailed studies on mineral-microbes interactions for better understanding.

Plants growing on contaminated mining soils often show growth inhibition compromised by nutrient depletion as well as by TE exposure.

Since those conditions threat their ability to survive, the bacterial potential to promote plant growth might be of high importance. The function and potential of the auxin IAA, which can be produced by plant-associated bacteria, is well known. IAA is involved in increasing root growth and root length, as well as in proliferation and elongation of root hairs (Taghavi et al., 2009). So, a more extended root system induced by bacterial IAA production might decrease the threat caused by nutrient depletion through the bigger soil volume that can be explored by the roots (Weyens et al., 2011). Several studies have shown that rhizosphere bacteria, phyllosphere bacteria and endophytes can improve phytoremediation efficiency by different mechanisms, including IAA production (Valls & de Lorenzo, 2002; Lebeau et al., 2008; Kidd et al., 2009; Mastretta et al., 2009; Rajkumar et al., 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). Considering our results about IAA production, *in vitro* 52% of the isolated bacterial strains are able to produce this hormone. Given the high number of native bacteria isolated from mining soils that are able to produce this hormone,

we hypothesize that this mechanism must be important for plant growth and development on these contaminated soils.

Besides directly inducing plant growth, IAA can also induce the transcription of ACC synthase, which is the enzyme that catalyzes the formation of 1-aminocyclopropane-1-carboxilic acid (ACC) (Glick, 2003). ACC is the immediate precursor of ethylene, which is also a plant growth regulating hormone, but only when present in very low amounts. At high levels, ethylene is a threat to plant growth and development since it causes growth inhibition and early senescence. So it seems important that plant-associated bacteria are able to cleave ACC by ACC deaminase. In that case, bacteria act as a sink for plant ACC, controlling the amount of ethylene released in the plant, and by consequence their growth and development (Stearns & Glick, 2003; Glick et al., 2007; Glick & Stearns, 2011). The high number (73%) of strains positive for ACC deaminase isolated from As-contaminated soils is remarkable. Our observations agree with Croes et al. (2013) and Truyens et al. (2014), which suggest that the TE contamination pressures bacteria selecting the TE tolerant, phosphorus solubilizing, nitrogen fixing and ACC deaminase and IAA producing genotypes.

Beside the remarkable potential of the native rhizobia isolated from As-contaminated soils, also the results we obtained for the different type or reference rhizobia strains are noteworthy (Table 2). Taking a look to their behaviour, we observed different strains from different rhizobia genera with positive results for most of all plant-growth promoting tests. These results make 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 tested for phytoremediation purposes in different soil contamination conditions (e.g. mining, smelting etc.).

In addition to their capacity to promote plant growth on TE-contaminated soils, it is really interesting if these bacteria possess multi-element resistance since almost all mining soils may have a multi-element pollution. The bacteria we have studied in this work came directly from mining soils, meaning they were exposed for many generations to a toxic environment and were forced to adapt to survive under this selective pressure.

All bacterial strains, without exception, isolated from As-contaminated soil are Zn- and Cd-resistant at both low and high [Zn] and

[Cd] (Table 4). Among those bacteria, we highlight the Zn- and Cd-resistance of *Rhizobium tropici* strain UFLA 05-16, which is highly As-resistant (Figure 1). Besides *Labrys monachus* strain UFLA 05-08 and *Inquilinus* sp. UFLA 05-07, which also significantly showed highly Zn- and Cd-resistance.

It is commonly known that microbial populations can notably be affected by toxic metal(oid)s, which threat microbial activity (Carneiro et al., 2008; Giller et al., 2009; Santos et al., 2013). However microbial resistance to toxic 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 & Phung, 2009; Croes et al., 2013).

5 Conclusion

The group of α-Proteobacteria harbours high bacterial diversity tolerant to As;

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

The intrinsic plant-growth promoting ability plus multi-element resistance of rhizobia increase the horizons for exploiting the symbiosis with different legume-plants on different conditions of contamination.

Acknowledgements

The authors are very grateful to Teotônio S. de Carvalho for kindly preparing the dendrograms using R software. This research was supported by the Brazilian National Council for the Scientific and Technological Development (CNPq), the Brazilian 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.

References

Aendekerk S, Ghysels B, Cornelis P, Baysse C. 2002. Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. Microbiology 148(8): 2371-2381.

Accioly AMA, Siqueira JO. 2000. Contaminação química e biorremediação do solo. In: Novais RF, Alvarez VH, Schaefer CE, eds. Tópicos em ciência do solo. Viçosa: Sociedade Brasileira de Ciência do Solo. v.1, p.299 - 352.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. 2006. Co-selection of antibiotic and metal resistance. Trends in Microbiology 14(4): 176-182.

Barkay T, Miller SM, Summers AO. 2003. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev. 27(2-3): 355-384.

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

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

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR. 2005. Cadmium-tolerant plant growth-promoting bacteria

associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem 37(2): 241-250.

Bissonnette L, St-Arnaud M, Labrecque M. 2010. Phytoextraction of heavymetals by two Salicaceae clones in symbiosis with arbuscular mycorrhizal fungi during the second year of a field trial. Plant Soil, 332(1): 55-67.

Bontidean I, Lloyd JR, Hobman JL, Wilson JR, Csöregi E, Mattiasson B, Brown NL. 2000. Bacterial metal-resistance proteins and their use in biosensors for the detection of bioavailable heavy metals. J. Inorg. Biochem. 79(1-4): 225-229.

Brear EM, Day DA, Smith PMC. 2013. Iron: an essential micronutrient for the legume-rhizobium symbiosis. Frontiers in Plant Science 4: 359.

Carneiro MAC, Siqueira JO, Moreira FMS, Soares ALL. 2008. Soil organic carbon, total nitrogen, microbial biomass and activity in two rehabilitation chronosequences after bauxite mining. Rev Bras Ciênc Solo 32(2):621-632.

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

Carvalho MTV, Amaral DC, Guilherme LRG, Aarts MGM. 2013. *Gomphrena claussenii*, the first South American metallophyte species with indicator-like Zn

and Cd accumulation and extreme metal tolerance. Front Plant Sci. 4: 180. doi: 10.3389/fpls.2013.00180

Costa GS, Franco AA, Damasceno RN, Faria SM. 2004. Nutrient input through litter in a degraded area revegetated with legume trees. Rev Bras Ciênc Solo 28(5): 919-927.

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

Cunningham JE, Kuiack C. 1992. Production of citric and oxalic acids and solubilization of calcium-phosphate by *Penicillium bilaii*. Applied Environmental Microbiology 58(5): 1451-1458.

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

Drewniak L, Dziewit L, Ciezkowska M, Gawor J, Sklodowska A. 2013. Structural and functional genomics of plasmid pSinA of *Sinorhizobium* sp. M14 enconding genes for arsenite oxidation and arsenic resistance. Journal of Biotechnology 164(4): 479-488.

Drewniak L, Rajpert L, Mantur A, Sklodowska A. 2014. Dissolution of Arsenic Minerals Mediated by Dissimilatory Arsenate Reducing Bacteria: Estimation of

the Physiological Potential for Arsenic Mobilization. BioMed Research International, vol. 2014, Article ID 841892, 12 pages, 2014. doi:10.1155/2014/841892

Empresa Brasileira de Pesquisa Agropecuária–EMBRAPA. Manual de métodos de análise de solos. 2 ed. Rio de Janeiro, 230 p. 2011.

Franco AA, Franco EFC, Campello EMR, da Silva , de Faria SM. Revegetação de Solos Degradados. Comunicado Técnico. In: EMBRAPA-CNPAB, Rio de Janeiro (1992), p. 9, n 9.

Franco AA, Dias LE, Faria SM, Campello EFC, Silva EMR. 1995. Use of nodulate and mycorrhizal forest leguminous trees as agents of recovery and maintenance of soil life: a technological model. Oecologia Brasiliensis. 1:459-467.

Fred EB, Waskman SA. 1928. Laboratory Manual of General Microbiology, McGraw-Hill Book Company, Inc., New York and London.

Giller KE, Witter E, McGrath SP. 2009. Heavy metals and soil microbes. Soil Biol Biochem 41(10): 2031-2037.

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

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

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

Hoagland DR, Arnon DL. The water culture methods for growing plants without soil. Berkeley: California Agriculture Experiment Station, 1950. 32p. (Bulletin, 347).

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

Jackson CR, Dugas SL, Harrison KG. 2005. Enumeration and characterization of arsenate-resistant bacteria in arsenic free soils. Soil Biol. Biochem. 37(12): 2319-2322.

Jesus EC, Moreira FMS, Florentino LA, Rodrigues MIR, Oliveira MS. 2005. Diversidade de bactérias que nodulam siratro em três sistemas de uso da terra da Amazônia Ocidental. Pesq. Agropec. Bras. 40(8):769-776.

Ji G, Silver S. 1992. Regulation and Expression of the Arsenic Resistance Operon from *Staphylococcus aureus* Plasmid p1258. Journal of Bacteriology 174(11): 3684 - 3694.

Kidd P, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R, Monterroso C. 2009. Trace elements behaviour at the root–soil interface: implications in phytoremediation. Environ Exp Bot 67(1): 243–259.

Kramer U, Pickering IJ, Prince RC, Raskin I, Salt DE. 2000. Subcellular localization and speculation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiol. 122(4): 1343–1353.

Lane DJ. 1991. 16S/23S rRNA sequencing, p 115-148. In: Stackebrandt E, 386 Goodfellow M. (ed.). Nucleic Acid Techniques in Bacterial Systematics, New York.

Lebeau T, Braud A, Jézéquel K. 2008. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: a review. Environ Pollut 153(3): 497–522.

Levy SB. 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. J. Appl. Microbiol. 92(31): 65-71.

Li TQ, Yang XE, Lu LL, Islam E, He ZL. 2009. Effect of Zn and Cd interactions on root morphology and metal translocation in a hyperaccumulating species under hydroponic conditions. J. Hazard. Mater. 169(1-3): 734-741.

