

Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn-contaminated soil and their effects on root growth

Peer-reviewed author version

MONTALBAN GINES, Blanca; CROES, Sarah; WEYENS, Nele; Carmen Lobo, Ma; Perez-Sanz, Araceli & VANGRONSVELD, Jaco (2016) Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn-contaminated soil and their effects on root growth. In: INTERNATIONAL JOURNAL OF PHYTOREMEDIATION, 18(10), p. 985-993.

DOI: 10.1080/15226514.2016.1183566

Handle: <http://hdl.handle.net/1942/22055>




Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn contaminated soil and their effects on root growth

Blanca Montalbán PhD, Sarah Croes, Nele Weyens, M Carmen Lobo, A. Pérez-Sanz & Jaco Vangronsveld PhD

To cite this article: Blanca Montalbán PhD, Sarah Croes, Nele Weyens, M Carmen Lobo, A. Pérez-Sanz & Jaco Vangronsveld PhD (2016): Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn contaminated soil and their effects on root growth, International Journal of Phytoremediation, DOI: [10.1080/15226514.2016.1183566](https://doi.org/10.1080/15226514.2016.1183566)


To link to this article: <http://dx.doi.org/10.1080/15226514.2016.1183566>

 View supplementary material 

 Accepted author version posted online: 09 May 2016.

 Submit your article to this journal 

 Article views: 6

 View related articles 

 View Crossmark data 

Characterization of bacterial communities associated with *Brassica napus* L. growing on a Zn contaminated soil and their effects on root growth

Blanca Montalbán PhD^{1,*}, Sarah Croes^{1,**}, Nele Weyens^{1,\$}, M Carmen Lobo², A. Pérez-Sanz³,
Jaco Vangronsveld Ph.D Professor⁴

¹Hasselt University, Centre for Environmental Sciences, Environmental Biology, Diepenbeek, Belgium

²IMIDRA, Finca “El Encin” A-2, Km 38,2, Alcalá de Henares, Spain, carmen.lobo@madrid.org

³IMIDRA, Agroenvironment Research, Finca “El Encín”, A-II km 38,200, Alcalá de Henares, 28800 Spain, araceli.perez@madrid.org

⁴Hasselt University, Environmental Biology, Agoralaan, building D, Diepenbeek, B-3590 Belgium, jaco.vangronsveld@uhasselt.be

*Corresponding Author E-mail: blanca.montalban@madrid.org

**Corresponding Author E-mail: sarah.croes@uhasselt.be

\$Corresponding Author E-mail: nele.weyens@uhasselt.be

Detailed phenotypic characterization of all the bacterial strains isolated can be found as supporting information.

The authors have no conflict of interest to declare

Abstract

The interaction between plant growth-promoting bacteria (PGPB) and plants can enhance biomass production and metal tolerance of the host plants. This work aimed at isolating and characterizing the cultivable bacterial community associated with *Brassica napus* growing on a

Zn contaminated site, for selecting cultivable PGPB that might enhance biomass production and metal tolerance of energy crops. The effects of some of these bacterial strains on root growth of *B. napus* exposed to increasing Zn and Cd concentrations were assessed. A total of 426 morphologically different bacterial strains were isolated from the soil, the rhizosphere, the roots and stems of *B. napus*. The diversity of the isolated bacterial populations was similar in rhizosphere and roots, but lower in soil and stem compartments. *Burkholderia*, *Alcaligenes*, *Agrococcus*, *Polaromonas*, *Stenotrophomonas*, *Serratia*, *Microbacterium* and *Caulobacter* were found as root endophytes exclusively. The inoculation of seeds with *Pseudomonas* sp. strains 228 and 256, and *Serratia* sp. strain 246 facilitated the root development of *B. napus* at 1000 μM Zn. *Arthrobacter* sp. strain 222, *Serratia* sp. strain 246, and *Pseudomonas* sp. 228 and 262 increased the root length at 300 μM Cd.

Key words

Plant-associated bacteria, endophytes, inoculation, plant growth-promoting bacteria, phytoremediation

1. Introduction

The number of areas polluted due to excess of metals and metalloids was growing due to the increase of anthropogenic activities such as mining, metallurgical industries, electroplating, manufacturing of plastics, paint pigments, alloy preparation and batteries, energy and fuel production and application of fertilizers and pesticides (Broadley *et al.*, 2007; Kirkham 2006; Panagos *et al.*, 2013). Several metals such as Cd can be accumulated in the food chain through uptake at the primary producer level and subsequently through transfer and bioaccumulation at higher trophic levels (Nagajyot *et al.*, 2010). Phytoextraction is a low cost technology that uses green plants to extract metals and metalloids from the soil and accumulate them in the harvestable plant parts. However, the low biomass production of most hyperaccumulator species, along with their low economic value (Vamerali *et al.*, 2010), led to a search for higher-biomass accumulator crops. Making use of these crops can provide renewable biomass that can be used for bioenergy production, ecomaterials or biocatalysis, and at the same time to remediate metal-contaminated soils that can no longer be used for food and feed production (Vangronsveld *et al.*, 2009; Witters *et al.*, 2012; Kidd *et al.*, 2015).

Metal availability, uptake and phytotoxicity are some of the main limiting factors of phytoextraction in metal-contaminated soils (Weyens *et al.*, 2009b). In case of high metal availability, energy crops that are effective in removing metals from soils can show a reduced growth due to phytotoxicity, thereby decreasing both the amount of marketable biomass and the remediation efficiency. The interaction between plant growth-promoting bacteria (PGPB) and plants can frequently enhance biomass production and metal tolerance of the plants (Germida *et al.*, 1998; Genrich *et al.*, 2000; Zhang *et al.*, 2012), diminishing symptoms of phytotoxicity.

Some metal tolerant PGPB bacteria from soil, rhizosphere or endophytes have the capacity to promote plant growth through mechanisms such as nitrogen fixation, production of siderophores and phytohormones (such as IAA, indole-3-acetic acid), solubilization of minerals like phosphorous, and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick *et al.*, 2003; Rajkumar *et al.*, 2009; Weyens *et al.*, 2009b; Sessitsch *et al.*, 2013). The latter enzyme has received increasing attention because of its role in lowering ethylene levels in a stressed plant. The presence of toxic amounts of metals, and other types of stress like high salt concentrations or phytopathogens induce elevated ethylene levels (mainly) causing inhibition of root growth and development (Glick, 2010; Schellingen *et al.*, 2014). Further, some bacteria can solubilize unavailable forms of potentially toxic elements in soils by excreting organic acids and siderophores (Ma *et al.*, 2009). On the other hand, some plant-associated bacteria can reduce the metal uptake by binding metals to anionic functional groups or to extracellular polymeric substances (Rouch *et al.*, 1995; Ma *et al.*, 2011; Rajkumar *et al.*, 2012). Extending our knowledge about these bacterial characteristics and the action mechanisms of PGPB is important for the development of more effective phytotechnologies for metal-contaminated sites (Zhang *et al.*, 2011; Sessitsch *et al.*, 2013).

Brassica napus L. (rapeseed) is a well-known high-biomass crop commonly used for bioenergy production. Many studies mentioned the tolerance of this crop to toxic amounts of metals, such as Cd and Zn, as well as its capacity to accumulate them in their tissues (Marchiol *et al.*, 2004; Grispen *et al.*, 2006; Hernández-Allica *et al.*, 2008). Based on its characteristics, *B. napus* can be a suitable candidate for phytoextraction purposes and to produce valorizable biomass on Cd/Zn-contaminated land (Marchiol *et al.*, 2004; Croes *et al.*, 2013).

