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DOI: 10.1080/15226514.2016.1183566
Handle: http://hdl.handle.net/1942/22055

International Journal of Phytoremediation

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

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To cite this article: 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

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

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

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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. *Burkoholderia*, *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 (Reynoso-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. From1982 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 m^2) 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 $Ca(NO_3)_2$ (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 MgSO4 (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), 10µL 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 QIAlquick 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 $MgSO_4$. Surface-sterilized seeds were immersed in a bacterial suspension (10^8 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 ($ZnSO_4.7H_2O$) and 50 and 300 µM of Cd ($CdSO_4.8H_2O$). 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 $MgSO_4$ 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 23ºC/12 ºC 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 $Ca(NO_3)_2$-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 Ca $(NO_3)_2$-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*, *Leifsoni a*, *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-hyperacumulator *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, Alacaligenes, 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 µM and 50 µM, respectively) and controls (Figure 3). However, root length decreased at higher doses of both metals (1000 µM Zn and 300 µM 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 1000µM 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 µM 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.

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Table 1. Total and Ca $(NO_3)_2$-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).

[b]Clean 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	$62\times10^4 \pm 52.8\times10^4$ (8) b
Rhizosphere	$30\times10^6 \pm 15.3\times10^6$ (20) a
Root	$13\times10^4 \pm 10.1\times10^4$ (17) b
Stem	$61\times10 \pm 57.0\times10$ (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 (N_2 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	Fe0µ	Fe0.25	O	A	IA	Ac	Ps	N
Rh	25	*Staphyloc*	++	+++	-	-	++	-	-	+	-	-
Soil	222	*Arthrobac*	++	+++	-	-	++	++	-	-	-	+
Root	228	*Pseudom*	++	++	+	+	+	++	+	-	++	-
Root	246	*Serratia*	++	+++	+	+	++	++	++	-	-	-
Root	256	*Pseudom*	++	+	+	+	-	+	++	+	++	+
Root	262	*Pseudom*	+	+	++	-	+	++	++	+	-	-

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.