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED. 2001. A fern that hyperaccumulates arsenic. Nature 409:579.

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

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

Mata MT, Baquero F, Pérez-Díaz JC. 2000. A multidrug efflux transporter in *Listeria monocytogenes*. FEMS Microbiology Letters 187(2):185-188.

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

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

McGrath, SP, Zhao FJ. 2003. Phytoextraction of metals and metalloids from contaminated soils. Curr. Opin. Biotechnol. 14(3): 277-282.

Meers E, Vandecasteele B, Ruttens A, Vangronsveld J, Tack FMG. 2007. Potential of five willow species (*Salix* spp.) for phytoextraction of heavy metals. Environ. Exp. Bot. 60(1): 57-68.

Melloni R, Nóbrega RSA, Moreira FMS, Siqueira JO. 2004. Densidade e diversidade fenotípica de bactérias diazotróficas endofíticas em solos de mineração de bauxita, em reabilitação. R. Bras. Ci. Solo 28(1):85-93.

Melloni R, Moreira FMS, Nóbrega RSA, Siqueira JO. 2006. Eficiência e Diversidade Fenotípica de Bactérias Diazotróficas que Nodulam Caupi [*Vigna unguiculata* (L.) Walp] e Feijoeiro (*Phaseolus vulgaris* L.) em Solos de Mineração de Bauxita em Reabilitação. R. Bras. Ci. Solo 30(2): 235-246.

Messens J, Silver S. 2006. Arsenate Reduction: Thiol Cascade Chemistry with Convergent Evolution. J. Mol. Biol. 362(1): 1-17.

Millar MR, Griffin N, Keyworth N. 1987. Pattern of antibiotic and heavy-metal ion resistance in recent hospital isolates of *Staphylococcus aureus*. Epidem. Inf. 99(2): 343-347.

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

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA. 1993. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacylamide gel electrophoresis of their total proteins. Systematic and Applied Microbiology 16(1):135-146.

Moreira FMS. 2010. Bactérias fixadoras de nitrogênio que nodulam espécies de Leguminosae. In: Moreira FMS, Huising EJ, Bignell DE, eds. Manual de Biologia dos solos tropicais – Amostragem e Caracterização da biodiversidade. Lavras: UFLA. p. 279-312.

Mukhopadhyay R, Rosen BP. 2002. Arsenate reductases in prokaryotes and eukaryotes. Environ. Health Perspect. 110(5): 745-748.

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

Nies DH, Silver S. 1995. Ion efflux systems involved in bacterial metal resistances. Journal of Industrial Microbiology 14(2): 186-199.

Nies D.H. 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microb Rev. 27(2-3): 33-39.

Novick RP, Roth C. 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J. Bacteriol. 95(4): 1335-1342.

Oliveira, A.; Pampulha, M. E.; Neto, M. M.; Almeida, A. C. (2009). Enumeration and Characterization of Arsenic-Tolerant Diazotrophic Bacteria in a Long-Term Heavy-Metal-Contaminated Soil. Water Air Soil Pollut 200(1):237-243.

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

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

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

Pulford ID, Watson C. 2003. Phytoremediation of heavy metal-contaminated land by trees: A review. Environ. Int. 29(4): 529-540.

Purchase D, Miles RJ, Young TWK. 1997. Cadmium uptake and nitrogen fixing ability in heavy metals resistant laboratory and field strains of *Rhizobium leguminosarum* biovar trifolii. FEMS Microbiol. Ecol. 22(1): 85-93.

Rajkumar M, Ae N, Freitas H. 2009. Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77(2): 153-160.

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

Ribeiro AC, Guimarães PTG, Alvarez VVH. 1999. Recomendações para o uso de corretivos e fertilizantes em Minas Gerais – 5ª aproximacão. Viçosa: UFV. 359 p.

Richmond MH, John M. 1964. Co-transduction by a Staphylococcal Phage of the Genes Responsible for Penicillinase Synthesis and Resistance to Mercury Salts. Nature 202: 1360-1361.

Rosen BP, Bhattacharjee H, Zhou T, Walmsley AR. 1999. Mechanism of the ArsA ATPase. Biochim. Biophys. Acta 1461(2): 207-215.

Ruiz N. 2003. The role of *Serratia marcescens* porins in antibiotic resistance. Microb. Drug Resist. 9(3): 257-264.

Sagiroglu A, Sasmaz A, Sen O. 2006. Hyperaccumulator plants of the Keban mining district and their possible impact on the environment. Pol. J. Environ. Stud. 15(2): 317-325.

Santos JV, Rangel WM, Guimarães AA, Jaramillo PMD, Rufini M, Marra LM, López MV, Silva MAP, Soares CRFS, Moreira FMS. 2013. Soil biological attributes in arsenic-contaminated gold mining sites after revegetation. Ecotoxicology 22(10):1526-1537.

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

Silver S. 1996. Bacterial heavymetal resistance: new surprises. Ann. Rev. Microb. 50: 753-789.

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

Small SK, Puri S, Sangwan I, O'Brian MR. 2009. Positive Control of Ferric Siderophore Receptor Gene Expression by the Irr Protein in *Bradyrhizobium japonicum*. Journal of Bacteriology 191(5): 1361-1368.

Sriprang R, Hayashi M, Yamashita M, Ono H, Saeki K, Murooka Y. 2002. A novel bioremediation system for heavy metals using the symbiosis between

leguminous plant and genetically engineered rhizobia. J Biotechnol., 99(3): 279-293.

Srivastava M, Ma LQ, Santos JAG. 2006. Three new arsenic hyperaccumulating ferns. Sci. Total Environ. 364(1-3): 24-31.

Stearns JC, Glick BR. 2003. Transgenic plants with altered ethylene biosynthesis or perception. Biotechnology Advances 21(3): 193-210.

Stephens JHG, Rask HM. 2000. Inoculant production and formulation. Field Crops Research 65(2-3): 249-258.

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

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

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

Trannin ICB, Siqueira JO, Moreira FMS, Lima AS. 2001. Tolerância de Estirpes e Isolados de *Bradyrhizobium* e de *Azorhizobium* a Zinco, Cádmio e Cobre "In Vitro". Revistra Brasileira de Ciência do Solo, 25(2): 305-316.

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

Valls M, Atrian S, de Lorenzo V, Fernández LA. 2000. Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for enhanced immobilization of heavy metals in soil. Nature Biotechnology, 18: 661-665.

Valls M, de Lorenzo V. 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol Rev 26(4): 327-338.

van der Lelie D, Taghavi S, Monchy S, Schwender J, Miller L, Ferrieri R, Rogers A, Wu X, Zhu W, Weyens N, Vangronsveld J, Newman L. 2009. Poplar and its bacterial endophytes: coexistence and harmony. Crit Rev Plant Sci 28(5): 346–358.

Vangronsveld J, Cunningham SD. 1998. Introduction to the concepts. In: Vangronsveld J, Cunningham SD, eds. Metal-Contaminated Soils In Situ Inactivation and Phytorestoration. Berlin: Springer-Verlag, p. 1-16.

Vincent JM. 1970. A manual for the practical study of root-nodule bacteria. Oxford: Blackwell Scientific Publications. 164 p.

Wang H, Lu Y, Li L, Liu S, Wang D, Sui S. 2000. Trimeric ring-like structure of ArsA ATPase. FEBS Letters 469(1): 105-110.

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

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

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

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

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

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

Wieshammer G, Unterbrunner R, Garcia T, Zivkovic M, Puschenreiter M, Wenzel W. 2007. Phytoextraction of Cd and Zn from agricultural soils by *Salix* spp and intercroppin of *Salix caprea* and *Arabidopsis halleri*. Plant Soil 298(1): 255-264.

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

Wright GD. 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv. Drug Deliv. Rev. 57(10): 1451-1470.

Wu CH, Wood TK, Mulchandani A, Chen W. 2006. Engineering Plant-Microbe Symbiosis for Rhizoremediation of Heavy Metals. *Appl. Environ. Microbiol.* 72(2): 1129-1134.

PAPER 2 - RHIZOBIA STRAINS ISOLATED FROM ZINC MINING SOIL ARE TOLERANT TO TRACE ELEMENTS AND SHOW VARIOUS POTENTIAL PLANT GROWTH PROMOTING TRAITS

According to *International Journal of Phytoremediation*

Rhizobia strains isolated from zinc mining soil are tolerant to trace elements and show various potential plant growth promoting traits

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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 studied as well. The *in vitro* screening indicated that rhizobia and other native genera have great potential for phytoremediation purposes, by establishing, 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, which is a promising set to be used for phytostabilization concern.

Keywords: Mining soils, rhizobia, plant-growth promoting, legume 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 et al., 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., 2013). 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., 2013). 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 et al. 2010 a,b; 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. Phosphorus and potassium were determined by Mehlich 1 extraction (HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}), and calcium, magnesium and aluminium by KCl extraction (1 mol L^{-1}). Potential acidity (H + Al) was estimated by SMP extraction and organic matter was determined by oxidation using $Na_2Cr_2O_7$ + H_2SO_4 (10N) (EMBRAPA, 2011). According to 5th Approach (Guidelines for lime and fertilizers use in Minas Gerais), soil active acidity was chemically classified as medium acid, phosphorus availability considering clay content and Prem value was categorized as very low, soil fertility (based on organic matter and cation exchange capacity) was also classified as very low for P and for potassium as well, low for calcium, medium for magnesium, low for aluminium and for

hydrogen+aluminium as well (Ribeiro et al., 1999). Soil physical parameters were determined by the pipette method according to Day (1965), and according to the classification of the normative guideline number 2 from Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) October 9th 2008, the soil texture was loam.

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			
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.

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 & 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 & Waksman, 1928), which is known as 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). The extraction kit protocol ZR Fungal/Bacterial DNA (Zymo Research Corp) was used for genomic DNA extraction from the cell cultures.

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).