This work aimed at isolating and characterizing the cultivable bacterial communities associated with *B. napus* growing on a Zn-contaminated site, for selecting cultivable PGP bacterial strains that might enhance biomass production and tolerance of *B. napus* on Zn-contaminated land. Long-term polluted soils are sources of metal-tolerant microorganisms and interesting interactions with the plants that are growing in these soils, such as PGP characteristics (Becerra-Castro *et al.*, 2012). A further objective is to evaluate the effects of PGP bacterial strains on root growth of *B. napus* in the presence of increasing concentrations of Zn or Cd, using vertical agar plates (VAPs). Here, VAPs will be used to perform preliminary inoculation experiments for selecting potential cultivable PGP bacteria having positive effects on root structure (Zhang *et al.*, 1998; Remans *et al.*, 2006), while avoiding competition with other microorganisms (Reynos-Cuevas *et al.*, 2008).

2. Experimental Procedures

2.1. Sampling

The experimental field (50°59'23''N, 5°14'21''E) was situated in Lummen (Belgium) near a zinc recycling plant that started its activities mid-1975. In the early days, only crude zinc ashes of hot-dip galvanizers were processed. From 1982 they started processing of zinc scrap to zinc metal. The contamination on the site that is causing phytotoxicity is limited to Zn, although total Pb concentrations are also clearly higher than background values (Table 1). Cadmium concentrations are within the range of background values in Flanders.

In September 2011, *B. napus* cultivar Remy seeds were sown (50-80 plants per m²) on this site. Two weeks before sowing, 3 cm of compost (based on horse and chicken manure), was applied

and worked in the soil using rototilling. Soils and plants were sampled in April 2012. Plants were sampled in the flowering stage.

2.2. Metal concentrations in soils and plants

Metal concentrations were determined in three replicates of soil and plant samples. The extractable fractions of metals in soil were estimated using 0.1 M $\text{Ca}(\text{NO}_3)_2$ (Mench *et al.*, 1994). (Pseudo)-total concentrations of metals in soil were determined by *aqua regia* digestion (Van Ranst *et al.*, 1999). Plants were washed with distilled water, separated into leaves, stems and roots, and then dried for 48 h at 65°C. Subsequently, the dried tissues were ground and digested according to Weyens *et al.* (2010). Metal concentrations in the extracts were determined using inductively coupled plasma optical emission spectrometry (ICP-OES). The quality of the digestion and analytical methods was tested including blanks and certified reference material (NIST Standard Reference Material 1570a, Trace elements in Spinach, U.S. Department of Commerce, National Institute of Standards and Technology) with every set of samples.

2.3. Isolation of cultivable bacterial strains

Cultivable bacteria were isolated from bulk soil, rhizosphere soil, roots and stems of *B. napus* following the sampling design of Croes *et al.* (2015). The isolation procedure was carried out according to Weyens *et al.* (2009a), but in this work, the sodium hypochlorite solution (1%) and time (1 min) during root surface sterilization were modified. Colony-forming units (cfu) were counted and calculated per gram soil or fresh plant weight. All morphologically different

strains were purified using five replicates and then stored at -70°C in a solution with 15% (w:v) glycerol and 0.85% (w:v) NaCl.

2.4. Phenotypic characterization

Purified bacterial strains were grown in 869 medium (Mergeay *et al.*, 1985), then washed twice with sterile 10mM MgSO_4 (Croes *et al.*, 2013) and tested for their Zn and Cd tolerance and potential plant growth-promoting (PGP) characteristics (phosphate solubilization, nitrogen fixation, ACC-deaminase activity and production of siderophores, organic acids, IAA and acetoin). Strains not able to grow in the test medium were considered as not detectable (nd). Media without bacteria were used as controls. The PGP characteristics were screened as described previously by Croes *et al.* (2013).

For testing the bacterial tolerance to either Zn or Cd all strains were grown on selective 284 medium with a C-mix (per liter medium, 0.52 g glucose, 0.35 g lactate, 0.66 g gluconate, 0.54 g fructose, and 0.81 g succinate) as described by Weyens *et al.* (2009a) and 1mM of Zn (added as ZnSO_4) or 0.8 mM of Cd (added as CdSO_4). Tolerance was assessed visually.

2.5. Genotypic characterization

Total genomic DNA was extracted from all purified morphologically different bacterial strains by the DNeasy® Blood and tissue kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes of the extracted DNA was carried out using the universal primers, 16S-prokaryotic-R (5'-ACGGGCGGTGTGTRC-3') and 16S-prokaryotic-F (5'-AGAGTTTGATCCTGGCTCAG-3') according to Weyens *et al.* (2009a).

PCR products were directly used for ARDRA and sequencing. For amplified 16S rDNA restriction analysis (ARDRA), 10 μ L of the PCR products were digested with the restriction endonuclease HpyCH4 IV (New England Biolabs, Beverly, MA, USA) and separated by electrophoresis (Weyens *et al.* 2009a). Bacterial strains with the same ARDRA patterns were grouped within each compartment (stem, root, rhizosphere, and soil). The PCR products of one representative strain per compartment were purified according QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified 16S rRNA genes were sent for sequencing by Macrogen (Korea) with an Automatic Sequencer 3730XL. Sequenced strains were identified by means of Sequence Match at the Ribosomal Database Project II. All strains had a sequence match score higher than 0.900.

Partial 16S-rDNA gene sequences were submitted to GenBank (National Center for Biotechnology Information, NCBI) with the accession numbers KT461822- KT461878.

2.6. *Effects of inoculation on root growth*

Certified seeds of *B. napus* L. cv. Nodari (Syngenta Seeds) were surface sterilized by immersing them in 0.1% sodium hypochlorite for 1 minute, then washed three times with Millipore water. Some surface-sterilized seeds were grown during 3 days at 30°C on 869 rich solid medium (Mergeay *et al.*, 1985) in order to verify the surface sterilization process. Seeds were considered surface sterilized when no bacterial growth was observed. Bacterial strains were grown on 869 liquid medium for 12h at 30°C, centrifuged at 1811g during 10 min, washed two times and resuspended in 10mM MgSO₄. Surface-sterilized seeds were immersed in a bacterial suspension (10⁸ cfu mL⁻¹) for 1h at room temperature. Subsequently, the seeds were placed in

Petri dishes with 1/10 diluted 869 solid medium for 1 day at 25°C in darkness. Finally, the inoculated seeds were put into square vertical plates (Zhang *et al.*, 1998) with MS medium spiked separately with 300 and 1000 µM of Zn (ZnSO₄.7H₂O) and 50 and 300 µM of Cd (CdSO₄.8H₂O). The metal doses used in this experiment were chosen according to the tolerance shown by *B. napus* in the presence of increasing doses of Cd and Zn under *in vitro* conditions (Montalbán *et al.*, 2014). Non-inoculated surface-sterilized seeds were immersed in 10mM MgSO₄ and were used as controls in plates with MS medium non-spiked (Control) and spiked (Non-inoculated). All plates were set up vertically in a growth chamber at 23°C/12 °C and 12 h of photoperiod. After 5 days, the root systems in the vertical plates were scanned, and root length was determined after analysis of scanned images using the Optimas Image Analysis Software 6.0 (MediaCybernetics) according to Remans *et al.* (2006).