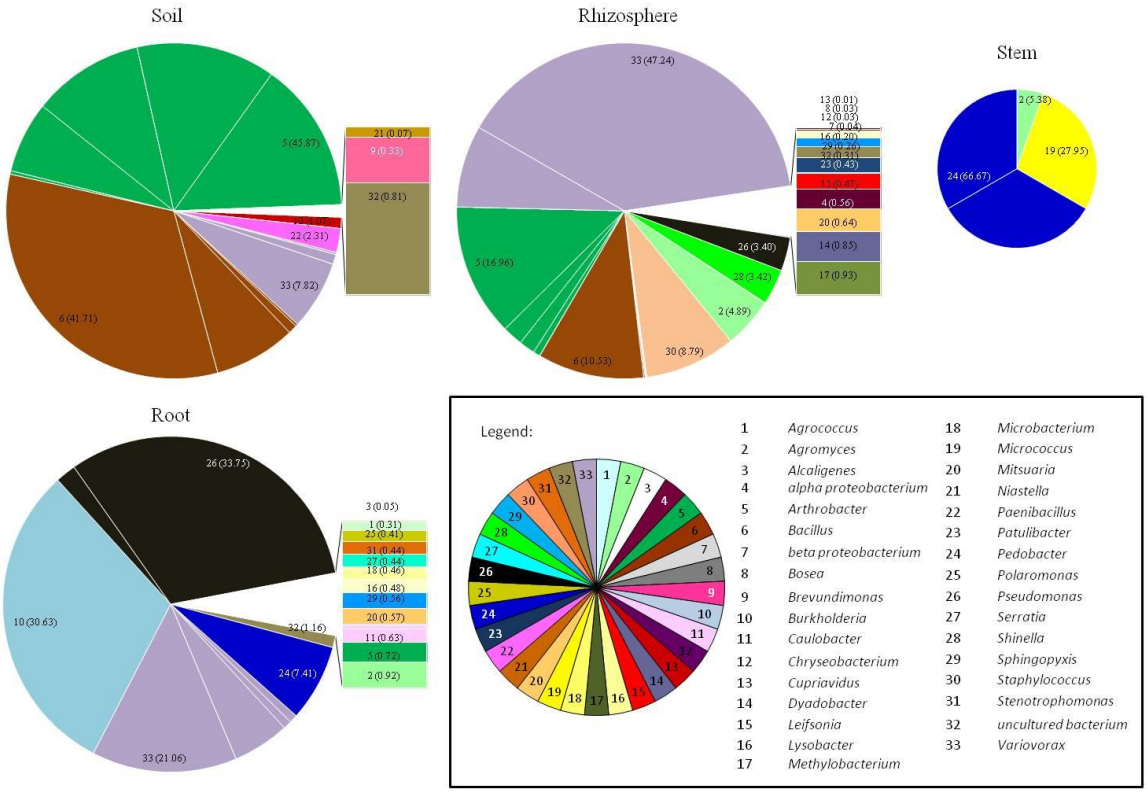

Fig.1. Diversity and abundance of cultivable bacteria isolated from bulk soil, rhizosphere, root and stem of *B. napus* plants grown in a Zn contaminated site. Abundance percentages are shown in parentheses. Bacterial strains with abundances lower than 1% are shown next to the pie diagram.