2.3 Phenotypic characterization

Plant growth promoting (PGP) traits such as production of organic acids (OA) and indole-3-acetic-acid (IAA), ACC deaminase activity (ACC), siderophore production (SID), and $Ca_3(PO_4)_2$ solubilization (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).

Solid medium was used to evaluate phosphate solubilization ability. According to Nautiyal (1999), 25 µl aliquots of inoculum were inoculated in holes with 0.5 cm diameter. Strains able to produce a clear zone around the hole were considered positive. Siderophore production was evaluated using the Schwyn and Neilands (1987) universal colorimetrical method, qualitatively measured by using blue chromium-azurol S (CAS) reagent. 50 µl inoculum were inoculated in 800 µl of selective 284 medium with a carbon mix (CMIX) and three different Fe concentrations: 0 (Fe-deficient), 0.25 (Fe-optimal) and 3 mM (Fe-over supply). Bacterial organic acid production was evaluated according to the Cunningham and Kuiack (1992) colorimetric method by adding alzarine red S pH indicator. 20 µl inoculum were inoculated in 800 µl of sucrose tryptone (ST) medium. Bacterial IAA production ability was tested using the Salkowski assay (adapted from Patten and Glick, 2002). ACC deaminase activity was evaluated according to a slightly modified protocol according to Belimov et al. (2005).

For testing trace element tolerance, all strains were plated on selective 284 medium (Weyens et al., 2013a) containing a carbon mix (CMIX) composed by (per litre of medium) 0.54 g of fructose, 0.66 g of gluconate, 0.52 g of glucose, 0.7 g of lactate and 0.81 g of succinate) and 0, 0.4 and 0.8 mM $CdSO_4$ or 0, 0.6 and 1.0 mM $ZnSO_4$. An aliquot of 20 µl was used and six repetitions were performed per plate. Tolerance was visually rated checking growth and polysaccharide (mucus) production on the plate. In this case, the same exopolysaccharide production pattern applied for rhizobia cultural characterization was used (*i.e.* scarce, low, moderate and abundant).

2.4 N_2-fixing nodulating bacteria and their potential to induce nodule formation on roots of their leguminous host plant (authentication)

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 *Leucaena 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. Each seed was inoculated with 1 mL of the bacterial inoculum containing about 10^8 cells at the moment of planting. One plant was grown in sterile plastic tubes for 65 days. Sand and vermiculite (1:1 ratio) were used as substrate and a four-fold dilution of modified Hoagland nutrient solution (Hoagland & 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 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 BR 822 strain

(*Sinorhizobium fredii*), 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 Performance of *L. leucocephala* in symbiosis with Cd- and Zn-tolerant strains, selected by *in vitro* screening, in a Zn-smelter contaminated soil (greenhouse assay)

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., 2008). 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* – experiment 1

In order to verify that the Lommel soil is free of native rhizobia to avoid their competition with our *in vitro* screened rhizobia able to establish symbiosis with *L. leucocephala*, a most probable number (MPN) experiment was performed. The contaminated soil inoculum, from a former agricultural field at 500 m northeast of a Zn-smelter in Lommel, Belgium, 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). 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 free of native rhizobia able to establish symbiosis with *L. leucocephala* – experiment 2

Mesorhizobium sp. (UFLA 01-765 strain) and *Rhizobium huautlense* (UFLA 01-775 strain) were isolated from a Zn mining area in the southeast of Brazil, using *L. leucocephala* as a trap plant. These rhizobia strains 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. To prepare the bacterial inoculum, the

strains were grown in 79 liquid medium (Fred & 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 et al. (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 1998).

During harvest, fresh shoot and root samples (at least 8 replicates for each condition) were washed with tap water to remove adherent soil particles. In order to remove trace elements present on the root surface, the roots were kept in 0.1 M HCl solution for 1 min (Tu & Ma, 2003). After that, roots were vigorously rinsed with distilled water to remove the HCl. 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. The digestion consisted of 3 cycles in 1 ml HNO_3 (65%) and 1 cycle in 1 ml HCl (37%) at 120 °C for 4h. Samples were then dissolved in HCl (37%) and diluted to a final volume of 5 ml (2% HCl). After harvesting the plants, soil was sampled from the pots to determine the pseudo-total Zn, Cd and Pb concentrations, which were estimated by aqua regia digestion. Metal concentrations were determined using ICP-OES.

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 et al., 2003; Saraswat and Rai, 2011). It was calculated by:

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

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* (see more details on authentication test and symbiotic efficiency assay at the "N_2-fixing nodulating bacteria and their potential to induce nodule formation on roots of their leguminous host plant" section).

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 leucocephala	UFLA 01-776	415	95%	*Mesorhizobium* sp. (HF931067.1)	*α-Proteobacteria*	+	-	+	++	I
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.**

3.2 Phenotypic characterization

It is clear that the 869 medium (Mergeay et al., 1985) also known as TY medium is not the best one for growing rhizobia. Rhizobia grow slowly and sometimes even do not grow, as we have noticed in this assay. In our first trial, using TY medium to activate our strains before the phenotypic tests, the number of positive strains for some tests was reduced to around 54% in comparison with the number of positive strains we got using the yeast mannitol medium, also known as 79 medium.

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 due to the production of polysaccharides on the medium, 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 et al., 1990; Brear et al., 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). Some rhizobia strains do not produce siderophores, however they 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 et al., 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 & de Lorenzo, 2002; Lebeau et al., 2008; Kidd et al., 2009; Mastretta et al., 2009; Rajkumar et al., 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 & Glick, 2003; Glick et al., 2007; Glick & 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.

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.

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 et al., 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., 2001a; Silver & 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 N_2-fixing nodulating bacteria and their potential to induce nodule formation on roots of their leguminous host plant

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.

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.

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 et al., 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. In future inoculation experiments, selected rhizobia will be tested on contaminated soil.

3.4 Performance of *L. leucocephala* in symbiosis with Cd- and Zn-tolerant strains selected by *in vitro* screening on a Zn-smelting contaminated soil

3.4.1 The Zn-smelter contaminated soil is free of native rhizobia able to establish symbiosis with *L. leucocephala*

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 1). 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.

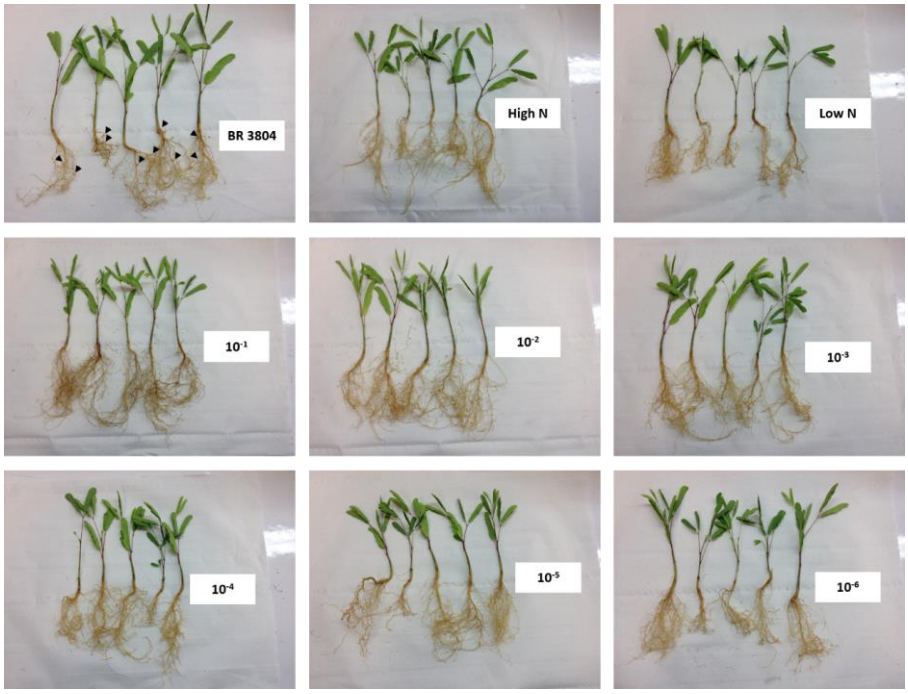

Figure 1. Most probable number (MPN) of rhizobia able to induce nodule formation on roots and to establish symbiosis with *Leucaena leucocephala* on Lommel soil.

3.4.2 Exploiting *L. leucocephala*-rhizobia symbiosis on a contaminated soil free of native rhizobia able to establish symbiosis with *L. leucocephala*

The numbers of fully expanded leaves on *L. leucocephala* at 30, 60 and 90 days after planting are shown in Figure 2.

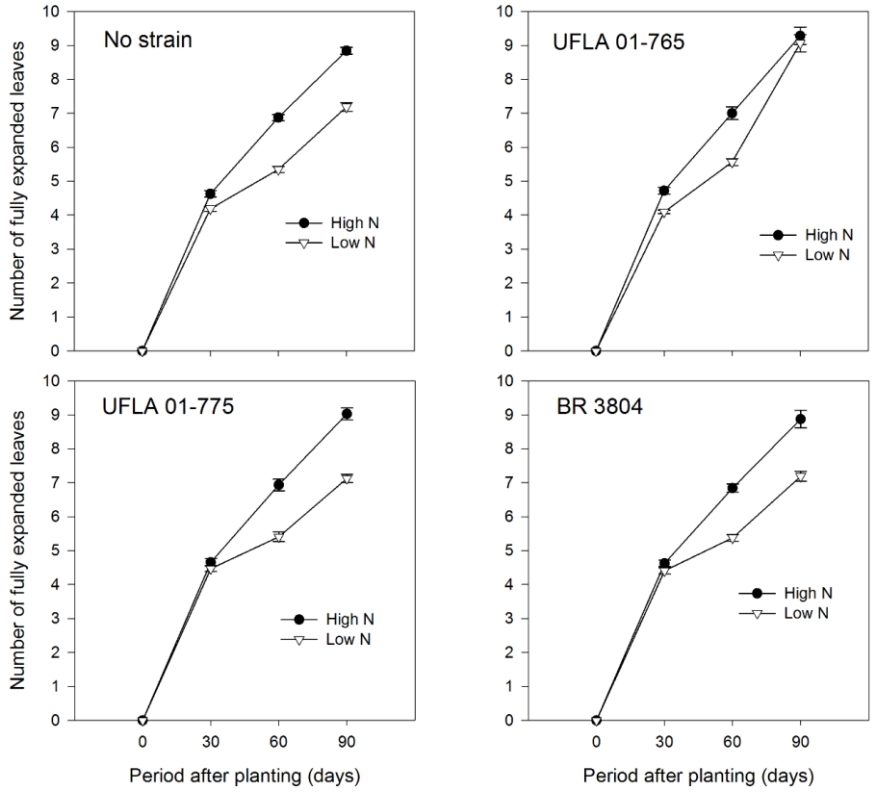

Figure 2. Increasing of the number of fully expanded leaves during the experimental period. $p<0.05$.