2.7. Statistical analysis

Statistical analysis of the VAPs results was done using the IBM SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) and Tukey's test were applied in this case. Differences at $p \leq 0.05$ level were considered significant. Genotypic information was subjected to correspondence analysis (CA), a principal component analysis related ordination technique based on chi-square distances, illustrating correlations between compartments to evaluate the isolation procedure (Croes *et al.*, 2015). CA was done using the statistical software package R (<http://cran.at.r-project.org>).

3. Results and discussion

3.1. Soil

The total and $\text{Ca}(\text{NO}_3)_2$ -extractable Zn and Cd concentrations in the soil are shown in Table 1. The soil presented sandy texture, pH 5.6 and 1.9% organic matter. As compared to background values and clean up values for metals in agricultural soils based on the Flemish legislation on soil remediation (VLAREBO, 2009), only total Zn concentrations were in excess. However the $\text{Ca}(\text{NO}_3)_2$ -extractable Zn concentration was high in comparison to non-polluted soils (unpublished results) ~~(try to compare with data in the literature notably leading to Zn phytotoxicity or Cd contamination of food; total soil Zn and Cd is not the most important; it is the labile pools at such acidic soil pH).~~

3.2. Bacteria isolated from *B. napus* growing on a Zn contaminated site

A total of 426 morphologically different cultivable bacterial strains were isolated from bulk soil, rhizosphere soil, roots and stems of the *B. napus* plants. The numbers of different genera were similar in rhizosphere (20) and root (17), but lower in soil (8) and stem (3) compartments (Table 2). The lower diversity of bacteria found in soil with respect to rhizosphere and roots can be explained as a rhizosphere selection by the plant on its surrounding bacterial community (Gomez-Balderas *et al.*, 2014). The number of cultivable strains found in rhizosphere soil was two orders of magnitude higher than in bulk soil and roots. The decline of the bacterial numbers from the rhizosphere to the roots and soil was previously reported by authors investigating *B. napus* associated bacteria (Germida *et al.*, 1998; Croes *et al.* 2013). This high density of cultivable bacteria in the rhizosphere is due to root exudates that provide high amounts of organic carbon directly to microbial populations, in comparison with the slow decomposition of recalcitrant organic matter in the bulk soil (Soderberg and Bååth, 1998). Microorganisms indeed

are attracted by carbohydrates, amino acids and organic acids that are present in the rhizosphere as root exudates and mucilage-derived components (Compant *et al.*, 2010).

On the other hand, the number of endophytic bacteria recovered from the roots was three orders of magnitude higher than in stem samples (Table 2). The numbers of endophytes found in roots and stems are in accordance with earlier reports (Lodewyckx *et al.*, 2002). This high density of bacteria in the lower parts of the plant with respect to upper parts was previously reported by Fisher *et al.* (1992). This supports the idea that at least part of the colonization of the plant interior occurred from the rhizosphere via the root system, through wound sites, root hairs and epidermal junctions (Weyens *et al.* 2009a; Becerra-Castro *et al.* 2011), and mainly during the first stages of root development when tissues are still undifferentiated (Hallmann *et al.*, 2001). Besides, some studies have suggested an active penetration of endophytes through enzymatic degradation of plant cells (Lodewyckx *et al.*, 2002; Truyens *et al.*, 2014).

3.3. Genotypic characterization

In total, 33 different bacterial genera were identified. The pie diagrams in Figure 1 show the diversity and relative abundance of bacterial genera present in each compartment. Each color and number relates to a different bacterial genus and subdivided colors represent bacterial genera with different accession numbers. Eight different genera of bacteria were identified in bulk soil, with *Arthrobacter* (45.9%) and *Bacillus* (41.7%) as dominant genera in this compartment. The high abundance of *Arthrobacter* sp. was not surprising, taking into account that it is considered as one of the most predominant members of cultivable soil microorganisms (Hanbo *et al.*, 2004). Moreover, this genus is in high abundance in Zn-polluted soils (Dell' Amico *et al.*, 2005). Also *Bacillus* was reported as a dominant genus in Cu-Pb-Zn contaminated soil (Ellis *et al.*,

2003). Twenty different genera of bacteria were identified in the rhizosphere, of which *Variovorax* (47.2%), *Arthrobacter* (16.9%), *Bacillus* (10.5%), and *Staphylococcus* (8.8%) were most dominant. The presence of these genera in the rhizosphere of *B. napus* was observed previously by other authors (Germida *et al.* 1998; Croes *et al.*, 2013).

Endophytic bacterial strains identified in roots belonged in particular to the genera *Pseudomonas* (33.8%), *Burkholderia* (30.6%), *Variovorax* (21.1%) and *Pedobacter* (7.4%). In stems, *Pedobacter* (66.7%) and *Micrococcus* (27.9%) dominated the cultivable bacterial population.

Although *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* are the most commonly isolated bacterial genera (Becerra-Castro *et al.*, 2011), in this work, *Enterobacter* and *Agrobacterium* were not isolated from any of the investigated compartments. Field studies on metal contaminated soils have shown that high levels of metals can modify the structure of microbial communities and decrease microbial diversity (Kelly *et al.* 2003, Dell' Amico *et al.*, 2005).

Paenibacillus, *Niastella* and *Brevundimonas* were only found in the bulk soil. Gomez-Balderas *et al.* (2014) and Croes *et al.*, (2013) also isolated the genus *Brevundimonas* from a Zn-Cd contaminated soil, but both studies did not report it in non-contaminated soils, indicating the eventual adaptation of this genus to Zn and Cd contaminated sites. On the contrary, *Arthrobacter* and *Variovorax* were present in all compartments studied, except in the stem.

Staphylococcus, *Shinella*, *Bosea*, *Chryseobacterium*, *Proteobacterium* a/b, *Patulibacter*, *Leifsonia*, *Dyadobacter* and *Methylobacter* were found only in the rhizosphere.

However, *Agromyces*, *Pseudomonas*, *Lysobacter*, *Sphingopyxis* and *Mitsuaria* were present in the rhizosphere and also as root endophytes. *Agromyces* was also found to be a stem endophyte. *Burkholderia*, *Alcaligenes*, *Agrococcus*, *Polaromonas*, *Stenotrophomonas*, *Serratia*, *Microbacterium* and *Caulobacter* were root endophytes exclusively. *Serratia* species were also found as root endophytes in the Cd-hyperaccumulator *Solanum nigrum* by Chen *et al.* (2012). Here, *Pedobacter* was found as root and stem endophyte, however, *Micrococcus* was exclusively present in the stem. *Micrococcus* sp. were also found by Velazquez *et al.* (2008) in stems of sugarcane and by Germida *et al.* (1998) in roots of *Brassica napus*, confirming the ability of this genus to colonize plant tissues.