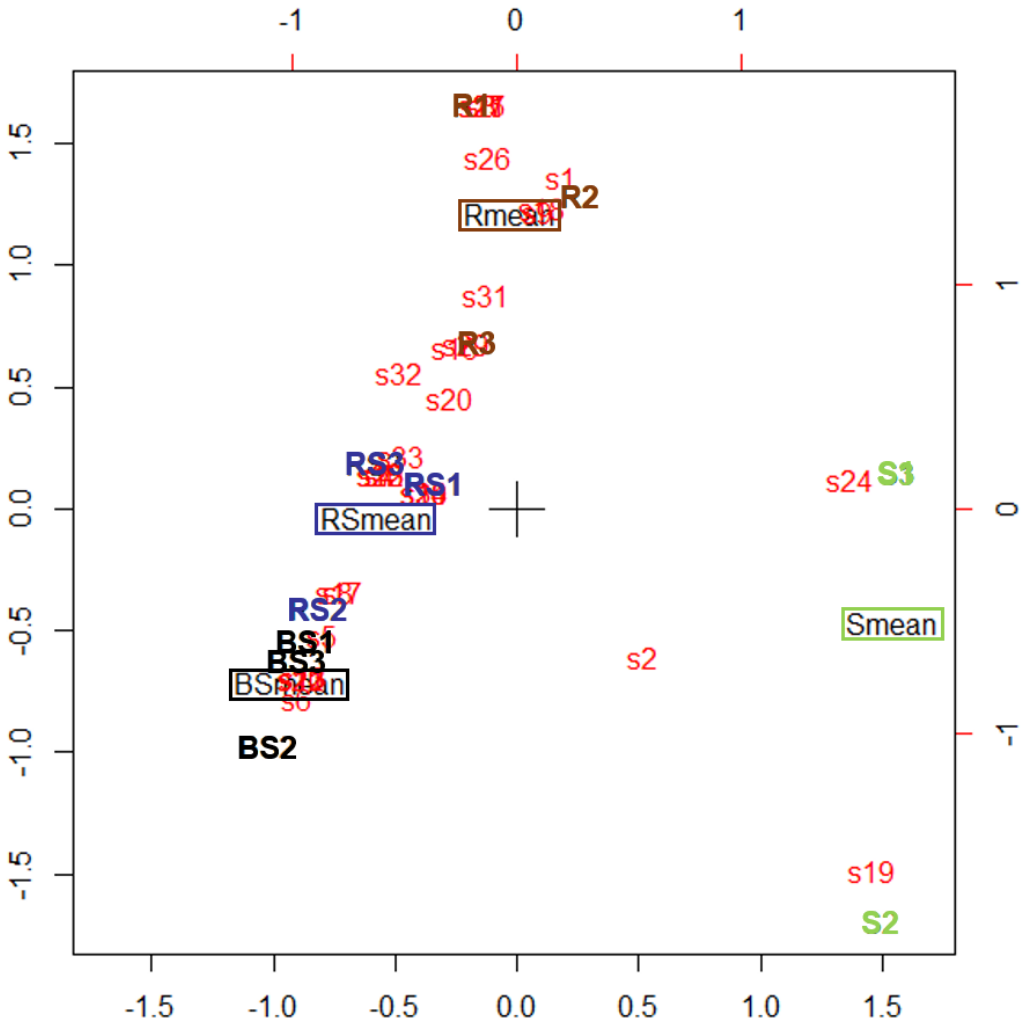

Fig.2. Correspondence analysis of bacterial communities isolated from soil, rhizosphere and *B. napus* samples. s1-s33 represent a bacterial genus (see legend in Figure 1). BS (Bulk soil), RS (Rhizosphere soil), R (Root), S (Stem). Three repetitions of each compartment were used to make the mixed sample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

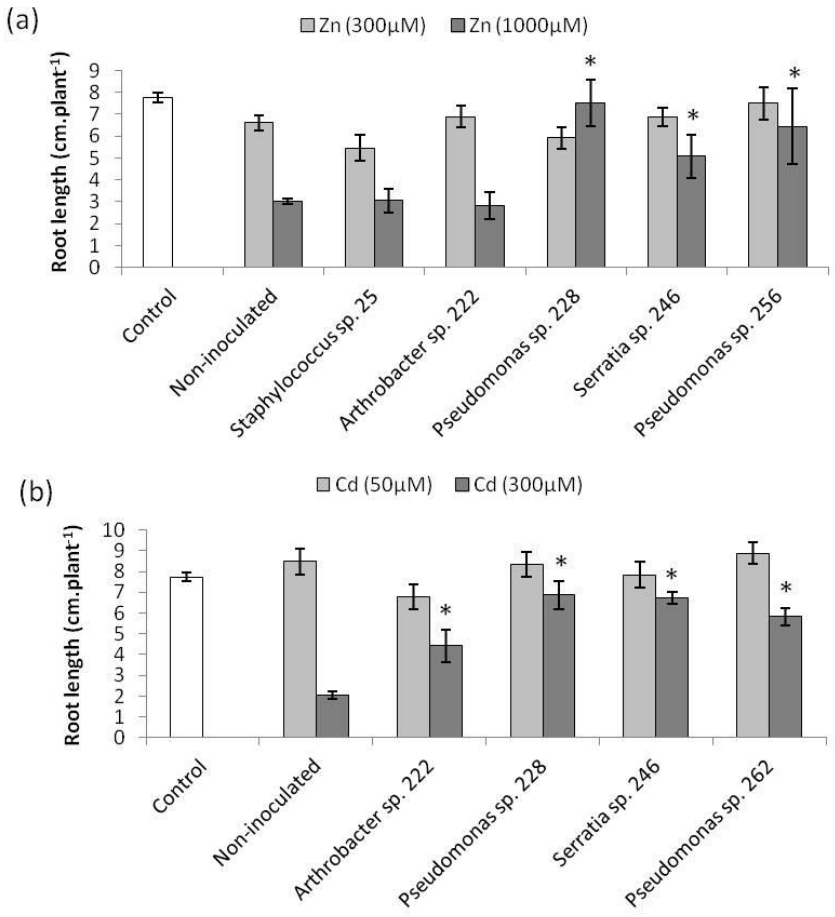

Fig.3. Root length of 5-days *B. napus* seedlings after inoculation of PGP bacterial strains in presence of (a) Zn and (b) Cd. Asterisks (*) represent significant differences between non-inoculated and inoculated plants after one-way ANOVA and Tukey's test ($p \leq 0.05$; mean values ± SE; n = 4).