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 et al., 2001b; Matsuda et al., 2002a-b; Chaudhary et al., 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 et al., 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 2, 3A and 5C respectively). Interestingly it also decreased the activities of several enzymes involved in antioxidative defence (Figure 10). Figure 3C 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 4C-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 4C - UFLA 01-765).

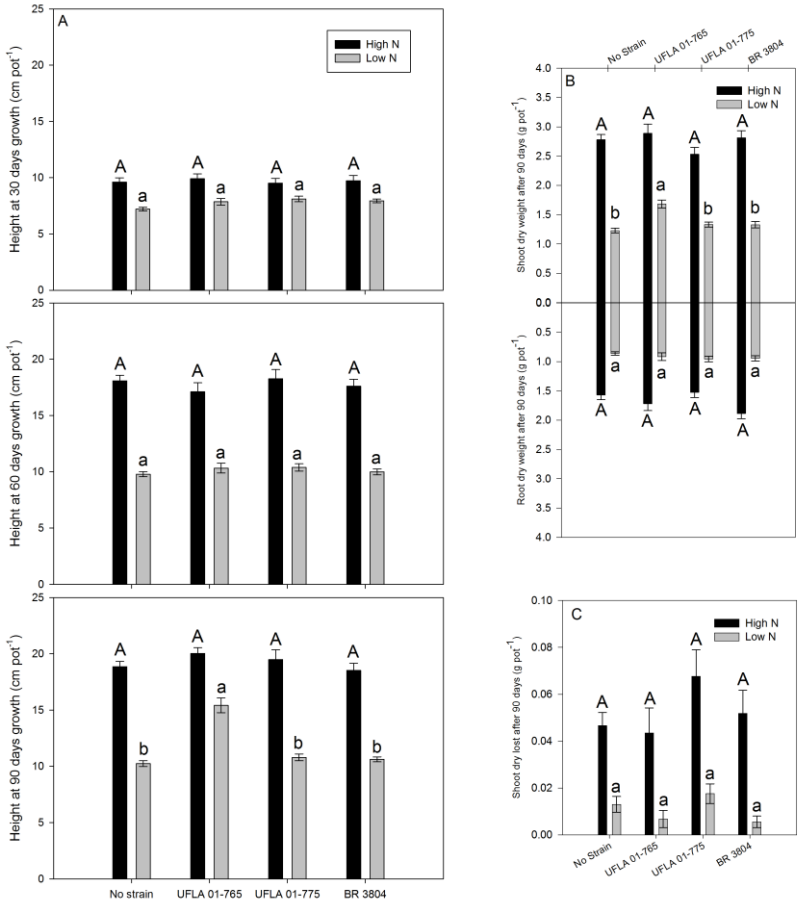

Figure 3. *Mesorhizobium* sp. (UFLA 01-765) isolated from Zn-mining soil contaminated with Zn and Cd is able to promote *Leucaena leucocephala* growth on Zn-smelting soil contaminated with Zn, Pb and Cd. A- Height. B- Dry biomass production (dry shoot and root weight). C- Dry shoot biomass lost after 90 days pot-experiment. Bars with the same upper or lower case letters do not differ by Scott-Knott test, $p<0.05$.

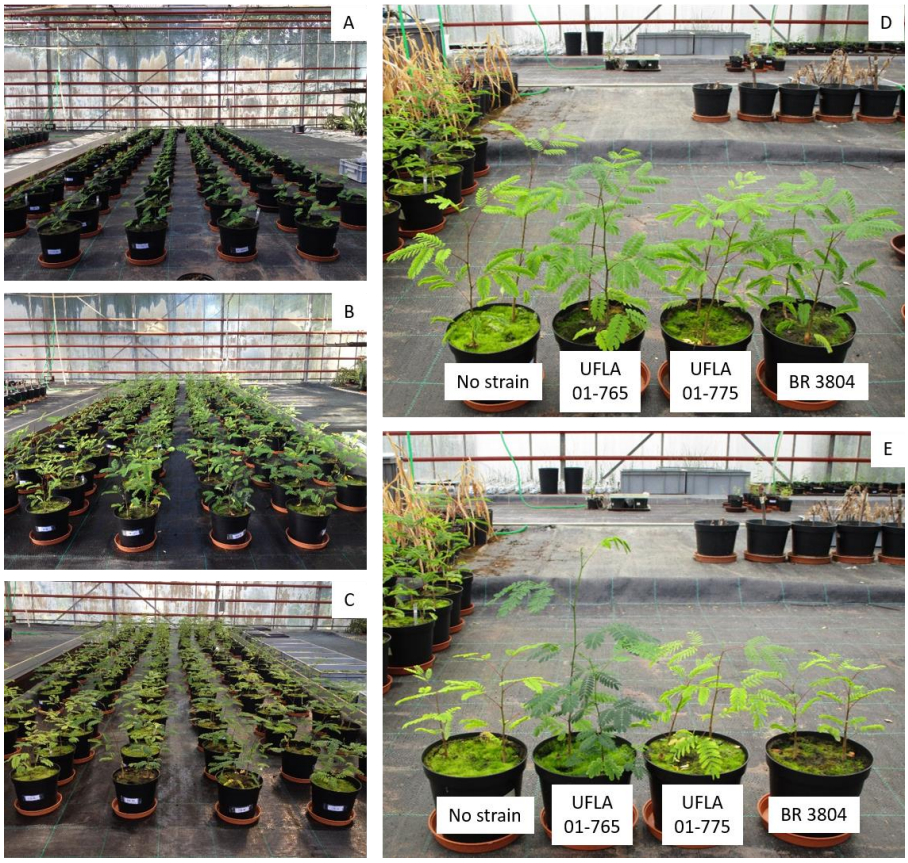

Figure 4. *Mesorhizobium* sp. (UFLA 01-765) isolated from Zn-mining soil contaminated with Zn and Cd is able to promote *Leucaena leucocephala* growth on Zn-smelting soil contaminated with Zn, Pb and Cd. A- Plant growth after 30 days; B- Plant growth after 60 days; C- Plant growth after 90 days; D- High N (150 mg N dm^{-3}) supplied plants at 90^{th} day; E- Low N (15 mg N dm^{-3}) supplied plants at 90^{th} day.

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 5D. 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 5C. 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 5B). 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 hypothesis 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 et al., 1988; Streeter, 1988; Vessey et al., 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.

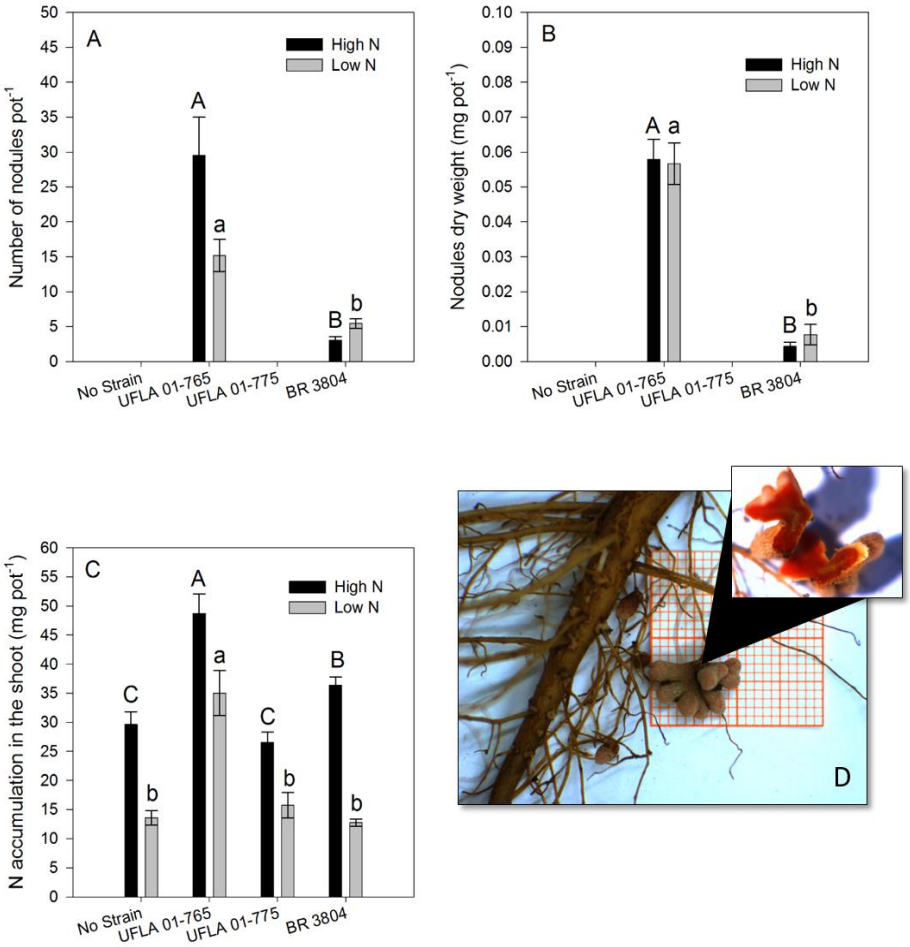

Figure 5. ***Mesorhizobium*** **sp. (UFLA 01-765) isolated from Zn-mining soil contaminated with Zn and Cd is able to efficiently fix N_2 in symbiosis with** ***Leucaena leucocephala*** **on Zn-smelting soil contaminated with Zn, Pb and Cd. A- Number of nodules. B- Nodules dry weight. C- N accumulation 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, $p<0.05$.**

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 gorwing in the Zn-, Pb- and Cd-contaminated soil from Lommel (Figure 5A). 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).