The genera *Cupriavidus*, *Niastella*, *Agromyces*, *Shinella*, *Bosea*, *Proteobacterium*, *Lysobacter*, *Shingopyxis*, *Patulibacter*, *Mitsuaria*, *Dyadobacter*, *Methylobacter*, *Alcaligenes*, *Agrococcus* and *Polaromonas* were isolated in this study (Figure 1), but were not previously reported for bacterial communities associated with *B. napus* (Croes *et al.*, 2013; Germida *et al.*, 1998). This can be due to (a) specific soil factors that could affect the rhizosphere populations (Bulgarelli *et al.*, 2012), and also (b) to the different concentrations of the (available) metals in the soil. Moreover, (c) the surface sterilization method employed, (d) the growth medium used for isolation (Lodewyckx *et al.*, 2002) and (e) the different growth stages of the host plants at the moment of sampling could also affect the bacterial populations (de Campos *et al.*, 2013; Croes, 2014).

Croes *et al.* (2013) reported that the most dominant root cultivable endophytes in *B. napus* were the genera *Pseudomonas*, *Pedobacter* and *Variovorax*. In this work, these genera were found in similar percentages. Taking into account that the seeds sown in our field originated from the

same seed stock as the one used by Croes *et al.* (2013), our results suggest that these endophytes remained present in the stored seeds..

According to the correspondence analysis ([Figure 2](#)), the mean cultivable rhizosphere bacterial community was more correlated (CC = 0.43) with the mean soil bacterial community than with the endophytic communities found in the roots and stems (CC = 0.35 and -0.08, respectively). This indicates that rhizosphere, bulk soil and root shared a high percentage of bacterial genera. However, the stem showed a higher number of genera specific for this compartment.. Root and stem bacterial communities showed a low correlation coefficient (CC = 0.06), indicating the presence of different bacterial genera. Several authors reported significant differences in bacterial communities between below-ground and above-ground plant parts, demonstrating that the organs of the plants have different bacterial communities associated with them (Lindow and Brandl, 2003; Izumi *et al.*, 2008; Weyens *et al.*, 2009a; Croes *et al.*, 2013).

3.4. Phenotypic characterization

A high percentage of stem endophytes showed tolerance to 1mM Zn, but they were not able to grow on 284 medium supplemented with C-mix and 0.8 mM Cd (Table 3). The Zn concentrations in leaf, stem and roots of *B. napus* collected in the field were 1013 ± 207 , 1301 ± 196 and 941 ± 138 mg kg dry weight⁻¹, respectively. The Cd concentrations were 1.4 ± 0.5 , 1.6 ± 0.6 and 1.2 ± 0.3 mg kg dry weight⁻¹ in leaf, stem and root, respectively. The high stem Zn concentration might explain why the endophytes were more frequently tolerant to 1 mM Zn. In contrast to the bacterial strains isolated from the stems, 21.7% of the root endophytes were tolerant to Cd. The percentages of rhizosphere and soil strains that were tolerant to 0.8mM Cd

and 1mM Zn were similar for both compartments. Croes *et al.* (2013) compared the metal tolerance of bacterial strains isolated from a Zn/Cd-contaminated field and a control field: the highest numbers of bacterial strains tolerant to 1.6 mM Cd and 2.5 mM Zn originated from the contaminated field. They suggested that the concentrations of metals present in the soil stimulated the presence of bacteria tolerant to both metals.

Stem endophytes could not solubilize phosphorus or produce siderophores (Table 3). Many of them were able to fix nitrogen and produce organic acids. The highest percentages of bacterial strains able to solubilize phosphorus and produce siderophores, IAA and ACC deaminase were found in the roots. In contrast, the highest percentage of bacterial strains capable of producing acetoin was found in the stems. Our results suggest that the production of organic acids, IAA and ACC deaminase are important bacterial characteristics in soil and roots, however, siderophore production occurs predominantly in root bacteria.

A comparison of phenotypic characteristics of bacterial communities that were isolated in different studies is complicated due to the large variation that exists between different plant species, growth conditions and concentrations of metals in the plant (Chen *et al.*, 2012). However, metal excess in soils can stimulate the production of bacterial siderophores that can decrease metal toxicity by providing iron to the plant (Dell' Amico *et al.*, 2005). Moreover, the production of IAA and ACC deaminase can stimulate root growth in presence of metals and also the root exudation that promotes the bacterial proliferation in the rhizosphere (Glick *et al.*, 2010). Our results support these hypotheses, due to the fact that most of the bacterial strains isolated from the Zn contaminated site show potential PGP characteristics (Table 3).

3.5. Inoculation of *B. napus* seeds with PGPB

Six Cd-Zn-tolerant bacterial strains (*Staphylococcus* sp. 25, *Arthrobacter* sp. 222, *Pseudomonas* sp. 228, *Serratia* sp. 246, *Pseudomonas* sp. 256, *Pseudomonas* sp. 262) isolated from bulk soil, rhizosphere and roots of *B. napus* growing on the Zn contaminated site) were selected based on their PGP characteristics (Table 4) to be inoculated on *B. napus* seeds. Root length was the parameter used to evaluate the effects of the bacteria on the growth and tolerance of the seedlings to both metals. The architecture of a root system is determined by the intrinsic developmental program but also, by external biotic and abiotic stimuli (Zhang *et al.*, 1998), such as the presence of toxic concentrations of metals in the growth medium (Remans *et al.*, 2012). Root growth has been often used to evaluate the plant tolerance to metals (Peralta *et al.*, 2001, Azevedo *et al.*, 2005), and it is one of the best markers to evaluate the effects of PGP bacteria on plant growth (Pattern and Glick, 2002).

B. napus did not exhibit significant differences in root length between seedlings grown with low concentrations of Zn or Cd (300 μ M and 50 μ M, respectively) and controls (Figure 3). However, root length decreased at higher doses of both metals (1000 μ M Zn and 300 μ M Cd). Cd can be highly phytotoxic, even at low concentrations (Groppa *et al.* 2008). On the contrary, zinc (Zn) is an essential micronutrient, required by plants to grow, but it becomes toxic at higher levels (Marques *et al.*, 2013). The positive effect of bacterial inoculation of the seeds on root growth was observed only at high doses of both metals. *Pseudomonas* sp. strain 228 and 256, and *Serratia* sp. strain 246, significantly increased root length of *B. napus* when exposed to 1000 μ M Zn (Figure 3a). *Arthrobacter* sp. strain 222, *Serratia* sp. strain 246, *Pseudomonas* sp. 228 and 262 significantly increased root length of *B. napus* seedlings at 300 μ M Cd in comparison to non-inoculated ones (Figure 3b).

In general, the inoculated bacterial strains were able to produce siderophores, indole-3-acetic acid (IAA) and exhibited ACC deaminase activity (Table 4). *Pseudomonas* sp. strain 228 and *Serratia* sp. strain 246 produced organic acids, and *Pseudomonas* sp. strain 256 could produce acetoin and fix nitrogen. Moreover, both *Pseudomonas* strains could solubilize phosphorous. *Arthrobacter* sp. strain 222 and *Pseudomonas* sp. strain 262 showed high ACC deaminase activities and moderate production of organic acids. *Pseudomonas* sp. strain 262 was also able to produce acetoin. *Arthrobacter* sp. strain 222 was the only strain that did not possess the capacity to produce siderophores or IAA, but was able of fixing nitrogen.

A positive effect due to inoculated bacterial strains on root growth of Cd-exposed *B. napus* was also observed by Sheng *et al.* (2006) after inoculation with *Pseudomonas* sp. and *Bacillus* sp., both being IAA producers. Many studies have reported positive effects of inoculated bacteria on plant growth (Glick, 2010). IAA (indole-3-acetic acid) and acetoin (3-hydroxy-2-butanone) production have been shown to stimulate root formation (Duan *et al.*, 2013), and ACC deaminase activity to protects against the growth inhibiting effects of various stresses such as toxic concentrations of metals (Glick, 2003). The PGP characteristics of the inoculated bacterial strains could play an important role in the root growth of *B. napus* seedlings in the presence of toxic concentrations of Zn and Cd. Further studies are necessary for investigating the growth-promoting properties of these bacterial strains in field soils where there is competition between indigenous microorganisms and where nutrients are present in more recalcitrant forms.

Conclusions

The isolation of bacteria associated with *B. napus* growing on a Zn contaminated site led to the identification of Zn- and/or Cd-resistant bacterial strains with potential PGP characteristics. Seed

inoculation of *Pseudomonas sp.* 228, *Serratia sp.* 246 and *Pseudomonas sp.* 256 improved root growth of *B. napus* seedlings at 1 mM Zn, and *Arthrobacter sp.* 222, *Pseudomonas sp.* 228, *Serratia sp.* 246 and *Pseudomonas sp.* 262 at 300µM Cd. Future work should be performed on soils from the field to evaluate the effects of these bacterial strains on plant growth and metal uptake, in order to select bacterial strains that can be used to improve remediation and biomass production on contaminated soils.

Acknowledgements

The authors would like to acknowledge INIA to support the grant of B. Montalbán (FPI-INIA 2010), EIADES PROGRAM S2009/AMB-1478, RTA000150-00-00-INIA project and UHasselt Methusalem project 08M03VGRJ. S. Croes and N. Weyens are grateful to the FWO (Fund for Scientific Research Flanders) for respectively a PhD and post-doc grant.

References

- Azevedo H, Pinto GGC, Fernandes J, Loureiro S, Santos C. 2005. Cadmium effects on sunflower growth and photosynthesis. *Journal of Plant Nutrition* 28: 2211-2220.
- 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 characterization. *Plant Soil* 340:413-433.
- Becerra-Castro B, Monterroso C, Prieto-Fernández A, Rodríguez-Lamas L, Loureiro-Viñas M, Acea MJ, Kidd PS. 2012. Pseudometallophytes colonising Pb/Zn mine tailings: A description of the plant–microorganism–rhizosphere soil system and isolation of metal-tolerant bacteria. *Journal of Hazardous Materials* 217– 218: 350-359.
- Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants. *New Phytologist* 173: 677-702.
- Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, Rauf P, Huettel B, Reinhardt R, Schmelzer E, Peplies J, Gloeckner FO, Amann R, Eickhorst T, Schulze-Lefert P. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488: 91-95.
- Chen L, Luo S, Chen J, Wan Y, Liu C, Liu Y, Pang X, Lai C, Zeng G. 2012. Diversity of endophytic bacterial populations associated with Cd hyperaccumulator plant *Solanum nigrum* L. grown in mine tailings. *Applied Soil Ecology* 62: 24-30.

Compant S, Clément C, Sessitsch A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization.

Soil Biol Biochem 42: 669-678.

Croes S, Weyens N, Janssen J, Vercampt H, Copaert 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: 371-384.

Croes S. 2014. The potential role of plant-associated bacteria to improve the efficiency of phytoremediation of metal-contaminated soils using rapeseed. Doctoral Dissertation. Hasselt University, Belgium.

Croes S, Weyens N, Colpaert J, Vangronsveld J. 2015. Characterization of the cultivable bacterial populations associated with field grown *Brassica napus* L.: an evaluation of sampling and isolation protocols. Environmental Microbiology DOI: 10.1111/1462-2920.12701.

de Campos SB, Youn JW, Farina R, Jaenicke S, Jünemann S, Szczepanowski R, Beneduzi A, Vargas LK, Goesmann A, Wendisch VF, Passaglia LM. 2013. Changes in root bacterial communities associated to two different development stages of canola (*Brassica napus* L. var *oleifera*) evaluated through next-generation sequencing technology. Microb Ecol 65:593–601.

Dell'Amico E, Cavalca L, Andreoni V. 2005. Analysis of rhizobacterial communities in perennial Gramineae from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. FEMS Microbiology Ecology 52: 153-162.

De Temmerman L, Vanongeval L, Boon W, Hoenig M, Geypens M. 2003. Heavy metal content of arable soils in northern Belgium. *Water, Air, and Soil Pollution* 148: 61-76.

Duan J, Jiang W, Cheng Z, Heikkila JJ, Glick BR. 2013. The Complete Genome Sequence of the Plant Growth-Promoting Bacterium *Pseudomonas* sp. UW4. *PLoS ONE* 8(3): e58640.

Ellis RJ, Morgan P, Weightman AJ, Fry JC. 2003. Cultivation-dependent and independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Applied and Environmental microbiology* 69(6): 3223-3230.

Fisher PJ, Petrini O, Scott HML. 1992. The distribution of some fungal and bacterial endophytes in maize. *New Phytol* 122: 299-305.

Genrich I, Dixon DG, Glick R. 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal of Microbiology* 46: 237-245.

Germida JJ, Siciliano SD, Renato de Freitas J, Seib AM. 1998. Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiol Ecol* 26: 43-50.

Glick BR. 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnology Advances* 21: 383–393.

Glick BR. 2010. Using soil bacteria to facilitate phytoremediation. *Biotechnology Advances* 28: 367-374.

Gomez-Balderas CDC, Cochet N, Bert V, Tarnaud E, Sarde CO. 2014. 16S rDNA analysis of bacterial communities associated with the hyperaccumulator *Arabidopsis halleri* grown on a Zn and Cd polluted soil. *European Journal of Soil Biology* 60: 16-23.

Grispen VMJ, Nelissen HJM, Verkleij JAC. 2006. Phytoextraction with *Brassica napus* L.: a tool for sustainable management of heavy metal contaminated soils. *Environ Pollut* 144: 77-83.

Groppa MD, Zawoznik MS, Tomaro ML, Benavides MP. 2008. Inhibition of root growth and polyamine metabolism in sunflower (*Helianthus annuus*) seedlings under cadmium and copper stress. *Biol Trace Elem Res* 126: 246-256.

Hallmann J. 2001. Plant interactions with endophytic bacteria. In: Jeger MJ, Spence NJ, eds. *Biotic Interactions in Plant-Pathogen Associations*. Wallingford, UK: CABI Publishing. p. 87-119.

Hanbo Z, Changqun D, Qiyong S, Weimin R, Tao S, Lizhong C, Zhiwei Z, Bin H. 2004. Genetic and physiological diversity of phylogenetically and geographically distinct groups of *Arthrobacter* isolated from lead-zinc mine tailings. *FEMS Microbiology Ecology* 49, 333-341.

Hernández-Allica J, Becerril JM, Garbisu C. 2008. Assessment of the phytoextraction potential of high biomass crop plants. *Environ. Pollut* 152: 32-40.

Izumi H, Anderson IC, Killham K, Moore ERB. 2008. Diversity of predominant endophytic bacteria in European deciduous and coniferous trees. *Can J Microbiol* 54: 173-179.

Kelly JJ, Häggblom MM, Tate RL. 2003. Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter as indicated by analysis of microbial community phospholipid fatty acid profiles. *Biol Fertil Soils* 38:65-71.