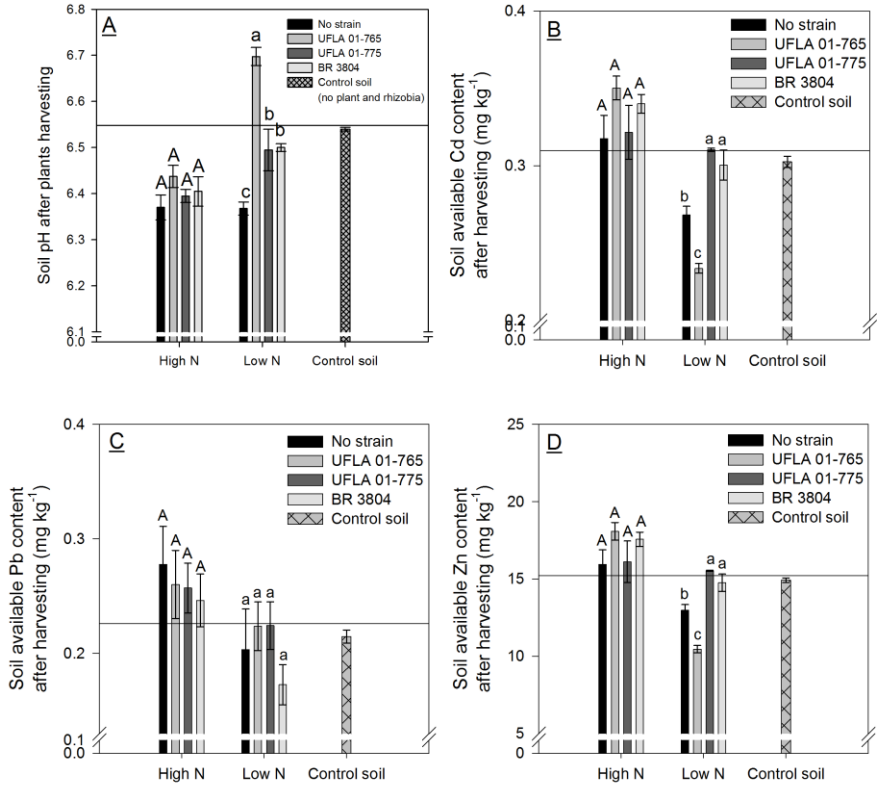

Figure 6. Soil pH-H_2O and available metal content after plant harvesting at 90 days and pH and metal content of the control soil without plant, rhizobia and NH_4NO_3. A- Soil pH-H_2O after plant harvesting. B- Cd available, C- Pb available and D- Zn available after plant harvesting. Bars with the same upper or lower case letters do not differ by Scott-Knott test, $p<0.05$.

Figure 6 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 6 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 6B-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, 2004). 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 6A-B and 6D). 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 8).

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 7).

This behavior of *L. leucocephala* was already reported by Saraswat and Rai (2011) and Ferreira et al. (2012).

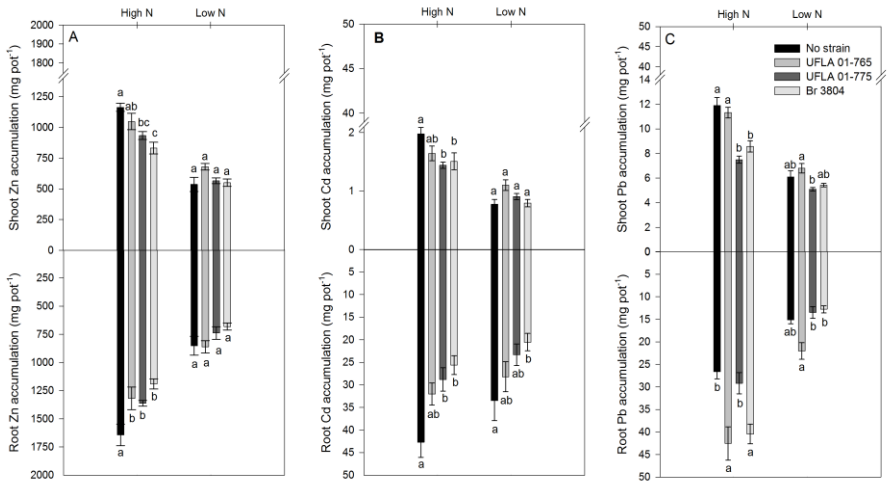

Figure 7. Zn, Cd and Pb accumulation by *Leucaena leucocephala* on a smelting Zn-, Pb- and Cd-contaminated soil after 90 days pot-experiment. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.

In general, metal accumulation in the shoots on low N did not differ among treatments (Figure 7), 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 8). 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 8). 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 9) and showed a better growth (Figures 3A-B and 4) and N accumulation (Figure 5C). 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.

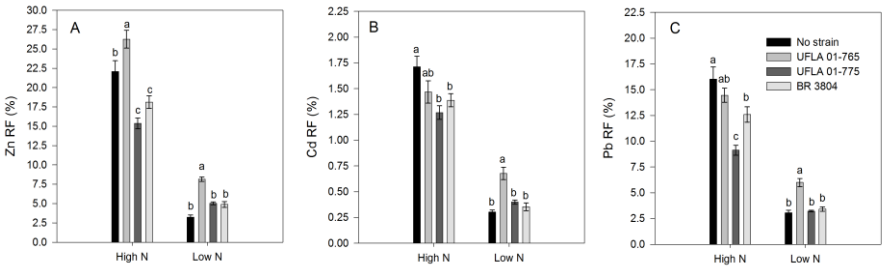

Figure 8. Zn, Cd and Pb remediation factor (RF) by *Leucaena leucocephala* on a smelting Zn-, Pb- and Cd-contaminated soil after 90 days pot-experiment. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.

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 $(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-}$

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.

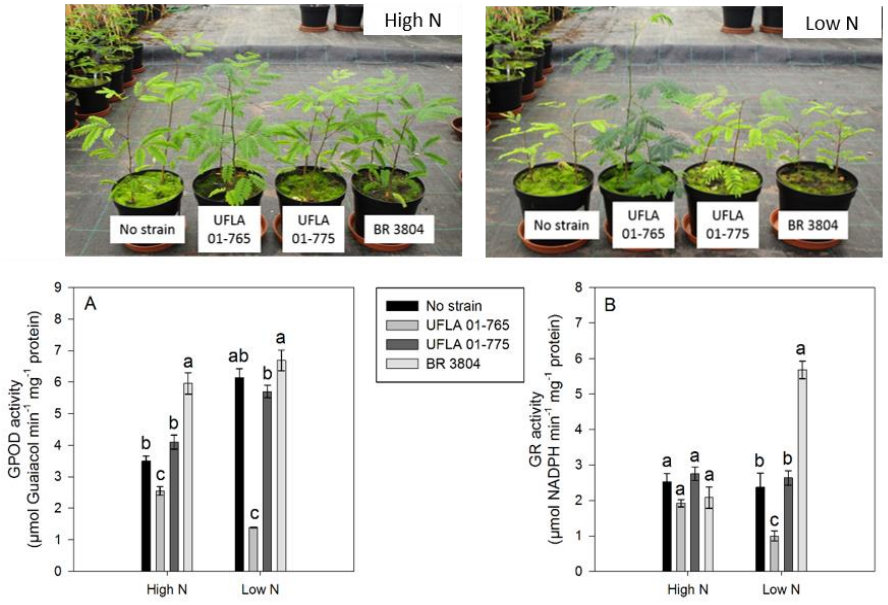

Figure 9. Shoot enzymatic activity of *Leucaena leucocephala* inoculated with rhizobia, and control without rhizobia, after 90 days pot-experiment on a Zn-, Pb- and Cd-contaminated soil. A- Guaiacol peroxidase (GPOD) activity. B- Glutathione reductase (GR) activity. Bars with the same upper or lower case letters do not differ by Scott-Knott test, p<0.05.

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 to André Janssen and Jorik Janssen for 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.

References

Abdel-Wahab, H. H.; Zahran, H. H.; Abd-Alla, M. H. 1996. Root-hair infection and nodulation of four grain legumes as affected by the form and the application time of nitrogen fertilizer. Folia Microbiol. 41:303-308.

Arreseigor, C.; Minchin, F. R.; Gordon, A. J.; Nath, A. K. 1997. Possible cause of the physiological decline in soybean nitrogen fixation in response to nitrate. J. Exp. Bot. 48:905-913.

Atkins, C. A.; Shelp, B. J.; Kuo, J.; Peoples, M. B.; Pate, T. S. 1984. Nitrogen nutrition and the development and senescence of nodules on cowpea seedlings. Planta 162:316-326.

Bacanambo, M.; Harper, J. E. 1996. Regulation of nitrogenase activity in *Bradyrhizobium japonicum*/soybean symbiosis by plant N status as determined by shoot C:N ratio. Physiol. Plant, 98: 529-538.

Bais, H. P.; Weir, T. L.; Perry, L. G.; Gilroy, S.; Vivanco, J. M. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology, 57: 233 - 266, 2006.

Baker, A. J. M.; McGrath, S. P.; Sidoli, C. M. D. and Reeves, R. D. (1994) The possibility of in situ heavy metal decontamination of polluted soils using crops

of metal-accumulating plants. Resources, Conservation and Recycling, 11: 41-49.