Kid P, Mench M, Álvarez-López V, Bert V, Dimitriou I, Friesl-Hanl W, Herzig R, Janssen J, Kolbas A, Müller I, Neu S, Renella G, Ruttens A, Vangronsveld J, Puschenreiter M. 2015. Agronomic practices for improving gentle remediation of trace element-contaminated soils. *International Journal of Phytoremediation* DOI: 10.1080/15226514.2014.1003788.

Kirkham MB. 2006. Cadmium in plants on polluted soils: Effects of soil factors, hyperaccumulation, and amendments. *Geoderma* 137: 19-32.

Lindow SE, Brandl MT. 2003. Microbiology of the Phyllosphere. *Appl Environ Microbiol* 69: 1875-1883.

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Thagavi S, van der Lelie D. 2002. Endophytic bacteria and their potential applications. *Crit Rev Plant Sci* 21:583–606.

Ma Y, Rajkumar M, Freitas H. 2009. Isolation and characterization of Ni mobilizing PGPB from serpentine soils and their potential in promoting plant growth and Ni accumulation by *Brassica* spp. *Chemosphere* 75: 719-725.

Ma Y, Prasad MNV, Rajkumar M, Freitas H. 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnology Advances* 29: 248-258.

Marchiol L, Sacco P, Assolari S, Zerbi G. 2004. Reclamation of polluted soil: phytoremediation potential of crop-related *Brassica* species. *Water Air Soil Pollut* 158: 345-356.

Marques APGC, Pires C, Moreira H, Rangel AOSS, Castro PML. 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biology & Biochemistry* 42: 1229-1235.

Mench M, Vangronsveld J, Didier V, Clijsters H. 1994. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed silty soil. *Environ Pollut* 86: 279-286.

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Van Gijsegem F. 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162: 328-334.

Montalbán B, García-Gonzalo P, Pradas del Real AE, Alonso J, Lobo MC, Pérez-Sanz A. 2014. *Brachypodium distachyon* tolerance to metals under in vitro conditions: a comparison with two metal-tolerant energy crops. Fresenius environmental bulletin 23(9): 2086-2092.

Nagajyoti PC, Lee KD, Sreekanth TVM. 2010. Heavy metals, occurrence and toxicity for plants: a review. Environ Chem Lett 8: 199-216.

Panagos P, Van Liedekerke M, Yigini Y, Montanarella L. 2013. Contaminated sites in Europe: Review of the current situation based on data collected through a European network. Journal of Environmental and Public Health, Article ID 158764, 11 pages.

Patten CL, Glick BR. 2002. The role of bacterial indoleacetic acid in the development of the host plant root system. Appl Environ Microbiol 68: 3795-801.

Peralta JR, Gardea-Torresdey JL, Tiemann KJ, Gomez E, Arteaga S, Rascon E, Parsons JG. 2001. Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa* L.). Bull Environ Contam Toxicol 66: 727-734.

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 MNV, Freitas H. 2012. Perspectives of plant-associated microbes in heavy metal phytoremediation. Biotechnology Advances 30: 1562-1574.

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A. 2006. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. *Plant Physiology* 140: 909-921.

Remans T, Thijs S, Truyens S, Weyens N, Schellingen K, Keunen E, Gielen H, Cuypers A, Vangronsveld J. 2012. Understanding the development of roots exposed to contaminants and the potential of plant-associated bacteria for optimization of growth. *Annals of Botany* 110: 239-252.

Reynoso-Cuevas L, Gallegos-Martínez ME, Cruz-Sosa F, Gutiérrez-Rojas M. 2008. *In vitro* evaluation of germination and growth of five plant species on medium supplemented with hydrocarbons associated with contaminated soils. *Bioresource Technology* 99: 6379-6385.

Rouch DA, Lee TOB, Morby AP. 1995. Understanding cellular responses to toxic agents: a model for mechanisms-choice in bacterial resistance. *J Ind Microbiol* 14: 132-141.

Schellingen K, Van Der Straeten D, Vandenbussche F, Prinsen E, Remans T, Vangronsveld J, Cuypers A. 2014. Cadmium-induced ethylene production and responses in *Arabidopsis thaliana* rely on ACS2 and ACS6 gene expression. *BMC Plant Biology* 14 DOI: 10.1186/s12870-014-0214-6.

Sessitsch A, Kuffner M, Kidd P, Vangronsveld J, Wenzel WW, Fallmann K, Puschenreiter M. 2013. The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biology & Biochemistry* 60: 182-194.

Sheng XF, Xia JJ. 2006. Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere* 64: 1036-1042.

Soderberg KH, Bååth E. 1998. Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. *Soil Biol Biochem* 30: 1259-1268.

Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2014. Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environmental Microbiology Reports*. DOI: 10.1111/1758-2229.12181.

Vamerali T, Bandiera M, Mosca G. 2010. Field crops for phytoremediation of metal-contaminated land. A review. *Environ Chem Lett* 8: 1-17.

Van Ranst E, Verloo M, Demeyer A, Pauwels JM. 1999. *Manual for the Soil Chemistry and Fertility Laboratory*. Ghent, Belgium: Ghent University, Faculty Agricultural and Applied Biological Sciences.

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. *Environ Sci Pollut Res Int* 16: 765-794.

Velázquez E, Rojas M, Lorite MJ, Rivas R, Zurdo-Piñeiro JL, Heydrich M, Bedmad EJ. 2008. Genetic diversity of endophytic bacteria which could be found in the apoplastic sap of the medullary parenchym of the stem of healthy sugarcane plants. *Journal of Basic Microbiology* 48: 118–124.

Vlarebo (Vlaams Reglement Bodemsanering). 2009. *Vlaams Reglement Betreffende bodemsanering en bodenbescherming*. Openbare Vlaamse Afvalstoffenmaatschappij. D/2005/5024/44. February 2009. 203 pp.

- Weyens N, Taghavi S, Barac T, van der Lelie D, Boulet J, Artois T, Carleer R, Vangronsveld J. 2009a. Bacteria associated with oak and ash on a TCE-contaminated site: characterization of isolates with potential to avoid evapotranspiration of TCE. *Environ Sci Pollut Res* 16: 830-843.
- Weyens N, Van der Lelie D, Taghavi S, Vangronsveld J. 2009b. Phytoremediation: plant-endophyte partnerships take the challenge. *Current Opinion in Biotechnology* 20: 248-254.
- Weyens N, Croes S, Dupae J, Newman L, van der Lelie D, Carleer R, Vangronsveld J. 2010. Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination. *Environ Pollut* 158: 2422-2427.
- Witters N, Mendelsohn RO, Van Slycken S, Weyens N, Schreurs E, Meers E, Tack F, Carleer R, Vangronsveld J. 2012. Phytoremediation, a sustainable remediation technology? Conclusions from a case study. I: Energy production and carbon dioxide abatement. *Biomass and bioenergy* 39: 454-469.
- Zhang H, Forde B. 1998. An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science* 279: 407-409.
- Zhang YZ, He LY, Chen ZJ, Wang QY, Quian M, Sheng XF. 2011. Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus*. *Chemosphere* 83: 57-62.
- Zhang WH, Huang Z, He LY, Sheng XF. 2012. Assessment of bacterial communities and characterization of lead-resistant bacteria in the rhizosphere soils of metal-tolerant *Chenopodium ambrosioides* grown on lead-zinc mine tailings. *Chemosphere* 87: 1171-1178.