Becerra-Castro, C., Kidd, P.S., Prieto-Fernández, A., Weyens, N., Acea, M.J., and Vangronsveld, J. (2011) Endophytic and rhizoplane bacteria associated with Cytisus striatus growing on hexachlorocyclohexane-contaminated soil: isolation and characterisation. Plant Soil 340: 413–433.

Becerra-Castro, C., Prieto-Fernández, A., Kidd, P.S., Weyens, N., Rodríguez-Garrido, B., Touceda-González, M., and Acea, M.J. (2012) Improving performance of Cytisus striatus on substrates contaminated with hexachlorocyclohexane (HCH) isomers using bacterial inoculants: developing a phytoremediation strategy. Plant Soil 362: 247–260.

Belimov, A.A., Hontzeas, N., Safronova, V.I., Demchinskaya, S.V., Piluzza, G., Bullitta, S., Glick, B.R. (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem 37: 241-250.

Bergmeyer, H.U., Gawenn, K., Grassl, M. (1974). Enzymes as biochemical reagents In: Methods in Enzymatic Analysis, Bergmeyer H.U. (ed.) Academic Press, New York, pp. 425 - 522.

Brear, E.M., Day, D.A., Smith, P.M.C. (2013) Iron: an essential micronutrient for the legume-rhizobium symbiosis. Frontiers in Plant Science 4: 359.

Carneiro, M.A.C., Siqueira, J.O., Moreira, F.M.S., Soares, A.L.L. (2008) Soil organic carbon, total nitrogen, microbial biomass and activity in two rehabilitation chronosequences after bauxite mining. R. Bras. Ci. Solo, 32:621-632.

Carvalho, T. S.; Moreira, F. M. S. Simbioses tripartites: leguminosas, fungos micorrízicos e bactérias fixadoras de nitrogênio nodulíferas. In: Siqueira, J. O.; Souza, F. A.; Cardoso, E. J. B. N.; Tsai, S. M. (eds.). Micorrizas: 30 anos de pesquisa no Brasil. 1ed. Lavras: Editora UFLA, 2010, p. 383-413.

Chaudhary, P., Dudeja, S.S., Kapoor, K.K. (2004). Effectivity of host-*Rhizobium leguminosarum* symbiosis in soils receiving sewage water containing heavy metals. Microbiological Research 159: 121–127.

Clemente, M.R., Bustos-Sanmamed, P., Loscos, J., James, E.K., Pérez-Rontomé, C., Navascués, J., Gay, M., Becana, M. (2012). Thiol synthetases of legumes: immunogold localization and differential gene regulation by phytohormones. J Exp Bot 63: 3923 – 3934.

Croes, S., Weyens, N., Janssen, J., Vercampt, H., Colpaert, J.V., Carleer, R., Vangronsveld, J. (2013) Bacterial communities associated with Brassica napus

L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microbial Biotechnology 6: 371-384.

Cunningham, J.E., Kuiack, C. 1992. Production of citric and oxalic acids and solubilization of calcium-phosphate by *Penicillium bilaii*. Applied Environmental Microbiology 58: 1451 - 1458.

Cunningham, S.D., Shann, J.R., Crowley, D.E., Anderson, T.A. 1997. Phytoremediation of contaminated water and soil. In: Kruger, E.L., Anderson, T.A., Coats, J.L., ed. Phytoremediation of soil and water contaminants. Washington: American Chemical Society, v.664, pp.2 - 17.

Davis, D.H., Doudoroff, M., Stanier, R.Y. (1969) Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. Int J System Bacteriol 19: 375-390.

Dias-Júnior, H.E., Moreira, F.M.S., Siqueira, J.O., Silva, R. 1998. Heavy metals, microbial density and activity in a soil contaminated by wastes from a zinc industry. Revista Brasileira de Ciência do Solo 22: 631-640.

Eaglesham, A. R. J. 1989. Nitrate inhibition of root nodule symbiosis in doubly rooted soybean plants. Crop Sci. 29:115-119.

Empresa Brasileira de Pesquisa Agropecuária–EMBRAPA. Serviço Nacional de Levantamento e Conservação do Solo. Manual de análises de solo. 2ed. Rio de Janeiro, 212 p. 1997.

Epstein, E.; Bloom, A. 2005. Mineral Nutrition of Plants: Principles and Perspectives. Sunderland: Sinauer. 2nd. 380 p.

Ferreira DF. (2011) SISVAR: A computer statistical analysis system. Ciência e Agrotecnologia 35: 1039-1042.

Ferreira, P.A.A., Lopes, G., Bomfeti, C.A., Longatti, S.M.O., Soares, C.R.F.S., Guilherme, L.R.G., Moreira, F.M.S. (2013). Leguminous plants nodulated by selected strains of *Cupriavidus necator* grow in heavy metal contaminated soils amended with calcium silicate. World J Microbiol Biotechnol 29: 2055–2066.

Ferreira, P.A.A., Bomfeti, C.A., Silva Júnior, R., Soares, B.L. Soares, C.R.F.S., Moreira, F.M.S. 2012. Symbiotic efficiency of *Cupriavidus necator* strains tolerant to zinc, cadmium, copper and lead. Pesquisa Agropecuária Brasileira 47: 85 - 95.

Franco, A.A.; Faria, S.M. (1997). The contribution of N2-fixing tree legumes to land reclamation and sustainability in the tropics. Soil Biology and Biochemistry, 29: 897-903.

Franco, A. A.; Campello, E. F.; Faria, S. M.; Dias, L. E. (2000) The importance of biological nitrogen fixation on land rehabilitation. In: Pedrosa, F. O.; Hungria, M.; Yates, G., eds. Nitrogen fixation: From molecules to crop productivity. Dordrecht, Kluwer Academic Publishers, p. 569-570.

Franco, A. A.; Balieiro, F. C. (2000) The role of biological nitrogen fixation in land reclamation, agroecology and sustainability of tropical agriculture. In: Rocha-Miranda, C. E., ed. Rio de Janeiro, Academia Brasileira de Ciências, p. 211-233.

Fred, E. B.; Waksman, S. A. (1928). Laboratory manual of general microbiology. New York: McGraw-Hill Book, 143 p.

Frérot, H., Lefèbvre, C., Gruber, W., Collin, C., Dos Santos, A., Escarré, J. (2006) Specific interactions between local metallicous plants improve the phytostabilisation of mine soils. Plant Soil 282:53–65

Giller, K.E., Witter, E., McGrath, S.P. (2009). Heavy metals and soil microbes. Soil Biol Biochem 41: 2031–203.

Glick, B.R. (2003) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv 21: 383-393.

Glick, B.R., Todorovic, B., Czarny, J., Cheng, Z., Duan, J., McConkey, B. (2007) Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26: 227–242.

Glick, B.R., and Stearns, J.C. (2011) Making Phytoremediation Work Better: Maximizing a Plant's Growth Potential in the Midst of Adversity. Int J Phytoremediation, 13: 4-16.

Gordon, A. J.; Skot, L.; James, C. L.; Minchin, F. R. 2002. Short-term metabolic response of soybean root nodule to nitrate. Journal of Experimental Botany 53: 423-428.

Grill, E., Gekeler, W., Winnacker, E.L., Zenk, H.H. (1986). Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing Fabales. FEBS Letters 205: 47–50.

Hedrich, R.; Schroeder J. I. 1989. The Physiology of ion channels and electrogenic pumps in higher plants. Annual Review of Plant Physiology. 40: 539-569

Hoagland, D.R.; Arnon, D.L. The water culture methods for growing plants without soil. Berkeley: California Agriculture Experiment Station, 1950. 32p. (Bulletin, 347).

Illmer, P., and Schinner, F. (1995) Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biol Biochem 27: 257-263.

Imsande, J. 1986. Inhibition of nodule development in soybean by nitrate or reduced nitrogen. J. Exp. Bot. 37:348-355.

Jesus EC, Moreira FMS, Florentino LA, Rodrigues MID, Oliveira MS. Diversidade de bactérias que nodulam siratro em três sistemas de uso da terra da

Amazônia Ocidental. Pesquisa Agropecuária Brasileira, Brasília, v. 40, p. 769-776, 2005.

Kanayama, Y.; Yamamoto, Y. 1990. Inhibition of nitrogen fixation in soybean plants supplied with nitrate I. Nitrite accumulation and formation of nitrosylleghemoglobin in nodules. Plant Cell Physiol 31: 341-346.

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., et al. (2009) Trace elements behaviour at the root–soil interface: implications in phytoremediation. Environ Exp Bot 67: 243–259.

Lebeau, T., Braud, A., and Jézéquel, K. (2008) Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: a review. Environ Pollut 153: 497–522.

van der Lelie, D., Taghavi, S., Monchy, S., Schwender, J., Miller, L., Ferrieri, R., et al. (2009) Poplar and its bacterial endophytes: coexistence and harmony. Crit Rev Plant Sci 28: 346–358.

Mahieu, S., Frérot, H., Vidal, C., Galiana, A., Heulin, K., Maure, L., Brunel, B., Lefèbvre, C., Escarré, J., Cleyet-Marel, J.C. (2011). *Anthyllis vulneraria* /*Mesorhizobium metallidurans*, an efficient symbiotic nitrogen fixing association able to grow in mine tailings highly contaminated by Zn, Pb and Cd. Plant Soil 342: 405–417.

Maimaiti, J., Zhang, Y., Yang, J., Cen, Y., Layzell, D.B., Peoples, M., Dong, Z. (2007) Isolation and characterization of hydrogen-oxidizing bacteria induced following exposure of soil to hydrogen gas and their impact on plant growth. Environ Microbiol 9: 435–444.

Mastretta, C., Taghavi, S., van der Lelie, D., Mengoni, A., Galardi, F., Gonnelli, C., et al. (2009) Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. Int J Phytoremediation 11: 251–267.

Matsuda, A., Moreira, F.M.S., Siqueira, J.O. 2002a. Tolerance of rhizobia genera from different origins to zinc, copper and cadmium. Pesquisa Agropecuária Brasileira 37: 343 - 355.