Table 1. Total and Ca (NO₃)₂-extractable metal concentrations (mg kg⁻¹ dry soil), pH and organic matter (OM) content of the soil in the experimental field. Results are mean ± SE of composite soil samples (depth: 0-25 cm).

| | Zn | Cd | Cu | Pb | OM | pH-KCl |
|--|------------------------|-------------|-------------|-------------|-----|-----------|
| | (mg.kg ⁻¹) | | | | (%) | |
| Total concentration | 343 ± 16 | 0.36 ± 0.04 | 35 ± 2 | 185 ± 10 | 1.9 | 5.6 ± 0.1 |
| Ca(NO ₃) ₂ -extractable | 81 ± 20 | 0.15 ± 0.02 | 0.18 ± 0.01 | 0.38 ± 0.02 | | |
| Background values ^a | 25-70 | 0.1-0.5 | 3-15 | 5-40 | 2.3 | |
| Remediation values ^b | 333 | 2 | 120 | 200 | 2 | |

^a'Common range' values in sandy soils in Flanders according to De Temmerman *et al.* (2003).

^bClean up values for remediation of a 'standard' agricultural soil (2% organic matter and 2% clay) according to the Flemish legislation on soil remediation (Vlarebo, 2009).

Table 2. Total numbers of colony-forming units (cfu) per gram fresh weight of soil, rhizosphere and *B. napus* tissues isolated from Zn contaminated site.

| Compartment | cfu g ⁻¹ fresh weight |
|-------------|--|
| Soil | 62×10 ⁴ ± 52.8×10 ⁴ (8) b |
| Rhizosphere | 30×10 ⁶ ± 15.3×10 ⁶ (20) a |
| Root | 13×10 ⁴ ± 10.1×10 ⁴ (17) b |
| Stem | 61×10 ± 57.0×10 (3) c |

Mean values ± SE, n = 3 independent replicates. The numbers of different bacterial genus are marked in parentheses. Letters represent significant differences between compartments after ANOVA and Tukey's test ($p \leq 0.05$)

Table 3. Phenotypic characterization of all purified bulk soil, rhizosphere, root and stem isolated bacteria.

| | Soil | Rhizosphere | Root | Stem |
|--------------------|-------|-------------|-------|-------|
| SID | 1.86 | 3.89 | 23.76 | 0.00 |
| OA | 36.68 | 5.53 | 20.79 | 53.79 |
| ACC | 68.39 | 18.94 | 69.25 | 0.26 |
| IAA | 35.38 | 6.49 | 47.21 | 26.76 |
| Acetoin | 5.57 | 10.32 | 7.07 | 20.00 |
| P sol | 4.09 | 1.63 | 17.39 | 0.00 |
| N ₂ fix | 1.05 | 1.05 | 1.20 | 53.33 |
| Zn (1 | 23.28 | 25.40 | 6.18 | 40.00 |
| Cd (0.8 | 22.27 | 25.07 | 21.68 | 0.00 |

Data are relative abundances expressed in percentages of the total number of cultivable bacteria isolated per gram fresh weight bulk soil, rhizosphere, roots and stem. Bacterial strains were tested for metal resistance (Cd or Zn) and potential plant growth-promoting characteristics: Phosphorus solubilization (P sol), nitrogen fixation (N₂ fix), production of siderophores (SID), organic acids (OA), ACC deaminase (ACC), indole-3-acetic acid (IAA) and acetoin (Acetoin).

Table 4. Metal tolerance and PGP characteristics of selected bacteria for inoculation in *B. napus* seeds.

| Com | Str | Identificat | Zn | Cd | Fe0 μ | Fe0.25 | O | A | IA | Ac | Ps | N |
|------|-----|------------------|----|-----|-----------|--------|----|----|----|----|----|---|
| Rh | 25 | <i>Staphyloc</i> | ++ | +++ | - | - | ++ | - | - | + | - | - |
| Soil | 222 | <i>Arthrobac</i> | ++ | +++ | - | - | ++ | ++ | - | - | - | + |
| Root | 228 | <i>Pseudom</i> | ++ | ++ | + | + | + | ++ | + | - | ++ | - |
| Root | 246 | <i>Serratia</i> | ++ | +++ | + | + | ++ | ++ | ++ | - | - | - |
| Root | 256 | <i>Pseudom</i> | ++ | + | + | + | - | + | ++ | + | ++ | + |
| Root | 262 | <i>Pseudom</i> | + | + | ++ | - | + | ++ | ++ | + | - | - |

Compartment (Comp.), Rhizosphere (Rh), Organic acids (OA), ACC (ACC deaminase activity), IAA (indole-3-acetic acid), Ace (Acetoin), phosphate solubilization (Psol), nitrogen fixation (N fix). + low, +++ high production.

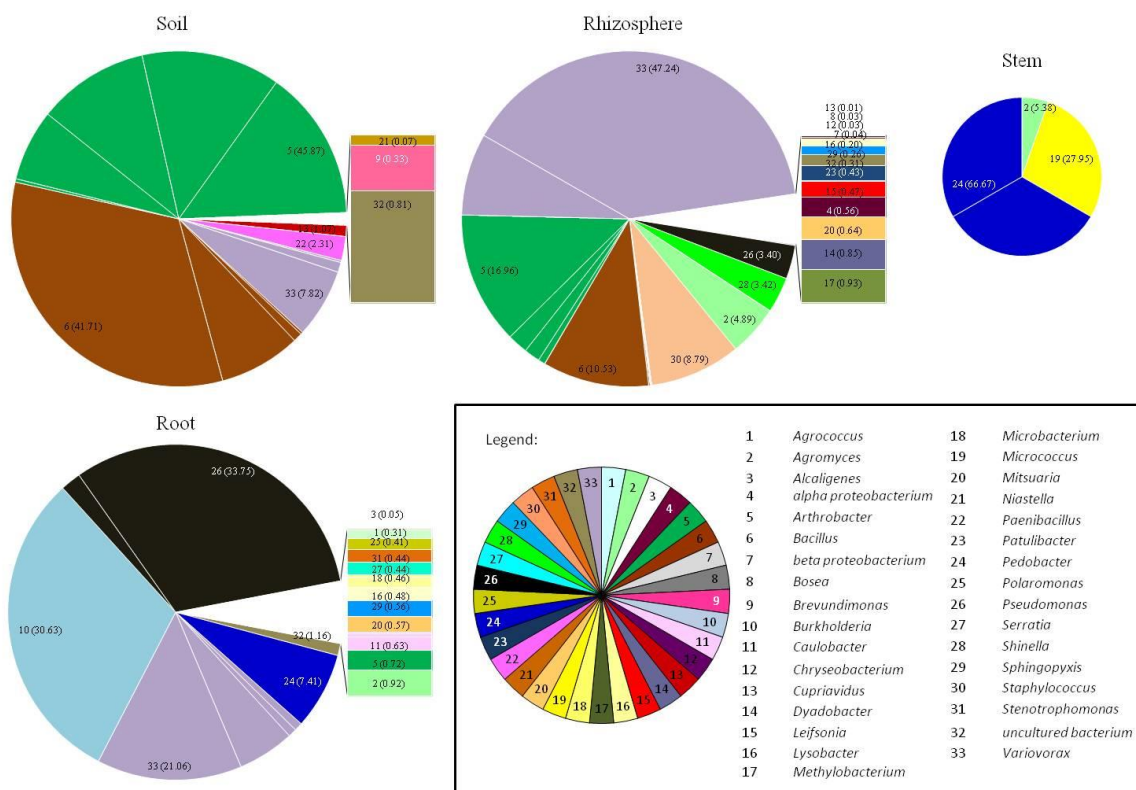


Fig.1. Diversity and abundance of cultivable bacteria isolated from bulk soil, rhizosphere, root and stem of *B. napus* plants grown in a Zn contaminated site. Abundance percentages are shown in parentheses. Bacterial strains with abundances lower than 1% are shown next to the pie diagram.