Matsuda, A., Moreira, F.M.S., Siqueira, J.O. 2002b. Survival of *Bradyrhizobium* and *Azorhizobium* in heavy metal contaminated soil. Revista Brasileira de Ciência do Solo 26: 249 - 256.

Meers, E., Van Slycken, S., Adriaensen, K., Ruttens, A., Vangronsveld, J., Du Laing, G. Witters, N., Thewys, T., Tack, F.M. 2010. The use of bio-energy crops (*Zea mays*) for 'phytoattenuation' of heavy metals on moderately contaminated soils: a field experiment. Chemosphere 78: 35 - 41.

Melloni, R.; Moreira, F.M.S.; Nóbrega, R.S.A.; Siqueira, J.O. (2006) Efficiency and phenotypic diversity among nitrogen-fixing bacteria that nodulate cowpea

[*Vigna unguiculata* (L.) WALP] and common bean (*Phaseolus vulgaris* L.) in bauxite-mined soils under rehabilitation. R. Bras. Ci. Solo, 30: 235-246.

Mergeay, M., Nies, D., Schlegel, H.G., Gerits, J., Charles, P., Van Gijsegem, F. (1985) *Alcaligenes eutrophus* CH34 Is a Fcultative Chemolithotroph with Plasmid-Bound Resistance to Heavy Metals. Jounal of Bacteriology 162: 328-334.

Moreira, F. M. S. et al. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacylamide gel electrophoresis of their total proteins. Systematic and Applied Microbiology, Stuttgart, v. 16, p. 135-146, 1993.

Moreira, F. M.S.; Carvalho, T. S.; Siqueira, J. O. Effect of fertilizers, lime, and inoculation with rhizobia and mycorrhizal fungi on the growth of four leguminous tree species in a low-fertility soil. Biology and Fertility of Soils, v. 46, p. 771-779, 2010a.

Moreira, F.M.S.; Faria, S.M.; Balieiro, F.C.; Florentino, L.A. Bactérias fixadoras de N_2 e fungos micorrízicos arbusculares em espécies florestais: avanços e aplicações biotecnológicas. In: Figueiredo, M.V.B.; Burity, H.A.; Oliveira, J.P.; Santos, C.E.R.S.; Stamford, N.P. (Org.). Biotecnologia aplicada a agricultura. Recife: Embrapa/IPA, 2010b, v. 1, p. 439-477.

Moreira, F.M.S.; Ferreira, P.A.A., Vilela, L.A.F., Carneiro, M.A.C. Symbioses of Plants with Rhizobia and Mycorrhizal Fungi in Heavy Metal-Contaminated Tropical Soils. In: Sherameti, I. and Varma, A. (eds.), Heavy Metal Contamination of Soils, Soil Biology. Springer International Publishing Switzerland, 2015.

Nautiyal, C.S. (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170: 265-270.

Neo, H. H.; Layzell, D. B. 1997. Phloem glutamine and the regulation of O2 diffusion in legume nodules. Physiologia Plantarum 113: 259-267.

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

Plessner, O., Klapatch, T., Guerinot, M.L. (1993) Siderophore utilization by *Bradyrhizobium japonicum*. Appl Environ Microbiol 59: 1688-1690.

Purcell, L. C.; Sinclair, T. R. 1990. Nitrogenase activity and nodule gas permeability response to rhizospheric NH3 in soybean. Plant Physiol. 92: 268–272.

Ribeiro, A.C., Guimarães, P.T.G., Alvarez, V.V.H. (1999). Recomendações para o uso de corretivos e fertilizantes em Minas Gerais – 5ª aproximacão. UFV, Viçosa, Minas Gerais.

Rajkumar, M., Ae, N., Freitas, H. (2009) Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77: 153–160.

Rajkumar, M.; Sandhya, S.; Prasad, M. N. V.; Freitas, H. Perspectives os plant-associated microbes in heavy metal phytoremediation. Biotechnology Advances 30: 1562-1574, 2013.

Rangel, W.M., Schneider, J., Costa, E.T.S., Soares, C.R.F.S., Guilherme, L.R.G., Moreira, F.M.S. 2014. Phytoprotective Effect of Arbuscular Mycorrhizal Fungi Species against Arsenic Toxicity in Tropical Leguminous Species. International Journal of Phytoremediation 16: 840 - 858.

Ruttens, A., Boulet, J., Weyens, N., Smeets, K., Adriaensen, K., Meers, E., Van Slycken, S., Tack, F., Meiresonne, L., Thewys, T., Witters, N., Carleer, R., Dupae, J., Vangronsveld, J. (2011). Short rotation coppice culture of willows and poplars as energy crops on metal contaminated agricultural soils. Int J Phytoremediation 13: 194–207.

Salomons, W. 1995. Environmental impact of metals derived from mining activities: Processes, predictions, prevention. Journal of Geochemic Exploration 52: 5 - 23.

Sanginga, N.; Wirkom, L. E.; Okogun, A.; Akobundu, I. O.; Carsky, R. J.; Tian, G. 1996. Nodulation and estimation of symbiotic nitrogen fixation by herbaceous and legumes in Guinea savanna in Nigeria. Biol. Fertil. Soils 23:441–448.

Santos, J.V., Rangel, W.M., Guimarães, A.A., Jaramillo, P.M.D., Rufini, M., Marra, L.M., López, M.V., Silva, M.A.P., Soares, C.R.F.S., Moreira, F.M.S. (2013) Soil biological attributes in arsenic-contaminated gold mining sites after revegetation. Ecotoxicology 22:1526–1537.

Saraswat, S., Rai, J.P.N. (2011) Prospective application of *Leucaena leucocephala* for phytoextraction of Cd and Zn and Nitrogen fixation in metal polluted soils. Int J Phytoremediation 13: 271–288.

Schuller, K. A.; Minchin, F. R.; Gresshoff, P. M. 1988. Nitrogenase activity and oxygen diffusion in nodules of soybean cv, Bragg and a supernodulating mutant: effects of nitrate. Journal of Experimental Botany 39: 865-877.

Schwyn, B., and Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160: 47-56.

Silver, S., Phung, L.T. 2009. Heavy metals, bacterial resistance. In Encyclopedia of Microbiology, pp. 220-227. Edited by M. Schaechter. Oxford: Elsevier.

Small, S.K., Puri, S., Sangwan, I., O'Brian, M.R. (2009) Positive Control of Ferric Siderophore Receptor Gene Expression by the Irr Protein in *Bradyrhizobium japonicum*. Journal of Bacteriology 191: 1361-1368.

Sodek, L.; Silva, D. M. 1996. Nitrate inhibits soybean nodulation andnodule activity when applied to root regions distant from the nodulation sites. R. Bras. Fisiol. Veg. 8: 187-191.

Stearns, J.C., and Glick, B.R. (2003) Transgenic plants with altered ethylene biosynthesis or perception. Biotechnology Advances 21: 193-210.

Streeter, J. G. 1988. Inhibition of legume nodule formation and N2 fixation by nitrate. CRC Crit. Rev. Plant Sci 7: 1-23.

Taghavi, S., Garafola, C., Monchy, S., Newman, L., Hoffman, A., Weyens, N., et al. (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl Environ Microbiol 75: 748–757.

Tang, C., Robson, A.D., Dilworth, M.J. (1990) The role of iron in nodulation and nitrogen fixation in *Lupinus angustifolius* L. New Phytol 114: 173-182.

Trannin, I.C.B.; Moreira, F.M.S.; Siqueira, J.O.; Lima, A. (2001a) Tolerance of *Bradyrhizobium* and *Azorhizobium* strains and isolates to Copper, Cadmium and Zinc "in vitro". R. Bras. Ci. Solo 25: 305-316.

Trannin, I.C.B., Moreira, F.M.S., Siqueira, J.O. (2001b). Growth and nodulation of *Acacia mangium*, *Enterolobium contortisiliquum* and *Sesbania virgata* in heavy metal contaminated soil. R Bras Ci Solo 25: 743–753.

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

Tu, S.; Ma, L.Q. (2003). Effects of arsenate and phosphate on their accumulation by an arsenic-hyperaccumulator *Pteris vittata* L. Plant and Soil, 249: 373–382.

Valls, M., de Lorenzo, V. 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol Rev 26: 327–338.

Van Slycken, S., Witters, N., Meers, E., Peene, A., Michels, E., Adriaensen, K., Ruttens, A., Vangronsveld, J., Du Laing, G., Wierink, I., Van Dael, M., Van Passel, S., Tack, F.M. 2013. Safe use of metal-contaminated agricultural land by cultivation of energy maize (*Zea mays*). Environmental Pollution 178: 375 - 380.

Vangronsveld, J., Clijsters, H. 1994. Toxic effects of metals. In Plants and the chemical elements. Biochemistry, uptake, tolerance and toxicity. Farago, M.E. (ed) VCH Publishers, Weinheim, pp. 150 - 177.

Vangronsveld, J., Colpaert, J.V., van Tichelen, K.K. 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physicochemical and biological evaluation of the durability of soil treatment and revegetation. Environmental Pollution 94: 131 - 140.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M. 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environmental Science and Pollution Research 16: 765–794.

Vessey, J. K.; Walsh, K. B.; Layzell, D. B. 1988. Can a limitation in phloem supply to nodules account for the inhibitory effect of nitrate on nitrogenase activity in soybean? Physiol Plant 74: 137-146.

Vessey, J. K.; Waterer, J. 1992. In search of the mechanism of nitrate inhibition of nitrogenase activity in legume nodules: Recent development. Physiologia Plantarum 84: 171-176.

Vincent, J.M. A manual for the practical study of root-nodule bacteria. Oxford: Blackwell, 1970. 164 p.

Vyslouzilova, M., Tlustos, P., Szakova, J. (2003). Zn and Cd phytoextraction potential of seven clones of *Salix* spp. planted on heavy metal contaminated soils. Plant Soil Environ 49: 542–547.

Wani P.A., Khan M.S., Zaidi A. (2007). Cadmium, chromium and copper in greengram plants. Agron Sustain Dev 27:145–153.

Wani P.A., Khan M.S., Zaidi A. (2008). Effects of heavy metal toxicity on growth, symbiosis, seed yield and metal uptake in pea grown in metal amended soil. Bull Environ Contam Toxicol. 81:152–158.

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

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

Weyens, N., van der Lelie, D., Artois, T., Smeets, K., Taghavi, S., Newman, L., et al. (2009c) Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation. Environ Sci Technol 43: 9413–9418.

Weyens, N., Truyens, S., Dupae, J., Newman, L., van der Lelie, D., Carleer, R., and Vangronsveld, J. (2010) Potential of *Pseudomonas putida* W619-TCE to

reduce TCE phytotoxicity and evapotranspiration in poplar cuttings. Environ Pollut 158: 2915–2919.

Weyens, N., Boulet, J., Adriaensen, D., Timmermans, J.-P., Prinsen, E., Van Oevelen, S., et al. (2011) Contrasting colonization and plant growth promoting capacity between wild type and a gfp-derative of the endophyte *Pseudomonas putida* W619 in hybrid poplar. Plant Soil 356: 217–230.

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

Weyens, N., Schellingen, K., Beckers, B., Janssen, J., Reinhart, C., van der Lelie, D., et al. (2013b) Potential of willow and its genetically engineered associated bacteria to remediate mixed Cd and toluene contamination. J Soils Sediments 13: 176–188.

Williams, L. E.; Miller, A. J. 2001. Transporters responsible for the uptake partitioning of nitrogenous solutes. Annual Review of Plant Physiology and Plant Molecular Biology. 52: 659-668.

Willems, A., de Ley, J., Gillis, M., Kersters, K. (1991) *Comamonadaceae*, a New Family Encompassing the Acidovorans rRNA Complex, Including

Variovorax paradoxus gen. nov. , comb. nov. for *Alcaligenes paradoxus* (Davis 1969). Int J System Bacteriol 41: 445-450.

PAPER 3 - DRAFT GENOME SEQUENCE OF *MESORHIZOBIUM* SP. UFLA 01-765, A MULTI-TOLERANT, EFFICIENT SYMBIONT AND PLANT-GROWTH PROMOTING STRAIN ISOLATED FROM ZN-MINING SOIL USING *LEUCAENA LEUCOCEPHALA* AS A TRAP PLANT

According to *Genome Announcements*

Draft genome sequence of Mesorhizobium sp. UFLA 01-765, a multi-tolerant, efficient symbiont and plant-growth promoting strain isolated from Zn-mining soil using Leucaena leucocephala as a trap plant

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Abstract

We report here the draft genome of *Mesorhizobium* sp. UFLA 01-765 strain, a Gram-negative bacterium of the Phyllobacteriaceae, isolated from a Zn-mining soil in Minas Gerais, Brazil. This strain is a promising plant-growth promoter, able to stablish symbiosis and efficiently fix N_2 with *Leucaena leucocephala* on multi-contaminated soil. Analysis of its 7.4-Mb draft genome will bring insights in land bioremediation applications in recovery of marginal land.

Introduction

Nowadays the global economic development is mainly supported by different mining activities, which is strongly linked to social

development, generating assets and wealth. On the other hand, mining exploitation, especially metal mining, causes huge environmental impacts. Native rhizobia from mining soils are promising candidates for land recover of those soils as they are probably more tolerant to these conditions.

An efficient N_2 fixing and plant-growth promoting strain was isolated from a Zn-mining soil in Minas Gerais, Brazil. This strain was identified as *Mesorhizobium* sp. UFLA 01-765 by partial 16S rDNA gene sequencing, the closest related partial 16S rDNA gene sequence (99%) in NCBI was from strain HF931067 (Genbank) (1).

Genomic DNA was isolated using a DNeasy blood and tissue kit (Qiagen, Venlo, Netherlands), treated with RNase I and purified by phenol:chloroform extraction. An IonTorrent PGM (Life Technologies Inc., Carlsbad, CA) used for sequencing after extracting DNA from stationary phase cells using standard techniques. DNA was digested and sequencing adaptors ligated using an Ion Xpress Plus Fragment Library Kit (Life Technologies Inc., Burlington, ON) according to the manufacturer's instructions. Adaptor-ligated DNA was size selected to a target length of 480 bp on a 2% E-Gel SizeSelect agarose gel, and Agencourt MAPure XP beads (Beckman Coulter, Mississauga, ON) were used for purification steps. An Ion Library Quantitation Kit was used to calculate the library dilution factor prior to amplification and enrichment with an Ion PGM Template OT2 400 kit on an Ion OneTouch 2 system. An Ion Sphere Quality Control Kit was used to quantify the percentage of

enriched Ion Sphere Particles prior to sequencing with an Ion PGM 400 Sequencing Kit.

In total, 1.2 million reads (mean length 291 bases) generated 351 Mb of data (> 305 M Q20 bases) in Torrent Suite 4.2.1. These were assembled using SPAdes 3.1.0 (2, 3) (uniform coverage mode; kmers 21, 33, 55, 77, 99) into 185 contigs greater than 500 bp, giving a consensus length of 7,464,539 bp (largest contig 366,840 bp; N50 = 123,481 bp). Open reading frame prediction and gene annotation were carried out using RAST (4). This strain has a GC content of 56.17% and 7423 coding genes arranged into 409 subsystems, 8 rRNAs (16s and 23s) and17tRNAs.

In symbiosis with *Leucaena leucocephala* on Cd-, Pb- and Zn-contaminated soil UFLA 01-765 strain promoted plant growth, increasing nitrogen accumulation and decreasing glutathione reductase (EC 1.8.1.7) and guaiacol peroxidase (EC 1.11.1.7) activities. Analysis of the draft genome confirmed the presence of genes coding for multi-resistance including metal-dependent hydrolases of the beta-lactamases superfamily I, Type I secretion outer membrane protein, DNA-binding heavy metal response regulator and cobalt-zinc-cadmium resistance protein CzcD. The cluster for metal-dependent hydrolases of the beta-lactamases superfamily I is similar to *Mesorhizobium* sp BNC1 and *Sinorhizobium meliloti* 1021, and cluster for DNA-binding heavy metal response regulator is similar to *Sinorhizobium meliloti* 1021. Several genes involved in the main mechanisms of plant-growth promotion (auxin biosynthesis, 1-aminocyclopropane-1-carboxylate deaminase activity, siderophore

production, phosphorus solubilisation and N_2 fixation) were found in the genome of this strain. The activity of these genes was confirmed by phenotypic assay. Moreover UFLA 01-765 produced IAA, ACC deaminase, siderophore, solubilized $Ca_3(PO_4)_2$, and utilized glycerol, glucose, fructose and sucrose as carbon sources, besides fixing N_2 in symbiosis with *L. leucocephala* on a multi contaminated soil.

The properties of the inorganic carbon (Ci)-uptake by carboxysome in proteobacteria are poorly understood. But it is well known that carboxysomes are specialized protein microcompartments within which some autotrophic bacteria concentrate CO_2 around their D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the primary carboxylating enzyme. This CO_2-concentrating mechanism (CCM) operate simultaneously with carbon dioxide and carbonate uptake transporters which accumulate carbonate in the cellular cytoplasm (5). Some rhizobia genera have been studied for autotrophic growth such as *Bradyrhizobium japonicum* USDA110, *Sinorhizobium meliloti* 1021, and the photosynthetic *Bradyrhizobium* sp. ORS278, and the presence of the RuBisCO gene at the genome was confirmed (5-6). Interestingly, up to now, *Mesorhizobium* genus has not any representant on the list still. Hence *Mesorhizobium loti* UFLA 01-765 strain is the first report for a chemoautotrophic growth within the genus. Its genome holds genes for the Calvin Benson Bassham (CBB) cycle, and genes upregulated under chemoautotrophic growth, such as RuBisCO and phosphoribulokinase.

In conclusion, *Mesorhizobium* sp. UFLA 01-765 is a promising strain as an inoculant for *L. leucocephala* to stimulate revegetation of Zn-

and Cd-contaminated sites, and it is a candidate as a type strain for the genus on studies about the CCM in chemoautotrophic rhizobia.

Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited at DDBJ/EMRL/GenBank under the accession XXXXXXXXXXXX. The version described in this paper is version XXXXXXXXXXXX.

Acknowledgements

This work was supported by a PhD grant to Wesley de Melo Rangel for doctoral training sandwich abroad (BEX: 13079/2013-01) from the Brazilian Commission for Improvement of Higher Education Staff (CAPES), and financial resource from 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).This work also has been financially supported by the Hasselt University Methusalem project 08M03VGRJ.

References

1. Armas-Capote, N., Pérez-Yépez, J., Martínez-Hidalgo, P., Garzón-Machado, V., del Arco-Aguilar, M., Velázquez, E., León-Barrios, M. 2014. Core and symbiotic genes reveal nine *Mesorhizobium*genospecies and three symbiotic lineages among the rhizobia nodulatingCicercanariense in its natural habitat (La Palma, Canary Islands). Syst. Appl. Microbiol. 37:140–148.

2. Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A. 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J. Comput. Biol. 19:455–477.

3. Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G. 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29:1072.

4. Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O. 2008. The RAST server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:1471–2164. http://dx.doi.org/10.1186/1471-2164-9-75.

5. Rae, B.D., Long, B.M., Badger, M.R., Price, G.D. 2013. Functions, Compositions, and Evolution of the Two Types ofCarboxysomes: Polyhedral Microcompartments That Facilitate CO_2Fixation in Cyanobacteria and Some Proteobacteria. Microbiology and Molecular Biology Reviews 77: 357–379.

6. Gourion, B., Delmotte, N., Bonaldi, K., Nouwen, N., Vorholt, J.A., Giraud, E. 2011. Bacterial RuBisCO Is Required for Efficient*Bradyrhizobium*/*Aeschynomene*Symbiosis. PLoSONE 6(7): e21900. doi:10.1371/journal.pone.0021900.