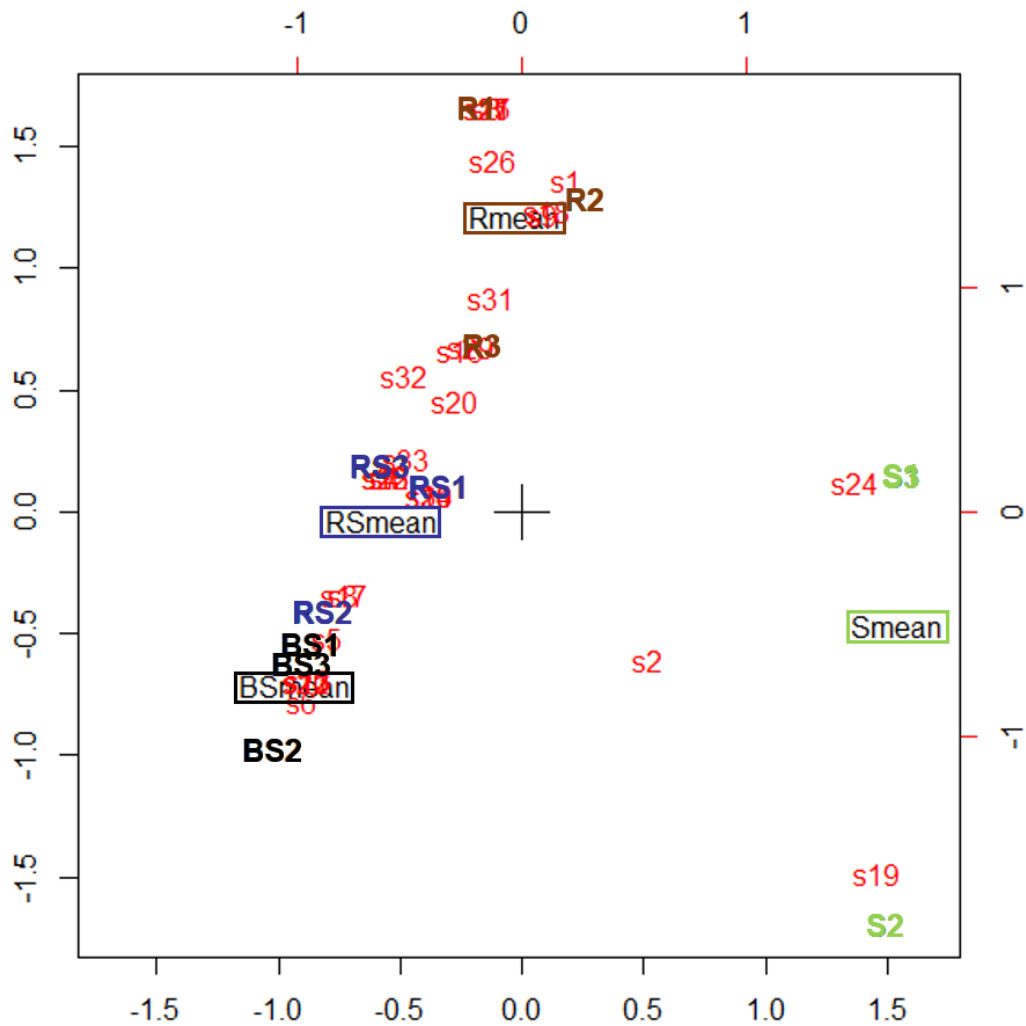


Fig.2. Correspondence analysis of bacterial communities isolated from soil, rhizosphere and *B. napus* samples. s1-s33 represent a bacterial genus (see legend in Figure 1). BS (Bulk soil), RS (Rhizosphere soil), R (Root), S (Stem). Three repetitions of each compartment were used to make the mixed sample (mean).

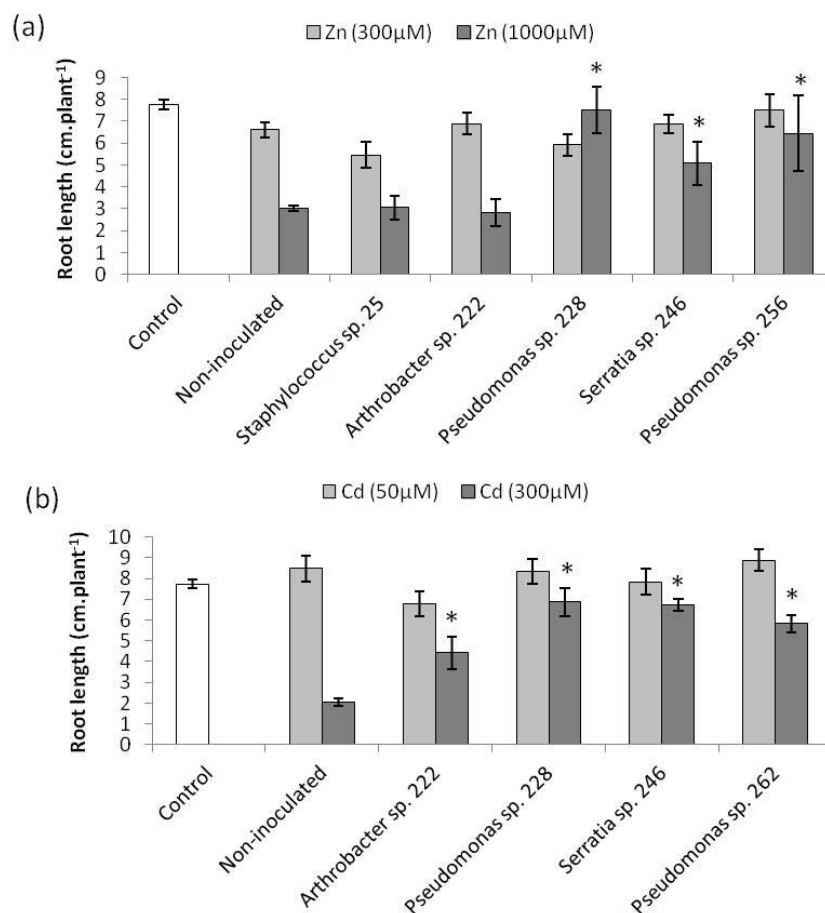


Fig.3. Root length of 5-days *B. napus* seedlings after inoculation of PGP bacterial strains in presence of (a) Zn and (b) Cd. Asterisks (*) represent significant differences between non-inoculated and inoculated plants after one-way ANOVA and Tukey's test ($p \leq 0.05$; mean values \pm SE; $n = 4$).